# CHARACTERISATION OF MEFLOQUINE ACCUMULATION IN *PLASMODIUM FALCIPARUM*

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## ABSTRACT

### Characterization of Mefloquine Accumulation in Plasmodium falciparum

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Mefloquine has been in use for over twenty years and still very little is known about its interaction with *Plasmodium falciparum*. In 1979, Fitch *et al* carried out the only other published extensive investigation of mefloquine accumulation, but were not able to demonstrate energy dependent uptake. They later indicated that an energy requirement may be being masked by mefloquine's ability to bind membrane phospholipids to a large extent (Chevli & Fitch, 1982).Until now no energy requirement for mefloquine accumulation has been uncovered.

This thesis investigates the relationship between chloroquine and mefloquine resistance, and characterizes the mechanism of mefloquine accumulation in *Plasmodium falciparum*. Conditions were established that enabled the amplification of the parasites' contribution to overall mefloquine accumulation in the parasitised erythrocyte. It was found that mefloquine accumulation is stimulated by glucose and is inhibited by the glycolysis inhibitor, iodoacetate, and also by incubation at low temperature. Mefloquine accumulation was also found to be partly dependent on the pH gradient between the acidic food vacuole and the external medium. It has also been determined that mefloquine-resistant *Plasmodium falciparum* accumulate approximately half the amount of mefloquine than do mefloquine-sensitive parasites.

It has been shown that the accumulation of both chloroquine and mefloquine have two components, a high affinity saturable component and a low affinity non-saturable component (Fitch *et al.*, 1979; Fitch *et al.*, 1974; Bray *et al.*, 1998). The saturable component has been well characterized, but until now the non-saturable component has not been identified. This thesis shows that chloroquine and mefloquine adsorption to synthetic β-haematin and pure isolated haemozoin is non-saturable. It is proposed that the malaria pigment is responsible for the low affinity, non-saturable component of chloroquine and mefloquine accumulation.

The effect of chloroquine, mefloquine and artemisinin on haemoglobin levels in parasitised erythrocytes was also measured. Chloroquine caused a buildup in haemoglobin and mefloquine caused a decrease in haemoglobin levels. This adds weight to previously published work (Famin & Ginsburg, 2002) suggesting that chloroquine prevents the degradation of haemoglobin, while mefloquine inhibits the endocytosis of haemoglobin.

## DECLARATION

I, Jason Walden, hereby declare that the work on which this thesis in based is my original work (except where acknowledgements indicate otherwise, and that neither the substance nor any part of this work has been, is being or is to be submitted for another degree at this university or at any other university.

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This thesis is presented for examination for the degree of Doctor of Philosophy.



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Date

## TABLE OF CONTENTS

## LIST OF FIGURES

### CHAPTER 1



### CHAPTER 2



### CHAPTER 3

**CHAPTER 4**



**CHAPTER 5**



**CHAPTER 6**

**CHAPTER 7**



**CHAPTER 9**

## LIST OF PLATES

### CHAPTER 2



### CHAPTER 3



### CHAPTER 6



### CHAPTER 7

## LIST OF TABLES

### CHAPTER 2



### CHAPTER 3



### CHAPTER 4



### CHAPTER 6



### CHAPTER 9

## LIST OF ABBREVIATIONS

| | |
|---|---|
| [ ] | Concentration |
| ABC | ATP-binding cassette |
| AO | Acridine Orange |
| APADH | 3-Acetylpyridine adenine dinucleotide |
| AQ | Amodiaquine |
| ART | Artemisinin |
| ATP | Adenosine Triphosphate |
| ATPase | Adenosine triphosphatase |
| BSA | Bovine serum albumin |
| CAR | Cellular Accumulation Ratio |
| CCCP | Carbonyl-cycanide meta-chlorophenyl-hydrozone |
| CPM | Counts per minute |
| CQ | Chloroquine |
| CQS | Chloroquine-sensitive |
| CQR | Chloroquine-resistant |
| DDT | Dichloro-diphenyl-trichloroethane |
| DNA | Deoxyribonucleic acid |
| DNase1 | Deoxyribonuclease 1 |
| FPIX | ferriprotoporphyrin IX |
| f | femto |
| G | Gauge |
| g | grams |
| GSH | Glutathione |
| h | Hour/s |
| HEPES | N-[2-hydroxyetyl]piperazine-N'-[2-ethansulphonic acid] |
| HF | Halofantrine |
| $IC_{50}$ | Drug concentration that inhibits the growth of 50 % of the parasite population |
| kDa | Kilodalton |
| $K_m$ | Michaelis-Menten constant. Concentration at which the velocity is half maximal. |

| | |
|---|---|
| M | Molar |
| m | Milli |
| μ | Micro |
| MDR | Multi-drug resistant |
| min | Minute/s |
| ml | Milliliter |
| MQ | Mefloquine |
| n | nano |
| NBD-Cl | 7-chloro-4-nitrobenzo-2-oxa-1,3-diazol |
| NBT | Nitro blue tetrazolium |
| NEM | N-ethylmaleimide |
| $NH_4Cl$ | Ammonium Chloride |
| NHE | $Na^+/H^+$ exchanger |
| °C | Degrees Celcius |
| p | pico |
| PBS | Phosphate buffered saline |
| PDN | Pyronaridine |
| *P.falciparum* | *Plasmodium falciparum* |
| PRBC | Parasitised red blood cell |
| Pst | Parasitemia |
| QUI | Quinine |
| RBC | Red blood cells |
| rpm | Revolutions per minute |
| SDS-PAGE | Sodium Dodecyl sulphate-polyacrylamide gel electrophoresis |
| trophs | Trophozoites |
| V-ATPase | Vacuolar-adenosine triphosphatase |
| $V_{max}$ | Maximum velocity |
| WHO | World Health Organisation |

# Chapter 1

# Introduction

### *1.1 Background*

Although cases of malaria have been documented in China as far back as 2700 B.C., malaria was only given its name in the early sixteenth century by the Italians. The disease's association with stagnant bodies of water prompted them to term the disease "mal'aria" (bad air). 200 years later "malaria" entered the English language to describe this scourge which kills between 1 and 2 million people annually. Malaria is caused by a protozoan parasite of the genus *Plasmodium,* 4 species infect humans and cause morbidity in 200 million people every year. *P.vivax*, *P.ovale* and *P.malariae* infect humans but are rarely lethal, while *P.falciparum* is responsible for almost all malaria fatalities, which are mostly in Africa (Foley & Tilley, 1998; Oaks *et al.*, 1991).



The immunological status of the individual determines the severity of an infection, therefore children and pregnant women are most at risk. Individuals living in malaria endemic regions, repeatedly exposed to malaria, develop semi-immunity. Many of these individuals remain asymptomatic, providing a large pool of undetected parasites, and contribute greatly to the transmission of malaria. Malaria manifests clinically in patients with shaking chills progressing to fevers and drenching sweats. Approximately 80% of deaths are as a result of

cerebral malaria caused by *P.falciparum*. Other complications that often result in death due to Plasmodium infection include renal failure, hypoglycaemia, severe anaemia, pulmonary oedema and shock (Oaks *et al.*, 1991).

## *1.2 Parasite Biology*

### *1.2.1 Life Cycle*

Plasmodium parasites have a complex life cycle involving at least 12 morphologically distinct stages within its two hosts. Two phases occur in the human host, and one in the female *Anopheles* mosquito. While the mosquito is biting a human to obtain its blood meal, malaria parasites are transmitted to the human host as sporozoites in the mosquito's saliva. Once in the bloodstream the sporozoites migrate rapidly to the liver where they invade hepatocytes. The sporozoites mature into hepatic schizonts within 5 to 15 days of infection. The schizonts divide asexually to produce 10 000 to 30 000 merozoites, infected liver cells lyse and the merozoites are released into the bloodstream. The targets of these merozoites are red blood cells. Once a merozoite has invaded a red blood cell it develops into a ring-stage parasite. The rings then mature into trophozoites and further into schizonts which again undergo asexual division to produce in 8 to 32 merozoites. The erythrocyte is lysed and the merozoites are released to invade additional red blood cells. This is the intra-erythrocytic cycle (Figure 1.1), the focus of a great deal of research because it is the phase that most antimalarial drugs target.

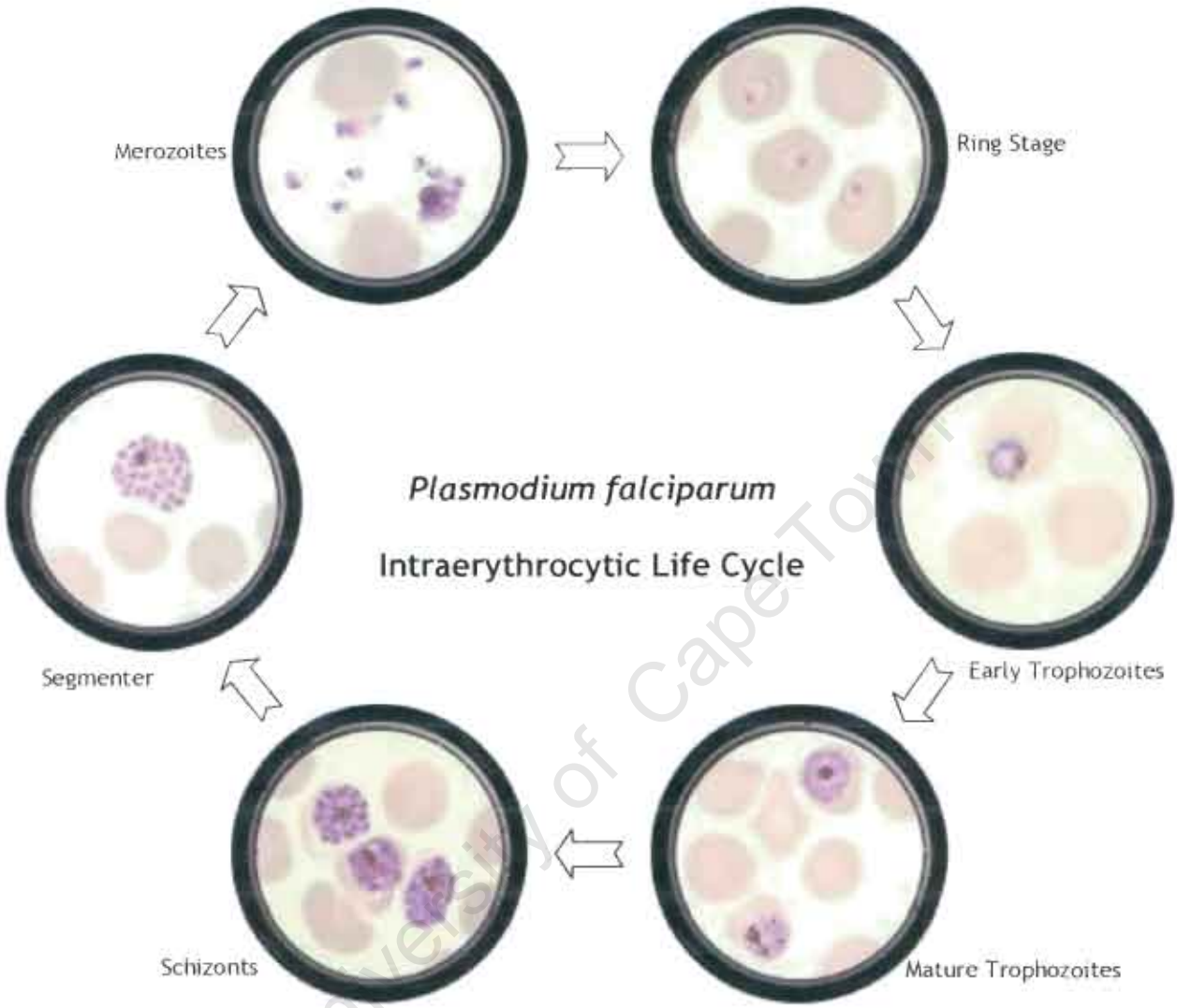





**Figure 1.1:** Diagram showing Giemsa-stained thin smears of the life cycle of *Plasmodium falciparum*.

Merozoites may differentiate and develop into gametocytes. It is this stage that survives ingestion by the mosquito during a blood meal. Male and female gametocytes fuse to become a zygote within the midgut of the mosquito. Over the next 12 to 48 hours an ookinete is

formed, which attaches to the mosquito stomach wall and penetrates the lining to become an oocyst. The parasites undergo a third round of asexual reproduction, during which the oocyst develops into more than 10 000 sporozoites. The oocyst then bursts and the sporozoites are released to migrate to the salivary glands, from which they will be injected into another human host during the mosquito's next blood meal.

### *1.3 Malaria Control*

From the mid-1940s through to the late 1950s a substantial malaria eradication campaign was undertaken. This campaign was successful in North America and Europe, but in malaria-endemic developing countries it became clear that eradication would be impossible, and there was a policy shift to malaria control instead (Foley & Tilley, 1997).

Malaria control requires a multi-pronged strategy, which may include a combination of drugs, vector control and vaccine components.



#### *1.3.1 Drugs*

The use of antimalarial drugs is divided into two categories, preventative and therapeutic. Some drugs, including doxycycline, proguanil, pyrimethamine and primaquine, target the liver stage parasites. Other drugs, such as chloroquine (CQ), mefloquine (MQ), quinine, sulfadoxine-pyrimethamine and artemisinin target the intraerythrocyctic cycle. In addition the artemisinin derivatives are able to kill gametocytes (Olliaro, 1999; Foley & Tilley, 1998). Clearly if one is able to successfully treat malaria cases, the parasite population within the

human population will decrease and thereby reduce the transmission of the disease. The number of effective drugs that are currently available for use is dwindling because of an increase in antimalarial drug resistance. Antimalarial drugs will be discussed in detail.

### *1.3.2 Vector Control*

There are a number of common ways that transmission by mosquitoes is interrupted:

- Insecticide Spraying - indoor and outdoor. However in many parts of the world mosquitos have become resistant to pyrethroid group of insecticides.
- Larviciding - the treatment of mosquito larval development sites with chemical or microbiological larvicides or with other biological agents such as lavivorous fishes.
- Source reduction - reducing larval development sites, mostly by removing stagnant bodies of water.
- Contact reduction - the use of protective clothing, mosquito coils, insect repellents and particularly insecticide-impregnated bednets and curtains have been shown to be effective in reducing human-mosquito contact (Oaks *et al.*, 1991).



### *1.3.3 Vaccines*

The rapid development of insecticide and drug resistance has resulted in increased interest in the development of a malaria vaccine. Indeed there is a great deal of work being carried out in this field (Anders & Saul, 2000). There are 3 stages in the complex *Plasmodium* life cycle that are being targeted:

- the pre-erythrocytic stage
- the sporozoites

- the liver stage

These are the best funded areas and therefore most studied, because there is more potential application for protection for short term travellers from developed countries to malaria areas. Putative vaccines against this stage have so far failed mainly due to large epitope polymorphisms and low immunogenicity (Anders & Saul, 2000).

Asexual blood stage vaccines hope to reproduce the semi-immunity that occurs in adults in endemic areas, via an antibody-mediated response to repeated exposure. A largely publicised candidate was SP166, which underwent large scale clinical trials and proved ineffective (Guerin *et al.*, 2002). Currently the leading candidates targeting the asexual intraerythrocytic stage are merozoite surface protein (MSP-1) and apical membrane antigen (AMA-1). Both have had some success in animal studies, but thus far have not been shown to be effective in immunising humans (Guerin *et al.*, 2002).

Vaccines that target the gamete stage of the parasite aim to prevent the mosquito from acquiring and transmitting the parasite. This benefits the community but not infected individuals. There have been some promising results in models but so far this approach is limited by low immunogenicity and a lack of natural boosting (Guerin *et al.*, 2002).

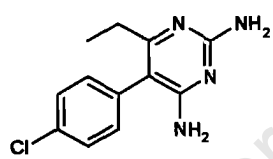
A number of serious vaccine candidates have been identified, and it is now necessary to test these in large scale clinical trials. One of the rate limiting factors of this area of research has been in the preparation of antigens for testing. Researchers have struggled to achieve high levels of expression of the proteins of interest (Anders & Saul, 2000).

### *1.4 Antimalarial Drugs*

The major classes of antimalarial drugs will be discussed briefly, and the quinoline antimalarials will then be discussed in detail.

#### *1.4.1 The folate inhibitors*

Pyrimethamine, sulfadoxine and proguanil are the most common antifolate drugs. These agents target the essential folate pathway of the parasite, more specifically the dihydrofolate reductase and dihydropteroate synthetase enzymes. Disruption of this pathway leads to the disruption of DNA synthesis. These molecular targets have long been established and the mechanisms of resistance are also well characterised (Hyde, 2002).



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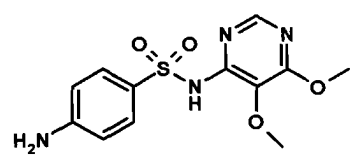

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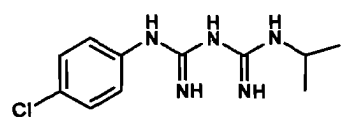


**(3)**

**Figure 1.3:** The chemical structures of pyrimethamine **(1)**, sulfadoxine **(2)** and proguanil **(3)**.

The failure of these drugs has been attributed almost entirely to a stepwise series of mutations in the genes coding for the above enzymes. As a result of the ease at which resistance develops these drugs are never administered separately; for example, pyrimethamine is almost always used in combination with PABA (para-amino benzoic acid) antimetabolites like sulfadoxine or dapsone.

### *1.4.2 Artemisinin derivatives*

The artemisinin class of antimalarials is derived from a Chinese herb (*Artemisia annua*) and reduces parasitemia faster than any other known antimalarial. Unlike the quinoline drugs, they are active against all of the asexual blood stages of the parasite and against gametocytes (Olliaro *et al.*, 2001).



These compounds are sesquiterpine lactones, which carry an epoxide bridge across the seven-membered ring (Figure 1.3). Studies have demonstrated the peroxide bridge is essential for the action of this class of drugs, and also that the modulation of oxidative stress may be the basis of its antimalarial action. The focus of work in this area has been to determine whether the peroxide bridge is able to act as a source of reactive oxygen species. The biological significance of the interaction of free radicals with biomolecules (e.g. haem, proteins, etc.), lipids and membrane bilayers is well established (Olliaro *et al.*, 2001; Meshnick, 1996). There is evidence that artemisinin is able to interact with free haem and haemozoin (Hong *et al.*, 1994). Relatively little is known about the exact mechanism of artemisinin action. Interestingly, despite there being very little clinical artemisinin resistance reported, there is evidence that there may be cross resistance with the mefloquine (MQ) and halofantrine (HF)

(Doury *et al.*, 1992). A study has indicated that these drugs may share aspects of their drug resistance mechanisms (Reed *et al.*, 2000).

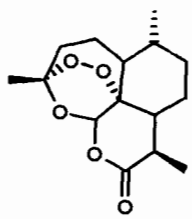


**Figure 1.3:** The chemical structure of artemisinin. Epoxide bridge indicated in red.



### *1.4.3 Quinoline Drugs*

For centuries the bark of the Chinchona tree was used by the indigenous people of Peru to treat fevers. In 1820, the active ingredient was isolated and identified as quinine, which became the mainstay of malaria treatment for the next 130 years (Egan, 2001).

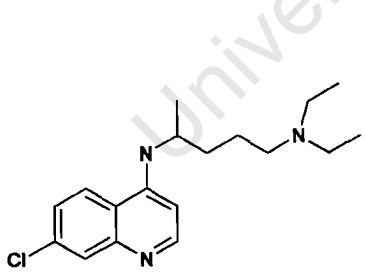
Since the synthesis of chloroquine in the 1940s, and other, safer quinoline derivatives, the use of quinine has decreased substantially and is now a last line treatment option for severe multidrug resistant malaria (Foley & Tilley, 1997; Raynes, 1999).

### *1.4.3.1 Chloroquine*

CQ was first synthesised in the 1920s as part of the German antimalarial medicinal chemistry program, but was rejected because it was thought to be too toxic. A 3-methyl analogue of CQ, called sontoquine, was instead chosen for development.

During World War II, German supplies of sontoquine were captured by the Allies. This resulted in renewed interest in the 4-aminoquinolines. Chloroquine was re-identified as an effective antimalarial by the American drug screening program during World War II. The drug was subsequently developed to become a safe, inexpensive and, in the absence of resistance, a highly effective antimalarial drug.

However, in the last 3 decades resistance has caused the efficacy of CQ to diminish remarkably, to the extent that in many areas of malaria endemicity it is largely ineffectual. It remains a first line treatment only in areas where financial and political factors do not allow the switch to a more effective drug (Foley & Tilley, 1998).

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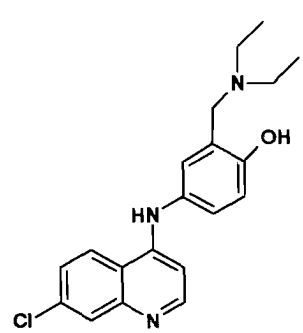



(2)

**Figure 1.3:** The chemical structures of the 4-aminoquinolines, chloroquine **(1)** and amodiaquine **(2)**.

#### *1.4.3.1.1 Mechanism/s of Accumulation of Chloroquine*

Chloroquine must enter the parasitised red blood cell (PRBC) in order to kill the malaria parasite. It is therefore important while discussing the mechanism of action of this drug to also consider its mechanism of entry and accumulation into the PRBC. It is clear that CQ accumulates in PRBCs to an extent at least two orders of magnitude greater than in uninfected erythrocytes (Yayon *et al.*, 1984b; Geary *et al.*, 1986; Krogstad *et al.*, 1987; Yayon *et al.*, 1984c). Furthermore, one of the few consistent distinctions between chloroquine-resistant (CQR) and chloroquine-sensitive (CQS) *P.falciparum* is that CQR parasites accumulate less CQ than CQS parasites (Krogstad *et al.*, 1987; Fitch *et al.*, 1974; Yayon *et al.*, 1984a). Therefore, the importance of CQ accumulation in the PRBC cannot be underestimated, in both the mechanism of action and the mechanisms of resistance to this drug. The mechanism of CQ accumulation is not fully understood; several theories are listed below and each will be discussed in detail.

- pH driven CQ accumulation
- Carrier mediated accumulation
- Haematin as an intracellular receptor driving the uptake of CQ



##### *1.4.3.1.1.1 pH-driven CQ accumulation*

An important factor in the accumulation of CQ is the fact that it is a diprotic weak base, with pKa values of 8.3 and 10.2. This is significant in the milieu of the PRBC because the pH of the parasite's cytoplasm and the extracellular environment is approximately 7.4. At this pH, a

proportion of the CQ is unprotonated and is therefore able to freely permeate membranes and distribute across all cellular compartments. However on entering the digestive food vacuole, whose pH is between 5.2 and 5.6 (Dzekunov *et al.*, 2000), the molecule will become mostly diprotonated. This will make it less membrane permeable, trapping it in the food vacuole. This proton gradient provides an effective CQ concentrating mechanism and accumulation will continue until the proton pumping capacity (buffering capacity) of the vacuole has been exceeded.

Once the buffering capacity of the vacuole is exceeded the pH of the food vacuole will increase. This pH increase, may inhibit the action of the vacuolar proteases (De Duve *et al.*, 1974), preventing the digestion of haemoglobin, which is an important nutrient supply for the parasite. The ability of CQ to accumulate due to this proton gradient is dependent on estimates of ΔpH, CQ concentration, the Henderson-Hasselbach equation and on the relative permeabilities of the neutral, mono- and diprotonated CQ species. There is a great deal of evidence supporting the contention that the accumulation of CQ is based predominantly on its weak base properties. Certain metabolic inhibitors, including the vacuologenic amine $NH_4Cl$ and the ionophore nigericin, have been shown to inhibit CQ accumulation. It was therefore proposed that an ATP driven pump controls pH and hence CQ uptake (Krogstad & Schlesinger, 1986; Vanderkooi *et al.*, 1988). This would occur in a similar manner to the mechanism by which mammalian lysosomal pH is maintained by translocation of protons to the interior of lysosomes at the expense of ATP (Schneider, 1981).

Evidence in support of this theory was provided by Choi and Mego when they reported that NEM and NBD-Cl, both classic proton pump inhibitors, inhibited an $Mg^{2+}$-dependent

ATPase isolated from the vacuolar membrance of *P. falciparum* (Choi.I & Mego, 1988). In addition two genes from *P. falciparum* were sequenced and showed significant homology with the A and B subunits of the vacuolar ATPase from a wide range of organisms (Karcz *et al.*, 1993b; Karcz *et al.*, 1994). Another important observation is that external pH has a profound effect on CQ accumulation. When the external pH is increased, the steady state accumulation increases, and conversely, when the external pH is decreased the CQ accumulated decreases (Yayon *et al.*, 1985). This influence of external pH on CQ accumulation is mirrored by the influence of external pH on the *in vitro* potency of CQ.

In addition, experiments designed to increase the pH of the vacuole itself have also provided evidence supporting the importance of the proton motive force in CQ accumulation. Bafilomycin A1, a proton pump inhibitor has been shown to reduce uptake and toxicity of CQ in *P. falciparum* (Bray *et al.*, 1992b). Ammonium chloride is a weak base, which accumulates in acidic compartments and increases the pH of the compartment. It has been shown to have a similar effect to Bafilomycin A1 (Yayon *et al.*, 1985). It is clear that there is substantial evidence supporting the theory that CQ uptake is solely dependent on the pH gradient across the vacuolar membrane. However, there are other studies that indicate that the extent of CQ accumulation cannot be explained by a predominantly pH driven mechanism (Krogstad & Schlesinger, 1986; Krogstad & Schlesinger, 1987; Bray *et al.*, 1996b; Hawley *et al.*, 1996). Some of the other proposed mechanisms of 4-aminoquinoline accumulation will be discussed below.

*1.4.3.1.1.2 Carrier mediated accumulation*

Carrier mediated accumulation is based on the assertion that the host cell pH is higher than the parasite cytosolic pH (Friedman, 1978; Mikkelson *et al.*, 1982). It has been proposed that the drug diffuses freely from outside the RBC (pH 7.4) into the RBC (pH 6.6) (Warhurst, 1988), where it becomes protonated and therefore membrane impermeable. A permease then mediates the transport of CQ into the parasite cytosol (pH 7.6) where it loses its protons. The drug is then able to travel down a pH gradient into the acidic food vacuole (pH 5.0), where it is once again protonated and trapped (Warhurst, 1986; Warhurst, 1988).

However, doubt has been cast on the validity of the above-mentioned theory. Several researchers have disputed the accuracy of the pH estimations (Yayon *et al.*, 1984a; Geary *et al.*, 1986). A decade later new support for carrier mediated transport was put forward by Sanchez and co-workers (Sanchez *et al.*, 1997) when they demonstrated that several amiloride derivatives, including EIPA (5-(N,N-dimethyl)amiloride), were able to competitively inhibit CQ uptake. In other eukaryotic systems, the amiloride derivatives specifically block the $Na^+$ binding site on $Na^+/H^+$ exchangers (NHE), interfering with the regulation of cytoplasmic pH and cell volume. It was proposed that CQ was imported via a NHE in exchange for protons (Wunsch *et al.*, 1998). It was claimed that this could account for the energy dependent, saturable uptake of CQ.

Strong evidence refuting this proposed mechanism of CQ uptake was provided when researchers carried out a series of CQ uptake experiments in sodium-free buffer. If the NHE

theory is correct then removal of the competing substrate ($Na^+$) would increase CQ uptake. However, it was shown that uptake decreased. Further, CQR and CQS parasites retained their relative accumulation characteristics and also the effect of chemosensitisers on the CQR strain was retained (Bray *et al.*, 1998; Bray *et al.*, 1999). It was also demonstrated that the amiloride derivatives, which are inhibitors of the NHE, displace CQ that is bound to ferriprotoporphyrin IX (FPIX) (Bray *et al.*, 1999). This may account for the findings that these drugs decrease the uptake of CQ into PRBCs.

*1.4.3.1.1.3 Haematin as an intracellular receptor driving the uptake of CQ*

The malaria parasite ingests haemoglobin by endocyctosis. It is transported to the food vacuole where it is digested by a several proteases to provide amino acids as nutrients to the parasite. A by-product of this haemoglobin metabolism is FPIX, much of which is crystallised to haemozoin.

Over thirty years ago, Fitch demonstrated that there are two components to CQ accumulation in malaria parasites. At nanomolar concentrations there is a saturable component and at higher concentrations there is a nonsaturable component (Fitch, 1970). It has been suggested that the nonsaturable component is due to low affinity binding of CQ to cyctosolic proteins (Menting *et al.*, 1997; Dorn *et al.*, 1998a). It was suggested as far back as 1980, by Chou *et al,* that the complexing of chloroquine to free monomeric FPIX may be responsible for the uptake of CQ into the food vacuole (Chou *et al.*, 1980b). It is clear that some chloroquine/FPIX binding occurs in the parasite.

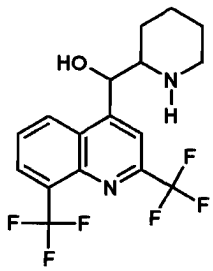
Numerous studies have shown that *in vitro* CQ is able to form a complex with FPIX (Chou *et al.*, 1980a; Warhurst, 1987; Blauer & Ginsburg, 1982). Radiolabelled quinolines were found to be associated with haemozoin after prolonged incubation with sublethal concentrations of the drugs (Sullivan *et al.*, 1996b). Also intracellular interactions between quinolines and FPIX have also been detected by photoaccoustic spectroscopy (Balasubbramanian *et al.*, 1984). Bray (1998) has provided convincing evidence that the binding of CQ to haematin may in fact be the driving force for the drug's accumulation, also that the high affinity saturable component of CQ accumulation is responsible for the antiplasmodial action of CQ.

Bray and co-workers made use of a specific inhibitor of haemoglobin digestion, Ro 40-4388. This compound inhibits Plasmepsin I, the enzyme responsible for the initial cleavage of haemoglobin and subsequent release of free heme, which gets oxidised immediately to haematin (Francis *et al.*, 1996). The action of this inhibitor effectively decreases that amount of free haematin available for CQ to bind. Bray and co-workers demonstrated that in the presence of Ro 40-4388, CQ accumulation in PRBC is decreased in a dose-dependent manner, demonstrating that it effectively reduces the number of CQ binding sites.

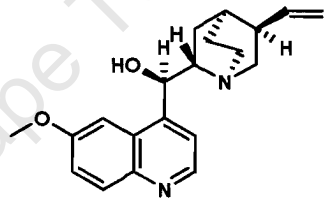
Ro 40-4388 has no effect on the accumulation of other weak bases that do not bind to FPIX. This disputes the contention that it is an increase in vacuolar pH that is the principle mechanism of action of these quinolines (Bray *et al.*, 1998).

### *1.4.3.2 Mefloquine*

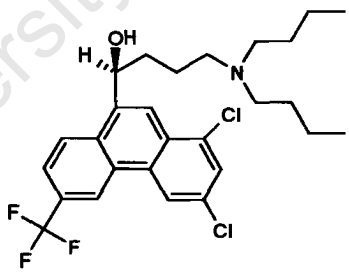
Because CQ failed to effectively protect American soldiers from malaria during the Vietnam War, the United States government started large scale screening of potential antimalarial drugs. Of the over 300 000 compounds screened, the best candidate was WR-142490, a quinolinemethanol structurally related to quinine (Peters *et al.*, 1977). This drug was subsequently named mefloquine (MQ) and has been used successfully for the last 30 years. Resistance has however developed in certain areas, and there have also been concerns regarding its toxicity (Foley & Tilley, 1998).




**(1)**




**(2)**




**(3)**

**Figure 1.4:** The chemical structures of the quinolinemethanols mefloquine **(1)** and quinine **(2)**, and the related phenanthrene, halofantrine **(3)**.

#### *1.4.3.2.1 Mechanism/s of Accumulation of Mefloquine*

The accumulation of weak bases in the food vacuole of the malaria parasite is dependent on the difference in pH between the extracellular medium and the food vacuole as well as the $pK_a$ values of the drugs concerned. A significant proportion of CQ is diprotonated at physiological pH, and since its accumulation is expected to be related to the square of the pH gradient, it should accumulate to a large extent. Mefloquine is mono-protonated and would be expected to accumulate to a much lesser degree than CQ. However, the accumulation of MQ in PRBC is a somewhat anomalous process, because MQ accumulates in the PRBC to a much larger extent than CQ (Vanderkooi *et al.*, 1988; Fitch *et al.*, 1979).

Fitch et al (1979) carried out MQ accumulation experiments in mouse erythrocytes infected with *Plasmodium berghei*. They observed that MQ accumulation was saturable, but was not inhibited by azide; iodoacetate; incubation at 2°C; or the absence of glucose. This would seem to indicate that the accumulation is not energy dependent. They also noted that uninfected erythrocytes accumulated more than half the amount of MQ accumulated by PRBCs, as opposed to CQ, which accumulates in trace amounts in uninfected RBCs. They also showed that CQ is able to inhibit MQ accumulation; MQ is able to inhibit CQ accumulation; and that CQS and CQR parasites accumulate the same amount of MQ. Fitch *et al.* hypothesised that this indicated that MQ had better access to quinoline receptors than CQ in CQR parasites (Fitch *et al.*, 1979).

In a stereospecific (using different enantiomers of MQ) study of MQ accumulation in RBCs, it was shown that MQ accumulation is complete in 5 seconds. MQ accumulation was also pH

dependent - at lower pH, less MQ accumulated. This could be explained by the fact that at lower pH MQ is more protonated ($pK_{a1}$ = 8.6), making it less membrane soluble. This could result in a decrease in passive diffusion (Vidriquin *et al.*, 1996). The authors also found that the MQ concentration inside RBCs increased when the accumulation experiments were performed in HEPES buffer as opposed to serum. This confirmed a high degree of serum protein binding by mefloquine. After RBC lysis it was shown that almost all of the MQ accumulated was accounted for by RBC membrane binding.

Vidrequin *et al.* also confirmed Fitch's finding that RBCs accumulate approximately half the amount of MQ taken up by PRBCs (Vidrequin *et al.*, 1996). Chevli and Fitch (1982) demonstrated that phospholipids account for almost all of the MQ that binds to membranes, whereas CQ does not appreciably bind to phospholipids. They also showed that MQ binds with high affinity to FPIX. This high degree of phospholipid binding may explain the large accumulation of MQ and also the fact that MQ accumulates similarly in CQR and CQS *P.berghei.* It was also suggested that high level of phospholipid binding may obscure an energy requirement for MQ accessing its FPIX receptor (Chevli & Fitch, 1982).

In an effort to distinguish between pH-dependent and pH-independent MQ and CQ accumulation, Vanderkooi and co-workers (1988) investigated drug accumulation in the presence and absence of a series of ionophores. After removing the proton gradient and electrical potentials across membranes, they claimed that 40-60% of MQ uptake and 90% of CQ uptake was pH dependent. It was also confirmed that MQ accumulates to a much greater extent than CQ in the PRBC. They proposed that MQ accumulation is driven not only by the

proton gradient, but also by an antiporter protein that couples drug uptake to proton outflow, but that CQ uptake is driven purely by the proton pump (Vanderkooi *et al.*, 1988).

CQ and MQ are both weak base quinoline drugs and it is apparent that there are similarities in their mechanisms of accumulation in PRBCs, the pH dependent nature of accumulation being the obvious similarity. The major difference in the accumulation of these related drugs is the fact that MQ is highly membrane-bound. This results in far more MQ accumulating in the PRBC than is predicted by the proton trapping hypothesis.

From the above discussions it is clear that an understanding of the mechanism of accumulation of these quinolines in the PRBC is of utmost importance in understanding the mechanism of action of the drugs. Another important process in parasite metabolism and also in the action of quinolines, is the breakdown of haemoglobin and the detoxification of its by-products. These processes will be discussed in detail below.



### *1.5 Haemoglobin Degradation in Plasmodium falciparum*

During the 48 hours of the pathogenic intraerythrocytic part of the *Plasmodium* life cycle, the parasite relies to a large extent on ingested haemoglobin as a source of amino acids. It is estimated that between 25% to 75% of the host RBC haemoglobin is degraded by the parasite (Zarchin *et al.*, 1986; Vander Jagt *et al.*, 1992; Gluzman *et al.*, 1994; Francis *et al.*, 1997; Krugliak *et al.*, 2002; Egan *et al.*, 2002). Most of the haemoglobin degradation occurs in the late trophozoite stage (Egan *et al.*, 2002; Goldberg & Slater, 1992).

The parasite ingests host cell cytoplasm, including haemoglobin by endocyctosis via the cytostome. A vesicle containing the host cyctoplasmic contents is pinched off from the terminal portion of the cytostome and is carried to the food vacuole (Slomianny, 1990). The transport vesicle then fuses with the food vacuole and the haemoglobin is emptied into the lumen of the vacuole. (Yayon *et al.*, 1984c; Krogstad *et al.*, 1985). There is debate as to whether the breakdown of haemoglobin starts in the transport vesicles, or whether it begins only inside the vacuole.

Haemoglobin subsequently undergoes a sequential degradative process involving two aspartic proteases (plasmepsin I and II), 1 cysteine protease (falcipain) and a metallopeptidase (falcilysin) (Goldberg & Slater, 1992; Francis *et al.*, 1997; Gluzman *et al.*, 1994; Eggleson *et al.*, 1999). The breakdown products are largely amino acids or short peptides, which are removed to the parasite cytoplasm for the synthesis of parasite proteins (Sherman, 1997; Zarchin *et al.*, 1986), and FPIX. FPIX, which is toxic to the parasite, must be dealt with before it is allowed to accumulate to levels that are lethal to the parasite. (Chou *et al.*, 1980a; Orjih *et al.*, 1981; Fitch *et al.*, 1982; Fitch *et al.*, 1983).



### *1.5.1 The detoxification of FPIX in P. falciparum*

In recent years there has been debate regarding how malaria parasites escape the lytic effects of haem. For many years it was known that free FPIX is incorporated into haemozoin or malaria pigment, and it was assumed that this was the predominant mechanism of haem detoxification in malaria parasites. However in 1998 Ginsberg claimed that only 30% of FPIX generated by haemoglobin breakdown is crystalised to haemozoin. The remaining 70%

of FPIX according to this postulate exits the food vacuole and is degraded by cytosolic GSH (Ginsburg *et al.*, 1998). Loria *et al* (1999) concurred, demonstrating that approximately a third of the free haem generated is incorporated into haemozoin. However, their theory argued that the haem remains in the food vacuole and is destroyed by peroxidative decomposition by $H_2O_2$, which is generated during the oxidation of Fe(II) haem to Fe(III) haem(Loria *et al.*, 1999).

