THE INTERACTION OF VERAPAMIL WITH THE HUMAN MALARIA PARASITE *Plasmodium falciparum*

Donelly Andrew Van Schalkwyk

Thesis presented for the degree of

DOCTOR OF PHILOSOPHY

in the Division of Pharmacology
Department of Medicine

UNIVERSITY OF CAPE TOWN

Supervisor
Associate Professor Peter John Smith

December 2004

DECLARATION

I, Donelly Andrew Van Schalkwyk, declare that the above thesis is my own unaided work, both in concept and execution, and that apart from the normal guidance from my supervisor, I have received no assistance except where acknowledgements indicate otherwise.

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

I grant the University of Cape Town free licence to reproduce the above thesis in whole or in part, for the purpose of research.

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

Signed by candidate

SIGNED:

27/12/2004

DATE:

ABSTRACT

The Interaction of Verapamil with the Human Malaria Parasite *Plasmodium falciparum*

Donelly Andrew Van Schalkwyk

Division of Pharmacology, Department of Medicine, University of Cape Town, South Africa

December 2004

Verapamil was the first compound identified to reverse resistance to chloroquine in *Plasmodium falciparum* (Martin et al, 1987). At concentrations that are non-toxic to parasites verapamil increases the accumulation of chloroquine into resistant parasites and thereby restores the parasite sensitivity to chloroquine. Verapamil lacks any effect on chloroquine action in sensitive strains. Drug accumulation and kinetic studies with radiolabeled drug analogues have been used in attempts to understand the mechanism by which verapamil reverses chloroquine resistance, but to date have yielded little information. A more comprehensive understanding of how verapamil interacts with the *Plasmodium* parasite and reverses chloroquine resistance may lead to the design of more effective chemosensitisers that would be clinically useful.

This thesis describes the accumulation of radiolabeled verapamil into various strains of *Plasmodium falciparum*. Verapamil accumulation was similar in chloroquine sensitive and resistant strains. Verapamil uptake was rapid, non-saturable over a large concentration range and was not energy-dependent.

There was no significant difference in the sensitivity to verapamil among the chloroquine sensitive and resistant parasites tested. Using western blots, the P-glycoprotein 1 (Pgh1) expression could not be correlated with the parasite sensitivity to verapamil. Verapamil had a concentration-dependent effect on increasing radiolabeled chloroquine accumulation in several resistant strains tested. Verapamil was able to reduce the rate of efflux of chloroquine within the first minute of measurement.

Verapamil was found to inhibit haem crystallization at very high concentrations *in vitro*. However, when verapamil was combined with chloroquine or quinine, it reduced the effect of these antimalarials on inhibiting haem crystallization *in vitro*.

At concentrations that are non-toxic to the parasite verapamil caused an accumulation of haemoglobin in chloroquine resistant strains. Using immunofluorescence microscopy this haemoglobin buildup was shown not to be associated with an increase in the number of endosomes in the parasite as is observed with chloroquine action.

ACKNOWLEDGEMENTS

Associate Professor Peter Smith, for your guidance and supervision. And for your many stories about your own experiences in the lab to remind me that science is a temperamental master.

To Associate Professor Tim Egan for your advice, discussions and for the collaborations that challenged me in different aspects of malaria research.

Dale Taylor, our friendship extends back to when I first started training as a scientist. Thanks for all the philosophical discussions, the jokes, the counseling. Good luck in all your endeavors, scientific or otherwise.

Doctor Uschi Wiehart for your friendship and the constant, unfaltering encouragement. Thank you for believing in me and for sharing your advice on how to get things done. Clearly I still have much to learn. Wood element!

Sandy, Susan, Jason, Justin, Quinton, Natalie, Grant for your friendship, encouragement and/or assistance at various stages during my degree.

Sumaya and Noor for keeping the tissue culture lab running so efficiently all the time.

My sisters, Mish and Dee, for putting up with me and always standing by me when times were tough.

My parents for urging me to keep on driving myself forward to achieve my ambitions. Thank you also for the financial or moral support you provided.

Thanks to the Medical Research Council of South Africa and the University of Cape Town Research Committee for their financial support during my degree.

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 10

LIST OF PLATES

CHAPTER 2

CHAPTER 7

CHAPTER 8

CHAPTER 10

LIST OF TABLES

CHAPTER 2

CHAPTER 5

CHAPTER 6

CHAPTER 7

CHAPTER 8

CHAPTER 10

LIST OF ABBREVIATIONS

^{3}H-CQ –	Tritiated chloroquine
^{3}H-VPL –	Tritiated verapamil
CQ –	Chloroquine
CQR –	Chloroquine resistant
CQS –	Chloroquine sensitive
CHF –	Chlorpheniramine
CHP –	Chlorpromazine
CYP –	Cyproheptadine
DAPI –	4',6-Diamidino-2-phenylindole Dihydrochloride
DBP –	Dibutyl Phthalate
DES –	Desipramine
DMEM –	Dulbecco's Modified Eagles Medium
DMSO –	Dimethyl Sulphoxide
EDTA –	Ethylelene diamine tetra-acetic acid
FPIX-	Ferriprotoporphyrin IX
Hb –	Haemoglobin
hc –	Haematocrit
HEPES –	Hydroxyethane piperazine sulphonic acid
HI_{50} –	The number of drug equivalents required to inhibit 50% of the β-haematin (haemozoin) formation.
IC_{50} –	The inhibitory concentration at which 50% of the cells are dead.
m –	Milli
μM –	Micromolar
M –	Molar
ml(s) –	Milliliter(s)
nM -	Nanomolar
NBT –	Nitroblue tetrazolium
OD –	Optical Density
PBS –	Phosphate Buffered Saline

PES –	Phenazine ethosulphate
P.falciparum-	*Plasmodium falciparum*
Pgp-	P-glycoprotein
Pgh1-	P-glycoprotein homologue 1
Pgh2-	P-glycoprotein homologue 2
pLDH –	Parasite lactate dehydrogenase
PRO –	Promethazine
pst –	Parasitaemia
PVDF –	Polyvinylidene difluoride
rpm –	Revolutions per minute
rcf –	Relative centrifugation factor
SDS-PAGE-	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
TRIF –	Trifluoperazine
Tris -	Tris(hydroxymethyl)aminomethane
VPL –	Verapamil

Chapter 1

Introduction

1.1 Perspective

Malaria is one of five diseases that cause the highest rates of mortality in man. Caused by the *Plasmodium* parasite, it is responsible for between 700,000 and 2.7 million deaths per year. Most of the mortality owing to malaria is attributable to the *P. falciparum* species but there are three other, less virulent species that affect man; namely *P. vivax*, *P. ovale* and *P. malariae*. Over 75% of the deaths attributable to malaria occur in African children and there are between 400-900 million febrile episodes in children under the age of 5 years (Breman, 2001).

1.2 The Life Cycle of the Malaria Parasites

Plasmodial infections are transmitted by the *Anopheles* mosquito. *Anopheles gambiae* and *Anopheles funestus* are the most effective vectors for *Plasmodium falciparum* transmission (Breman, 2001). The *Plasmodium* parasite undergoes a complex life cycle both in the human host and in the anopheline mosquito.

When a female mosquito bites a human host, sporozoites enter the bloodstream. They then travel to and infect hepatic cells. Here the sporozoites undergo asexual fission to release a new form of the parasite called the merozoite. Merozoites, released from the liver cells, can now enter the asexual erythrocytic stage of their growth in the human host. They infect erythrocytes and form a ring stage which later matures into the trophozoite stage. At this point the parasite is rapidly ingesting large amounts of the host cell's haemoglobin (Goldberg et al, 1990). The trophozoite nucleus then divides asexually to form a schizont containing between 6-24 nuclei. These

schizonts then divide and release merozoites when the erythrocyte is ruptured for the life cycle to continue.

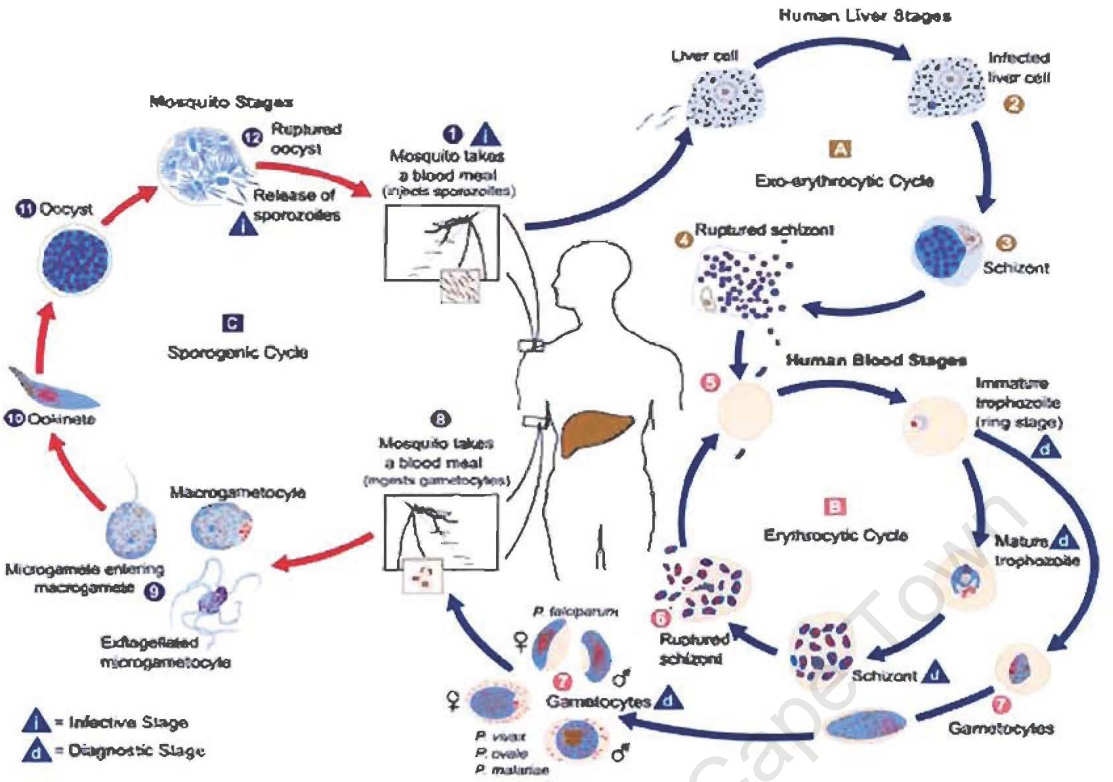


Figure 1.1: The life cycle of the malaria parasite. Image taken from http://www.cdc.gov/malaria/biology/life_cycle.htm

Sometimes the merozoites develop into gametocytes within the erythrocyte. When the mosquito ingests these cells during a blood meal the gametocytes take on either a male or female form. When the erythrocyte is lysed, the male and female forms fuse to form an ookinete. The ookinete migrates to the gut wall of the mosquito where it forms an oocyst. This oocyst divides to form the sporozoites that will migrate to the mosquito saliva to continue the cycle when the next human host is bitten (Prescott, 1993).

1.3 Antimalarial Drugs that Target the Blood Stages

Most of the antimalarial drugs used today act on the erythrocyte stages of the parasite life cycle. The three main classes of drugs involved in targeting the erythrocyte stages are:

1) The folate antagonists (sulphadoxine, pyrimethamine, proguanil)

2) The quinoline based drugs (chloroquine, mefloquine, quinine)

3) The artemisinins (artemether, artesunate, artemisinin)

The drugs that will be focused on here are the quinoline-based antimalarials and more specifically the action of chloroquine (CQ). CQ had been the mainstay of malaria treatment throughout malaria endemic areas for over 50 years. However, since the first cases of resistance to CQ were reported in the early sixties, there has a steady decline in the use of this cheap and relatively safe antimalarial. Today the prevalence of chloroquine resistance (CQR) has made the drug virtually useless worldwide as an antimalarial therapy.

1.4 Action of Chloroquine

The action of CQ on the *Plasmodium* parasites is stage specific. CQ exerts its toxic effects on the trophozoite stage of parasite life cycle (Geary et al, 1989). This is the period during which the parasite is most rapidly taking up haemoglobin from the erythrocyte.

1.4.1 Altered pH (Alkalinisation) in the Acidic Vesicle (Vacuole)

Early work on determination of pH in vacuoles of macrophages using the uptake of fluorescent dextran estimated the pH at between 4.8-5.2 (Okhuma and Poole, 1978). Exposure to CQ in this system led to an increase in the vacuolar pH.

The parasite cytoplasm has a pH of 7.4 and its vacuole has a pH of between 5.2-5.7 (Krogstad et al, 1985; Dzekunov et al, 2000). CQ is a weak base with pKas of 8.1 and 10.2. It would be expected to move into acidic compartments like the parasite's digestive vacuole down the pH gradient. Within the vacuole CQ becomes diprotonated. This charged species will not easily cross membranes and will thus accumulate within the vacuole. It has been estimated that CQ can reach micromolar concentrations in the parasite and millimolar concentrations in the vacuole (Yayon et al, 1984b). The accumulation would continue until the buffering capacity of the vacuole is exceeded. Then the pH of the vacuole would increase and the function of enzymes crucial to the

parasite's survival would be undermined (De Duve et al, 1974). Krogstad et al (1985) also used fluorescent dextran uptake to demonstrate that CQ could increase the vacuolar pH of *Plasmodium falciparum* parasites. This increase in vacuolar pH caused by CQ was confirmed by other groups (Yayon et al, 1985).

1.4.2 Interaction with Ferriprotoporphyrin IX (FPIX)

The *Plasmodium* parasite takes up haemoglobin from the host erythrocyte which is digested by means of a series of proteases into amino acids and peptide fragments. This haemoglobin breakdown also leads to the release and buildup of toxic haem or ferriprotoporphyrin IX. The parasite detoxifies haem by converting it to the non-toxic crystal haemozoin. The structure of this crystal has recently been elucidated using X-ray powder diffraction (Pagola et al, 2000). Chou et al (1980) proposed that CQ can interact with haem. CQ accumulation was shown to correlate well with the amounts of free haem available in the parasites (Bray et al, 1999). CQ binding to haem causes an inhibition of crystallization which in turn leads to the buildup of toxic haem. This free haem can induce membrane permeabilization that can lead to haemolysis (Chou et al, 1980).

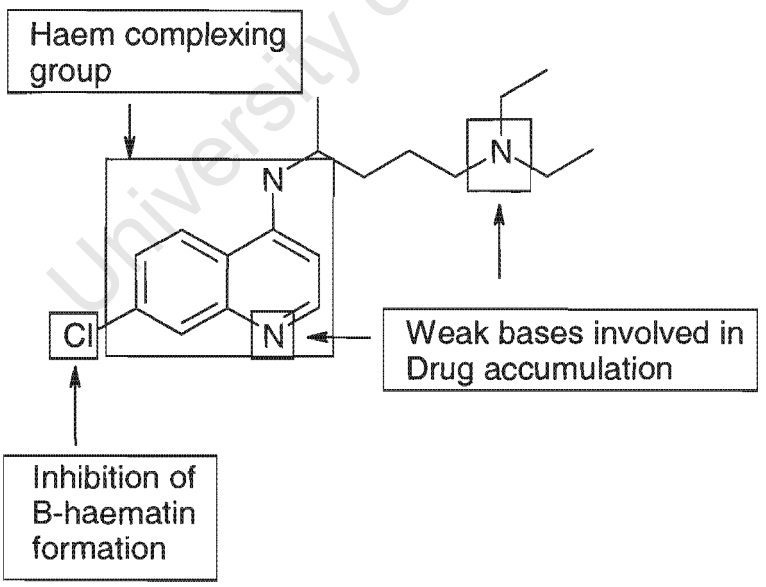


Figure 1.2: The structure of chloroquine (Egan et al, 2000).

Structure-activity studies have given insight into the interaction between CQ and haem. By modifying the structure of CQ and testing its effect on the inhibition of crystallization, the important structural characteristics have been identified (Egan et al, 2000; Kaschula et al, 2002). The planar 4-aminoquinoline ring is important in binding to the haem structure and the chloride group is important in inhibiting crystallization. The basic side chain is important in the accumulation of CQ into the acidic vesicle via pH trapping where it can interact with the haem (Egan et al, 2000).

1.4.3 Inhibition of Glutathione-Linked- or Peroxidative- Degradation of FPIX

While it is known that haem can be detoxified by crystallization into haemozoin, two groups proposed that this was not the only method of removing free haem.

Ginsburg et al (1998) reported that only about 30% of the haem is converted to haemozoin. They propose that the majority of the haem is then exported into the parasite cytoplasm where it is degraded by glutathione. They showed that both CQ and amodiaquine were able to inhibit the glutathione mediated degradation of haem both in solution or when membrane-bound.

Loria et al (1999) also suggest that only a third of the haem is converted to haemozoin. Haem is able to react with H_2O_2 causing its own breakdown in a process referred to as peroxidative decomposition. By mimicking the conditions in the food vacuole, they were able to show that peroxidative decomposition could occur quite rapidly. They then showed that CQ and other antimalarials inhibited the peroxidative decomposition of haem. They propose that this may be one of the ways in which CQ exerts its toxicity towards the parasite.

The results presented in the two above-mentioned articles have been questioned on quantitative grounds. Egan et al (2002) determined the fate of haem iron in the Plasmodial parasites. They found that 61% of the iron present in parasitised erythrocytes was found in the trophozoite. This correlated well with the 62% of the haemoglobin that was taken up by the parasite from the erythrocyte. Over 90% of the iron within the parasite was found in the food vacuole and almost 88.9% of this iron was in the form of haemozoin. This contradicts the theories of both Ginsburg

et al (1998) and Loria et al (1999) that only about 30% of the haem is converted into haemozoin. This suggests that the inhibition by CQ of haem crystallization was the main action of this antimalarial.

1.4.4 Other Possible Mechanisms of Chloroquine Action

It has recently been reported that CQ causes a buildup of haemoglobin in *Plasmodium falciparum* trophozoites (Famin and Ginsburg., 2002). This haemoglobin was identified in endosomes outside the vacuole using various fluorescent techniques (Hoppe et al, 2004). Previously it had been shown using electron microscopy that, in the presence of CQ, endosomes accumulate within the digestive vacuole of the parasites (Yayon et al, 1984a). It was proposed that one of the effects of CQ was to inhibit the fusion of the endosomes with the vacuole membrane. Whether this is a primary action of CQ or a secondary result of one of the actions mentioned above is not known.

CQ has also been suggested to inhibit a haem polymerase enzyme. Slater and Cerami (1992) suggested that the formation of haemozoin from free haem is catalyzed by a haem polymerase enzyme. They then demonstrated that CQ was able to inhibit the enzyme in a concentration dependent manner. This theory came under scrutiny when it was shown that the formation of β-haematin from haem could occur in the absence of proteins (Dorn et al, 1995). Also CQ is able to inhibit the β-haematin formation in the absence of protein *in vitro* (Egan et al, 1994).

1.5 Chloroquine Resistance

Resistance to a drug can be brought about in several ways. Firstly the drug may be altered to become less active and thus less toxic to the cell concerned. Secondly the drug target may be altered so that the drug cannot exert its effect on the modified target. Thirdly the target may be overexpressed thereby reducing the effect of the toxin. Fourthly the drug may be denied access to its target by either a reduction in its accumulation or an increase in its efflux out of the cell or just away from its site of action.

Fitch was the first to report that CQR *Plasmodium falciparum* strains accumulate less CQ than do CQS strains (1970). This reduced accumulation of CQ forms the basis for all the theories for resistance to CQ. The manner in which this is achieved though has been widely debated. Another distinct characteristic of resistance to CQ is the ability to reverse its resistance using agents like verapamil (VPL). However recently discovered strains appear to lack this characteristic as will be described later.

