EVALUATION OF ANTIHISTAMINES FOR *in vitro* ANTIMALARIAL ACTIVITY AGAINST *Plasmodium falciparum*

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A full thesis submitted in fulfilment of the requirements for the degree of Magister Pharmaceuticiae in the Faculty of Natural Sciences, School of Pharmacy, University of the Western Cape

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Plasmodium falciparum

Antihistamines

Chloroquine resistance

Synergism

Cyproheptadine

Ketotifen

Chlorpheniramine

H_3 antagonists

Morphology

Haemoglobin

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I declare that *Evaluation of Antihistamines for in vitro Antimalarial Activity against Plasmodium falciparum* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Full name.................................... Date.................................

Signed...

ABSTRACT

Evaluation of Antihistamines for *in vitro* Antimalarial Activity against *Plasmodium falciparum*

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The declining efficacy of antimalarial drugs against resistant *Plasmodium falciparum* strains in several endemic regions has amplified the world's burden of neglected diseases. This has highlighted the need for alternate strategies for chemotherapy and chemoprophylaxis. Since malaria is prevalent primarily in third world countries, it is critical for novel therapies to be affordable. Previous research has found that some antihistamines possess inherent antimalarial activity and cause a marked reversal of chloroquine resistance *in vitro* and *in vivo.* Promising results have been demonstrated when chlorpheniramine was combined with chloroquine to reverse chloroquine resistance in two African studies (Sowunmi *et al*, 1997; Abok., 1997). Recently, astemizole and its principle human metabolite desmethylastemizole were identified as potent inhibitors of *Plasmodium falciparum* at sub-micromolar concentrations in both chloroquine sensitive and chloroquine resistant parasites, showing efficacy *in vitro* and in two mouse models. The promising results observed with these studies warrant a more comprehensive understanding of how antihistamines interact with the malaria parasite. Additionally, analysing the different structural and mechanistic characteristics of antihistamines may lead to the design and development of effective and affordable antimalarial agents or chloroquine resistance modulators.

This thesis describes the antimalarial activity of mainly off-patent (generic) antihistamines by comparing the efficacy of a total of 24 antihistamines, representing histamine$_1$, histamine$_2$, and histamine$_3$ receptor antagonists, against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum.* Cyproheptadine, ketotifen, loratadine, desloratadine, 3-(1H-Imidazol-4-yl) propyldi (p-fluorophenyl) methyl ether hydrochloride and ciproxifan display IC_{50} values less than 4µg/ml. There was no significant difference in the sensitivity to antihistamines among the chloroquine sensitive and resistant parasites tested. A tricyclic nucleus appears to be an important structural scaffold for antihistamines which exhibit low IC_{50} values.

Synergistic studies indicate that enhancement of the antimalarial effect of chloroquine on *P. falciparum* was observed with the ethanolamines against the chloroquine sensitive parasites. Cyproheptadine, ketotifen and desloratadine exerted a marked synergistic action with

chloroquine against chloroquine sensitive and resistant parasites. Chlorpheniramine exhibited synergism with chloroquine against resistant parasites only.

Microscopic studies illustrate the effect of antihistamines on parasite morphology when compared to control. Using immunofluorescence microscopy, it was seen that ketotifen decreases haemoglobin localization while cyproheptadine increases haemoglobin localization in the parasite's food vacuole. Western blots have confirmed these results, in addition to indicating that chlorpheniramine decreases the haemoglobin content in the parasite.

The results confirm that certain antihistamines do indeed cause a reduction in the growth of malaria parasites. Furthermore, the histamine$_1$ and histamine$_3$ receptor antagonists are most active while histamine$_2$ receptor antagonists have no antimalarial activity. Microscopic studies suggest that antihistamines do not exert their antimalarial effect via a single mechanism of action.

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Chapter 1

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

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AH - Antihistamine

AHs – Antihistamines

CQ – Chloroquine

CQR – Chloroquine resistant

CQS - Chloroquine sensitive

DMSO – Dimethyl sulfoxide

FIC – Fractional Inhibitory Concentration

FPIX - Ferriprotoporphyrin IX

H_1 - Histamine 1

H_2 - Histamine 2

H_3 - Histamine 3

H_4 - Histamine 4

hc - Haematocrit

HEPES – Hydroxyethane piperazine sulphonic acid

IC_{50} – The inhibitory concentration at which 50% of the parasites are dead.

m – Milli

µM – Micromolar

µg – microgram

M – Molar

ml(s) – Millilitre(s)

ng – nanogram

NBT – Nitroblue tetrazolium

PBS – Phosphate Buffered Saline

PES – Phenazine ethosulphate

P. falciparum – Plasmodium falciparum

Pgp – P-glycoprotein

Phg 1 – P glycoprotein homologue 1

pLDH - Parasite lactate dehydrogenase

pst – Parasitaemia

pRBC's – Parasitized red blood cell

SAR – Structure Activity Relationship

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

RBC – Red Blood Cell

QSAR – Quantitative Structure Activity Relationship

Chapter 1

Introduction

1.1 Background

Malaria is undoubtedly the most important parasitic disease in terms of human suffering. About 3.3 billion people, that is, half of the world's population are at risk of malaria. In 2008, there were 247 million cases of malaria which resulted in one million deaths, mostly among children living in Africa. A child dies every 45 seconds of malaria in Africa and the disease accounts for 20% of all childhood deaths (World Health Organisation, 2009). The control of malaria is threatened by inadequate resources and drug resistance. Worldwide it is Africa that carries the greatest burden of *falciparum* malaria mortality and morbidity and it is also Africa that is most resource- limited (Winstanley, 2003).

Studies published in 2003 estimate an average cost of approximately $800 million to bring a drug with a New Chemical Entity to market (DiMasi *et al*, 2003). A study published in 2006 estimates that costs vary from around $500 million to $2 billion depending on the therapy or the developing firm (Adams and Brantner, 2006). With the cost of new drug discovery rising, novel therapeutics for diseases, such as malaria which are endemic to the third world seems out of reach for the people in dire need of a cure.

1.2 Life Cycle of the Malaria Parasite

Human malaria is caused by four species of the *Plasmodium* parasite, namely *P. falciparum, P. vivax, P. ovale, and P. malaria* (Mueller *et al*, 2007). The infected female Anopheles mosquito feeds on human blood and injects the parasites in the form of sporozoites into the bloodstream. The sporozoites travel to the liver and invade hepatocytes. Over 5-15 days, the sporozoites grow, divide, and produce tens of thousands of haploid forms, called merozoites, per liver cell. Some

malaria parasite species remain dormant for extended periods in the liver, causing infection and relapses, weeks or months later. The merozoites exit the liver cells and re-enter the bloodstream, beginning a cycle of invasion of red blood cells, asexual replication, and release of newly formed merozoites from the red blood cells repeatedly over 1-3 days.The merozoite develops within the erythrocyte through ring, trophozoite and schizont stages (Fujioka and Aikawa, 2002).

After entering the erythrocyte the parasite undergoes a trophic period. The young trophozoite is often called a ‘ring’ due to its morphology. As the parasite increases in size this 'ring' morphology disappears and it is referred to as trophozoite. After a period of growth the trophozoite undergoes an asexual dividing process of erythrocytic schizogony. The nucleus divides 3-5 times into a variable number of small nuclei. This is soon followed by the division of cytoplasm forming a schizont. Mature schizonts are fully developed forms in which, as a result of segmentation of the nucleus and the cytoplasm a number of small rounded merozoites are produced. The erythrocyte containing the segmented schizonts eventually ruptures and releases the newly formed merozoites that invade new erythrocytes. Concomitantly, a small portion of the parasites differentiate from newly invaded merozoites into sexual forms, which are macrogametocyte (female) and microgametocyte (male) (Bannister and Mitchell, 2003; Ibezim and Odo, 2006).

When a mosquito bites an infected human, it ingests the gametocytes. In the mosquito gut, the infected human blood cells burst, releasing the gametocytes, which develop further into mature sex cells called gametes. Male and female gametes fuse to form diploid zygotes, which develop into actively moving ookinetes that burrow into the mosquito midgut wall and form oocysts. Growth and division of each oocyst produces thousands of active haploid forms called sporozoites. After 8-15 days, the oocyst bursts, releasing sporozoites into the body cavity of the mosquito, from which they travel and invade the mosquito salivary glands. The cycle of human infection re-starts when the mosquito takes a blood meal, injecting the sporozoites from its salivary glands into the human bloodstream (Fujioka and Aikawa, 2002). Figure 1.1. outlines the complete life cycle of the malaria parasite.

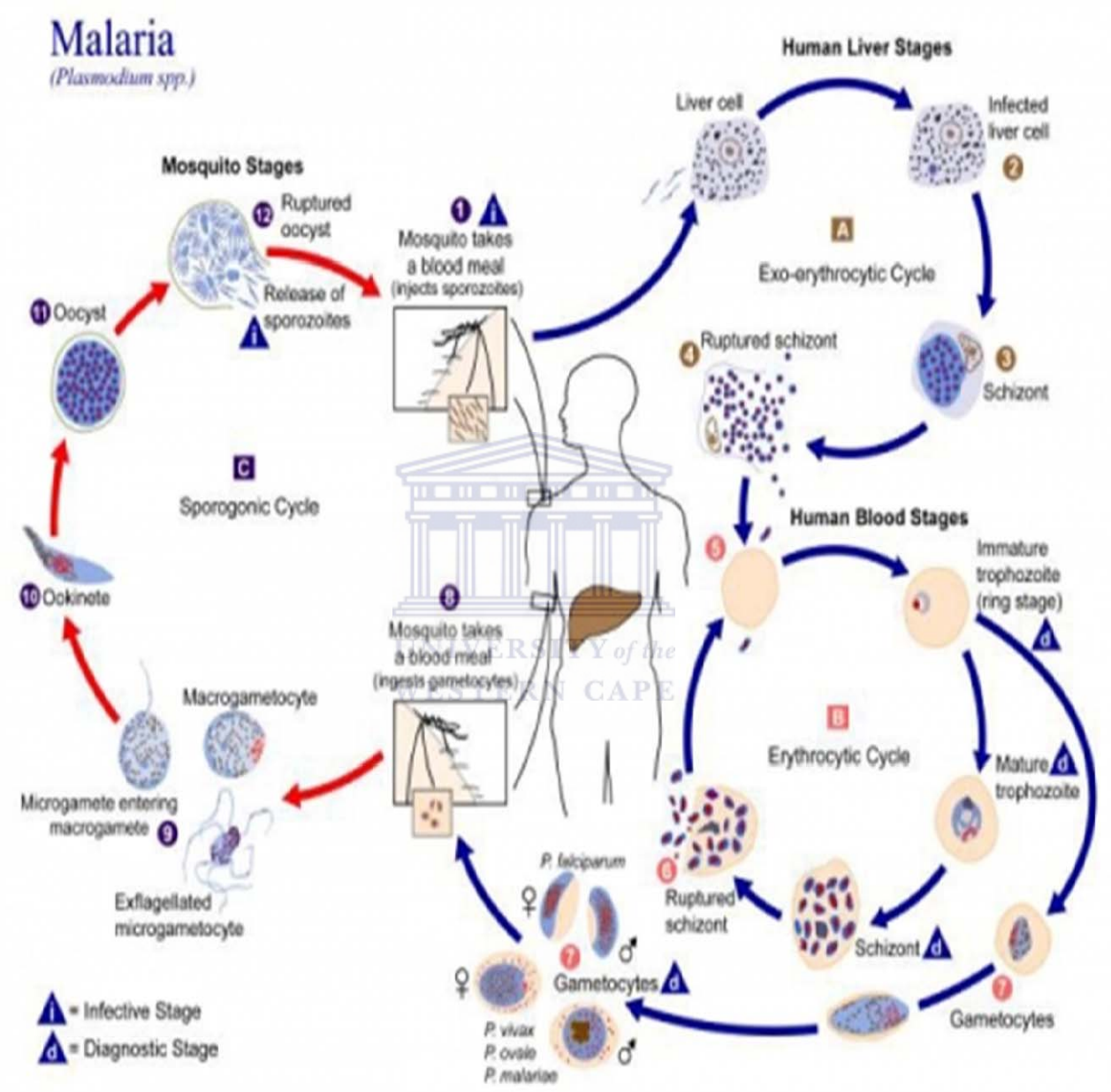


Figure 1.1: The life cycle of the malaria parasite. Image taken from

http:// www.cdc.gov/malaria/biology/life_cycle.htm

1.3 Antimalarial Drugs that Target the Blood Stages

The search for effective antimalarial agents is ongoing; however, current antimalarial drugs can be grouped into three major classes:

1.3.1. Quinolines: (e.g. chloroquine (CQ), mefloquine, quinine, amodiaquine) and related aryl alcohols have been the mainstay of antimalarial therapy and prevention (Robert *et al*, 2001).

1.3.2. Antifolates: (pyrimethamine, cycloguanil) that inhibit nucleotide biosynthesis by limiting essential folate cofactors (Ouellette, 2001; Robert *et al*, 2001).

1.3.3. Artemisinin and derivatives: (artemether, arteether, artenusate) are sesquiterpene lactone peroxides and breakdown of the labile peroxide bridge within the sesquiterpene lactone molecule generates free radicals that are toxic parasite (De Vries and Dien, 1996).

Resistance to the first two classes is widespread, and resistance to the third is proceeding much more rapidly than initially expected.

The structures of the main antimalarial drugs are shown in figure 1.2 for reference.

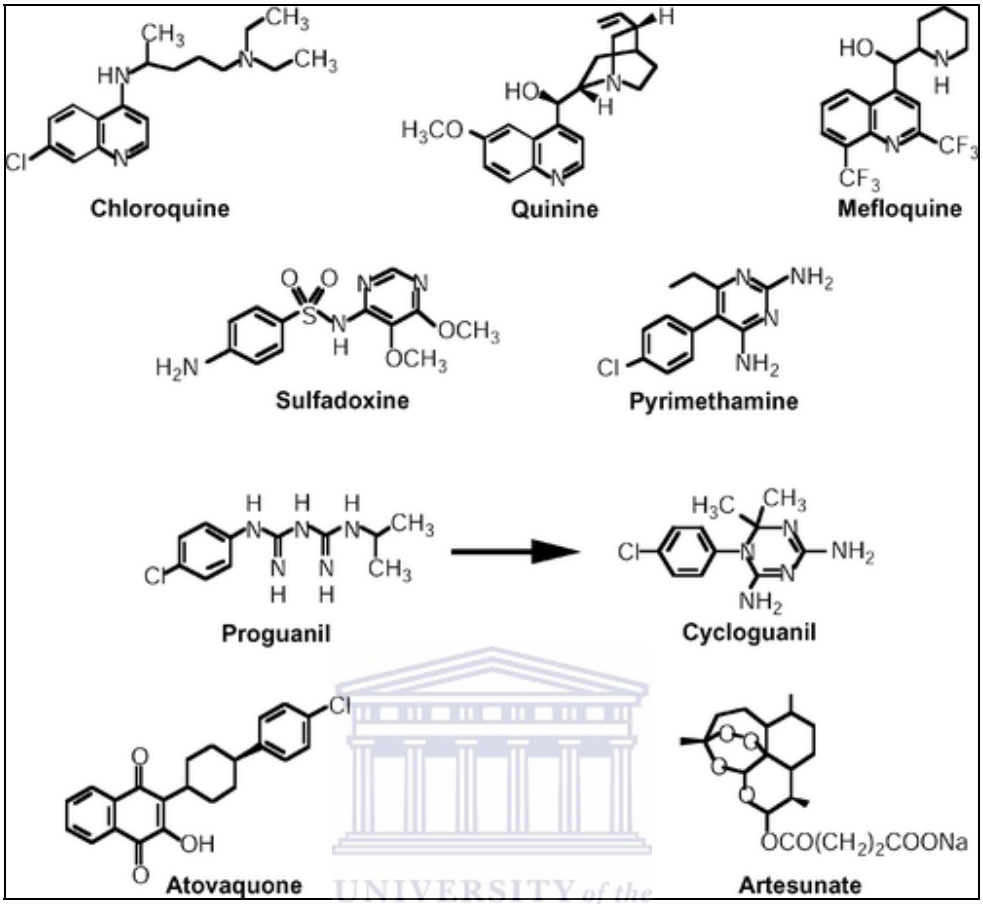


Figure 1.2: Structures of common antimalarial drugs

1.4. Chloroquine (CQ)

CQ was discovered 75 years ago and for a long time was the drug of choice against malaria. However, CQ resistance has increased to the point where it is virtually ineffective in many malarious regions. Since CQ is the most efficacious low-cost drug available, CQ resistance has been exceptionally detrimental in third-world countries where the spread of malaria is rampant (Ouellette, 2001).

CQ is postulated to have several mechanisms of action. Amongst those which receive the most support are the following:

1.4.1 Altered pH in the Acidic Food Vacuole

The malaria parasite contains an acidic digestive food vacuole, similar to mammalian lysosomes (Krogstad *et al*, 1985). Intraerythrocytic malaria parasites ingest the erythrocyte haemoglobin via endocytosis and deliver it to the parasite food vacuole in endosomes (Hoppe *et al*, 2004; Roberts, *et al,* 2008). CQ, taken up with haemoglobin accumulates in the acidic food vacuole of intraerythrocytic malaria parasites by virtue of its weak base properties (Sullivan *et al*, 1996; Yayon *et al*, 1984). CQ is a diprotic weak base with pKa's of 8.1 and 10.2. In its uncharged form, CQ diffuses freely through the membrane into the parasites cytoplasm which has a pH of 7.4. The parasites food vacuole has a pH of 5.2-5.4 and CQ diffuses into acidic compartments of the malaria parasite down this pH gradient (Yayon *et al*, 1984). Once inside the food vacuole CQ becomes diprotonated and membrane impermeable. Therefore, the extent of CQ accumulation depends on the difference in pH between the parasite food vacuole and the extracellular environment (Krogstad *et al*, 1985; Yayon *et al*, 1985). Krogstad *et al* (1985) verified that antimalarials, CQ, quinine, and mefloquine as well as NH_4Cl inhibited parasite growth at concentrations virtually identical to those that increased parasite vacuole pH. The increase in vacuolar pH disrupts metabolic processes such as receptor mediated endocytosis, lysosomal enzyme targeting and enzyme mediated haemoglobin degradation which ultimately leads to starvation of the parasite.

1.4.2 Interaction with Ferriprotoporphyrin IX (FPIX)

The haemoglobin inside the parasite food vacuole is hydrolysed by aspartic proteases to free haem (Fe^{+3}) or ferriprotoporphyrin IX and denatured globin. The denatured globin is further hydrolysed into amino acids which are used by the parasite for protein synthesis (Kumar *et al* 2007). Free haem is toxic due to its ability to destabilise and lyse membranes (Chou and Fitch, 1981). To overcome free haem (Fe^{+3}) toxicity, the malaria parasite detoxifies haem by

polymerization of FPIX to an inert crystal called malaria pigment or haemozoin. (Jani *et al*, 2008). This involves dimerization of FPIX to ß-haematin which is promoted by unsaturated lipids. The lipids serve in concentrating monomeric FPIX, thereby maintaining a state favourable for dimerization (Fitch *et al*, 2003). CQ interferes with FPIX detoxification by masking unsaturated lipids in parasitized erythrocytes rendering them unavailable for FPIX dimerization. Fitch and Russel (2006) propose that CQ binds with high affinity to FPIX removing it from globin. Consequently, FPIX complexes with CQ and an excess of denatured globin could be produced in endosomes. The FPIX-CQ complex has membrane toxicity and it inhibits endosomal maturation which results in a reduction in haemoglobin degradation and cause formation of haemoglobin-laden vesicles with double membranes. Therefore, inhibition of endosomal maturation could allow unsaturated membrane lipids to remain masked and unavailable to promote FPIX dimerization. Furthermore, Sullivan *et al* (1996) suggest that a CQ-FPIX complex incorporates into the growing polymer of haemozoin to terminate chain extension, blocking further sequestration of toxic haem.

1.4.3 Inhibition of Glutathione - dependent or Peroxidative degradation of FPIX

Ginsburg *et al* (1998) proposed that CQ prevents degradation of haem by glutathione. They suggest that only 30% of the free haem is converted into haemozoin and that the remainder of the haem that exits in the food vacuole is degraded by gluthatione in the parasite's cytosol. They show that CQ and amodiaquine competitively inhibit the degradation of haem by glutathione, thus allowing haem to accumulate in membranes.

Loria *et al* (1999) suggest that free haem moieties are by-products of haemoglobin degradation. The haem in oxyhaemoglobin is oxidized from the Fe (II) state to the (Fe III) state with the consequent production of H_2O_2. They suggest that only one third of the haem is polymerized to form haemozoin and that free haem is degraded by reacting with H_2O_2 causing its own peroxidative decomposition. CQ would accumulate in the food vacuole where it would inhibit haem peroxidative decomposition, promoting the association of haem with membranes. This

would lead to a build up of toxic moieties which would irreversibly damage the proteins and lipids of the food vacuole.

The results presented in the two above-mentioned studies were questioned by Egan *et al* (2002). They concluded that more than 95% of the haem iron released from host haemoglobin was in the form of haemozoin. Spectroscopic analysis showed that haemozoin was the only detectable iron species in parasites. This contradicts the studies of Ginsburg *et al* (1998) and Loria *et al* (1999).