Recently Egan *et al* (2002) accounted for 95% of the haem iron in *P. falciparum*. They showed that 61% of the iron within the PRBC is in the trophozoite, and that 92% of that iron is found inside the food vacuole. 88% of the vacuolar iron was shown to be associated with the haemozoin. It was also demonstrated by Mossbauer spectroscopy that haemozoin is the only detectable iron species in the trophozoite. These conclusions were confirmed by electron spectroscopic imaging. This is convincing evidence that the incorporation of haem into the innocuous haemozoin is overwhelmingly the predominant method of FPIX detoxification (Egan *et al.*, 2002).

Recently, the structure of haemozoin has been elucidated. Haem molecules form dimers via reciprocal iron-carboxylate bonds and these dimers are in turn linked to each other by hydrogen bonds to form a crystal (Pagola *et al.*, 2000). However the actual *in situ* process of this biomineralisation is still unclear. It was initially thought that the process was mediated by an enzyme (Slater & Cerami, 1992). It has since been demonstrated that haemozoin formation can occur without the presence of an enzyme (Egan *et al.*, 1994; Dorn *et al.*, 1995). A number of biological entities have been shown to support the formation of malaria pigment *in vitro*. They include preformed haemozoin (Dorn *et al.*, 1995), β-haematin (Egan *et al.*,

1994), synthetic lipids (Fitch *et al.*, 1999), and *P. falciparum* histidine-rich proteins (PfHRP) (Sullivan *et al.*, 1996a).

The degradation of haemoglobin and subsequent incorporation of FPIX into the haemozoin crystal are clearly vital processes for the malaria parasite and present target sites for antimalarials.

### *1.6 How do the aminoquinolines kill malaria parasites?*

Having outlined in detail the essential processes of haemoglobin degradation and haem detoxification, it is now appropriate to discuss how the aminoquinolines may interfere with these processes, in order to kill the parasite.



#### *1.6.1 The toxicity of FPIX*

There is ample evidence that FPIX is toxic to the malaria parasite and that an accumulation of this porphyrin will kill it (Chou *et al.*, 1980a; Orjih *et al.*, 1981; Fitch *et al.*, 1982; Fitch *et al.*, 1983). A logical postulation is that CQ complexes to the free FPIX, preventing it from being incorporated into the haemozoin crystal. It has been demonstrated that there is a good correlation between the binding of drugs to FPIX *in vitro* and the binding of drugs to the receptor of intact malaria parasites (Chou *et al.*, 1980b). There is also substantial evidence that quinoline antimalarials can interact with FPIX and as a result, inhibit the formation of both synthetic β-haematin and haemozoin in both chemical and biological systems. Egan *et al*

first demonstrated that quinolines that are able to bind to haem are also able to chemically inhibit β-haematin formation *in vitro*, but quinolines that cannot bind haem had no such effect (Egan *et al.*, 1994; Egan *et al.*, 1999). Various researchers have since shown that there is a correlation between inhibition of haematin crystalisation, binding to haem and inhibition of parasite growth for a large array of antimalarial quinolines (Dorn *et al.*, 1998b; Dorn *et al.*, 1995; Dorn *et al.*, 1998c; Egan *et al.*, 1999; Basilico *et al.*, 1997; Hawley *et al.*, 1998; Sullivan *et al.*, 1999; Egan *et al.*, 2000).

The weight of evidence in the literature supports the hypothesis that the quinolines primarily act by causing the accumulation of free haem within the parasite, resulting in the parasite being poisoned by its own waste products. MQ binds free haem and inhibits β-haematin formation but interestingly MQ's interaction with haem has been shown to be much weaker than CQ's interaction with haem (Chou *et al.*, 1980b; Chevli & Fitch, 1982; Egan *et al.*, 1999). This seems contrary to what one might expect, given MQ's high potency and the fact that MQ is not expected to accumulate in the vacuole to the same extent as CQ, due to its single positive charge. This might be an indication that haem induced cell lysis may not be the primary target of MQ. How CQ, and possibly MQ, prevent the formation of the haemozoin crystal is still unclear. Several mechanisms have been proposed, which will be discussed below.



*1.6.2 Haem polymerase*

In the early 1990s several researchers put forward evidence that an enzyme is required for the formation of haemozoin. It was shown that a trophozoite extract was able to initiate haemozoin formation and that this process could be inhibited by certain quinolines (Slater &

Cerami, 1992). This work was supported by other researchers who carried out similar experiments in *P. berghei* (Chou & Fitch, 1993) and also in *P. falciparum* (Orjih & Fitch, 1993). However there is considerable evidence in argument against the presence of a haem polymerase. In 1994 it was demonstrated that haematin can spontaneously occur in an acid environment at temperatures between 6°C and 65°C, in the absence of any protein (Egan *et al.*, 1994). Further they showed that the process is sensitive to the antimalarials CQ, AQ and QUI, but not epiquinine or hydroxquinoline which are not active against malaria. It has also been observed that parasite lysates that have been boiled or exposed to proteinases retain their haem polymerase activity. This evidence argues strongly that a haem polymerase is not involved in the polymerisation process. This in turn suggests that the quinolines do not act by inhibiting a haem polymerase enzyme.



*1.6.3 Haemozoin capping*

Sullivan *et al* (1996; 1998) have proposed that the obstruction of haemozoin formation by quinolines may be due to the capping of the elongating haemozoin crystal by the CQ-FPIX complex. This would prevent further growth of the crystal and allow FPIX to accumulate. They observed by electron microscopy and subcellular fractionation that after incubation with sub-inhibitory concentrations of radiolabelled CQ and quinidine, the drugs became associated with haemozoin (Sullivan *et al.*, 1996b). Binding assays, where the association between the quinoline-haem complex and haemozoin was investigated, revealed that the binding of haem-complexed CQ, MQ and quinidine to haemozoin was saturable and specific. They claimed that this binding to haemozoin was dependent on the presence of free haem (Sullivan *et al.*, 1998).

*1.6.4 Alkalinisation of the food vacuole*

It has been suggested that the accumulation of the weakly basic aminoquinoline antimalarials results in an increase of the pH in the vacuole (Homewood *et al.*, 1972). The functioning of vacuolar enzymes could be drastically affected by this alkalinisation. As discussed earlier in this chapter (Section1.5), the degradation of haemoglobin is controlled by a series of enzymes that reduce the haemoglobin to amino acids and monomeric haem. The disruption of this vital process would effectively starve the parasite of essential amino acids required for protein synthesis.

Goldberg *et al* demonstrated that the digestion of haemoglobin is pH dependent, maximal between pH 5 and 5.5 and minimal at pH 6 (Goldberg *et al.*, 1990). It has been reported that therapeutic concentrations of CQ, MQ, QUI and $NH_4Cl$ increase the pH of the vacuole (Krogstad *et al.*, 1985).

However, other researchers have refuted this claim. Ginsberg *et al* showed that the concentrations at which vacuolar alkalinsation occurs are one to two orders of magnitude higher than pharmacological drug levels (Ginsburg *et al.*, 1989). Yayon et al reported that vacuolar pH remained unchanged at therapeutic concentrations of CQ (Yayon *et al.*, 1985). If drug-induced alkalinisation of the food vacuole is correct then it would be expected that the antimalarial potency of the drug would correlate with its $pK_a$ - the major determinant of a drug's ability to accumulate in an acidic compartment by ion-trapping.

A study in 1991 showed that two CQ analogues with almost identical $pK_a$ values exhibited very different levels of antimalarial potency (Veignie & Moreau, 1991). MQ is a more potent antimalarial than CQ, but it is mono-protonated as opposed to CQ which is di-protonated inside the vacuole; its potency does not seem to be related to its charge. This casts doubt on its role in the alkalinisation of the food vacuole. It has also been proposed that CQ directly affects vacuolar proteases (Vander Jagt *et al.*, 1986), but this is unlikely because the concentration of CQ needed to inhibit these enzymes is higher than can be achieved in the food vacuole.

*1.6.5 Inhibition of haemoglobin degradation*

It has been shown that exposure of malaria parasites to CQ causes less FPIX to be incorporated in haemozoin (Orjih & Fitch, 1993). Additionally, vesicles of undigested haemoglobin have been observed in CQ treated parasites. Yayon *et al* indicated by electron microscopy that these vesicles are inside the vacuole (Yayon *et al.*, 1984c). However in another electron microscopic study these vesicles were observed within the cytoplasm of the parasite (Egan *et al.*, 2001).

Recently it has been demonstrated that CQ and AQ cause an accumulation of haemoglobin within the trophozoite, but MQ and QUI have been shown to decrease the buildup of haemoglobin. These researchers proposed that MQ and QUI may act by inhibiting the endocyctosis of host cell haemoglobin, whereas CQ and AQ prevent haemoglobin degradation (Famin & Ginsburg, 2002). Hoppe and co-workers have recently developed an assay for endocytosis in malaria parasites and initial findings indicate that MQ inhibits

endocytosis, which CQ does not (Hoppe, 2003). Another possibility, in light of the fact that a build up of haemoglobin containing vesicles has been observed in the cytoplasm after CQ treatment (Egan *et al.*, 2001), is that CQ prevents vesicle fusion with the food vacuole.

It has also been demonstrated that in CQ treated malaria parasites, the transport vesicles are unable to discard their contents into the vacuole (Yayon & Ginsburg, 1983). Geary and Ginsberg (1997) proposed that CQ inhibits a phospholipase, that is responsible for the breakdown of the membrane of the endocyctosed vesicle on fusion with the food vacuole (Ginsburg & Geary, 1997). In 1992 CQ was shown to directly inhibit phospholipase activity in a *P. falciparum* extract (Ginsburg & Krugliak, 1992). However the concentrations required were in the millimolar range, which are in excess of what can be achieved within the parasite.

It is doubtful whether phospholipase is the actual target for inhibition of vesicle fusion by CQ. The inhibition of endocytosis by MQ and the inhibition of vesicle fusion by CQ provide interesting and novel alternative mechanisms for the action of these drugs. Both of these mechanisms imply that the site of action of these quinolines is independent of the food vacuole - the focus of the bulk of research in this area. Whatever the details of these proposals, the end result would be starvation of the parasite by quinolines, as opposed to the parasite being killed through the inhibition of the detoxification of haem. However, it is possible that both CQ and MQ have more than one mode of action.

*1.6.6 The inhibition of protein synthesis*

A study in 1991 showed that at 15 μM, haem is able to stimulate cell-free protein synthesis in trophozoite extracts. The same study demonstrated that CQ (3 μM) is able to inhibit this synthesis by binding tightly to haem (Surolia & Padmanaban, 1991). But this is unlikely to be the true mechanism of CQ action. It has been demonstrated repeatedly that the concentration of haem required for stimulation of protein synthesis would be too toxic to cells (Fitch *et al.*, 1983; Chou & Fitch, 1980).

*1.6.7 Inhibition of catalase activity of FPIX or inhibition of GSH mediated haem degradation?*

As mentioned in Section 1.5.1 of this chapter it has been proposed that approximately two thirds of the haem generated from haemoglobin digestion is degraded by its interaction with hydrogen peroxide (Loria *et al.*, 1999). When oxy-haemoglobin enters the acidic environment of the food vacuole it is rapidly converted to methaemoglobin, a by-product of which is $H_2O_2$, which is toxic to the parasite. Haem exhibits some catalase and peroxidase activity, and when involved in peroxidative reactions the porphryin ring can be destroyed. Loria *et al* proposed that the interaction of $H_2O_2$ and haem results in the degradation of the haem and also the breakdown of the reactive oxygen species. They further demonstrated that CQ, MQ and quinacrine are able to inhibit the peroxidative degradation of haem. Loria *et al* also found that exposure to these drugs caused an increase in membrane associated haem, which is thought to greatly affect the integrity of the parasite (Loria *et al.*, 1999).

Another proposal that aims to account for the two thirds of haemoglobin-derived haem that is apparently not incorporated into haemozoin is that this haem travels out of the food vacuole and is degraded by a GSH dependent process (Ginsburg *et al.*, 1998). This process is also able to degrade haem and CQ, MQ and AQ are able to inhibit this degradation *in vitro.* Increasing the glutathione levels of the parasites in dose-response experiments resulted in increased resistance to CQ and decreasing GSH levels caused an increased sensitivity to CQ. It was suggested that CQ complexes to haem and is able to competitively inhibit the degradation of the haem (Ginsburg *et al.*, 1998).

Both of these hypotheses have been challenged on quantitative grounds. As mentioned previously, it has been convincingly shown that 95 % of iron in *P. falciparum* is in the haem form (Egan *et al.*, 2002). This does not discount either of the above theories, because up to 5 % of the parasite's iron could not be accounted for. However, in terms of relative importance to the survival of the parasite, haemozoin formation seems to be paramount.



*1.6.8 RNA breakdown and DNA binding*

A completely alternative mechanism has been proposed, involving CQ interaction with nucleic acids. It has been demonstrated that the 4-aminoquinolines are able to interact with both plasmodial and mammalian DNA *in vitro* (Parker & Irwin, 1952), and that the exposure of *P.knowlesi* to CQ caused a breakdown of ribosomal RNA (Warhurst & Williamson, 1968). Interaction of CQ with nucleic acids as a mechanism of action has largely been rejected for the following reasons:

- DNA binding by CQ enantiomers correlates poorly with their antimalarial activity (Parker & Irwin, 1952) ;
- The concentrations required for the quinolines to exert these effects are in the high micromolar range, which is higher than would be required for antimalarial potency (Krogstad & Schlesinger, 1986);
- The *Plasmodium* genome is AT-rich (75%) and it has been shown that CQ has a higher affinity for GC-rich sequences. CQ has a higher affinity for the host DNA than the parasite DNA, therefore the specificity of CQ toxicity is not explained (Ginsburg & Krugliak, 1992);
- Other quinolines, such as MQ, do not interact with DNA (Peters *et al.*, 1977).

### *1.7 Quinoline Resistance*

With the introduction of CQ and DDT it was thought that the spread of malaria would be halted. Efforts to eradicate malaria resulted in widespread and indiscriminate use of CQ. In 1961, for example, the World Health Organisation (WHO) supplied Brazil with 84 000 tons of CQ for inclusion in table salt. It was not long before the first incidences of CQ resistance were reported in South America and South East Asia. CQ resistance has now spread to virtually every malaria area (Foley & Tilley, 1998).

### *1.7.1 Chloroquine resistance*

#### *1.7.1.1 Mechanisms of chloroquine resistance*

Despite a great deal of research into the mechanism of CQ resistance, there is still debate as to what physiological changes take place that allow malaria parasites to survive at therapeutic concentrations of CQ. There primary phenotypic characteristic of CQR parasites is that CQR parasites accumulate less drug than CQS parasites (Fitch, 1970) and that this phenomenon originates at the food vacuole (Saliba *et al.*, 1998). Malaria researchers have been investigating the nature of the physiological changes, which result in the above phenomena, for the past four decades.



##### *1.7.1.1.1 Increased efflux*

The basis of drug resistance in many types of cancer cells is the extent to which they accumulate the cancer drugs. The mechanism by which resistant cells accumulate less drug, involves the efflux of these drugs from the cells. This process prevents toxic levels of drug being reached within the cell (Fojo *et al.*, 1985). The mechanism that is responsible for this drug extrusion involves an ATP-dependent transport protein called P-glycoprotein (Pgp). Multi-drug resistant (MDR) cancer cells overexpress this protein, resulting in increased drug efflux from the MDR cell. Another feature of this MDR phenomenon is that it is reversible on exposure to verapamil (Gottesman & Pastan, 1993). There is evidence that the mechanism of CQ resistance may be similar to MDR cancer cells, as CQR parasites accumulate less drug than CQS parasites (Fitch, 1970), and that verapamil can reverse CQ resistance (Martin *et al.*, 1987). Further evidence established that CQR parasites efflux CQ 40–50 times more rapidly

than CQS parasites (Krogstad *et al.*, 1987). CQ efflux was also shown to be an ATP-dependent process (Krogstad *et al.*, 1992).

The discovery and amplification of 2 pgp-related genes, *pfmdr1* (Foote *et al.*, 1989) and *pfmdr2* (Rubio & Cowman, 1994), in the malaria genome gave great support to the efflux hypothesis. The *pfmdr2* gene was quickly discounted as a CQ resistance protein because no correlation with CQ resistance could be found (Rubio & Cowman, 1994).

However a great deal of work has been done to characterise the *pfmdr1* gene product, Pgh1. It is a member of the ATP-binding cassette (ABC) family of transport proteins (Foote *et al.*, 1989; Foote *et al.*, 1990). It has also been localised to the membrane of the food vacuole (Cowman *et al.*, 1991). This evidence and initial reports that indicated a correlation between certain *pfmdr1* alleles and CQ resistance, provided some evidence for an efflux mechanism (Foote *et al.*, 1989; Foote *et al.*, 1990). The role of mutations in *pfmdr1* and the implications for CQ resistance are discussed later in this chapter (section 1.7.1.2.1).



There are, conversely, also strong arguments against the efflux hypothesis. Firstly, Pgh1 is located on the food vacuole membrane (Cowman *et al.*, 1991). This means that the orientation of the protein is such that the ATP binding site faces into the cytoplasm of the parasite, which suggests a role in pumping substrates into the food vacuole rather than out of the vacuole (Karcz *et al.*, 1993a). Secondly, this increased efflux phenotype in CQR parasites is not observed by other researchers. The rapid efflux phenotype has been observed in both CQR and CQS strains (Bray *et al.*, 1992a). Several research groups have put forth observations that the decreased accumulation in CQR strains is due to the decrease in uptake

of the drug and not by increased efflux (Bray *et al.*, 1992a; Bray *et al.*, 1996a; Geary *et al.*, 1990; Ginsburg & Stein, 1991).

*1.7.1.1.2 Increase in vacuolar pH*

An important component of CQ accumulation is the passage of the drug down a pH gradient into the acidic food vacuole where it it is trapped by protonation (Chapter 1, section 1.4.1.1.1). If this process is disrupted, less CQ will accumulate in the food vacuole. Geary *et al* developed a kinetic model for CQ accumulation and applied it to CQR and CQS parasites They calculated that there was a larger pH gradient in the CQS strain than in the CQR strain (Geary *et al.*, 1990). One explanation for the above finding is that the proton pump, which maintains intravacuolar pH may be weakened, therefore causing less protons to enter the vacuole resulting in an overall rise in pH.

Bray *et al* tested this explanation and they found that bafilomycin A1, a specific proton pump inhibitor, decreased CQ accumulation in PRBC and this effect was more marked in CQR strains. In addition, they found that when used in combination with CQ in dose-response experiments, bafilomycin A1 caused a decrease in sensitivity to CQ (Bray *et al.*, 1992b).

A kinetic modelling study of the uptake of CQ in PRBC supports the existence of a weakened proton pump (Ginsburg & Stein, 1991). The A and B sub-units of a vacuolar [H+]ATPase (proton pump) have been identified and cloned. However no mutations have yet been found that may result in CQ resistance (Karcz *et al.*, 1993b; Karcz *et al.*, 1994).

In an effort to discern whether the vacuoles of CQR parasites have a higher pH than those of CQS parasites Dzekunov and co-workers carried out single cell pH measurements using the lysomotropic dye acridine orange (AO). In contrast to the generally accepted hypothesis that CQR parasites may have an elevated pH, they found that CQR parasites had a pH lower than CQS parasites. It is thought that a drop in pH would shift the haem equilibrium in favour of the insoluble haem. It is the soluble form of haem that is proposed to be the target of CQ. Therefore, a lower vacuole pH in CQR strains would imply a decrease in CQ target and hence resistance (Dzekunov *et al.*, 2000).

These researchers also showed that CQ exposure altered the vacuolar pH of the resistant strain, but not the sensitive strain. Also, they found that verapamil normalised the vacuole pH of the CQR strain to a pH similar to the CQS strain (Ursos *et al.*, 2000). Transfection of mutant forms of *pfcrt*, a gene recently linked to CQ resistance, into malaria parasites led to increased acidification of the food vacuole. AO was also used to show this pH change (Fidock *et al.*, 2001b).

This work has been strongly challenged by Bray *et al.* They claim that AO does not accumulate in the food vacuole, but rather in the parasite cytoplasm. These researchers used co-localisation of AO with other vacuole-specific fluorescent markers in an effort to pinpoint where AO is located within the PRBC. They concluded that there is no AO associated with the food vacuole - all the AO fluorescence was situated in the parasite cytosol (Bray *et al.*, 2002a; Bray *et al.*, 2002b).

There has been rigorous debate around these diametrically opposed findings, the debate mostly focuses on result interpretation and technical issues. It will be interesting to follow the resolution of this debate.

*1.7.1.1.3 Access to an intravacuolar receptor*

In section 1.4.1.1.3 of this chapter, it was shown that there is good evidence to suggest that the binding of CQ to FPIX could be the dominant factor driving CQ accumulation. According to the Bray/Fitch model of CQ accumulation, CQ accumulates primarily due to its high affinity to the intravacuolar receptor, FPIX. This group also demonstrated in the application of their model that CQR and CQS strains have the same saturable binding capacity for CQ, but the key difference between them is the affinity (apparent $K_d$) of CQ binding. The CQR resistant strain has approximately nine times higher binding affinity than the CQS strain. They explain further that the action of verapamil is to increase the binding affinity of the CQR strain to a level approaching that of the CQS strain (Bray *et al.*, 1998; Bray *et al.*, 1999).

### *1.7.1.2 Genetics of chloroquine resistance*

#### *1.7.1.2.1 Pfmdr1 - chloroquine*

The discovery and amplification of *pfmdr1* (Foote *et al.*, 1989) resulted in a significant research focus to implicate its protein product, Pgh1 in CQ resistance. All the initial clues indicated that Pgh1 could be involved in CQ transport: it was localised to the food vacuole membrane, which is the site of CQ accumulation (Cowman *et al.*, 1991); it belongs to the ABC family of transport proteins; and it is homologous to the Pgp in MDR cancer cells (Foote *et al.*, 1989; Foote *et al.*, 1990). The initial reports also suggested an overexpression of the *pfmdr1* gene in CQR strains (Foote *et al.*, 1989; Foote *et al.*, 1990). However subsequent studies failed to confirm any correlation of Pgh1 expression with CQ resistance (Cowman *et al.*, 1994). Two mutant alleles of *pfmdr1* were identified by Foote and colleagues (1990):

- K1 mutation – $Asn^{86}$ to $Tyr^{86}$
- 7G8 mutation – $Ser^{1034}$ to $Cys^{1034}$; $Asn^{1042}$ to $Asp^{1042}$; $Asp^{1246}$ to $Tyr^{1246}$

Using the above alleles they were able to predict the resistance status of 34 out of 36 *P. falciparum* isolates. Field studies in Africa reported correlation between the $Asn^{86}$ to $Tyr^{86}$ mutation and CQ resistance (Basco *et al.*, 1995). The *pfmdr1* gene has been expressed in mammalian cells and it was shown to confer increased CQ sensitivity to these cells. The introduction of mutated *pfmdr1* did not however, provide this increased CQ sensitivity (van Es *et al.*, 1994).

Subsequently, several reports have provided evidence that Pgh1 does not have a direct role in CQ resistance. In a genetic cross experiment between a CQR strain and a CQS strain, *pfmdr1* did not segregate with the CQ resistance phenotype (Wellems *et al.*, 1990; Wellems *et al.*, 1991b). Furthermore no correlation was found in field isolates between the resistance alleles in *pmdr1* and chloroquine resistance (Wilson *et al.*, 1993). Photoaffinity labelling with a photoreactive analogue of CQ did not demonstrate any interaction with Pgh1 (Foley *et al.*, 1994).

The evidence related to whether Pgh1 is involved in CQ resistance has been inconsistent and contradictory. In a study in Uganda in children with malaria, researchers were unable to find an absolute correlation with *pfmdr1* alleles and CQ resistance. However, they claimed that at least one of the point mutations was found in 90% of the clinically resistant samples. They speculated that although not the definitive marker for CQ resistance, *pfmdr1* in combination with other "unknown co-factors" and "genetic alterations" is connected with the CQ resistance phenotype (Flueck *et al.*, 2000). Recently researchers were able for the first time, to directly examine the effect of the mutant *pfmdr1* alleles by transfection in *P. falciparum*. They showed that inserting the three 7G8 mutations into CQS parasites (D10) did not affect the $IC_{50}$ of this strain. However, when they inserted the wild type (D10) allele into the CQR strain (7G8) the $IC_{50}$ was halved. This indicated that although *pfmdr1* mutations cannot confer resistance to a CQS strain, they can have a cumulative effect in conjunction with other genes and confer a higher level of resistance in the CQR strain (Reed *et al.*, 2000).

Although not definitive, this to some extent explains the contradictory evidence surrounding Pgh1's role in CQR resistance. This manipulation (insertion of the D10 allele in 7G8) also

resulted in an increase in CQ accumulation, providing the first direct evidence for the involvement of Pgh1 in CQ accumulation (Reed *et al.*, 2000).

*1.7.1.2.2 Cg2*

When Wellems and colleagues carried out their genetic cross experiment between a CQS strain and a CQR strain, they found no linkage with *pfmdr1*. However, they did find that the progeny exhibited strict phenotypic characteristics of either the CQR or CQS sensitive parent They believed that another single locus must be responsible for CQ resistance (Wellems *et al.*, 1990; Wellems *et al.*, 1991b). Later this locus was mapped to a 36 kb segment of chromosome 7 that included the cg2 gene (Su *et al.*, 1997). Polymorphisms in this gene have been linked to CQ resistance (Su *et al.*, 1997; Duraisingh *et al.*, 2000b).

Other studies have not been able to demonstrate a link between *cg2* polymorphisms and CQ resistance. In DNA transfection and allelic modification experiments, when *cg2* sequences from CQS parasites replaced sequences in CQR parasites there was no change in sensitivity to CQ (Fidock *et al.*, 2001a). These results seem to rule out a role for *cg2* as a mediator of CQ resistance.

*1.7.1.2.3 Pfcrt*

In 1991 Wellem's crossover study indicated that a locus on chromosome 7 was linked to chloroquine resistance (Wellems *et al.*, 1991a). Almost a decade later, Fidock (2001)

identified a CQ resistance gene within this locus, near *cg2,* to be *pfcrt.* This gene encodes the transmembrane protein PfCRT. It is situated on the food vacuole membrane (Fidock *et al.*, 2001b). It was demonstrated that sets of point mutations in this gene were associated with chloroquine resistant laboratory isolates from diverse geographical origins. Two mutations proved to be invariant:

- K76T – $Thr^{76}$ to $Lys^{76}$
- A220S – $Ala^{220}$ to $Ser^{220}$

These were present in all the CQR isolates. Furthermore in transfection experiments, it was demonstrated that CQ resistance may be conferred to CQS strains by plasmids expressing mutant forms of the *pfcrt* gene (Fidock *et al.*, 2001b). There have been several studies indicating that *pfcrt* may be a good marker for CQ resistance. In a trial focusing on children in Mali, it was found that, in 100% of post-treatment infections, the K76T mutation was present as opposed to a random pre-treatment incidence of 40% (Djimde *et al.*, 2001). Studies in non-immune travellers (Durand *et al.*, 2001), in patients from Cameroon (Basco & Ringwald, 2001), Brazil (Vieira *et al.*, 2001), Papua New Guinea and Thailand and in laboratory strains (Warhurst, 2001), have demonstrated that the K76T mutation in *pfcrt* is a good marker for CQ resistance. There have also been studies that indicate that, in some areas, the association of *pfcrt* mutations with CQ resistance is not complete, and that other factors such as mutations in other genes or host immunity also play a role in the development of CQ resistance (Babiker *et al.*, 2001; Mayor *et al.*, 2001; Dorsey *et al.*, 2001).

Recently several *pfcrt* "knockdown" clones of the CQR 7G8 strain of *Plasmodium falciparum* were produced. These clones expressed 30 -40 % less PfCRT than the parent clone. The knockdown clones were more sensitive to CQ and had altered intracellular pH, providing further evidence for a relationship between PfCRT and chloroquine resistance. The increased expression of PfCRT had no effect on the sensitivity of these clones to either MQ or QUI (Waller *et al.*, 2003).

### *1.7.2 Mefloquine Resistance*

MQ was developed and introduced as a result of the widespread development of CQ resistance. Resistance to MQ was reported within 5 years of its introduction in some areas (Nosten *et al.*, 1991). In an effort to prolong the effective use of MQ it is being used in combination with artesunate or artemether (Price *et al.*, 1995).

#### *1.7.2.1 Mechanisms of mefloquine resistance*

As previously discussed in this chapter (sections 1.7.1.1.1 and 7.1.2.1), the *pfmdr1* and its gene product Pgh1 were initially thought to be involved in a CQ resistance mechanism that resembled that found in MDR cancer cells. However, an association between Pgh1 and CQ resistance has been difficult to prove and only recently with Reed and co-workers' transfection experiments (Reed *et al.*, 2000) has a role for Pgh1 been found. The CQR mutant is not able to confer resistance on its own, but seems to be responsible for conferring a higher level of CQ resistance.

The link between MQ resistance and Pgh1 has proved to be more consistent and there is ample evidence implicating Pgh1 in the development of MQ resistance. Several studies in patient isolates have demonstrated that mefloquine resistance is associated with an amplification of the *pfmdr1* gene (Peel *et al.*, 1993; Wilson *et al.*, 1993; Cowman *et al.*, 1994) as well as an over-expression of Pgh1 (Cowman *et al.*, 1994).

In several studies where *P. falciparum* cultures were subjected to MQ drug pressure the resultant parasite strains were shown to amplify and over-express the *pfmdr1* gene as well as its protein product Pgh1. (Cowman *et al.*, 1994; Peel *et al.*, 1994; Wilson *et al.*, 1989; Barnes *et al.*, 1992). However, two studies have demonstrated that after drug pressure MQ resistance can be achieved without a concomitant increase in expression of Pgh1 (Lim *et al.*, 1996; Ritchie *et al.*, 1996). Good evidence has been presented to demonstrate that MQ interacts with Pgh1. MQ's ability to interact with the MDR1 P-glycoprotein (a homologue of Pgh1) in cancer cells was assessed. This assessment demonstrated that MQ competed with verapamil for binding to MDR1, whereas CQ did not. MQ was also able to inhibit the efflux of known MDR1 substrates from cancer cells and to even reverse resistance to neoplastic agents (Riffkin *et al.*, 1996).

The *pfmdr1* gene has been transfected into *Saccharomyces cerevisae ste6* knockout cells. The *ste6* mutation in these yeast cells prevents the transport of *a*-mating factor. The incorporation of *pfmdr1* complemented the *ste6* mutation and allowed the transport of the peptide, indicating that Pgh1 can function as a transporter. Mutated *pfmdr1*, containing mutations that have been implicated in drug resistance, was transfected into the yeast cells and the transport function was abolished. Expression of the wild-type *pfmdr1* in the yeast cells conferred

resistance to MQ, QU, HF and quinacrine. The above study provides excellent evidence that links *pfmdr1* to MQ, QUI and HF resistance. Expression of the mutant *pfmdr1* did not confer resistance. CQ was not examined because the yeast cells were not CQS (Volkman *et al.*, 1995).

Duraisingh and colleagues (2000) provided good evidence that *pfmdr1* is implicated in resistance to both MQ and ART. They examined the progeny of a genetic cross between HB3 and 3D7, both CQS, but with differing sensitivities to MQ and ART. They showed a complete allelic association between the HB3-like *pfmdr1* allele and increased sensitivity to MQ and ART. They also showed that in a battery of unrelated laboratory strains this association was consistent. In molecular modelling experiments in which they compared Pgh1 with the mammalian MDR3 protein they determined that one of the variant amino acid residues ($Asn^{1042}$ to $Asp^{1042}$) in Pgh1 was homologous to a residue in MDR3. This corresponding residue, when mutated, decreased the ability of MDR3 to confer multidrug resistance to cancer cells (Duraisingh *et al.*, 2000a).

In the transfection experiments of Reed *et al* (2000), the results were more dramatic for MQ, HF and ART than they were for CQ (section 1.7.1.2.1). The introduction of the 7G8 alleles into the MQ, HF and ART resistant D10 strain resulted in an increased susceptibility to all 3 of these drugs. Conversely, when the D10 alleles were introduced into the MQ, HF and ART sensitive 7G8 strain, resistance to each of these drugs was conferred. The link between Pgh1 and MQ resistance is now well established; however the exact physiological role of Pgh1 in MQ's mechanism of action and mechanism of resistance remains unclear.

### *1.7.3 Quinoline cross-resistance*

The 4-aminoquinoline AQ was introduced in response to CQ resistance, however there is substantial cross resistance between these related 4-aminoquinolines (Bray *et al.*, 1996b; Bray *et al.*, 1996a). Considerable data also demonstrates cross resistance between the quinoline methanols MQ and QUI, and also with the closely related phenanthrene, HF (Wilson *et al.*, 1993; Bray *et al.*, 1994; Cowman *et al.*, 1994; Peel *et al.*, 1994). Cross resistance within these two groups of quinolines is not surprising due to the close structural relationship between compounds within the groups. In fact, one might expect that cross resistance between these two groups should also be evident. However cross resistance between the 4-aminoquinolines and the quinoline methanols does not easily develop and there seems to be a paradoxical relationship between the parasite sensitivities to the two groups of drugs.

Numerous studies have shown that the result of MQ drug pressure was an increase in MQ resistance and a concomitant increase in sensitivity to CQ (Cowman *et al.*, 1994; Peel *et al.*, 1994; Peel *et al.*, 1993; Lambros & Notsch, 1984; Merkli & Richle, 1980). Equally, CQ drug pressure not only causes an increase in CQ resistance, but also an increase in MQ sensitivity (Merkli & Richle, 1980; Lambros & Notsch, 1984). This interesting cross resistance pattern highlights the dissimilarity in the nature and action of these related drugs and will be discussed in detail in Chapter 2 (Section 2.1).

### *1.7.4 Reversal of quinoline resistance*

Since Martin and co-workers first demonstrated verapamil's ability to confer CQ sensitivity to CQR strains (Martin *et al.*, 1987), a whole battery of compounds have been shown to reverse resistance. These include the tricyclic antidepressant desipramine (Bitonti *et al.*, 1988; Basco & Le Bras, 1990), the SSRI (selective serotonin reuptake inhibitor) antidepressant fluoxetine (Gerena *et al.*, 1992) the antihistamine chlorpheniramine (Basco & Le Bras, 1994) and others. Interestingly, none of the compounds that are able to chemosensitise CQR parasites are able to do the same in MQR parasites. Only one molecule has shown any MQ resistance reversing activity. Penfluridol, a piperidine neuroleptic, drug has been shown to reverse resistance to MQ *in vivo* and *in vitro*, but it has no effect on CQR parasites (Oduola *et al.*, 1993; Peters & Robinson, 1991; Kyle *et al.*, 1988). The fact that there is no overlap in the compounds that reverse CQ and MQ resistance is a further indication that these drugs have disparate mechanisms of resistance.



## *1.8 Scope of Study*

Despite the fact the MQ has been used clinically for in excess of twenty years very little is known about it's mechanism of action and more specifically about its accumulation in *Plasmodium*-infected erythrocytes. One of the primary reasons for this is that it is a difficult drug to work with in the laboratory, because it is highly hydrophobic. This hydrophobicity causes external non-specific binding of MQ to bind to serum proteins and to membranes to a large degree. This high degree of extraneous binding may result in important drug-related

processes being obscured in the investigation of MQ's interaction with the parasite (Chevli & Fitch, 1982).

It is the intention of this study to ascertain the nature of the interaction of MQ with *Plasmodium falciparum*. This study will: (a) investigate the relationship between MQR and CQR, and how the expression of Pgh1 correlates with these two phenomena, (b) determine how MQ is distributed within the PRBC and characterise the various components of MQ accumulation, (c) determine whether there is an energy requirement for MQ accumulation, (d) determine whether MQR parasites handle MQ differently to MQS parasites, (e) investigate the effect of MQ and CQ on haemoglobin levels in PRBCs.

# Chapter 2

# Drug Susceptibilities of *Plasmodium falciparum* strains

## *2.1 Introduction*

In Chapter 1 (Section 1.7.3) the paradoxical relationship between 4-aminoquinoline resistance and quinoline methanol resistance was discussed briefly. Several investigations have clearly demonstrated that drug pressurized laboratory strains have inverse relationships with respect to their sensitivities to CQ and MQ. The first studies involving the use of drug pressure to bring about changes that result in MQ resistance were carried out in murine models (*Plasmodium berghei*). In these early studies no obvious inverse relationship between CQ and MQ sensitivity was observed, but no cross resistance was observed either (Merkli & Richle, 1980; Peters *et al.*, 1977).

Observations in clinical isolates in several studies from Africa and Thailand provided the initial indications that there may be an inverse relationship between MQ and CQ resistance (Webster *et al.*, 1985; Childs *et al.*, 1991; Basco & Le Bras, 1992).

In 1984, the first experiments involving MQ drug pressure on *Plasmodium falciparum* were carried out. CQR and CQS isolates were subjected to MQ drug pressure. The CQS isolate (Camp strain) became MQ resistant but its CQ sensitivity did not change. The CQR Smith

isolate also became MQ resistant but in addition, it became more CQ susceptible (Lambros & Notsch, 1984). The fact that patient isolates were used in the above study means that the MQ may have selected for a population of parasites that were innately resistant to the drug. Therefore, the use of heterogeneous populations in this study may not have been ideal to investigate the genetic or biochemical changes that occur as a result of MQ drug pressure.

To obtain a genetically homogeneous population of *falciparum* parasites, Oduola *et al* used a single RBC manipulation technique to produce the W2 clone (Oduola *et al.*, 1988a). They drug pressurized this MQS and CQR W2 clone (MQ $IC_{50}$ = 4.5 ng/ml and CQ $IC_{50}$ = 122.5 mg/ml) with increasing concentrations of MQ. The resulting progeny, W2mef, became resistant to MQ ($IC_{50}$ = 30.4 ng/ml) and more susceptible to CQ ($IC_{50}$ = 88.6 ng/ml). Note that the CQ $IC_{50}$ did not decrease to the level of a fully CQS strain (Oduola *et al.*, 1988b).

Barnes *et al* (1992) subjected two *P.falciparum* clones to CQ drug pressure. They used the progeny from the above mentioned study, W2mef, and another clone FAC8. FAC8 had similar characteristics to W2mef. Both the W2mef and FAC8 clones had a CQ $IC_{50}$ of 83 ng/ml and MQ $IC_{50}$s of 46 ng/ml and 38 ng/ml respectively. The resulting progeny after CQ pressure had similar sensitivities to these drugs. FAC8's progeny, FAC860.2, became CQ resistant with an $IC_{50}$ of 195 ng/ml and the $IC_{50}$ of W2mef's progeny, $W2mef^{CQ}$, increased to 161 ng/ml. The MQ $IC_{50}$s for FAC860.2 and $W2mef^{CQ}$ decreased to 11 ng/ml and 24 ng/ml respectively. In that study, when the levels of *pfmdr1* were measured in the FAC8 strain and its CQ-pressured progeny, FAC860.2, it was found the increase CQ resistance (and concomitant increase in MQ sensitivity) correlated with a decrease in *pfmdr1* (Barnes *et al.*, 1992).