1.5.1 Reduced Import/Uptake of Chloroquine

Sanchez et al (1997) and Wunsch et al (1998) proposed that a Na^+/H^+ exchanger was responsible for importing CQ into the malaria parasite. They suggested that CQ was exchanged for protons via the Na^+/H^+ exchanger leading to its accumulation into the parasites. CQR parasites possess an impaired Na^+/H^+ exchanger compared with CQS parasites. CQR parasites thus have a reduced capacity to import CQ. This theory was challenged by Bray et al (1998/1999). They used sodium-free media to show that CQ was not exchanged for protons via the Na^+/H^+ exchanger (Bray et al, 1998). They also showed that amiloride, an inhibitor of the Na^+/H^+ antiport, and its derivatives were competing with CQ for haematin, the intravacuolar target of CQ. This would explain why CQ uptake was reduced in the presence of these inhibitors of the Na^+/H^+ exchanger as opposed to any altered function of the exchanger.

1.5.2 Increased Efflux of Chloroquine

Originally it was demonstrated that CQR strains efflux CQ far more rapidly than do CQS strains (Krogstad et al, 1987). CQ was shown to be effluxed from CQR strains at a rate 40-50 times faster than that seen in a CQS strain. Other groups have also reported a similar finding (Bayoumi et al, 1994). The efflux mechanism has been shown to be energy dependant as it is significantly less effective in the absence of glucose (Krogstad et al, 1992). More recently, it was once again affirmed that the efflux mechanism is responsible for reduced accumulation and that this was an energy dependent process (Sanchez et al, 2003). However two groups have questioned these hypotheses. Firstly Bray et al (1992a) showed that both CQS and CQR strains have similar efflux rates with the CQS strain showing a marginally faster rate of efflux. They proposed that a

reduced accumulation mechanism would better explain the differences between CQS and CQR strains. This was confirmed by Martiney et al (1995) who also could not distinguish between the efflux rates of CQS and CQR strains. The role of efflux in the resistance to CQ will be further discussed in Chapter 5.

1.5.3 Alteration of pH in the Digestive Vacuole

Since CQ accumulation is in part due to the pH gradient between the parasite cytoplasm and the digestive vacuole, any reduction in this gradient would lead to less CQ being taken up by the parasites. Initial theories suggested that in chloroquine-resistant parasites there was an increased vacuolar pH. This was attributed to a weakened vacuolar proton pump and was based on evidence from kinetic modeling (Ginsburg and Stein, 1991) and the use of pH altering agents (Bray et al, 1992b). However there is no concrete evidence supporting this theory.

Using single cell-level analysis of digestive vacuolar pH, Dzekunov et al (2000) were able to show that the CQR strain (Dd2) has a lower pH than the CQS strain (HB3) tested. Using the fluorescence intensity of acridine orange in the vacuole to quantify the absorption of the weak base and thus the pH, they calculated the pH of the CQS strain at 5.64. Their CQR strain had a pH of 5.21. Previous literature had suggested that CQR strains have a higher vacuolar pH (Krogstad et al, 1985; Yayon et al, 1984b). Since CQ accumulates partly by virtue of the weak base effect, the pH gradient would be smaller in CQR strains and thus less CQ would accumulate. This evidence suggested the opposite was occurring. To explain this anomaly, Ursos et al (2000) proposed that the lower pH in the CQR parasites reduces the amount of soluble haem and that the availability of free haem is highly pH dependent. Since CQ accumulation is determined by its access to free haem (Bray et al, 1998), the CQR parasites will accumulate less CQ at this lower pH.

This evidence was questioned from an experimental aspect by Bray et al (2002). They suggested that the acridine orange measured was not vacuolar fluorescence and was in fact due to cytosolic fluorescence. They also argued that any fluorescence in the vacuole owing to acridine orange would, to a certain degree, be quenched by the ferriprotoporphyrin IX.

Later studies have tried to iron out the experimental questions raised in the above paragraph (Dzekunov, et al, 2002; Waller et al, 2003). This theory for explaining the resistance to CQ remains controversial (Kirk and Saliba, 2001). A general change in vacuolar pH would be expected to cause cross-resistance to a range of quinoline-like antimalarials that also accumulate by means of their interaction with haem. Also other CQ analogues with shorter or longer side-chains are equally effective against both CQR and CQS strains (De et al, 1996; Ridley et al, 1996). These analogues should accumulate similarly to CQ and thus a change in pH should also induce cross-resistance to these compounds. The structural specificity of CQ resistance suggests a specific transporter is involved (Ridley, 1998).

1.5.4 Access to Haematin

Bray et al (1998) have shown that CQ uptake is dependent on its access to haem. They used an inhibitor of haemoglobin digestion, RO 40-4388, to show that in the absence of free haem there is a decrease in the amount of CQ accumulated. They also found that the capacity for CQ-haematin binding is similar for both CQS and CQR parasites. They propose, however, that CQR strains have a reduced accessibility of haematin for CQ. They also showed that VPL, a resistance reverser, was able to increase the affinity of CQ for the haem in CQR strains.

1.6 Genes/Proteins in Chloroquine Resistance in *Plasmodium*

Several genes have been implicated in the resistance to CQ in *P.falciparum*. Originally because of the similarities between CQR in *P.falciparum* and MDR in cancer cells and the chemosensitisation in both instances by VPL, it was thought that a homologue of P-glycoprotein was involved. Using a Pgp specific monoclonal antibody (C219), Krogstad et al (1991) detected a 40-42 kDa protein band in two CQS strains and one CQR strain of *P.falciparum* and a 240 kDa band in one of the CQS strains. The lower molecular weight band was also observed in two strains of *P.berghei* where it was overexpressed in the CQR line relative to the CQS line (Krogstad et al, 1991). However this result with the C219 antibody could not be reproduced in a later study (Cremer et al, 1995).

1.6.1 P-glycoprotein (Pgp) Homologues in Plasmodium falciparum

Two genes encoding P-glycoprotein homologues have been discovered in *P.falciparum*; *pfmdr1* and *pfmdr2* (Foote et al, 1989; Wilson et al, 1989). An amplification of the *pfmdr1* gene was detected in some of the CQR strains tested suggesting a link to CQR but the association was not absolute (Foote et al, 1989; Wilson et al, 1989). The protein product of *pfmdr1*, the P-glycoprotein homologue 1 (Pgh1), is a 160 kDa protein located primarily on the digestive vacuole of the parasite. It is expressed throughout the erythrocytic cycle including the gametocyte stage (Cowman et al, 1991). Pgh1 expression levels were not always associated with CQR since equal amounts were detected in both the CQS and CQR strains tested. The only strain showing an increased expression, FAC8, was no more resistant to CQ than any other CQR strain (Cowman et al, 1991). In a separate study, several progeny from a cross between the CQS HB3 and CQR Dd2 strains were compared in their sensitivity to CQ and mefloquine, CQ efflux halftimes, *pfmdr1* copy number and restriction fragment length polymorphism (RFLPs). No linkage could be found between the CQR phenotype and the *pfmdr1* gene (Wellems et al, 1990). It was later demonstrated that high level CQR was incompatible with increased Pgh1 expression (Barnes et al, 1992). CQ pressure on a strain expressing 3 copies of Pgh1 (FAC8) caused a deamplification of the *pfmdr1* gene and loss of Pgh1 expression. Also no amino acid change was seen in Pgh1. However the cells deficient in Pgh1 became more sensitive to mefloquine. Thus *pfmdr1* copy number may be linked to mefloquine resistance (Barnes et al, 1992). When mefloquine was used to pressure this strain (FAC8), the new highly mefloquine resistant lines had no further increase in Pgh1 expression (Lim et al, 1996). Also there were no changes in amino acid composition of *pfmdr1*. Thus mutations or changes in amplification or expression of *pfmdr1* are not essential in either CQR or increased resistance to mefloquine. However it was recently demonstrated that Pgh1 can modulate sensitivity of *Plasmodium* to several antimalarials (Reed et al, 2000). Although mutations in Pgh1 can not confer CQR alone, they can lead to increased CQR through decreased drug accumulation. This increase in resistance is susceptible to verapamil. Thus Pgh1 mutations are involved in modulation of CQR in some strains of *P.falciparum* (Reed et al, 2000).

Pfmdr2 is a 110 kDa protein with 10 hydrophobic domains. It is located in the plasma membrane of mainly the trophozoites and schizonts of *P.falciparum*. However there is no difference in expression between the CQS and CQR strains (Rubio and Cowman, 1994). There was no difference in gene copy number between CQS and CQR strains tested (Wilson et al, 1989). Also Wellems et al (1990) detected neither amplification of nor polymorphisms within the *pfmdr2* gene in CQR strains when compared to CQS strains in his genetic cross. Thus the *pfmdr2* gene and its protein product are not involved in CQR in *P.falciparum*.

1.6.2 CG2

In 1990, Wellems et al used a genetic cross to show that the *pfmdr1* and *pfmdr2* genes were not linked to the chloroquine resistance phenotype in *Plasmodium falciparum*. Using the same genetic cross they were able to link the chloroquine resistance phenotype to a 36 kb segment of chromosome 7 in *Plasmodium falciparum* (Wellems et al, 1991). In 1997, Su et al identified two genes with complex polymorphisms that were closely linked to CQR *P.falciparum*. These two genes were called cg1 and cg2.

However Fidock et al (2000a) were able to demonstrate that cg1 and cg2 do not alter the response of *P.falciparum* parasites to CQ. They used DNA transfection and allelic exchange to replace the polymorphisms in either cg1 or cg2 from CQR strains with those of the CQS HB3 strain. All the CQR strains remained resistant to CQ and all maintained their verapamil reversibility irrespective of substitutions in either the cg1 or cg2 genes. However the close association between the polymorphisms within these genes and the CQR strains suggested that a gene nearby might be responsible for the CQR phenotype. This then led to the identification of PfCRT (Fidock et al, 2000b).

1.6.3 PfCRT

PfCRT is a 48.6kDa protein containing 10 transmembrane domains but lacking an ATP-binding motif. It was localized to the digestive vacuole membrane using western blotting and immuofluoresence techniques (Fidock et al, 2000b). CQR strains with mutations in PfCRT at

position 76 ($^{K}76^{T}$ or $^{K}76^{I}$) linked with resistance to CQ were associated with an increased vacuolar acidification. Another mutation strongly associated with CQR involved a change of an asparigine to a serine ($^{A}220^{S}$).

The mutation in PfCRT that appears to be most important in determining the CQR phenotype involves the change of a charged amino acid, lysine, to an uncharged amino acid, threonine ($^{K}76^{T}$). Other mutations also involve changes in charge from the lysine to asparagine ($^{K}76^{N}$) or the lysine to isoleucine ($^{K}76^{I}$) mutation. These mutations all suggest a specific protein target is involved in the resistance to CQ as well as its reversal by chemosensitisers (Cooper et al, 2002).

Using pfcrt-modified "knockdown" clones, Waller et al (2003) were able to decrease pfcrt transcription levels and PfCRT protein expression levels. This resulted in a drop in CQ IC_{50} relative to the 7G8 parent line. These knockdown clones also had an altered digestive vacuolar pH similar to that found in CQS lines. This emphasized the link between expression of PfCRT, sensitivity to CQ and altered intracellular pH in *Plasmodium falciparum*.

Changes in pfcrt expression had no effect though on parasite sensitivity to quinine and mefloquine. Disruption of the pfcrt gene was found to be deleterious to parasite viability indicating that PfCRT is important in maintaining parasite growth (Waller et al, 2003).

Two papers have reported unique mutations in CQR strains from South America, Papua New Guinea ($^{C}72^{S}/^{K}76^{T}$) and the Philippines ($^{K}76^{T}/^{A}144^{T}/^{L}160^{Y}/^{N}326^{D}$) that are associated with a loss of verapamil reversibility of CQ (Mehlotra et al, 2001; Chen et al, 2003). Resistance to CQ is assumed to have developed independently from different geographical foci which are broadly classified as the Asian/African Dd2 type (VPL-reversible) and the South American/Oceanic 7G8 (VPL-insensitive) types. Both CQR phenotypes have a reduced vacuolar pH relative to the CQS strains. In the presence of VPL the Dd2 type parasites experienced an increase in vacuolar pH (alkalinized) whereas the 7G8 type showed no change in vacuolar pH when exposed to 5μM VPL (Bennett et al, 2004).

CQ has recently been demonstrated to bind directly to PfCRT (Zhang et al, 2004). However no strong evidence was shown that quinine is also bound although PfCRT is able to modulate quinine resistance. Through comparing the sequence of PfCRT with other 10 transmembrane transporter sequences, Tran and Saier (2004) have found significant homology with a nucleotide sugar transporter of the drug/metabolite transporter superfamily. They suggest thus that PfCRT's function is as a transporter.

1.6.4 Multidrug Resistance Associated Protein (MRP) homologues in Plasmodium falciparum*?*

Most recently a homologue of the multidrug resistance associated protein (MRP) has been identified in *Plasmodium falciparum* (Klokouzas et al, 2004). MRP, like Pgp, is a large 180-195kDa protein located mainly in the plasma membrane of cancer cells and can cause resistance to a range of hydrophobic drugs. It decreases the accumulation of antitumour agents in the cells by increasing their efflux from the cells (Zaman et al, 1994). The precise role of this *Pf*MRP in *Plasmodium falciparum* has not been established but it has been hypothesized that it may act to transport glutathione or its conjugates in the plasma membrane (Klokouzas et al, 2004). It is not yet known if this protein plays any role in drug resistance in malaria.

1.6.5 Other Proteins Possibly Involved in Resistance to Chloroquine

Photoaffinity analogues have been extensively used to identify drug targets in MDR cancer cells (for review, see Safa, 1999) and *P.falciparum* (Ye et al, 1989; Foley et al, 1994; Desneves et al, 1996). Azidopine, used to identify Pgp in MDR cells (Safa et al, 1987), was demonstrated to bind to a protein of 155-170 kDa in *P.falciparum* (Ye et al, 1989). This binding was competed for by both CQ and VPL. Smaller proteins were also identified in both the CQR and CQS strain. Foley et al (1994) used CQ photoaffinity analogues to identify two proteins of molecular weights 42 and 33 kDa in CQR and CQS *P.falciparum*. There was no difference in levels of labeling between the two strains and VPL did not compete for binding. Thus it was proposed that neither of these proteins were involved in the resistance to CQ. Later it was discovered that the 33 kDa protein was the parasite lactate dehydrogenase (Menting et al, 1997).

Several candidate proteins have also been identified in the CQR rodent *Plasmodium berghei* (Li et al, 1993) and *Plasmodium chabaudi* (Carlton et al, 1998, Hunt et al, 2004). Li et al (1993) identified a 53 kDa protein that was overexpressed in the CQR parasites when compared to the CQS parasites. The precise role of this protein in the resistance to CQ has yet to be established. A homologue of *pfmdr1* found in *P.chabaudi* (*pcmdr1*) was not associated with CQR in the rodent malaria (Carlton et al, 1998). The authors predict that the gene responsible for CQR in *P.chabaudi* is probably located on chromosome 11. More recently, orthologues of *pfcrt* and *pfmdr1* were identified in *Plasmodium chabaudi* (Hunt et al, 2004). The genes, *pccg10* and *pcmdr1*, were very similar to their respective gene sequences in the CQS *Plasmodium falciparum*. However these sequences were not altered in three clones with different levels of resistance to CQ. Moreover genetic crosses showed no linkage between the two genes and CQ resistance. Thus it is unlikely that these genes are implicated in the resistance to CQ in *P.chabaudi* (Hunt et al, 2004).

1.7 Quinoline Resistance Reversal in *Plasmodium falciparum*

1.7.1 Reversal of Mefloquine Resistance

Resistance reversal in *Plasmodium falciparum* has been observed for several of the quinoline-based antimalarials. Reversal of mefloquine resistance has been observed with only one chemosensitiser, penfluridol (Peters and Robinson, 1991; Oduola et al, 1993). As with CQR strains, the shift in sensitivity with penfluridol was only observed in the mefloquine resistant strain with no effect being seen in the mefloquine sensitive strain. Verapamil had no effect on modulating mefloquine resistance either *in vivo* (Peters and Robinson, 1991) or *in vitro* (Oduola et al, 1993) nor does penfluridol modulate CQ resistance. It is not known whether penfluridol's effect of enhancing mefloquine action is linked with an increase in mefloquine accumulation as is the case with CQ in CQ resistance reversal (Oduola et al, 1993). Recently there has been a report of reversal of mefloquine resistance by NP30, a nonylphenolethoxylate (Ciach et al, 2003). Since these agents are also able to reverse CQR (Crandall et al, 2000) it is unlikely that the method of resistance reversal is similar to that of penfluridol or verapamil.

1.7.2 Quinine and Quinidine Resistance Reversal

Quinine and Quinidine resistance can also be modulated by verapamil (Cooper et al, 2002). Plasmodial strains expressing the $^{K}76^{T}$ mutation are both CQR and less sensitive to quinine and quinidine. Verapamil is able to sensitize the parasites to all three of these quinolines. In strains carrying the $^{K}76^{N}$ mutation, the parasites are resistant to CQ and quinine but show no change in quinidine IC_{50} compared with a sensitive strain. Yet verapamil is still able to increase the susceptibility of the parasite to all three compounds. Lastly a mutation at $^{K}76^{I}$ causes resistance to CQ and quinidine but drastically reduces the parasites susceptibility to quinine. Verapamil can sensitize these parasites to both CQ and quinidine but reduces the sensitivity of the parasites to quinine (Cooper et al, 2002). Other compounds that reverse CQR also reverse resistance to quinine and quinidine. Various calcium channel antagonists can reverse resistance *in vitro* (Kyle et al, 1990). It would thus appear that CQR reversal is very closely linked to the reversal of quinine and quinidine resistance.

1.7.3 Reversal of Chloroquine Resistance

Since the discovery of the chemosensitising potential of VPL in 1987 (Martin et al, 1987; Krogstad et al, 1987), there have been a wide range of structurally and functionally diverse agents described that also demonstrate the ability to reverse CQ resistance. Other calcium channel inhibitors were shown to reverse CQR *in vitro* (Ye and Van Dyke, 1988; Adovelande et al, 1998) and *in vivo* (Tanabe et al, 1990). Studies in Aotus monkeys with CQR *Plasmodium falciparum* could not demonstrate a cure with a verapamil analogue but this could be due to impaired pharmacokinetics or bioavailability (Williams et al, 1992).

Reversal of CQR has also been shown with tricyclic antidepressants both *in vitro* (Gerena et al, 1992; Taylor et al, 2000; Bhattacharjee et al, 2001) and *in vivo* in mice (Miki et al, 1992) and Aotus monkeys (Bitonti et al, 1988). Antihistamines are of greatest interest clinically because they are commonly co-prescribed with CQ to treat pruritus. Several studies using antihistamines show great promise in potentially restoring CQ efficacy in humans (Abok et al, 1997; Sowunmi

et al, 1997; Oduola et al, 1998). The diversity of structure and function of these resistance reversers can be extended to the antipsychotic phenothiazines (Kyle et al, 1993; Kalkinidis et al, 2002; Van Schalkwyk et al, 2001; Singh et al, 2000) and even plant derived compounds (Haruki et al, 2000).

The reversible component of CQR is by no means universal as was demonstrated in recent literature. *Plasmodium berghei* strains that exhibit very high level resistance to CQ were observed to have lost the potentiation of CQ by VPL and nicardipine (Platel et al, 1998). These strains lacked haemozoin. The lack of effect of VPL on parasites with no haemozoin had been previously reported by Fitch in 1989. New Brazilian strains of *Plasmodium falciparum* appear to be hypersensitive to a number of structurally diverse resistance reversers (Menezes et al, 2002; Menezes et al, 2003). These agents also lack the ability to enhance CQ activity in these strains. The reason for this uncharacteristic response to chemosensitisation is unknown but may be linked to mutations in PfCRT (Mehlotra et al, 2001).