There is often a lack of general consensus regarding the precise mechanism of action of CQ; however, it is evident that CQ interferes with crystallization of free haem monomers released during proteolysis of haemoglobin by binding to them causing a build up of toxic haem.

1.5. Chloroquine Resistance

CQ resistance was first observed in Thailand in 1957 and on the Colombian-Venezuelan border in 1959. By 1988 resistance had spread to essentially all of sub-Saharan Africa and today CQ has lost its efficacy in all but a few areas of the world (World Health Organisation, 2009).

Mechanism of Resistance

The mechanism of CQ resistance (CQR) is still not fully understood. Many studies demonstrate the central role of two proteins, namely Pgh1 (P-glycoprotein homolog 1) and CRT (CQ resistance transporter) in mediating CQR. Both are essential membrane proteins, localized to the parasite's food vacuole membrane.

1.5.1. Access to haematin: Basis of CQR

Resistant strains of *P. falciparum* have developed a mechanism that reduces access of CQ to FPIX, subsequently decreasing the accumulation of CQ in the parasites food vacuole (Bray *et al*., 1998). Verapamil and other structurally unrelated drugs selectively chemosensitize or "reverse" CQR by inhibiting this mechanism and increasing the accumulation of CQ within the parasites (Bray *et al*, 1999)

1.5.2 *Plasmodium falciparum* CQ Resistant Transporter

The *Plasmodium falciparum* CQ resistant transporter (*pfCRT*) protein localizes to the digestive vacuole membrane and contains 10 putative transmembrane domains. Mutations within the *pfCRT*, particularly a K76T (lysine to threonine) amino acid substitution at codon 76 in a predicted transmembrane region appear to reduce the binding of CQ to FPIX. Transfection experiments done previously have shown that mutant *pfcrt* alleles can confer CQR to a CQ-sensitive (CQS) strain and that resistance is dependent on the presence of the *PfCRT* K76T mutation (Sidhu *et al*, 2002).

Recent bioinformatic analysis reveals that CRT protein is a member of a previously undefined family of proteins, falling within the drug and metabolite transporters (DMT) super-family (Martin and Kirk, 2004). The amount of CQ imported into the parasite is dependant upon the integrity of the proton gradient maintained across the vacuolar membrane (Bray *et al*, 2006).€ However, the pH of the food vacuole does not play a primary role in CQR, since there was no significant difference in food vacuole pH of CQS and CQR parasites (Hayward *et al*, 2006). Bray *et al* (2006) studied the energy coupling and subcellular localization of CQ uptake process and they demonstrated that the mutant *PfCRT* functions as a gated channel or pore, resulting in charged CQ species to leak out of the parasites food vacuole which reduces the binding to FPIX.

1.5.3 *Plasmodium falciparum* Multi- Drug resistant (MDR) Gene

In both micro-organisms and tumours, drug resistance can arise from the presence of P-glycoprotein (P-gp) that are capable of expelling a variety of structurally and functionally unrelated drugs. P-gp belongs to the ABC (ATP-binding cassette) transporter super-family and is encoded by MDR genes. *P. falciparum* possesses an MDR homologue (*pfmdr1*) whose gene product, Pgh-1, is expressed during intraerythrocytic development of the parasite. The malarial protein, Pgh1 or MDR1, is a 162 kDa ABC-type transporter with 12 predicted transmembrane domains and two ATP-binding folds thought to be involved in importing solutes into the food vacuole, including the drugs mefloquine, halofantrine and artemisinin (Rohrbach *et al*, 2006).

Point mutations in *pfmdr1* have been associated with changes in parasite susceptibility to CQ, quinine, mefloquine, and artemisinin derivatives, in both laboratory cell lines and clinical isolates. Studies associating *pfmdr1*'s role in decreasing CQ susceptibility has been inconsistent and unlikely to be accountable for all cases of CQR (Price *et al*, 1999). *Pfmdr*1 copy number and the expression of Pgh1 are closely correlated with resistance to mefloquine rather than to CQR in field isolates and drug-pressured laboratory cell lines (Price *et al*, 1999; Nelson *et al*, 2005).

Recent studies by Johnson *et al* (2008), however, have presented novel evidence that pre-treatment with phenobarbitone can elicit decreased susceptibility to CQ in both CQ-resistant and CQ-sensitive parasite lines and that this is associated with the increased expression of the drug transporter Pgh1. The proximal promoter regions from both *pfmdr* and *pfcrt* were investigated and numerous putative binding sites for nuclear receptors with sequence similarities to regions known to be activated by phenobarbitone in mammals were identified. These findings raise the novel theory that nuclear-receptor-mediated responses to drug exposure might be a method of gene regulation in *P. falciparum.*

1.6. Non- Antibiotics with Antimalarial Activity

The antimicrobial activity of synthetic, non- chemotherapeutic compounds such as phenothiazine and methylene blue has been known since the time of German scientist, Paul Ehrlich (1854-1915). A variety of compounds, which are employed in the management of pathological conditions of a non-infectious aetiology have displayed broad spectrum antimicrobial activity against bacteria and other micro-organisms. Drugs that are neither antibiotic nor antimicrobial chemotherapeutic agents but which possess antimicrobial properties are termed non-antibiotics (Kristiansen and Amaral, 1997). It has been noted from numerous studies that phenothiazines, thioxanthenes, other neuroleptics, antihistamines (AHs), and some cardiovascular agents are among many drugs capable of inhibiting diverse classes of microbes at different dosing levels (Amaral *et al*, 2001; Kristiansen and Amaral, 1997).

Similarly, many studies have identified compounds ranging from diverse pharmacological and structural classes to possess intrinsic antimalarial activity.

The antimalarial properties of phenothiazines have been well- established. The concentrations required for promoting these effects are clinically feasible and non-toxic. However, the use of phenothiazines has not materialised as antimalarials due to their ability to produce serious side effects (Amaral *et al*, 2001).

Weisman *et al* (2006) employed high throughput screening to a library of known drugs, which indicated that two drugs, namely the anti-arrhythmic propafenone and the antipsychotic thioridazine, exhibit growth inhibition activity against *P. falciparum* in the same concentration range as exhibited in patient serum levels at therapeutic dosing for their indicated uses.

The effects of a series of bisphosphonates on the inhibition of growth of *P. falciparum in vitro* were studied. The most active compounds were n-alkyl bisphosphonates. Five compounds were selected for *in vivo* investigation in a *Plasmodium berghei* mouse suppressive test. The most active compound caused an 80% reduction in parasitaemia with no overt toxicity (Martin *et al*, 2001; Ghosh *et al*, 2004).

A recent study demonstrated that anti-arthritic gold (I) drug; Auranofin causes a strong and nearly complete inhibition of *P. falciparum* growth *in vitro*, at very low concentrations (Sannella *et al*, 2008).

1.6.1 Intrinsic Antimalarial Activity of Antihistamines

An early study on the antimalarial activity of five antihistaminic compounds against *P. falciparum in vitro* and *in vivo,* demonstrated that cyproheptadine, ketotifen, pizotyline, loratadine and azatadine possessed inherent antimalarial activity *in vitro* against a CQS strain. Cyproheptadine, pizotyline and loratadine were slightly more active in the CQR strain *in vitro*, while ketotifen and azatadine were significantly less active. The *in vivo* results established that cyproheptadine, ketotifen and pizotyline were active against the CQS strain while the activity of these compounds was enhanced against the CQR strain (Peters *et al*, 1990).

Singh and Puri (1998) evaluated five tricyclic AHs for prophylactic activity against *Plasmodium yoelii nigeriensis* infection in mice. Treatment with cyproheptadine, ketotifen and terfenadine completely prevented the establishment of patent infection in mice while partial activity was noted for azatadine and loratadine as there was a marginal delay in the development of patent infection.

A clinical drug library screen identified astemizole and its principle human metabolite desmethylastemizole as potent inhibitors of *P. falciparum* at sub-micromolar concentrations in both CQS and CQR parasites, showing efficacy *in vitro* and in two mouse models. Furthermore,

experimental evidence suggested that astemizole and desmethylastemizole inhibit haem crystallization, and co-purify with haemozoin within the *P. falciparum* food vacuole in both CQR and CQS parasites. However, in 1999 astemizole was voluntarily withdrawn from the United States and Europe after decreased sales due to warnings about its safety, yet it is currently sold in generic form in over 30 countries, including Cambodia, Thailand and Vietnam, which are malaria endemic (Chong *et al*, 2006).

1.7 Quinoline Resistance Reversal

It is well established that certain compounds have the ability to restore the anti-neoplastic effect in MDR cancer cells (Martin *et al*, 1987; Martiney *et al*, 1995). This phenomenon was suggested as a possible mechanism to explain *P. falciparum* resistance to CQ.

1.7.1 Calcium Channel Blockers

The modulation of CQ resistance with calcium channel blocker, verapamil was first reported in 1987 (Martin *et al*, 1987). Since the discovery of the chemosensitizing potential of verapamil, several structurally unrelated compounds have also demonstrated resistance reversal potential. Other cardiovascular agents, namely diltiazem, nifedipine, nicardipine, amlodipine and verapamil analogues were shown to restore CQ sensitivity in CQR *P. falciparum* strains (Adovelande *et al*, 1998; Menezes *et al*, 2003). Studies in *Aotus* monkeys demonstrated that verapamil did not reverse CQ resistance, but this could be due to pharmacokinetics or drug metabolism in the host. The cardiovascular side effects of verapamil in the concentration range utilized by Martin *et al* (1987) and the mortality observed in the *Aotus* monkeys may also have militated against the use of verapamil (Kyle *et al*, 1993). The non-classic calcium channel blockers, fantofarone was combined with verapamil, and have shown that these two agents act synergistically as a significant lowering of the required verapamil concentration was observed (Adovelande *et al*, 1998).

1.7.2 Tricyclic Antidepressants

Tricyclic antidepressants were also identified as modulating agents in CQR *P. falciparum,* both *in vitro* and *in vivo*. Desipramine and imipramine reversed CQ resistance *in vitro* at concentrations observed in the plasma of patients using antidepressants. Furthermore, the parasitaemias of owl monkeys treated with CQ-desipramine were considerably suppressed (Bitonti *et al*, 1988; Menenzes *et al*, 1997). However, desipramine did not enhance efficacy of CQ in clinical trials, possibly due to the ability of this drug to bind to plasma proteins, in particular to the α_1- acid glycoprotein, highly present in malaria patients (Boulter *et al*, 1993). Promising results were observed in a study that examined the efficacy of imipramine-CQ combination and its bioavailability among Sudanese patients with CQR *P. falciparum* infection, with mild to moderate side effects. They attributed these results to variation of plasma proteins among malaria patients (Adam *et al*, 2004).

Fluoxetine, a serotonin re-uptake inhibitor, was able to modify CQ resistance *in vitro*; however, possible toxicity precludes clinical utility (Gerena *et al*, 1992). Additionally, citalopram, which is an extremely potent inhibitor of neuronal serotonin reuptake, enhanced the activity of CQ in resistant *plasmodium in vitro* and *in vivo*, and exhibited minimal toxicity when compared to other psychotropic drugs. Detailed toxicology and pharmacokinetic studies are still required (Evans *et al*, 1998).

The *vitro* modulating effect of antidepressant drugs was considered in terms of its particular re-uptake blocking effect in relation to its ability to increase CQ accumulation, as well as to increase CQ sensitivity when resistant parasites are incubated in the presence of antidepressants. Amitripyline reversed CQ resistance at doses comparable to steady state serum levels reported in patients undergoing treatment (Taylor *et al*, 2000).

1.7.3 Phenothiazines (Antipsychotic Drugs)

Antipsychotic agents, specifically phenothiazine drugs: chlorpromazine, trifluoperazine, prochlorperazine, methotrimeprazine, and fluphenazine have demonstrated *in vitro* resistance reversal of CQ (Basco and Le Bras, 1992; Kalkanidis *et al*, 2002; Menenzes *et al*, 2002). Combinations of chlorpromazine or prochlorperazine with CQ reversed resistance as was evident by cures obtained in *Aotus* monkeys infected with CQR *P. falciparum.* However, the doses required to obtain these results are unwarranted for human indication (Kyle *et al*, 1993). Notably, phenothiazine drugs would reverse the CQR South American parasites with a lesser degree of susceptibility, compared to the African and Southeast Asian isolates as was the case with verapamil (Menezes *et al*, 2002).

1.8. Synergism between Antihistamines and Quinolines

AHs are of clinical significance because they are commonly co-prescribed with CQ, to relieve CQ-induced pruritis and as an anti-emetic (Oduola *et al*, 1998; Fehintola *et al*, 2004). Various studies have correlated increased levels of histamine with disease severity in *P. falciparum* infection. The use of AHs has also been suggested as preventative therapy to reduce the risk of progression to severe malaria (Beghdadi *et al*, 2008 and 2009).

Several studies have confirmed that tricyclic histamine (H_1) receptor antagonists reverse CQ resistance in *P. falciparum in vitro* and *in vivo.* Antihistaminic agents, namely ketotifen, pizotyline, azatadine and cyproheptadine, reversed CQ resistance in mice infected with *P. yoelii ssp*. and in *P. falciparum in vitro* (Peters *et al*, 1990; Valecha *et al*, 1992). Cyproheptadine and ketotifen significantly potentiated the efficacy of halofantrine against halofantrine resistant *P. yoelli nigeriensis* (Singh and Puri, 2000a). A similar study suggested that cyproheptadine in combination with CQ produced a curative response in CQR *P. yoelli nigeriensis*, while ketotifen, azatadine, and pheniramine produced a moderate effect in combination with CQ (Singh and Puri, 2000b). However, cyproheptadine was not effective in reversing CQ resistance in Aotus monkeys as probable drug toxicity was observed (Kyle *et al*, 1993).

Agrawal *et al* (2002) investigated the effect of cyproheptadine, on the formation of haemozoin by MDR *P. yoelii*, both *in vitro* and *in vivo.* The data suggests that cyproheptadine inhibited haem polymerization (enzymatic reaction involving haem polymerase) activity in the cell-free parasite extract and cause a significant decrease in intact erythrocytes. Disruption of the haem detoxification process by cyproheptadine was also confirmed *in vivo.* Notably, the dose of cyproheptadine used in this study was higher than that of the therapeutic range.

The use of chlorpheniramine, another tricyclic AH, in combination with CQ has demonstrated promising results in clinical studies to reverse CQR in humans. A clinical trial in Nigeria evaluated the efficacy of CQ-chlorpheniramine combination in 98 children with acute uncomplicated *P. falciparum*. The cure rate on day 14 was 81% among those children receiving CQ-chlorpheniramine (Sowunmi and Oduola, 1997). Another study compared the pharmacokinetics of CQ when given alone or concomitantly with chlorpheniramine to Nigerian children with *P. falciparum* malaria. Whole-blood CQ concentrations were monitored by means of high-performance liquid chromatography (HPLC) analysis of blood dried on filter paper. Pharmacokinetic analysis suggested that the peak whole-blood CQ concentration (C_{max}) and the area under the first-moment drug-concentration-time curve (AUMC) were notably increased in the presence of chlorpheniramine. The time to achieve the peak (T_{max}) was reduced when administered with chlorpheniramine. A shorter parasite clearance time and higher cure rate was observed with CQ- chlorpheniramine combination when compared to treatment with CQ alone, which provides additional data on the concomitant use of both these drugs (Okonkwo *et al*, 1999).

Chlorpheniramine also demonstrated potentiation with mefloquine, quinine and pyronaridine in addition to CQ as previously established, against a MDR *P. falciparum* strain and a CQR *P. falciparum* clone (Nakornchai and Konthiang, 2006).

A combination of promethazine and CQ was analysed *in vitro* against several CQR strains, in the *P. falciparum Aotus* monkey model and in bioassays from volunteers given promethazine. The results from this study indicate that promethazine is a potent modulator of CQR both *in vitro* and *in vivo*. Significant reduction in IC_{50} was found with plasma samples obtained from volunteers, 3-4 hours after administration of promethazine. Consequently, promethazine has the potential to reach plasma levels that enhance the effect of CQ (Oduola *et al*, 1998).

A recent study compared the pharmacokinetic interactions between CQ- chlorpheniramine and CQ-promethazine in 15 healthy volunteers. Chlorpheniramine demonstrated a statistically significant increase in the erythrocytic accumulation of CQ, and in the bioavailability and half life of erythrocytic CQ. Promethazine, on the other hand, did not possess any significant effect although this may be due to the small number of volunteers or host related factors (Gbotosho *et al*, 2008).

The utility of chlorpheniramine can be extended to include potentiation of amodiaquine in amodiaquine resistant parasites harbouring mutant *pfcrtT76* and *pfmdr1Y86* alleles. Five children who failed CQ and/or amodiaquine treatment were successfully retreated with concomitant use of chlorpheniramine and amodiaquine (Sowunmi *et al*, 2007).

Astemizole displayed an additive effect when combined with CQ, quinidine and artemisinin in both CQS and CQR strains (Chong *et al*, 2006).

With the exception of chlorpheniramine, resistance reversal agents have not been used clinically due to the requirement of higher concentrations than established therapeutic doses, mainly attributable to serum α_1-acid glycoprotein binding.

1.9 Structural link to Antimalarial Activity

The antimalarial activity and inhibition of the formation of ß- haematin by phenothiazine compounds were investigated by synthesizing a series of novel chlorpromazine analogues. The increase in antimalarial activity of certain phenothiazine analogues were correlated to an increase in the basicity of the alkyl amino side chains which ultimately may increase uptake into the acidic parasitic digestive food vacuole. Compounds which inhibited ß-haematin formation possessed alkyl amino side chains linked to the ring nitrogen. The most potent analogue had a branched tribasic side chain substitution and was reported to be 100 times better in activity than that of chlorpromazine against a CQS strain (Kalkanidis *et al*, 2002).

1.10. Structural link to Resistance and Resistance reversal

Despite the functional and structural assortment of the resistance reversal agents discussed above, most of them have common structural characteristics. Lipid solubility, two planar aromatic rings, a cationic charge and tertiary nitrogen were found to be important features (Gerena *et al*., 1992). Loratadine is an AH that is structurally related to azatadine, cyproheptadine, and pizotyline but it was reported that loratadine lacks *in vitro* (Peters *et al*, 1990) and *in vivo* (Singh and Puri, 2000) resistance modulating effects against *P. falciparum* and *P. yoelli*, respectively. Loratadine has the N atom as part of a non-basic carbamate group instead of a basic amine group, and consequently the charge at this proximal amine group is significant as can be exemplified by loratadine lacking significant resistance reversal properties.

Cyclosporine A, ivermectin and progesterone have no effect on reversing CQR in *P. falciparum* (Van Schalkwyk *et al*, 2001) but they are very effective resistance reversal agents in mammalian- P-glycoprotein (Pouliot *et al*, 1997). Therefore, it is apparent that certain structural features are a requisite for CQR reversal.

Several studies have aimed to highlight the structure-activity relationships of resistance reversal agents. A study comparing the abilities of tricyclic imipramine and its analogues suggested that the direction of the dipole moment vector was essential for CQR reversal properties. Their analysis demonstrated that the calculated dipole moment vectors directed towards the aromatic ring or the nitrogen atom of the tricyclic ring in certain active analogues emphasize the critical function of the aromatic ring in relation to resistance reversal activity (Bhattacharjee *et al*, 2001).