Two later studies carried out drug pressure on W2mef with MQ (Peel *et al.*, 1993) and with CQ (Peel *et al.*, 1994). After the application of MQ pressure, the resulting clone (Mef 2.4) became more MQ resistant and the $IC_{50}$ shifted from 14.49 ng/ml to 20.17 ng/ml. There was also a shift in the CQ $IC_{50}$s as a result of the MQ pressure, the CQ $IC_{50}$ decreased from 62 ng/ml to 48 ng/ml (Peel *et al.*, 1993). In the second study, CQ pressure caused W2mef to become more CQ resistant, with a CQ $IC_{50}$ shift from 62 ng/ml to 102 ng/ml in the progenitor, $W2mef^{CQ200}$. CQ exposure caused the MQ $IC_{50}$ of W2mef to decrease from 14 ng/ml to 2 ng/ml in $W2mef^{CQ200}$ The levels of Pgh1 in the original parent strain, W2, and the resultant progenitor strains was measured. It was found that the MQ pressured W2mef strain had overexpression of Pgh1 relative to W2, furthermore, the CQ pressured $W2mef^{CQ200}$ showed a loss of Pgh1 amplification (Peel *et al.*, 1994).

Another study performed further MQ pressure on W2mef and also on K1, a highly CQ-resistant strain. MQ pressure caused MQ resistance in these strains to increase, and CQ resistance to decrease. This study also showed that with increasing MQ resistance there was an amplification of the *pfmdr1* gene and overexpression of its gene product Pgh1 (Cowman *et al.*, 1994).

It is clear that in laboratory strains subjected to drug pressure there is an inverse relationship between CQ resistance and MQ resistance. When the shift to MQ resistance occurs, although the CQ $IC_{50}$ decreases, it is not normally to the level of a fully sensitive strain.

Studies of patient isolates have also shown that the correlation between MQ resistance and increased Pgh1 expression is not limited to laboratory strains. One study showed that in eleven patient isolates of *Plasmodium falciparum,* MQ resistance was associated with

increased gene copy number and increased expression of *pfmdr1 (Wilson et al., 1993)*. As recently as 2001 it was show that malaria parasites isolated from a Japanese MQR patient expressed 7.2 times more Pgh1 than a MQS control strain (Kim *et al.*, 2001).

Several studies have also shown that MQ resistance can be achieved in certain laboratory strains, with no increase in Pgh1 expression (Lim *et al.*, 1996; Ritchie *et al.*, 1996). This shows that the correlation between MQ resistance and Pgh1 expression is not absolute and that there may be other mechanisms in the malaria parasite by which it can become resistant to MQ.

This chapter evaluates the MQ and CQ susceptibilities of a range of *Plasmodium falciparum* clones and isolates and examines the apparent paradoxical relationship between MQ resistance and CQ resistance. The expression of Pgh1 is also assessed in several strains.

## *2.2 Results*

### *2.2.1 Characterization of Plasmodium falciparum strains with respect to CQ and MQ sensitivity.*

A range of *Plasmodium falciparum* clones and isolates were evaluated for their sensitivity to CQ and MQ. The parasite lactate dehydrogenase activity after 48 hours of drug exposure was used as a measure of parasite viability (Makler *et al.*, 1993) See Chapter 9, section 9.3 for details. It was determined that six out of the nine available strains of *P.falciparum* were CQR and three were CQS. From figure 2.1 it is easy to distinguish the CQR from the CQS strains, it is clear that each of the strains fall into either the CQS set (broken line) or the CQR set (solid line).

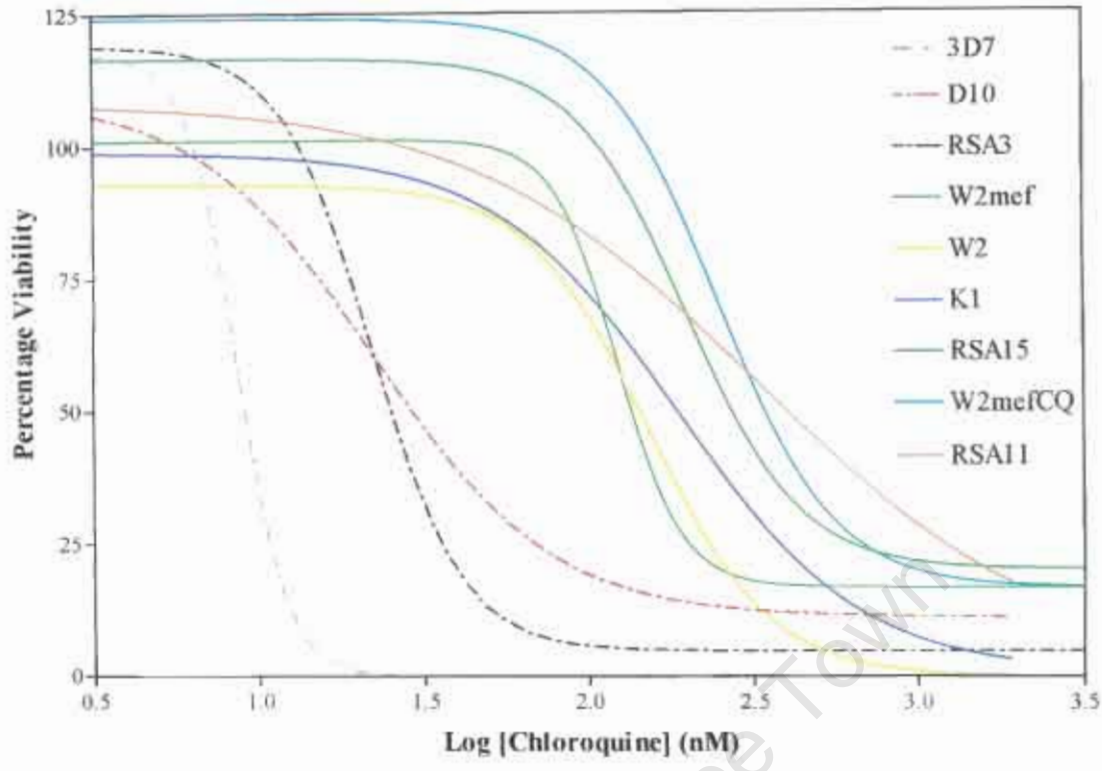



**Figure 2.1**: Chloroquine dose-response curves for 9 strains of *Plasmodium falciparum*. The broken curves signify CQS strains and the CQR strains are represented by solid lines. The CQ concentration range was between 2 nM to 2000 nM. Each curve reflects data from at least 2 separate experiments performed in duplicate (see Table 2.1). The curves were fitted using the Sigma Plot 3.0 sigmoidal dose-response curve non-linear regression equation.

The distinction between MQ resistance and sensitivity is less clear (Figure 2.2). The threshold for in vitro MQ sensitivity is considered to be 19 nM. This was determined from the correlation of clinical and *in vitro* data by the Walter Reed Army Institute of Research (Peel *et al.*, 1994) and Webster *et al* (Webster *et al.*, 1985). It is interesting to observe that the only strains whose $IC_{50}$s surpass this threshold, and can be defined as MQR, are D10 and RSA3, the two CQS strains (Table 2.1).

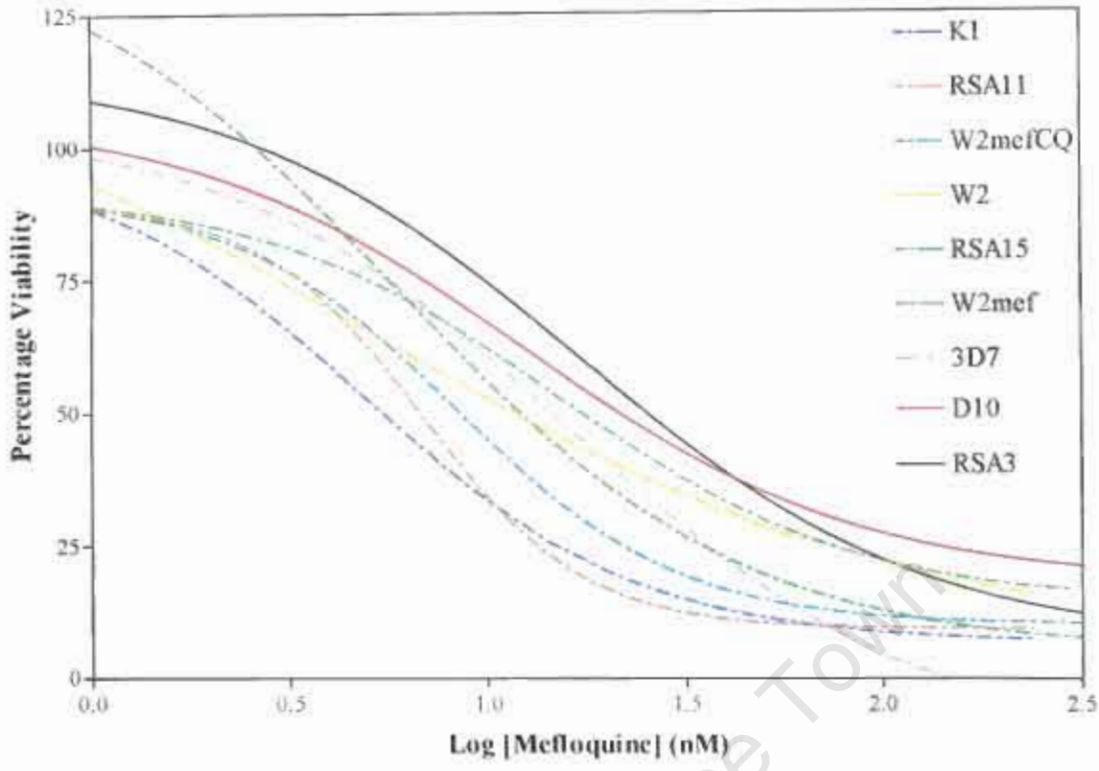



**Figure 2.2:** Mefloquine dose-response curves for 9 strains of *Plasmodium falciparum*. The broken curves signify MQS strains and the MQR strains are represented by solid lines. The MQ concentration range was between 0.5 nM and 250 nM. Each curve reflects data from at least 2 separate experiments performed in duplicate (see Table 2.1). The curves were fitted using the Sigma Plot 3.0 sigmoidal dose-response curve non-linear regression equation.



### *2.2.2 The relationship between CQ resistance and MQ resistance*

In an effort to further illustrate the inverse relationship between MQ and CQ sensitivities a graph of the MQ $IC_{50}$s for each strain was plotted against the corresponding CQ $IC_{50}$s (figure 2.5). Linear regression was performed on the data. Although no statistically significant negative correlation could be demonstrated ($r = 0.764$), it is clear that there is a trend

supporting the existing evidence that MQ resistance is inversely proportional to CQ resistance. See Table 2.1 for $IC_{50}$ values.

**Table 2.1:** Dose response curves for CQ and MQ in 9 strains of *Plasmodium falciparum*.

| Strain | CQ $IC_{50}$ (nM) | | MQ $IC_{50}$ (nM) | |
|---|---|---|---|---|
| D10 | 20.21 ± 4.50 | (n = 4) | 20.58 ± 2.57 | (n = 3) |
| RSA 11 | 320.27 ± 14.53 | (n = 3) | 4.26 ± 1.27 | (n = 3) |
| W2 | 171.85 ± 57.85 | (n = 2) | 5.39 ± 0.81 | (n = 2) |
| W2mef | 110.23 ± 16.45 | (n = 5) | 14.59 ± 1.39 | (n = 5) |
| $W2mef^{CQ}$ | 305.15 ± 54.03 | (n = 4) | 8.85 ± 2.17 | (n = 7) |
| K1 | 114.20 ± 13.10 | (n = 2) | 4.86 ± 0.9 | (n = 3) |
| RSA15 | 246.93 ± 34.55 | (n = 4) | 9.75 ± 2.5 | (n = 4) |
| RSA3 | 24.21 ± 2.28 | (n = 3) | 24.00 ± 3.49 | (n = 3) |
| 3D7 | 8.489 ± 0.314 | (n = 1) | 16.65 ± 3.18 | (n = 1) |

The $IC_{50}$ values reflect the mean and SEM for at least 2 separate experiments performed in duplicate, except for the results for the 3D7 strain which is the result of one experiment performed in duplicate.

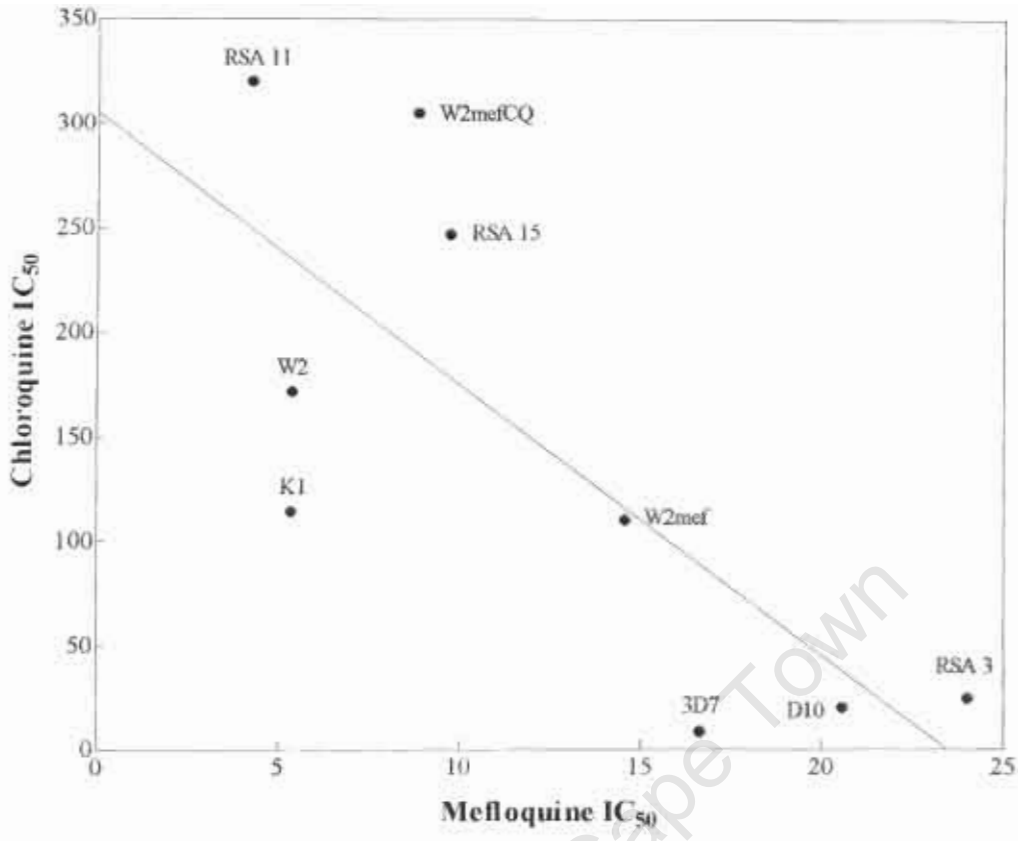



**Figure 2.3:** A plot of the $IC_{50}$ of CQ against the $IC_{50}$ of MQ. Linear regression of the data was performed (r = 0.764). See table 2.1 for $IC_{50}$ values.



### *2.2.3 The relationship between Pgh1, CQ resistance and MQ resistance*

Pgh1 expression levels were measured in the D10, RSA11, W2, W2mef, K1 and 3D7 strains of *Plasmodium falciparum*. Isolated trophozoites were run on SDS PAGE and then transferred to a nitrocellulose membrane by Western blotting and the Pgh1 protein bands were detected by autoradiography (see Chapter 9, Sections 9.15 to 9.17 for details). Plate 2.1 shows the autoradiograph of the Pgh1 bands from the Western Blot. In figure 2.4 the intensities of each band are shown. Cowman et al (1994) used 3D7 as a reference strain, against which they compared the other strains tested. They used this strain because it has a

single copy of *pfmdr1*. Their experiments showed that K1 and 3D7 express similar amounts of Pgh1; the same was shown in this study (figure 2.4). RSA11 and W2 both expressed similar amount of Pgh1 to 3D7, both are MQS. Cowman *et al* (1994) determined that W2mef expressed 2.6-fold more Pgh1 than 3D7, this study also indicates W2mef overexpresses Pgh1 relative to 3D7, by 2.1-fold. Other work has shown that mRNA expression levels in W2mef are two to three times higher than in W2 (Peel *et al.*, 1994; Wilson *et al.*, 1993). This work shows that W2mef expresses 1.8-fold more Pgh1 than W2. The only other strain to over express Pgh1 relative to 3D7 was D10 (1.5-fold), the other MQR strain tested. Strain 3D7 has an $IC_{50}$ is similar to that of W2mef (16.65 ± 3.18 nM and 14.59 ± 1.39 nM respectively), but its expression levels are half that of W2mef.

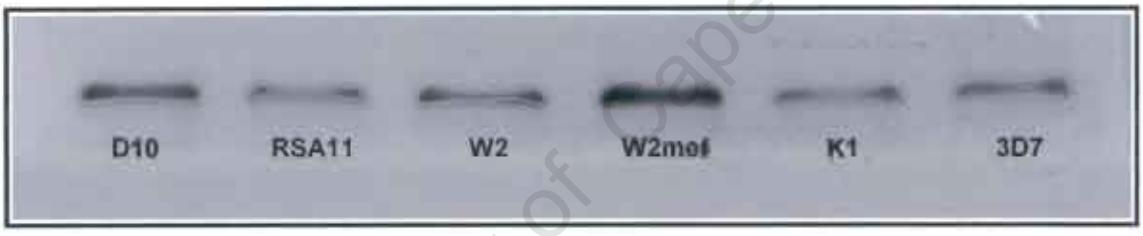



**Plate 2.1**: Autoradiograph from a Western Blot showing Pgh1 expression levels in the D10, RSA11, W2, W2mef, K1 and 3D7 strains of *Plasmodium falciparum*. The experiment was performed twice on separate occasions.

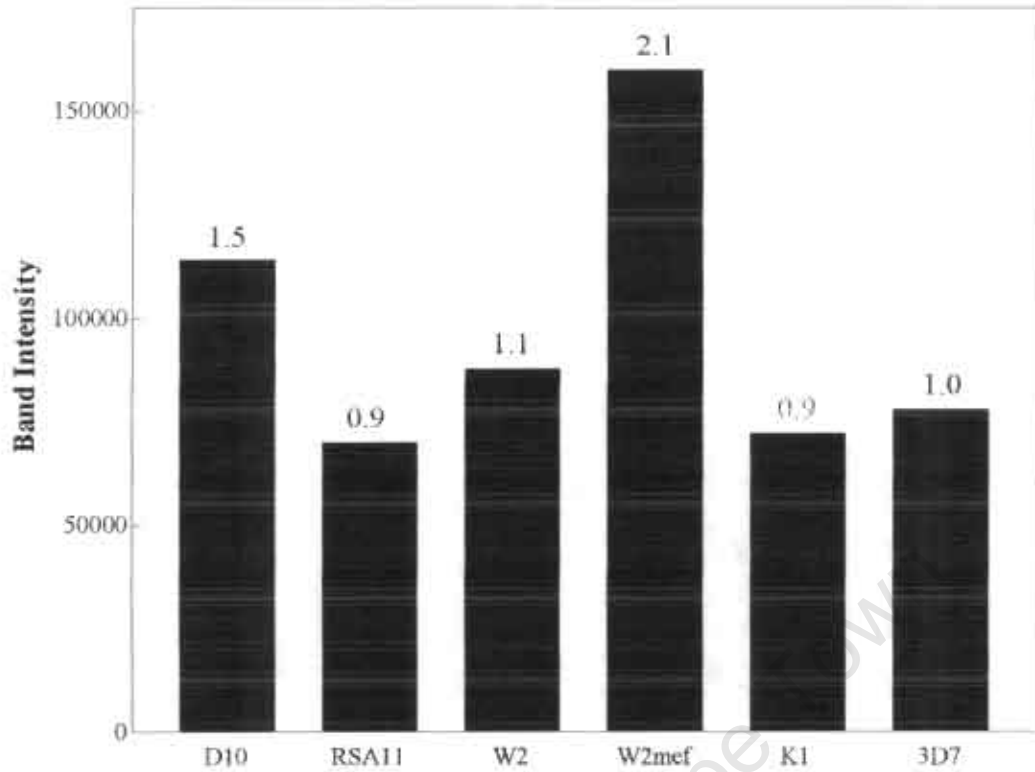



**Figure 2.4:** Shows the Pgh1 band intensities for the D10, RSA11, W2, W2mef, K1 and 3D7 strains of *Plasmodium falciparum* (from the autoradiograph in plate 2.1). The intensity of each band was measured using Kodak 1D Image Analysis Software Program. The experiment was performed twice on separate occasions. The numbers above the bar reflect the fold increase in expression from the 3D7 strain.

In an effort to illustrate that relationship between Pgh1 expression levels and drug resistance, the $IC_{50}$s for CQ and MQ were plotted against Pgh1 protein band intensities (figure 2.5). Linear regression was performed on the data. Although statistically no correlation could be shown in this small sample, the correlation for MQ ($r = 0.2916$) is better than that of CQ ($r = 0.0903$).

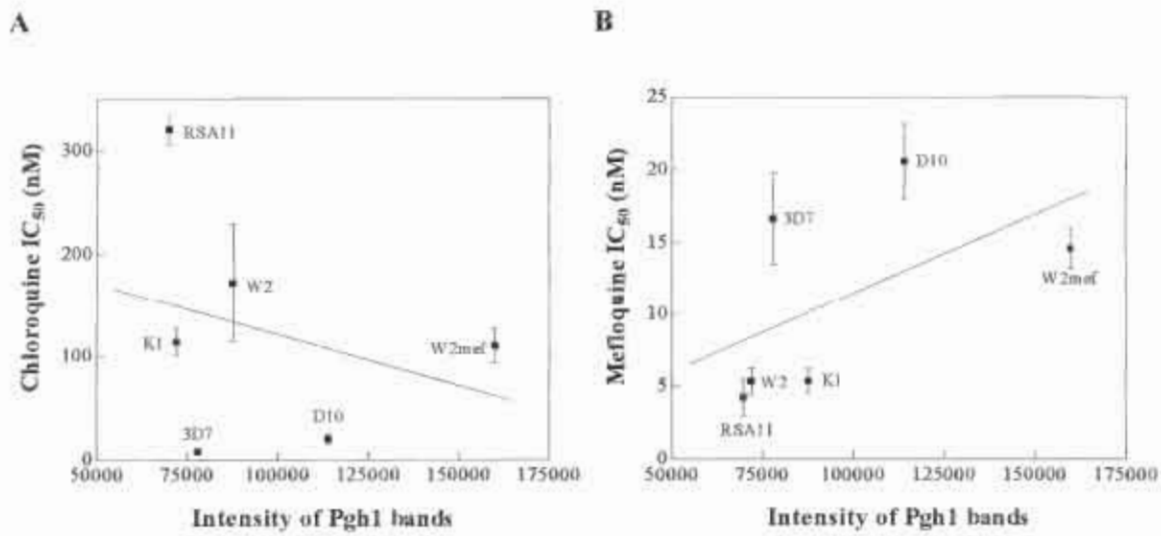



**Figure 2.5:** Plots of $IC_{50}$ for CQ (**A**) and MQ (**B**) against Pgh1 band intensity for the six strains tested. Linear regression was performed on the data. The co-efficients of correlation for CQ and MQ were -0.3005 and 0.5400 respectively.

### *2.3 Discussion*

This chapter confirms previous work indicating there is a paradoxical relationship between MQ resistance and CQ resistance (Barnes *et al.*, 1992; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Peel *et al.*, 1993; Peel *et al.*, 1994). A sample of nine *Plasmodium falciparum* isolates and clones were evaluated with respect to their susceptibility to CQ and MQ. Figure 2.3 shows that there is a statistically weak inverse relationship ($r = 0.764$) between the CQ and MQ $IC_{50}$s in the strains tested.

The W2, W2mef, K1 and 3D7 have been previously investigated for Pgh1 expression levels (Cowman *et al.*, 1994; Wilson *et al.*, 1993). The relative amounts of Pgh1 expressed in these strains, in this study, correspond with previous work. D10 and RSA11 had not been tested before. D10 was the most MQ resistant strain tested and it overexpressed Pgh1. But, not as much as W2mef, which is not as MQ resistant as D10. RSA11 is MQS and expressed similar amounts of Pgh1 to the 3D7 strain, which has previously been shown to have only one gene copy of *pfmdr1*. 3D7 has only one copy of the *pfmdr1* gene and therefore expresses relatively little Pgh1. This is incongruous with the theory that increased levels of Pgh1 is responsible for MQ resistance. In this study it was shown to have a similar susceptibility to MQ as W2mef. This could indicate that there is either more than one mechanism of resistance to MQ in malaria parasites or that mutations in Pgh1 can confer MQ resistance to MQS Plasmodium falciparum. There is also strong evidence to support the involvement of Pgh1 mutations in MQ resistance (Reed *et al.*, 2000).

Looking at the CQ dose response curves (Figure 2.1), it is also interesting to note that 3 parasite lines (3D7, K1 and RSA11) exhibit shallower slopes than the other strains. Theoretically the shallower slopes could indicate that these strains could have multiple targets. These strains also exhibited the lowest levels of Pgh1 expression (Figure 2.4).

# Chapter 3

# Characterisation of [$^3$H]-Mefloquine accumulation in *Plasmodium falciparum*

### *3.1 Introduction*

There is a dearth of information on the accumulation of MQ by *Plasmodium falciparum* relative to the substantial data on CQ accumulation. Mefloquine is a hydrophobic drug that binds with high affinity to uninfected erythrocytes (San George *et al.*, 1984). It partitions from saline solution in erythrocyte membranes with a partition coefficient of 60 (San George *et al.*, 1984; Mu *et al.*, 1975). As opposed to CQ which is taken up by uninfected RBCs to a very limited extent (Fitch *et al.*, 1979), MQ binds both phospholipids ((Chevli & Fitch, 1982) and stomatin (Desneves *et al.*, 1996) within the RBC membrane. MQ also binds tightly to serum components and specifically to the high density lipoprotein, Apo-A1 (Desneves *et al.*, 1996). This drug equilibrates between plasma and RBCs at a ratio of 1:1.7 (Vidriquin *et al.*, 1996).

Thus far there is minimal direct evidence that MQ accumulation in malaria parasite is energy dependent. There also appears to be no published data measuring the energy dependence of MQ accumulation in *Plasmodium falciparum*. Over 20 years ago Fitch *et al* (1979) carried out the first experiments that investigated the energy dependence of MQ accumulation. These experiments were carried out in the murine parasite, *Plasmodium berghei*. They firstly

performed experiments in the presence and absence of glucose and were not able to show that glucose stimulated MQ accumulation. They were also not able to inhibit MQ accumulation by incubating the PRBC at low temperature (2 °C). Furthermore, the metabolic inhibitors, azide and iodoacetate (both at 1 M) were not able to appreciably decrease the uptake of MQ. These experiments were carried out with PRBC preparations with parasitemias of less that 50 %. In this work, MQ accumulation was also measured as a function of the external MQ concentration, the authors described the resulting curves as "curvilinear", meaning that the best fit of the data results from a regression equation that contains terms for a rectangular hyperbola and a straight line. The authors claimed that this was indicative that there are two components to MQ accumulation, one saturable and the other, non-saturable (Fitch *et al.*, 1979).

Three years later Chevli and Fitch (1982) discovered that MQ binds with high affinity to membrane phospholipids and this binding accounts for a great deal of the MQ accumulated in PRBCs (Chevli & Fitch, 1982). These authors suggested that this vast degree of MQ binding may explain why, in their previous experiments (Fitch *et al.*, 1979), no energy-dependent MQ accumulation was detected. They explained that the phospholipid binding may have obscured a relatively small energy requirement for MQ accumulation (Chevli & Fitch, 1982).

The only other investigation into the energy requirement for MQ accumulation was also carried out in murine parasites, but this study used *Plasmodium chaubaudi*. Vanderkooi *et al* (1988) focussed on the reliance of CQ and MQ accumulation on intra-vacuolar pH. They used a series of ionophores to distinguish between overall MQ and CQ accumulation, and accumulation that is dependent on a pH gradient. The ionophores used included carbonyl-cycanide meta-chlorophenyl-hydrozone (CCCP), which increases proton permeability,

valinomycin, which makes membranes permeable to $K^+$ and nigericin, which allows electroneutral exchange of $H^+$ and $K^+$ ions. It demonstrated that both MQ and CQ accumulation are sensitive to these agents. However, the accumulation of CQ was more sensitive to these ionophores than MQ accumulation. The study showed that approximately 90 % of CQ accumulation is dependent on a pH gradient, whereas only 60 % of the MQ accumulation requires a pH gradient. These experiments were carried out at 0 °C and at 25 °C, and it was found that the pH gradient-dependent component on the drugs' accumulation was significantly reduced by incubation at low temperature. From this, the authors' inferred that the majority of the pH-dependent drug accumulation is an active process, requiring energy.

Two points of evidence indicate that there may be a protein involved in the transport of MQ into the PRBC. Firstly, according to the weak base mechanism of drug accumulation, monoprotic MQ is anticipated to accumulate to a much lesser extent, than diprotic CQ, in the acidic food vacuole (Chapter 1, Section 1.4.2.1). However, this is not the case, MQ accumulates in the PRBC to a larger extent than CQ. MQ is also a more potent antimalarial than CQ (Fitch *et al.*, 1979; Vanderkooi *et al.*, 1988). Chevli and Fitch (1982) demonstrated that a large proportion of this accumulation is due to membrane phospholipid binding. However, the presence of an additional factor involved in the accumulation of MQ cannot be discounted.

Further evidence for a MQ transporter protein is the fact that the p-glycoprotein analogue, Pgh1 has been implicated in MQ resistance. Investigations have shown that an increased expression of Pgh1 and also several mutations in this protein are associated with MQ

resistance (Peel *et al.*, 1993; Wilson *et al.*, 1993; Cowman *et al.*, 1994) (See Chapter 1, Section 1.7.2.1 and Chapter 2, Section 2.1 for details).

In this chapter, optimum conditions were established to investigate the accumulation of MQ in PRBC. Experiments were then carried out to characterise the nature of MQ accumulation in *Plasmodium falciparum*. Firstly, the energy dependence of MQ accumulation was established, by using metabolic inhibitors, incubation at low temperature and by energy deprivation. Secondly the pH dependence of MQ accumulation was determined by investigating the effect of an ionophore, a proton pump inhibitor, vacuologenic amine and external pH on MQ accumulation. The effect of a range of other antimalarials on MQ accumulation was also determined. The role of Pgh1 in MQ accumulation was also probed using P-type ATPase inhibitors and substrates. The effect of chemosensitisers on MQ accumulation was also investigated.

### *3.2 Results*

#### *3.2.1 Establishment of conditions for the accumulation of [$^3$H]-mefloquine in Plasmodium falciparum infected erythrocytes.*

Vidrequin et al (1996) carried out RBC MQ accumulation experiments in HEPES buffer and also in serum-containing complete medium. They found that the RBCs incubated in the HEPES buffer, contained significantly more MQ than the RBCs in complete medium. This was attributed to MQ binding to serum proteins, which thereby reduced the amount of drug available for uptake by the RBCs (Vidriquin *et al.*, 1996). The effect of serum protein binding on overall MQ and CQ accumulation was evaluated. Erythrocytes infected with *Plasmodium falciparum* (D10 strain) were incubated in culture medium with and without 0.5 % Albumax, in the presence of radiolabelled CQ and MQ, and the accumulation of the PRBC measured. Albumax is the serum substitute, rich in lipids and protein, used in the continuous culture of malaria parasites (See Chapter 7, Section 7.1.2).

Figure 3.1 shows that removal of Albumax from the culture medium in the CQ accumulation experiment did not have an effect on the amount of CQ accumulated by the PRBC. This also confirms that CQ does not bind appreciably to serum proteins. There was an almost 60% increase in the amount of MQ accumulated when the Albumax was removed from the medium. This means that serum proteins significantly decrease the amount of MQ available for uptake by the parasite. To ensure that the maximum amount of MQ is available to the parasites, in all furthur accumulation experiments the Albumax was removed from the uptake medium. The fact that CQ accumulation was not affected by the removal of the Albumax

indicates that the parasites viability and inherent ability to accumulate drug has not been inhibited. This is probably because the incubation period is only 60 minutes.

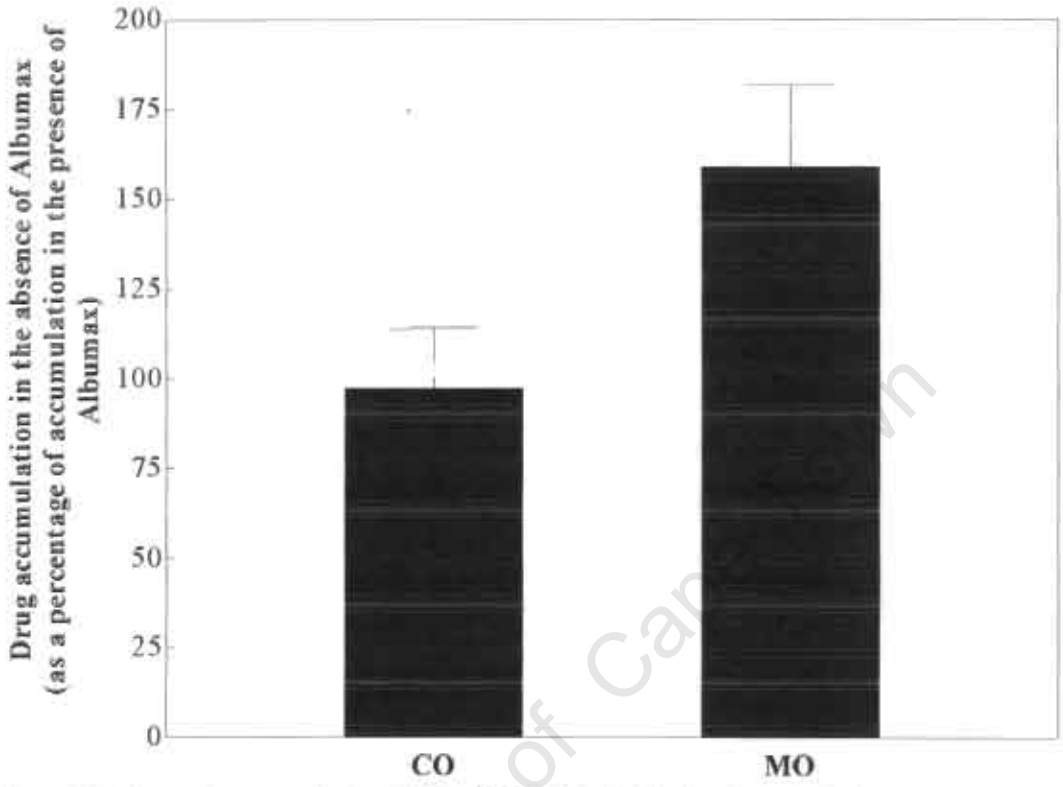



**Figure 3.1**: Shows the accumulation of CQ and MQ (10 nM) in the absence of Albumax, as a percentage of accumulation in the presence of 0.5 % Albumax. Error bars represent the standard error of the mean from 3 separate experiments performed in duplicate or triplicate.

It was thought that by increasing the parasitemia of the culture used in the accumulation experiments, it might be possible to further distinguish between MQ accumulation in uninfected erythrocytes and *Plasmodium*-infected erythrocytes. By adapting a method published by Ginsburg (1998), it was possible to enrich parasite cultures and consistently achieve parasitemias of greater than 85 % (see Chapter 7, Section 7.4) (Ginsburg *et al.*, 1998). Plate 3.1 is a photograph of a thin smear of a typical *in vitro Plasmodium falciparum* culture, the parasitemia is approximately 8 %.

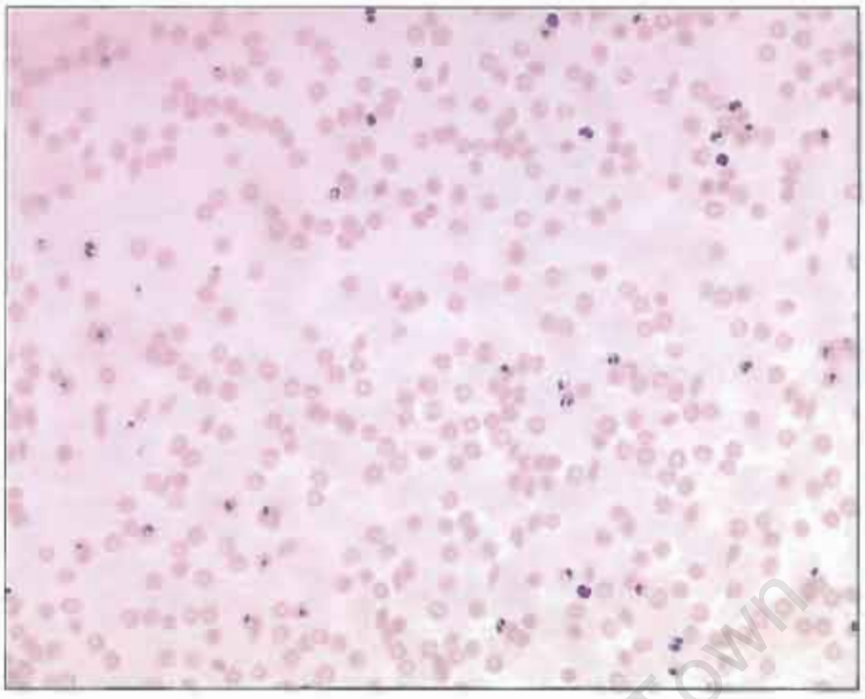


**Plate 3.1:** A Giemsa stained thin smear representing an *in vitro Plasmodium falciparum* culture under normal culture conditions. (parasitemia equals approximately 8 %) (40 X magnification)

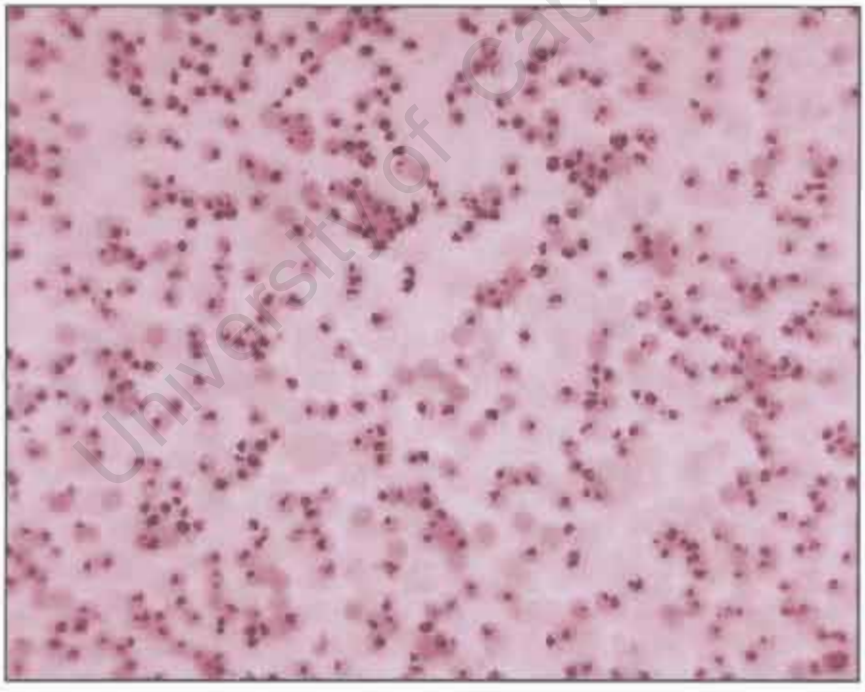


**Plate 3.2:** A Giemsa stained smear representing an *in vitro Plasmodium falciparum* culture after trophozoite enrichment following centrifugation through Percol (See Chapter 8, Section 8.4). (parasitemia equals approximately 90 %) (40 X magnification)

Plate 3.2 shows the culture after the trophozoite enrichment, the parasitemia is approximately 90 %. Under light microscopy the parasites appear to be morphologically intact.