1.8 Drug Resistance in Multidrug Resistant Cancer Cells

The phenomenon of chloroquine resistance reversal was discovered from similarities observed with the reversal of resistance to anticancer agents in Multidrug Resistant (MDR) cancer cells (Martin et al, 1987). Verapamil was first discovered to reverse resistance to antitumour agents in MDR cancer cells over 20 years ago (Yusa and Tsuruo, 1981). These MDR cells were found to accumulate a much reduced amount of the antitumour agents (Tsuruo et al, 1982; Fojo et al, 1985) and thereby evade their toxicity. Verapamil was shown to inhibit efflux of the antitumour agents from the cells and in so doing it led to a restoration of the toxic accumulation of the antitumour agents (Rogan et al, 1984). It was observed that these cells were cross-resistant to a large number of other antitumour agents that were structurally and functionally different (Fojo et al, 1985). The important determinant in this resistance was discovered to be a plasma membrane protein called P-glycoprotein (Pgp) (Juliano & Ling, 1976). This 170,000 kDa protein is characterized by 12 transmembrane domains and two ATP-binding domains. It belongs to a family of transmembrane proteins called the ATP-binding cassette (ABC) proteins and is overexpressed in MDR cells (Juliano and Ling, 1976). Using photoaffinity analogues, several

groups have been able to show direct binding of these antitumour agents to P-glycoprotein (Safa et al, 1986). Since the initial discovery of the chemosensitiser VPL, a range of other chemosensitisers has been identified. These include cyclosporine A, ivermectin and azidopine (Zacherl et al, 1994; Pouliot et al, 1997; Safa et al, 1987) These are also structurally and functionally distinct from each other (Warr et al, 1988). Some have been shown to bind directly to P-glycoprotein though photoaffinity labeling (Safa et al, 1987; Safa et al, 1988). The antitumour agents and chemosensitisers thus are both are substrates for Pgp. Pgp has been demonstrated to efflux antitumour agents out of the MDR cells. Resistance reversal is achieved by competition of the chemosensitisers for efflux, which leads to a restoration of the accumulation of the antitumour agents to toxic levels in the cells.

Certain of the cell lines that are resistant to antitumour agents and overexpress Pgp also exhibit hypersensitivity to VPL (Warr et al, 1988; Cano-Gauchi et al, 1987)

1.9 Verapamil

Verapamil is a monoprotic weak base with a dissociation constant (pKa) of 9.2 (Bray et al, 1992a). It is a α_1 antagonist and blocks L-type calcium channels in smooth and cardiac muscle. It is 90% protein bound in plasma (Clarke EC, 1986) and has a very low partition coefficient in erythrocytes suggesting there is no significant accumulation in this compartment (Czejka et al, 1992). The binding of verapamil to albumin was not saturable between 0.04-17μM suggesting that the binding sites are of high capacity and low affinity. The binding is also stereoselective with the (-)-isomer of verapamil less bound to albumin than the (+)-isomer (Gross et al, 1988).

1.9.1 Verapamil as a Calcium Channel Blocker

The action of verapamil in malaria as a chemosensitiser has been demonstrated to be independent of calcium channels. Whereas calcium channels are specifically blocked by the (S)-(-) isomer, the resistance reversal activity in malaria can be achieved by both the (R)-(+) and the (S)-(-) isomer (Ye and Van dyke, 1988). Also verapamil, at concentrations that inhibit parasite growth (10-20μM), does not decrease the levels of free calcium within the parasites. This suggests that calcium movement into the parasite or the vacuole does not depend on the verapamil-sensitive

calcium channels (Adovelande et al, 1993). This observation is dissimilar to the role of verapamil in reversing multidrug resistance in cancer cells where verapamil was found to alter the calcium levels in cells where it reversed resistance to antitumour agents (Canu-Gauci and Riordan, 1987). It was suggested that verapamil may have a direct inhibitory effect on plasmodial calmodulin, a calcium dependent protein important for cellular metabolic activities. Verapamil caused significant antagonism of known calmodulin antagonists, R24571 and W-7, in growth assays on the multidrug resistant Indochina strain FCB_{k+}. This would suggest the compounds may be competing for the same receptor binding site (Scheibel et al, 1987).

1.9.2 Effect of Verapamil on Other Quinolines

Verapamil is able to enhance the action of CQ in CQR strains but has no effect on CQS strains (Martin et al, 1987). Verapamil is also able to modulate quinine sensitivity (Waller et al, 2003; Cooper et al, 2002). In plasmodial strains carrying the $^{K}76^{T}$ or the $^{K}76^{N}$ mutation in PfCRT verapamil was able to decrease the IC_{50} of quinine to levels seen in the quinine sensitive strain. In the strain with the $^{K}76^{I}$ mutation, a strain that exhibited hypersensitivity to quinine, verapamil increased the IC_{50} five fold (Cooper et al, 2002). This effect appeared to be stereospecific since quinidine maintained its verapamil reversible component in the all strains carrying mutations at position 76 of PfCRT (Cooper et al, 2002).

1.9.3 Hypersensitivity of Plasmodium falciparum *to Verapamil*

Whereas the early work showed verapamil had a chloroquine resistance reversal effect at concentrations that were non-toxic to the parasites (Martin et al, 1987), recent work has shown that certain Brazilian CQR strains are hypersensitive to verapamil (Menezes et al, 2003). The toxicity of verapamil is usually 100-1000 times greater than that of chloroquine (Martin et al, 1987, Jacobs et al, 1988) and most CQR strains have a verapamil reversible component. However verapamil and other calcium channel blockers as well as certain phenothiazines previously shown to be resistance reversers exhibit antimalarial activity in a concentration range similar to CQ in the new Brazilian CQR strains and lack the ability to potentiate CQ action (Menezes et al, 2002, Menezes et al, 2003).

1.9.4. Effects of Verapamil in the Absence of Chloroquine

In an ultrastructural study of the effect of verapamil on parasite growth, it was demonstrated that 100μM added for 24 hours to both CQS and CQR strains caused significant food vacuole enlargement although the vacuole membrane remained intact (Jacobs et al, 1988). There were dispersed crystalline pigment granules and some membranous debris. Swollen mitochondria were also observed. In contrast, drug pressure on either the CQS or CQR strains with 1μM verapamil showed only a few parasites with vacuolar swelling and increased granular matrix (Jacobs et al, 1988). Though this is the concentration used to reverse CQ resistance, no chloroquine like effects were observed. This suggests the action of verapamil as a resistance reverser is independent of its toxic effect and is different to CQ antimalarial activity. CQ alone caused food vacuolar swelling and increased granular matrix at 63nM in CQS *Plasmodium falciparum* (Jacobs et al, 1988). There was also some clumping of pigment granules and disintegration of the food vacuole membrane. Similar morphological changes were observed in CQS *Plasmodium chabaudi* when infected mice were administered CQ at 6mg/kg (Ohsawa et al, 1991). While CQ at these concentrations mentioned above had no effect on the morphology CQR parasites, addition of verapamil brought about morphological changes similar to those seen in the sensitive parasites in the absence of verapamil (Jacobs et al, 1988; Ohsawa et al, 1991).

The effect of 10μM verapamil on the parasites is to arrest growth in the trophozoite stage (Adovelande et al, 1993). This growth inhibition of the parasites is partially reversible if the verapamil is removed by washing before schizogony begins. If the drug pressure is maintained at 20μM verapamil until the 48th hour then the parasite growth is completely inhibited and they become morphologically abnormal (Adovelande et al, 1993).

The effect of VPL on the pH of the digestive vacuole of *Plasmodium* parasites has also recently been reported (Ursos et al, 2000). CQS parasites have a higher vacuolar pH than CQR parasites. When VPL (either 1μM or 5μM) was added to CQS parasites there was no effect on changing the vacuolar pH. However when VPL was added to CQR parasites there was a 0.26 and 0.35 pH unit increase at 1μM and 5μM respectively. These values approached the pH values for the CQS

strain in the absence of VPL but never quite reached the same levels. This suggests that VPL may be reversing resistance to CQ partly by altering the pH of the digestive vacuole to mimic that of the CQS strain.

1.10 Structural Link to Resistance and Resistance Reversal

Although many functionally and structurally different compounds are able to reverse CQR in *Plasmodium falciparum*, there appear to be certain specific characteristics that are important in chemosensitisation. These are: a cationic charge, two planar rings, lipophilicity and a tertiary nitrogen (Gerena et al, 1992). Certain compounds that are highly effective at reversing resistance in MDR cancer cells (cyclosporin A, ivermectin, progesterone) have no effect on reversing CQR in *Plasmodium falciparum* (Van Schalkwyk et al, 2001). This supports the theory that specific features are important given that the three aforementioned examples lack some or all of the characteristics listed above yet are highly effective inhibitors of the mammalian P-glycoprotein (Pouliot et al, 1997; Zacherl et al, 1994).

1.10.1 Specific Determinants of Chemosensitisers

The side chain length of CQ analogues was shown to be important in determining the resistance profile to these drugs (De et al, 1996). More specifically, the distance of the tertiary amine from the planar rings may be the important determinant in chloroquine resistance. Any distance between 4 and 8 carbons separation from the planar rings and the CQR strains exhibited varying degrees of resistance to the compound. Anything distance longer than 8 or shorter than 4 carbons from the rings and the compounds were equally effective against the CQS and CQR strains (De et al, 1992).

Recently there have been some Structure Activity Relationship (SAR) studies performed on chloroquine resistance reversers (Pradines et al, 2002; Guan et al, 2002, Bhattacharjee et al, 2001, Bhattacharjee et al, 2002). There appears to be a requirement for a secondary or tertiary amine linked to the planar structure via a carbon bridge of between 2-4 atoms (Bhattacharjee et al, 2001, Bhattacharjee et al, 2002; Guan et al, 2002). The nature of the amine group on the proximal

nitrogen is also important in the resistance reversal properties. In a study by Pradines et al (2002) it was observed that most of the compounds with amino groups could increase CQ accumulation. Only one of the 13 compounds with an amido group could increase accumulation. All the compounds that exerted an effect had a protonatable nitrogen at physiological pH (Pradines et al, 2002). This importance of the charge at the proximal amine group can also be illustrated through the ineffectiveness of loratadine (Peters et al, 1990; Singh and Puri, 2000). Loratadine, an antihistamine, is structurally very closely related to azatadine, cyproheptadine and pizotyline. Yet it had no effect either *in vitro* on *Plasmodium falciparum* (Peters et al, 1990) or *in vivo* on *Plasmodium yoelii* (Peters et al, 1990; Singh and Puri, 2000). Lipophilicity (Log P) has been associated with the resistance reversal (Bhattacharjee et al, 2002). Increased resistance reversal activity was strongly associated with increased lipophilicity. But the importance of lipophilicity has been questioned in other work (Pradines et al, 2002).

1.10.2 Possible Link between Chemosensitiser and Antimalarial Activity

The close structural relationship between chemosensitisers and the antimalarial chloroquine have been more highlighted when Kalkinidis et al (2002) were able to modify a chemosensitiser thereby converting it into an antimalarial. However the most effective antimalarial analogue synthesized from the chemosensitiser scaffold was found to have lost most of its modulating action on CQ in the CQR strain. It has been suggested that the weakened synergism with CQ in the CQR strain could be a result of competition for the antimalarial target haematin (Egan, 2002).

1.10.3 Compounds Lacking "Typical" Features of Chemosensitisers

There have been some compounds shown to reverse CQR (e.g. NP30, probenecid and cimetidine) that do not have the structural characteristics mentioned above (Crandall et al, 2000; Nzila et al, 2003; Ndifor et al, 1993). Moreover some of these compounds are able to reverse the resistance to both CQ and mefloquine which is not typical of the "conventional" chemosensitisers mentioned above (Ciach et al, 2003). Some compounds that reduce intracellular glutathione levels are also able to sensitize the parasites to CQ (Ginsburg et al, 1998; Deharo et al, 2003). It

remains to be established how these agents can reverse resistance and whether it is specific for CQ alone.

1.11 Clinical Relevance of Chloroquine Resistance Reversal

Ever since verapamil was first discovered to reverse CQR (Martin et al, 1987), there has been a question about the therapeutic viability of resistance reversal. Ye and Van Dyke (1988) suggested that one could avoid verapamil toxicity to calcium channels by using the (R)-(+) isomer since calcium channels are specifically blocked by the (S)-(-) isomer.

1.11.1 Toxicity

Many of these chemosensitisers block P-glycoprotein from secreting or excreting toxins from certain normal tissue types. This has the potential to lead to a toxic accumulation of CQ and the reversing agent in these cell types (Watt et al, 1990). Although CQ and verapamil were non-toxic to HEP-G2 liver cells individually, these compounds became toxic when combined.

1.11.2 Bioavailability

Another important factor is the binding of the chemosensitisers to plasma proteins. Many of the *in vitro* studies in *Plasmodium falciparum* do not accurately mimic the conditions in human blood. While desipramine was able to reverse CQR *in vitro* and in Aotus monkeys (Bitonti et al, 1988), no such effect was seen in a trial in humans (Warsame et al, 1992). Boulter et al (1993) demonstrated that desipramine's effect of enhancing CQ action was reduced in the presence of plasma proteins. Verapamil is 90% protein bound in plasma (Clarke, 1986). It binds to both albumin and α_1-acid glycoprotein (Gross, 1988). A study in MDR cancer cells has shown that serum protein binding can severely deplete the medium of the chemosensitiser and thus undermine the effect on resistance reversal (Lehnert et al, 1996). It has been suggested that the reason for the more successful trial of chloroquine combined with chlorpheniramine in humans was due to the reduced capacity of the chlorpheniramine to bind to plasma proteins (Sowunmi et al, 1997).

1.11.3 Successes

Some promising results have been observed when chlorpheniramine was combined with chloroquine to reverse CQR in two African studies (Sowunmi et al, 1997; Abok, 1997). In the one case, a doctor supplemented CQ therapy in his patients with a treatment of chlorpheniramine. The parasite clearance improved from 30% in the chloroquine only group to 70% in the chemosensitisers-supplemented therapy after 4 days (Abok, 1997). Sowunmi et al (1997) performed a clinical trial in children with acute uncomplicated falciparum malaria. They found that 85.4% of the children treated with the CQ-chlorpheniramine combination were cured at day 14 compared to 75.5% in the group treated with CQ alone. The combination was also well-tolerated suggesting promise as a new treatment regimen. Lastly, promethazine (1μM) was shown to enhance CQ action *in vitro* against several CQR Nigerian strains and in Aotus monkeys infected with CQR falciparum malaria (Oduola et al, 1998). They also showed that plasma, taken at various time points after the administration of promethazine to human volunteers, could reverse CQR *in vitro*. They found the largest reduction in IC_{50} with the plasma sample taken between 3-4 hours after promethazine administration. This suggests that promethazine can reach levels high enough in the bloodstream to enhance CQ activity.

1.11.4 Chemosensitiser Cocktails

The use of "cocktails" of chemosensitisers in combination with chloroquine to reverse resistance has been proposed (Adovelande et al, 1998; Van Schalkwyk et al, 2001). While most of the individual chemosensitisers are toxic to humans at concentrations that optimally reverse CQR, these authors suggest combining the chemosensitisers at concentrations which are suboptimal in reversing resistance. The chemosensitisers act additively in enhancing CQ action. In this manner the toxicity of any single agent can be avoided. The "cocktail" would have to contain agents that are functionally different since using two or more calcium channel blockers, for instance, would have the same toxic effect of using one at a high dose.

1.12 Purpose of this Study

Despite the extensive information on the ability of verapamil to reverse CQ resistance, its mechanism of action remains unknown. The purpose of this study is to characterize the interaction of VPL with chloroquine sensitive and resistant strains of *Plasmodium falciparum* and more specifically to:

(1) Relate the sensitivity of VPL in several parasite strains to the expression levels of Pgh1.

(2) Investigate the energy- and concentration dependence of VPL uptake.

(3) Use tritiated analogues of CQ and VPL to investigate the kinetics of uptake and efflux in resistance reversal.

(4) Determine the effect of verapamil on haem crystallization.

(5) Determine the effect of VPL in altering a buildup of haemoglobin in CQS and CQR strains of *Plasmodium falciparum.*

Chapter 2

The Differential Sensitivity of *Plasmodium falciparum* to Verapamil and Chloroquine and the Relationship with Pgh1 Expression.

2.1 Introduction

The effect of VPL on the reversal of CQ resistance has been well-established. VPL acts synergistically with CQ to reduce its IC_{50} in CQR strains (Martin et al, 1987). This effect is specific to CQR strains and is linked to an increased accumulation of CQ (Krogstad et al, 1987). The phenomenon of resistance reversal by VPL appeared to be universal in CQR strains, irrespective of their geographical origins, but exceptions have recently been reported (Platel et al, 1998; Menezes et al, 2003).

Verapamil has been used extensively *in vitro* to reverse chloroquine resistance since it was first identified as a chemosensitiser in 1987 (Martin et al, 1987). The IC_{50} of verapamil in plasmodial strains is 100-1000 times greater than that of chloroquine (Martin et al, 1987). This finding is consistent in a variety of strains (Bray et al, 1998; Peters et al, 1990; Adovelande et al, 1998) but has recently been challenged. Several new Brazilian strains have shown an enhanced susceptibility to VPL and other chemosensitisers at concentrations that are similar to CQ activity (Menezes et al, 2003; Menezes et al, 2002). These chemosensitisers lack the ability to modulate CQ activity in these CQR strains (Menezes et al, 2002; Menezes et al, 2003). Thus there are phenotypic variations to CQ resistance reversal between strains of different geographical regions. This is not surprising given that CQR is proposed to have evolved independently from two separate foci in South East Asia and South America (Payne et al, 1987).

The intrinsic antimalarial activity of VPL varies in CQR and CQS strains of *P.falciparum*. In some cases CQR strains are more sensitive to VPL than CQS strains (Adovelande et al, 1998;

Martiney et al, 1995; Gerena et al, 1992; Martin et al, 1987). In other instances VPL sensitivity profiles appear to match the sensitivity to CQ (Peters et al, 1990). Bray et al (1998) showed that in a range of strains with varying sensitivity to CQ there is a lack of cross-resistance of CQ with the resistance modulators VPL, primaquine and daunomycin. While CQR strains can have a 10-fold difference in sensitivity to CQ from the CQS strains, this large difference in sensitivity has not been observed with VPL between strains until the recent report of the verapamil sensitivity of the newly discovered Brazilian strains (Menezes et al, 2003).

Cowman et al (1991) demonstrated that increased Pgh1 expression is not essential in the CQR phenotype since many CQR strains lacked any increased Pgh1 levels compared to CQS strains. Barnes et al (1992) showed that increased Pgh1 expression is incompatible with high level CQ resistance. Pgh1 is nonetheless important in modulating the CQR phenotype (Reed et al, 2000). It is important to note that mechanism of resistance reversal is not completely understood. Although both PfCRT and Pgh1 have been implicated in CQR phenotype, the potential role of these proteins in resistance reversal is not known.

To date no comparison has been made between the expression of Pgh1 and the intrinsic antimalarial activity of VPL or that of the ability of VPL to reverse CQ resistance. Mefloquine resistance has been reported to be very closely linked to *pfmdr1* gene copy number (Cowman et al, 1994; Price et al, 2004). Moreover mefloquine has been shown to inhibit the binding of VPL to P-glycoprotein in mammalian cells (Riffkin et al, 1996). Given that Pgh1 is a homologue of mammalian P-glycoprotein, it may be possible to link the antimalarial activity of VPL to Pgh1 expression.

This chapter will determine and compare the intrinsic antimalarial activity of VPL in strains of varying geographical origin and the degree to which VPL can modify CQ activity. It will also compare the intrinsic toxicity of VPL to the expression of Pgh1 in *P.falciparum*.

2.2 Results and Discussion

Table 2.1 shows the geographical origin of the strains/clones used in this study. 3D7 is assumed to be an African strain although it was isolated from an airport worker in Amsterdam (Crandall et al, 2000; Pradines et al, 2002). FAC8 was derived from a Brazilian isolate ITG2F6 (Biggs et al, 1989). The CQR strains represent all three continents (Asia, Africa and South America) where cases of malaria are most often found. Thus the parasites used in this study represent a geographically diverse group.