Another study by Bhattacharjee *et al* (2002) expressed the generation of a dependable chemical function-based 3D-QSAR model for CQR reversal by comparing the tricyclic compounds, imipramine and desipramine, as well as 15 of their analogues. Several quantitative structure-activity relationship studies to determine the role of calculated stereo-electronic properties in aid of elucidating a pharmacophoric model for CQR reversal was performed. The results conclude that two aromatic hydrophobic sites and a hydrogen bond acceptor site, preferably at a side chain nitrogen atom is fundamental for resistance reversal (See Figure 1.3). Nine of the eleven CQR reversal agents from a group of structurally diverse compounds including cyproheptadine, ketotifen, azatadine, promethazine and chlorpheniramine, display the pharmacophore as illustrated on the 3D QSAR pharmacophore model. Electronic properties, specifically; frontier orbital energies and the intrinsic basicity of the nitrogen atom in the tricyclic heterocycle can be associated with CQR reversal. Structure-activity relationships accentuated lipophilicity and density of the molecule with significant resistance reversal properties

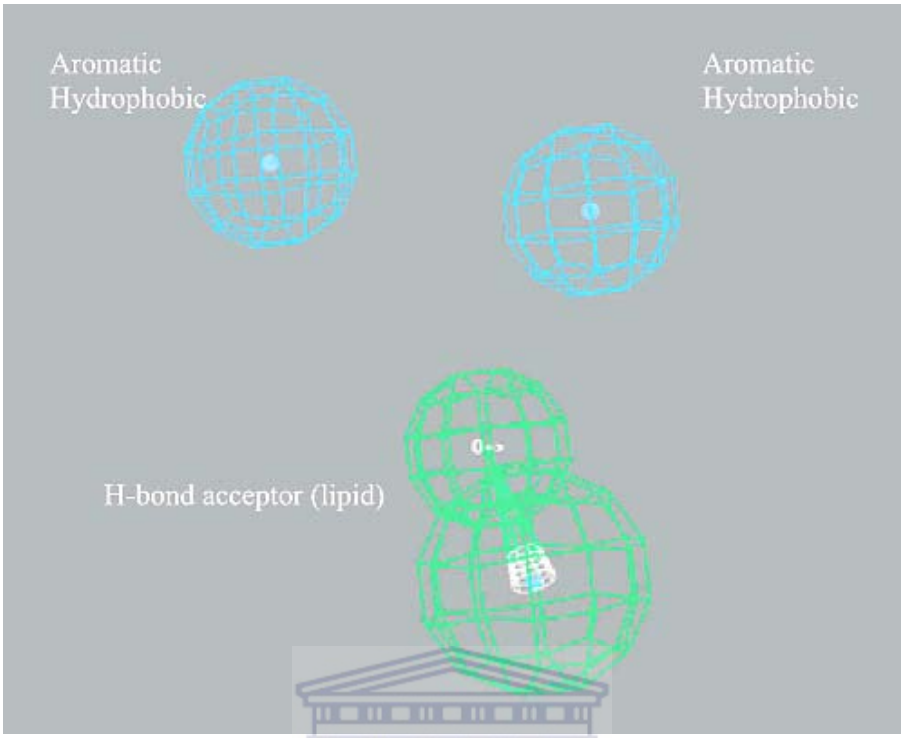


Figure.1.3: Pharmacophore model for CQ modulating agents based on that proposed by Bhattacharjee *et al*, (2002)

Structure-activity relationship studies on a series of synthetic compounds capable of modulating CQR in *P. falciparum* were investigated. Four amine ring systems, specifically phenothiazine, iminodibenzyl, iminostilbene, and diphenylamine's were prepared to study the effect of a polycyclic aromatic nucleus on resistance reversal activity. Phenothiazine analogues displayed the best resistance modulation in comparison to alternate ring systems. The length of the alkyl bridge is an important consideration as increasing the side chain from 3 carbons to 4-6 carbons enhances resistance reversal, with a 4 carbon side chain exhibiting potent activity. Steric tolerance was also explored by adding cyclic and non cyclic aliphatic amines. The bulky substituents at the side chain amino group resulted in a loss of activity. Conclusively, this study has highlighted 3 structural features of a compound that demonstrated superior chemosensitizing activity in comparison to verapamil. These features include a phenothiazine ring, a pyrrolidinyl group joined by a four carbon alkyl bridge (Guan *et al*, 2002).

Further insight into the structure activity relationships of resistance reversers was presented by investigating the effects of a series of dihydroethano-and ethenoanthracene derivatives on CQ accumulation in CQS and CQR strains. Synthetic 9, 10-dihydroethanoanthracene derivatives have been shown to reverse resistance to CQ in several strains and isolates.

The nature of the basic group was linked to an increase in CQ accumulation and an amino group was a prevalent structural feature in many derivatives that successfully induced CQ accumulation (Pradines *et al*, 2002).

A corresponding study by Alibert *et al* (2002) explored structure-activity relationships and molecular modeling studies of dihydroanthracene derivatives in an attempt to delineate a pharmacophoric moiety for resistance reversal. Owing to the fact that maprotiline (antidepressant that is chemically similar to tricyclic antidepressants) has shown anti-MDR activity on cancer cells and *P. falciparum*, a novel set of rigid 9, 10-dihydro-9, 10-ethano or ethenoanthracene (DEEA) derivatives were synthesized. Structural attributes, namely, an amino group, the nature of the group associated with the amino group, the chain length carrying these functions, and the presence of an ethano bridge vs. that of an etheno bridge were significant for resistance reversal. The resistance modulation effect was also reliant on their possession of protonatable nitrogen at physiological pH. Lipophilicity was correlated to reversal activity as the calculated log*D* values of the active compounds ranged between -1.50 and 2.61 whereas a compound having a log*D* value lower than -1.5 lacked resistance reversal capability.

1.11 Quinoline based Hybrid Drugs

Recent studies have highlighted the advantage of combining two drugs into a single hybrid molecule; combining CQ with resistance reversing agents and astemizole.

An innovative class of such hybrid molecules was synthesized by Burgess *et al* (2006). A molecule that combined a 4-amino-7-chloroquinolone nucleus with a resistance reversal group, based on imipramine, (Figure.1.4) displayed potent activity against both CQS and CQR strains of *P. falciparum in vitro* and in *P. chabaudi* in mice with no obvious signs of toxicity. However, this prototype molecule is too hydrophobic to have adequate oral bioavailability in humans. These hybrid molecules were termed "reversed CQs" (RCQ's) and they present a strategy that may prove viable upon further detailed studies

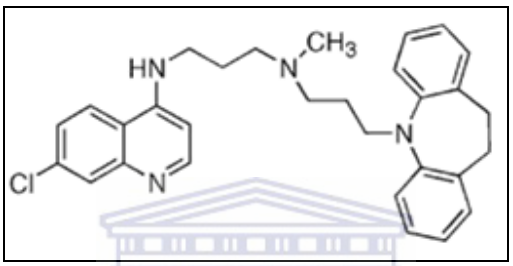


Figure.1.4: Prototype hybrid model for CQ modulating agents based on that proposed by Burgess *et al*, (2006)

Musonda *et al* (2009) conducted a hybridization study that combined the core portions of two structurally distinct moieties of CQ and astemizole, via an appropriate linker, into a single molecule. Some of these hybrid compounds were up to 10-fold more active than CQ against CQR *P. falciparum in vitro* however this effect was not observed *in vivo*. Further work in this area was suggested to improve drug-like properties of hybrid compounds.

Recently, a study conducted by Andrews *et al* (2010), has shown that linking several resistance reversal-like moieties to a 4-amino-7-chloroquinoline core may prove to be advantageous against CQS and CQR strains, depending on linker and reversal agent modifications. This study aimed to further the concept of synthesizing a "reversed CQ molecule" presented by Burgess *et al* (2006).

1.12 Histamine and Antihistamines

Histamine, a ß-imidazole-ethylamine is a low-molecular-weight amine synthesized from l-histidine exclusively by histidine decarboxylase, an enzyme that is expressed in cells throughout the body, including central nervous system neurons, gastric-mucosa parietal cells, mast cells, and basophils. Histamine has an important role in human health, exerting its diverse biologic effects through four types of receptors. Histamine is a chemical mediator participating in many cell physiology processes, among them allergic reactions, inflammation, gastric acid secretion and -probably- central and peripheral neurotransmission (Simons, 2004). The four major types of histamine receptors, H_1, H_2, H_3, and H_4-receptors, differ in their expression, signal transduction, and function. H_1 and H_2 receptors are widely expressed, in contrast to H_3 and H_4 receptors (Nunes, 2006).

AHs are described according to the histamine receptor with which they interact. AHs consist of a diverse class of pharmacological agents that encompass first generation and second generation H_1 receptor inverse agonists, which are used in the treatment of allergy and inflammatory disorders. H_2 inverse agonists act primarily on gastric mucosa, inhibiting gastric acid secretion, and are consequently being used for the treatment of gastrointestinal and related disorders (Neslson, 2007; Simons, 2004). Newer, experimental H_3 receptor antagonists and H_4 receptor antagonists do not have a defined clinical use, although a number of drugs are in clinical trials. H_3 receptors are presynaptic auto and hetero-receptors that control the release of histamine and other neurotransmitters in the brain. H_3-antagonists have a stimulant action and are said to have a nootropic effect. Therefore their role in Alzheimer's disease, schizophrenia, obesity, epilepsy, sleep disorders and Attention deficit hyperactivity disorder (ADHD) are being investigated. H_4 receptors have been identified on hematopoietic cells; therefore H_4 antagonists appear to have an immuno-modulatory role and are being investigated as possible anti- inflammatory and immuno-modulatory agents (Nelson, 2007).

H_1 and H_2 AHs have been redefined as inverse agonists that combine with and stabilize the inactive form of the H_1-receptor, shifting the equilibrium toward the inactive state (Simons, 2004).

H_1 (first generation) AHs have been classified into six chemical groups: the ethanolamines, ethylenediamines, alkylamines, piperazines, piperidines, and phenothiazines (Simons, 2004). The classification according to function of first-generation H_1 -AHs, which are sedating, as compared with second-generation compounds, which are relatively non-sedating, is now more commonly used. The second generation H_1 AHs does not penetrate the blood-brain barrier significantly due to their amphoteric nature (Nelson, 2007).

1.13 General Objectives of this Study

An approach to antimalarial chemotherapy that has been explored is to identify agents that are developed or marketed as treatments for other diseases. Considering the difficulties of funding antimalarial drug discovery, the advantage of these compounds is that whatever their mechanism of action, they have an established safety profile. Consequently it will be inexpensive to develop them as antimalarials, as this does not necessitate research and development costs. Furthermore, this approach will be of greater economic feasibility, after patents have expired. Therefore the first objective of this study was to:

1.13.1 Screen mainly off-patent (generic) Antihistamines for antimalarial activity.

The comparative efficacy of a total of 24 antihistamines, representing Histamine$_1$ (H_1), Histamine$_2$ (H_2), and Histamine$_3$ (H_3) receptor antagonists, will be evaluated against CQ-sensitive (CQS) and CQ- resistant (CQR) strains of *Plasmodium falciparum.*

Combining previously effective agents with compounds that reverse parasite resistance to these agents offers another approach to chemotherapy. It is evident that many drugs, particularly antihistamines, show promise in resistance reversal. The second objective of this study was therefore to:

1.13.2 Investigate if synergistic interactions could occur between antihistamines and CQ that may enhance the activity of CQ in the CQS and CQR strain. Hence, while CQ appears to have failed as a first line antimalarial, it may be revived by a combination with an effective resistance modulator.

Progress towards characterization of the parasite's biology and morphology, in addition to determining the mechanism by which antihistamines exert their antimalarial effects are essential for the process of developing new therapeutic alternatives as well as optimization of lead compounds. Consequently, the third objective of this study was to:

1.13.3 Study morphological changes in the parasite induced by active antihistamines and to determine the effect of active antihistamines on haemoglobin uptake by the parasite using immunofluorescence microscopy and Western blot analysis.

Chapter2

Intrinsic Antimalarial Activity of Antihistamines on Chloroquine Sensitive and Chloroquine Resistant Strains of *Plasmodium falciparum*

2.1 Introduction

The intrinsic antimalarial effect of AHs was explored previously both *in vitro* and *in vivo.* Results published by several investigators suggest that the tricyclic AHs possess inherent antimalarial activity. These investigations clearly demonstrated that AHs such as cyproheptadine, ketotifen, pizotyline, loratadine, azatadine and terfenadine somewhat inhibited the *plasmodium* parasite (Peters *et al*, 1990; Singh and Puri, 1998). More recent studies demonstrated that astemizole could be a viable option in the fight against malaria (Chong *et al*, 2006). These studies provide clear evidence that screening existing drugs for unknown activities could have many feasible outcomes.

This chapter will determine and compare the intrinsic antimalarial activity of AHs in a CQS strain and a CQR strain. In doing so, a clear comparison of the differential sensitivity of CQS or CQR *P. falciparum* strains to various classes of AHs can be deduced. Given that AHs can be categorized into various structural and hence pharmacological subclasses, each possessing different physicochemical properties it may be possible to link the antimalarial activity to a specific structural class of AHs.

2.2 Materials and Methods

2.2.1 Drugs used in this Study

All the compounds were dissolved in the solvents described in Table 2.1 and stored as stipulated, either at ambient temperature or 2-8ºC. For experiments with parasites the compounds were dissolved in methanol, dimethyl sulfoxide (DMSO) or millipore water and diluted until the solvent concentration was less than 1% which is non-toxic to the parasites.

2.2.2. In vitro *Plasmodium falciparum* culture

Two strains were selected for experimental work: D10, a CQS strain and DD2, a CQR strain. The *plasmodium* parasites were cultured using a modified Trager and Jenson method (1976). The parasites were maintained in RPMI 1640 culture medium (Biowhittaker) supplemented with 25mM sodium bicarbonate, gentamycin sulphate (50µg/l), 22mM glucose, and 25mM HEPES (Hydroxyethane Piperazine Sulphonic Acid), 5.0g/l Albumax II and 323µM hypoxanthine. The cells were routinely maintained at 2% haematocrit (hc) and between 5% - 10% parasitaemia (pst). The culture medium was changed daily and cells were fed with O^+ human erythrocytes (Western Province Blood Transfusion Service) in the trophozoite stage. The cells were stored in sealed 50ml flasks under a gas mixture of 3% O_2; 4% CO_2; 93% N_2. The flasks were kept in an incubator at 37ºC.

The parasites were viewed under oil immersions using Giemsa-stained thin blood smears. Briefly, Giemsa stain (Merck) was diluted 1:10 in phosphate-buffered saline (PBS). The thin blood smears were fixed onto a microscope slide with methanol and were exposed to the Giemsa stain for 10 minutes. The slide was washed briefly with water and dried before being examined under the microscope.

Parasitaemia was determined by counting the number of parasitised erythrocytes cells and expressed as a fraction of the total number of erythrocytes (parasitised + unparasitised).

2.2.3. Synchronization of *Plasmodium falciparum* Parasites

The parasite culture was synchronized in the ring stage using the method of Lambros and Vanderberg (1979). 15 ml of a 5% (w/v) D-sorbitol solution (37ºC) was added to the parasite pellet and left to stand for 10 minutes at 37ºC. The parasites were centrifuged and the supernatant removed before returning the parasites to the culture medium.

2.2.4. Parasite Lactate Dehydrogenase Assay for determining Parasite Viability

Culture-derived parasitised erythrocytes were mixed with fresh culture medium and erythrocytes to yield a 1% parasitaemia and 1% haematocrit suspension and distributed in 96 well microtitre plates. The blank for the assay was the unparasitised erythrocytes without drug and the control was the parasitized erythrocytes without drug. The experiment involved incubating the parasites in a volume of 200µl along with the particular drug at defined concentration of 100µg/ml. Each drug concentration was measured in quadruplicate. The parasites were incubated for 48 hours in the plate, in desiccator cabinets under the gas mixture as described previously.

The Lactate dehydrogenase activity is used as a measure of parasite viability. The Malstat™ reagent contains lactate to initiate the reaction and APAD (3-acetyl pyridine adenine dinucleotide) which the parasites selectively use as a cofactor in the enzymatic reaction instead of NAD. The NBT/PES is a solution containing Nitro Blue Tetrazolium (1.6mg/ml) and Phenazine Etho Sulphate (0.08mg/ml). As the reaction proceeds, the yellow tetrazolium ion is reduced to a purple formazan salt.

Once the 48 hours incubation is complete, 100µl of Malstat™ reagent and 25 µl of NBT/PES solution were added to each well of a new flat bottomed 96-well microtitre plate. The sample

was resuspended in each of the wells of the original plate and 15µl was taken from each well and added to the corresponding well of the Malstat™ plate, thus initiating the lactate dehydrogenase reaction. Colour development was monitored at 620nm as the reaction proceeded (Makler *et al*, 1993).The average absorbance of the blank (unparasitised erythrocytes) was subtracted from all the parasitised samples. The percentage viability was calculated by dividing the absorbance of the drug-exposed parasites by the absorbance of the control parasites (drug free) and multiplying this value by 100.

2.2.5. Dose–response Data Analysis

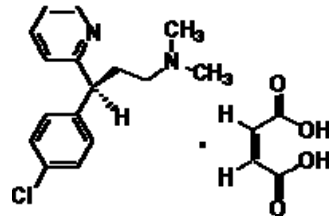
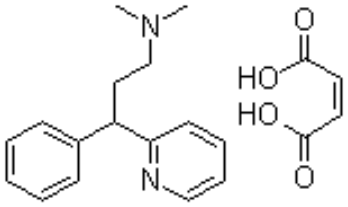
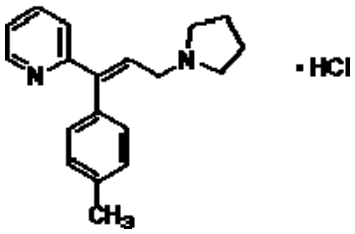
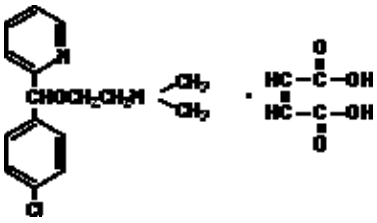
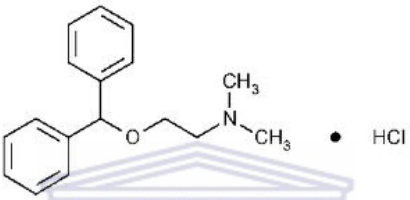
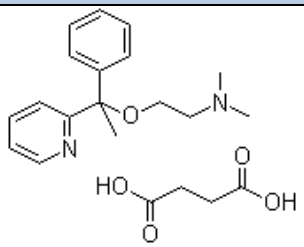
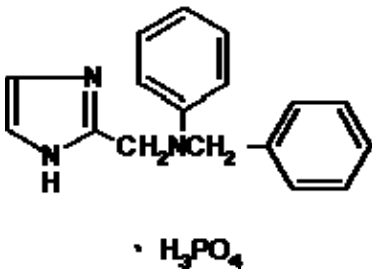
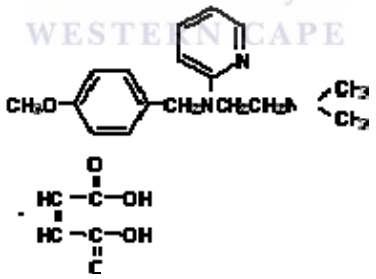
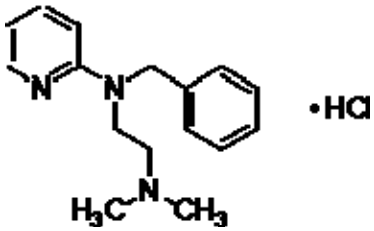
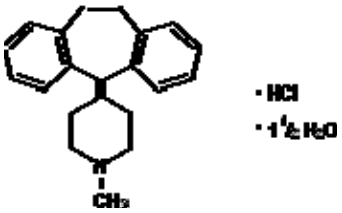
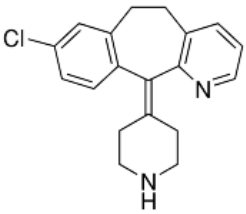
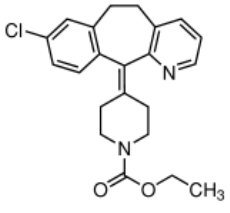
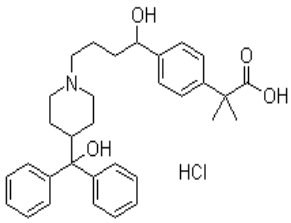
All dose–response data was analysed using Graphpad Prism version 4.01 for Windows (GraphPad Software, San Diego, California USA; www.graphpad.com). Absorbance values were converted to percentage parasite viability for the lactate dehydrogenase assay and non-linear regression (sigmoidal curve with variable slope) was performed on the dose–response data. Total growth was determined from control wells with no AH added and the concentration required to bring the curve down to a point half way between the top and bottom plateaus of the curve (IC_{50}) was calculated.

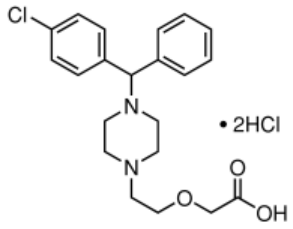
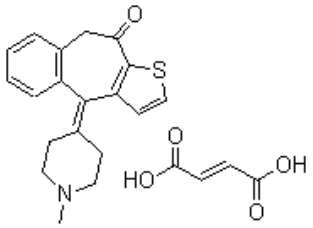
Table 2.1. Compounds used in this study along with their molecular weights, solvents and source.

Compound Name	Molecular Weight	Solvent	Source
H_1 receptor antagonists			
Chlorpheniramine maleate	274.788	water	Sigma
Pheniramine	240.3434	water	Adcock Ingram
Triprolidine	278.1783	water	Adcock Ingram
Carbinoxamine maleate	290.1186	water	Sigma
Diphenhydramine HCl	255.1623	water	Sigma
Doxylamine succinate	270.3694	water	Sigma

Pyrilamine maleate	285.384	water	Sigma
Tripelenamine HCl	255.358	water	Sigma
Anatazoline phosphate	363.348	water	Sigma
Cyproheptadine HCl	287.3981	methanol	Sigma
Cetirizine dihydrochloride	388.888	water	Sigma
Loratadine	382.883	methanol	Sigma
Desloratadine	310.821	methanol	Sigma
Ketotifen	309.425	DMSO	Sigma
Fexofenadine HCl	501.2879	methanol	Sigma
H_2 receptor antagonists			
Cimetidine	252.1157	methanol	Sigma
Ranitidine HCl	314.404	water	Sigma
Nizitadine	331.257	methanol	Sigma
Famotidine	337.445	DMSO	Sigma
H_3 receptor antagonists			
Ciproxifan HCl	270.3263	DMSO	Sigma
Thioperamide maleate	292.4428	DMSO	Sigma
3-(1H-imidazol-4-yl)propyl-di(p-fluorophenyl)methyl ether HCl	364.82	DMSO	Sigma
2-((3-trifluoromethyl)phenyl) histamine dimaleate	487.38	DMSO	Sigma

The chemical structures of all antihistamines used in this study are shown below.