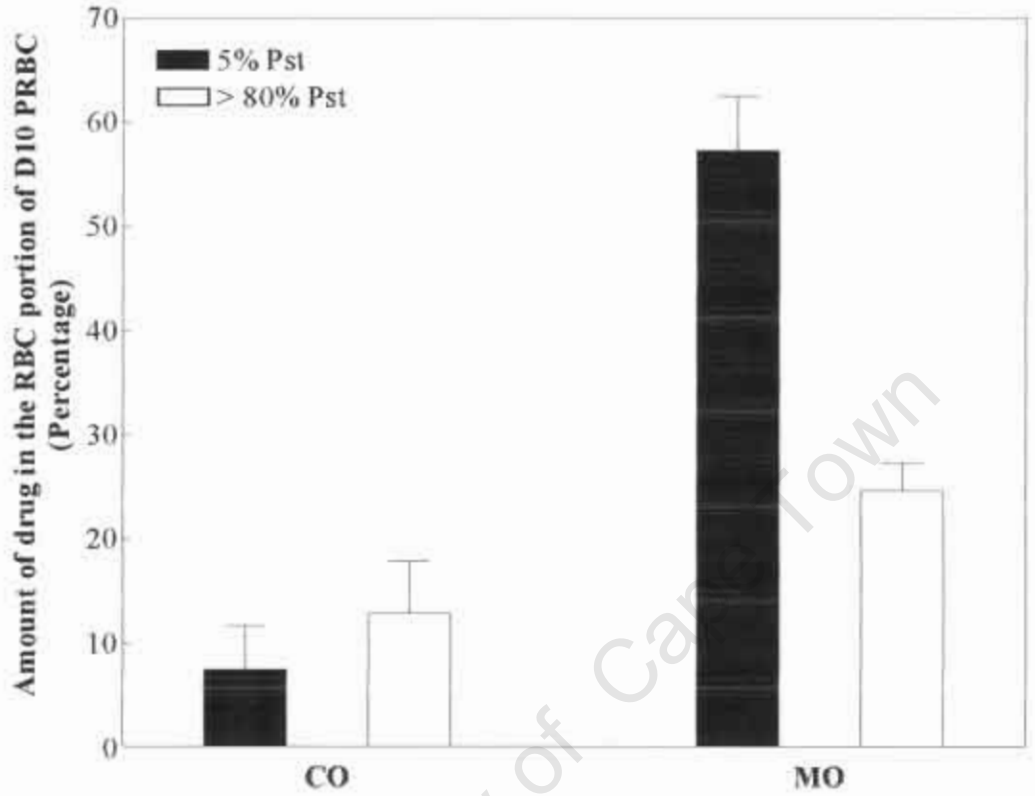



**Figure 3.2:** Shows the drug accumulation attributed to RBC as a percentage of the total drug uptake in D10 PRBC using culture of 5% and > 80%. Error bars represent the standard error of the mean from at least 3 separate experiments performed in triplicate.



Figure 3.2 shows the contribution of RBC to the overall accumulation of CQ and MQ inside PRBCs. There was no difference in the RBC portion of CQ accumulation at 5 % parasitemia and at over 80 % parasitemia ($p = 0.4750$). This was expected because it has been demonstrated many times that CQ accumulation in uninfected erythrocytes is minimal (Fitch *et al.*, 1979).

The percentage of MQ accumulation that could be attributed to RBCs was significantly less when the parasitemia was higher than 80 % than when the parasitemia was 5 % (p = 0000262). At 5 % parasitemia the RBCs contributed nearly 60 % of the total MQ accumulation. When the parasitemia was over 80 %, this figure fell to only 25 %.

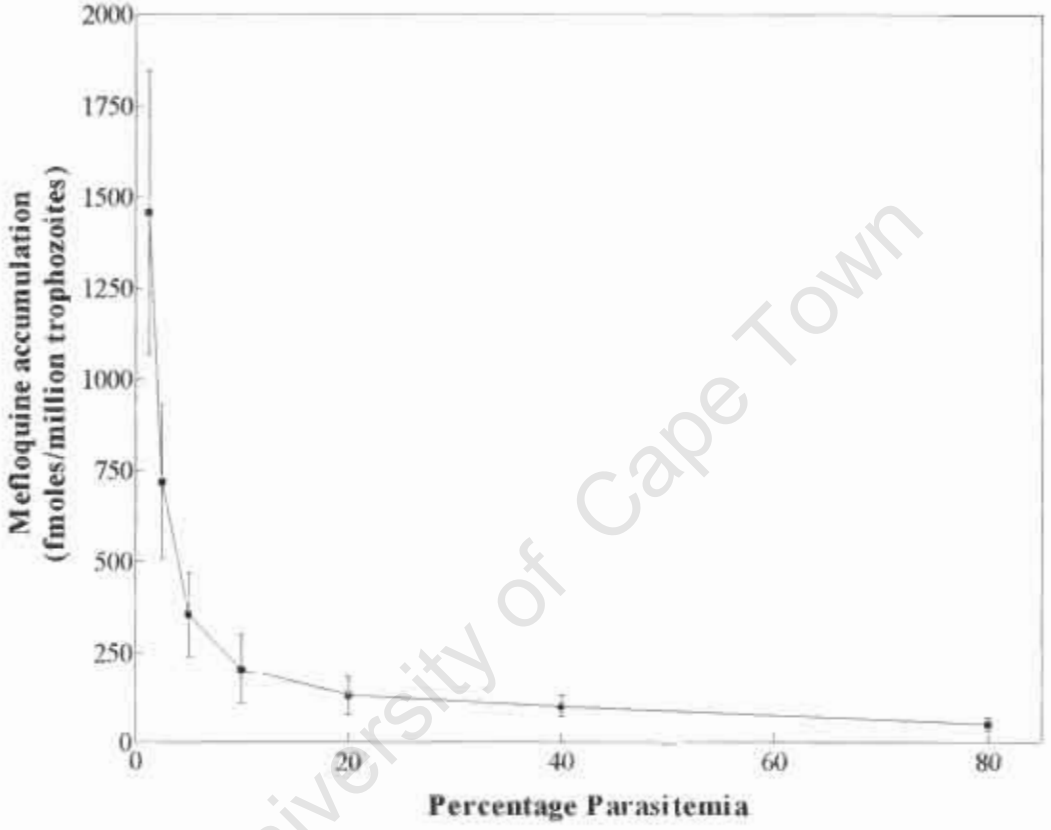





**Figure 3.3:** Shows the effect of inoculum size on drug accumulation in RSA11 PRBCs. PRBCs were incubated in radiolabelled MQ (10 nM) over a range of parasitemias from 1.25 % to 80 % at an haematocrit of 0.1 %. Error bars represent the standard deviation from two separate experiments performed in triplicate.

MQ accumulation experiments were carried out over a range of parasitemias from 1.25 % to 80 %, to investigate the effect of innoculum size. Figure 3.3 shows that as the parasitemia increased, the amount of MQ that accumulated in each individual PRBC decreased. This

effect was profound at the lower parasitemias. From 20 % parasitemia to 80 % parasitemia the effect was less marked.

### *3.2.2 Time dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes.*

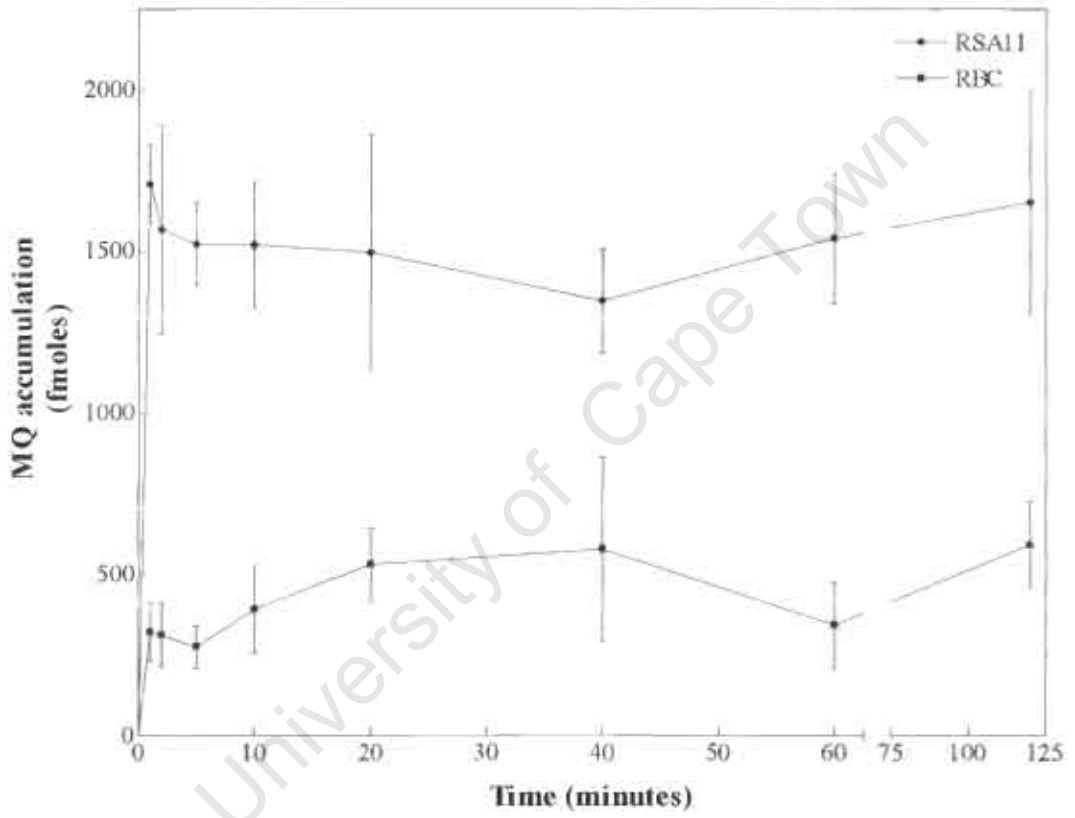





**Figure 3.4**: Time course of [$^3$H]-MQ accumulation in RSA 11 PRBCs and uninfected RBCs, expressed in fmoles, at an external MQ concentration of 10 nM. Incubation time varied from 1 minute to 120 minutes. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.

In figure 3.4. the time course for MQ accumulation is shown. RSA11 PRBCs and uninfected RBCs were allowed to accumulate 10 nM [$^3$H]-MQ for 1 to 120 minutes. MQ accumulation

in both RBCs and PRBCs is very rapid and is complete in one minute. There was no change in the amount of MQ accumulated from 1 minute to 120 minutes. There was no significant difference in accumulation between the 1 minute time point and any of the other time points (all p values > 0.05). This was true for both RBCs and PRBCs. Over all the time points the PRBCs accumulated approximately 4 times more MQ than the uninfected RBCs.

### *3.2.3 Concentration dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

The dependence of MQ accumulation on the external MQ concentration was investigated and is plotted in figure 3.5. The data were fitted to an equation similar to the equations used by other researchers to describe the two components of MQ and CQ accumulation. Bray *et al* (1998) modelled the saturable and non-saturable uptake of CQ in a similar manner to that which Fitch *et al* (1970) had done previously with CQ and MQ (Fitch, 1970; Fitch, 1973; Fitch *et al.*, 1979). The data was fitted by computer to the equation below using an iterative procedure (Graphpad Prism) (see Appendix 1 for the derivation).

$$[TD] = \frac{[ED] \cdot Cap}{[ED] + Kd} + m \cdot [ED]$$

The data fitted the above equation, which incorporates both rectangular hyperbola and straight line terms ($r^2 = 0.978$). This was better than when the data was fitted to regression equations for a straight line ($r^2 = 0.8107$) or a rectangular hyperbola ($r^2 = 0.8717$) individually. This indicates that the assumptions made in deriving the "curvilinear" equation

are correct. That is, there are saturable and non-saturable components to MQ accumulation. Despite the good fit of the data to the model, there was considerable scatter at the various concentration points, particularly at the higher concentrations. This meant that the values for Cap and $K_d$ had extremely large 95% confidence intervals, rendering the values obtained for these fitted parameters statistically meaningless.

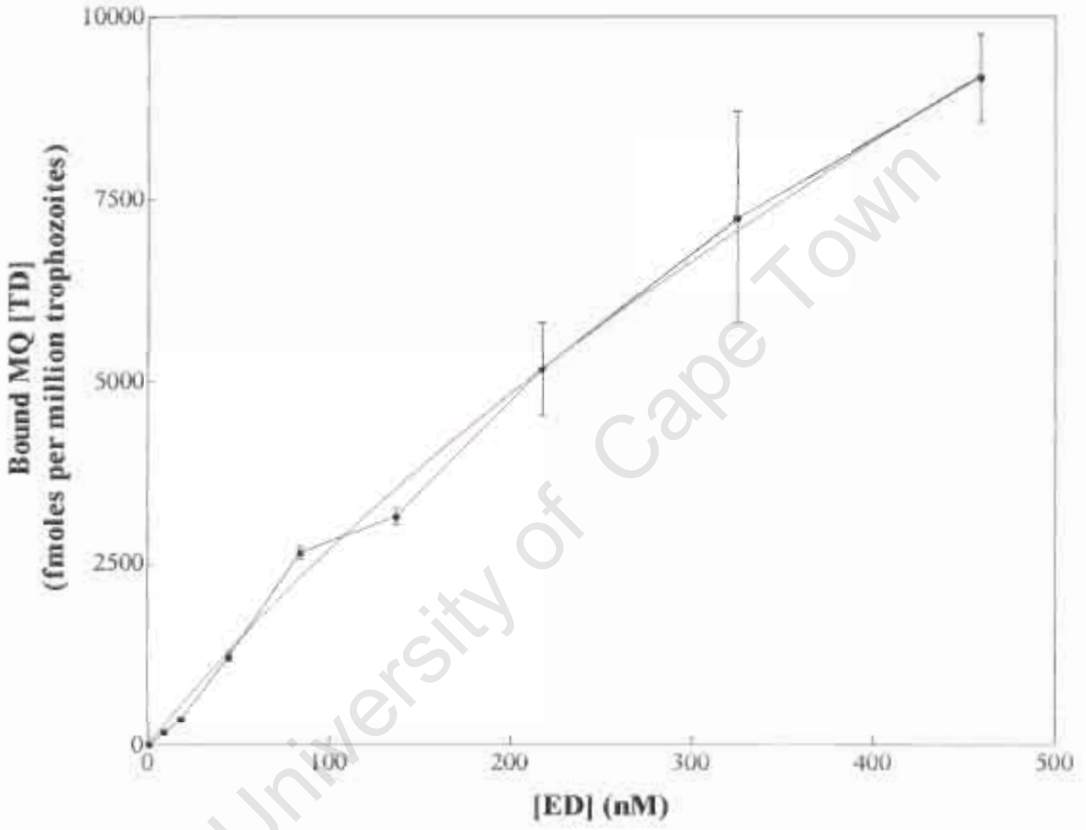





**Figure 3.5:** [$^3$H]-MQ accumulation in RSA 11 PRBC, expressed as fmoles/million trophozoite-infected RBCs, over a range of external MQ concentrations (1 nM to 500 nM). Nonlinear regression was performed on the data (red line) using the equation, [TD] = (([ED]·Cap)/([ED]+Kd)) + m·[ED] that describes a curvilinear relationship ($r^2 = 0.978$). Error bars represent the standard deviation from 3 separate experiments performed in triplicate.

### *3.2.4 Effect of metabolic inhibitors on the accumulation of [$^3$H]-mefloquine in Plasmodium falciparum infected erythrocytes*

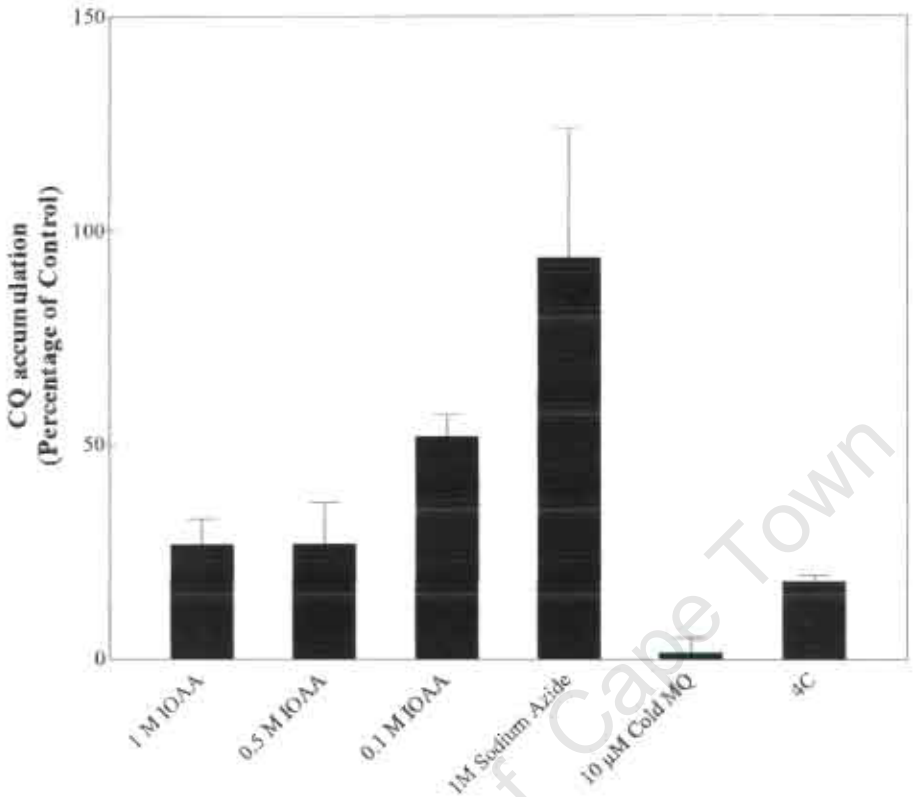



**Figure 3.6:** The effect of iodoacetate (1 M, 0.5 M and 0.1 M), Sodium Azide (1 M), 1000-fold excess unlabelled MQ (10 µM) and incubation at 4°C on the accumulation of 10 nM [$^3$H]-CQ in RSA 11 PRBC. Results are expressed as a percentage of the control. Control vials contained only PRBCs and [$^3$H]-CQ. Accumulation due to RBCs has been subtracted. Error bars represent the standard deviation from 2 separate experiments performed in duplicate.



Although the parasite has a single mitochondrion, it does not have a functional citric acid cycle, it is therefore thought that the parasite is dependent entirely on glycolysis for its energy requirements (Kirk, 2001). In Figure 3.6 and 3.7 the effect of metabolic inhibition on CQ and MQ accumulation in RSA11 PRBC, respectively, was investigated.

As might be expected, sodium azide, which inhibits oxidative phosphorylation, did not have a large effect on CQ accumulation; it caused only a 24 % decrease. The glycolysis inhibitor, iodoacetate, inhibited CQ accumulation to a much larger extent. At concentrations of 1 M, 0.5 M and 0.1 M CQ, accumulation was inhibited by 73 %, 73 % and 48 % respectively. This agrees with the assertion that glycolyis is the major energy supply for the parasite (Kirk, 2001). CQ accumulation was diminished to 18 % by incubation at 4°C. These results confirm previous work demonstrating the high energy requirement for CQ accumulation (Fitch *et al.*, 1974b; Fitch *et al.*, 1974a; Bray *et al.*, 1992a; Krogstad *et al.*, 1992). It should be noted that these experiments were carried out in RSA11, a CQR strain of *P.falciparum*. It has been shown that CQR strains accumulate less CQ than CQS strains (Krogstad *et al.*, 1987; Fitch *et al.*, 1974b; Yayon *et al.*, 1984). Also accumulation in CQR strains is less energy dependent than in CQS strains (Bray *et al.*, 1992b; Fitch *et al.*, 1974a). In the presence of 10 μM unlabelled MQ, CQ accumulation was abolished to 2 % of the control.

Figure 3.7 shows that sodium azide did not affect the accumulation of MQ, as accumulation was 97 % of the control. However, the glycolysis inhibitor, iodoacetate decreased the accumulation of MQ at all three of the concentrations used. 0.5 M iodoacetate decreased MQ accumulation by 43% and was the most effective concentration. Concentrations of 1 M and 0.1 M also decreased MQ accumulation (27 % and 32 % respectively). Incubating the PRBC at 4°C resulted in a 24 % decrease in MQ accumulation. These experiments provide the first direct evidence of energy-dependent MQ accumulation. The presence of a 10 μM unlabelled MQ inhibited [$^3$H]-MQ accumulation by 91 %, indicating that there may be specific uptake of MQ in the malaria parasite.

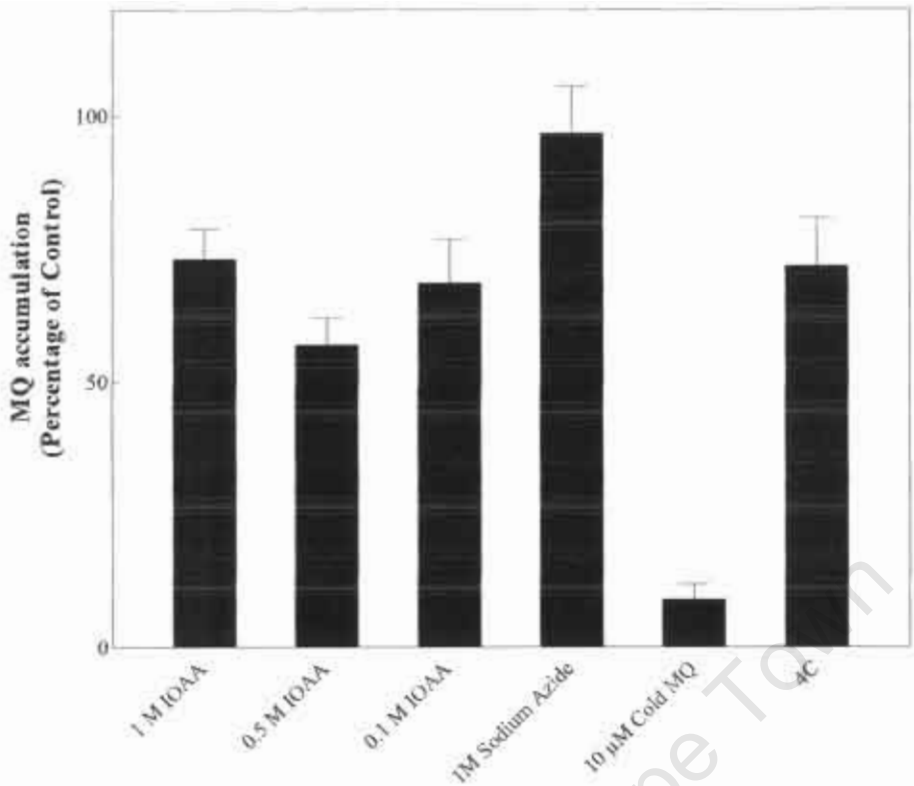



**Figure 3.7**: The effect of iodoacetate (1 M, 0.5 M and 0.1 M), Sodium Azide (1 M), 1000-fold excess unlabelled MQ (10 µM) and incubation at 4°C on the accumulation of [$^3$H]-MQ in RSA 11 PRBC. Results are expressed as a percentage of the control. Control vials contained only PRBCs and [$^3$H]-MQ. Accumulation due to RBCs has been subtracted. Error bars represent the standard deviation from 3 separate experiments performed in triplicate.



### *2.2.5 Glucose dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

To further determine the extent to which MQ accumulation is energy dependent, experiments were carried out in phosphate-buffered saline (PBS) in the presence and absence of 10 mM glucose. To check the experimental conditions, CQ accumulation was measured in the CQS D10 strain in the presence and absence of glucose (figure 3.8). It was confirmed that CQ

accumulation is highly dependent on glucose as an energy source, CQ accumulation was stimulated from 10.5 to 63 fmoles per million PRBC, a six-fold increase. This glucose dependence has been proven in numerous other studies (Fitch *et al.*, 1974b; Fitch *et al.*, 1974a; Bray *et al.*, 1992a; Krogstad *et al.*, 1992).

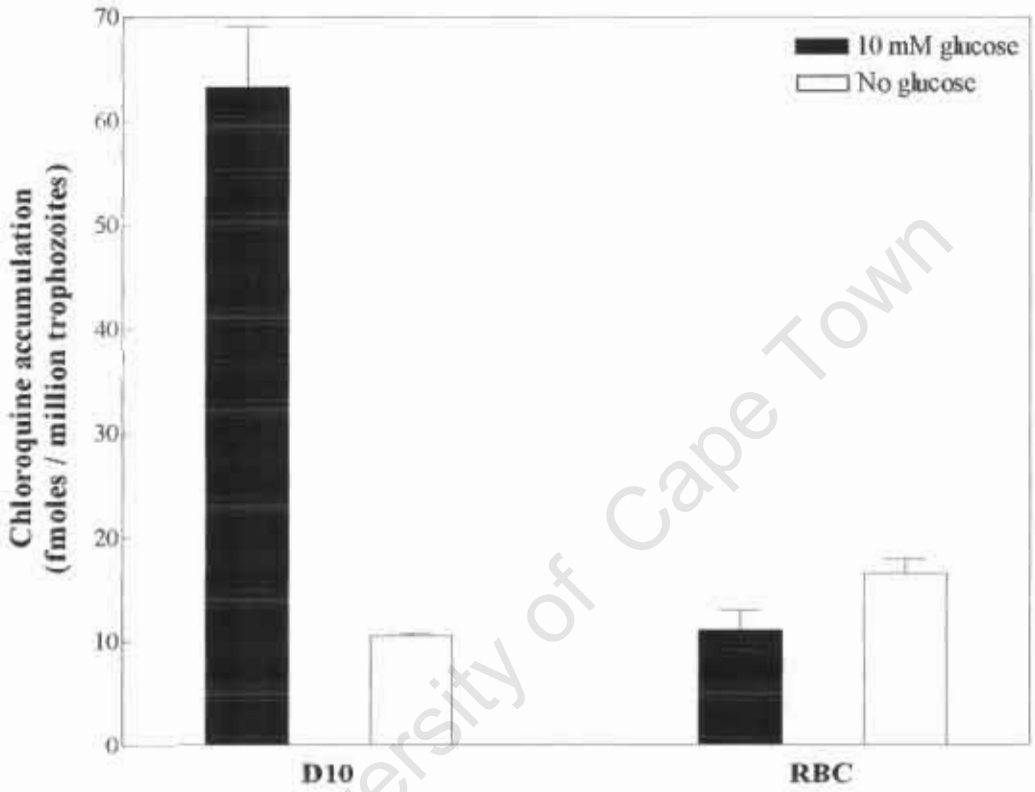



**Figure 3.8:** [$^3$H]-CQ accumulation in CQS D10 PRBC and uninfected erythrocytes, in the presence and absence of 10 mM glucose. Results are expressed as a fmoles per million trophozoites. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.



Under the above-mentioned experimental conditions MQ accumulation was, for the first time, shown to be dependent on glucose, and hence energy dependent (figure 3.9). Accumulation in uninfected erythrocytes was the same whether or not glucose was present ($p = 0.9625$). In both the MQR D10 strain and the MQS RSA 11 strain the absence of glucose caused a decrease in the amount of MQ accumulated. The presence of glucose caused a 1.8-

fold increase in MQ accumulation, from 49 to 90 fmoles per million PRBCs. RSA11 PRBC accumulation 2.5-fold more MQ in the presence of glucose, accumulation rose from 19 to 47 fmoles per million PRBCs. This experiment also confirms that MQR D10 accumulates less MQ than MQS RSA11.

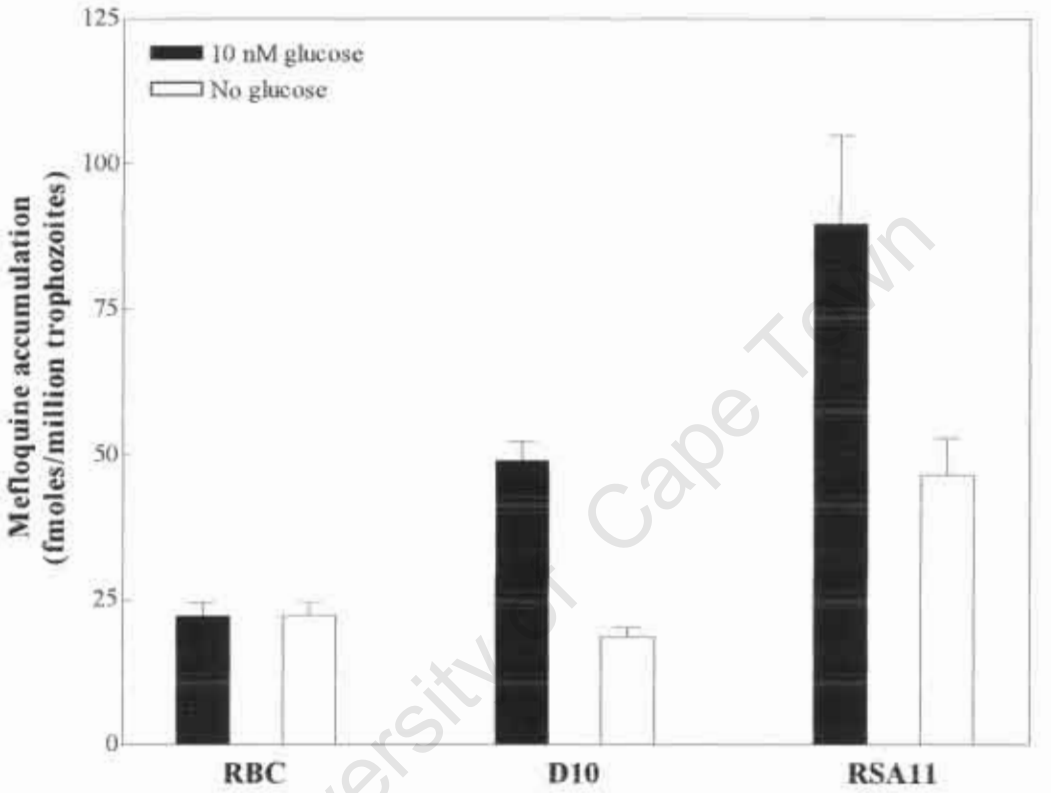



**Figure 3.9:** [$^3$H]-MQ (10 nM) accumulation in uninfected erythrocytes, MQR D10 and MQS RSA11 PRBC, in the presence and absence of 10 mM glucose. Results are expressed as a fmoles per million trophozoites. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.

### *3.2.6 pH dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

MQ accumulation was measured in RSA11 parasites over a range of external pHs, from pH 5 to pH 9. Figure 3.10 shows that accumulation was sensitive to both an increase and a decrease in external pH, with maximal accumulation occurring at pH 7.5. When the external pH increased by one unit to 8.5 , MQ accumulation decreased from 330.6 ± 58.5 to 249 ± 76.7 fmoles/million PRBCs. When the medium pH was decreased by one pH unit (pH 6.5), MQ accumulation decreased to 162.1 ± 76.7 fmoles/million PRBCs.

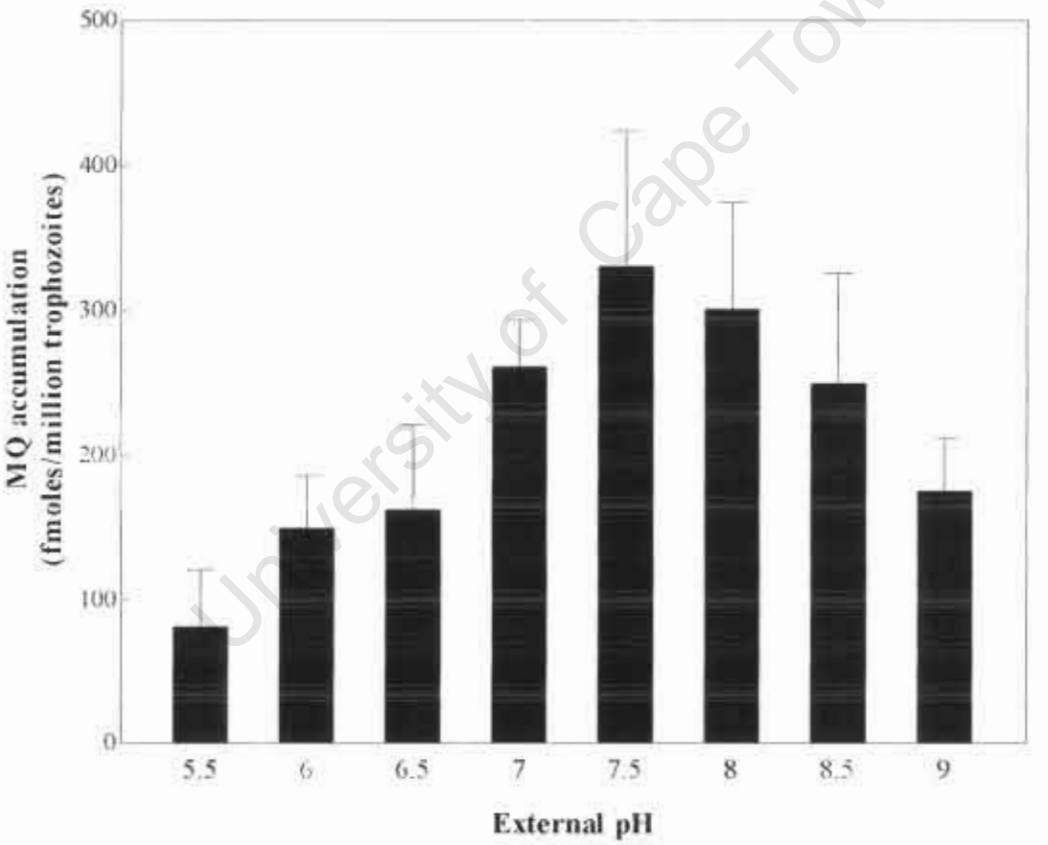



**Figure 3.10**: The effect of external pH on the accumulation of [$^3$H]-MQ in RSA 11 PRBC. Results are expressed in fmoles per million trophozoite infected RBCs. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.

To further examine the nature of MQ accumulation, PRBCs were exposed to a range of pH gradient modulators. Bafilomycin A1 and NEM, specific vacuolar proton pump inhibitors, caused MQ accumulation to decrease by 37 and 59 % respectively. The ionophore nigericin caused a 74 % decrease in MQ accumulation, this is in agreement with Vanderkooi *et al* (1988). They showed that 40 to 60 % decrease in MQ accumulation occurs in the presence of nigericin (Vanderkooi *et al.*, 1988). Ammonium chloride caused a 10 % decrease in MQ accumulation.

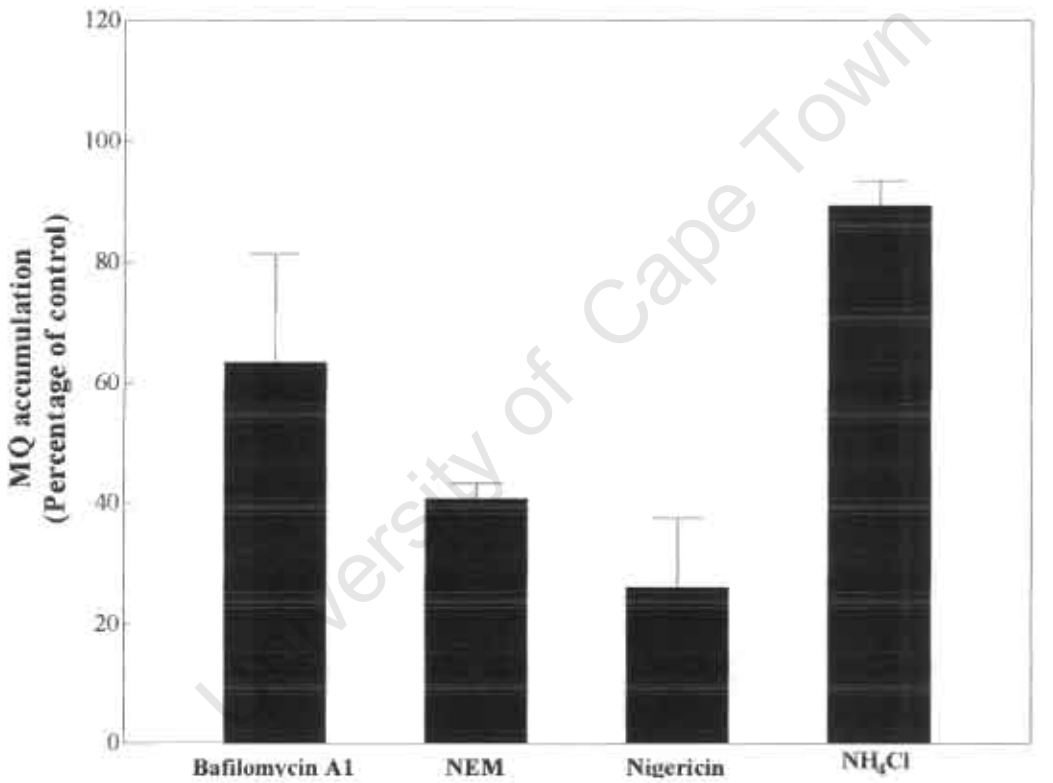





**Figure 3.11**: The effect of the vacuolar pH modulators, bafilomycin A1 (1 μM), nigericin (10 μg/ml), N-ethylmaleimide (10 μM) and ammonium chloride (10 mM) on the accumulation of [$^3$H]-MQ in RSA 11 PRBC. , Results are expressed as a percentage of the control. Control vials contained only PRBCs and [$^3$H]-MQ. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.