Table 2.1. The CQ sensitivity of the strains used in this study and their geographical origin.

Strain/Clone	Chloroquine sensitivity	Geographical origin
3D7	CQS	Africa
D10	CQS	Papua New Guinea
FAC8	CQR	Brazil
K1	CQR	South East Asia
RSA11	CQR	Africa
W2	CQR	Indochina/Asia

In vitro resistance to CQ was defined as an IC_{50} > 100nM while CQS strains exhibited IC_{50}s < 100nM in line with previous reported literature (Basco & Le Bras, 1994; Cremer et al, 1995). For the purpose of this study, complete reversal of CQ resistance was defined as a reduction in the IC_{50} to a level below 100nM for the CQR strains.

The parasite lactate dehydrogenase assay was used to determine drug sensitivity to CQ and VPL (See Chapter 10, Section 10.5). The parasites (2% parasitaemia and 1% haematocrit) were incubated with either CQ or VPL that had been serially diluted. For the resistance reversal results, the CQ was serially diluted and a fixed concentration of VPL (1μM) was added to each well of the plate. The assay was performed over 48 hours (Makler et al, 1993).

2.2.1 *Characterization of the sensitivity of the* P.falciparum *strains to CQ*

The CQR strains used in this study differ between 6-fold and 17-fold in sensitivity to CQ over the CQS strains used (see Table 2.2). The FAC8 strain had an IC_{50} of 155.8nM (80.38ng/ml) which compares well with the reported literature value of 83ng/ml (Barnes et al, 1992).

Table 2.2 The IC_{50}s of CQ, VPL and CQ combined with 1μM VPL on 6 strains of *Plasmodium falciparum* with varying CQ sensitivity profiles. The Activity Enhancement Index (AEI) was determined by dividing the CQ IC_{50} by the CQ + 1μM VPL IC_{50}. Any value around 1 suggests no enhancement, a value lower than 1 suggests antagonism and values greater than 1 suggest synergism.

Strain/Clone	Chloroquine sensitivity	IC_{50} (nM)		AEI	IC_{50} (μM)
		CQ alone	CQ + 1μM VPL		VPL alone
3D7	CQS	21.40±0.937	21.45±1.170	0.998	17.72±3.020
D10	CQS	26.48±2.612	27.00±2.539	0.981	17.51±1.476
FAC8	CQR	155.8±39.58	43.12±13.70	3.613	12.82±3.558
K1	CQR	255.0±25.80	62.82±8.405	4.059	12.52±0.875
RSA11	CQR	354.0±38.60	42.55±5.458	8.320	14.39±3.354
W2	CQR	361.4±27.07	99.72±12.63	3.624	14.47±5.534

The RSA11 and W2 strains are the most CQ resistant and have different geographical origins. FAC8 is a South American clone and it has the lowest IC_{50} for the CQR strains with the K1 strain IC_{50} being intermediate between the highly CQR and least CQR strains. The two CQS strains show little difference in IC_{50} despite their different origins.

FAC8 has been shown to over-express Pgh1 and it has been suggested that over-expression of Pgh1 is deleterious to high levels of CQR (Barnes et al, 1992). This is confirmed here since FAC8 has the lowest CQ IC_{50} of all the CQR strains tested. The CQS strains exhibited very similar IC_{50}s. There is no clear trend linking the geographical origin to CQ sensitivity but this may be a result of the small sample size.

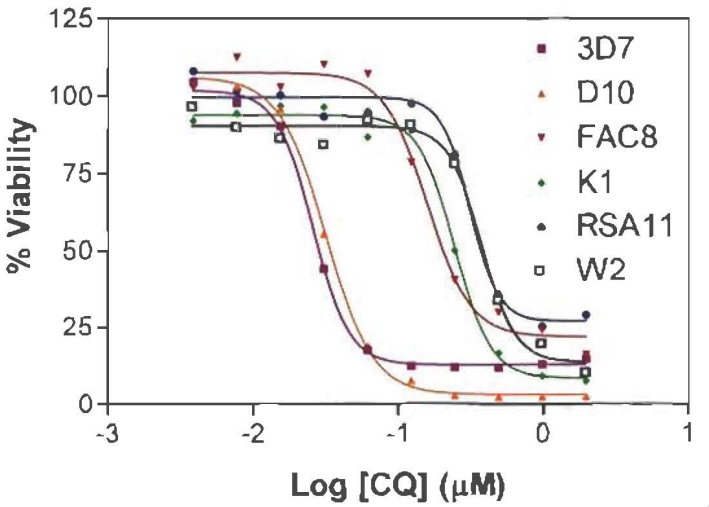


Figure 2.1 The differences in the sensitivity to CQ for the strains used in this study. The curves represent the data from at least three experiments each performed in duplicate. The curves were generated using the Prism 3.0 non-linear regression function (sigmoidal dose response equation).

2.2.2 Characterization of the effect of 1μM VPL on CQ sensitivity for the strains used in this study

The addition of 1μM VPL had no effect on altering the sensitivity to CQ in either of the CQS strains. VPL reversed resistance to CQ in all four CQR strains. The level of chemosensitization was similar in three of the four CQR strains. This is evident in the Activity Enhancement Index (AEI; see Table 2.2). FAC8, K1 and W2 all show an enhanced activity of between 3.6-4 times with 1μM VPL. However RSA11 has an AEI approximately twice that of the other CQR strains. Thus for three of the four CQR strains it would appear that the VPL sensitive component is constant and the main difference lies in their level of resistance to CQ.

RSA11 is the only African CQR strain in this study. It would be interesting to screen other African strains to determine if they also are more sensitive to VPL reversibility.

None of the CQR strains had their IC_{50} reduced to a value close to those of the CQS strains tested. However all the IC_{50}s were reduced to levels below those for the cutoff (100nM) in defining the CQS strain from the CQR strain.

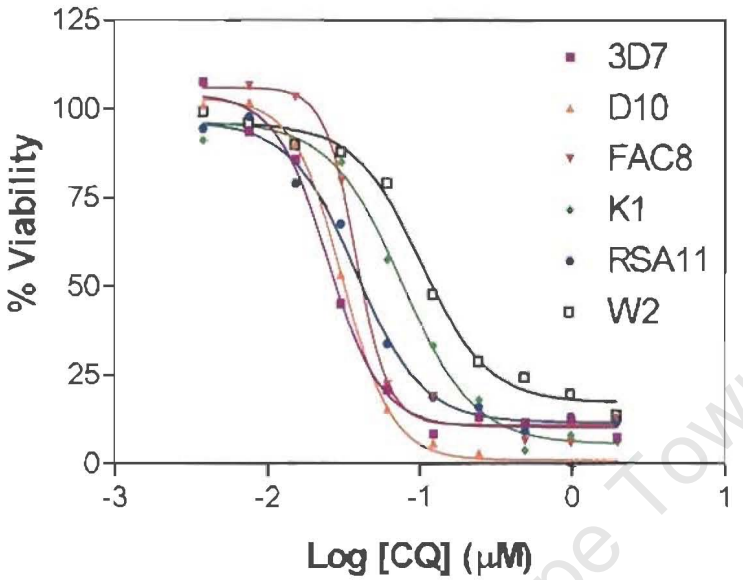


Figure 2.2 The differences in the sensitivity to CQ in the presence of 1μM VPL for the strains used in this study. The curves represent the data from at least three experiments each performed in duplicate. The curves were generated using the Prism 3.0 non-linear regression function (sigmoidal dose response equation).

Although the AEIs for 3 of the 4 CQR strains are similar, their initial IC_{50}s for CQ differ more than two-fold. Thus although the VPL effect was constant, the overall effect on reversing resistance was diminished as the resistance to CQ increased. Thus the CQ IC_{50} with VPL in the W2 strain fell marginally within the threshold distinguishing CQS strains from CQR strains. Strains more resistant to CQ than W2 may not be sensitized sufficiently in the presence of VPL to make the effect clinically relevant. This observation of the diminution of the VPL reversal effect in highly CQR strains has been reported (Platel et al, 1998). However this is not true for all strains since the RSA11 strain had a far more marked decrease in IC_{50} in the presence of VPL despite its IC_{50} being similar to that of the W2 strain.

The differences in AEI for the CQR strains is not unique. The ability of VPL to have a greater effect on some strains has previously been reported by Reed et al (2000) with strains expressing different mutations in Pgh1. Their 7G8-mdr^{7G8} transfectant was more resistant to CQ (IC_{50} = 389nM) when compared to the other two CQR transfectants, 7G8-mdr$^{D10/c1}$ (IC_{50} = 204nM) and 7G8-mdr$^{D10/c2}$ (IC_{50} = 215nM). The 7G8-mdr^{7G8} transfectant accumulated less CQ than the other two transfectants (7G8-mdr$^{D10/c1}$ or 7G8-mdr$^{D10/c2}$) but in the presence of VPL, its accumulation increased 7-fold while the others accumulated 4-fold more CQ. Also in the presence of VPL, all three CQR transfectants used by them had their IC_{50} values reduced to similar level. Thus the RSA11 strain used in this study may contain mutations in Pgh1 similar to those seen by Reed et al (2000) in their 7G8 strain while the other strains may be expressing alternate mutations in Pgh1 that cause them to respond similarly to those seen in the 7G8-mdr$^{D10/c1}$ or 7G8-mdr$^{D10/c2}$ transfectants.

None of the AEI's showed any antagonism by VPL on CQ. This may imply that VPL is not competing with CQ for the intracellular receptor responsible for CQ accumulation.

2.2.3 Characterization of the sensitivity of the P.falciparum *strains to VPL.*

Given the increased AEI for the African RSA11 strain when compared to the other CQR strains, we tested the intrinsic antimalarial activity of VPL in all the strains. This would distinguish whether the increased AEI was due to a true resistance reversal effect or a possible synergistic effect from an increased sensitivity of the strain to VPL.

While there is a difference of between 6-17-fold in the CQ sensitivity of these strains, there is no such difference in VPL sensitivity. The IC_{50}s range between 12-18μM (see Table 2.2). Although the two CQS strains show the highest IC_{50}s for VPL, they do not differ significantly from the most CQR strains ($p>0.05$). Also there is no significant difference in VPL IC_{50} between any of the CQR strains ($p>0.05$).

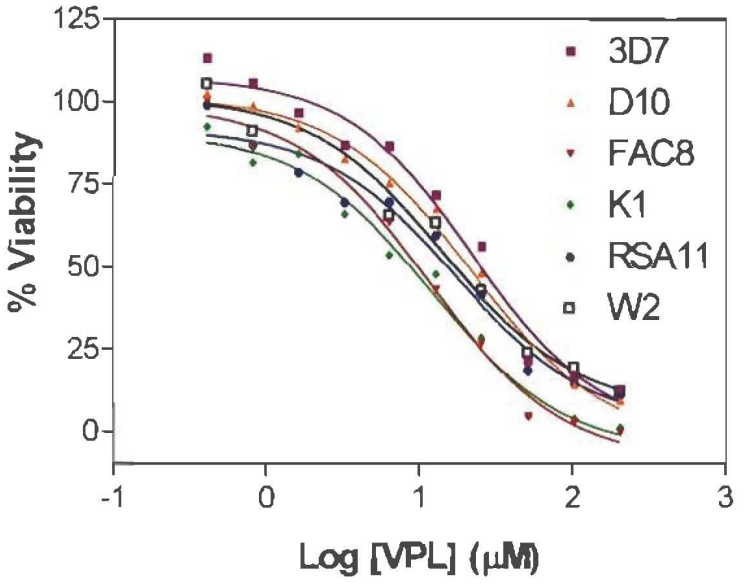


Figure 2.3 The differences in the sensitivity to VPL for the strains used in this study. The curves represent the data from at least three experiments each performed in duplicate. The curves were generated using the Prism 3.0 non-linear regression function (sigmoidal dose response equation).

VPL IC_{50}s in plasmodial strains are an order of magnitude higher than CQ IC_{50}s against the CQS strains There is no significant difference in VPL IC_{50} between CQS and CQR strains ($p>0.05$) nor is there a difference between strains from different geographical regions. This supports the theory of Bray et al (1998) that there is no cross-resistance between VPL and CQ. This would suggest that the mechanism regulating sensitivity of *Plasmodium falciparum* to VPL is separate from the mechanism causing resistance to CQ.

Some of the reported IC_{50}s for VPL have suggested that CQR strains are more sensitive to VPL than CQS strains (Gerena et al, 1992; Martiney et al, 1995, Adovelande et al, 1998). Hypersensitivity to VPL has been observed in certain multidrug resistant tumour cells (Cano-Gauci and Riordan, 1987; Warr et al, 1988). Even though the MDR cells accumulated less VPL than the wild type cells they were 40 times more sensitive to VPL toxicity (2μM vs. 80μM) (Cano-Gauci and Riordan, 1987). Martiney et al (1995) suggested that VPL in their CQR *P.falciparum* strain was, on average, 3.2 fold more toxic than in their CQS strain. These effects

may simply be strain specific as other authors report very small differences (Martin et al, 1987; Van Schalkwyk et al, 2001).

Verapamil slows down plasmodial growth at 10μM or blocks growth at 20μM (Adovelande et al, 1993). It was demonstrated that at these two concentrations verapamil does not change the free calcium concentration of the parasites. Thus it is likely that verapamil does not exert its toxicity via calcium channel blocking (Adovelande et al, 1993). Since verapamil is able to antagonize the action of certain calmodulin inhibitors, it was assumed that some of verapamil's toxicity is a product of its interaction with calmodulin itself (Scheibel et al, 1987). Ultrastructural studies show markedly different effects of verapamil on the parasite morphology when compared to CQ. Food vacuole enlargement and dispersed pigment granules were observed when parasites are exposed to 100μM but the vacuole membrane remained intact (Jacobs et al, 1988). These effects coupled with the fact that verapamil has an IC_{50} well above that of conventional quinoline-based antimalarials suggest that verapamil toxicity is dissimilar to the toxicity exerted by conventional antimalarials.

In addition, Martiney et al (1995) reported that VPL IC_{50} values did not differ within strains much when measured between pH 6.4-7.9. This suggests that VPL is not exerting an effect as a lysosomal weak base. They also demonstrated that over a large VPL concentration range (up to 40μM) there was still no change in the CQ IC_{50} in the CQS strain. These concentrations should be highly toxic to the parasites. This confirms that the VPL toxicity is unrelated to its ability to modulate CQ sensitivity.

The large difference in VPL IC_{50} values relative to CQ IC_{50} values in our CQR strains differ from the results reported in new Brazilian strains confirming that there are significant geographical differences in the phenotype of CQR strains (Menezes et al, 2003). In the Brazilian strains VPL toxicity was observed from 34nM and there was no synergism observed with CQ. In the CQR strains tested in this study, VPL IC_{50} values were much higher than CQ IC_{50} values and CQ activity was enhanced in each instance in CQR strains.

2.2.4 The expression of Pgh1 and its relationship to sensitivity to VPL

The correlation between Pgh1 expression and CQ or MQ sensitivity is already well-established. Pgh1 levels have not been correlated with VPL sensitivity nor has it been reported for the African strain RSA11.

Comparison of the Pgh1 levels between the known strains and the African strain (RSA11) might;

a) aid in explaining the increased AEI observed for the RSA11 strain and

b) reveal a possible correlation between VPL sensitivity and Pgh1 expression.

Vacuoles were used in the determination of the Pgh1 levels because it is known that most of the Pgh1 protein resides in the parasite food vacuole membrane (Cowman et al, 1991). Equal numbers of vacuoles isolated from trophozoites (See Section 10.10 for methodology) were run using sodium dodecyl polyacrylamide electrophoresis (SDS-PAGE). The proteins were transferred to a PVDF membrane by western blotting and probed with anti-Pgh1 antibodies raised to either the N-terminal ATP-binding site or to a C-terminal region (See sections 10.15-10.17).

3D7 D10 FAC8 K1 RSA11 3D7 D10 FAC8 K1 RSA11

Panel A Panel B

Plate 2.1 Western Blot results showing the expression of Pgh1 from food vacuoles of several strains of *Plasmodium falciparum*. In Panel A the membrane was incubated with anti N-terminal ATP-binding site antiserum. In panel B the membrane was incubated with the anti-C-terminal region antiserum. The strains tested from left to right are: 3D7, D10, FAC8, K1 and RSA11. W2 was not tested.

The P-glycoprotein homologue (Pgh1) was detected in vacuoles from all 5 strains tested and was recognized by both the antisera tested. Equal numbers of vacuoles were loaded onto the gel and it

is evident that the FAC8 strain has a more intense band than the other strains tested. Using the Multi-Analyst® PC software from Bio-Rad and selecting FAC8 as the reference strain the band intensities were calculated for comparison.

Table 2.3 The band intensities for Pgh1 expression in the isolated vacuoles from 5 plasmodial strains of varying sensitivity to CQ. The results represent the data from 3 separate experiments performed with antisera to both the C-terminal region and the ATP-binding domain.

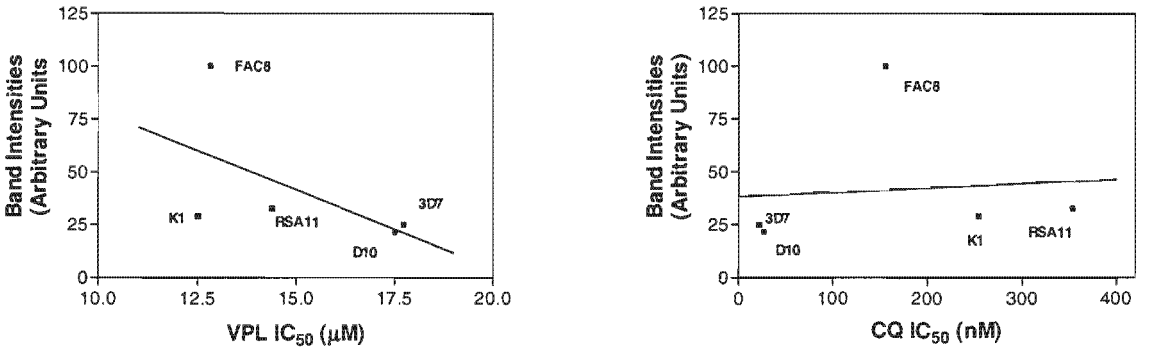
Strain	Average Band Intensity (arbitrary units)	Relative decrease in band intensity compared to FAC8[a]
3D7	24.75±10.17	4.040
D10	21.38±12.91	4.667
FAC8	100.0±0.000	1.000
K1	28.81±14.08	3.471
RSA11	32.19±16.30	3.107

[a] The relative decrease in band intensity is determined by dividing the FAC8 intensity by the individual band intensity for each strain.

No significant difference was observed in vacuolar Pgh1 levels in any of the strains apart from FAC8 which showed levels of Pgh1 of between 3.1-4.7 times higher than the other 4 strains. This result is similar to that reported for the FAC8 strain reported by Cowman et al (1991) and Price et al (2004). As mentioned earlier, overexpression of Pgh1 was shown to be deleterious to high levels of CQR (Barnes et al, 1992). This would appear to be confirmed since both the RSA11 and K1 strain contain only one copy of Pgh1 and have IC_{50}s more than twice as high as the FAC8 IC_{50}.

There is no correlation between Pgh1 expression and sensitivity of *P.falciparum* to VPL (Figure 2.4 A). Both mefloquine and VPL are monoprotic weak bases. Given that mefloquine is known to compete for VPL binding to P-glycoprotein in mammalian cells (Riffkin et al, 1996) and the increased P-glycoprotein homologue 1 expression has been associated with resistance to

mefloquine (Cowman et al, 1994), one might have expected that the FAC8 strain would be more resistant to VPL than the strains with only one copy of Pgh1. This is clearly not the case.



A B

Figure 2.4. Association between (A) VPL IC_{50} and Pgh1 expression and (B) CQ IC_{50} and Pgh1 expression in various strains of differing CQR profiles. The correlation coefficients are (A) $r^2 = 0.3196$ and (B) $r^2 = 0.0080$.