Alkylamines		
		
Chlorpheniramine maleate	Pheniramine maleate	Triprolidine HCl
Ethanolamines		
		
Carboxamine maleate	Diphenhydramine HCl	Doxylamine succinate
Ethylenediamines		
		
Antazoline phosphate	Pyrilamine maleate	Tripelenamine HCl
Piperidines		
			
Cyproheptadine HCl sesquihydrate	Desloratadine	Loratadine	Fexofenadine HCl

Piperazines	Mast cell Stabilizer
 Cetirizine dihydrochloride	 Ketotifen fumarate
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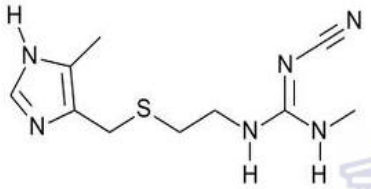
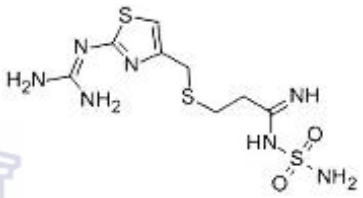
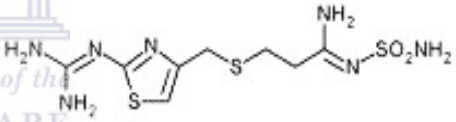
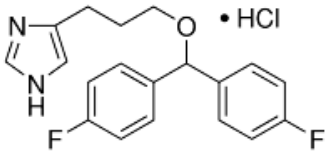
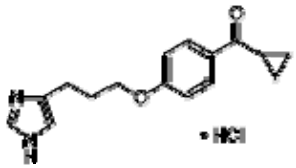
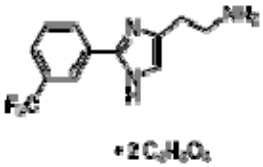
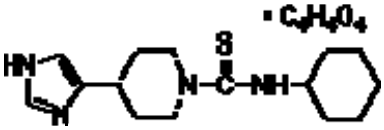
 Cimetidine	 Famotidine
 Nizatidine	 Ranitidine HCl
Chemical Structures of Histamine$_3$ receptor antagonists	
 3-(1H-Imidazol-4-yl)propyldi(p-fluorophenyl)methyl ether	 Ciproxifan hydrochloride
 2-((3Trifluoromethyl) phenyl) histamine dimaleate	 Thioperamide maleate

2.3 Results

Sensitive and resistant strains of *P. falciparum* were exposed to a wide concentration range of AHs to establish whether any of the drugs possess intrinsic antimalarial activity. The results are plotted as percentage viability of the parasites *versus* logarithm of the drug concentration in micro molar (µM). AHs with IC_{50} > 150 µM were not considered for antimalarial activity. Each experiment was performed in triplicate, and each drug concentration was tested in quadruplicate.

Table 2.2. IC_{50} values for sensitive and resistant strains of *P. falciparum*

Compound Tested	IC_{50} in (µM)	
	D10	**DD2**
Histamine$_1$ Receptor Antagonists		
CQ diphosphate	0.02067±0.007	0.1889±0.067
Chlorpheniramine maleate	65.58±3.66	60.48±0.082
Pheniramine maleate	>150	>150
Triprolidine hydrochloride	82.21±8.68	36.09±0.023
Carbinoxamine maleate	45.91±3.08	72.02±8.75
Diphenhydramine hydrochloride	52.59±3.01	103.56±11.11
Doxylamine succinate	>150	>150
Antazoline phosphate	33.14±0.14	33.51±1.46
Pyrilamine maleate	21.37±3.81	21.31±4.55
Tripelenamine hydrochloride	90.74±4.16	>150
Cyproheptadine hydrochloride	4.99±0.15	8.78±0.48
Ketotifen fumarate	6.748±0.24	63.02±1.55
Cetirizine dihydrochloride	55.21±0.38	56.29±3.16
Desloratadine	6.34±0.38	12.86±4.56
Fexofenadine hydrochloride	>150	>150
Loratadine	5.88±0.39	4.86±1.28

Table 2.2. IC_{50} values for sensitive and resistant strains of *P. falciparum* (cont.)

Histamine$_2$ Receptor Antagonists		
Cimetidine	>150	>150
Famotidine	>150	>150
Nizatidine	>150	>150
Ranitidine hydrochloride	>150	>150
Histamine3 Receptor Antagonists		
3-(1H-Imidazol-4-yl)propyldi(p-fluorophenyl)methylether hydrochloride	6.41±0.96	15.02±0.72
Ciproxifan hydrochloride	13.49±0.75	14.27±1.94
Thioperamide maleate	>150	>150
2-((3Trifluoromethyl)phenyl) histamine dimaleate	12.11±0.57	25.80±0.94

Table 2.2 shows the inherent antimalarial activities of the AHs tested. All the test values were far greater than the IC_{50} for CQ. The IC_{50} range for CQ was approximately 0.020-0.021 µM against the CQS strain 0.19- 0.20 µM for the CQR strain. Amongst the first generation of H_1 receptor antagonists, cyproheptadine and ketotifen (IC_{50} 5.0- 6.8 µM), were the most potent in the CQS strain. The 2nd generation H_1 receptor antagonist loratadine and its active metabolite, desloratadine, had the highest activity (IC_{50} 4.0-12 µM). None of the H_2 receptor antagonists had any antimalarial effects. Amongst the H_3 receptor antagonists three of the four compounds tested exhibited significant activity against both strains (IC_{50} 6.0-26 µM)

Dose response curves were plotted of the antimalarial activity of active AHs and CQ vs. the percentage viability of *P. falciparum*

Dose response curves of Antihistamines for D10 and DD2 plasmodial strains

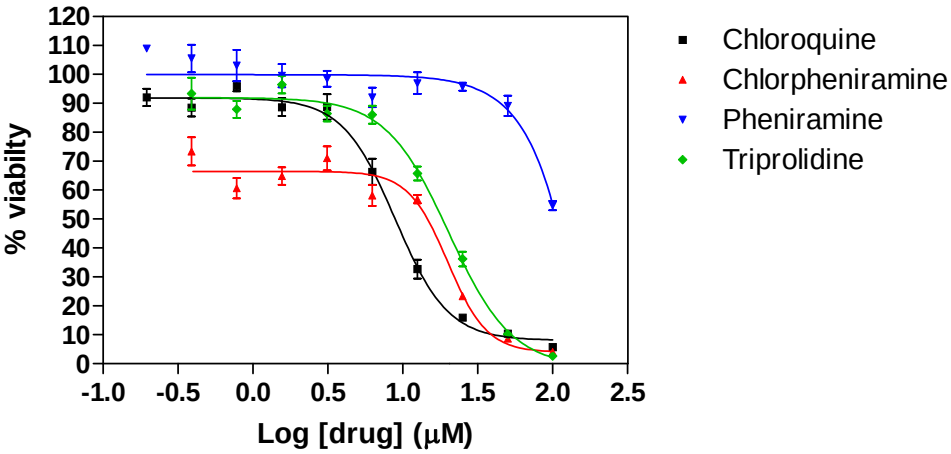


Fig. (2.1). Antimalarial effect of CQ and Alkylamines on D10

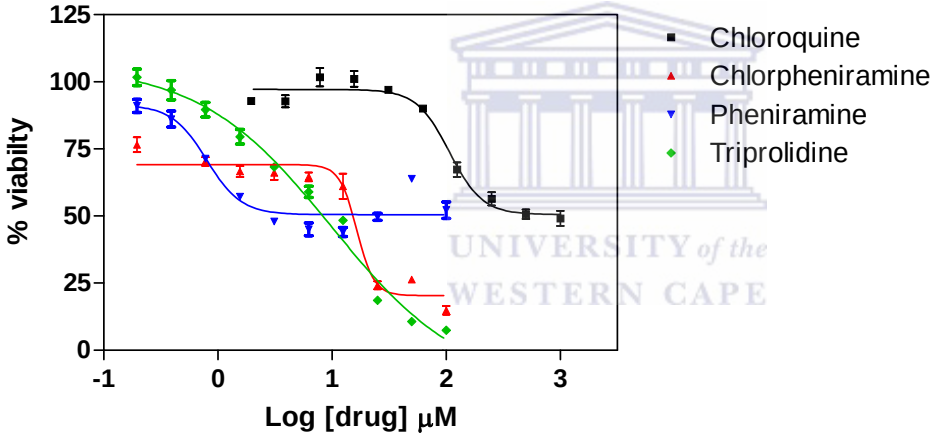


Fig. (2.2). Antimalarial effect of CQ and Alkylamines on DD2

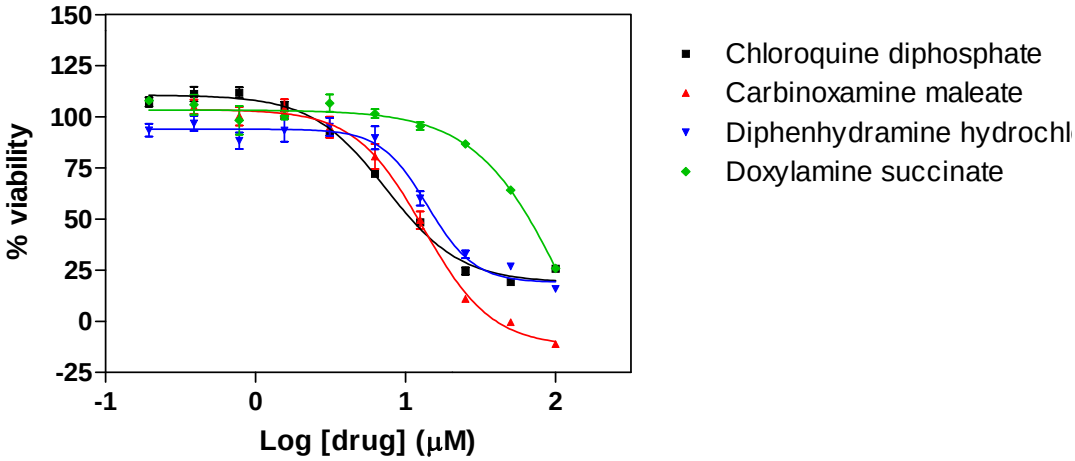


Fig. (2.3). Antimalarial effect of CQ and ethanolamines on D10

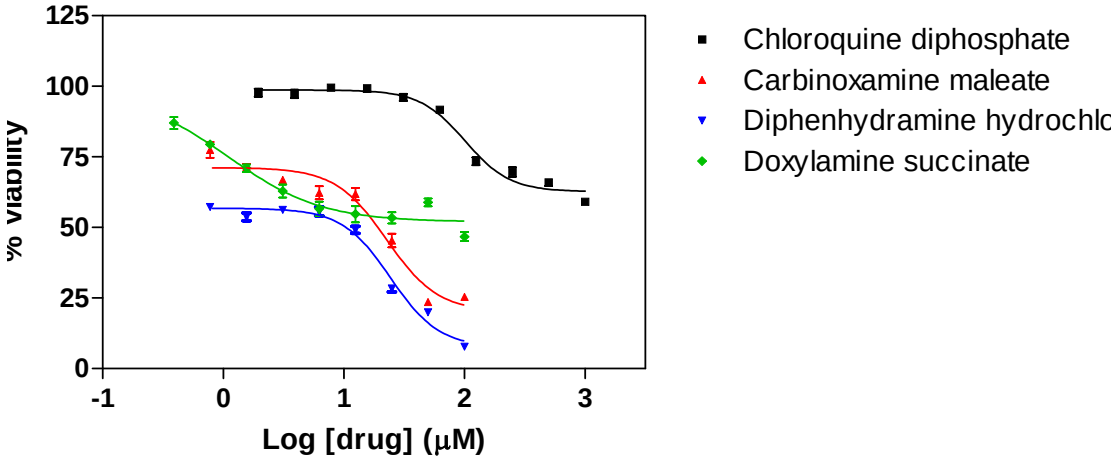


Fig. (2.4). Antimalarial effect of CQ and ethanolamines on DD2

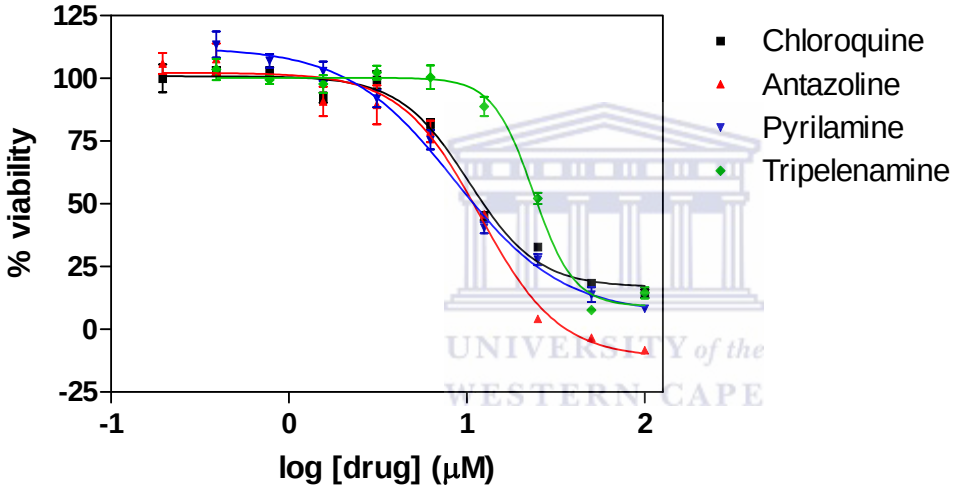


Fig. (2.5). Antimalarial effect of CQ and ethylenediamines on D10

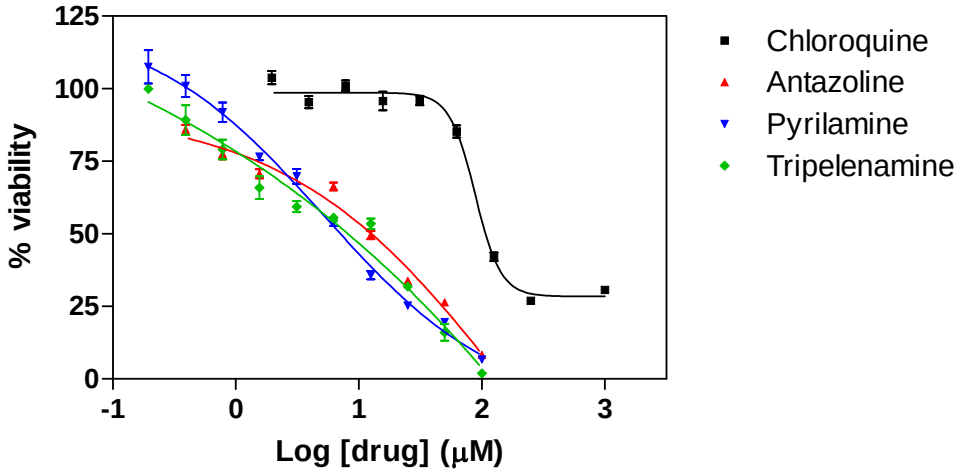


Fig. (2.6). Antimalarial effect of CQ and ethylenediamines on DD2

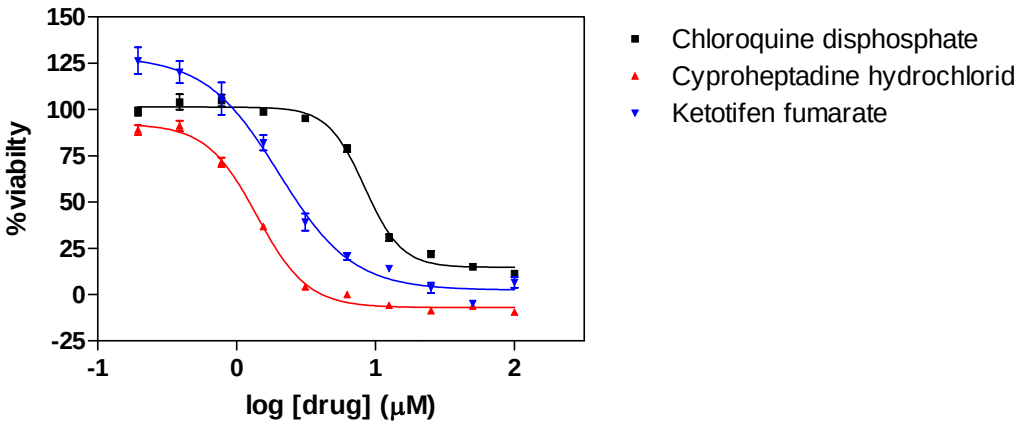


Fig. (2.7). Antimalarial effect of CQ, Cyproheptadine and Ketotifen on D10

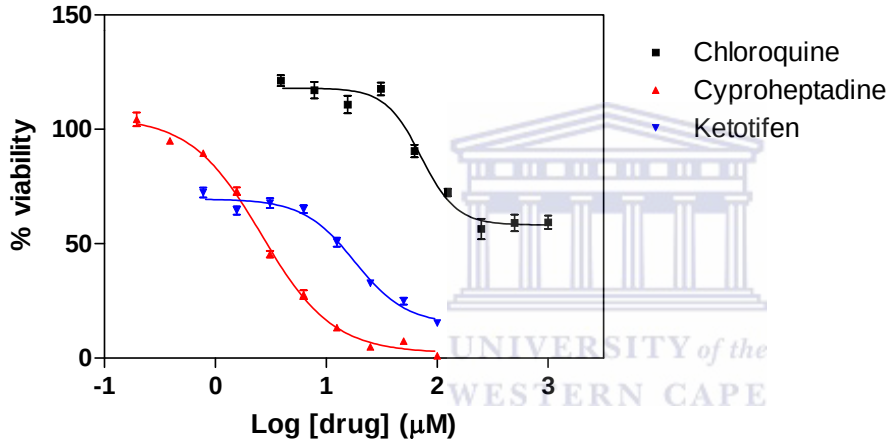


Fig. (2.8). Antimalarial effect of CQ, Cyproheptadine and Ketotifen on DD2

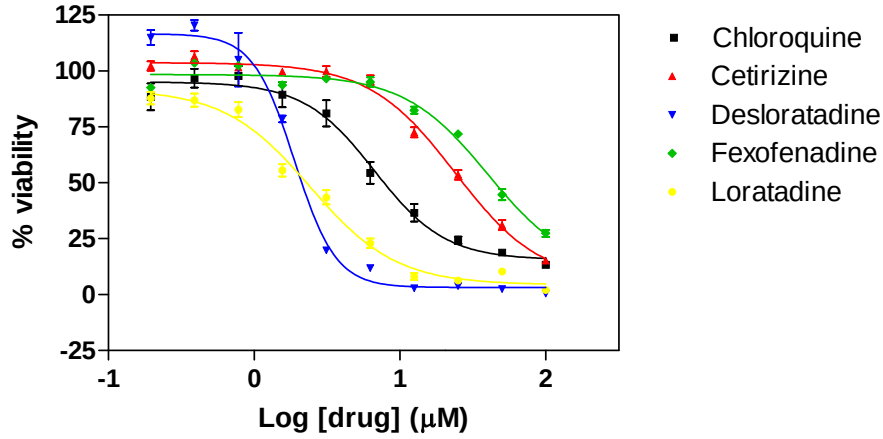


Fig. (2.9). Antimalarial effect of CQ and 2nd generation H_1 receptor antagonists on D10

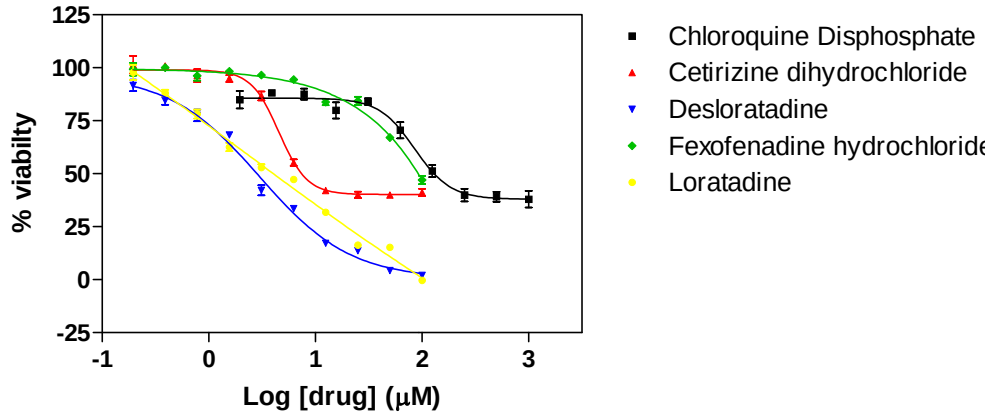


Fig. (2.10). Antimalarial effect of CQ and 2nd generation H_1 receptor antagonists on DD2