### *3.2.7 Effect of P-glycoprotein inhibitors and substrates on [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

Vanadate is a phosphate transition-state analogue and a P-type ATPase inhibitor (Harvey, 1992). Vanadate inhibits CQ accumulation in digestive vacuoles isolated from *P.falciparum (Saliba et al., 1997).* Progesterone reverses P-gp-mediated drug resistance in MDR cancer cells by increasing drug accumulation in the cells (Huang Yang *et al.*, 1989). It is also able to increase CQ accumulation in *P.falciparum* isolated digestive vacuoles *(Saliba et al., 1997).* Rhodamine 123 is a well characterised P-gp substrate (Gottesman & Pastan, 1993). Figure 3.10 shows that none of these P-gp modulators have any effect on MQ accumulation in *P.falciparum.*

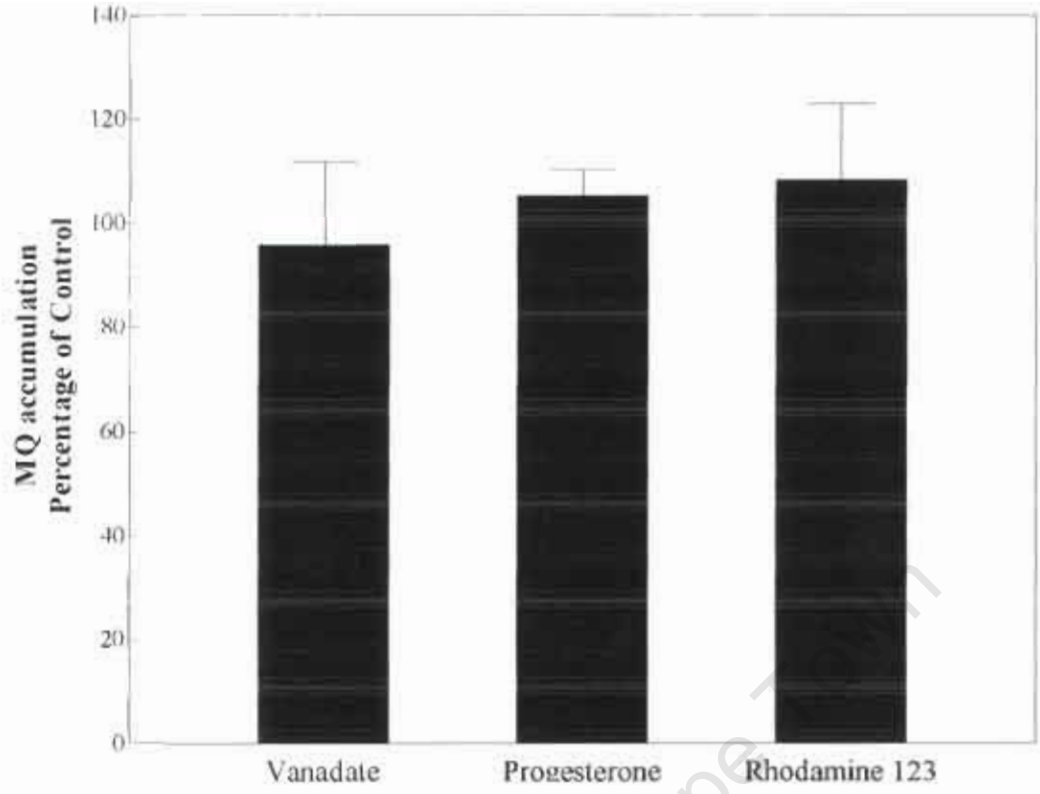



**Figure 3.12:** The effect of vanadate (100 μM), progesterone (100 μM) and rhodamine 123 (1 μg/ml) on the accumulation of [$^3$H]-MQ in RSA 11 PRBC. Results are expressed as a percentage of control in the absence of drug. Error bars represent the standard deviation from 2 separate experiments performed in triplicate



### *3.2.8 Effect of antimalarials on [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

A range of antimalarials were tested for their ability to compete out [$^3$H]-MQ accumulation. Unlabelled MQ was able to significantly inhibit [$^3$H]-MQ at both 100-fold ($p = 0.0023$) and 1000-fold ($p < 0.0001$) excess. However, none of chloroquine, quinine, amodiaquine, pyronaridine or artemisinin were able to significantly affect MQ accumulation (all pvalues >

0.01). Interestingly 10 μM MQ was able to abolish [$^3$H]-CQ accumulation to only 4 % of the control.

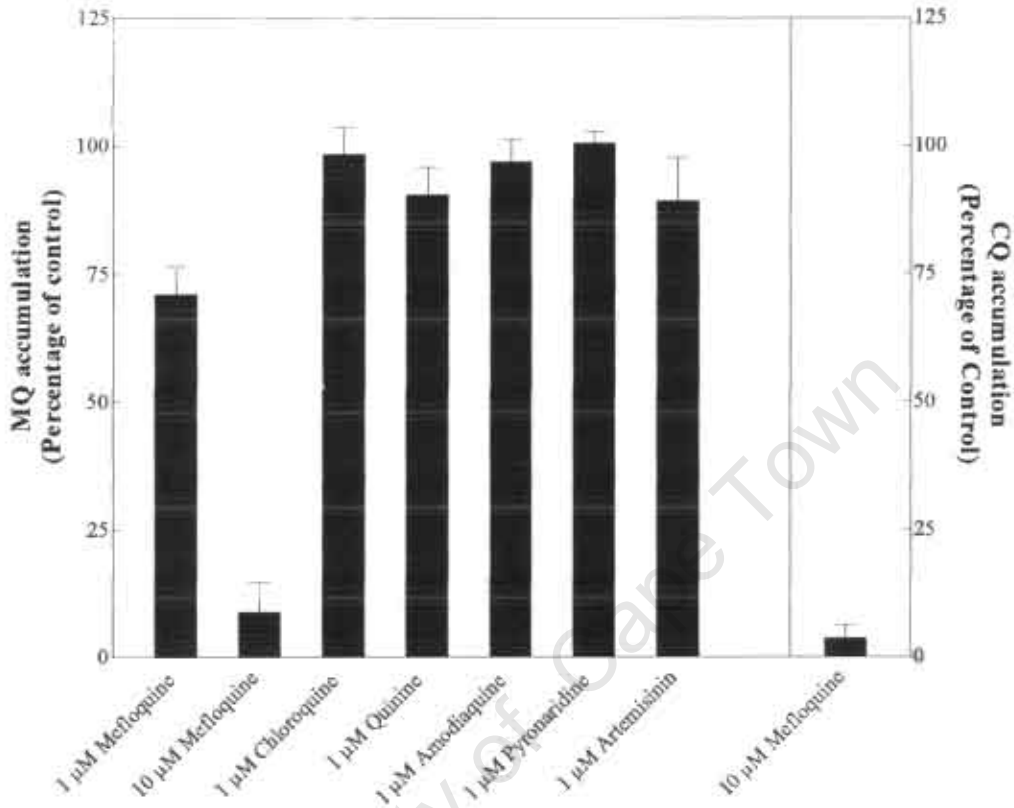



**Figure 3.13:** The effect of unlabelled mefloquine, chloroquine, quinine, amodiaquine, pyronaridine and artemisinin on the accumulation of [$^3$H]-MQ in RSA 11 PRBC. The effect of 10 μM unlabelled MQ on [$^3$H]-CQ accumulation is also shown. Results are expressed as a percentage of control in the absence of drug. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.



### *3.2.9 Effect of resistance reversers on [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

Verapamil is a calcium channel blocker that is able to increase CQ accumulation in CQR parasites bringing about a reversal of resistance (Martin *et al.*, 1987). The effect of verapamil

on MQ accumulation was investigated in both MQS and MQR strains. Verapamil was not able to increase MQ accumulation, in fact a slight decrease was observed in both strains (Figure 3.14). Verapamil has previously been shown to have no effect on MQR strains (Oduola *et al.*, 1993). Penfluridol is the only agent that has been shown to reverse resistance in MQR strains (Oduola *et al.*, 1993; Peters & Robinson, 1991). This was confirmed in the current study, because 0.5 μ M penfluridol decreased the MQ $IC_{50}$ of the MQR D10 strain from 7.5 nM to 3 nM (Table 3.1). Penfluridol at the same sub-inhibitory concentration (0.5 μM) used by Oduola (1993), did not cause an increase in MQ accumulation (Figure 3.14).

**Table 3.1:** The effect of penfluridol on the $IC_{50}$ of the MQR strain of *Plasmodium falciparum*, D10.

| | **MQ IC50 (nM)** | |
|---|---|---|
| | **MQ alone** | **MQ + 0.5 μM penfluridol** |
| **D10 Strain** | 7.500 ± 1.414 | 3.006 ± 1.723 |

Values are mean and standard deviations from two separate experiments performed in duplicate.

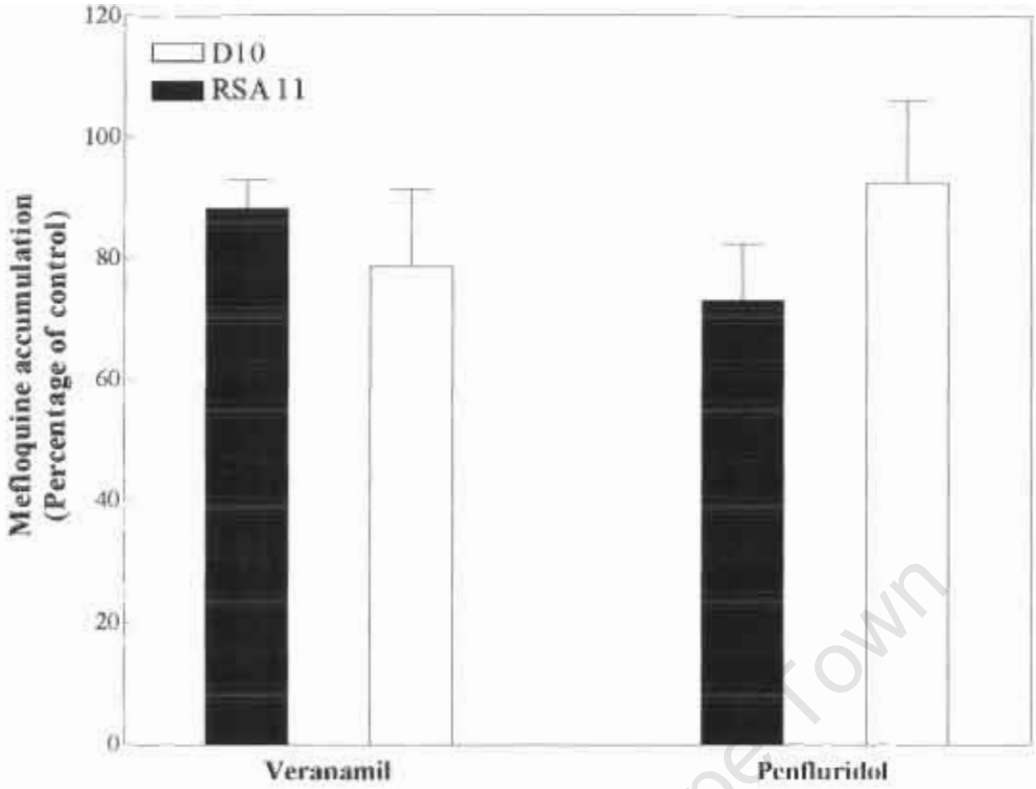



**Figure 3.14**: The effect of verapamil (5 μM) and penfluridol (0.5 μM) on the accumulation of [$^3$H]-MQ in RSA 11 (MQS) and D10 (MQR) PRBC. Results are expressed as a percentage of control in the absence of drug. Error bars represent the standard deviation from 2 separate experiments performed in triplicate.

## *3.3 Discussion*

### *3.3.1 Establishment of conditions for the accumulation of [$^3$H]-mefloquine in Plasmodium falciparum infected erythrocytes.*

On discovering that MQ binds to a large extent to membrane phospholipids Chevli and Fitch (1982) suggested that this sequestration to the membranes may obscure an energy dependent component of MQ accumulation (Chevli & Fitch, 1982). They assert that this may explain their earlier findings, where they could not detect energy dependent MQ accumulation (Fitch *et al.*, 1979). Until now no energy dependent MQ accumulation has been discovered. In Section 3.2.1 experimental conditions were established that enabled further distinction between parasite-dependent and erythrocyte-dependent MQ accumulation. The rationale for this was to amplify any parasite-dependent effect on MQ accumulation, in an effort to reveal the energy dependent component alluded to by Chevli and Fitch (1992).

By removing the serum components (Albumax) from the uptake medium it was possible to make more non protein bound MQ available for uptake by the parasite (Figure 3.1). In the absence of Albumax CQ accumulation was not affected, indicating that over the sixty minutes of incubation the parasites were not adversely affected and were able to accumulate drug normally.

To further increase the influence of parasite-dependent MQ accumulation, experiments were carried out using cultures with parasitemias of greater than 80 %. By using parasitemias of

80 %, as opposed to the normal 5 %, it was possible to decrease the influence of the erythrocytes on MQ accumulation from 60 % of total MQ uptake to 25 % (Figure 3.2).

It has been demonstrated frequently that inoculum size has a profound effect on the potency of quinolines. The fractional volume of PRBCs is directly proportional to the $IC_{50}$. This phenomenon is the inoculum effect. As the number of parasites in a culture increases more drug is depleted from the medium by uptake into each cell, thereby reducing the total amount of drug that is able to accumulate in each cell, to mediate its killing effect. The profound influence of the inoculum effect of MQ accumulation was shown in figure 3.3. As the parasitemia was increased from 1.25 % to 10 % there was a drastic decrease in the amount to drug that accumulated in each PRBC. There appeared to be less of an effect from 20 % to 80 % parasitemia. As a result of this inoculum effect, it was evident that removing the Albumax from the uptake medium was important in ensuring that as much MQ as possible is available for uptake by the parasites.

It is accepted that the above mentioned manipulations of the experimental conditions are more "artificial" than the normally used conditions of complete medium and low parasitemia. However, they were necessary in order to focus on the parasite-dependent accumulation of MQ.

### *3.3.2 Time dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes.*

MQ accumulation was shown to be very rapid and maximal accumulation was evident at 1 minute and did not change from that level up to an incubation time of 120 minutes. It has been shown previously that MQ passage into uninfected erythrocytes is rapid. Vidriquin *et al* showed that accumulation is complete within 5 seconds (Vidriquin *et al.*, 1996). Vanderkooi *et al* (1988) carried out time course experiments in uninfected mouse RBCs and in *P.chabaudi*-infected RBCs from 1 minute to 15 minutes. They claimed that maximal RBC accumulation occurred at 1 minute and the infected RBCs accumulated MQ maximally after 5 minutes. However their data indicate that at one minute the amount of MQ accumulated was only very slightly below the amount accumulated at 5 minutes, and no errors bars were shown. They also showed that CQ accumulation in *P.chabaudi*-infected RBCs is slower than MQ accumulation, CQ accumulation was maximal after 10 minutes. Other studies in *Plasmodium falciparum* have shown that steady state CQ accumulation is only achieved after only 60 minutes (Geary *et al.*, 1986). It is clear that MQ accumulates far more rapidly that CQ does.



### *3.3.3 Concentration dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

There is ample evidence supporting the existence of both saturating and non-saturating components in the accumulation of CQ (Fitch, 1970; Fitch, 1973; Bray *et al.*, 1998). However, only Fitch's study, in 1979, has shown that MQ may also have these two elements

in its uptake. In section 3.2.2, it was shown that the data describing the dependence of MQ accumulation on the external MQ concentration fits an equation describing a "curvilinear" plot better than either a straight line regression or a rectangular hyperbola regression. This is very similar to Fitch's (1979) findings that MQ's accumulation does in fact have a similar pattern to that of CQ. The saturable component of CQ accumulation was identified to be the binding of CQ to haem (Bray *et al.*, 1998). It is conceivable that haem is also responsible for the saturable component of MQ accumulation, because it has been shown to both bind haem and also to inhibit β-haematin formation *in vitro* (Egan *et al., 1997;* Egan *et al., 1999)*.. However it should be noted that the affinity of MQ for haem is much lower than that of CQ (Log Ks are 3.9 and 5.52 respectively).

Also the fact that MQ has only one protonation site compared with two for CQ, means that according to weak base hypothesis much less MQ should accumulate in the acidic food vacuole and be available for haem binding. However, it has been shown in this work (Chapter 4, Section 4.2.1) and elsewhere (Fitch *et al.*, 1979; Vanderkooi *et al.*, 1988) that PRBCs accumulate more MQ than CQ. This higher uptake is probably due to the large extent to which MQ binds to membrane phospholipids (Chevli & Fitch, 1982), which may at least in part be responsible for the saturable uptake of MQ.

The identity of the non-saturable component of uptake of both CQ and MQ is still unknown.

### *3.3.4 pH dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

In Section 3.2.4 it was demonstrated in figure 3.8 that the accumulation of MQ is sensitive to changes in the external medium pH. The importance of the pH gradient between the external medium and food vacuole of the parasite was reinforced by the experiments in which the parasite was exposed to several pH gradient modulators. The fact that both of the proton pump inhibitors (bafilomycin A1 and NEM) as well as the ionophore, nigericin, caused a decrease in MQ accumulation, proves that MQ accumulation is, in part, dependent on a pH gradient. This concurs with the work of Vanderkooi *et al* (1988), who used a series of ionophores, including nigericin, to show that 40 to 60 % of MQ accumulation is pH-dependent. The same study showed that CQ accumulation is approximately 90 % pH-dependent (Vanderkooi *et al.*, 1988). CQ has two protonation sites as opposed to MQ which has one, therefore according to the proton-trapping hypothesis, CQ should accumulate in a more pH-driven manner (See Chapter 1, Section 1.4.1.1.1). There is much evidence supporting the assertion that CQ accumulation is highly pH-dependent (Choi.I & Mego, 1988; Krogstad & Schlesinger, 1986; Yayon *et al.*, 1985; Bray *et al.*, 1992b). The current study, along with the work of Vanderkooi *et al* (1988), proves that that MQ accumulation in *Plasmodium*-infected RBCs is only partly dependent on the pH gradient.

It should be noted that there may be another explanation for the accumulation pattern observed between pH 5.5 to pH 9. Phosphofructokinase is the most important rate-limiting enzyme in glycolysis, this enzyme is exquisitely sensitive to pH, since the parasite is dependent solely on glycolysis for ATP production, and interference in this process would

have an effect on the functioning of the parasites. Therefore the changes in pH over the range may also reflect different ATP levels and therefore also parasite function

### *3.3.5 Effect of P-glycoprotein inhibitors and substrates on [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

There is abundant evidence that Pgh1 is involved in MQ resistance. Increased expression of Pgh1 (Peel *et al.*, 1994; Wilson *et al.*, 1993; Cowman *et al.*, 1994; Barnes *et al.*, 1992) and mutations in Pgh1 (Reed *et al.*, 2000; Duraisingh *et al.*, 2000) have been linked with MQ resistance. Expression of Pgh1 in yeast conferred MQ resistance to those cells (Volkman *et al.*, 1995) and transfection of certain Pgh1 alleles into malaria parasites changed the MQ sensitivity of those parasites (Reed *et al.*, 2000) (for more detail on the link between MQ resistance and Pgh1, see Chapter 1, Section 1.7.2.1). To determine whether the role of Pgh1 in MQ resistance is related to an ability to interfere with the accumulation of the drug, MQ accumulation experiments were carried out in the presence of several compounds which are known to interact with Pgh1 or its homologue, Pgp.



Vanadate, is an inibitor of Pgp, by virtue of the fact that it is a phosphate transition state analogue (Harvey, 1992). It has also been shown to abolish CQ accumulation in isolated food vacuoles *(*Saliba *et al., 1997)*. It had no effect on MQ accumulation (figure 3.10). Huang Yang et al (1989) showed that progesterone is able to reverse Pgp-mediated multi-drug resistance by increasing drug accumulation in MDR cancer cells (Huang Yang *et al.*, 1989); it also increases CQ accumulation in isolated food vacuoles *(*Saliba *et al., 1997)*. Figure 3.10

shows that this steroid chemosensitiser had no effect on MQ accumulation. Rhodamine 123 is a fluorescent compound that has been used in several studies for the determination of the transport function of Pgp, because it is an substrate for Pgp. No alteration in MQ accumulation was observed when the parasites were exposed to Rhodamine 123 (Figure 3.10)

Verapamil's ability to reverse resistance in CQR strains of *Plasmodium falciparum* is thought to be predominantly due to it's ability to increase the amount of CQ that accumulates in CQR parasites. It has been demonstrated that verapamil is unable to shift the $IC_{50}$ of MQR strain down to that of MQS strains (Oduola *et al.*, 1993; Peters & Robinson, 1991). The finding in this study (figure 3.11) that verapamil has no effect on MQ accumulation, explains why this compound is unable to reverse resistance in MQR strains.

Penfluridol is the only compound that has been shown to chemosensitise MQR strains. Peters and Robinson (1991) showed that mefloquine, when administered with penfluridol, cured mice infected with MQR *Plasmodium yoelii* (Peters & Robinson, 1991). *In vitro* reversal of mefloquine resistance has also been demonstrated (Oduola *et al.*, 1993). This was confirmed in this study (Table 3.1). The experiment represented in Figure 3.14 shows the first investigation of penfluridol's action on MQ accumulation. 0.5 μM penfluridol was used by Oduola to shift MQ $IC_{50}$s (Oduola *et al.*, 1993), this concentration did not affect MQ accumulation. There are two possible explanations for this. Firstly, the mechanism by which penfluridol reverses resistance is not related to the accumulation of MQ. Secondly, the shifts in MQ $IC_{50}$ observed by Oduola *et al* and in Section 2.2.7 were small, the best shift demonstrated was a shift from 6.85 to 2.38 ng/ml (Oduola *et al.*, 1993). It is possible that the current experimental conditions were not able to reflect a change in MQ accumulation that might be responsible for the small shifts in $IC_{50}$ observed in this study and by Oduola *et al.*

### *3.3.6 Effect of antimalarials on [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

The effect of a 100-fold excess of unlabelled MQ, CQ, QUI, AQ, PDN and ART on [$^3$H]-MQ accumulation were investigated. It might be expected that the action of the quinoline antimalarials could be two-fold: Firstly to decrease MQ accumulation because they are weak bases and their accumulation in the food vacuole would cause vacuolar acidification, resulting in a reduction in the pH gradient; secondly a decrease could occur due to competitive inhibition of a specific uptake mechanism or receptor because these compounds are structurally related. In Section 3.2.6 it was shown that only MQ itself, at 100-fold and 1000-fold excess, was able to reduce [$^3$H]-MQ accumulation. None of the other antimalarials had an effect on [$^3$H]-MQ accumulation.

It may not be surprising that artemisinin, being structurally unrelated, did not affect MQ accumulation. The lack of cross resistance between CQ, AQ and PDN with MQ (Cowman *et al.*, 1994; Peel *et al.*, 1994; Peel *et al.*, 1993; Lambros & Notsch, 1984; Merkli & Richle, 1980), may explain the lack of effect on MQ accumulation observed.

It is well documented that there is cross resistance between MQ and QUI (Wilson *et al.*, 1993; Bray *et al.*, 1994; Cowman *et al.*, 1994; Peel *et al.*, 1994), it is somewhat anomalous that QUI did not affect MQ accumulation. Only MQ has been shown to bind phospholipids to a large extent (Chevli & Fitch, 1982). Therefore, one would not expect the other antimalarials to compete out [$^3$H]-MQ-phospholipid binding. It is therefore possible that despite the

improved experimental conditions, the high degree of [$^3$H]-MQ-phospholipid binding is obscuring any specific inhibition of MQ accumulation by the other antimalarials.

The ability of MQ to abolish CQ accumulation is also anomalous. MQ's ability to bind to a large extent and with high affinity to membrane phospholipids may interfere with CQ's passage through the RBC membrane, the parasitiphorous vacuolar membrane, the plasma membrane and the food vacuole membrane, down the pH gradient into the food vacuole.

### *3.3.7 The energy dependence of [$^3$H]-mefloquine accumulation in Plasmodium falciparum infected erythrocytes*

In 1979 Fitch *et al* were unable to demonstrate energy dependent MQ accumulation in *P.berghei* (Fitch *et al.*, 1979). Three years later these authors discovered that MQ binds membrane phospholipids to a large extent. This finding prompted the suggestion that this phospholipid binding may have obscured any energy requirement for MQ accumulation in their previous experiments (Chevli & Fitch, 1982). Until now, the only evidence supporting this contention was provided by Vanderkooi et al (1988) who performed an experiment that showed that MQ accumulation in murine malaria parasites was partly temperature dependent. When the temperature of the incubation medium was decreased from 25 °C to 0 °C, MQ accumulation decreased by 40 % (Vanderkooi *et al.*, 1988).



The glycolysis inhibitor, iodoacetate, inhibited MQ accumulation at all three concentrations used, where as the oxidative phosphorylation inhibitor, sodium azide, did not have an effect. This not only indicates that there is an energy requirement for MQ accumulation but also

confirms that the parasite is dependent on glycolysis for its energy supply. Incubation of the parasites at 4 °C decreased MQ accumulation to similar levels to those caused by iodoacetate. Glucose deprivation resulted in a decrease in MQ accumulation in MQR and MQS strains of 40 % and 55 % respectively. Interestingly CQ accumulation in CQR strains have also been shown to be less sensitive to metabolic inhibition (Fitch *et al.*, 1975).

It appears that in this system between approximately 25 % and 45 % of MQ accumulation is energy dependent

# Chapter 4

# Mefloquine accumulation in mefloquine-resistant and mefloquine-sensitive *Plasmodium falciparum.*

## *4.1 Introduction*

One of the few consistent phenotypic differences between CQR and CQS *Plasmodium* strains is that resistant parasites accumulate less CQ than sensitive parasites (Fitch, 1969; Fitch, 1970). The only previous study directly measuring MQ accumulation in strains with different resistance profiles was carried out by Fitch et al (1979). This study, which compared the accumulation of MQ in CQR and CQS strains of *Plasmodium berghi*, found that there was no difference in the amount of MQ taken up by the strains tested. The sensitivity of the parasites to MQ was not determined in that study (Fitch *et al.*, 1979).

An indirect method of measuring drug accumulation is by using the inoculum effect (Geary *et al.*, 1990; Bray *et al.*, 1996; Hawley *et al.*, 1996). Reed *et al*s' (2000) (see Chapter 1, Section 1.7.1.2.1) transfection study estimated the MQ cellular accumulation ratio (CAR) in their transfected lines using the inoculum effect. They found that parasites transfected with the 7G8 allele became MQS. Using this indirect method of measuring MQ uptake they also showed that these parasites accumulated more MQ than the parasites containing the D10 allele (Reed *et al*, 2000).

This chapter compares the MQ accumulation capabilities of strains of *Plasmodium falciparum* with different resistance profiles. MQ accumulation was firstly measured directly by measuring the uptake of radiolabelled MQ in the MQS RSA11 and the MQR D10 strains. Then the inoculum effect was used to measure the cellular accumulation ratio in these strains.

*4.2 Results*

### *4.2.1 Accumulation of [$^3$H]-chloroquine and [$^3$H]-mefloquine in mefloquine-resistant and mefloquine-sensitive Plasmodium falciparum*

In figure 4.1 the accumulation of both radiolabelled CQ and MQ is shown. The CQS D10 strain accumulates almost 14-fold more CQ than the CQR strain (61.70 ± 13.67 and 4.46 ± 0.69 fmoles per million trophozoites respectively). This is in agreement with previous work (Fitch, 1969; Fitch, 1970). Furthermore, MQ was shown to accumulate 2.8-fold more in the MQS RSA11 strain (223.34 ± 21.05 fmoles per million trophozoites) than in the MQR D10 strain (77.63 ± 9.64). This experiment also confirms previous work that MQ accumulates to a greater extent than CQ in malaria parasites (Fitch *et al.*, 1979; Vanderkooi *et al.*, 1988). In comparing the MQS RSA11 strain with the CQS D10 strain, MQ accumulates 3.6-fold more than CQ.

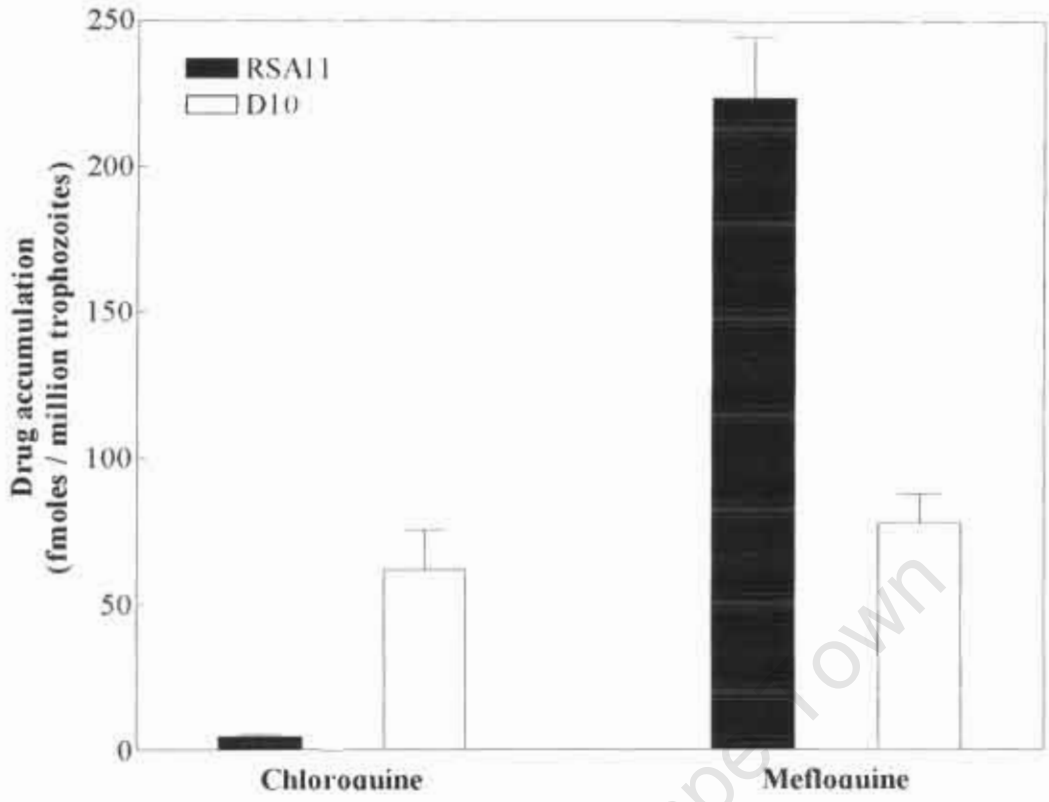



**Figure 4.1:** Compares the accumulation of [$^3$H]-CQ and [$^3$H]-MQ in the D10 and RSA11 strains. Accumulation was carried out at an external concentration of 10 nM for both drugs, for 1 hour. Errors bars represent the SEM from 4 separate experiments performed in duplicate or triplicate.



### *4.2.2 Measurement of the cellular accumulation ratio in mefloquine-resistant and mefloquine-sensitive Plasmodium falciparum*

An alternative method of evaluating the ability of malaria parasites to accumulate quiniline antimalarial is to take advantage of a phenomenon called the inoculum effect. The inoculum effect is discussed in detail in Chapter 3, Section 3.3.1. Briefly, because of significant depletion of drug from the external medium, the measured drug $IC_{50}$ for a strain increases as the inoculum size increases.

Inoculum size can also be described as the fractional volume of the PRBC:

$$\text{Fractional volume of the PRBC} = \text{parasitemia (\%)} \times \text{haematocrit (\%)} \times 10^{-4}$$

A plot of $IC_{50}$ against the fractional volume of PRBC gives a straight line. The y-intercept is the absolute $IC_{50}$, it is the $IC_{50}$ of the strain to the particular drug at an inoculum size of zero. The equation for the calculation of cellular drug accumulation ratio is (Ginsberg, 1990; Hawley, 1996; Bray, 1998):

$$\text{Accumulation ratio} = \frac{IC_{50}\text{ measured} - IC_{50}\text{ absolute}}{IC_{50}\text{ absolute} \times \text{fractional volume of PRBC}} \qquad \textbf{(1)}$$

The CQ and MQ $IC_{50}$s were determined from dose-response curves for D10 and RSA11 at inoculum sizes ranging between 0.00005 and 0.0004 (figures 4.2 A and 4.5 A). The fractional PRBC volume was then plotted against the measured $IC_{50}$ and linear regression was performed to determine the absolute $IC_{50}$s (figures 4.2 B and 4.5 B; table 4.1 and 4.2). The cellular accumulation ratio (CAR) for each strain was then determined using equation 1 (Figures 4.4 and 4.7).

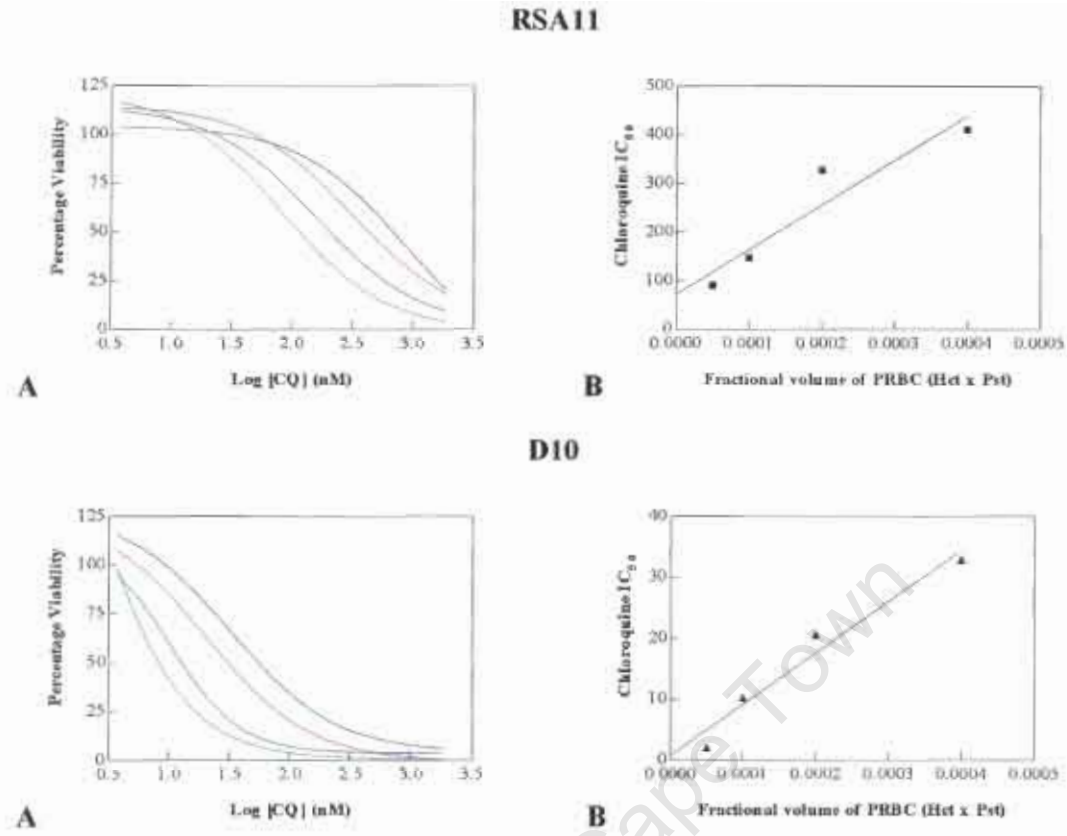



**Figure 4.2:** Graphs demonstrating the effect of inoculum size on CQ potency against the RSA11 and D10 strains of *P. falciparum*. **(A)** CQ dose-response curves with inoculum size of 0.00005 (green); 0.0001 (blue); 0.0002 (red) and 0.0004 (black) over a CQ concentration range from 2 nM to 2 µM. **(B)** The corresponding graphs of measured $IC_{50}$ versus inoculum size for each strain. Each experiment was performed twice in duplicate.

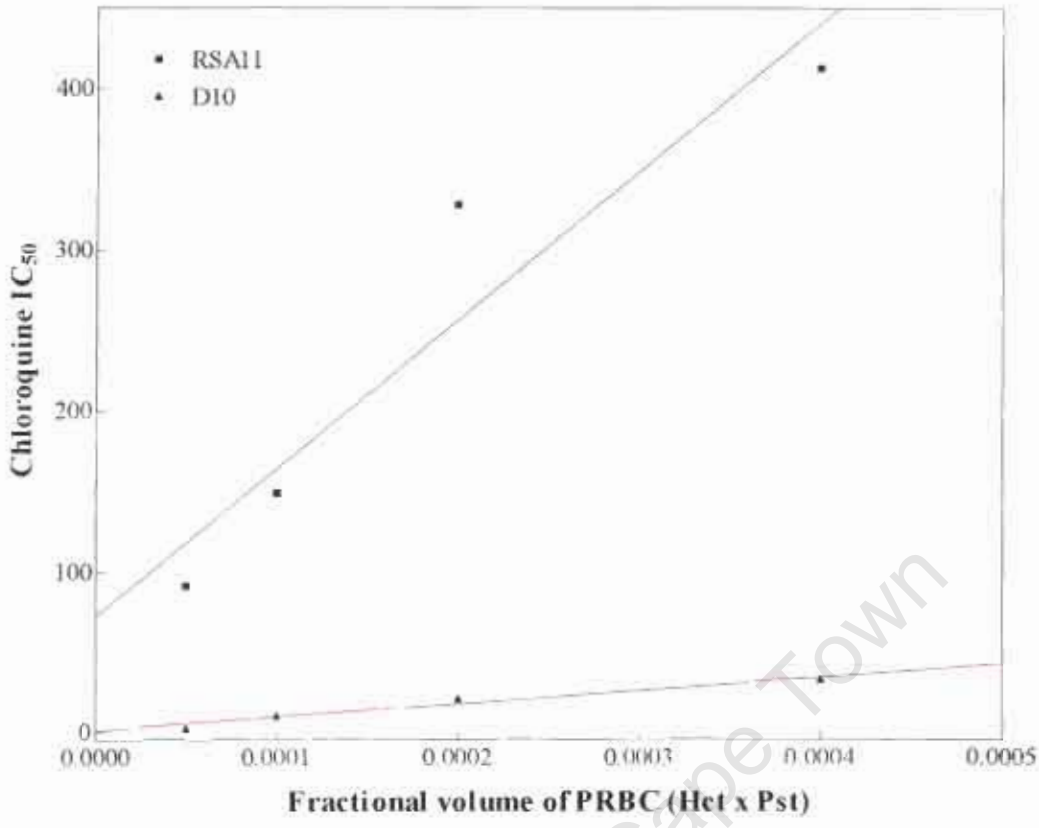



**Figure 4.3**: Comparative graph of measured CQ $IC_{50}$ versus inoculum size for RSA11 (black), D10 (red). Results of the regression lines are given in Table x.1. Each experiment was performed twice in duplicate.