The results of the association between the Pgh1 expression and CQ IC_{50}s show that there is no correlation between them (Figure 2.4 B). This confirms the results of Cowman et al (1991). Strains that were either CQS or more resistant to CQ than FAC8 expressed the same amount of Pgh1.

While Pgh1 overexpression has been demonstrated to be important in the resistance to mefloquine, it does not determine the sensitivity to either CQ or its resistance modulator VPL. The role of Pgh1 in modulating CQR appears thus to be more a function of mutations in the gene rather than expression of the protein (Reed et al, 2000).

Chapter 3

Characterization of the Effect of Verapamil on the Accumulation of Tritiated Chloroquine in Multiple Strains of *Plasmodium falciparum*

3.1 Introduction

In 1970, Fitch demonstrated that CQR strains of *P.falciparum* accumulate less CQ than CQS strains. The majority of the accumulated CQ is localized to the digestive vacuole in the CQS parasites (Aikawa, 1972, Yayon et al, 1984b). Accumulation of CQ was found to be energy dependent in both whole CQS parasites (Krogstad et al, 1992; Bray et al, 1992) and isolated digestive vacuoles (Saliba et al, 1998). In CQR strains the accumulation of CQ increases under conditions of metabolic deprivation (Krogstad et al, 1992; Bray et al, 1992). This is consistent with the theory that there is an energy-dependent efflux pump in the CQR strains (Krogstad et al, 1992). The sensitivity of plasmodial strains to CQ and other 4-aminoquinolines is highly dependent on their accumulation into the parasites (Bray et al, 1992; Hawley et al, 1996; Hawley et al, 1998).

Martin et al (1987) showed that VPL was able to reverse resistance to CQ in *Plasmodium falciparum* strains. That same year Krogstad et al (1987) demonstrated that several resistance reversers, including VPL, could increase the accumulation of CQ into CQR parasites over a large concentration range. This increase in accumulation was attributed to an inhibition of the efflux of CQ from the CQR strain. VPL did not increase CQ accumulation in the CQS strain over the same concentration range (Krogstad et al, 1987). These observations were later confirmed in other strains (Wellems et al, 1990).

Martiney et al (1995) found that VPL could also increase the accumulation of CQ into the CQS strain (D10) at concentrations between 0.5-1μM. However the increase in accumulation was not

associated with a change in sensitivity as was observed for CQR strains. Moreover in CQR strains they found that CQ accumulation did not correlate with CQ sensitivity. When CQR strains were manipulated to accumulate more CQ than CQS strains by altering the external medium pH, their IC_{50}s were still higher than those of the CQS strain.

This chapter will examine the effect of increasing concentrations of VPL on the accumulation of ^{3}H-CQ in several strains of *Plasmodium falciparum* with varying sensitivity to CQ. It will also compare the effect of various other chemosensitisers on ^{3}H-CQ accumulation at equal molar concentrations. It will then examine the role of temperature and energy dependence on VPL-dependent ^{3}H-CQ accumulation.

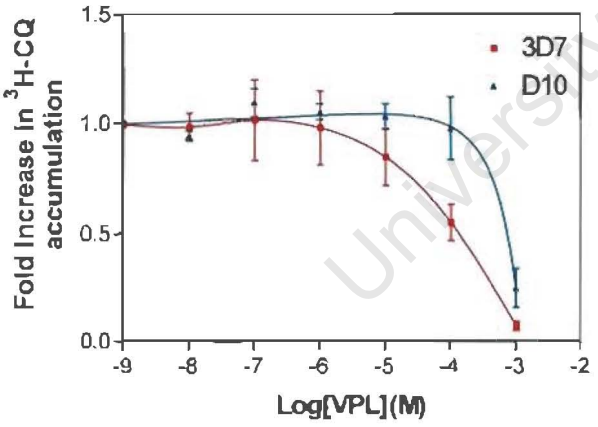
Although much of the following work is similar to results published previously, some reports have been contradictory and some effects may be strain specific. The experiments described herein were deemed necessary to either explain or compare with results reported in later chapters. It also allows for a direct comparison between the accumulation of ^{3}H-CQ and ^{3}H-VPL (described in chapter 4) within this study. The comparison of the effect of VPL and the other chemosensitisers on the plasmodial parasites described herein will also form an important point of discussion for experiments presented in chapters 6 and 7.

3.2 Results and Discussion

In all of the following sections the accumulation of ^{3}H-CQ was measured in parasites (1% haematocrit, 5% parasitaemia) over 1 hour. VPL and other chemosensitisers were added 15 minutes before the addition of the ^{3}H-CQ. For a detailed description of the methodology, refer to section 10.6.

3.2.1 The effect of increasing VPL concentrations on ^{3}H-CQ accumulation in CQS strains

Figure 3.1 clearly illustrates that VPL had no effect on increasing CQ accumulation in the CQS strains 3D7 and D10 at concentrations reported to reverse resistance (1-10μM). At higher concentrations (100μM-1mM) there was a significant drop in CQ accumulation in both strains. The 3D7 strain appeared to be more sensitive to the effects of the higher VPL concentrations on CQ accumulation than did D10.



Strain/Clone	IC_{50} (nM)		AEI
	CQ alone	CQ + 1μM VPL	
3D7	21.40	21.45	0.998
D10	26.48	27.00	0.981

Figure 3.1 The dose dependent effect of VPL on the accumulation of ^{3}H-CQ (2nM) in the CQS strains 3D7 and D10. Each experiment was performed in triplicate on three separate occasions. The curves were generated using the Prism 3.0 Spline curve function. The IC_{50}s provided on the right are taken from Table 2.2.

The drop in ^{3}H-CQ accumulation seen in the CQS strains at the high VPL concentrations (100μM) was also demonstrated in the original papers describing the action of VPL (Krogstad et al, 1987; Wellems et al, 1990). There was also no increase in CQ accumulation between 0.5-1μM as reported by Martiney et al (1995).

Most of the early literature showed a lack of effect of VPL both on the accumulation of CQ and on changing the sensitivity to CQ in CQS strains (Martin et al, 1987; Krogstad et al, 1987; Wellems et al, 1990). We also have found no increase in CQ accumulation in either of the CQS strains used in this study (including the D10 strain) over a large concentration range. These results contrast with those of Martiney et al (1995).

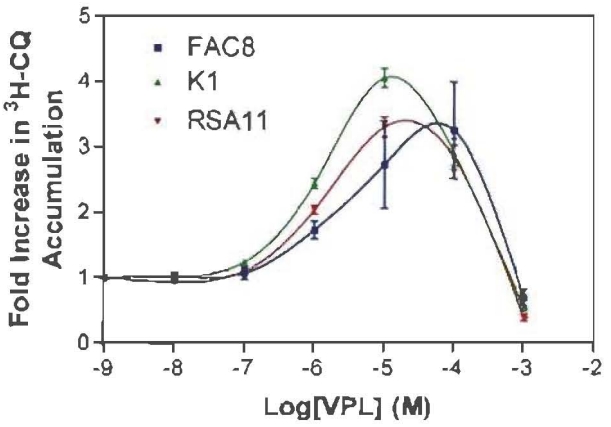
A decrease in CQ accumulation was observed at higher concentrations of VPL. 3D7 was more sensitive to this decrease in accumulation than D10. VPL has been shown to increase membrane permeability in some mammalian cells (Drori et al, 1995). This may cause leakage of accumulated CQ at the higher concentrations. However these concentrations are well above the VPL IC_{50}s for 3D7 and D10 so the reduction in accumulation could be due to general cytotoxicity.

Some erythrocyte lysis was also observed in these strains at 1mM as indicated by a reddening of the extracellular media after the samples were centrifuged. This would suggest that some permeabilisation or disintegration of the membranes (parasite and erythrocyte) may also have occurred.

3.2.2 The effect of increasing VPL concentrations on ^{3}H-CQ accumulation in CQR strains

Figure 3.2 illustrates a comparison of the effects of VPL on CQ accumulation in the 3 CQR strains tested. The K1 and RSA11 strains reached their peak CQ accumulation at roughly the same VPL concentration but at a lower concentration than that of the FAC8 strain. All the CQR

strains accumulated between 3-4 times the amount of CQ maximally relative to the control and all demonstrated a sharp decline in CQ accumulation at 1mM similar to the CQS strains.



Strain/Clone	IC_{50} (nM)		AEI
	CQ alone	CQ + 1μM VPL	
FAC8	155.8	43.12	3.613
K1	255.0	62.82	4.059
RSA11	354.0	42.55	8.320

Figure 3.2 The dose dependent effect of VPL on the accumulation of ^{3}H-CQ (2nM) in the CQR strains FAC8, K1 and RSA11. Each experiment was performed in triplicate on three separate occasions. The curves were generated using the Prism 3.0 Spline curve function. The IC_{50}s provided on the right are taken from Table 2.2.

There did not appear to be any correlation between the amount of CQ accumulated and the shift in sensitivity to CQ. RSA11 showed a marked shift in sensitivity to CQ (8-fold) but did not accumulate any more CQ than did K1 or FAC8. At 1μM VPL there was a very small difference in the amount of ^{3}H-CQ accumulated.

All three of the CQR strains exhibited a maximal accumulation of between 3-4 times the control value at VPL concentrations between 10-100μM. FAC8 reached its maximal accumulation "later" than the other two strains but its level of resistance reversal with 1μM VPL is not markedly different from the other CQR strains. FAC8 expresses higher levels of Pgh1 than the other two strains. However it is difficult to predict how this might have a bearing on the response to VPL reversal given that the function of Pgh1 is currently not known.

The difficulty in extrapolating accumulation data to changes in sensitivity has been previously reported (Bray et al, 1994). The ^{3}H-CQ accumulation assay was measured over 1 hour while the sensitivity assays were only read after 48 hours. The effect of VPL on reversing CQ resistance is often observed at much lower concentrations than those found to cause maximal ^{3}H-CQ accumulation. All three CQR strains have a similar capacity to increase CQ accumulation yet their IC_{50}s are modulated to different degrees in the presence of VPL (see Figure 3.2).

All the strains show a drop in accumulation of CQ at 1mM VPL and, as with the CQS strain, this is probably due to some permeabilization or disintegration of the membranes.

3.2.3 The effect of various chemosensitisers on ^{3}H-CQ accumulation at the same concentration (1μM) in the CQS strains

There are many structurally and functionally different compounds that reverse CQ resistance. These include promethazine (antihistamine), chlorpromazine (antipsychotic), chlorpheniramine (antihistamine) and desipramine (antidepressant). It was decided to investigate the action of these widely known chemosensitisers on ^{3}H-CQ uptake for comparison with VPL.

The concentration of 1μM was used for all the chemosensitisers tested because it reverses CQ resistance significantly and it is non-toxic to both the CQS and CQR strains (D. Taylor, personal communication). In Figure 3.3 it can be seen that different chemosensitisers all lack the ability to increase CQ accumulation in the CQS strains. This is in agreement with the AEI found in Table 2.2 where VPL showed no enhancement of CQ activity. This confirms the observation that their effect is specific to CQR strains.

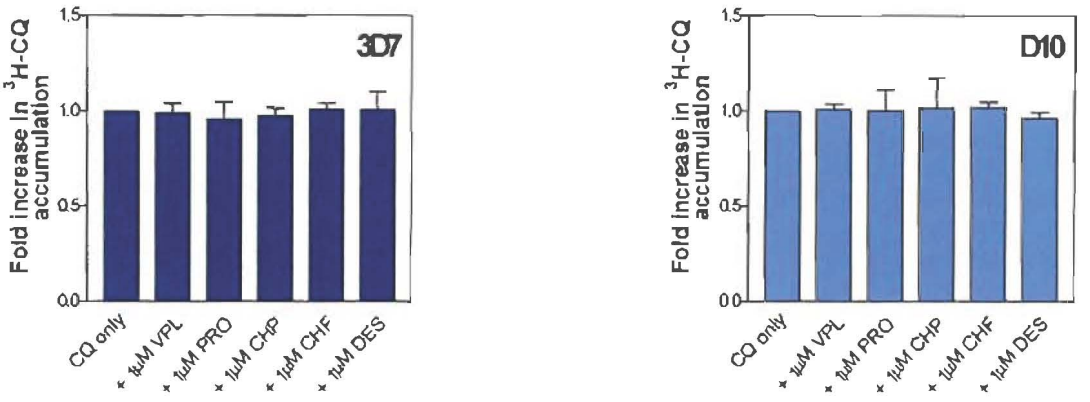


Figure 3.3 The effect of a range of chemosensitisers on the accumulation of ^{3}H-CQ (2nM) in the CQS strains, 3D7 and D10, at a fixed concentration of 1μM. The bars represent fold increases over the controls. Each experiment was performed in triplicate on two separate occasions. The drugs examined were: verapamil (VPL), promethazine (PRO), chlorpromazine (CHP), chlorpheniramine (CHF) and desipramine (DES).

All concentrations used were non-toxic to the parasites and thus would not be expected to exert any effect on the CQS parasites. Kalkinidis et al (2002) showed that chlorpromazine (CHP) can inhibit β-haematin formation similarly to CQ. If the binding to haem and subsequent inhibition of β-haematin formation is linked to CQ uptake (Bray et al, 1998) then CHP might be expected to compete for CQ accumulation. However the concentrations used here are clearly not high enough to compete for CQ accumulation. CHP is monoprotic and thus will not accumulate as much as CQ did and it has a lower capacity for inhibiting β-haematin formation than did CQ (Kalkinidis et al, 2002).

3.2.4 The effect of various chemosensitisers on ^{3}H-CQ accumulation at the same concentration (1μM) in the CQR strains

The effect of VPL at 1μM did not differ significantly from other chemosensitisers tested at same concentration ($p>0.05$) in either CQR strain. Chlorpheniramine had a consistently smaller increase in accumulation than the other chemosensitisers tested albeit statistically insignificant ($p>0.05$). All the chemosensitisers increased CQ accumulation between 2-3 fold in both strains. For RSA11, 1μM VPL enhanced CQ sensitivity 8.3 fold with a 2.67 fold increase in CQ

accumulation. Similarly for the K1 strain, an AEI of 4.059 was only accompanied by a 2.043 fold increase in CQ accumulation. Similar results have been previously reported (Bray et al, 1994).

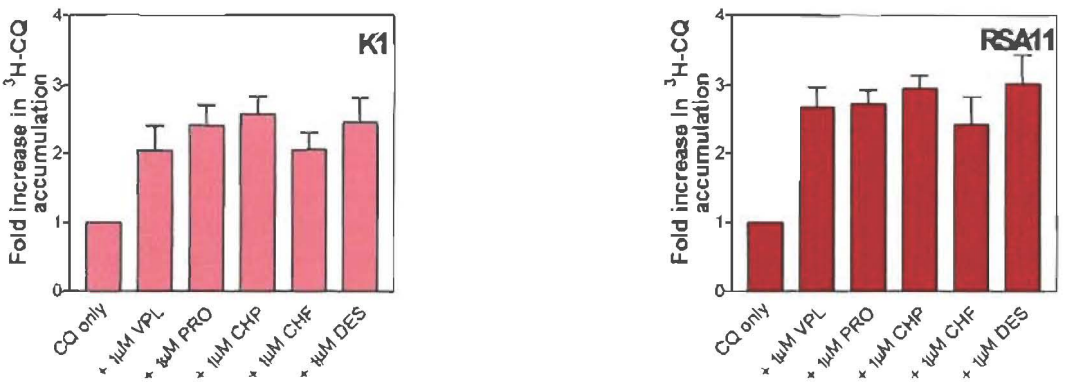


Figure 3.4 The effect of a range of chemosensitisers on the accumulation of ^{3}H-CQ (2nM) in the CQR strains, K1 and RSA11, at a fixed concentration of 1μM. The bars represent fold increases over the controls. Each experiment was performed in triplicate on two separate occasions. The drugs examined were: verapamil (VPL), promethazine (PRO), chlorpromazine (CHP), chlorpheniramine (CHF) and desipramine (DES).

All the chemosensitisers are monoprotic with similar pKa's (see table 10.1) and would thus be expected to accumulate to the same levels as VPL did in the CQR strain. All appear to have an equal capacity to increase CQ accumulation at this concentration. Although the chemosensitisers are structurally and functionally different, their similar behaviour on ^{3}H-CQ accumulation points to a common mode of action in *Plasmodium falciparum.* Thus the work on VPL can likely be extrapolated to the other chemosensitisers.

3.2.5 The temperature dependence of the VPL effect on ^{3}H-CQ accumulation

While it is known that ^{3}H-CQ accumulation is temperature and energy dependent, not much is known about the temperature and energy dependence of the resistance reversal effect by VPL.

VPL is able to increase the accumulation of ^{3}H-CQ at 37°C in the CQR strain (K1). At 4°C there was a decrease in CQ accumulated in the CQR strain and VPL did not increase the amount of CQ accumulated at this temperature (see Figure 3.5).

There was also a large decrease in the CQ accumulated at 4°C in the CQS strain. As expected VPL had no effect on CQ accumulation in the CQS strain at either 37°C or at 4°C.

The demonstration of the temperature dependence of CQ accumulation has been previously reported (Sanchez et al, 1997). In both their CQS strain (HB3) and their CQR strain (Dd2), there was a decrease in the CQ accumulated at 4°C when compared to that accumulated at 37°C.

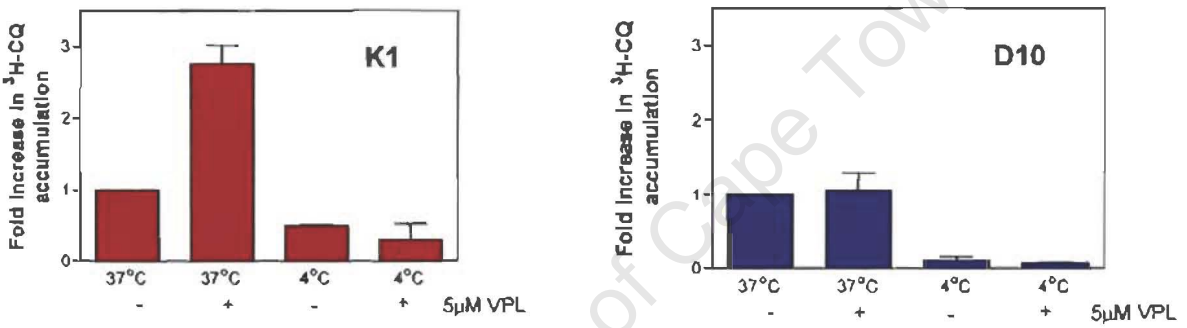


Figure 3.5 The temperature dependence of VPL on ^{3}H-CQ accumulation in a CQR (K1) and CQS (D10) strain of *Plasmodium falciparum*. All values were determined relative to the control (37°C; No VPL). The experiment was performed twice in triplicate.

Sanchez et al (1997) suggest that the temperature dependence of CQ accumulation implies a transport mechanism is responsible for the uptake of CQ in *Plasmodium falciparum*. However this could also imply that an efflux pump is not operating efficiently given that most of the parasite enzymes or energy-dependent processes should only be optimal at 37°C. VPL did not affect the uptake of CQ in the CQR strain at 4°C. This result is consistent with the mode of action of chemosensitisers being energy dependent. It is thus unlikely that the action of VPL is a general changing of the fluidity of the membrane as has been suggesting for function of VPL in some MDR cancer cells (Drori et al, 1995).

3.2.6 The effect of metabolic deprivation on the VPL effect on ^{3}H-CQ accumulation

Azide inhibits oxidative phosphorylation and malaria parasites need an external source of glucose for glycolysis (Krogstad et al, 1992). Thus cells treated with azide and deprived of glucose were considered energy depleted. This method was also used by Bray et al (1992) to examine metabolic deprivation.

The accumulation of CQ in the CQS strain (D10) was greatly reduced in the absence of glucose. The addition of VPL had no effect on CQ accumulation under any conditions as expected for the CQS strain.