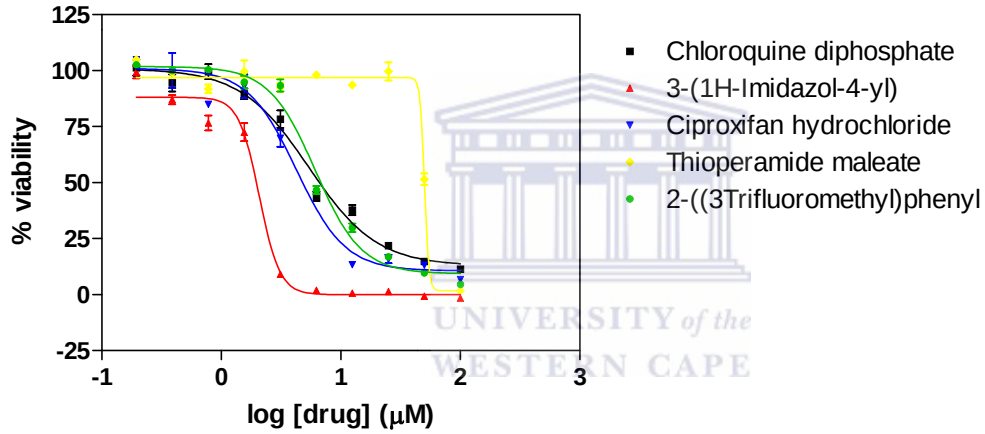


Fig. (2.11). Antimalarial effect of CQ and H_3 receptor antagonists on D10

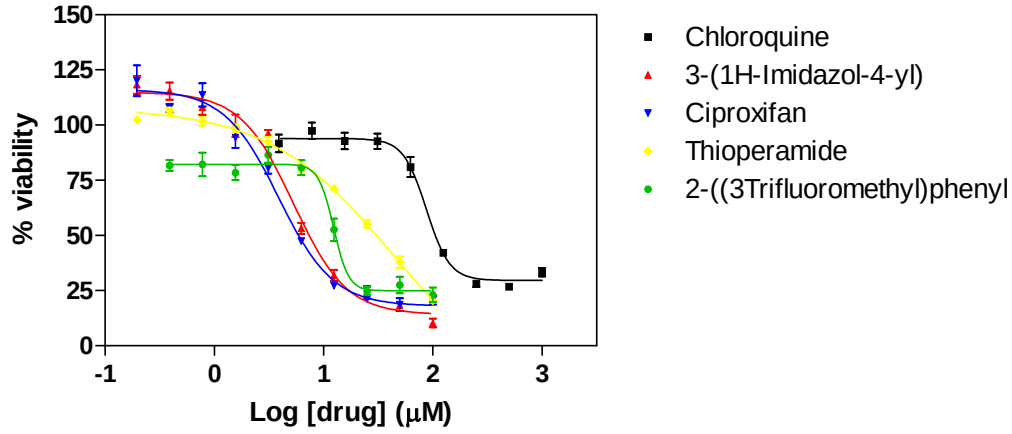


Fig. (2.12). Antimalarial effect of CQ and H_3 receptor antagonists on DD2

Table 2.3. Log *D* values of Antihistamines

Antihistamine	Log *D* at pH 7
Chlorpheniramine maleate	1.13
Triprolidine hydrochloride	2.36
Carbinoxamine maleate	1.12
Diphenhydramine hydrochloride	1.92
Cyproheptadine hydrochloride	4.93
Ketotifen fumarate	3.25
Cetirizine dihydrochloride	-0.02
Desloratadine	2.95
Fexofenadine hydrochloride	2.68
Loratadine	6.23
Cimetidine	-0.11
Famotidine	-3.02
Nizatidine	0.75
Ranitidine hydrochloride	-0.13

Log*D* values taken from Chemical Abstracts Service, American Chemical Society and were calculated by using Advanced Chemistry Development (ACD) Software Solaris V4.67.

2.3 Discussion

Twenty four AHs acting on 3 different histamine receptor subtypes were evaluated for antimalarial activity. The H_1 and H_3 receptor antagonists have been identified as agents that are able to inhibit the growth of the parasites. The IC_{50} range of AHs against the D10 and DD2 plasmodial strain is about 100-1000 times less active than that of CQ.

However, it is worthy to note that Menezes *et al* (1997, 2002, and 2003) tested a number of drugs against several newly discovered Brazilian CQR plasmodium isolates at concentrations that are used therapeutically. These agents have been typically defined as resistance reversers based on results from numerous studies. Imipramine, desipramine, phenothiazines and its analogues, as well as verapamil and other cardiovascular drugs were evaluated for antimalarial activity in addition to CQ resistance reversal. The results established that the drugs tested were

not able to reverse CQ resistance in Brazilian strains but possessed intrinsic antimalarial activity at concentrations similar to CQ against resistant parasites. Therefore, CQR Brazilian isolates appear to have a higher susceptibility to many different resistance reversal agents marketed for other indications. Thus, it is probable that the AHs presenting with low antimalarial activity in this study could also possess significantly lower IC_{50} values depending on the geographical phenotype of the CQR strain. The antimalarial activity of verapamil was previously reported at 10- 20μM (Adovelande *et al,* 1993*).* Contrastingly, Menezes *et al* (2003) reported IC_{50} values in the range of 0.034- 2.2 μM for the new CQR Brazilian isolates. In the context of this study, AHs exhibiting IC_{50} values lower than 4 μM may have significantly lower IC_{50} values against other strains, i.e., they may be more potent and have fewer side- or toxic effects because they are effective at lower concentrations.

AH sensitivity to *P. falciparum* appears to favour the D10 strain. The dose response curves indicate that 100% kill is not achieved in many of the AHs tested against the DD2 strain, even at high concentrations. This is evident from the sigmoidal nature of the dose response curves for the D10 strain, which indicates high potency in contrast to that for the DD2 resistant strain.

The antihistamines that possess intrinsic antimalarial activity belong to the ethylenediamine, ethanolamine and the alkylamine classes of H_1 receptor antagonists. The two aromatic groups linked through a short chain to a tertiary aliphatic amine are structural features of H_1 antihistamines. Correlations can therefore be proposed between the structure of H_1 antihistamines displaying an inhibitory effect and CQ. Structure–activity studies have given insight into the interaction between quinoline and free haem. The 4 aminoquinoline nucleus of CQ and other antimalarials are responsible for complexing free haem (Egan 2004). The aminoalkyl side chain of quinoline drugs is responsible for their antimalarial activity and change in the length of this side chain does not influence activity in the CQS strain however increases activity in a CQR strain (Egan *et al*, 2004). Therefore, the antimalarial action of some antihistamines may be due to interaction with free haem, thereby inhibiting haemozoin

formation. The lack of antimalarial activity of the H_2 receptor may be due to the lack of two aromatic systems.

The obvious structural similarity of the AHs that display the lowest IC_{50} is their tricyclic ring systems. Cyproheptadine, loratadine, desloratadine and ketotifen share a common tricyclic nucleus. It was suggested that cyproheptadine's tricyclic moiety serves as a high affinity ligand for different receptor subtypes (Fedi *et al*, 2008). This could explain the activity against *P. falciparum* in addition to the numerous reports suggesting that the tricyclic phenothiazines compounds are active against a wide array of micro-organisms. Kalkinidis *et al* (2002) synthesized a number of potent antimalarial analogues based on the tricyclic structure of chlorpromazine. The findings from this study confirm the importance of the tricyclic nucleus as foundation for antimalarial drug discovery.

The results obtained in this study correlate well with the findings made by Peters *et al*, (1990) for AHs; cyprohepatadine, ketotifen and loratadine. Peters *et al* (1990) suggested that ketotifen is more active *in vivo* in the rodent model. They theorized that this may be because in humans the drug undergoes three principle types of metabolism, to reduce the ketone while rats lack the N-glucoronidation metabolic process. It is also worthy to note that despite loratadine possessing inherent antimalarial activity, it was found to be inactive *in vivo* (Peters *et al*, 1990).

The H_3 receptor antagonists have activity comparable to the H_1 receptor antagonists. Many of these structures share a common pharmacophore of amine-alkylene-oxygen-aryl chain. The structural similarities between the H_1 and H_3 antagonists suggest that certain AH structural features are a requisite for antimalarial activity.

The imidazole ring is a common structural feature in almost all H_3 antihistamines. The N3 of the imidazole compounds have the potential to interact with the haem iron atom of ferric cytochrome

P450. The imidazole containing antifungal, clotrimazole displays potent antimalarial (IC_{50} 0.2-1.1 μM) activity in CQS and CQR strains (Tiffert *et al*, 2000). Huy *et al* (2002a) found that clotrimazole can remove haem from histidine rich peptide-haem protein complex which, initiates haem-polymerization and from glutathione- haem complex in parasites. Additionally, they show that ketoconazole and miconazole behave similarly to clotrimazole in binding to haem. These azole antifungals form stable haem-azole complexes with two nitrogenous ligands derived from the imidazole moieties.

A corresponding study by Huy *et al* (2002, b) reported that clotrimazole has high binding affinity for ferric haem to form a haem-clotrimazole complex, which subsequently disturbs haem degradation by glutathione and the complex damages the cell membrane more than free haem. The effect of the haem-azole complex disintegrates the parasites cell membrane and this is thought to play an important role in the antimalarial mechanism of action. Therefore, the haem-clotrimazole complex prevents the conversion of free haem into haemozoin. Nitrogen heterocycle chytochrome P450 inhibitors, offer antimalarial activity via inhibition of haemozoin formation, however, the triazole, fluconazole did not bind to free haem or inhibit haemozoin formation (Chong and Sullivan, 2003). It is likely, therefore, that the antimalarial activity of H_3 antihistamines may also be mediated through the imidazole moiety of these compounds, similar to that of clotrimazole.

The lipophilicity of a drug is a pivotal parameter that may explain biological activity. In an attempt to correlate different physicochemical properties to antimalarial activity, it was found that the antimalarial activity of AHs correlated strongly with the log *D* values of the test drugs. Log *D* (distribution coefficient) describes the log *P* of an ionisable compound at a particular pH. The log *D* values of test AHs at physiological pH 7.0 are presented in table 2.2. With most of the drugs, it appears as if, a higher log *D* value correlates with a low IC_{50} value. The H_2 receptor antagonists tested have log *D* values in the range of -3.02 to 0.75, whereas the AHs presenting with substantial antimalarial activity have higher log *D* values. This could explain the H_2 antagonist's antimalarial profile as these drugs may not have the ability to penetrate the parasites

cellular membrane to exert an inhibitory effect. Substantial physicochemical data on the H_3 receptor antagonists is not available as these compounds are still in an experimental phase. Previous studies have established that there is no direct correlation between antimalarial activity and inhibition of haemozoin unless pKa and lipophilicity of the test compound have been considered (Egan *et al*, 2000; Egan, 2004)

There were no significant differences in the antimalarial activity between the CQS and CQR strains used. However it is interesting to note that the antimalarial activity of ketotifen in the CQR strain is 10 fold higher than that for the CQS strain. This interesting observation warrants further investigation to determine if this difference is due to the resistant parasite's ability to accumulate ketotifen.

In relation to toxicity and side effects of AHs, it is important to consider that if AHs were to be used as antimalarial drugs or used in combination with current antimalarial agents, then treatment is most likely to be acute rather than chronic and patients in poorer countries where malaria is endemic are less likely to consume interacting medications. Hence, the incidence of side effects and toxicity are likely to be low.

The data presented above certainly do not support the premise that any of the AHs tested could be clinically implemented as an alternative to CQ or any other current antimalarial for both CQR and CQS plasmodia. However, it is clear that the findings made highlight the value of a tricyclic core and imidazole moiety in relation to antimalarial activity. The H_3 receptor antagonists have not been previously explored for intrinsic antimalarial activity. These results demonstrate the need to enhance the literature on the effects of the newly discovered H_3 and H_4 receptor antagonists on plasmodium parasites. Furthermore these results also confirm that H_2 receptor antagonists are inactive against malaria parasites.

Chapter 3

The Effect of Antihistamines in Combination with Chloroquine against Chloroquine Sensitive and Chloroquine Resistant Strains of *Plasmodium falciparum*

3.1. Introduction

The rapid development and widespread resistance to CQ and other antimalarials, in addition to the exorbitant cost of drug development has accentuated the necessity to optimise the use of existing antimalarial drugs. In an attempt to preserve the efficacy of CQ, a number of agents belonging to diverse pharmacological classes have been explored for their potential to reverse the resistance to CQ by *Plasmodium species.* These agents include calcium channel blockers (Martin *et al*, 1987; Kyle *et al*, 1993), tricyclic antidepressants (Bitonti *et al*, 1988), antipsychotic phenothiazines (Basco and Le Bras, 1992; Kalkanidis *et al,* 2002) and AHs (Peters *et al*, 1990). However, the clinical use of these agents has been limited due to high protein binding and toxicity at the elevated concentrations required to reverse resistance (Boulter *et al*, 1993).

AHs are particularly important since, chlorpheniramine, in combination with CQ, successfully reversed CQR in two African clinical trials (Sowunmi and Oduola, 1997; Okonkwo *et al*, 1999). Additionally, AHs are routinely co-prescribed with CQ to treat CQ induced adverse effects (Oduola *et al*, 1998; Fehintola *et al*, 2004). AHs are widely available at low-cost in malaria endemic zones and are often prescribed to children above 2 years of age (Sowunmi and Oduola, 1997)

CQ is accumulated to lower levels in resistant parasites and hence, the reversal of CQ resistance has been credited to the ability of chemosensitising agents to increase the amount of CQ accumulated by the resistant parasite (Martiney *et al*, 1995; Bray *et al,* 1992).

It is noteworthy that Martiney *et al* (1995) found that verapamil also increased the accumulation of CQ into the CQS D10 strain at concentrations between 0.5-1μM. However, the accumulation of verapamil in CQS strain did not change the sensitivity to CQ as is observed with CQR parasites.

The work reported in this chapter was done to examine the effect of combining AHs with CQ, over a wide concentration range, on the sensitivity of both CQS and CQR strains to CQ. Although several studies have shown that AHs, particularly chlorpheniramine, can serve as chemosensitizers to CQ in CQR strains, none has reported on possible synergistic activity between different structural classes of AHs and CQ. The aim of this study was, thus, to determine if AHs can enhance the effect of CQ on CQS and CQR *P. falciparum* strains and, if so, to compare the extent of synergism amongst representatives of the different AH classes.

3.2 Materials and Methods

3.2.1 In vitro *Plasmodium falciparum* culture

Cultures were maintained according to the methods of Trager and Jenson (1976). (See Chapter 2, Section 2.2.2)

3.2.2 Drug dilutions

CQ and AHs were dissolved to the desired concentrations in appropriate solvents (refer to table 2.1). The initial concentration used for CQ was 100 ng/ml for the CQS strain and a 1000 ng/ml for the CQR strain. The concentration used for all AHs was 100 µg/ml. Testing the activity of the AHs in the presence of CQ was performed at the following concentration percentage ratios: 75% CQ and 25% AH, 50% CQ and 50% AH, 25% CQ and 75% AH for both strains.

3.2.3 *In vitro* drug activity measurement

The parasite lactate dehydrogenase assay (pLDH) was used to measure CQ-AH activity. (See Chapter 2, Section 2.2.4)

3.2.4 Isobologram preparation and data analysis

For each combination assay, IC_{50} was calculated from four sets of concentration response graphs, each containing the AH or compound alone curve, and 3 combination curves. Fractional inhibitory concentration values of compound A (FICA) and compound B (FICB) were calculated separately in each combination. FIC values were calculated by the following equation to plot isobolograms:

$$FIC = \frac{\text{Fraction of drug concentration required to produce IC50 when used in combination}}{\text{Fraction of drug concentration required to produce IC50 when used alone}}$$

The fractional inhibitory concentration (FIC) for 5 preparations in quadruplicate was calculated to plot isobolograms with mean FIC. The sum FIC (FIC Index) value for each of the preparations was determined to classify the drug–drug interaction (Berenbaum, 1978; Gupta et al., 2002). A FIC of > 1.0 and < 4.0 represents additivity (indifferent), a FIC less than 1.0 represents synergy, and a FIC greater than 4.0 represents antagonism (Odds, 2003; Nduati and Kamau, 2006; Bhattacharya et al., 2008). (See Appendix 1 and Appendix 2 for $IC_{50,}$ FIC and Sum FIC (FIC index) values used to plot isobolograms).

3.3 Results

3.3.1 Potentiation/ Synergism of CQ in the CQ Sensitive Strain

Combinations of AHs with CQ were tested against CQS P. *falciparum.* The ethanolamine class of AHs including carbinoxamine, diphenhydramine and doxylamine showed slight synergistic effects with CQ in the CQS strain. Cyproheptadine, ketotifen and desloratadine also proved to have a synergistic action when combined with CQ in CQS parasites. However, loratadine displayed an antagonistic response with CQ in the CQS strain. The other H_1 AHs, as well as the H_2 and H_3 receptor antagonists were indifferent in combination with CQ in the sensitive strain (results not shown). The isobolograms below depict the effect of each AH tested at each of the chosen concentrations with CQ for the D10 sensitive strain. The points below depict the FIC index or Sum FIC of CQ/AH combination. (See appendix1 and appendix 2)

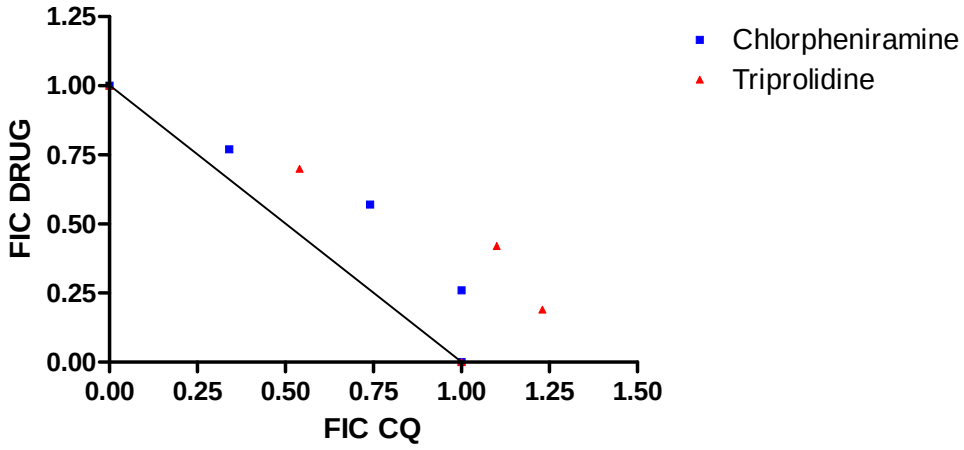


Fig.3.1 Isobologram illustrating the interaction between CQ and Alkylamines on D10

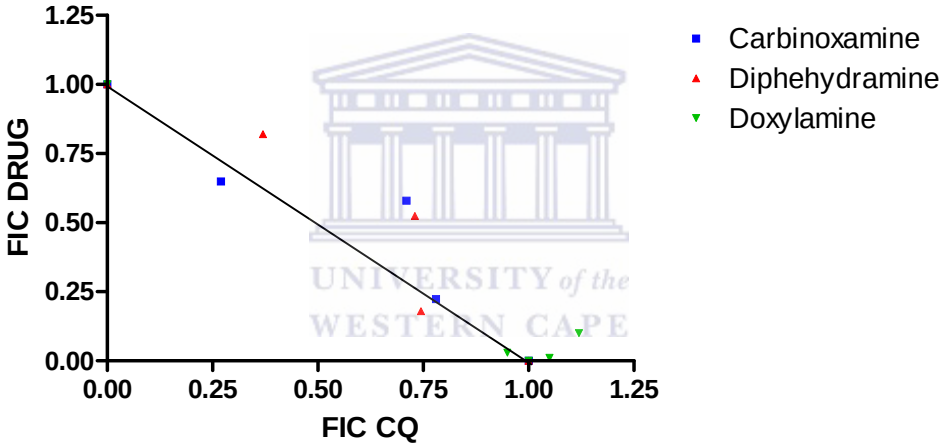


Fig.3.2. Isobologram illustrating the interaction between CQ and Ethanolamines on D10

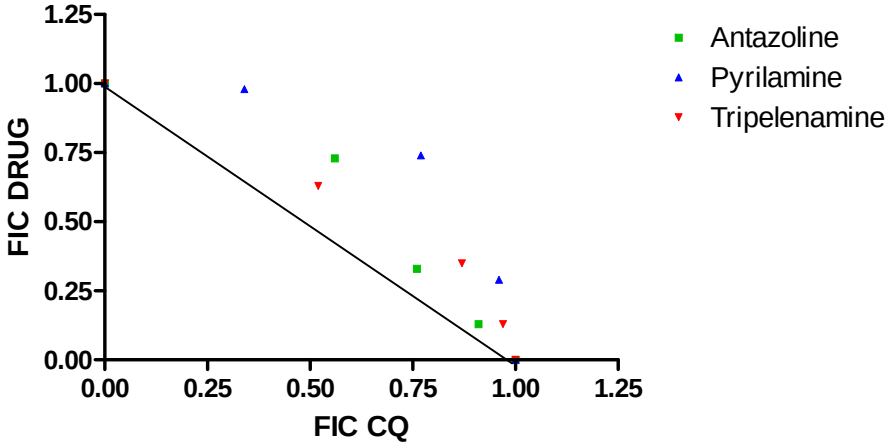


Fig.3.3 Isobologram illustrating the interaction between CQ and Ethylenediamines on D10