The CQ absolute $IC_{50}$s for RSA11 and D10 confirm that D10 is more sensitive to CQ than RSA11 (0.7070 ± 2.762 and 72.23 ± 50.40 nM respectively). The CAR for D10 was 130081.3 ± 14343.82 nM which almost 10-fold higher than the CAR for RSA 11 (13342.68 ± 3807.46 nM) (table 4.1). This confirms the results obtained in this study, by direct measurement of the accumulation of radiolabelled CQ, where 14-times more CQ accumulatied in D10 (Section 4.2.1).

**Table 4.1:** Regression parameters of chloroquine $IC_{50}$ versus inoculum size regression lines

| Parameter | Strains | |
|---|---|---|
| | RSA11 | D10 |
| Absolute $IC_{50}$ * | 72.23 ± 50.40 | 0.7070 ± 2.762 |
| Slope | 920900 ± 218700 | 84110 ± 11980 |
| R [†] | 0.948 | 0.9808 |
| CAR [‡] | 13342.68 ± 3807.46 | 130081.3 ± 14343.82 |

Values are mean and standard deviations from two separate experiments performed in duplicate. Data were calculated from figures 4.2 and 4.3.

* y-intercept (nM)

[†] Coefficient of correlation

[‡] Cellular accumulation ratio

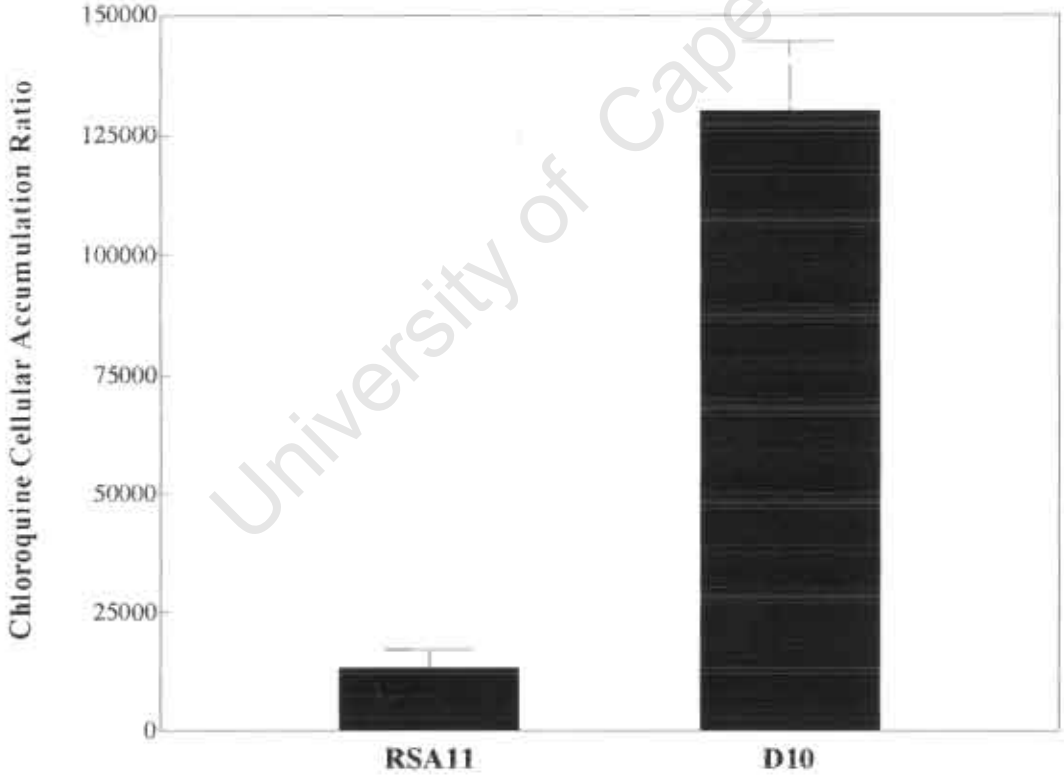



**Figure 4.4**: Chloroquine cellular accumulation ratios for RSA11 and D10 as estimated by the inoculum effect.

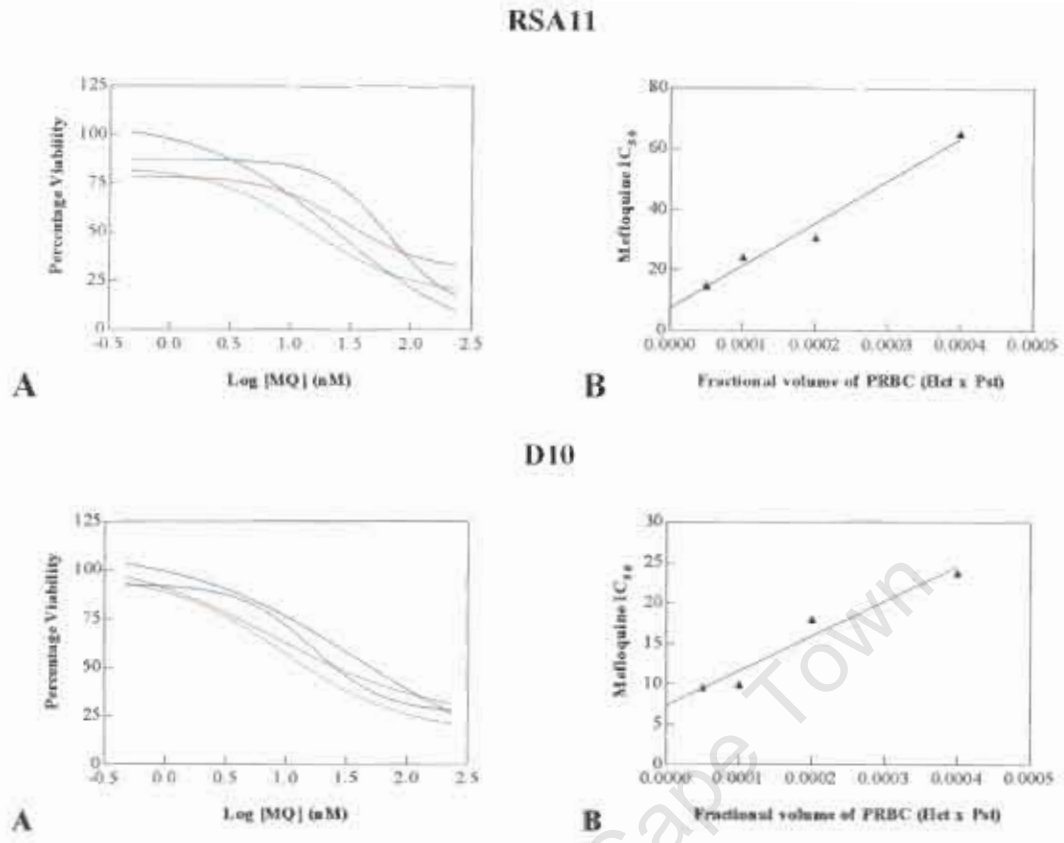



**Figure 4.5**: Graphs demonstrating the effect of inoculum size on MQ potency against the RSA11 and D10 strains of *P. falciparum*. **(A)** MQ dose-response curves with innoculum size of 0. 0.00005 (green); 0.0001 (blue); 0.0002 (red) and 0.0004 (black) over a MQ concentration range from 0.5 nM to 240 nM. **(B)** The corresponding graphs of measured $IC_{50}$ versus inoculum size for each strain. Each experiment was performed twice in duplicate.

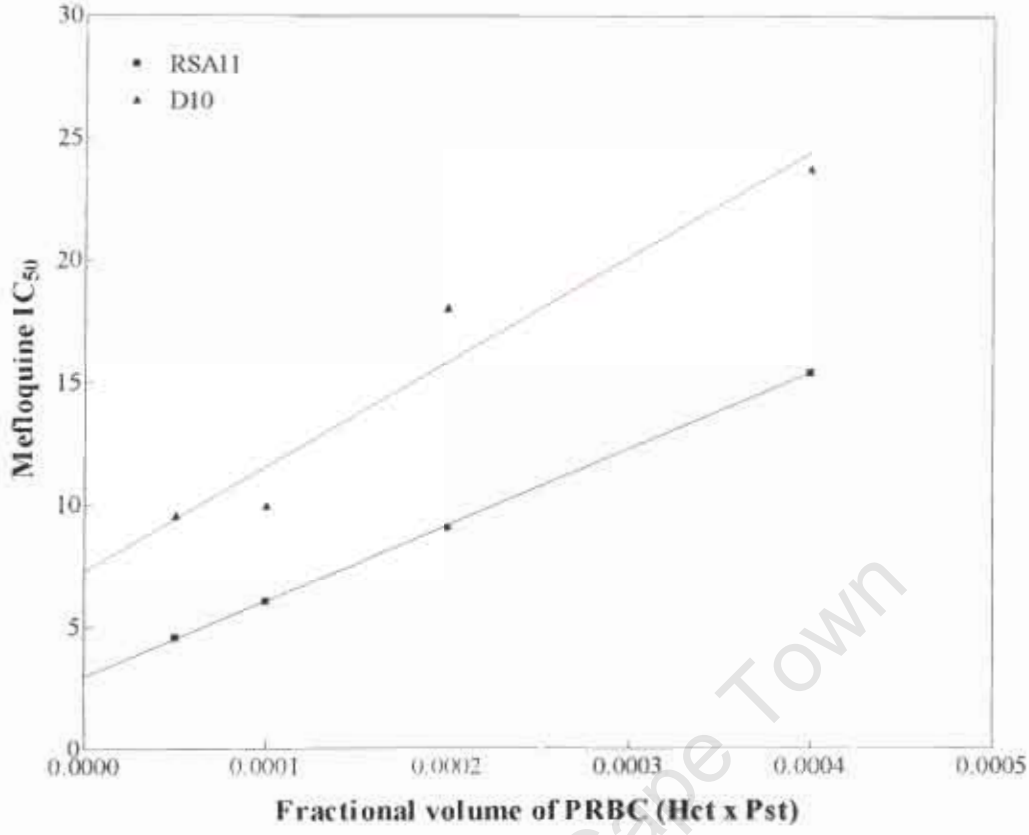



**Figure 4.6:** Comparitive graph of measured MQ $IC_{50}$ versus inoculum size for RSA11 (black), D10 (red). Results of the regression lines are given in Table 4.2. Each experiment was performed twice in duplicate.

The absolute $IC_{50}$ for MQ in RSA11 (2.954 ± 0.0811 nM) was less than half the absolute $IC_{50}$ in D10 (7.290 ± 1.708 nM), confirming that D10 is less sensitive to MQ than RSA11 (Table 4.2). The calculation of the CARs, by the inoculum effect, for these strains confirmed that the MQR D10 strain (5732.85 ±1553.63 nM) accumulates less MQ than the MQS RSA11 strain (10572.95 ± 262.68 nM) (Figure 4.7)

**Table 4.2:** Regression parameters of mefloquine $IC_{50}$ versus inoculum size regression lines for MQ (see Graphs 4.7 and 4.8)

| Parameter | Strains | |
|---|---|---|
| | RSA11 | D10 |
| Absolute $IC_{50}$ * | 2.954 ± 0.0811 | 7.290 ± 1.708 |
| Slope | 31020 ± 351.7 | 42870 ± 7411 |
| R † | 0.99985 | 0.971391 |
| CAR ‡ | 10572.95 ± 262.68 | 5732.85 ±1553.63 |

Values are mean and standard deviations from two separate experiments performed in duplicate. Data were calculated from Graphs 4.2 and 4.3.

* y-intercept (nM)

† Coefficient of correlation

‡ Cellular accumulation ratio

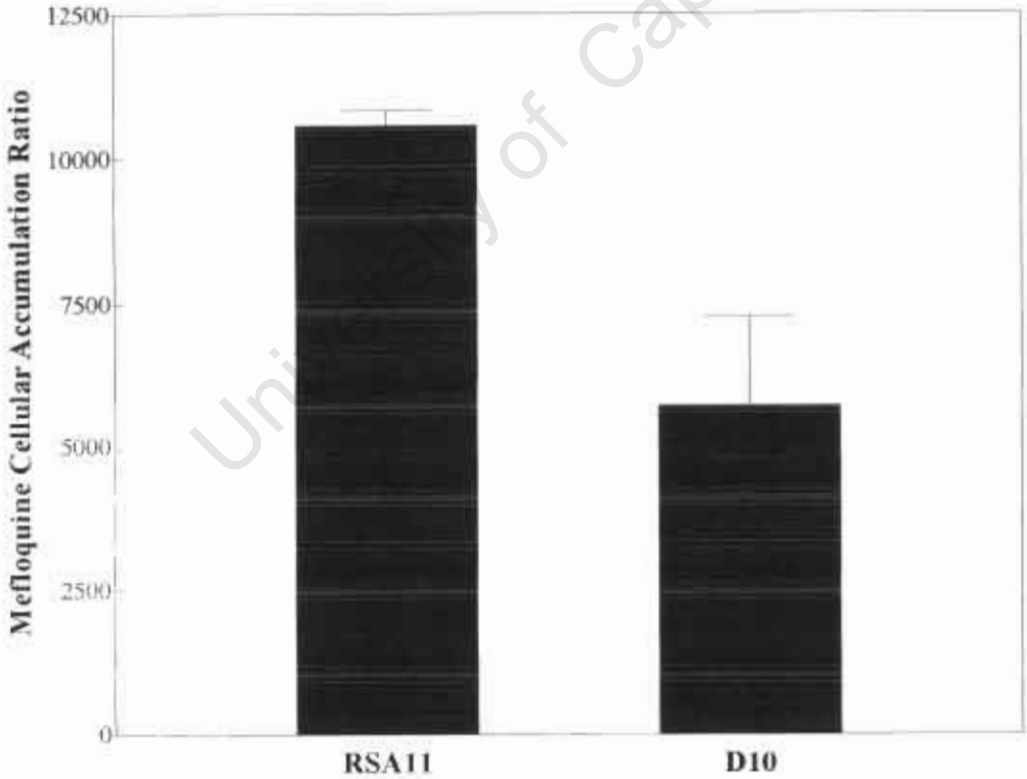



**Figure 4.7:** Mefloquine cellular accumulation ratios for RSA11 and D10 as estimated by the inoculum effect.

### *4.3 Discussion*

The MQ accumulation capabilities of a MQR strain and a MQS strain of *P. falciparum* were evaluated using two different methods. Firstly, MQ accumulation was measured directly using tritiated MQ. It was demonstrated that the MQR strain, D10 (IC50 = 20.58 ± 2.57) accumulated 2.8-times more MQ than the MQS RSA11 strain (IC50 = 4.26 ± 1.27). In control experiments, tritiated CQ accumulation was carried out in these strains and the results obtained confirmed previous work (Fitch, 1969; Fitch, 1970; Yayon *et al.*, 1984) showing that CQR malaria parasites accumulate less CQ than CQS parasites.

By obtaining the absolute $IC_{50}$s for the two strains for both CQ and MQ the relative sensitivities of the strains were confirmed. Using the absolute $IC_{50}$s and equation 1 the CARs of the two drugs in both strains were calculated. CQ, once again, served as a control and confirmed that more CQ accumulates in CQS parasites than in CQR parasites. In comparing the CARs of D10 (MQR) and RSA11 (MQS), it was observed that RSA11 accumulates almost double the amount of MQ than D10 does. This confirmed that results obtained with tritiated MQ, that MQR *Plasmodium falciparum* accumulate less MQ than MQS *Plasmodium falciparum*.

# Chapter 5

# Characterisation [$^3$H]-Mefloquine Accumulation in Isolated Digestive Vacuoles

## *5.1 Introduction*

It is well established that *Plasmodium falciparum*-infected erythrocytes is able to concentrate CQ several thousand-fold in the food vacuole, which is the primary site of accumulation of CQ (De Duve *et al.* 1974;Yayon *et al.* 1984). It has also been demonstrated that the food vacuole is able to accumulate CQ in an ATP-dependent manner (Saliba *et al.* 1998).

In Chapter 3, it was established that MQ accumulation, in erythrocytes infected with *P. falciparum*, is at least partially energy-dependent. It was shown that inhibition of glycolysis; incubation at low temperature; and glucose depravation all caused a decrease in MQ accumulation. It was also shown that the accumulation of MQ is sensitive to perturbation of the pH gradient. This chapter attempts to determine whether the energy-dependent component of MQ accumulation originates in the food vacuole and also whether vacuolar accumulation is dependent on the pH gradient.

*5.2 Results*

### *5.2.1 Adenosine Triphosophate dependence of [$^3$H]-mefloquine accumulation in isolated digestive vacuoles of Plasmodium falciparum*

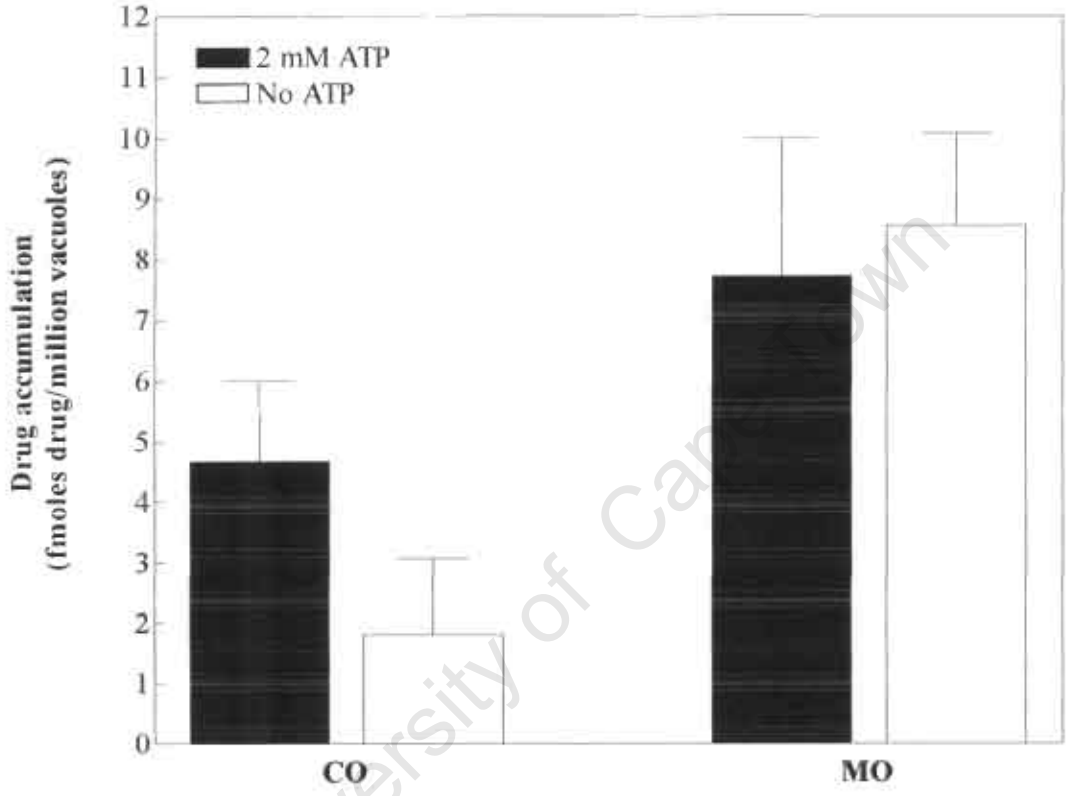



**Figure 5.1:** Shows the accumulation of [$^3$H]-MQ and [$^3$H]-CQ in food vacuoles isolated from RSA 11 in the presence and absence of 2 mM ATP. The external drug concentration was 10 nM for both drugs. The graph represents the mean and standard deviations from 6 separate experiments performed in duplicate for CQ and in triplicate for MQ.

Food vacuoles were isolated from MQS RSA11 parasites according to the method of Saliba et al (1998). This method provides pure, intact and functional food vacuoles. As previously demonstrated by Saliba et al (1998), Figure 5.1 confirms that the isolated food vacuoles accumulate significantly more CQ in the presence of 2 mM ATP ($p = 0.0005$).

It was observed that ATP did not stimulate MQ accumulation, there was no significant difference in MQ accumulation in the presence or absence of ATP ($p = 0.3559$) (Figure 5.1).

### *5.2.2 pH dependence of [$^3$H]-mefloquine accumulation in isolated digestive vacuoles of Plasmodium falciparum*

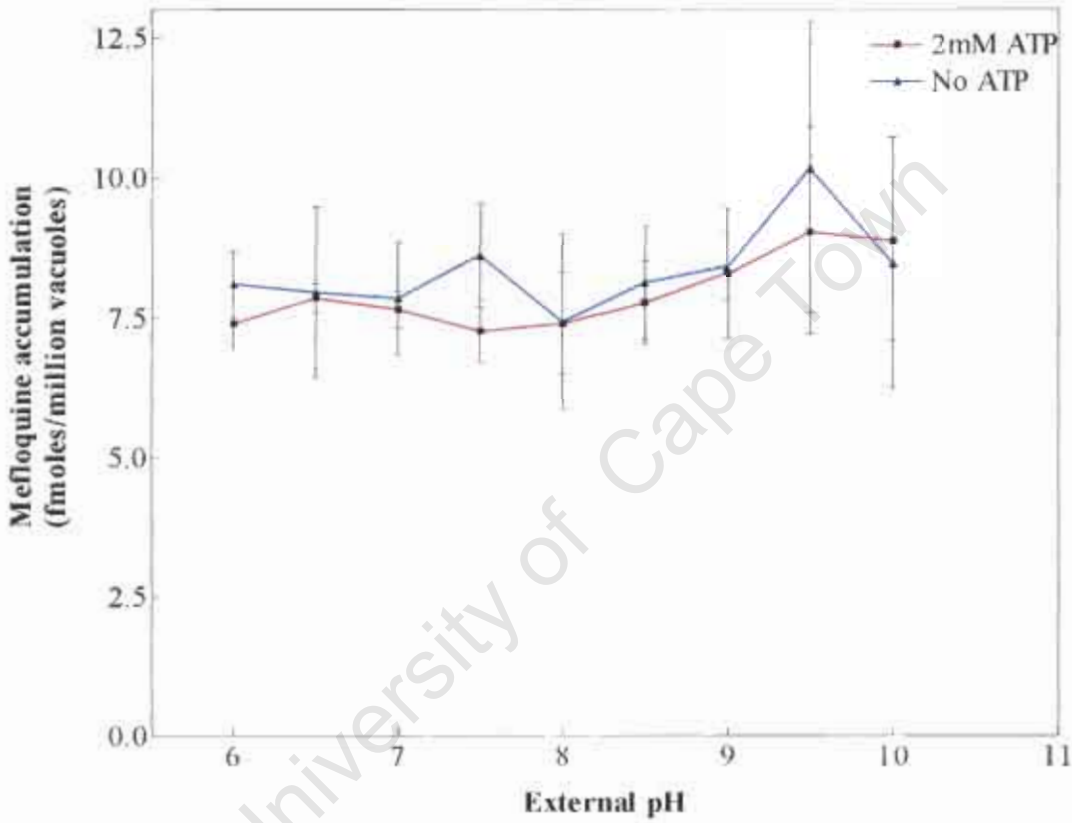





**Figure 5.2:** Shows the accumulation of [$^3$H]-MQ in RSA 11 food vacuoles, in the presence and absence of 2 mM ATP, over a pH range between pH 6 and pH 10, after 1 hour incubation. The graph represents the mean and standard deviations from 2 separate experiments performed in duplicate.

MQ accumulation in PRBCs was shown to be sensitive to changes in the pH of the external medium, with maximal accumulation taking place at pH 7.5, close to physiological pH (Figure 3.8). Figure 5.2 demonstrates that MQ accumulation in isolated vacuoles is

unaffected by changes in the pH of the external medium. Vacuoles incubated in the presence of 2 mM ATP did not accumulate significantly more MQ that those incubated without ATP, at any of the pHs from 6 to 10 (all p values > 0.01).

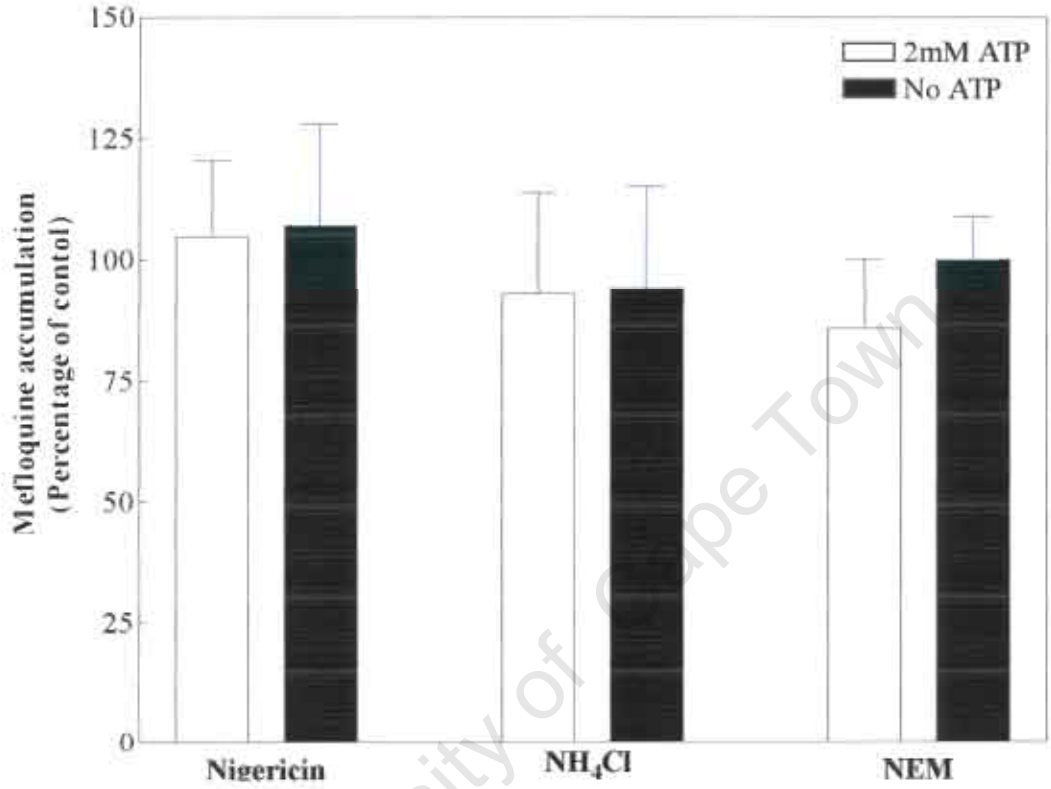



**Figure 5.3**: Shows the effect of nigericin (10 µg/ml), ammonium chloride (10 mM) and N-ethylmaleimide (10 µM) on the accumulation of [$^3$H]-MQ in RSA 11 food vacuoles, in the presence and absence of 2 mM ATP. The graph represents the mean and standard deviations from 2 separate experiments performed in duplicate.



Various pH gradient modulators were found to decrease MQ accumulation in PRBC (Figure 3.9). The effect of these compounds on MQ accumulation in isolated vacuoles was investigated. None of nigericin, $NH_4Cl$ or NEM caused the accumulation of MQ to differ from the control experiments (all p values > 0.01).

*5.3 Discussion*

The experiments carried out in PRBCs (Chapter 3) showed that, by optimising the uptake conditions to amplify the contribution of the parasites to MQ accumulation, the energy requirement for accumulation could be uncovered. Fitch *et al* (1982) were correct in their assertion that the energy-dependent component was obscured because of the large amount of "background" MQ binding to the four membranes in the PRBC system.

In the isolated food vacuole there is only one membrane in the experimental preparation. It was expected that the "background" MQ binding would be reduced and that energy dependent vacuole uptake would be revealed.

However this was not the case, no ATP dependent accumulation was evident in this system. There could be several explanations for this. Firstly, that there is no ATP dependent transport across the vacuole membrane and that the energy-dependence observed in PRBC is at the plasma membrane level. Secondly it is possible that the isolated food vacuole system is not efficient enough to demonstrate ATP-dependent MQ accumulation.

In Figure 3.11 six times more CQ accumulated in the PRBC, in the presence of glucose. When CQ accumulation was carried out in vacuoles only two and a half times more CQ accumulated when ATP was present (Figure 5.1). This means that there is a "loss" of energy dependence going from the PRBC system to the vacuole system. In the PRBC system there was only a two to two and a half-fold increase in MQ accumulation in the presence of glucose. If there is an experimental "loss" in the vacuole system, the relatively small energy

dependence in the PRBC system for MQ accumulation, may have been lost due to an imperfect vacuolar system.

# Chapter 6

# [3H]-Mefloquine and [3H]-Chloroquine Adsorption to Haemozoin and β-haematin

## *6.1 Introduction*

As discussed in Chapter 1 (Section 1.4.1.1.3) Fitch proposed in the 1970s that there are two components to CQ accumulation in the PRBC. He suggested a high-affinity saturable component and also a non-saturable lower affinity component. He hypothesised that the CQR Monterey strain may have a deficiency in high-affinity CQ receptor sites compared to the CQS Camp strain (Fitch, 1970; Fitch *et al.*, 1974). The theory of receptor-driven CQ accumulation lost favour due to abundant data being published supporting the proton trapping hypothesis.

However, in 1998 Bray *et al* identified and characterised the high affinity, saturable component of CQ accumulation. They used Ro 40-4388, a potent and specific inhibitor of plasmepsin I, the first enzyme in the haemoglobin degradation pathway (See Chapter 1, Section 1.5). The action of this inhibitor is to effectively decrease the amount of haematin available due to decreased haemoglobin breakdown. Ro 40-4388 was able to decrease CQ accumulation in a dose-dependent manner suggesting that CQ accumulation is dependent on the presence of haematin. These investigators also determined that CQR and CQS

*P.falciparum* strains have the same number of binding sites but the affinity for these binding sites is significantly lower in CQR strains (Bray *et al.*, 1998).

Sullivan et al carried out experiments in which they incubated *P. falciparum* parasites for 20 hours in the presence of tritiated CQ, quinidine (Sullivan *et al.*, 1996) and MQ (Sullivan *et al.*, 1998), and then by electron microscope autoradiography and sub-cellular fractionation, determined the amount of drug associated with the haemozoin fractions. The autoradiographs of parasites after incubation indicated that the majority of CQ in the parasite is associated with the haemozoin crystals. Sub-cellular fractionation indicated that 35 % to 75 % of the quinolines were associated with the haemozoin pellets. Under conditions designed to facilitate the formation of haemozoin these investigators found that these three quinolines associated with newly formed haemozoin only if free monomeric haem was present.

The non-saturable component of CQ accumulation has not been identified. There have been suggestions that it may be due to binding to abundant cytosolic proteins (Foley *et al.*, 1994; Dorn *et al.*, 1998; Bray *et al.*, 1998). Foley *et al* found that a photoreactive analogue of CQ bound specifically to two proteins with molecular weights of 42 kDa and 33 kDa (Foley *et al.*, 1994). The 33 kDa protein was later found to be lactate dehydrogenase (Menting *et al.*, 1997). These studies did not determine what proportion of the CQ that accumulates in the PRBC, is bound to the proteins. It does not seem likely that significant CQ binds to proteins in the cytosol because by far the majority of CQ accumulates in the acidic food vacuole (Yayon *et al.*, 1984), whether it be due to ion-trapping (Ginsburg *et al.*, 1989) or receptor-binding (Bray *et al.*, 1998). It seems far more likely that the non-saturable component of CQ accumulation will be found in the food vacuole.

This chapter investigates the distribution of CQ and MQ within the isolated food vacuole of *P. falciparum* and identifies of the non-saturable component of CQ and MQ accumulation. We accumulated drug in isolated food vacuoles and measured the amount of drug associated with a crude haemozoin fraction and the remaining membrane and lumen fraction. We also measured the extent to which CQ and MQ are able to bind to synthetic β-haematin and pure isolated haemozoin.

## *6.2 Results*

### *6.2.1 [$^3$H]-CQ and [$^3$H]-MQ distribution in the isolated food vacuole*

*P. falciparum* food vacuoles were isolated using the method of Saliba *et al* (see Chapter 7, Section 7.2) (Saliba *et al.*, 1998). Vacuoles were incubated for 1 hour in buffer containing 2 mM ATP and either 10 nM [$^3$H]-CQ or 10 nM [$^3$H]-MQ at 37°C (Chapter 9, Section 9.6). The food vacuoles were then fractionated as described in Chapter 9 (Section 9.7). Briefly, after lysis by nitrogen cavitation, two preparations were made:

- The lysed vacuoles were centrifuged for 4 minutes at 13 000 rpm, resulting in a crude haemozoin pellet.
- The lysed vacuoles were centrifuged through a Percol cushion to remove vacuolar membranes, resulting in a more purified haemozoin pellet.

The radioactivity in both the pellet and the supernatant of each of these preparations was counted.

Figure 6.1 shows the percentage of pre-accumulated CQ that is associated with haemozoin after fractionation. The crude fractionation and the Percol fractionation resulted in comparable amounts of haemozoin-associated CQ (75% and 73% respectively).

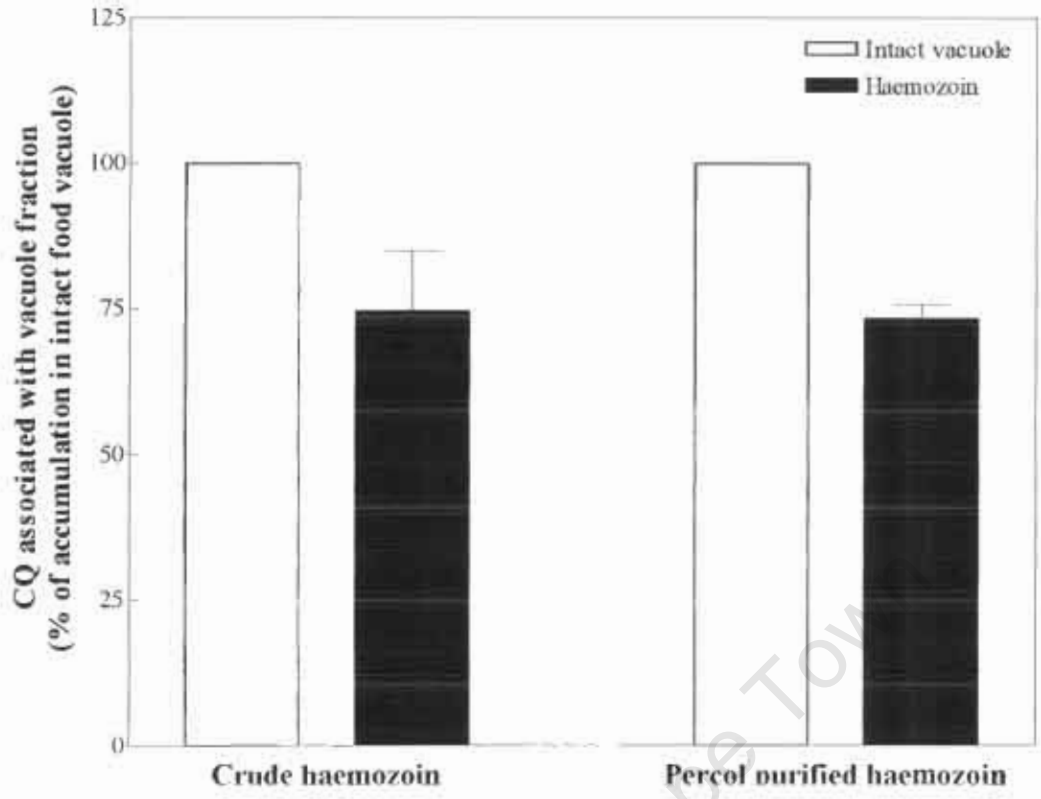



**Figure 6.1:** Shows the distribution of pre-accumulated [$^3$H]-CQ after sub-fractionation of the isolated food vacuole. Control vials with only uptake buffer and radioactivity were treated in exactly the same way as the experimental vials and no radioactivity could be detected below the Percol cushion after centrifugation. Data represent the mean and SEM of 2 separate experiments performed in duplicate.

The distribution of MQ in the isolated food vacuole is shown in figure 6.2. The crude haemozoin pellet bound a similar amount of MQ compared to the Percol-purified pellet (45 % and 42 % respectively).

Overall, the percentage of these quinolines associated with haemozoin is similar to that found by Sullivan et al (Sullivan *et al.*, 1996; Sullivan *et al.*, 1998). In comparing the distribution of CQ and MQ there are some interesting features. In the case of CQ most of the drug is associated with the haemozoin (75 %). However, in the case of MQ, the haemozoin fraction contains less than 50 % of the accumulated drug.

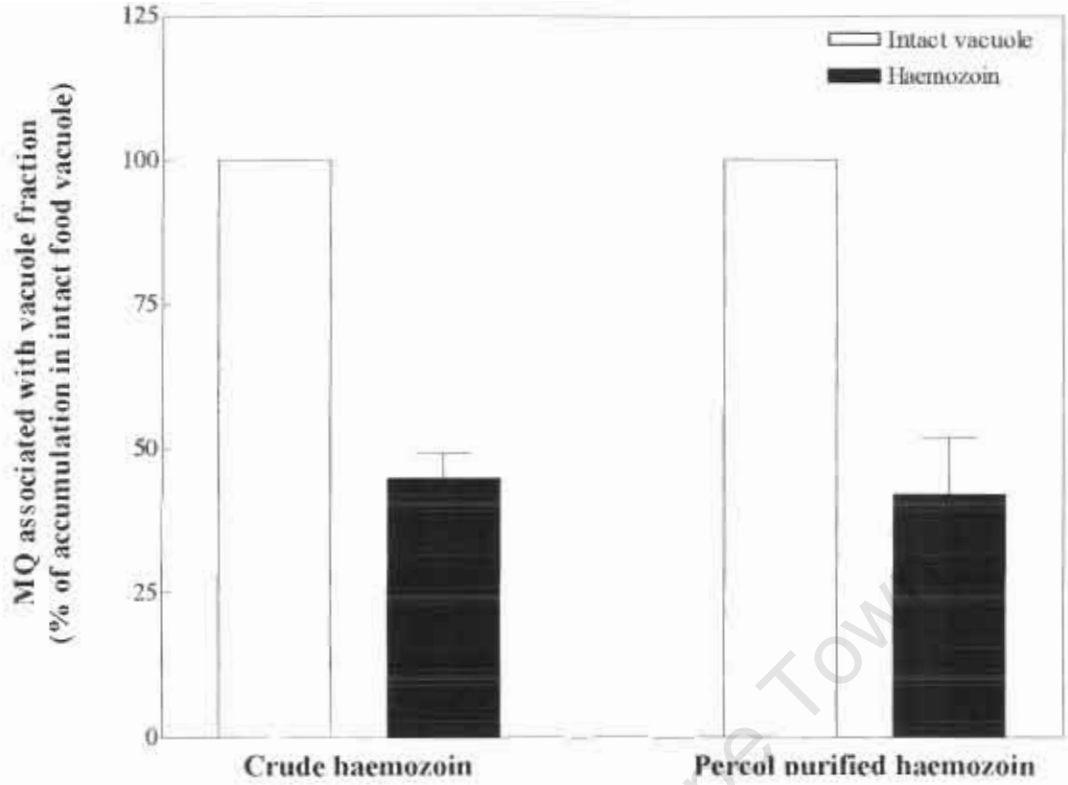



**Figure 6.2:** Shows the distribution of pre-accumulated [$^3$H]-MQ after sub-fractionation of the isolated food vacuole. Control vials with only uptake buffer and radioactivity were treated in exactly the same way as the experimental vials and no radioactivity could be detected below the Percol cushion after centrifugation. Data represent the mean and SEM of 2 separate experiments performed in duplicate.