In the CQR strain there was an increase in CQ accumulation in the absence of glucose (see Figure 3.6). VPL increased CQ accumulation when glucose was present, but decreased the CQ accumulation in the absence of glucose, albeit insignificant ($p = 0.0892$). The increase in CQ accumulation in the absence of glucose was similar to the amount of CQ accumulated in the presence of VPL and glucose.

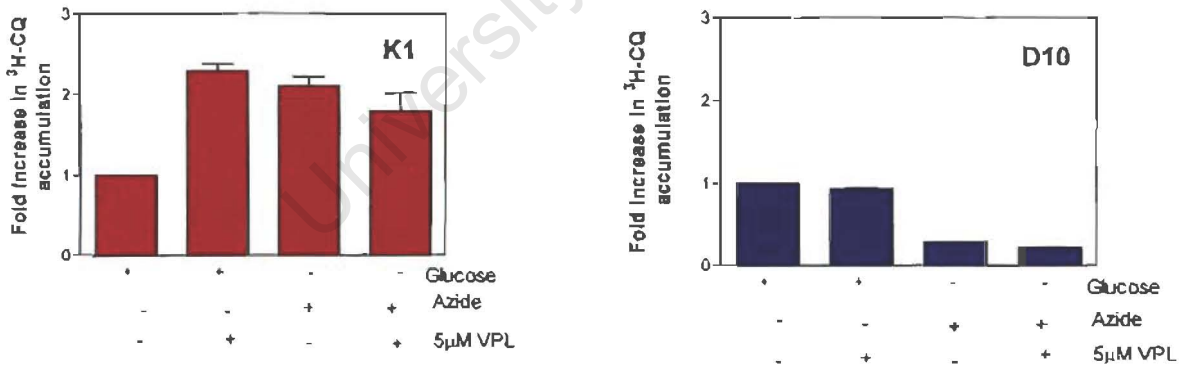


Figure 3.6 The effect of metabolic deprivation on the VPL effect on ^{3}H-CQ accumulation at 37°C in a CQR (K1) and a CQS (D10) strain of *Plasmodium falciparum*. All values were determined relative to the control (PBS + 1mg/ml glucose; No VPL). The azide concentration was 10mM. The experiment was performed once in triplicate.

CQ accumulation decreases in conditions of metabolic deprivation in the CQS strain but increases under the same conditions in the CQR strain. The results reported here agree with those of Krogstad et al (1992) and Bray et al (1992). Krogstad et al (1992) used the lack of metabolizable substrates to demonstrate that efflux is an energy dependent process. Bray et al (1996) found dissimilar results for their CQR K1 strain when they substituted glucose with 2-deoxy-D-glucose. They found no significant difference between the energy-depleted and energy-present samples, but did not propose a mechanism to explain this effect. What is evident from that paper was that neither VPL nor vinblastine could increase the uptake of CQ under conditions of metabolic deprivation (Bray et al, 1996). This observation was confirmed in this chapter. VPL had no effect on CQ accumulation in the absence of glucose once again suggesting that its action is associated with an energy-dependent putative transporter.

What is interesting to note is that the accumulation of CQ in the absence of glucose is similar to the accumulation of CQ in the presence of glucose and 5μM VPL. Krogstad et al (1992) reported that the amount of CQ taken up by the CQR strain in the absence of glucose was roughly twice as much as that taken up with glucose present. This is similar to our findings. The addition of 5μM VPL also caused an approximately 2-fold increase in CQ accumulation in the presence of glucose. If, in the absence of glucose, a putative efflux pump is inhibited, then these results are consistent with the proposal that VPL inhibits the pump in the presence of glucose.

Not surprisingly VPL had no effect under any conditions on the CQS strain.

The results in this chapter also concur with the results of Sanchez et al (2003) where it was demonstrated that after the addition of glucose there was an increase in CQ accumulated in the CQS strain and a decrease in the amount of CQ accumulated in the CQR strain. This was assumed to support the theory of an energy dependent efflux pump present in CQR strains (Sanchez et al 2003).

In conclusion the results reported here support the theory of a temperature- and energy-dependent putative CQ transporter in the CQR strains of *P.falciparum*. The results presented also

demonstrate that VPL and a diverse range chemosensitisers exert similar effects on CQ accumulation in the parasites at sublethal equimolar concentrations.

Chapter 4

Characterization of Tritiated Verapamil Accumulation in *Plasmodium falciparum*

4.1 Introduction

Although VPL can increase the accumulation of anticancer agents into MDR cells, VPL itself has different uptake kinetics in some MDR cancer cells. VPL accumulates in lysosomes of mammalian cells (Lelong et al, 1991) and can bind to membranes of certain cells (Cornwell et al, 1987).

Studies with tritiated [^{3}H] verapamil demonstrate different uptake kinetics into MDR cells relative to their drug sensitive counterparts (Yusa and Tsuruo, 1989). In the drug sensitive human erythrocyte leukemic K562 cell line, VPL uptake was 3-fold higher than in the Adriamycin resistant line K562/ADM. The antitumour agent vincristine (5μM) was able to increase the accumulation of VPL in the MDR cell line suggesting that VPL is transported by a similar carrier to vincristine (Yusa and Tsuruo, 1989). VPL was also demonstrated to bind to P-glycoprotein directly using photoactive analogues (Yusa and Tsuruo, 1989, Safa et al. 1988). Thus VPL is effluxed by P-glycoprotein and reverses multidrug resistance in cancer cells by competitively inhibiting the drug transport through the P-glycoprotein.

However, the differential accumulation of VPL in drug-sensitive versus drug-resistant tumour cells is not universal. Both VPL and azidopine were observed to accumulate to similar levels in wild type and daunorubicin resistant Ehlich ascites tumour cells (Sehested et al, 1990). Despite this, vincristine was shown to increase the accumulation of VPL in the resistant line only. Also, the photoreactive azidopine was shown to label P-glycoprotein and verapamil could inhibit the

labeling. So VPL may inhibit P-glycoprotein without competing for drug efflux (Sehested et al, 1990).

To date only limited work has been published on verapamil uptake by *Plasmodium falciparum* using tritiated analogues of verapamil. Bray et al (1992) demonstrated the effect of energy depletion and competition with 100nM CQ on ^{3}H-VPL accumulation in a CQS and CQR strain. When glucose was removed and with the metabolic inhibitor sodium azide added, both the CQS and CQR strain experienced a drop in VPL accumulation. This suggests that VPL accumulation, similar to CQ, is energy dependent. Moreover CQ at 100nM increased the accumulation of ^{3}H-VPL in both CQS and CQR strains by 42% and 43% respectively. The authors suggest that this implies an efflux pump that has a similar capacity in the two strains. They also found that the accumulation ratios for the two strains were far in excess of the predicted values for monoprotic weak bases to accumulate in the acidic food vacuole. They concluded that a large amount of the verapamil probably accumulates in the cytosol of the parasites. In 1998, Bray et al showed that a proteinase inhibitor had no effect on ^{3}H-VPL accumulation while it drastically reduced the ^{3}H-CQ accumulation. This was attributed to the inhibition of CQ's binding to haematin and it is assumed therefore that the uptake of VPL is not dependent on its binding to haematin.

This chapter will deal with the characterization of the accumulation of ^{3}H-VPL into uninfected and *Plasmodium falciparum* infected erythrocytes. It will demonstrate the energy and concentration dependence on ^{3}H-VPL uptake and the effect of competition with a large molar excess of CQ and VPL.

4.2 Results and Discussion

4.2.1 Accumulation of ^{3}H-VPL by erythrocytes and parasitized erythrocytes

The uptake of ^{3}H-VPL (1nM) was examined in both parasitised and unparasitised erythrocytes. The experiment was performed over 1 hour. Thereafter the cells were spun at 13000rpm for 1 minute, the supernatant removed and the pellet processed for scintillation counting. The complete experimental procedure can be found in section 10.6 of the Methodology chapter.

From Figure 4.1 it is evident that VPL is rapidly lost from the pellet of either unparasitized or parasitized erythrocytes when it is washed once with PBS, suggesting a low affinity binding site, if any. The accumulation of ^{3}H-VPL is not significantly different between the unparasitized or the parasitized erythrocytes for both the washed ($p = 0.1249$) and unwashed samples ($p = 0.8787$).

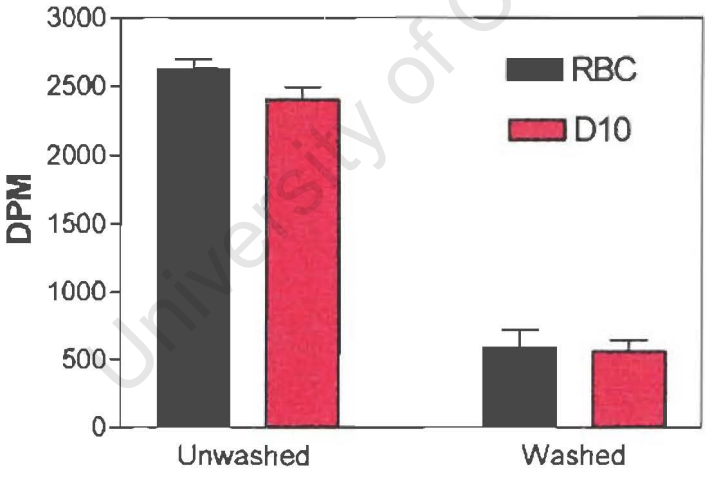


Figure 4.1 The accumulation of ^{3}H-VPL (1μM) into unparasitized and parasitized erythrocytes over 1hour and the effect of washing the pellet with PBS. Both erythrocyte and D10 infected erythrocyte samples were at 1% haematocrit with the D10 parasitaemia at 5%. This experiment was performed twice in triplicate.

This experiment was performed using the standard conditions of 1% haematocrit and 5% parasitaemia. Under these conditions CQ accumulates to a far greater extent in parasitized

erythrocytes than in uninfected erythrocytes. The reason for this is that CQ accumulation into unparasitized erythrocytes occurs by means of passive diffusion (Ferrari and Cutler, 1990) while CQ accumulation into parasitized erythrocytes is dependent on its access to haematin (Bray et al, 1998; Bray et al, 1999) and/or proton trapping (Yayon et al, 1984b). The accumulation of VPL thus differs significantly from that of CQ. This illustrates that there is no specific uptake of VPL by parasitised erythrocytes.

Czejka et al (1992) demonstrated that VPL has a low partition coefficient in erythrocytes and that the interaction of VPL with erythrocytes is weak and reversible. This would appear to be confirmed in this chapter.

For all the subsequent experiments the unparasitized and parasitized erythrocytes were spun through dibutyl phthalate to separate them from the external medium. To distinguish between parasite specific accumulation and erythrocyte accumulation it was decided not to use the conventional 5% parasitaemia and 1% haematocrit. Instead the parasites were enriched according to the method of Ginsburg et al (1999). Parasitised erythrocytes were layered on a Percoll gradient (60%: 90%) in medium containing 3% alanine and spun for 20 minutes at 10000 rpm. Trophozoites were separated from the unparasitised erythrocytes and partitioned between the 60%: 90% Percoll layer. The experiments were then run at a 0.1% haematocrit. The parasitaemia of the enriched culture was consistently greater than 90% (see section 10.8 of the Methodology chapter).

4.2.2 The energy dependence of ^{3}H-VPL accumulation into enriched trophozoites

There is no significant difference in ^{3}H-VPL accumulation between the CQS and CQR strains when glucose is present ($p = 0.9195$) (See Figure 4.2). Although there appears to be a decrease in ^{3}H-VPL accumulation in the absence of glucose, these values are not significantly different from those where glucose is present ($p = 0.8846$ for D10; $p = 0.9376$ for K1). The accumulation in the absence of glucose also does not differ significantly between strains ($p = 0.7671$). Thus metabolic deprivation does not affect the accumulation of ^{3}H-VPL whereas it has a profound effect on the

accumulation of ^{3}H-CQ (see Figure 3.6). These results are similar to those obtained by Bray et al (1992).

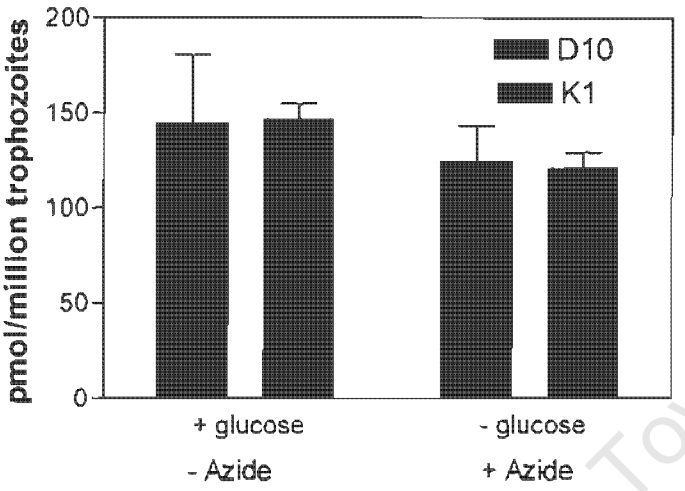


Figure 4.2 A comparison of the energy dependence of ^{3}H-VPL accumulation in enriched trophozoites from a CQS and CQR strain of *Plasmodium falciparum* (0.1% hc). The experiment was run in either PBS + 1mg/ml glucose (+ glucose) or PBS + 10mM sodium azide (+ azide). The experiment was run in triplicate on two separate occasions.

The lack of effect of metabolic deprivation on ^{3}H-VPL accumulation is another significant difference from the accumulation of CQ in *Plasmodium* parasites. The glucose-dependent changes in CQ accumulation were suggested to support evidence for a putative efflux pump in CQR strains (Krogstad et al, 1992). If VPL is also a substrate for this pump then one might expect similar effects to those seen with CQ (e.g. a decreased accumulation in the absence of glucose). Clearly, though, the differences in the methods of accumulation between CQ and VPL make this difficult to verify.

These differences in the uptake of a cytotoxic agent and a chemosensitiser are not without precedent. There have been reports of similar uptake levels and kinetics for VPL in mammalian cell lines either sensitive or resistant to daunorubicin (Sehested et al, 1990). In their tumour cells

VPL accumulated at a similar rate and to the same levels in both the drug sensitive and MDR cells. This occurred despite it being a substrate for the P-glycoprotein efflux pump.

4.2.3 The concentration dependence of ^{3}H-VPL uptake into a CQR and CQS strain of Plasmodium falciparum *at 4°C and 37°C.*

The equilibration of ^{3}H-VPL in the CQS strain is not saturable over a 1 million fold concentration range. The experiment was performed at both 37°C and at 4°C. There is no difference between the two curves and they are almost superimposable. This represents a fundamental difference between the accumulation of CQ and VPL in the *Plasmodium* parasite.

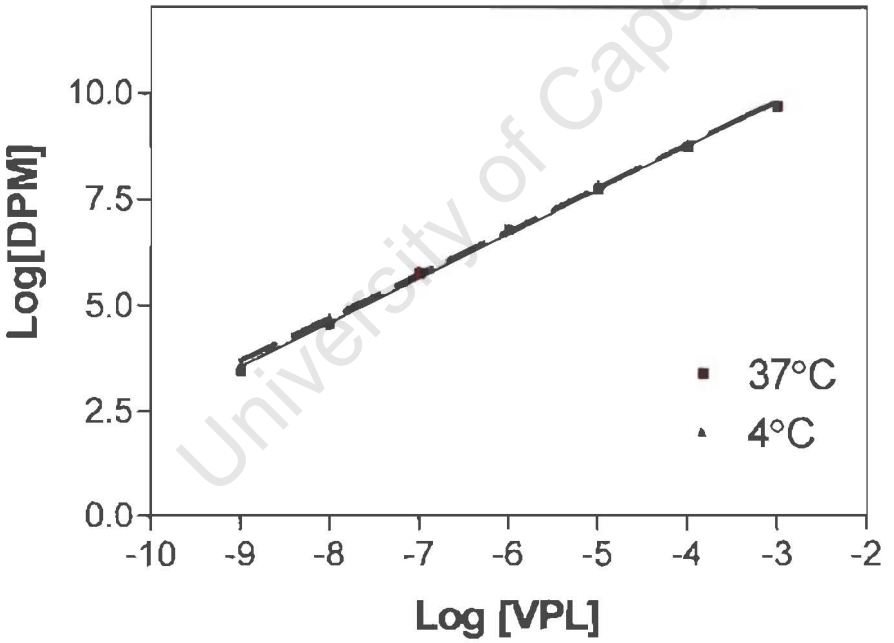


Figure 4.3 A comparison of the concentration dependence of ^{3}H-VPL accumulation at 37°C and 4°C in enriched trophozoites from the CQS strain (D10). The experiments were performed in triplicate on 2 (4°C) or 3 (37°C) separate occasions. The linear regressions were generated using Prism 3.0. The linear correlation coefficients for the two curves are: $r^2 = 0.9987$ for the experiment performed at 4°C and $r^2 = 0.9980$ for the experiment performed at 37°C.

Figure 4.4 shows the same pattern for the CQR strains as for the CQS strain. There is no saturation over the large concentration range (1nM to 1mM). Also there is no difference between

the experiments when performed at 4°C and when performed at 37°C. These curves are also almost identical to those of the CQS strain.

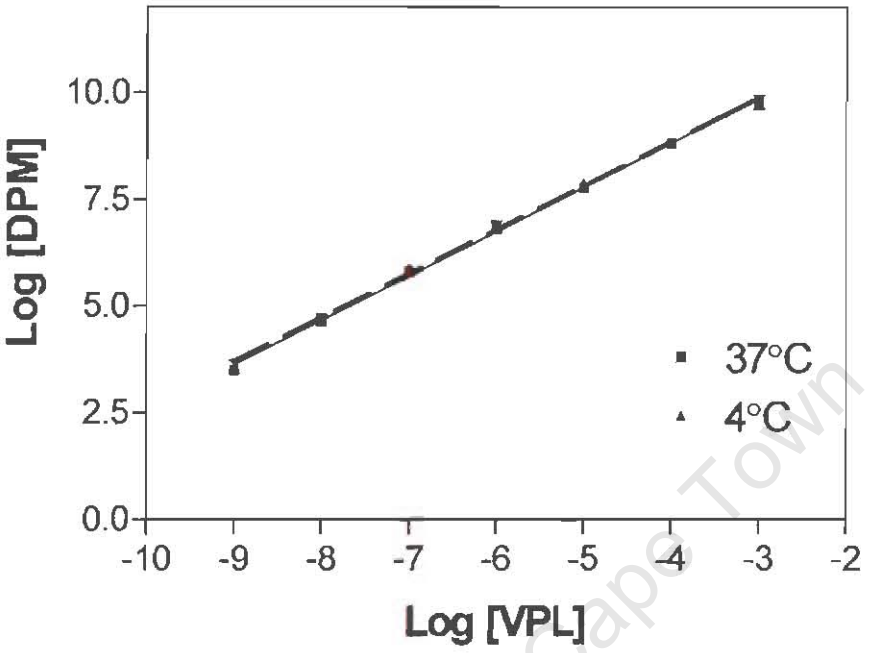


Figure 4.4 A comparison of the concentration dependence of ^{3}H-VPL accumulation at 37°C and 4°C in enriched trophozoites from the CQR strain (K1). The experiments were performed in triplicate on 2 (4°C) or 3 (37°C) separate occasions. The linear regressions were generated using Prism 3.0. The linear correlation coefficients for the two curves are: $r^2 = 0.9983$ for the experiment performed at 4°C and $r^2 = 0.9983$ for the experiment performed at 37°C.

^{3}H-VPL equilibration is not saturable over a large concentration range suggesting that any binding sites present are of high capacity and low affinity. The similar accumulation profiles at both 4°C and 37°C and the lack of energy dependence argue against an active uptake. Thus VPL appears to accumulate by means of diffusion only. VPL does not inhibit β-haematin formation at 10 equivalents (Warhurst et al, 2003) and thus probably does not bind to haematin. CQ uptake is highly dependent on its binding and access to haematin (Bray et al, 1998).