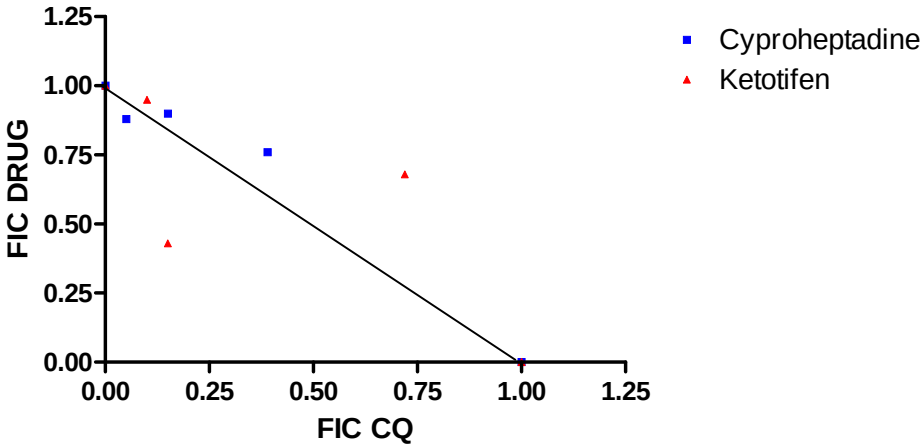


Fig.3.4 Isobologram illustrating the interaction between CQ and Cyproheptadine, Ketotifen on D10

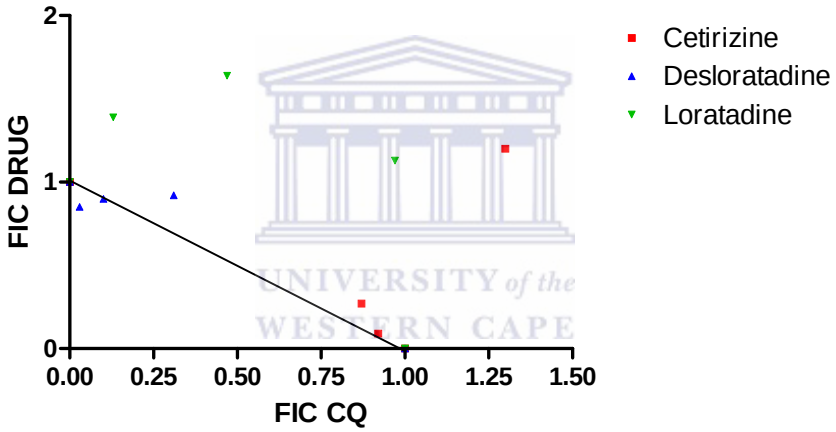


Fig.3.5 Isobologram illustrating the interaction between CQ and cetirizine, desloratadine, loratadine on D10

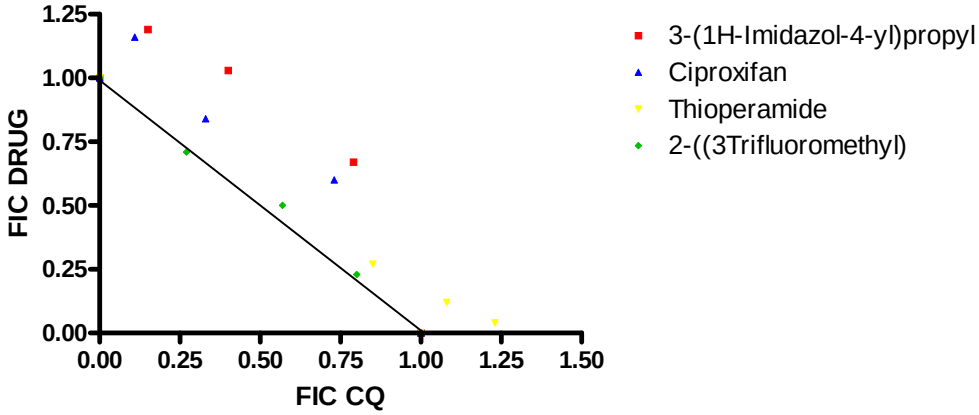


Fig.3.6 Isobologram illustrating the interaction between CQ, and H_3 receptor antagonists on D10

3.3.2 Potentiation / Synergism in the CQ Resistant Strain

Certain AHs were selected on the basis of their intrinsic antimalarial activity profile and tested in combination with CQ. A synergistic effect was observed with chlorpheniramine, cyproheptadine, ketotifen and desloratadine while loratadine and cetirizine had an antagonistic effect. The H_3 receptor antagonists were indifferent in combination with CQ in the resistant strain as well. The isobolograms below depict the effect of each AH tested at each of the chosen concentrations with CQ for the DD2 resistant strain.

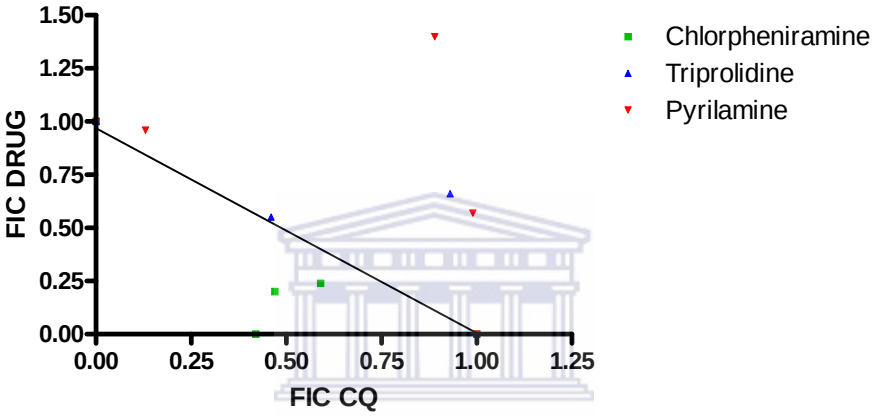


Fig. 3.7 Isobologram illustrating the interaction between CQ and chlorpheniramine, triprolidine, pyrilamine on DD2

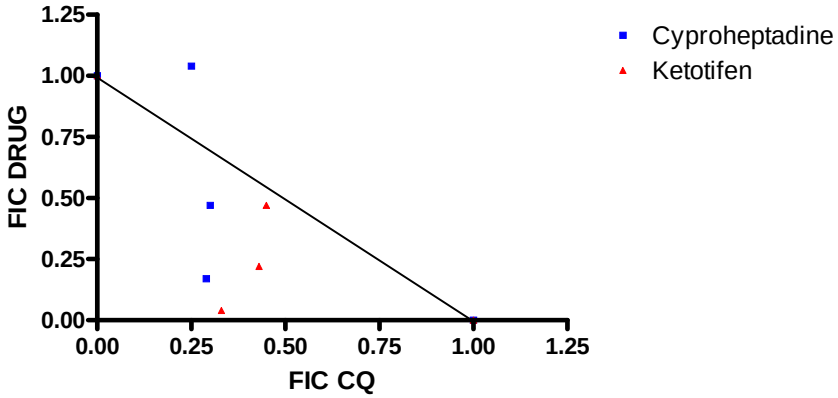


Fig.3.8 Isobologram illustrating the interaction between CQ and cyproheptadine, ketotifen on DD2

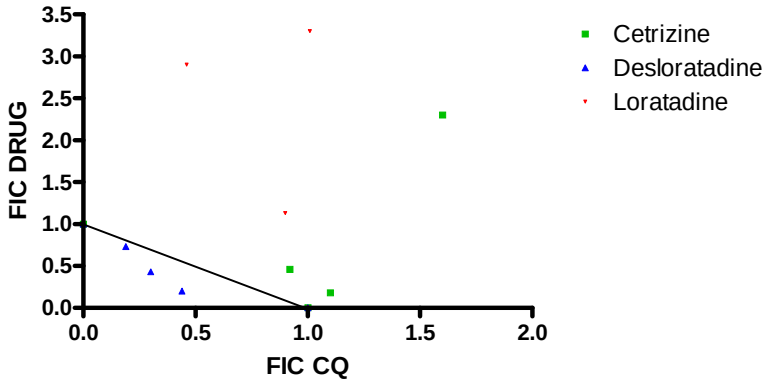


Fig. 3.9 Isobologram illustrating the interaction between CQ and cetirizine, desloratadine, loratadine on DD2

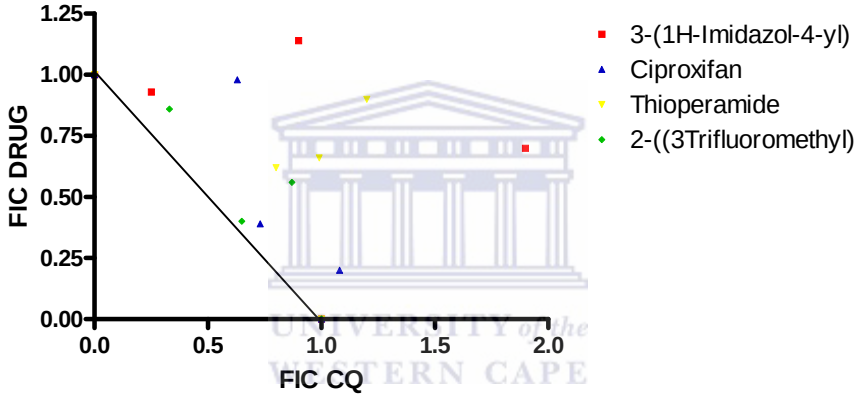


Fig 3.10. Isobologram illustrating the interaction between CQ, and H_3 receptor antagonists on DD2

3.4 Discussion

The derived isobolograms provide clear evidence of synergistic interactions between CQ and several AHs. Although the mechanism of action of CQ on sensitive strains is not clearly established, there is evidence that resistant strains accumulate less CQ than sensitive strains (Martiney *et al*, 1995; Bray *et al,* 1992). This reduced accumulation is attributed to an enhanced efflux of CQ from resistant parasites (Martin *et al,* 1987). Krogstad *et al* (1987) demonstrated that several resistance reversers increased the accumulation of CQ into CQR parasites; however this was not seen with the CQS strain over the same concentration range.

The results of this study indicate that certain AHs, in particular the ethanolamines exert a synergistic effect when combined with CQ in the CQS strain. The results above suggest that these compounds possess moderate antimalarial activity with the exception of doxylamine. It is therefore important to note that despite doxylamine possessing no antimalarial activity; it is able produce a synergistic response. The aminoalkyl ether AHs (ethanolamines) can be structurally highlighted for further SAR studies, seeing that carbinoxamine, doxylamine and diphenhydramine produced a slight synergistic response with CQ in the CQS strain. Other therapeutically useful derivatives of diphenhydramine reported to have superior therapeutic profiles relative to diphenhydramine may serve as potential candidates for synergism studies

Additionally, cyproheptadine, ketotifen and desloratadine also had a similar effect. Such findings have also been reported with AHs by Peters *et al* (1989), in a CQS isolate, at a similar inhibitory concentration, with the exception of desloratadine. The reason for the synergism between CQ and AHs in the sensitive strain remains unknown. However, the synergistic effect could be due the independent antimalarial effect of antihistamines and the independent effect of CQ. When combined, CQ and AHs produce a synergistic response

There is a substantial body of literature suggesting tricyclic H_1 receptor antagonists like cyproheptadine, promethazine, ketotifen or azatadine, reverse *in vitro* CQ resistance in *P. falciparum* ((Peters *et al*, 1990; Valecha *et al*, 1992; Kyle *et al*, 1993). This study shows that cyproheptadine, ketotifen and desloratadine enhances the activity of CQ in CQR parasites, however, loratadine has no effect on the activity of CQ as noted previously by Peters *et al* (1990) and Singh and Puri (2000). Despite the *in vitro* results observed by previous studies and similar results found in this study, cyproheptadine and ketotifen did not prove beneficial in reversing CQR *in vivo*, due to its high level of protein binding. However, desloratadine is not commonly reported as a resistance reverser in the literature, the finding made in this study, that desloratadine caused synergistic response with CQ in CQR strain of *P. falciparum* may, thus, require further investigation. The tricyclic structure was common to all AHs that exhibited synergism. Structural manipulation, in addition to unravelling molecular mechanisms for cyproheptadine, ketotifen and desloratadine, may prove worthwhile. The data showing enhancement of CQ activity *in vitro* by selected AHs are significant. Confirmation with *in vivo* studies is now required.

Chlorpheniramine exerted a synergistic action with CQ in CQR parasites. There was no effect seen in the CQS strain. This observation suggests that the synergistic effect of chlorpheniramine is limited to resistant isolates, possibly via a distinct mechanism enabling CQ accumulation, as chlorpheniramine has weak antimalarial activity.

The common structural features among chemosensitisers have been modelled and compared with *in vitro* resistance reversal activity by many SAR studies (Bhattercharjee *et al*, 2001; Alibert *et al*, 2002; Bhattercharjee *et al*, 2002; Guan *et al*, 2002). Pharmacophores consist of two aromatic rings and aliphatic nitrogen, which appears to be essential for the reversal of CQ resistance. Many AHs fulfil these requirements which may indicate that the synergistic results presented here may be due to an increase in accumulation of CQ.

Additionally, most chemosensitisers, like the aminoquinolones, have fused rings; however chlorpheniramine and verapamil consists of two unfused planar rings separated by an alkyl chain, yet according to literature, chlorpheniramine is the most promising resistance reverser.

There are many structurally and pharmacologically diverse compounds that reverse CQ resistance. All chemosensitisers reported in the literature including all AHs which were found to act synergistically with CQ in both strains are monoprotic weak bases with similar pKa's. This physicochemical characteristic is a key factor for intravesicular accumulation. However, a diprotic weak base such as CQ is concentrated more than simple monoprotic weak bases within acid vesicles. Consequently it can be assumed that these compounds have the ability to accumulate CQ in CQR strains and could indicate that AHs and other chemosensitisers may, thus, have a common mechanism of action in *P. falciparum*.

The above results suggest that certain AHs, possessing weak antimalarial activity may enhance the action of CQ in CQR strains, as is the case with chlorpheniramine. In a study by Kalkinidis *et al*, (2002) phenothiazine-based chemosensitisers were modified to make them more effective at inhibiting ß-haematin. Although the modifications proved successful at increasing their plasmodicidal activity, the compounds appeared to lose their ability to reverse CQ resistance. This strongly suggests that the concept of antimalarial action and resistance reversal are two distinct opposing activities. This may explain the observation that all H_3 receptor antagonists lacked the ability to enhance the action of CQ, despite having considerable intrinsic antimalarial activity.

It is essential that the pharmacokinetic parameters are considered in CQR reversal therapy. Since the activity of CQ is dependent on the presence of the chemosensitiser, both compounds should be ideally matched for their absorption and half-lives. A novel strategy employed by Burgess *et al* (2006) and Adrews *et al* (2010) is a hybrid molecule in which the chemosensitiser is conjugated to CQ. Since chlorpheniramine was found to enhance the pharmacokinetic properties

of CQ (Okonkwo *et al*, 1999; Gbotosho *et al*, 2008), a hybrid molecule consisting of CQ and chlorpheniramine may prove to be an ideal candidate for further investigation.

The work described in this chapter confirms the synergistic potential of selective AHs. Results showed that the H_3 receptor antagonists, although moderately active as antimalarials, are ineffective at enhancing the activity of CQ in both sensitive and resistant strains of *P. falciparum* compared to the H_1 receptor antagonists. The mechanism involving CQ resistance reversal is complex and many factors such as structural and physicochemical characteristics, including molecular interactions may play a role. While the mode of antimalarial and chemosensitiser effects of these AHs have not been elucidated, characterising parasite morphology in the presence of AHs, notably H_1 and H_3 blockers, may help explain some of the observations made in this study. This approach was followed and the findings are reported in the next chapter.

Chapter 4

The Effect of Antihistamines on the Morphology of *Plasmodium falciparum*

4.1 Introduction

The parasite's asexual erythrocytic cycle begins with merozoite recognition of, binding to, and invasion of an erythrocyte. Once erythrocytes have been invaded, the parasite is found inside the parasitophorous vacuole (Shermin, 1979). During the 48 h cycle the parasite develops through three distinct stages: ring (0-24 h post-invasion), trophozoite (24-36 h) and schizont (36-48 h). These stages are distinguished by specific morphological characteristics (Elmendorf and Haldar, 1993). At the schizont stage, the parasite divides within the vacuole to produce approximately 16 daughter merozoites which, upon rupture of the red blood cell, reinvade new erythrocytes. Mature red blood cells are quiescent, lacking all intracellular organelles (Elmendorf and Haldar, 1994). However, parasite infected erythrocytes undergo several biochemical and ultrastructural modifications. The development of new membrane structures in the infected erythrocyte cytoplasm is a prominent alteration. Parasite-derived proteins are exported into the erythrocyte cytoplasm and membrane, while several proteins are localised to the parasitophorous vacuolar membrane and the tubular/vesicular membranous network structures surrounding the parasite (Halder *et al*, 2001)

The trophozoite survives intracellularly due to supply of nutrients from the host cell and disposal of waste products, both of which are accomplished by an increase in solute transport across the host plasma membrane. Haem, which is toxic to the parasite, is rendered harmless by crystallization into particles of dark pigment, referred to as haemozoin crystals, which is

scattered within the food vacuole (Fujioka and Aikawa, 2002). These golden-brown to black granules within the food vacuole can be recognized as a dense clump of granules by light microscopy. (See Fig.4.1)

In an attempt to explain the mechanism of action of the active AH test drugs, the morphology of the intra-erythrocytic parasite was investigated with light microscopy in the presence and absence of the test agents. The objective was to determine if the drugs exerted any structural changes in the parasite during its maturation from the trophozoite to the schizont stage. The drugs that were included in this study were cyproheptadine, ketotifen, and chlorpheniramine.

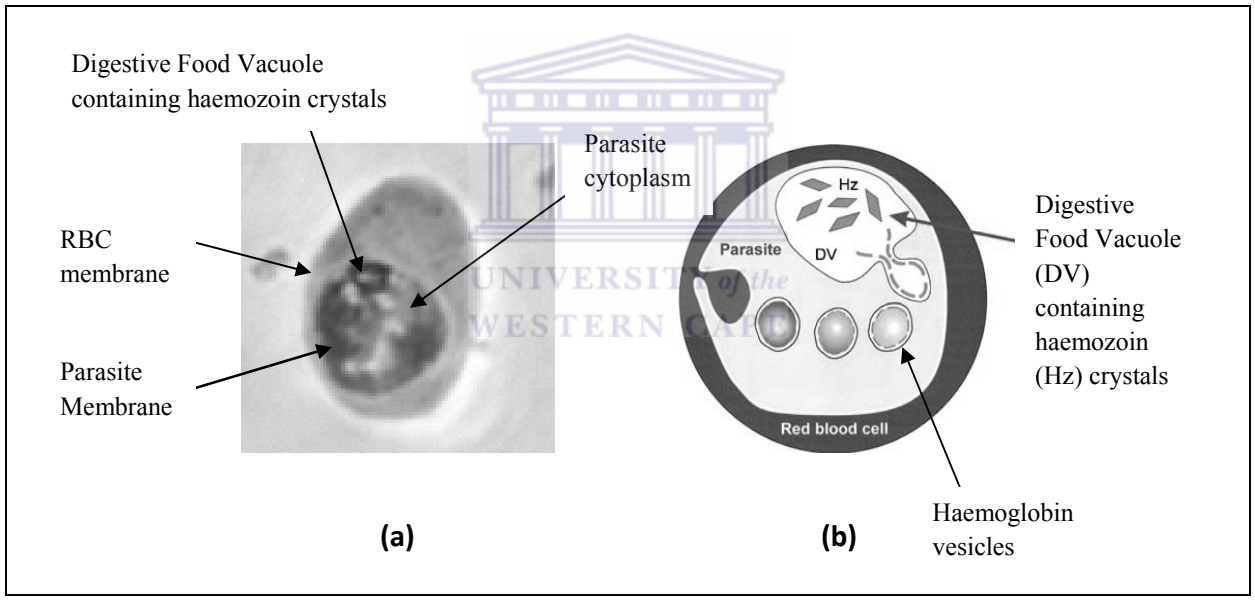


Fig (4.1) Representation of parasite within red blood cell; a) Light microscope image of trophozoite
b) Representation of image (a)

4.2 Materials and Methods

4.2.1 In vitro *Plasmodium falciparum* culture

Cultures were maintained according to the methods of Trager and Jenson (1976). (See Chapter 2, Section 2.2.2)

4.2.2 Drug Treatments

AHs were added to *P. falciparum* cultures at concentrations approximately 5 times their IC_{50} values to help ensure that the effects were observed within a short time interval. Drug pressurisation was performed over a 5 hour period during which parasites matured from early to late trophozoite stage. Giemsa stained blood smears were prepared at specific time intervals and examined by light microscopy.

4.2.3. Light Microscopy

The Giemsa stained blood smears were examined with a Nikon Eclipse E600 fluorescence microscope fitted with a 100x Apochromat objective. The images were captured with a Media Cybernetics CoolSNAP-Pro monochrome cooled charge-couple device camera.