### *6.2.2 [$^3$H]-CQ and [$^3$H]-MQ adsorption onto synthetic β-haematin*

In light of the above results that indicate that a large percentage of accumulated CQ and MQ associates with haemozoin, experiments were carried out to assess the extent to which CQ and MQ are able to bind to preformed haemozoin. β-haematin, which is chemically identical to native haemozoin, was synthesized according to the method of Egan et al (Egan *et al.*, 1999a). The binding of both CQ and MQ was found to be dependent on the pH of the incubation medium. For both CQ and MQ, approximately twice as much drug bound to β-haematin at pH 4.5, than at pH 7.4 (Figure 6.3).

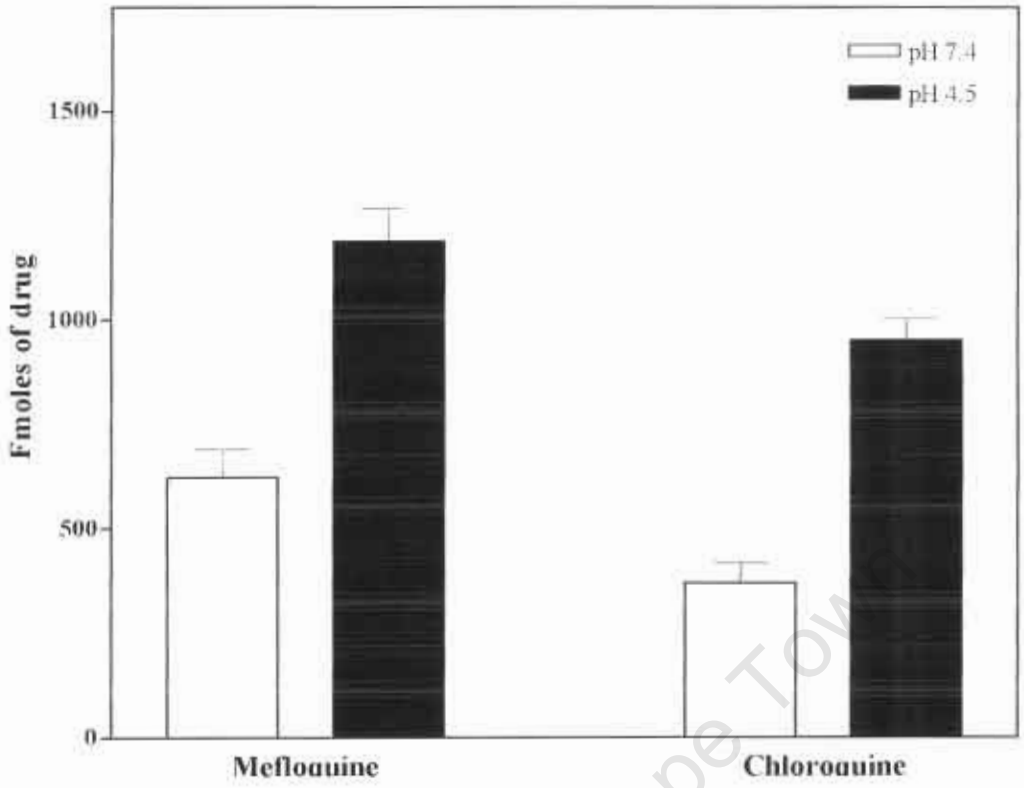



**Figure 6.3**: Shows the pH dependence of drug adsorption onto β-haematin. Equal amounts (50 µg) of β-haematin were incubated in the presence of either 10 nM [3H]-CQ or 10 nM [3H]-MQ for 30 minutes at pH 4.5 (black bars) or pH 7.4 (white bars). Data represent the mean and standard deviation of a single experiment performed in duplicate.



The binding of chloroquine to β-haematin was measured over a concentration range from 2 nM to 2 mM. Initially 500 µg of β-haematin was incubated at 37 °C for 30 minutes in 1 ml of buffer containing the drug (Figure 6.4). It was evident that this amount of β-haematin did not saturate CQ binding. In an effort to produce the expected saturation curve the amount of β-haematin was decreased ten-fold and the concentration range extended to 20 mM. The result was a plot very similar in shape to the previous experiment using 500 µg, there was no evidence of saturation using 50 µg of β-haematin. At each CQ concentration, except 2nM, the amount of drug bound to the 500 µg samples was more than the amount bound to the 50 µg

samples. However, only at the 2mM concentration point could the 500 µg sample be shown to bind significantly more CQ than the 50 µg sample ($p = 0.0159$).

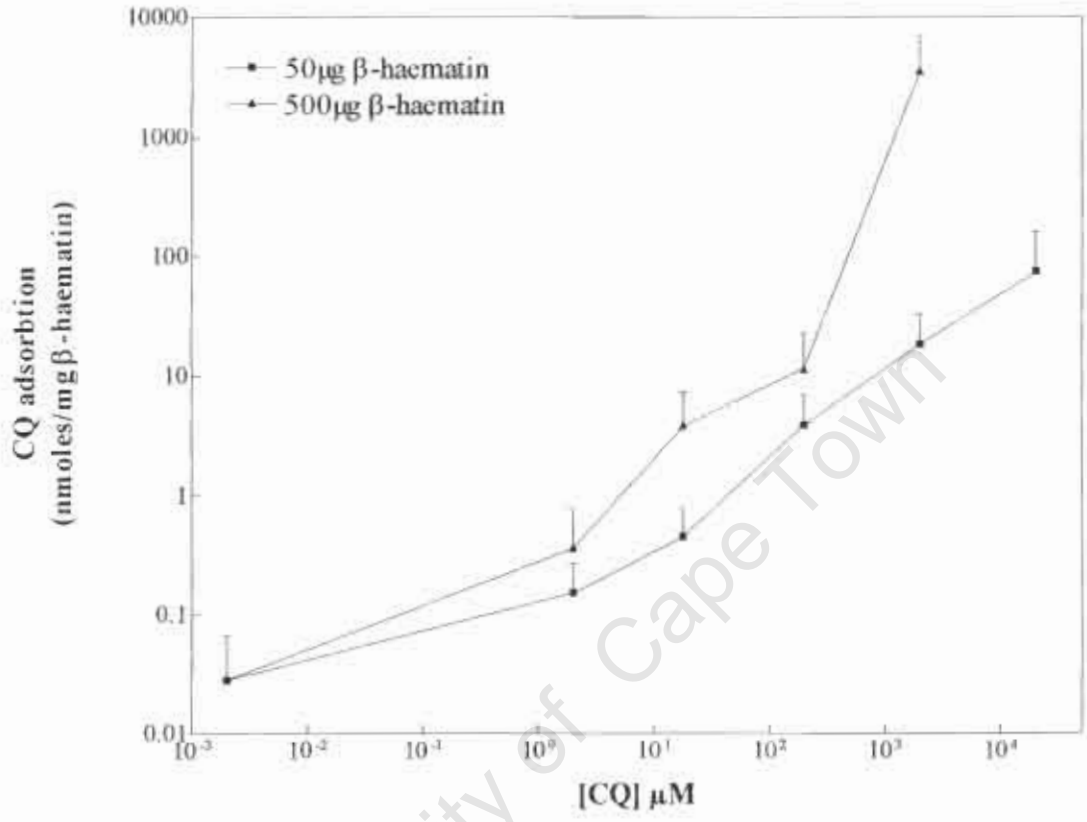



**Figure 6.4**: Shows the extent of adsorption of [$^3$H]-CQ onto β-haematin. 50 µg (■) or 500 µg (▲) of β-haematin was incubated in 1 ml of PBS at pH 5 containing [$^3$H]-CQ. The CQ concentration range was between 2 nM and 20 mM. The data represent the means and standard deviations from three separate experiments performed in duplicate.

MQ binding to β-haematin was also investigated under the same conditions as the CQ experiments above (Figure 6.5). It was observed that MQ behaves in a very similar manner to CQ and there is no saturation of MQ binding. The actual amount of MQ that bound to the β-haematin at each concentration point was not significantly different to the amount of CQ that bound at each point (all p values > 0.1).

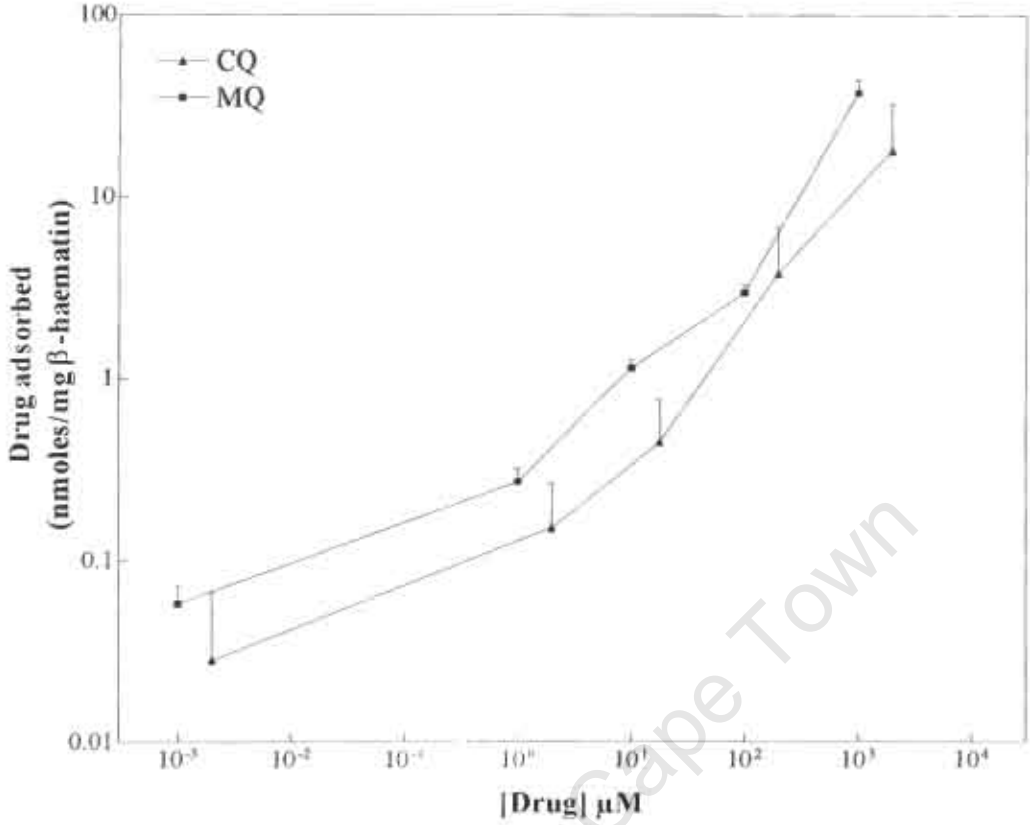



**Figure 6.5**: Compares the extent of adsorption of CQ (▲) and MQ (■) onto β-haematin. 50 μg of β-haematin was incubated in 1 ml of PBS at pH 5 containing [$^3$H]-CQ or [$^3$H]-MQ. The CQ concentration range was between 2 nM and 2 mM and the MQ concentration range was between 1 nM and 1 mM. The data represent the means and standard deviations from three separate experiments performed in duplicate.



### *6.2.3 Purity of isolated haemozoin*

β-haematin was prepared according to the method of Egan *et al* (Egan *et al.*, 1999) (see Chapter 8, Section 7.8). It has been conclusively demonstrated that the product of this synthesis is chemically and structurally identical to haemozoin. X-ray diffraction, infrared spectroscopy and elemental analysis have proven that β-haematin is the only species present after synthesis, and no haematin is evident in the resultant preparation (Egan *et al.*, 1999;

Egan *et al.*, 2001). Nonetheless, it was decided to compare the behavior of haemozoin to that of β-haematin. Haemozoin was isolated from D10 PRBCs using the protocol outlined in Chapter 7 (Section 7.9). The isolation protocol involved DNase I and Proteinase K digestions, to remove DNA and proteins. The preparation was also incubated overnight in 2.5 % SDS to solubilise any monomeric haematin. Scanning electron microscopy and infrared spectroscopy was used to verify that only haemozoin crystals were present in the preparation. From the scanning electron micrographs of synthetic β-haematin (Plates 6.1) and isolated haemozoin (Plate 6.2), it is clear that morphologically the two species are indistinguishable, the size and shape of the crystals are very similar. Furthermore no extraneous cell debris can be observed on the electron micrographs indicating that the haemozoin preparations are pure. Supplementary evidence of the purity of the haemozoin was provided by comparing the infrared spectra of pure synthetic β-haematin with the spectra of the isolated haemozoin. The spectra are identical; there are the characteristic peaks for β-haematin at 1660 $cm^{-1}$ and 1207 $cm^{-1}$. There is also no evidence of contamination either by protein or, importantly, by monomeric haematin.

**Plate 6.1:** Scanning electron micrograph of synthetic β-haematin. Synthesised according to the method of Egan et al (Egan *et al.*, 1999a). (magnification: x 50 000)

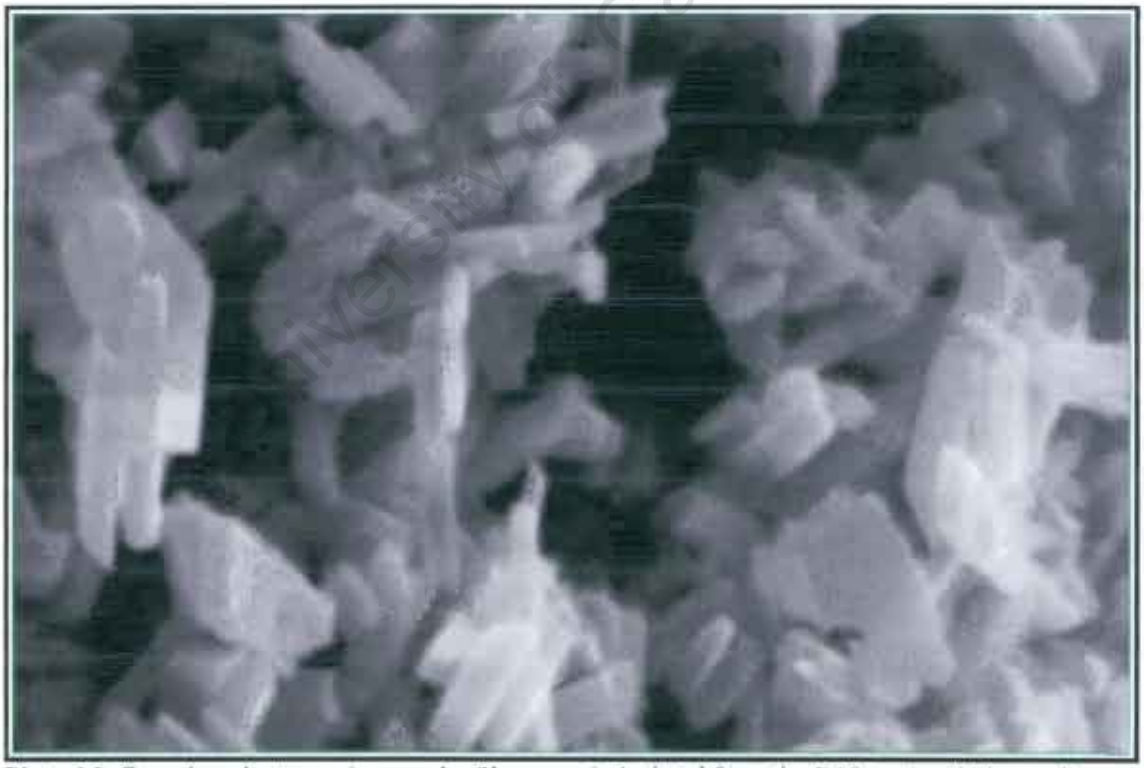


**Plate 6.2:** Scanning electron micrograph of haemozoin isolated from the D10 strain of *Plasmodium falciparum* according to the method outlined in Chapter 7, Section 7.8. (magnification: x 50 000)

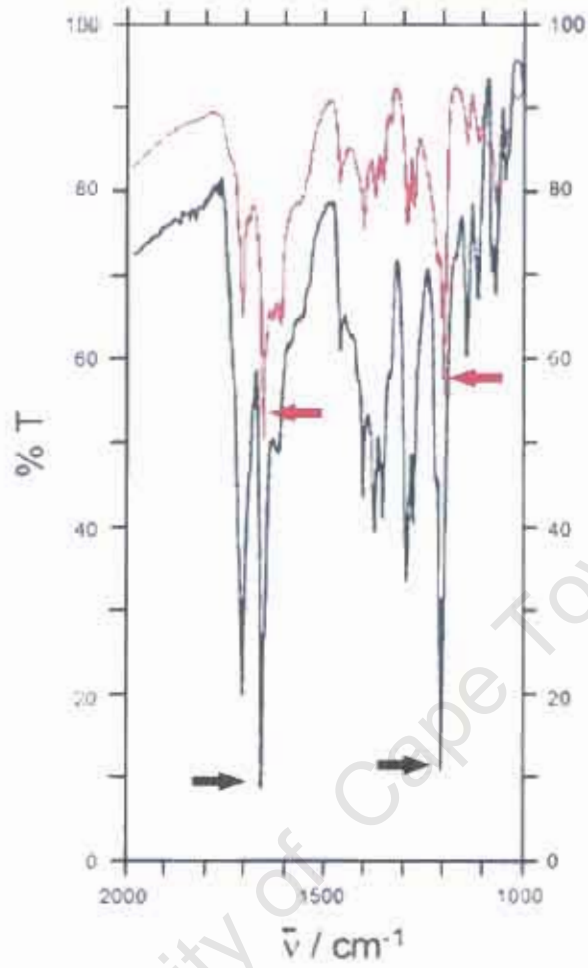



**Figure 6.6**: Shows the infrared spectra of synthetic β-haematin (red line) and isolated haemozoin (black line). Arrows indicate peaks at 1660 $cm^{-1}$ and 1207 $cm^{-1}$ for each spectrum.



### *6.2.4 Modelling of the interaction of CQ and MQ with β-haematin and isolated haemozoin*

After confirming that the isolated haemozoin crystals were pure and intact, drug binding experiments were performed. As described above in Section 6.2.2 fixed amounts of isolated haemozoin (50 µg) were incubated in increasing concentrations of [$^3$H]-CQ and [$^3$H]-MQ from 2 nM to 20 mM. The results were similar to the β-haematin binding experiments.

Binding did not saturate at high drug concentrations. There is no evidence that the binding of either drug is saturating up to a concentration of 20 mM.

In an effort to investigate the nature of the interaction of CQ and MQ with the malaria pigment, the data was fitted to two adsorption isotherms. The Langmuir isotherm is a model that describes the adsorption of a substrate on a surface, where the substrate forms monolayer on the surface, which has homogeneous binding sites.

The Langmuir adsorption isotherm can be derived to describe the adsorption of a solute onto a surface from solution (Adamson, 1975). The resulting equation is as follows

$$\frac{c_2}{n_2^s} = \frac{1}{n^s b} + \frac{c_2}{n^s}$$

$c_2$ = concentration of solute in solution

$n_2^s$ = number of moles of solute bound to the surface of the crystal

$n^s$ = number of moles of binding sites on the surface per gram of crystal

A plot of $c_2/n_2^s$ versus $c_2$ should give a straight line when the solute forms a monolayer with homogeneous binding sites on the surface. The binding data from the above experiments (Section 6.2.2 and 6.2.4) was fitted to the Langmuir isotherm. The data did not fit the model well (data not shown). This was not surprising, for two reasons:

- The Langmuir isotherm assumes one type of binding site on the solid's surface.
- The molar amount of drug associated with the malaria pigment crystal is in excess of what could be explained by a monolayer of drug coating the crystal surface.

The Langmuir isotherm can be modified to take into account heterogeneous binding sites. One version of this modified form is the Freundlich adsorption isotherm. The equation for the Freundlich isotherm is as follows:

$$\ell n n_2^s = \left(\frac{1}{n}\right) \ell n\, c_2 + \ell n\, an^s$$

A plot of $\ell n\, n_2^s$ against $\ell n\, c_2$ should give a straight line if the data fits the model. The constant $\ell n\, an^s$ is the y-intercept, and provides an empirical measure of the capacity of the solid to adsorb solute. $n^s$ is the number of moles of binding sites on the crystal surface and 'a' is a thermodynamic term related to the free energy of association of the solute to the various binding sites on the surface of the crystal. The slope is given by 1/n and gives an empirical gauge of the intensity or strength of the binding (Adamson, 1975).



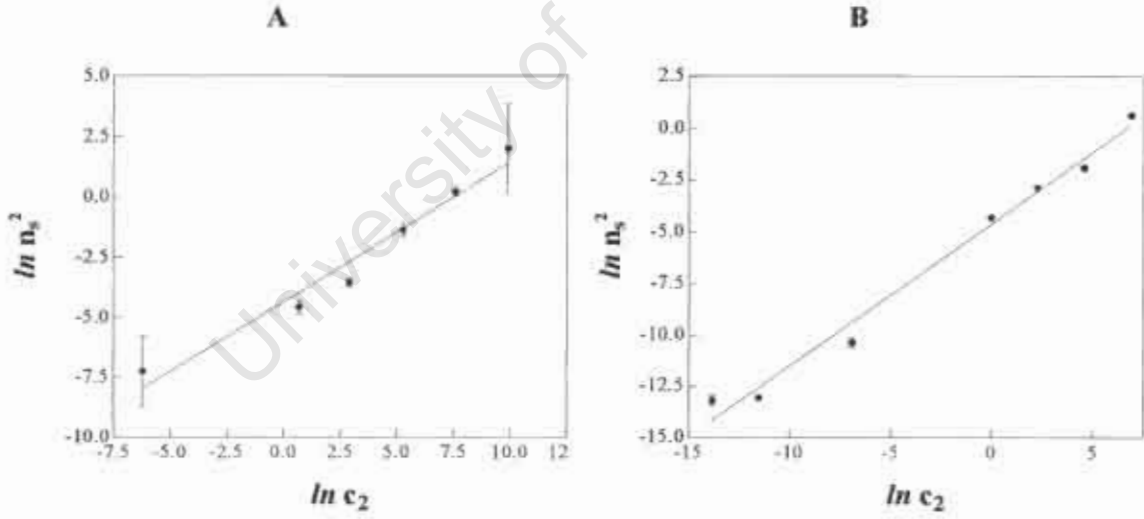



**Figure 6.7**: The β-haematin binding data has been fitted to the Freundlich Isotherm. Graph **A** represents CQ binding to β-haematin. Graph **B** represents the data for MQ binding to β-haematin. The graphs represent the data from three experiments performed in triplicate. The regression parameters for the data are shown in Table 6.1

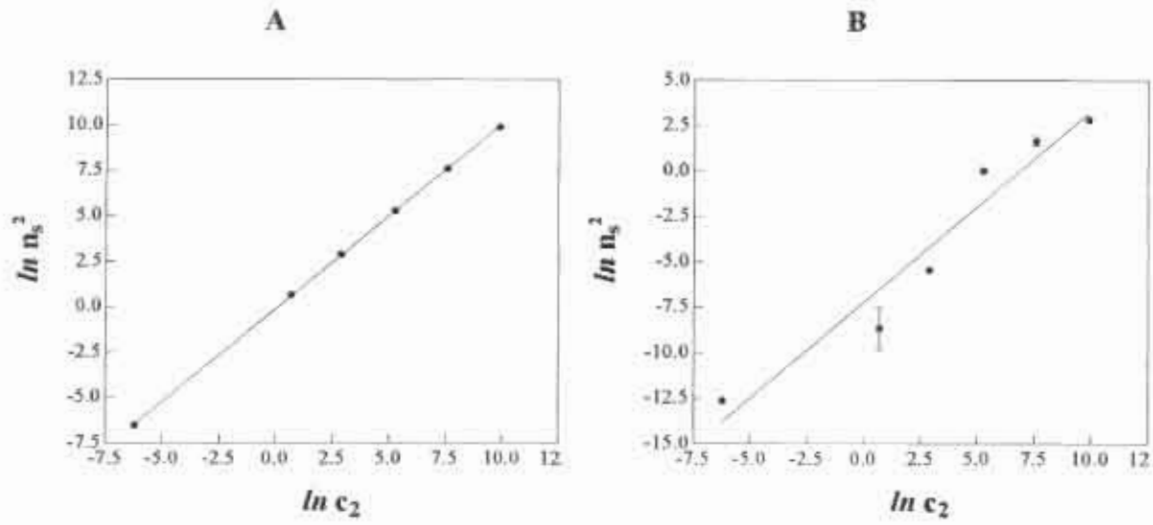



**Figure 6.8:** The haemozoin binding data has been fitted to the Freundlich Isotherm. Graph **A** represents CQ binding to β-haematin. Graph **B** represents the data for MQ binding to isolated haemozoin. The graphs represent data from a single experiment performed in quadruplicate. Errors bars represent standard deviations. The regression parameters for the data are shown in Table 6.1.

Figures 6.8 and 6.9 show the drug binding data from above (Sections 6.2.2 and 6.2.4) fitted to the Freundlich Isotherm. The data for the adsorption of CQ and MQ to both haemozoin and β-haematin result in straight lines with excellent correlation coefficients, indicating that the interaction of these drugs with these crystals obeys the Freundlich isotherm. Linear regression was performed on the data and the regression parameters are shown in Table 6.1.

The strength of binding of CQ and MQ to β-haematin appears to be very similar; the slopes are 0.58 and 0.69 respectively. The capacity of β-haematin to adsorb these drugs is also similar, the y-intercept of the regression lines are -4.4 for CQ and -4.6 for MQ. The strength of binding of the two drugs to the isolated haemozoin is similar, 1.0 for CQ and 1.1 for MQ. These values are higher for the drugs' interaction with haemozoin than their interaction with

β-haematin. The capacity of haemozoin to bind CQ appears to be very much higher than its capacity to bind MQ; the y-intercepts were -0.12 and -7.2 respectively.

**Table 6.1:** Regression parameters for the Freundlich isotherm (see Figures 6.8 and 6.9)

| Parameter | CQ | | MQ | |
|---|---|---|---|---|
| | β-haematin | Haemozoin | β-haematin | Haemozoin |
| R [†] | 0.9507 | 0.9999 | 0.9928 | 0.9738 |
| Slope (1/n) | 0.5797 ± 0.04729 | 1.018 ± 0.002745 | 0.6919 ± 0.02415 | 1.055 ± 0.05845 |
| y-intercept | -4.352 ± 0.2936 | -0.1160 ± 0.0171 | -4.617 ± 0.1927 | -7.232 ± 0.3630 |

Data were calculated from the graphs in Figures 6.8 and 6.9.

[†] ***Coefficient of correlation***

## *6.3 Discussion*

### *6.3.1 [$^{3}$H]-CQ and [$^{3}$H]-MQ distribution in the isolated food vacuole*

Approximately 75 % of pre-accumulated CQ was found to be associated with haemozoin after fractionation of the food vacuole, whereas less that 50 % of MQ that accumulated in the food vacuole was bound to the haemozoin fraction. Given the hydrophobic nature of MQ and its high non-specific binding this might be considered unexpected. However, it could be explained by the fact that MQ also binds to a large degree to membrane phospholipids and CQ does not (Chevli & Fitch, 1982), this sequestration to the membranes could result in less MQ being available to adsorb to the surface of haemozoin.

### *6.3.2 Purity of isolated haemozoin*

It was important to establish the purity of the haemozoin preparations. This was done using scanning electron microscopy and infrared spectroscopy. This was especially important for the MQ binding experiments, because of the drug's ability to bind to such a large extent to proteins, membranes and monomeric haematin. There was no artifactual binding to any other extraneous biological material. The fact that the nature and extent of drug binding to both the synthetic and native species are so similar, provide further proof that these crystals are chemically similar and that both preparations are free of contamination by monomeric haem.

### *6.3.3 [$^3$H]-CQ and [$^3$H]-MQ adsorption to synthetic β-haematin and pure isolated haemozoin*

In Chapter 3 (Figures 3.10 and 3.11) it was demonstrated that MQ accumulation is reduced when the pH gradient across the vacuolar membrane is dissipated by nigericin. The dissipation of this gradient would result in an increase in the vacuolar pH due to the electroneutral exchange of $H^+$ and $K^+$ ions. This would result in a decrease in the amount of MQ taken up due to the weak-base effect. Also, figure 6.3 showed that when the pH of the buffer surrounding the β-haematin was increased from 4.5 to 7.4 the amount of MQ that was bound to the β-haematin decreased by half. This decline in the ability of haemozoin to bind MQ may also contribute to the reduction of MQ accumulation seen when to the pH gradient is dissipated by nigericin.

CQ accumulation is also inhibited by exposure to nigericin , in fact the effect of nigericin is greater on CQ accumulation than MQ accumulation (Vanderkooi *et al.*, 1988). This is probably due to the greater role played by the weak base effect on CQ because it is doubly charged.

It is clear that both synthetic β-haematin and pure isolated haemozoin have a large capacity to bind both CQ and MQ. There is sufficient evidence in the literature demonstrating two components to CQ accumulation (Bray *et al.*, 1998; Fitch, 1970; Chou *et al.*, 1980; Fitch & Chevli, 1981). A well-characterized, high-affinity, saturable component and a non-saturable, low affinity component, which until now has been unidentified.

The work in this chapter has identified the component of CQ accumulation that is non-saturable. Exposure of both synthetic β-haematin and isolated haemozoin to various concentrations of radiolabelled CQ has demonstrated that the binding of these drugs is non-saturable. Furthermore by fitting the binding data to the Freundlich isotherm, it was shown that at high concentrations these quinolines adsorb to the malaria pigment in a multi-layered fashion.

### *6.3.4 Modelling of the interaction of CQ and MQ with β-haematin and isolated haemozoin*

In section 6.2.5 it was discovered that the interaction of both CQ and MQ with synthetic β-haematin and isolated haemozoin obeys the Freundlich isotherm. The implication of this is that these drugs are able to adsorb to the surface of the crystal not as a monolayer with homogeneous binding sites but rather in multiple layers over the solid surface which has heterogeneous binding sites (Adamson, 1975). This is consistent with the evidence indicating that haemozoin crystals are needlelike in shape, having several different crystal surfaces (Egan, 2002; Buller, 2002).

The regression parameters, from the straight lines resulting from fitting the data to the Freundlich adsorption isotherm, provide insight into the nature of CQ and MQ's interaction with malaria pigment. Because the parameters are largely empirical absolute quantification of the capacity and strength of adsorption is not possible, but they are useful for comparative analyses. The results represented in figure 6.8 show that CQ and MQ have similar capacities to bind β-haematin and also that the strength of the interactions are similar.

The results for adsorption to haemozoin are incongruent with the results obtained for β-haematin. The capacity of the isolated haemozoin to bind both CQ and MQ was higher than the capacity of β-haematin. One explanation might be that despite the exhaustive isolation and purification procedure, the haemozoin preparation might be contaminated by membranes or proteins, to which the drugs may bind. However, this seems unlikely because CQ and MQ bind both proteins and membranes to different extents and the capacity of the two drugs to adsorb to both crystals was very similar. The strength of binding of the two drugs to haemozoin was not only different from the β-haematin results, but, were also different from each other. CQ and MQ were found to interact equally strongly with the synthetic β-haematin. The β-haematin experiments were each performed on three separate occasions in duplicate. The haemozoin experiments were only performed once, due to insufficient material, because the yield of haemozoin after the isolation procedure was very low (see Chapter 9, Section 9.9). Therefore the results obtained from the β-haematin experiments are more reliable than those obtained with haemozoin. Thus the discrepancy may be attributable to experimental error in the haemozoin results.

Nevertheless despite the discrepancies between the β-haematin and haemozoin results several important observations have been made. The surface of malaria pigment crystals have heterogeneous binding sites for CQ and MQ, and after forming an initial drug layer around the crystal the drugs are able to self-associate to form multiple layers of drug above the crystal surface. The result of this is that the binding of both CQ and MQ to malaria pigment is non-saturable. It is proposed that haemozoin is responsible for the low affinity, non-saturable component of CQ and MQ accumulation that has been demonstrated in previous work and in this study (Bray *et al.*, 1998; Fitch *et al.*, 1979).

# Chapter 7

## Acceleration of haemoglobin degradation: an alternative mechanism of mefloquine action

***Introduction***

In Chapter 1 (Section 1.6.5) an alternative mode of action for the quinoline antimalarials was discussed. It was proposed that CQ may act by preventing the degradation of haemoglobin, whereas MQ's action may be to prevent endocyctosis of haemoglobin (Famin & Ginsburg, 2002). It has been shown that haemoglobin vesicles are formed after parasites are exposed to CQ, there are conflicting reports as to whether these vesicles are in the food vacuole or the cytoplasm of the parasite (Yayon *et al.*, 1984; Egan *et al.*, 2001).

It was recently demonstrated that CQ and AQ caused a build up of haemoglobin in trophozoites and that MQ and QUI cause a decrease in the amount of haemoglobin (Famin & Ginsburg, 2002). These experiments were carried out by exposing PRBC in culture to the various drugs and then measuring the haemoglobin in trophozoite extracts in two ways. Firstly, extracts were run on SDS-PAGE and *O*-dianisidine chloride used to stain FPIX. Stained bands were compared to an adjacent haemoglobin standard. Secondly the haemoglobin levels in trophozoite extracts were measured spectrophotometrically by monitoring the Soret band (412 nm). These authors proposed that CQ and AQ may interfere with the degradation of haemoglobin and that MQ and QUI may interfere with the ingestion process (Famin & Ginsburg, 2002).

In this chapter, experiments similar to those of Famin and Ginsberg (2002) were carried out. The effect of CQ and MQ on trophozoite haemoglobin levels was measured using a different method. The experiment was carried out in both MQS (CQR) and MQR (CQS) strains of *Plasmodium falciparum*.

***Results***

The effect of CQ and MQ on haemoglobin levels in D10 and RSA11 trophozoites was measured. Synchronous cultures of *P.falciparum* in the early trophozoite stage were incubated for six hours in the presence of drug. The concentrations used were equivalent to five times the $IC_{50}$ of the drugs for the strains used. An aliquot of PRBCs was removed from each of the cultures and the trophozoites were isolated by saponin lysis and extensive washing in PBS. The trophozoites were then run on SDS-PAGE and then using a haemoglobin antibody, Western Blotting and autoradiography, the relative amounts of haemoglobin in each parasite preparation was determined (see Chapter 9, Section 7.13 for more details). Results are shown in plate 7.1.


**Plate 7.1:** Autoradiograph from a Western Blot showing the amount of haemoglobin in D10 (black text) and RSA11 (white text) trophozoites after 6 hours of exposure, in the early trophozoite stage, to **CQ** and **MQ**. The concentrations of drug used were equal to approximately five times the $IC_{50}$ of the drug for each strain. See Chapter 9, Section 9.1.3 for details. The control (**CON**) parasite preparations were not exposed to any drug. Pure haemoglobin (**Hb**) was also run on the gel as a standard molecular marker. The experiment was performed twice, on two separate occasions.

The intensity of each band was quantified using image analysis software (Kodak 1D). Figure 7.1 shows the intensity of each band from the Western Blot in plate 7.1. This data shows that exposure to CQ causes a 2.6- and 2.9-fold increases of haemoglobin in D10 and RSA11 trophozoites respectively compared to the untreated control.

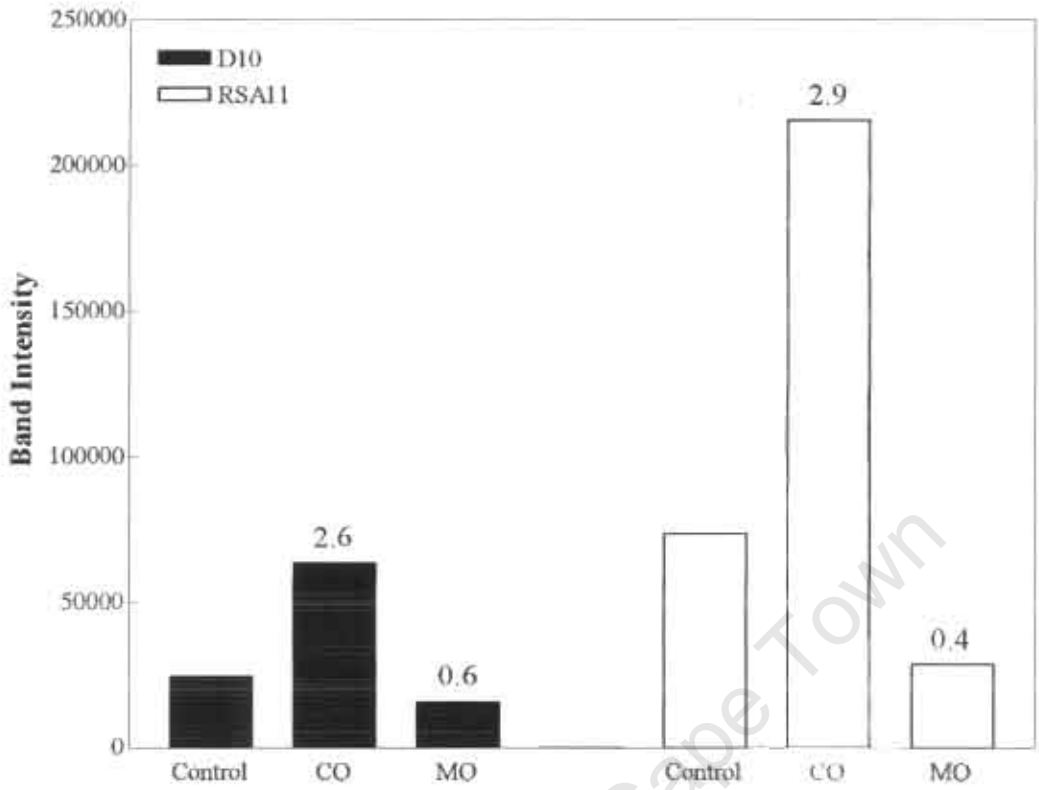



**Figure 7.1:** Shows the intensity of each band from Plate 7.1. The intensity of each band was measured using Kodak 1D Image Analysis Software Program. The number above each bar is the fold increase in haemoglobin relative to the untreated control.

Treatment of parasites with MQ caused a decrease in haemoglobin levels in D10 and RSA11 by 40 and 60 %, respectively. The action of CQ and MQ is similar in both strains.