The lack of any significant difference in the saturable accumulation of VPL between the two strains is supported by the lack of any significant difference between the IC_{50}s for VPL in the strains. The difference in CQ accumulation is very closely linked to the difference in its IC_{50} between CQS and CQR strains.

4.2.4 The effect of a large excess of CQ and VPL on ^{3}H-VPL accumulation in CQS and CQR strains of Plasmodium falciparum.

From Figure 4.5 it is observed that neither an excess of CQ nor an excess of VPL has any significant effect on the accumulation of ^{3}H-VPL in the CQS strain. Although there appears to be a slight decrease in accumulation of ^{3}H-VPL, this decrease is not significant ($p > 0.05$). An excess of CQ causes a decrease in the accumulation of ^{3}H-CQ in the CQS strain while, as expected, VPL has no effect on ^{3}H-CQ accumulation in the CQS strain.

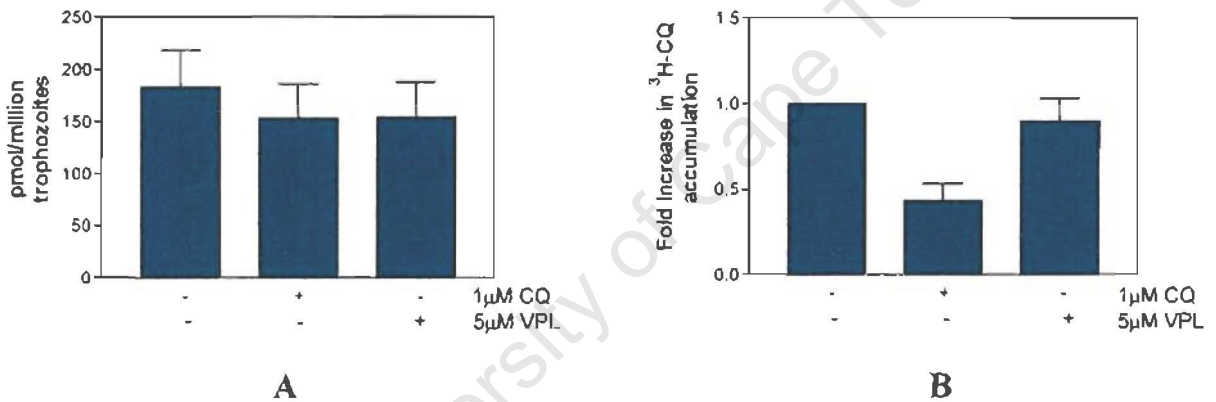
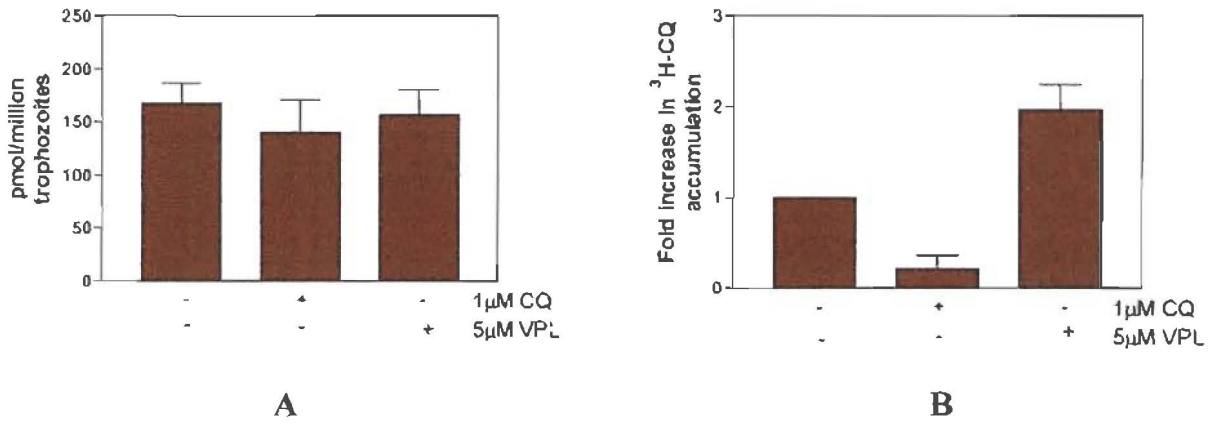


Figure 4.5 The effect of a large molar excess of CQ or VPL on the accumulation of 1nM ^{3}H-VPL (A) or 1nM ^{3}H-CQ (B) in enriched trophozoites from the CQS strain (D10). The experiment was performed three times in triplicate.

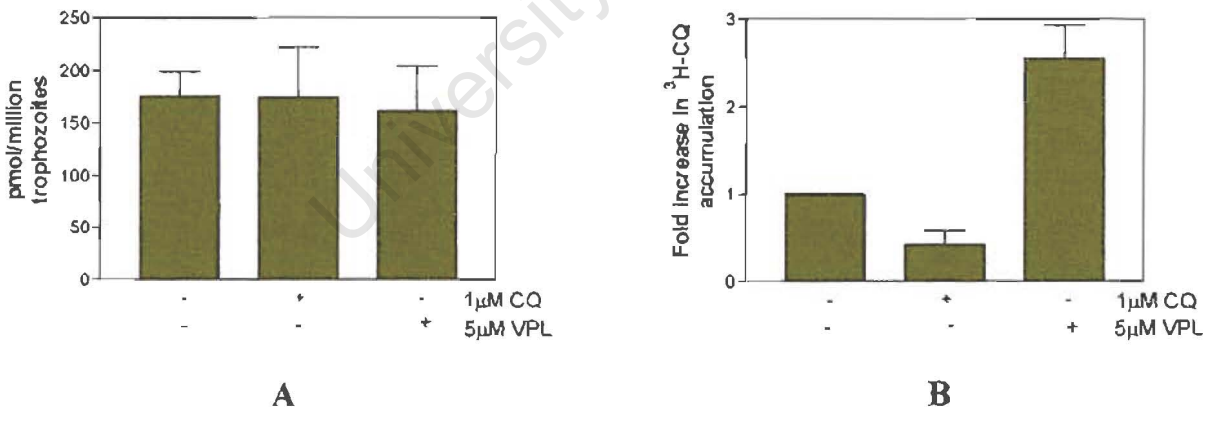
In the CQR RSA11 strain, there is also no significant change in the accumulation of ^{3}H-VPL in the presence of a large excess of either CQ or VPL. This differs greatly from the effect of these two drugs on the accumulation of ^{3}H-CQ in the same strain. There is a large decrease in ^{3}H-CQ accumulation in the presence of 1μM CQ while there is a 2-fold increase in ^{3}H-CQ accumulated when 5μM VPL is present.



A B

Figure 4.6 The effect of a large molar excess of CQ or VPL on the accumulation of 1nM ^{3}H-VPL (A) or 1nM ^{3}H-CQ (B) in enriched trophozoites from the CQR strain (RSA11). The experiment was performed three times in triplicate.

The results for another CQR strain W2 were similar to those of the CQR strain RSA11. Again there is no change in the accumulation of ^{3}H-VPL with either a large excess of unlabelled CQ or VPL. The ^{3}H-CQ accumulation is greatly altered under the same conditions.



A B

Figure 4.7 The effect of a large molar excess of CQ or VPL on the accumulation of 1nM ^{3}H-VPL (A) or 1nM ^{3}H-CQ (B) in enriched trophozoites from the CQR strain (W2). The experiment was performed three times in triplicate.

This illustrates the differences in the accumulation of CQ relative to that of VPL in strains of varying sensitivity to CQ. There is no difference in the VPL accumulation in either the CQS or

CQR strain when incubated with excess CQ or VPL. The accumulation of CQ varies significantly under the same conditions depending on the strains used.

The results presented here differ from those of Bray et al (1992) which showed that CQ (at 100nM) can increase the accumulation of ^{3}H-VPL to the same degree in both the CQS and CQR strains. They suggest that if VPL was inhibiting efflux of CQ by acting as a substrate, then one would expect an increase in ^{3}H-VPL accumulation in the presence of CQ. Thus the CQS and CQR strains both have an efflux pump of similar capacity.

A large excess of CQ competes for ^{3}H-CQ binding as has been previously demonstrated (Sanchez et al, 1997). Since the accumulation of ^{3}H-VPL is not saturable over a large concentration range (see Figure 4.4 and Figure 4.5), it is not surprising that unlabelled VPL does not compete for its own binding. It also explains the lack of effect of CQ on its accumulation. The ^{3}H-VPL concentration used in this study differs from that used by Bray et al (1992) but this should not affect the result given that VPL is non-saturable over a much larger concentration range.

These results are not incompatible with the efflux pump theory. Instead this may suggest why a relatively large concentration of VPL is necessary to see an effect on CQ uptake in the CQR strain. Some mammalian cells also show a similar VPL uptake profile between drug sensitive and multidrug resistant cell lines (Sehested et al, 1990). Yet VPL still inhibited efflux specifically from the multidrug resistant cell line.

Clearly VPL does not have similar accumulation properties to CQ for two main reasons:

1) It is monoprotic and thus will not accumulate as much as the diprotic CQ and
2) It does not inhibit haem crystallization at similar levels to CQ and thus probably does not bind to haematin which has been proposed as one of the reasons for the significant uptake of CQ in *Plasmodium falciparum*.

These two reasons may provide the explanation for the vastly different effects of VPL uptake when compared to the well-established effects of CQ accumulation.

Chapter 5

The Accumulation and Efflux of Tritiated Chloroquine and Verapamil in *Plasmodium falciparum* and Mammalian Cell Lines

5.1 Introduction

Differences in import and export kinetics have long been debated as a reason for the reduced accumulation and thus resistance of *Plasmodium falciparum* to CQ.

5.1.1 Chloroquine Accumulation over Time

Krogstad et al (1987) demonstrated that during the initial 5 minutes of CQ accumulation there is no difference in the accumulation rates between the CQS and CQR strains. They suggest that the most important determinant for resistance to CQ is the enhanced efflux capacity of CQR strains.

Bray et al (1994) showed the initial rate of CQ accumulation is greatly reduced in the CQR strains relative to the CQS strain. The CQR strain reached steady state faster than the CQS strain. They suggest that this indicates an enhanced efflux but the lower net accumulation of CQ is suggested to be a product of both reduced uptake and increased efflux. VPL slowed the CQ uptake so that the steady state was reached at the same time as the CQS strain. However the amount of CQ accumulated at steady state in the CQR strain never reached the levels of the CQS strain. Also VPL (5μM) did not exert any effect at a higher CQ concentration (100nM). This implied that the exporter had a low capacity and was being swamped at the higher CQ concentration (Bray et al, 1994). Bray et al (1996) later showed that 5μM VPL increased the initial uptake rate 2.5 fold in the CQR strain (within 6-7mins).

Martiney et al (1995) found contradictory results. They observed that by 45-60s there is a clear difference between CQ accumulation in CQS and CQR parasites. But the VPL related increase in

CQ accumulation in CQR strains only took effect after 5 minutes. They suggest VPL does not affect CQ uptake during the time period in which the differences between CQS and CQR strains are first observed. They also showed that VPL increased CQ accumulation rates in both CQR and CQS strains (Martiney et al, 1995).

Sanchez et al (1997) reported a biphasic accumulation of CQ in the CQR strain Dd2. During the first 15 minutes of exposure to CQ there was a gradual increase in CQ accumulated although not as much as is taken up in the CQS strain over the same time period. Thereafter they observed a decrease in the CQ accumulated in the CQR strain. They assumed this to be a product of the efflux mechanism.

In studies with CQ in uninfected erythrocytes, VPL had no effect on the kinetics of CQ uptake. This suggests that the putative VPL-sensitive CQ carrier is not present in the uninfected erythrocyte membrane (Ferrari and Cutler, 1990).

5.1.2 Chloroquine Efflux over Time

Krogstad et al (1987) were first to propose that a rapid CQ efflux phenotype exists in CQR strains of *P.falciparum*. They demonstrated that there was a 40-50 fold difference in the rate of efflux of ^{3}H-CQ from the CQR strain when compared to the CQS strain. This trend was confirmed by Wellems et al (1990) using different strains. Both groups reported a rapid efflux of between 1-2 minutes for their CQR strains and slower efflux (>60 minutes) in the CQS strains. Since CQR strains accumulate less CQ than CQS strains, the difference may simply be due to a difference in starting concentration. Therefore Krogstad et al (1992) increased the external CQ concentration so that both strains had the same ^{3}H-CQ concentration at time zero. Despite this alteration the CQS strain was still 40-50 times slower at releasing CQ than the CQR strain. They also showed that efflux was an energy-dependent process by removing glucose and adding vanadate. This had the effect of slowing down the efflux half-life and increasing CQ accumulation in the CQR strain (Krogstad et al, 1992). Resistance reversers like VPL, diltiazem and daunomycin also slowed the rate of efflux in the CQR strain (Krogstad et al, 1987). This rapid efflux phenotype has also been reported elsewhere (Bayoumi et al, 1994; Sanchez et al,

2003). In CQR African clones the time required to release half of the ^{3}H-CQ (t½) was less than 10 minutes while the CQS clones all had t½ of greater than 20 minutes (Bayoumi et al, 1994).

However the rapid efflux phenomenon was questioned by Bray et al (1992). They demonstrated in a range of strains that under their conditions there was no significant difference in the rate of efflux irrespective of their CQ sensitivity. In addition VPL slowed the efflux rates in both the CQS and CQR strains. They found the efflux was biphasic consisting of an initial rapid phase followed by an equilibrium phase. Martiney et al (1995) reported similar findings when they demonstrated that there is no difference in the efflux rates between their CQS (D10) strain and their CQR (Dd2) strain. Efflux rates were measured after preloading with 4nM CQ for between 45-60mins. After 10mins their CQS strain had effluxed 32±14% of the ^{3}H-CQ and the CQR strain had effluxed 43±3%.

Ginsburg and Stein (1991) used kinetic modeling to suggest that a weakened vacuolar proton pump was responsible for the differences in sensitivity to CQ. This would raise the baseline vacuole pH in the CQR strain. Krogstad et al (1985) showed the baseline pH between CQS and CQR strains was similar and recent evidence suggests that the vacuolar pH in CQR strains is lower than that of CQS strains (Dzekunov et al, 2000). Krogstad et al (1992) and Sanchez et al (2003) have demonstrated an energy-dependence of the efflux carrier thereby arguing against the weakened proton pump theory or an altered CQ importer (Ferrari and Cutler, 1991).

Lastly Sanchez et al (2003) used trans-stimulation conditions in the presence and absence of glucose to demonstrate that within CQR strains there is an energy-dependent transporter responsible for the efflux of CQ away from its binding sites within the parasites. They suggest that their model for a CQ efflux carrier is compatible with previously reported accumulation kinetics reported by Bray et al (1998). They show that the efflux half-life is 6 minutes in the CQR strain which is much slower than their measured accumulation half-lives of between 1-2 minutes.

Studying the kinetics of drug uptake of both anticancer agents and resistance reversers has been important in identifying the role of P-glycoprotein in MDR cancer cells. No protein target has yet been conclusively implicated in CQR *Plasmodium falciparum*. Yet kinetic studies are still

valuable in describing the action of a putative CQ transporter. Given the many contradictory reports on the accumulation and efflux of CQ from *Plasmodium* parasites, some of these experiments have been performed in the presence and absence of VPL to further examine the action of resistance reversers on the kinetics of CQ uptake and efflux.

This chapter deals with the characterization of the accumulation and efflux of ^{3}H-CQ and ^{3}H-VPL in a CQS and CQR strain of *Plasmodium falciparum*. The accumulation of ^{3}H-VPL in *P.falciparum* is compared with the accumulation of ^{3}H-CQ for both CQS and CQR strains. The accumulation of ^{3}H-VPL in *P.falciparum* is also compared to ^{3}H-VPL accumulation into mammalian cell lines. The chapter also examines the role of VPL in altering the rates of efflux of ^{3}H-CQ when exposed to parasites under different conditions.

5.2 Results and Discussion

5.2.1.1 Time course of ^{3}H-CQ uptake into CQS and CQR strains and the effect of VPL.

From Figure 5.1.a it is clear that the CQS strain (D10) accumulated more CQ after 60 minutes than the CQR strain (K1). When the time course was performed on the CQS strain in the presence of 5μM VPL there was a slight decrease in the CQ accumulated after 60 minutes although this difference was not significant ($p>0.05$). The curves are almost identical during the first 15 minutes before the slight divergence is observed. This confirmed that VPL had no effect on the time course of CQ uptake in the CQS strain. In the CQR strain VPL increased the net CQ uptake at 60 minutes (3.75 fold).

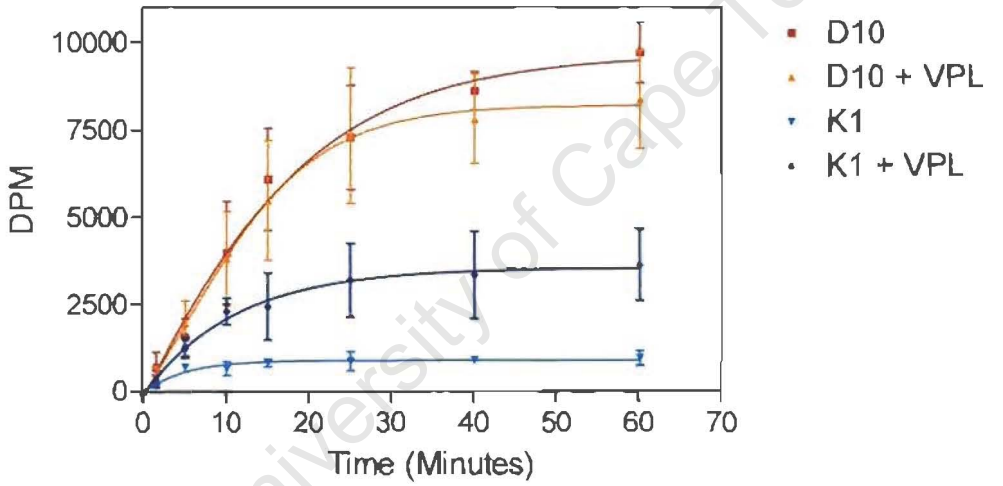


Figure 5.1.a A time course comparing the accumulation of 1nM ^{3}H-CQ in the presence and absence of 5μM VPL in a CQS and CQR strain of *Plasmodium falciparum*. Each experiment was performed in duplicate on 3 separate occasions. The curves were generated in Prism 3.0.

Steady state was achieved when the rate of uptake equaled the rate of efflux. Steady state was reached much sooner in the CQR strain (see Figure 5.1.b). The time taken to reach half the steady state values (T½) was 4.55±1.44 mins for the CQR strain and 12.4±4.32mins for the CQS strain in the absence of VPL. VPL slowed the accumulation of CQ to steady state by almost two-fold in the CQR strain (7.95±0.99mins). Interestingly VPL enhanced the time to steady state for the CQS

strain (10.9±3.37mins) albeit not statistically significant (p = 0.66). These observations are very similar to those of Bray et al (1994) although the actual values differ. They found that VPL slowed the accumulation to such an extent that the T½ of the CQR strain was identical to that of the CQS strain.