4.3 Results

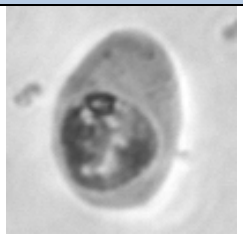
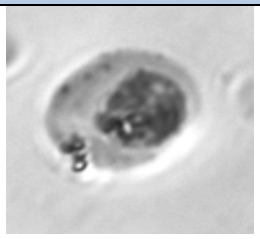
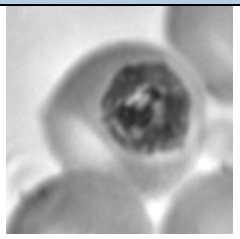
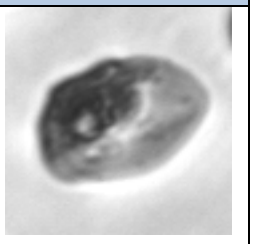
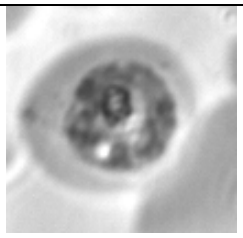
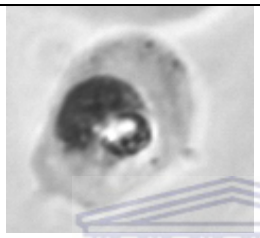
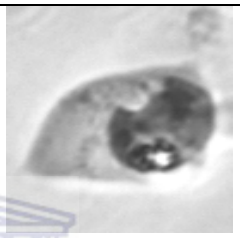
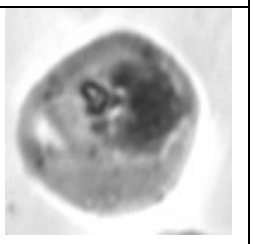
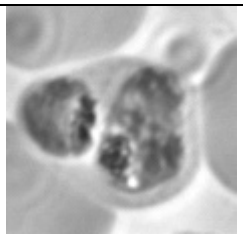
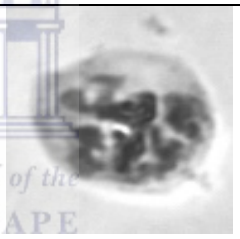
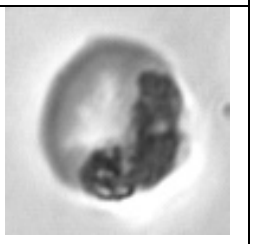
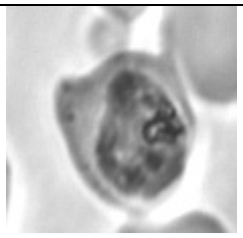
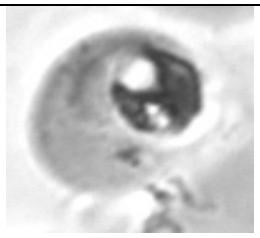
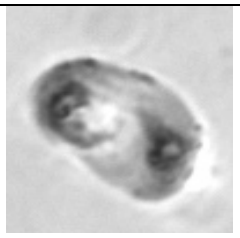
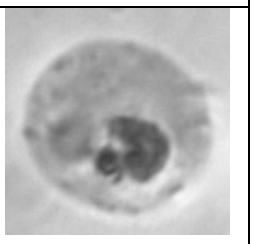
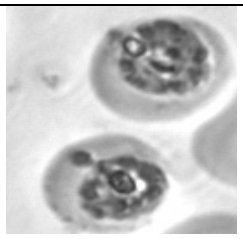
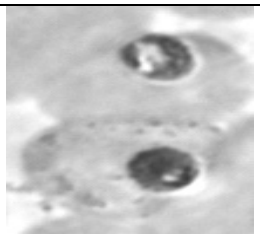
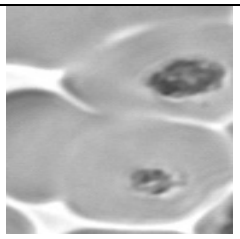
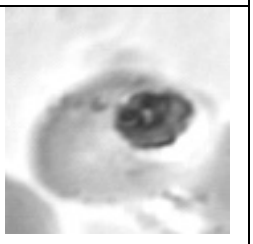
	a) Control D10	b) Chlorpheniramine	c) Cyproheptadine	d) Ketotifen
10min				
30min				
1hrs				
2hrs				
5hrs				

Fig (4.2) Representative smears of parasites drug pressured for 10min-5hrs (a) serves as respective controls b) chlorpheniramine, c) cyproheptadine, d) ketotifen

The parasites shown in figure (4.2) are a representation of a number of parasites demonstrating similar morphological changes. Compared to the untreated control, parasites treated with chlorpheniramine, cyproheptadine and ketotifen show a number of morphological changes. After only 10 minutes parasites treated with chlorpheniramine appear smaller compared to the control. The parasite membrane is not as well defined as that of the control and there is no clear food vacuole. After 30 minutes the membrane damage is very evident and it appears that the parasite cytoplasm is leaking into the host cell. Big cytoplasm vacuoles become visible and the parasite seems to fragment between 1 and 2 hours of drug treatment. Unlike the control, the chlorpheniramine treated parasites do not advance to schizont stage after 5 hours, but are small and inconspicuous.

After 10 minutes parasites treated with cyproheptadine also appear smaller compared to control. The parasite cytoplasm appears granular and the food vacuole and haemozoin crystal is not easily recognised. After 30 minutes the parasite membrane has disintegrated and after one to two hours the parasite had completely fragmented. At 5 hours the parasites are small and barely discernible and it is evident that they will not develop into schizonts unlike the control.

Parasites treated with ketotifen display a granular cytoplasm after 10 minutes. The food vacuole is small and the membrane is not well defined. After 30 minutes the parasite membrane shows further damage. At 1 hour the parasite appears irregular and the food vacuole is no longer visible. After a time period of 1-5 hours the parasite is drastically smaller when compared to control.

4.4 Discussion

The repeated cycles of parasite invasion and destruction of erythrocytes are central to malaria disease. After invasion of erythrocytes, Plasmodium spp. drastically changes their biology and start to export and remodel proteins (Waller *et al*. 2000; Wickham *et al*. 2001). The parasite is then able to mature into different erythrocytic forms. The test AHs were selected on the basis of their intrinsic antimalarial activity or synergistic effect with CQ.

The lack of maturation and changes in parasite morphology may be the effects of either direct or indirect influences of chlorpheniramine, ketotifen and cyproheptadine on the erythrocytic stage of the parasite's life cycle. The disintegration of the parasites membrane and cytoplasm is preventing many processes that enable the parasite to feed and dispose of its waste products. The total disappearance of the schizont stage and disintegration of parasitised cells observed after 5 hours suggests that antihistamines could possibly interfere with haemoglobin digestion or uptake thereby starving the parasite and preventing its development into the schizont stage. This can be seen in the abnormal appearance of the haemozoin containing digestive food vacuole.

The high pKa's of AHs further indicates that their active forms are uncharged lipid soluble bases. This may suggest that the drugs concentrate in the lipid cytoplasmic membrane of the parasites. If present in high enough concentrations, they may disrupt membrane proteins, reducing the integrity of the membrane which will ultimately lead to cell lysis. This could be possible explanations for the morphological changes observed, particularly after 30 minutes of incubation with the drugs, when membrane damage was clearly observed and the parasite cytoplasm was leaking out from the cells. The effect of antihistamines may also be less specific in that their accumulation in membranes may physically disorder the membrane resulting in loss of integrity, causing increased membrane permeability.

The possible CQ-sensitising effect of some AHs may also be related to their ability to accumulate in the cytoplasmic membrane of CQR *Plasmodium spp*. If the mechanism of resistance is due to a membrane-bound efflux pump, such as a P-glycoprotein type

transmembrane protein that actively pumps CQ out of the cell, (Rohrbach *et al*, 2006) binding of AHs to the protein may alter its higher-order structure to such an extent that it may become inactive. It has been found, for example, that H_1 and H_2 receptor agonists and antagonists can bind to cell membranes of mast cells in low concentrations, stabilising the membrane and preventing the release of histamine (Lau and Pearce, 1990). Hence, AHs which have been found to be resistance reversers, but without antimalarial activity, may possibly exert their effect by this mechanism. Further studies are needed to support this contention.

This is the first report describing alterations in parasite morphology with the use of AHs. However, it will be useful to determine the effect of these AHs on haemoglobin content and localisation, in order to better understand the mechanistic interactions between the plasmodium parasite and AHs. This approach was followed and the findings are reported in the next chapter.

Chapter 5

The Effect of Antihistamines on the Localization of Haemoglobin and on Haemoglobin Content in *Plasmodium falciparum*

5.1. Introduction

Intraerthrocytic malarial parasites are known to endocytose large quantities of surrounding RBC cytoplasm. Haemoglobin is the main constituent of erythrocyte cytoplasm and represents the bulk of the endocytic material in malaria parasites. The haemoglobin is transported to the parasite's digestive food vacuole via endocytic vesicles (Egan *et al*, 2002). Haemoglobin digestion in the food vacuole provides amino acids for parasite protein synthesis and is required to maintain osmotic integrity of the host cell. Proteolytic digestion of haemoglobin releases toxic FPIX (haem). The parasite is equipped with a detoxifying mechanism that crystallizes the haem monomers into an insoluble substance known as haemozoin (Egan *et al,* 2002; Sullivan, 2002). CQ interferes with the crystallization of the free haem monomers released during haemoglobin proteolysis by binding to them, accumulating toxic FPIX or FPIX-CQ complexes, which inhibit key enzymes as well as compromise membrane integrity (Fitch and Russel, 2006)

Previous studies have suggested that CQ blocks haemoglobin degradation and should therefore produce an increase in undigested haemoglobin in malaria parasites (Famin & Ginsburg, 2002; Hoppe *et al*, 2004). It has been demonstrated that different quinoline antimalarials affect the parasite's ability to take up haemoglobin in different ways (Famin & Ginsburg, 2002; Hoppe *et al*, 2004; Roberts *et al,* 2008). CQ and amodiaquine inhibited haemoglobin degradation and this inhibition was related to parasite death. Mefloquine and quinine did not exhibit the same effects on haemoglobin (Famin & Ginsburg, 2002).

Hoppe *et al.* (2004) was able to demonstrate that mefloquine inhibits the process of endocytosis while CQ inhibits the fusion of the endocytosed vesicles with the food vacuole. This inhibition of

fusion by CQ leads to a build up of haemoglobin containing vesicles in the cytoplasm of the parasites causing parasite starvation.

It is not known what effect AHs have on these processes when used alone. Since the action of CQ is related to the inhibition of haemoglobin degradation in the CQS parasites, it was decided to investigate the effect of AHs, namely, cyproheptadine, ketotifen and chlorpheniramine on haemoglobin levels in CQS parasites using Western blots. Additionally, this chapter examines the effect of specific AHs, namely cyproheptadine and ketotifen on changing the amount of haemoglobin present in CQS *P. falciparum* using immunofluorescence techniques.

5.2. Materials and Methods

5.2.1 Parasite culture

The D10 strain of *P. falciparum* was cultured according to the methods of Trager and Jenson (1976) (See Chapter 2, Section 2.2.2).

5.2.2 Drug treatments

Early trophozoite- stage cultures of *P. falciparum* were incubated for 5 hours with selected AHs at concentrations 5 times their IC_{50}.

5.2.3 Immunofluorescence assay

Glass coverslips were coated in polylysine and distributed into a 24-well plate containing PBS. Twenty microliters of infected RBCs were resuspended in 200 µl of PBS, and 40 µl of the suspension was pipetted into each plate well. The cells were pelleted onto the polylysine coverslips by centrifugation at 100 × *g* for 2 min. Excess, unbound RBCs were flushed from the

coverslips by pipetting. RBC membranes were lysed and excess haemoglobin was removed by briefly rinsing the coverslips in PBS containing 0.04% (w/v) saponin and additional washing in PBS. The coverslips were fixed in methanol and incubated for 30 min in blocking solution (PBS containing 1 Mm $CaCl_2$, 1 mM$MgCl_2$, 2%[w/v] bovine serum albumin [BSA], and 10% [v/v] fetal calf serum), followed by incubation for 1 h in blocking solution containing rabbit antihaemoglobin antiserum (dilution, 1:500). After several washes in PBS the coverslips were incubated in blocking solution containing rhodamine-conjugated goat anti-rabbit Ig secondary antibody (1:250), washed in PBS, rinsed in water, mounted in mounting medium, and examined by fluorescence microscopy.

5.2.4 Fluorescence microscopy

The microscope slides were examined with a Nikon Eclipse E600 fluorescence microscope fitted with a ×100 Apochromat objective. The images were captured with a Media Cybernetics CoolSNAP-Pro monochrome cooled charge-couple device camera.

5.2.5 Western blotting

RBC's from a 10 ml-culture were suspended in 1 ml of 0.25% (w/v) saponin in PBS to lyse the RBC membranes. The released parasites were pelleted at 1,500 × *g* for 3 min and washed five times in cold PBS to remove excess haemoglobin. The parasite pellet was solubilised in 150 µl of reducing SDS-PAGE sample buffer, 10-µl aliquots run on an SDS–11% polyacrylamide gel, and the resolved proteins transblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). The membranes were incubated in blocking buffer (20 mM Tris and 0.15 M NaCl [pH 7.4] containing 0.1% [v/v] Tween 20, 1% [w/v] BSA, and 2% [wt/vol] fat-free milk powder), followed by incubation in blocking buffer containing rabbit antihaemoglobin antiserum (1:5,000) and blocking buffer containing peroxidase-conjugated goat anti-rabbit IgG (1:5,000). The membranes were washed, soaked with an enhanced chemiluminescence Western blotting detection reagent (Amersham Pharmacia Biotech), and exposed to Kodak BioMax Light autoradiography film to detect bound secondary antibodies. Images of the developed

autoradiographs were captured with a Kodak EDAS 290 gel documentation system that incorporates a Kodak DC290 digital camera. The images were analysed, and the net intensities of individual bands were determined with Kodak 1D image analysis software (version 3.5).

5.3 Results

5.3.1 The effect of AHs on the localisation of haemoglobin in the parasitized erythrocytes of *Plasmodium falciparum*

To determine the subcellular location of haemoglobin in parasites, control parasites and parasites cultured with cyproheptadine and ketotifen for 5 hours, were fixed and reacted with antihaemoglobin antiserum in an immunofluorescence assay. In control parasites, haemoglobin was located prominently in the food vacuole (Fig. 5.1 (a), arrow). The RBC surrounding the parasite is not visible in these images, due to a brief saponin lysis which is carried out prior to parasite fixation in order to remove extraparasitic haemoglobin. Cyproheptadine treated parasites exhibited a prominent increase in haemoglobin in the food vacuole (Fig. 5.1 (b), arrow). In contrast to the controls, ketotifen treated parasites displayed reduced fluorescence in the food vacuole (Fig. 5.1 (c), arrow).

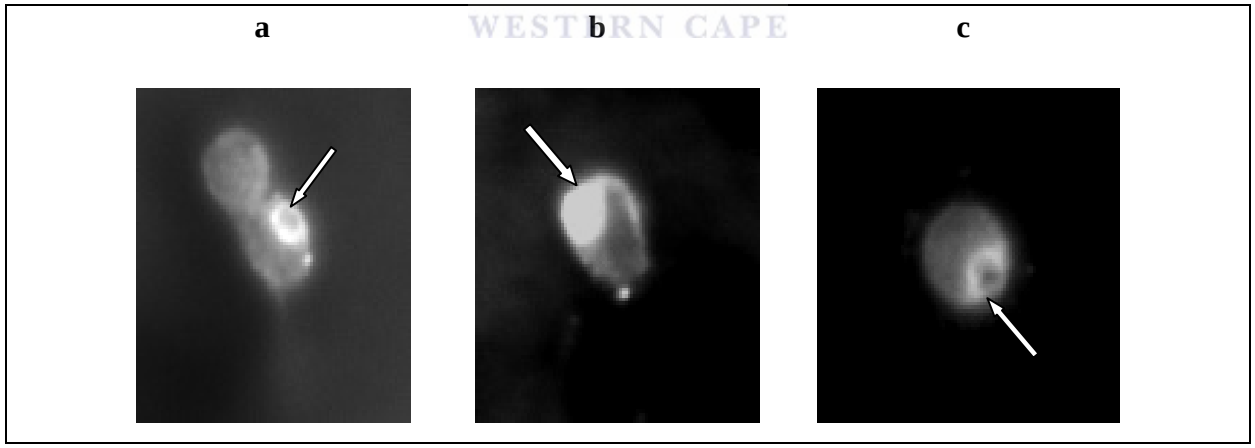


Fig 5.1 Subcellular localisation of haemoglobin by immunofluorescence. pRBCs from a control culture (a) and one treated with cyproheptadine (b) and ketotifen (c) for 5hrs

5.3.2 The effect of AHs on haemoglobin levels in *Plasmodium falciparum*

To determine the effects of the selected AHs on haemoglobin levels in the D10 strain of *P. falciparum*, parasites were incubated with cyproheptadine, chlorpheniramine and ketotifen for 5 hours; released from the RBC's by saponin treatment, and washed extensively to remove extraneous haemoglobin. The results confirm that cyproheptadine treatment [fig 5.2 (a)] leads to a discernible increase in haemoglobin levels compared to those in the control parasites. Ketotifen treatment [fig 5.2 (b)] decreased the haemoglobin levels and chlorpheniramine [fig 5.2 (c)] appeared to decrease parasite haemoglobin levels compared to those in the controls.

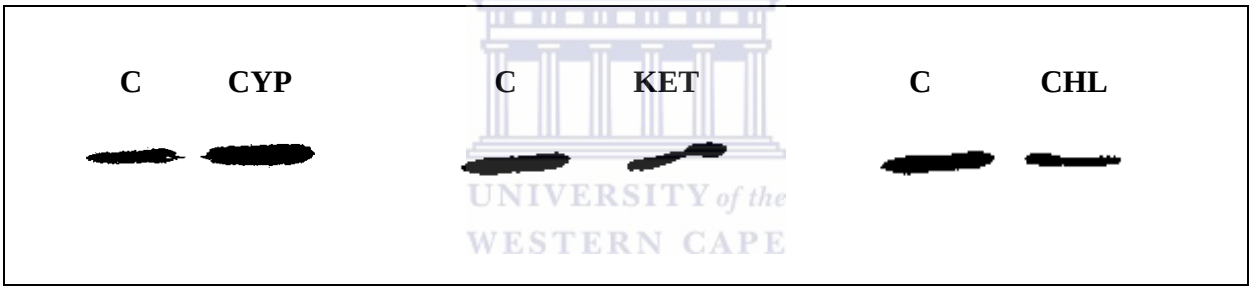


Fig 5.2 Haemoglobin accumulation in parasites; Parasite cultures were left untreated (control; C) or were incubated with (a) cyproheptadine (CYP), (b)ketotifen (KET) and (c)chlorpheniramine (CHL) for 5 h

5.4 Discussion

The results above suggest that selected AHs which may be synergistic in combination with CQ, may be acting via different mechanisms in *P. falciparum*. Cyproheptadine treated parasites shows an increase in haemoglobin using immunofluorescence. This supports the Western blot data which also demonstrated that there was an increase in haemoglobin. Although the increase in haemoglobin levels in cyproheptadine treated parasites probably reflects a block in haemoglobin digestion, an alternative explanation could be a stimulation of endocytosis or uptake of haemoglobin from the infected erythrocytes.

Malaria parasites would detoxify free haem from haemoglobin via haem polymerization that results in haemozoin. This process was shown to be an enzymatic reaction involving haem polymerase, however the mechanism of formation of haemozoin is still under debate. Agrawal *et al* (2002) reported that the action of cyproheptadine involved inhibition of the haem polymerization function thereby decreasing haemozoin. In context of this study, this may be a possible explanation of the cyproheptadine induced increase of haemoglobin.

The increase in haemoglobin may suggest that cyproheptadine's effect may be confined to the digestive food vacuole. Cyproheptadine is a weak base and therefore it would increase the pH of the parasite food vacuole. Given that the function of enzymes within the food vacuole is pH dependant, any change in pH of the vacuole may slow down the activity of proteins and enzymes that may break down haemoglobin. This may be a possible explanation to the increased levels of haemoglobin in the parasite after being treated with cyproheptadine.

Conversely, the fact that ketotifen and chlorpheniramine treated parasites results in a reduction in parasite haemoglobin levels could be interpreted as either a stimulation of haemoglobin digestion or a block in haemoglobin endocytosis. In each case, the parasites will presumably starve as the haemoglobin is not being broken down for the production of parasite proteins. A decrease in haemoglobin is probably also a result of toxic effects of the drug on the parasites, since the concentration used is significantly above the IC_{50} for chlorpheniramine and ketotifen.

Krugliak *et al* (2002) quantitatively tested the degradation of haemoglobin and the increase in parasite protein content as a function of parasite maturation in cultures of *P. falciparum*. They demonstrated that the parasite does not require all the haemoglobin taken up form the erythrocyte cytoplasm. The parasite digested up to 65% of the host cell's haemoglobin but utilized only up to about 16% of the amino acids derived from haemoglobin digestion. Therefore if AHs are slowing down the breakdown of haemoglobin causing it to increase in the vacuole, as is seen with cyproheptadine, this may not necessarily be lethal to the parasites at low concentrations. It may be able to survive on the haemoglobin that it can break down. On the contrary, if AHs increased haemoglobin breakdown causing a decrease relative to the control , the parasite would pump the excess amino acids out of the erythrocyte as its would normally discard its excess. This may explain the moderate antimalarial activity of antihistamines when compared to CQ.

The action of antihistamines appears to differ from the action of CQ where the build-up of haemoglobin is not within the food vacuole. Hoppe *et al* (2004) demonstrated that haemoglobin was present in cytoplasmic transport vesicles rather than undigested vesicles in the food vacuole when treated with CQ.