*Discussion*

The results shown, in plate 7.1 and figure 7.1, verify the findings of Famin and Ginsberg (2002). CQ causes a buildup of haemoglobin in the parasite and MQ decreases the amount of haemoglobin in the parasite. The two strains used in this experiment have very different resistance profiles, D10 is MQR and CQS whereas RSA11 is MQS and CQR (see Chapter 2, Section 2.2.1). In figure 7.1, although no quantitative assessment between the strains can be made, it is clear that the effect of each of the drugs on haemoglobin levels, was similar in both strains.

There are two mechanisms that could explain why CQ causes an increase in haemoglobin in the isolated trophozoite. Firstly, CQ may be directly preventing the degradation of haemoglobin. This could be achieved in a number of ways:

- CQ directly inhibits the proteases that are responsible for the degradation of haemoglobin, this inhibition is unlikely because very high concentrations are necessary. At 1 mM CQ decreased the activity of one protease by less than 30 % (Vander Jagt *et al.*, 1986).
- CQ inhibits the formation of haemozoin, allowing toxic FPIX to accumulate, which is able to inhibit proteases, as is the CQ-FPIX complex (Vander Jagt *et al.*, 1986; Blauer & Ginsburg, 1982).

Secondly, CQ may be preventing the fusion of the haemoglobin-containing transport vesicles with the digestive food vacuole. Strong evidence in support of this theory has been provided

by electron microscopic studies. Two studies have shown that CQ-treated parasites have a buildup of haemoglobin containing vesicles. However they disagree on the location of the vesicles. One study has shown that the vesicles are inside the food vacuole (Yayon *et al.*, 1984) and the other places them in the parasite cytosol (Egan *et al.*, 2001).

Famin and Ginsberg (2002) proposed that MQ inhibits the ingestion of haemoglobin. It is known that MQ is able to inhibit phagocytosis in human leucocytes (Kharazmi & Erikson, 1986) and neutrophils (Labra & Babin-Chevaye, 1988). In an ultrastructural study of the effect of MQ on malaria parasites, the authors reported vacuolar swelling and a decrease in the number of haemozoin crystals in both *P.berghei* and *P.falciparum* PRBCs after exposure to MQ (Jacobs *et al.*, 1987). Examination of the published electronmicrograph of MQ-treated *P.falciparum* shows that the vacuole is indeed swollen and there is little haemozoin. There is also no evidence of haemoglobin-containing vesicles in either the cytosol or the food vacuole. It has also now been shown, using an assay for endocytosis in *Plasmodium falciparum*, that MQ does inhibit this essential process in the malaria parasite (Hoppe, 2003). Because MQ interacts with phospholipids to such a large extent (Chevli & Fitch, 1982), it is not difficult to envisage membrane-bound MQ interfering with the complex processs of endocyctosis.

# Chapter 8

## Discussion, Conclusions and Perspectives

With the development of CQ resistance in almost every malaria endemic area of the world, the importance of the few alternative antimalarials available, is greater than ever before. MQ has been in use for over twenty years and is still effective for treatment and prophylaxis against CQR malaria parasites in many areas. Despite this there is very little published data on the interaction of this drug with *P.falciparum*. The major reason for this scarcity of information on MQ's action, is the fact that it is highly hydrophobic and therefore binds to serum proteins and membranes to a high degree. The consequence of this when performing radiolabelled drug accumulation experiments is that there is massive background binding in the PRBC system and it is difficult to evaluate parasite specific MQ accumulation.

This thesis has focused on the development of a system that makes possible the examination of MQ's interaction with *P.falciparum*-infected erythrocytes. It was found that removing the 'serum' components (Albumax) from the uptake medium allows for more MQ to be available for accumulation into PRBCs. Uninfected erythrocytes were separated from trophozoite-infected erythrocytes by centrifugation through Percol. This resulted in parasite preparations with trophozoite parasitemias that were consistently above 85 %. These modifications to the uptake methodology allowed a better differentiation between accumulation in uninfected RBCs and

PRBCs, effectively amplifying the parasite dependent component of MQ accumulation.

Having established conditions that allow the measurement of parasite-dependent MQ accumulation, a battery of experiments were performed in order to characterize the nature of this drug uptake. MQ accumulation was found to be complete in one minute, which is faster than CQ uptake in PRBCs, which is maximal after 30 minutes.

Previous work investigating the energy dependence of MQ accumulation yielded negative results, no energy dependence could be demonstrated (Fitch *et al.*, 1979). These same authors explained their negative results, three years later, when they demonstrated that MQ binds membrane phospholipids to a high degree. They indicated that this binding may obscure an energy requirement for MQ accumulation (Chevli & Fitch, 1982). The current study has shown that there is an energy requirement for MQ accumulation. The glycolysis inhibitor, iodoacetate, was able to inhibit MQ accumulation by approximately 25 %, as was incubation at 4 °C. Furthermore glucose deprivation reduced MQ uptake by half.

MQ accumulation was also shown to be partly dependent on the pH gradient between the external medium and the acidic food vacuole. This is in agreement with previous work that found that 40 – 60 % of MQ accumulation was pH-dependent (Vanderkooi *et al.*, 1988).

No antimalarials, at 100-fold excess, were able to compete out MQ accumulation. However, MQ was able to abolish CQ accumulation. This may be explained in the

context of an alternative mechanism of action, where MQ inhibits the ingestion of host cell haemoglobin. It has been published recently that MQ causes a decrease in the amount of haemoglobin in the trophozoite and CQ causes a build-up of haemoglobin (Famin & Ginsburg, 2002). This was confirmed in this study by a different method. If CQ accumulation is driven by binding to haematin in the food vacuole and MQ inhibits haemoglobin ingestion, then the effect of MQ will be to decrease the amount of haematin available for CQ binding and therefore decrease the accumulation of CQ.. This alternative or additional mechanism may also explain the higher potency of MQ against malaria parasites and its effectiveness again CQR strains. However, it is questionable whether a one hour incubation of the parasites with MQ would sufficiently deplete haematin levels to affect CQ accumulation Another, more simplistic explanation is that MQ's extensive membrane binding may impede the passage of CQ through the various membranes to the food vacuole. This might also explain why MQ is not affected by CQ, because CQ does not significantly bind membranes.

The MQ and CQ sensitivities of nine strains of *P.falciparum* were evaluated. Although no statistical relationship could be proven between MQ and CQ resistance, there was a trend supporting the many published studies indicating that there is a paradoxical relationship between MQ and CQ resistance.

The expression of Pgh1 was measured in six strains and compared to their CQ and MQ sensitivities. No statististical correlation could be proved but there was more of an association between Pgh1 expression and MQ resistance, than with CQ resistance.

This is in agreement with many clinical and laboratory studies indicating a link between MQ resistance and overexpression of Pgh1. It was somewhat anomalous that the Pgp modulators had no effect on the accumulation of MQ.

It was demonstrated that MQR *P.falciparum* accumulate half as much radiolabelled MQ than do MQS *P.falciparum*. This was confirmed by measuring the cellular accumulation ratio (CAR) using the inoculum effect method, the CAR in the MQS strain was approximately double that of the MQR strain.

MQ accumulation in PRBC was measured as a function of the external MQ concentration, and this data was fitted to a curvilinear nonlinear regression equation. This indicated that there are saturable and non-saturable components to MQ accumulation. This is in agreement with previous work with MQ (Fitch *et al.*, 1979). Similar studies have been performed with CQ and they also show a two-component model of accumulation (Fitch, 1970; Bray *et al.*, 1998). There is persuasive evidence indicating that the saturable uptake of CQ is driven by binding to haematin in the food vacuole (Bray *et al.*, 1998). Until now, the identity of the non-saturable component of drug uptake has remained unknown.

To further dissect the interaction of MQ with the parasite, MQ accumulation was measured in pure and intact food vacuoles. ATP-dependent CQ accumulation was demonstrated but no ATP-dependent MQ accumulation was observed. Therefore either the energy-dependent component, measured in PRBCs, is not at the level of the vacuole or the vacuolar system is not sensitive enough to expose the energy requirement for MQ uptake.

The distribution of both MQ and CQ in the isolated food vacuole was determined by sub-cellular fractionation. It was observed that slightly under 50 % of the MQ and 75 % of the CQ in the food vacuole was associated with haemozoin. In light of the high degree of drug association with the haemozoin fraction of the vacuole, it was decided to examine the binding of these quinolines to both synthetic β-haematin and isolated haemozoin. It was observed that neither MQ nor CQ adsorption to malaria pigment could be saturated. By fitting the adsorption data to the Freundlich adsorption isotherm it was determined that both the capacity and affinity of MQ and CQ to bind β-haematin was similar. The fact that the adsorption data obeys the Freundlich isotherm also indicates that there are heterogeneous binding sites on the pigment crystal and that these drugs form multiple layers above the crystal surface, explaining the non-saturability of MQ and CQ adsorption to haemozoin.

In conclusion, this thesis provides the first direct evidence that there is an energy requirement for MQ accumulation. Furthermore it has, for the first time, been demonstrated that MQR malaria parasites accumulate less MQ than MQS malaria parasites. The non-saturable component of both CQ and MQ accumulation has now been identified to be the malaria pigment, haemozoin.

Additional studies that may help further elucidate the mechanism of MQ accumulation include:

- More MQ accumulation experiments on a range of strains of varying degrees of MQ accumulation.
- It would be enlightening to carry out MQ accumulation experiments in the transfectant strains created by Reed et al, (2000), to further assess the role of *pfmdr1* in MQ resistance.

- More precise study of the distribution of MQ within the PRBC, by doing MQ accumulation and then subcellular fractionation.

# Chapter 9

# Materials and Methodology

## *9.1 Plasmodium falciparum Parasite Culture*

### *9.1.1 Plasmodium falciparum strains used:*

- D10 was derived from FQC-27 from Papua New Guinea (Ekong *et al.*, 1993)
- FAC8 was derived from the cloned isolate ITG2 (Biggs *et al.*, 1989)
- W2 clone was derived from a mixture of Sierra Leone I/CDC and Indochina III/CDC lines (Oduola *et al.*, 1988a)
- W2mef was derived by mefloquine pressure from W2 (Oduola *et al.*, 1988b)
- $W2mef^{CQ}$ was derived from W2mef by CQ pressure (Barnes *et al.*, 1992)
- K1 is a strain from Thailand (Thaithong & Beale, 1981)
- 3D7 is a clone from the NF54 strain, isolated from an airport worker in Amsterdam
- *RSA 11 was isolated in Kwa-Zulu Natal, South Africa (Freese et al., 1991)*
- RSA 15 *was isolated in Kwa-Zulu Natal, South Africa (Freese et al., 1991)*
- RSA 3 *was isolated in Kwa-Zulu Natal, South Africa (Freese et al., 1991)*

*9.1.2 Culture conditions*

The maintenance of *P. falciparum* was fundamentally the same as that described by Trager and Jensen (Trager & Jensen, 1976). The parasite culture was maintained in RPMI 1640 culture medium with glutamine, supplemented with 25mM $NaHCO_3$, 22.2 mM glucose, 0.323 mM hypoxanthine, 25mM HEPES (N-[2-hydroxyethyl]piperazine-N'-[2-ethansulfonic acid], 50 µg/ml gentamcin and 0.5% Albumax II. The parasites were maintained in tissue culture flasks or petri dishes in desicator cabinets at an haematocrit of 4% and a parasitemia of between 5-15%. Human $O^+$ erythrocytes, were washed twice with culture medium without Albumax to remove the Buffy coat, and were added to the parasite culture to maintain the haematocrit and parasitemia. The cultures were kept under an atmosphere of 93% $N_2$, 4% $CO_2$, 3% $O_2$ at 37°C. The culture medium was changed daily.

*9.1.3 Preparation of thin blood smears*

Daily observation of the parasites was done by preparing thin blood smears. The smears were fixed with 100% methanol and stained with 10% Giemsa (Sigma) in phosphate buffered saline (PBS) (C.A. Milsch) for 10 minutes at room temperature. The slides were then rinsed with water and dried. They were then viewed under Leitz Laborlux 12 microscope with a 100-x oil (1250 centistokes) immersion objective.

*9.1.4 Cryostorage of Parasites*

1 volume of glycerolyte medium was added to 1 volume of ring stage PRBCs drop-wise, swirling constantly, and was left to stand for 5 minutes. Then a further 2 volumes of glycerolyte medium was added while swirling. This preparation was then divided into 2 ml aliquots and transferred to cryotubes and placed at –80°C overnight and then transferred to liquid nitrogen. Glycerolyte medium consists of sodium lactate (16 g/L), potassium chloride (300 mg/L), sodium dihydrogen phosphate (13.8 g/L), glycerol (570g/L) (Analar) at pH 6.8.

*9.1.5 Thawing*

For re-introduction to culture, the parasites are thawed at 37°C and transferred to a 50 ml centrifuge tube. To 2 ml of sample, 400 µL of a 12% NaCl solution was added drop-wise while swirling, and left to stand for 5 minutes. Then 1ml of a 1.8% NaCl solution was added drop-wise with swirling. The culture was then centrifuged for 5 minutes at 400g. After the supernatant was aspirated, 10 ml of a solution containing 0.9% NaCl and 0.2% glucose was added drop-wise with swirling. The culture was centrifuged again for 5 minutes at 400 g. The pellet was washed again using culture medium without Albumax II, and equal volume of fresh RBCs was added and the parasites were transferred to a culture flask, and placed under normal culture conditions (see Section above).

*9.1.6 Synchronisation*

Parasite cultures were routinely synchronised in the ring stage. The PRBC pellet was exposed to 5 volumes of 5% D-sorbitol for 10 minutes and then centrifuged at 600g for 5 minutes. The sorbitol causes those erythrocytes containing trophozoites and schizonts to be lysed, thereby leaving a synchronised ring-stage culture pellet, which was then put back into culture (Lambros & Vanderberg, 1979).

***9.2 Digestive Vacuole Isolation***

The method of Saliba *et al* (1998) was used to isolate pure and intact food vacuoles from *P. falciparum*. Digestive vacuoles were harvested from parasites in the late trophozoite stage. A highly synchronous culture with at least 10% parasitemia was used. Approximately 5 ml of culture pellet was resuspended in 50ml of PBS containing 0.5 mg/ml saponin, and left to stand for 2 minutes. The suspension was then centrifuged at 1500g for 10 minutes. The resulting supernatant was discarded, and the pellet, containing trophozoites was washed twice with cold PBS. The pellet was then resuspended in 1ml ice-cold hypotonic lysis buffer ($H_20$ at pH 4.5). The suspension was triturated through a 27-G 1.2cm needle, after which it was centrifuged for 2 minutes on a microfuge at 13 000 rpm. The crude vacuole pellet was then resuspended in 1 ml of uptake buffer containing 0.05mg/ml DNase I and then incubated at 37°C for 5 minutes, after which it was centrifuged for 2 minutes at 13 000 rpm.

Uptake buffer contained: 2 mM MgSO4, 100 mM KCl, 10 mM NaCl, 25 mM HEPES, 25 mM NaHCO3 and 5 mM sodium phosphate at pH 7.4. This pellet was then resuspended in 200 µl of ice-cold uptake buffer and triturated twice through a 27-G 1.2 cm needle, 1.3 ml ice-cold Percol solution was added and the suspension microfuged at 13 000 rpm at 4°C for 10 minutes. The pure vacuoles were in the pellet, which was washed twice with uptake buffer and then resuspended in uptake buffer to aliquot for experiments (Saliba *et al.*, 1998).

### *9.3 In vitro Plasmodium falciparum Cytotoxicity Assay*

The assay used for the evaluation of the toxicity of drugs on strains of *Plasmodium falciparum* is based on the activity of parasite lactate dehydrogenase. Makler *et al* developed a method of distinguishing between host (RBC) lactate dehydrogenase and parasite lactate dehydrogenase (Makler *et al.*, 1993). The assay is dependent on the ability of pLDH to rapidly utilise 3-acetyl pyridine NAD (APAD) as a coenzyme in converting lactate to pyruvate. The host RBC LDH catalyses this reaction extremely slowly (Makler & Hinrichs, 1993).



The assay was carried out in 96 well, flat-bottomed microtitre plates. The plates are labelled 1 to 12 across the top of the plate and A to G down the plate. To all wells except column 3, 100µl of complete medium was added. Drug stock solutions, in complete medium, were made up to double the required starting concentration and 200µl of this stock was added to column 3. Using a multichannel Gilson pipette, half serial dilutions were made across the plate by transferring 100µl of drug solution well by well from column 3 through to column 12, discarding 100µl from the final column.

Stock parasite cultures of 2% Pst and 2% Hct were prepared, as well as a RBC stock of 2% Hct. 100µl of the RBC stock was added to all the wells in column 1, this served as the blank. 100µl of the PRBC stock was added to every other well. Note that column 2 was the control, it contained the PRBC culture in the absence of drug. The final Hct in every well was 1% and the final Pst in the PRBC wells was 2%. The completed plate was then placed in a desiccator cabinet, gassed (93% $N_2$, 4% $CO_2$, 3% $O_2$) and placed in an incubator at 37°C for 48 hours.

At the end of the 48 hour incubation period a second flat bottomed 96 well microtitre plate was prepared by adding 100µl of Malstat™ and 25µl of 0.24mM PES/1.96mM NBT solution to every well. The initial experimental plate containing the parasite culture was removed from the incubator and the cells in every well resuspended using a multichannel Gilson pipette. To start the pLDH assay reaction 15µl from each well of the culture plate was transferred to the corresponding well in the Malstat plate. The optical density of each well is read at a wavelength of 620 nm. A colour change from yellow to blue/purple indicates parasite growth (Makler *et al.*, 1993). The percentage viability in each well containing drug was calculated using the following formula:

$$\text{Percentage viability} = \frac{\text{OD drug containing wells - Mean OD of blank wells}}{\text{Mean OD control wells - Mean OD of blanks wells}} \times 100$$

The dose-response curve is constructed by plotting the percentage viability against the log of the drug concentration.

### *9.4 Trophozoite Enrichment*

The procedure for separating trophozoite infected erythrocytes from uninfected erythrocytes is an adaptation from the method published by Ginsberg et al (Ginsburg *et al.*, 1998). Two Percol solutions were prepared, 90% and 60% in RPMI 1640 culture medium containing 5% DL-Alanine. To an 1.5 ml microfuge tube 700µl of the 90% Percol solution was added, 500µl of the 60% Percol solution was carefully layered above the 90% solution. 300µl of parasitised erythrocytes were layered on top of the 60% Percol layer. The vials were then centrifuged at 10 000 rpm for 20 minutes.

The uninfected erythrocytes and ring-infected erythrocytes formed a layer in the 90% Percol layer near the bottom of the vial. The trophozoite infected erythrocytes formed a layer higher up in the 60% layer. The trophozoite layer is harvested to a centrifuge tube containing 10ml of complete RPMI 1640 medium and the alanine allowed to equilibrate for 10 minutes and then centrifuged at 600g for 5 minutes. The resulting pellet was then ready for use. This method results in an enriched parasite culture with a parasitemia of 95% or greater.



### *9.5 Drug Accumulation in Enriched Parasitised Red Blood Cells*

Parasitised erythrocytes were harvested after the enrichment procedure and a stock suspension of the cells was made up according to the experimental conditions needed. The volume of the stock suspension was adjusted so that 50µl could be removed from the stock and added to the experimental vial (containing complete medium), resulting

in the required parasitemia and haematocrit. In experiments where modulators or inhibitors were added, the PRBC culture was pre-incubated for 10 minutes in the presence of these compounds, prior to the addition of the radiolabelled drug. The haematocrit for these drug accumulation experiments was usually set at 0.1% unless otherwise stated. The Eppendorf vial containing 1 ml of the experimental parasite culture was routinely incubated in a 37°C water bath for one hour, unless otherwise stated.

After the incubation 200µl of dibutyl phthalate was added to each of the microfuge tubes and immediately centrifuged for 2 minute at 13 000 rpm. This resulted in a parasitised erythrocyte pellet at the bottom of the tube separated from the culture medium, containing free drug, by the dibutyl phthalate layer. The supernatant was removed and the tip of the microfuge tube cut off and placed in a 4 ml scintillation vial, and processed for scintillation counting.

To minimise the effect of quenching by the haemoglobin the PRBC pellet was processed as follows. 100µl of the tissue solubiliser Solvable (Packard) was added to the tip containing the PRBC pellet. The scintillation vial was then incubated at 60°C for 30 minutes, after which 25µl of 0.1 M EDTA was added to the tip inside the vial and left to stand for 5 minutes. 100 µl of 30% hydrogen peroxide ($H_2O_2$) was then added to the tip and the vial was again incubated for 30 minutes at 60°C. Finally 2 ml of scintillation fluid was added to the scintillation vial, hand shaken and counted in Tri-Carb 2100TR Liquid Scintillation Analyser (Packard) at least 60 minutes after adding the scintillation fluid.

### *9.6 Drug Accumulation in Isolated Digestive Vacuoles*

The food vacuoles were isolated as described above and suspended in uptake buffer on ice, this constituted the vacuole stock suspension. The volume of the stock suspension was adjusted so that 50µl could be removed from the stock and added to the experimental vial (containing the radiolabelled drug in uptake buffer). The total volume in the tube was 1ml. An extra vial containing only vacuoles and uptake buffer was included for counting the number of vacuoles per vial. The vacuoles were counted using a haemocytometer. The microfuge tubes containing the isolated vacuoles for drug accumulation were incubated in a 37°C water bath for 1 hour, unless otherwise stated.

After 60 minutes the microfuge tubes were spun at 13 000 rpm for 2 minutes and the supernatant aspirated. 1 ml of drug-free uptake buffer was then added to each of the vials containing the vacuole pellet and the pellet was resuspended and centrifuged again at 13 000 rpm for 2 minutes. The supernatant was aspirated and the tip of the tube was cut off and placed in a 4ml scintillation vial. 50 µl of Solvable (Packard) was added to each tip and incubated at 60°C for 15 minutes, then 25 µl of EDTA was added to the tip, which was left to stand for 5 minutes. 50 µl of $H_2O_2$ was added to each tip and incubated again at 60°C for 15 minutes. Finally, 2 ml of scintillation fluid was added to the scintillation vial, which was then hand shaken and allowed to stand at room temperature for 60 minutes before counting on a Tri-Carb 2100TR Liquid Scintillation Analyser (Packard).

### *9.7 Vacuolar fractionation*

After allowing either [$^3$H]-CQ or [$^3$H]-MQ to accumulate in six 1 ml aliquots of isolated food vacuoles for one hour as described above (Section 7.6), two of the 1 ml aliquots were immediately processed for scintillation counting as described in Section 7.6 above. The remaining loaded vacuoles were lysed by subjecting them to nitrogen cavitation for 1 hour at 750 kPa. Two of the remaining 1 ml aliquots were centrifuged for 4 minutes at 13 000 rpm. 500 µl of the supernatant was removed from each of these vials for scintillation counting. The remaining supernatant was removed and the tip of the vial containing the crude haemozoin pellet was cut off and also processed for scintillation counting. The final two 1 ml aliquots of lysed vacuoles were layered onto a 20 % Percol cushion and centrifuged for 15 minutes at 13 000 rpm. 500 µl of the supernatant was removed from each of these vials for scintillation counting. The remaining supernatant was aspirated and the tip of the vial containing the slightly purified haemozoin was cut off and processed for scintillation counting.



### *9.8 Synthesis of β-Haematin*

β-haematin was prepared according to the method of Egan *et al* (Egan *et al.*, 1999). Haematin (hydroxo-Fe(III) PPIX) was prepared by dissolving 200 mg of haemin in 40 ml 0.1 M NaOH. The haematin solution was stirred in a thermostat controlled titration cell. 23.2 ml of Glacial Acetic acid and 4 ml of 1.0 M HCl was added to the haematin solution and allowed to stir at 60°C for 60 minutes. The resulting slurry was then filtered on a 0.1 µm cellulose nitrate filter disc (Whatman) and then thoroughly washed with deionised water and dried over $P_2O_5$. The dried precipitate was recovered

from the filter disc and ground to a fine powder with an agate mortar and pestle and stored in a desiccator.

### *9.9 Isolation of Haemozoin*

D10 strain parasite cultures with at least 10% late trophozoite parasitemia were harvested regularly from continuous culture and stored at –80°C. The frozen PRBC pellet was thawed in a 37°C water bath. The volume was made up to 50 ml with Millipore water and sonicated for 30 minutes. The sample was then centrifuged at 4°C at 27000g and the supernatant was discarded. The pellet was then resuspended in 20mM Tris buffer containing 0.02mg/ml DNaseI and incubated at 37°C for 10 minutes. Proteinase K (0.5 mg/ml) was added to the haemozoin suspension and incubated for 30 minutes at 37°C. The preparation was centrifuged for 30 minutes at 27000g, and again the supernatant was discarded. The resultant pellet was resuspended in a 25mM Tris buffer containing 2.5% SDS at pH 7.8, at left overnight (16 hours) at room temperature.

The sample was then centrifuged at 4°C for 30 minutes and the supernatant discarded. The pure haemozoin pellet was washed twice with Millipore water and the final suspension was lyophilised and weighed. The yield from approximately 85 ml of spun down parasite culture at a minimum of 10 % parasitemia at approximately 3 mg of pure haemozoin.

### *9.10 Drug binding to β-Haematin and Haemozoin*

In each microfuge tube 50 µg of β-haematin or haemozoin was incubated for 30 minutes at pH 5 in 1 ml PBS containing the appropriate concentration of radiolabelled drug. When the concentration of CQ and MQ exceeded 2 nM and 1 nM respectively, unlabelled drug was included. At the end of the incubation the above suspension was layered on top of 300 µl of 1.5 M sucrose in a microfuge tube and centrifuged at 13000 rpm for 20 minutes.

The malaria pigment passed through the sucrose and was separated from the free drug in solution. After the separation 500 µl of the supernatant was removed for scintillation counting and the remaining supernatant aspirated and discarded. The tip containing the pigment pellet was cut off and placed in a scintillation vial. 2 ml of scintillation fluid (Optima Gold, Packard) was added to the vial containing the supernatant, and the vial was vortexed. To each vial containing the pellet 50µl NaOH was added and the vial shaken to dissolve the malaria pigment. 1 ml of 3.5 % (m/v) sodium hypochlorite was then added to bleach the sample. 2 ml of scintillation fluid was then added and the vials were vigorously shaken. The samples were then left in the dark overnight, before being counted on a Tri-Carb 2100TR Liquid Scintillation Analyser (Packard).

### *9.11 Electron Microscopy of β-Haematin and Haemozoin*

The samples, which were dry powders, were sprinkled onto a microscope stub that had been coated with carbon graphite glue. The excess powder was tapped off to give a single layer of powder. The stubs were then sputter coated with gold/palladium alloy and then examined with an analytical Leo S440 scanning electron microscope.

### *9.12 Infrared Spectroscopy of β-Haematin and Haemozoin*

A 16 mm diameter matrix disc of the synthesised β-haematin and the isolated haemozoin was obtained by grinding 1 mg of sample with 250 mg of KBr in a mortar and pestle and pressing the mixture under 10 tons psi of pressure for 1 minute. An Erkin-Elmer 983 infrared spectrophotometer was used to obtain an infrared spectrum between 1000 $cm^{-1}$ and 2000 $cm^{-1}$. The presence of sharp bands at 1660 $cm^{-1}$ and 1207 $cm^{-1}$ indicate that β-haematin is present. (Egan *et al.*, 2001).

### *9.13 The effect of drugs on haemoglobin content of Plasmodium falciparum trophozoites*

In the early trophozoite stage, a culture of *P. falciparum* was split into four equal aliquots and placed in four new culture flasks under normal culture conditions. The drug concentrations used, corresponded to approximately five times the $IC_{50}$ of the specific strain to the various drugs (Table 7.1). The final control flask was left free of drug.

**Table 9.1:** Drug concentrations used for haemoglobin experiment. Parasites were incubated for 6 hours in a drug concentration five times higher that the strain's $IC_{50}$ for the drug.

| Drug | D10 | RSA 11 |
|---|---|---|
| Chloroquine | 175 nM | 1.75 µM |
| Mefloquine | 100 nM | 100 nM |
| Artemisinin | 150 nM | 150 nM |

The four flasks were gassed and incubated at 37°C for 6 hours, until the late trophozoite stage. The cultures were removed from the incubator and centrifuged at 600g for 5 minutes and the supernatant aspirated and discarded. 200µl was removed from each pellet and resuspended in 1 ml of PBS in a microfuge tube. To lyse the RBC membrane saponin was added to a final concentration of 0.05%. After briefly vortexing the samples they were centrifuged at 8000 rpm for 3 minutes, the supernatant was discarded and the isolated trophozoite sample was washed in PBS 6 times. 1 ml of water and 400 µl sample application buffer was added to the sample and the tube placed in boiling water for 5 minutes. The samples were then centrifuged at 13000 rpm for 5 minutes. 10 µl of the supernatant was added to each lane on an 11% SDS PAGE gel.



### *9.14 Protein Determination*

A protein standard curve was first set up using bovine serum albumin. A 1 mg/ml stock solution of bovine serum albumin in water was made up. From this, 10 standard solutions of 800 µl were obtained in a concentration range from 5 µg to 50 µg of

protein. To each of these vials, 200 µl of Biorad Protein Assay Dye was added and the vials vortexed. 200µl of the protein standards were transferred to a 96-well flat-bottomed microtitre plate. The experiment was performed in quadruplicate. The absorbance of each protein solution was read at 620 nm on a 7520 Microplate Reader (Cambridge Technology Inc). The standard curve was constructed by plotting protein (in µg) against absorbance (Figure 9.1).

To determine that amount of protein in trophozoite samples, the preparations were resuspended in water and 5 or 10 µl aliquots removed and treated exactly as above and the protein concentration determined from the standard curve.



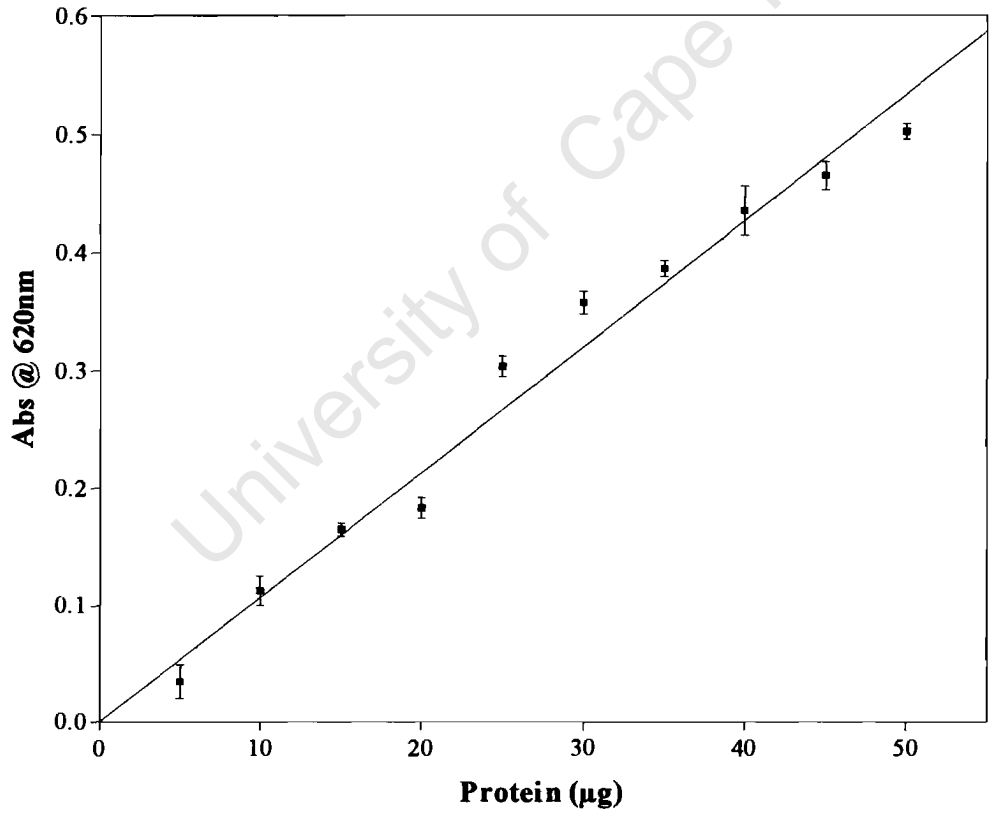



**Figure 9.1:** Protein standard curve for bovine serum albumin over a range from 5 µg to 50 µg of protein. Linear regression yielded a coefficient of correlation of 0.9882.

### *9.15 Parasite harvesting and sample preparation for Pgh1 experiments*

Approximately 2 ml of PRBC pellet (10 % parasitemia) was added to 45 ml of PBS containing 0.05 % saponin, the sample was briefly mixed and then centrifuged at 1500 g for 10 minutes. The resulting supernatant was discarded and the remaining pellet, containing isolated trophozoites, was harvested to a microfuge tube and resuspended with 1 ml of PBS. The sample was then centrifuged at 13000 rpm for 1 minute and the supernatant discarded, the sample was washed in this manner 5 times. A protein determination was carried out on the sample, then diluted so that all samples contained 1 mg/ml of protein. The preparations were placed in boiling water for 5 minutes and centrifuged at 13 000 rpm for 5 minutes. 10µl of the supernatant of each sample was loaded on the gel.

### *9.16 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*

Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis was carried out using a Biorad Mini-PROTEAN® 3 apparatus. Samples were run on 1-1.5 mm thick gels, the stacking gel used was 4.3% acrylamide. The acrylamide content of the running gel was either 10% (pgh1 experiments) or 11% (haemoglobin experiments). Electrophoresis was performed at a constant current of 40 mA until the dye front reached the bottom of the gel. The gels were then either used for Western Blotting or were stained with Coomassie brilliant blue R-250. Prestained molecular weight markers were also run on the stained gel to estimate sample molecular weights.

### *9.17 Western blotting*

The protein sample were transferred from gels onto 0.2 µm Immun-Blot™ PVDF membrane (Bio-Rad) using the Biorad Mini Trans Blot system. The transfer buffer consisted of 24.9 mM Tris, 1.9 M glycine, 10 % SDS and 20% methanol and the transfer was done at a constant voltage of 120V for 1.5 hours. The nitrocellulose was was blocked overnight in blocking solution containing a 5 % solution of milk powder and 0.1 % Tween 20. The membrane was then incubated in the presence of the primary antibody (α-Pgh1 antibody or α-haemoglobin antibody) for one hour with gentle shaking. The primary antibody solution was removed and the nitrocellulose washed 3 times with PBS containing 0.1% Tween 20. The nitrocellulose was exposed to the secondary antibody (α-rabbit antibody) for 1 hour with gentle shaking. The membrane was then washed 3 times. The protein side of the nitrocellulose was then bathed with ECL Western Blotting Analysis System™ (Amersham Sciences) solution for 1 minute, after which it was exposed to X-ray film for 45 seconds to visualise the protein bands of interest.



### *9.18 Data Analysis*

Statistical significance (p values) was established using the nonparamentric Mann-Whitney test using the computer program Graphpad Prism 3.0. The level of significance was set at $p \leq 0.05$. Linear regression was performed using the Graphpad Prism 3.0 program and the regression parameter calculated by the program. The data from dose-response curves was fitted to the built-in sigmoidal nonlinear regression curve in Graphpad Prism 3.0, and the $IC_{50}$s were calculated by the program. The data

from the concentration dependence of MQ accumulation (section 3.2.2) was fitted to the derived equation in Appendix 1, using an iterative procedure in the Graphpad Prism 3.0 program.

***Appendix 1***

**Assumption 1:** There are two forms of MQ binding, one saturating and the other non-saturating. The total MQ bound is equal to the sum of these two components.

**Assumption 2:** The concentration of bound MQ due to the non-saturating component is directly proportional to the concentration of external drug.

We can now formulate equilibrium equations relating both saturating and non-saturating bound drug concentration to external drug concentration.

$$\text{Non-saturating} = m \cdot [ED] \qquad (1)$$

Where [ED] = concentration of external drug in the medium

Saturating bound drug ([BD]): given by the law of mass action

$$[BD] \overset{K_d}{\Leftrightarrow} \text{Free sites} + [ED]$$

[Free sites] = Cap – [BD] where Cap is the total number of binding sites in the cell (both free and occupied).

Thus

$$Kd = \frac{[ED](Cap - [BD]}{[BD]}$$

so $$[BD]([ED + Kd) = [ED] \cdot Cap$$

and $$[BD] = \frac{[ED] \cdot Cap}{[ED] + Kd} \quad (2)$$

The total concentration of bound drug ([TD]) is then:

$$[TD] = [BD] + m \cdot [ED] \quad (3)$$

$$[TD] = \frac{[ED] \cdot Cap}{[ED] + Kd} + m \cdot [ED] \quad (4)$$

*Appendix 2 – Reagent List*

| **Reagent** | **Source** | **Storage conditions** |
|---|---|---|
| RPMI 1640 | Sigma | -4°C |
| $NaHCO_3$ | Sigma | 24°C (room temp) |
| Glucose | Sigma | 24°C (room temp) |
| Hypoxanthine | Sigma | -4°C |
| HEPES | Sigma | 24°C (room temp) |
| Albumax II | Sigma | -4°C |
| Giemsa | Sigma | 24°C (room temp) |
| Immersion oil (1250 centistokes) | Sigma | 24°C (room temp) |
| Sodium lactate | Sigma | 24°C (room temp) |
| Potassium chloride | Sigma | 24°C (room temp) |
| Sodium dihydrogen phosphate | Sigma | 24°C (room temp) |
| Glycerol | Analar | 24°C (room temp) |
| Sodium Chloride | Sigma | 24°C (room temp) |
| D-sorbitol | Sigma | 24°C (room temp) |
| Saponin | Sigma | 24°C (room temp) |
| DNase I | Sigma | -4°C |
| 3-acetyl pyridine NAD (APAD) | Sigma | -4°C |
| Percol | Sigma | -4°C |
| Chloroquine diphosphate | Sigma | 24°C (room temp) |
| Chloroquine diphosphate (triated) | Sigma | -8°C |
| Mefloquine Hydochloride | Roche | 24°C (room temp) |
| Mefloquine Hydrochloride (tritiated) | Roche | -8°C |
| Dibutyl phthalate | Sigma | 24°C (room temp) |
| Solvable | Packard | 24°C (room temp) |
| EDTA | Sigma | 24°C (room temp) |
| Hydrogen peroxide | Sigma | -4°C |
| Haemin | Sigma | 24°C (room temp) |
| $P_2O_5$ | Sigma | 24°C (room temp) |
| Proteinase K | Sigma | -4°C |
| Sucrose | Sigma | 24°C (room temp) |
| Optima Gold scintillation fluid | Packard | 24°C (room temp) |
| KBr | Sigma | 24°C (room temp) |
| Bovine serum albumin | Sigma | -4°C |
| Acrylamide | Sigma | -4°C |
| Coomassie brilliant blue R-250 | Sigma | 24°C (room temp) |
| Tween 20 | Sigma | 24°C (room temp) |

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