Further mechanistic studies are required to fully understand the interaction between AHs and *P. falciparum*. Nonetheless, the results suggest that AHs used in this study has an effect on the parasite's ability to digest haemoglobin, which ultimately may induce parasite starvation.

Chapter 6

Summary and Conclusion

The increasing resistance by the *plasmodium* parasite against first line and second line antimalarial drugs demands a continuous effort to develop new antimalarial agents. Development of new antimalarials has not yielded much success. The identification of antimalarial effects of drugs already in use for other clinical indications represents a cost effective approach with potentially rapid clinical application. Another approach involves restoring the activity of chloroquine action by combining it with agents that are able to modulate chloroquine resistance. There are structurally and pharmacologically a diverse group of agents that have been identified to either possess intrinsic antimalarial activity or resistance reversal capability. Such characteristics have been reported with certain antihistamines, with the successful clinical implementation of chlorpheniramine.

Hence, this thesis describes the antimalarial activity of selected antihistamines on both chloroquine-sensitive and chloroquine-resistant *P. falciparum* alone and in combination with chloroquine. The effect of selected antihistamines on parasite morphology was studied and the observation that haemoglobin levels are altered in the presence of active antihistamines is also reported.

The H_1 and H_3 antihistamines tested have IC_{50} values in low micromolar concentrations. The intrinsic antimalarial activity of antihistamines was not significantly different among the chloroquine-sensitive and chloroquine- resistant *P. falciparum* strains. This would suggest that the action of these antihistamines in killing the parasite in the absence of chloroquine remains the same. The hypersensitivity of new chloroquine-resistant Brazilian strains to known chemosentizers may suggest that antihistamines could be given at lower concentrations to such *P. falciparum* isolates and that geographical diversity may influence the effect of antihistamines.

The comparative investigation of antihistamines acting on three histamine receptor subtypes has given insight into specific structural features of antihistamines displaying low micromolar antimalarial activity. It appears that two aromatic ring systems are important and fused aromatic systems, like the tricyclic antihistamines display the lowest IC_{50} values. The imidazole moiety of the H_3 antagonists also appears to be correlated with antimalarial activity. This strongly supports the rationale for drug design of a molecule that contains these essential moieties. This study has also highlighted Log *D* as an important physicochemical parameter in determining the antimalarial activity of antihistamines.

This thesis has described the effect of combining antihistamines with chloroquine at varying concentrations against chloroquine-sensitive and chloroquine resistant strains. Synergism was observed in the chloroquine-sensitive strain with the ethanolamines and tricyclic antihistamines. This may suggest that their independent antimalarial effects produce a synergistic response. Synergism was observed in the chloroquine-resistant strain with the tricyclic antihistamines and chlorpheniramine. Chlorpheniramine did not elicit a synergistic action in the chloroquine-sensitive strain and the H_3 antagonists lacked the ability to elicit any synergistic response in both strains. Hence, the concept of intrinsic antimalarial activity and resistance modulation are two distinct unrelated activities. The isobolograms show that synergism occurs at specific concentrations for different antihistamines.

This work strongly supports the concept that an improved drug can be made by combining elements of CQ and a reversal agent, such as chlorpheniramine. The most interesting of these are the new hybrid molecules containing both antimalarial and resistance reversal pharmacophores. A recommended study would be to test dual acting H_1/H_3 antihistamines for antimalarial and resistance modulation capacity. Work is currently being done with these dual acting antihistamines, including a chlorpheniramine analogue that incorporates the imidazole alkylamine group of many H_3 antagonists (Nelson, 2007). Taken in context of this study, a dual acting H_1/H_3 molecule could be both antimalarial and a resistance reverser.

The antihistamines tested have an effect on parasite morphology exhibiting membrane effects in addition to disintegrating the parasite thereby preventing further growth.

The precise mechanism by which antihistamines inhibit and interact with *P. falciparum* remains unclear, however the experiments described above show that antihistamines appear to act on haemoglobin in the parasites digestive food vacuole, either increasing or decreasing haemoglobin content. This shows that the effect of antihistamines is different to that of chloroquine. It also suggests that different antihistamines may display different modes of action against malaria parasites. Further mechanistic studies are recommended to understand the interaction between antihistamines and the parasite.

In conclusion, this investigation is a comparative study of the potential of different antihistamine classes, including novel H_3 antagonists to combat drug resistant malaria. While much is known about the chemosensitizing effect of these agents, little is known of how antihistamines act in the absence if chloroquine. Thus, this thesis describes for the first time the effect of active antihistamines on the biochemistry and morphology of the plasmodium parasite.

Taken together, the results suggest that the combination of an active antihistamine, either as a fixed-dose preparation or as a covalent conjugate complex with a 4-aminoquinoline can enhance the mode of antimalarial activity of chloroquine. The main objectives of this study have therefore been met.

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APPENDIX 1: D10 (µmol)

1. CHLORPHENIRAMINE

	CQ	75%CQ 25%CHLORPHENIRAMINE	50%CQ 50% CHLORPHENIRAMINE	25%CQ 75%CHLORPHENIRAMINE
IC 50	0.031	0.0315	0.0231	0.0105
FIC CQ		1	0.74	0.34
	DRUG	25%CHLORPHENIRAMINE 75%CQ	50% CHLORPHENIRAMINE 50%CQ	75%CHLORPHENIRAMINE 25%CQ
IC 50	75.8	20.07	43.4	58.4
FIC DRUG		0.26	0.57	0.77
FIC INDEX		**1.26**	**1.31**	**1.11**

2. TRIPROLIDINE

	CQ	75%CQ 25%TRIPROLIDINE	50%CQ 50% TRIPROLIDINE	25%CQ 75% TRIPROLIDINE
IC 50	0.020	0.025	0.022	0.011
FIC CQ		1.25	1.1	0.55
	DRUG	25% TRIPROLIDINE 75%CQ	50% TRIPROLIDINE 50%CQ	75% TRIPROLIDINE 25%CQ
IC 50	83.4	15.4	35.3	58.8
FIC DRUG		0.19	0.42	0.70
FIC INDEX		**1.44**	**1.52**	**1.25**

3. CARBINOXAMINE

	CQ	75%CQ 25%CARBINOXAMINE	50%CQ 50% CARBINOXAMINE	25%CQ 75% CARBINOXAMINE
IC 50	0.019	0.016	0.014	0.0052
FIC CQ		0.84	0.73	0.27
	DRUG	25% CARBINOXAMINE 75%CQ	50% CARBINOXAMINE 50%CQ	75% CARBINOXAMINE 25%CQ
IC 50	42.99	9.57	25.1	28.1
FIC DRUG		0.22	0.58	0.65
FIC INDEX		**1.06**	**1.31**	**0.92**

4. DIPHENHYDRAMINE

	CQ	75%CQ 25%DIPHENHYDRAMINE	50%CQ 50% DIPHENHYDRAMINE	25%CQ 75%DIPHENHYDRAMINE
IC 50	0.019	0.014	0.014	0.007
FIC CQ		0.74	0.74	0.37
	DRUG	25% DIPHENYDRAMINE 75%CQ	50% DIPHENHYDRAMINE 50%CQ	75%DIPHENHYDRAMINE 25%CQ
IC 50	54.0	9.5	28.3	44.29
FIC DRUG		0.17	0.52	0.82
FIC INDEX		**0.91**	**1.26**	**1.19**

5. DOXYLAMINE

	CQ	75%CQ 25%DOXYLAMINE	50%CQ 50% DOXYLAMINE	25%CQ 75% DOXYLAMINE
IC 50	0.011	0.012	0.011	0.013
FIC CQ		1.09	1	1.18
	DRUG	25% DOXYLAMINE 75%CQ	50% DOXYLAMINE 50%CQ	75% DOXYLAMINE 25%CQ
IC 50	683.9	7.73	21.3	73.8
FIC DRUG		0.011	0.03	0.10
FIC INDEX		**1.011**	**1.03**	**1.28**

6. ANTAZOLINE

	CQ	75%CQ 25%ANTAZOLINE	50%CQ 50% ANTAZOLINE	25%CQ 75% ANTAZOLINE
IC 50	0.011	0.011	0.008	0.006
FIC CQ		1	0.73	0.55
	DRUG	25% ANTAZOLINE 75%CQ	50% ANTAZOLINE 50%CQ	75% ANTAZOLINE 25%CQ
IC 50	38.8	5.01	12.6	28.18
FIC DRUG		0.13	0.32	0.73
FIC INDEX		**1.13**	**1.05**	**1.28**

7. PYRILAMINE

	CQ	75%CQ 25%PYRILAMINE	50%CQ 50% PYRILAMINE	25%CQ 75% PYRILAMINE
IC 50	0.014	0.013	0.011	0.005
FIC CQ		0.93	0.79	0.36
	DRUG	25% PYRILAMINE 75%CQ	50% PYRILAMINE 50%CQ	75% PYRILAMINE 25%CQ
IC 50	25.856	7.642	19.146	25.355
FIC DRUG		0.30	0.74	0.98
FIC INDEX		**1.23**	**1.53**	**1.34**

8. TRIPELENAMINE

	CQ	75%CQ 25%TRIPELENAMINE	50%CQ 50% TRIPELENAMINE	25%CQ 75% TRIPELENAMINE
IC 50	0.017	0.017	0.015	0.009
FIC CQ		1	0.88	0.53
	DRUG	25% TRIPELENAMINE 75%CQ	50% TRIPELENAMINE 50%CQ	75% TRIPELENAMINE 25%CQ
IC 50	84.744	11.032	29.899	53.376
FIC DRUG		0.13	0.35	0.63
FIC INDEX		**1.13**	**1.23**	**1.16**

9. CYPROHEPTADINE

	CQ	75%CQ 25%CYPROHEPTADINE	50%CQ 50% CYPROHETADINE	25%CQ 75% CYPROHEPTADINE
IC 50	0.02	0.008	0.003	0.001
FIC CQ		0.4	0.15	0.05
	DRUG	25% CYPROHEPTADINE 75%CQ	50% CYPROHEPTADINE 50%CQ	75% CYPROHEPTADINE 25%CQ
IC 50	6.006	4.569	5.428	5.327
FIC DRUG		0.76	0.90	0.89
FIC INDEX		**1.16**	**1.05**	**0.94**

10. KETOTIFEN

	CQ	75%CQ 25% CYPROHETADINE	50%CQ 50% CYPROHETADINE	25%CQ 75% CYPROHETADINE
IC 50	0.010	0.007	0.002	0.001
FIC CQ		0.7	0.2	0.1
	DRUG	25% CYPROHETADINE 75%CQ	50% CYPROHETADINE 50%CQ	75% CYPROHETADINE 25%CQ
IC 50	5.953	4.027	2.555	5.636
FIC DRUG		0.68	0.43	1
FIC INDEX		**1.38**	**0.63**	**1.1**

11. CETIRIZINE

	CQ	75%CQ 25%CETIRIZINE	50%CQ 50% CETIRIZINE	25%CQ 75% CETIRIZINE
IC 50	0.010	0.009	0.009	0.013
FIC CQ		0.9	0.9	1.3
	DRUG	25% CETIRIZINE 75%CQ	50% CETIRIZINE 50%CQ	75% CETIRIZINE 25%CQ
IC 50	44.177	4.161	11.857	51.274
FIC DRUG		0.09	0.27	1.16
FIC INDEX		**0.99**	**1.17**	**2.46**

12. DESLORATADINE

	CQ	75%CQ 25%DESLORATADINE	50%CQ 50% DESLORATADINE	25%CQ 75% DESLORATADINE
IC 50	0.030	0.009	0.003	0.001
FIC CQ		0.3	0.1	0.03
	DRUG	25% DESLORATADINE 75%CQ	50% DESLORATADINE 50%CQ	75% DESLORATADINE 25%CQ
IC 50	5.415	4.974	4.855	4.591
FIC DRUG		0.92	0.90	0.85
FIC INDEX		**1.22**	**1.00**	**0.88**

13. LORATADINE

	CQ	75%CQ 25%LORATADINE	50%CQ 50% LORATADINE	25%CQ 75% LORATADINE
IC 50	0.019	0.018	0.009	0.002
FIC CQ		0.95	0.47	0.11
	DRUG	25% LORATADINE 75%CQ	/50% LORATADINE 50%CQ	75% LORATADINE 25%CQ
IC 50	7.201	8.125	11.808	10.003
FIC DRUG		1.13	1.64	1.39
FIC INDEX		**2.08**	**2.11**	**1.50**

14. 3-(1H-IMIDAZOL-4-YL)PROPYL-DI(P-FLUOROPHENYL)METHYL ETHER HCL (3H)

	CQ	75%CQ25% (3H)	50%CQ 50% (3H)	25%CQ 75% (3H)
IC 50	0.013	0.010	0.005	0.002
FIC CQ		0.77	0.38	0.15
	DRUG	25%(3H) 75%CQ	50%(3H) 50%CQ	75% (3H)25%CQ
IC 50	7.088	4.759	7.289	8.462
FIC DRUG		0.67	1.03	1.19
FIC INDEX		**1.44**	**1.41**	**1.34**

15. CIPROXIFAN

	CQ	75%CQ 25%CIPROXIFAN	50%CQ 50% CIPROXIFAN	25%CQ 75% CIPROXIFAN
IC 50	0.018	0.013	0.006	0.002
FIC CQ		0.72	0.33	0.11
	DRUG	25% CIPROXIFAN 75%CQ	50% CIPROXIFAN 50%CQ	75% CIPROXIFAN 25%CQ
IC 50	14.009	8.449	11.704	16.240
FIC DRUG		0.60	0.84	1.16
FIC INDEX		**1.32**	**1.17**	**1.27**

16. THIOPERAMIDE

	CQ	75%CQ 25%THIOPERAMIDE	50%CQ 50% THIOPERAMIDE	25%CQ 75% THIOPERAMIDE
IC 50	0.011	0.013	0.012	0.009
FIC CQ		1.18	1.09	0.82
	DRUG	25% THIOPERAMIDE 75%CQ	50% THIOPERAMIDE 50%CQ	75% THIOPERAMIDE 25%CQ
IC 50	171.521	8.097	20.425	47.565
FIC DRUG		0.07	0.17	0.40
FIC INDEX		**1.25**	**1.26**	**1.22**

17. 2-((3-TRIFLUOROMETHYL)PHENYL)HISTAMINE DIMALEATE (TF)

	CQ	75%CQ 25% (TF)	50%CQ 50% (TF)	25%CQ 75% (TF)
IC 50	0.010	0.008	0.006	0.003
FIC CQ		0.8	0.6	0.3
	DRUG	25% (TF) 75%CQ	50% (TF) 50%CQ	75%(TF) 25%CQ
IC 50	12.032	2.733	5.930	8.579
FIC DRUG		0.23	0.49	0.71
FIC INDEX		**1.03**	**1.09**	**1.01**

APPENDIX 2: DD2 (µmol)

1. CHLORPHENIRAMINE

	CQ	75%CQ 25%CHLORPHENIRAMINE	50%CQ 50% CHLORPHENIRAMINE	25%CQ 75%CHLORPHENIRAMINE
IC 50	0.159	0.066	0.094	0.074
FIC CQ		0.42	0.59	0.47
	DRUG	25%CHLORPHENIRAMINE 75%CQ	50% CHLORPHENIRAMINE 50%CQ	75%CHLORPHENIRAMINE 25%CQ
IC 50	202.811	3.887	49.456	40.941
FIC DRUG		0.02	0.24	0.20
FIC INDEX		**0.44**	**0.83**	**0.67**

2. TRIPROLIDINE

	CQ	75%CQ 25%TRIPROLIDINE	50%CQ 50% TRIPROLIDINE	25%CQ 75% TRIPROLIDINE
IC 50	0.206	0.095	0.134	0.066
FIC CQ		0.46	0.65	0.32
	DRUG	25% TRIPROLIDINE 75%CQ	50% TRIPROLIDINE 50%CQ	75% TRIPROLIDINE 25%CQ
IC 50	35.948	5.759	23.891	60.465
FIC DRUG		0.16	0.66	1.68
FIC INDEX		**0.62**	**1.31**	**2.00**

3. PYRILAMINE

	CQ	75%CQ 25%PYRILAMINE	50%CQ 50% PYRILAMINE	25%CQ 75% PYRILAMINE
IC 50	0.196	0.194	0.176	0.027
FIC CQ		0.99	0.90	0.14
	DRUG	25% PYRILAMINE 75%CQ	50% PYRILAMINE 50%CQ	75% PYRILAMINE 25%CQ
IC 50	18.221	10.863	26.659	17.650
FIC DRUG		0.60	1.46	0.97
FIC INDEX		**1.59**	**2.36**	**1.11**

4. CYPROHEPTADINE

	CQ	75%CQ 25%CYPROHETADINE	50%CQ 50% CYPROHETADINE	25%CQ 75% CYPROHETADINE
IC 50	0.117	0.034	0.035	0.030
FIC CQ		0.29	0.30	0.26
	DRUG	25% CYPROHETADINE 75%CQ	50% CYPROHETADINE 50%CQ	75% CYPROHETADINE 25%CQ
IC 50	13.525	2.261	6.392	14.040

FIC DRUG		0.17	0.47	1.04
FIC INDEX		**0.46**	**0.77**	**1.30**

5. KETOTIFEN

	CQ	75%CQ 25% CYPROHETADINE	50%CQ 50% CYPROHETADINE	25%CQ 75% CYPROHETADINE
IC 50	0.139	0.050	0.059	0.063
FIC CQ		0.36	0.42	0.45
	DRUG	25% CYPROHETADINE 75%CQ	50% CYPROHETADINE 50%CQ	75% CYPROHETADINE 25%CQ
IC 50	62.018	2.706	13.658	29.623
FIC DRUG		0.04	0.22	0.48
FIC INDEX		**0.40**	**0.64**	**0.93**

6. CETIRIZINE

	CQ	75%CQ 25%CETIRIZINE	50%CQ 50% CETIRIZINE	25%CQ 75% CETIRIZINE
IC 50	0.154	0.170	0.143	0.260
FIC CQ		1.10	0.93	1.69
	DRUG	25% CETIRIZINE 75%CQ	50% CETIRIZINE 50%CQ	75% CETIRIZINE 25%CQ
IC 50	41.529	7.444	18.969	97.535
FIC DRUG		0.18	0.46	2.35
FIC INDEX		**1.28**	**1.39**	**4.04**

7. DESLORATADINE

	CQ	75%CQ 25%DESLORATADINE	50%CQ 50% DESLORATADINE	25%CQ 75% DESLORATADINE
IC 50	0.162	0.072	0.055	0.031
FIC CQ		0.44	0.34	0.19
	DRUG	25% DESLORATADINE 75%CQ	50% DESLORATADINE 50%CQ	75% DESLORATADINE 25%CQ
IC 50	21.945	4.559	9.616	16.09
FIC DRUG		0.21	0.44	0.73
FIC INDEX		**0.65**	**0.78**	**0.92**

8. LORATADINE

	CQ	75%CQ 25%LORATADINE	50%CQ 50% LORATADINE	25%CQ 75% LORATADINE
IC 50	0.172	0.156	0.174	0.078
FIC CQ		0.91	1.01	0.45
	DRUG	25% LORATADINE	50% LORATADINE	75% LORATADINE

		75%CQ	50%CQ	25%CQ
IC 50	6.27	7.09	20.954	21.252
FIC DRUG		1.13	3.34	3.39
FIC INDEX		**2.04**	**4.35**	**3.84**

9. 3-(1H-IMIDAZOL-4-YL)PROPYL-DI(P-FLUOROPHENYL)METHYL ETHER HCL

	CQ	75%CQ 25% (3H)	50%CQ 50% (3H)	25%CQ 75% (3H)
IC 50	0.131	0.252	0.125	0.033
FIC CQ		1.92	0.95	0.25
	DRUG	25% (3H) 75%CQ	50% (3H) 50%CQ	75% (3H) 25%CQ
IC 50	15.523	11.973	17.713	14.202
FIC DRUG		0.77	1.14	0.91
FIC INDEX		**2.69**	**2.09**	**1.16**

10. CIPROXIFAN

	CQ	75%CQ 25%CIPROXIFAN	50%CQ 50% CIPROXIFAN	25%CQ 75% CIPROXIFAN
IC 50	0.035	0.032	0.026	0.022
FIC CQ		0.91	0.74	0.63
	DRUG	25% CIPROXIFAN 75%CQ	50% CIPROXIFAN 50%CQ	75% CIPROXIFAN 25%CQ
IC 50	13.110	2.585	5.135	12.888
FIC DRUG		0.20	0.39	0.98
FIC INDEX		**1.11**	**1.13**	**1.61**

11. THIOPERAMIDE

	CQ	75%CQ 25%THIOPERAMIDE	50%CQ 50% THIOPERAMIDE	25%CQ 75% THIOPERAMIDE
IC 50	0.055	0.054	0.044	0.069
FIC CQ		0.98	0.80	1.25
	DRUG	25% THIOPERAMIDE 75%CQ	50% THIOPERAMIDE 50%CQ	75% THIOPERAMIDE 25%CQ
IC 50	12.416	31.316	7.756	36.281
FIC DRUG		2.52	0.62	2.92
FIC INDEX		**3.50**	**1.42**	**4.17**