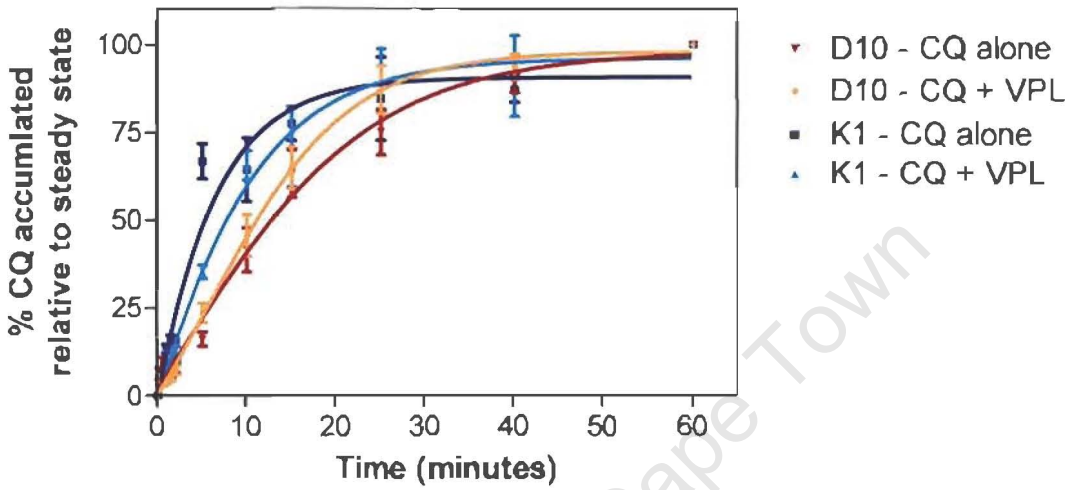


Figure 5.1.b A measure of the rate of CQ uptake to steady state based on Figure 5.1.a. The curves were generated in Prism 3.0 using a nonlinear regression analysis.

The accumulation of ^{3}H-CQ was time-dependent in both CQS and CQR strains. The CQS strain exhibited a greatly increased rate of uptake within the first five minutes when compared to the CQR strain. These results contrast with those of Krogstad et al (1987) but are similar to those of Bray et al (1994), who found that steady state was reached sooner in the CQR strain when compared to the CQS strain. VPL slows down the time to steady state in the CQR strain although not to the same extent. Bray et al (1994) suggest that their results indicate an enhanced efflux capability in the CQR strain and that VPL inhibits this efflux. They also propose that the lower accumulation in the CQR strain is thus a product of both a reduced uptake and enhanced efflux. In a later study they showed that VPL increased the initial CQ uptake rate 2.5 fold (Bray et al, 1996) once again supporting their assumption that changes in uptake rates are important in determining resistance to CQ.

Martiney et al (1995) found that VPL increased CQ accumulation rates in both the CQS and CQR strains. This was not observed in this study. The effect of VPL on enhancing CQ accumulation was limited to the CQR strain. The reason for this discrepancy is not clear since they used the same CQS strain (D10). Also they found the VPL effect on CQ accumulation was only observed after 5 minutes in the CQR strain. Again this was not evident in this work. The VPL effect was observed very early on in both the increase in rate of uptake and the slowing of the time taken to reach steady state.

There was no indication of biphasic CQ accumulation reported by Sanchez et al (1997) in the CQR strain used in this study. Once the steady state was achieved there was no drop in accumulation of CQ. Sanchez et al (1997) used the same CQR strain (Dd2) as Martiney et al (1995) yet they report very different accumulation profiles.

5.2.1.2 Time course of ^{3}H-VPL uptake into a CQS and CQR strain of Plasmodium falciparum.

In contrast to CQ, ^{3}H-VPL accumulation was complete within 1 minute in both the CQS and CQR strains. There was no significant difference in the amount of VPL accumulated at any subsequent time points throughout the experiment for each strain ($p>0.05$).

For ^{3}H-VPL this is more indicative of an equilibration of the drug from external medium into the parasite rather than active accumulation. Thus the term accumulation will be synonymous with equilibration when used to describe ^{3}H-VPL in *P.falciparum*.

A large CQ concentration had no effect on the accumulation of ^{3}H-VPL in either the CQS or the CQR strain at any time point. This result is in contrast with that found by Bray et al (1992) who showed that CQ reduced ^{3}H-VPL accumulation after 60 minutes. It is clear that VPL accumulation differs from CQ accumulation in *P.falciparum* strains. There was no significant strain dependent stimulation or reduction in ^{3}H-VPL accumulation by a high molar excess of CQ as has been observed for ^{3}H-CQ in the presence of VPL in CQR strains.

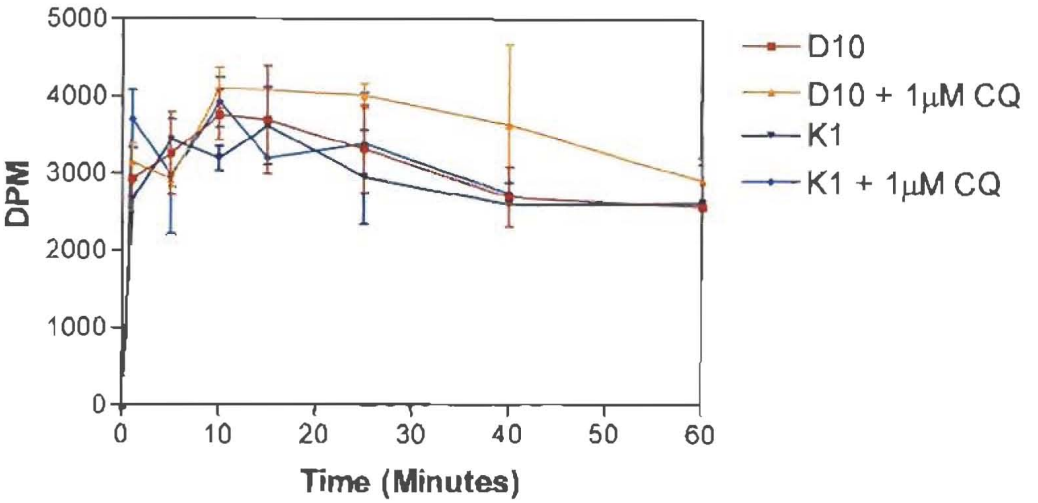


Figure 5.2 A time course comparing the accumulation of 1nM ^{3}H-VPL in the presence and absence of 1μM CQ in a CQS and CQR strain of *Plasmodium falciparum*. Each experiment was performed in duplicate on 2 separate occasions.

The rapid accumulation of ^{3}H-VPL into both CQS and CQR strains of *P.falciparum* contrasted markedly from ^{3}H-CQ accumulation where steady state was achieved much later and where there was a clear difference between the two strains. This lack of time-dependent accumulation was most probably due to rapid diffusion of VPL into the cells. Kinetic differences of CQ are closely related to the differences in sensitivity to CQ. The lack of differences in kinetics of VPL may also explain why its IC_{50}s are similar between CQS and CQR strains.

The lack of competition for the accumulation of ^{3}H-VPL by a high molar excess of CQ in either the CQS or the CQR strain contrasts with results reported by Bray et al (1992). They suggest that CQ does compete for ^{3}H-VPL accumulation. CQ is known to bind to FPIX (Chou et al, 1980; Bray et al, 1998) and to PfCRT (Zhang et al, 2004) but it is not known if there is a receptor for VPL. Any putative VPL receptor would have to be of high capacity and low affinity.

5.2.1.3 Time course of ^{3}H-VPL uptake into mammalian cell lines.

The accumulation of ^{3}H-VPL was measured in two mammalian cell lines that are cultured routinely in our laboratory. Neither cell line overexpresses P-glycoprotein. The cells were plated into a 24-well microtitre plate. When cells became confluent they were exposed to ^{3}H-VPL

(1nM). This is the same concentration used in the *Plasmodium falciparum* experiments. The medium was removed at various time points and the cells processed for scintillation counting as described in Section 10.13.

It is clear that VPL was taken up rapidly by two mammalian cell lines (see Figure 5.3). Accumulation occured most rapidly within the first 5 minutes and thereafter reached saturation. Steady state was reached within 1 hour. Both these cell lines had similar uptake curves.

The pattern of VPL accumulation in mammalian cells differed from that observed in *P.falciparum* strains. In the mammalian cells the uptake was time dependent whereas the VPL diffused/equilibrated rapidly into the *Plasmodium* strains. This pattern of VPL uptake into mammalian cells was more similar to that of CQ uptake in *Plasmodium falciparum*.

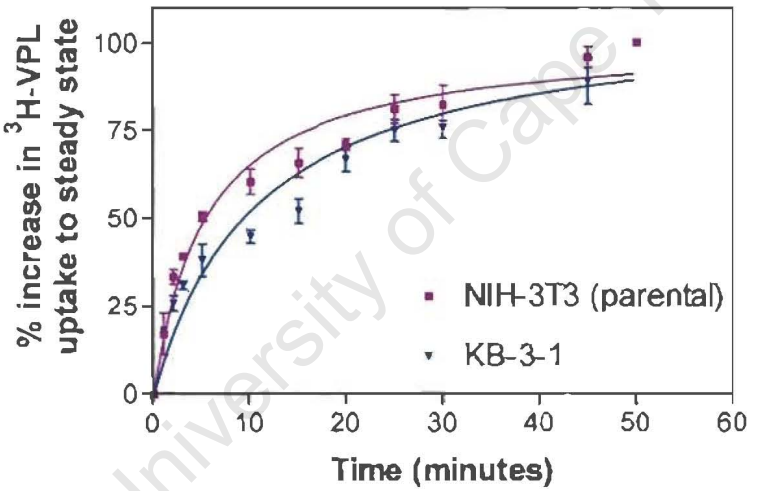


Figure 5.3 Time course for the uptake of ^{3}H-VPL (1nM) into two mammalian cell lines. Each experiment was performed in duplicate on 2 separate occasions. The curves were generated in Prism 3.0 using a non-linear regression analysis.

VPL accumulates into acidic lysosomes in mammalian cells and its accumulation can be reduced by raising the intralysosomal pH with CQ (Lelong et al, 1991). VPL also binds to membrane vesicles of certain cell types (Cornwell et al, 1987).

The accumulation of ^{3}H-VPL into mammalian cell lines was clearly time dependent. There is a rapid uptake phase within the first 10 minutes followed by a saturation phase as the steady state is reached. This pattern of uptake is similar to other reports on VPL uptake (Cornwell et al, 1987; Warr et al, 1988; Yusa and Tsuruo, 1989; Sehested et al, 1990). The cells used here did not overexpress P-glycoprotein and did not carry the multidrug resistance phenotype. Multidrug resistant cells accumulate less VPL and at a slower rate than wild type cells (Yusa and Tsuruo, 1989; Warr et al, 1988) but this is not always consistent (Sehested et al, 1990). Yusa and Tsuruo (1989) showed that VPL uptake in multidrug resistant cells is increased and more rapid when it is co-incubated with an anticancer agent or another chemosensitiser. This suggests that VPL is reversing multidrug resistance by competitively inhibiting drug transport.

No such effect was observed when ^{3}H-VPL was co-incubated with CQ in the CQR strain of *Plasmodium falciparum*. Clearly the uptake kinetics differ between these cell types and thus it is not possible to determine if any competition is occurring between CQ and ^{3}H-VPL in *P.falciparum* under these conditions as it was demonstrated for anticancer agents and ^{3}H-VPL in MDR cells (Yusa and Tsuruo, 1989).

5.2.2 The Effect of VPL on the Time Course of ^{3}H-CQ Efflux from the CQS and CQR Strains.

Given the importance of CQ efflux in defining the CQR phenotype, it was decided to examine the role of VPL in modulating the efflux in both a CQR and a CQS strain. Since there are significant differences reported by various groups for the effect of VPL on CQ efflux (see section 5.1.2), we examined the effect of VPL on both CQ accumulation and efflux under various experimental conditions, in which VPL was either present or absent from the incubation medium. A detailed description of the experimental procedures can be found in section 10.7.2 of the Methodology chapter.

Figure 5.4 shows the lack of effect of VPL on ^{3}H-CQ efflux from the CQS strain under any conditions. The presence or absence of VPL in either the accumulation or efflux buffer had no significant effect on CQ efflux rates. The efflux curves are shown as the time taken to equilibrium. The time taken to efflux half the ^{3}H-CQ (T½: see Table 5.1) from the CQS strain is

significantly faster than the results of Krogstad et al (1987). However it was slower than the efflux rates observed in the CQR strain (see Figure 5.5).

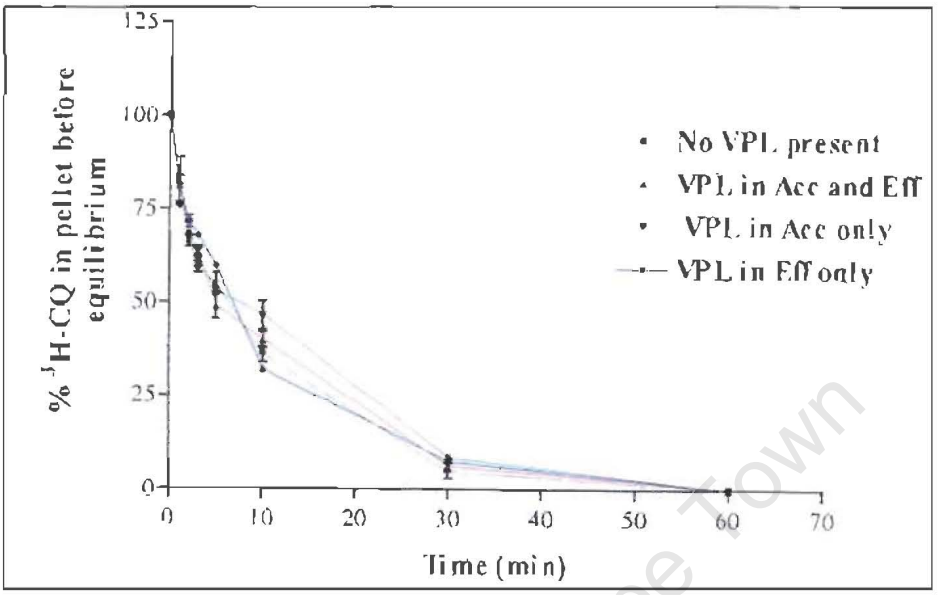


Figure 5.4. Comparison of the efflux patterns for ^{3}H-CQ in parasites exposed to 5μM VPL under various experimental conditions in the CQS strain D10. Each point is measured as a percentage of the T=0 value after subtraction of the equilibrium value at 60 minutes.

It is clear that under these experimental conditions VPL has no effect on the efflux of ^{3}H-CQ from the CQS strain. This is in contrast to the findings of Bray et al (1992) who reported that VPL could slow efflux from both CQS and CQR strains.

In Figure 5.5 the effect of VPL on slowing the efflux of ^{3}H-CQ can be observed for the CQR strain. In both experiments VPL was absent from the accumulation buffer. In one experiment 5μM VPL was added after CQ had reached steady state and was only present once efflux had been initiated. In the other experiment VPL was absent during efflux. VPL had no effect on CQ efflux in the first minute but slowed efflux between 1-10 minutes.

The efflux pattern in the absence of VPL is very rapid with most of the CQ removed within the first minute. This result agrees with previous data on the rapid efflux of CQ from CQR parasites (Krogstad et al, 1987; Bayoumi et al, 1994).

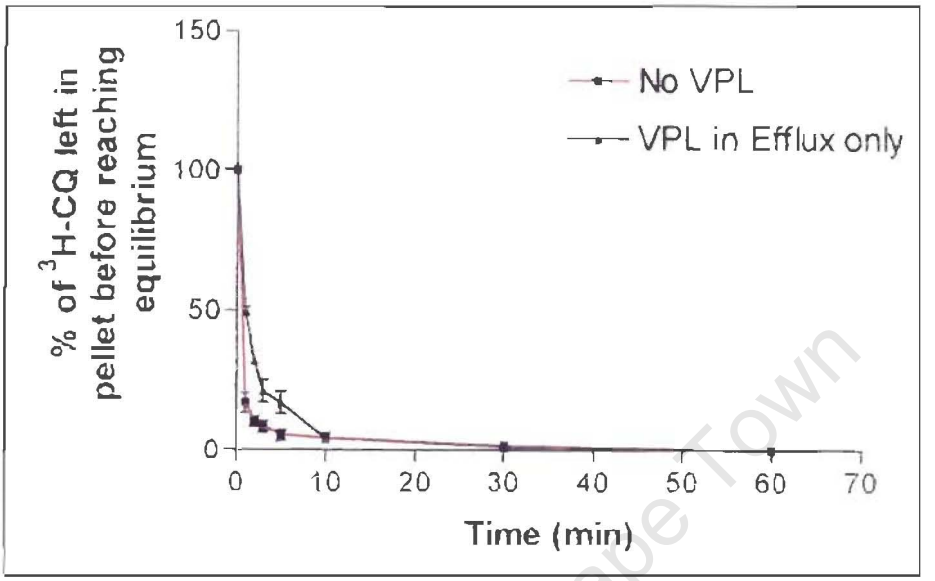


Figure 5.5 Comparison of the efflux patterns for ^{3}H-CQ in parasites in the absence or presence of 5μM VPL during the efflux phase in the CQR strain K1. Each point is measured as a percentage of the T=0 value after subtraction of the equilibrium value at 60 minutes.

When VPL was present in both the accumulation and efflux buffers it significantly inhibited CQ efflux from the CQR parasite but still not to the rates observed in the CQS strain (see Figure 5.6/ Table 5.1). This may explain why the accumulation of CQ in CQR strains never reaches the levels seen in the CQS strains.

When VPL was removed during the efflux phase there was an increase in the efflux rate. This was probably due to the rapid movement of VPL from the parasite into the external medium. This would reduce any interaction between VPL and a putative pump. It also suggests a low binding affinity of VPL for the putative receptor. However enough VPL must still have been present during the initial efflux phase when efflux is most rapid as it still had a significant effect in retarding the efflux rate (see Table 5.1).

When VPL was present during the CQ accumulation stage but absent during the efflux stage there was a slight decrease in the efflux rates compared to when it was present at all stages. However the VPL concentration must have been high enough during the crucial first minutes of measurement to exert the effect of slowing the rate down. Since VPL accumulates rapidly possibly through diffusion, it is likely that it will diffuse out of the cells as quickly and equilibrate with the external medium.

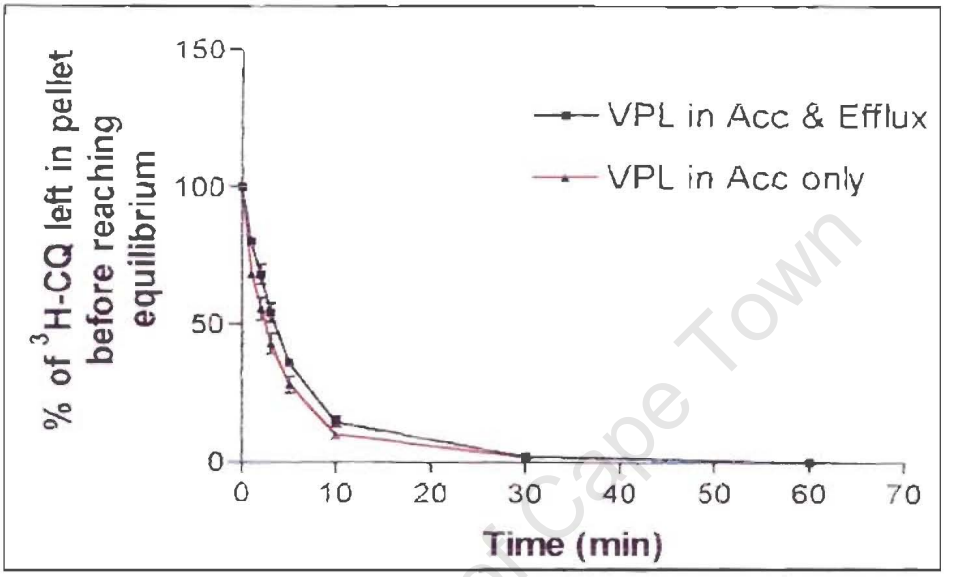


Figure 5.6 Comparison of the efflux patterns for ^{3}H-CQ in parasites in the absence or presence of 5μM VPL during the efflux phase in the CQR strain K1. Both samples contained 5μM VPL during the 1 hour accumulation phase with the ^{3}H-CQ. Each point is measured as a percentage of the T=0 value after subtraction of the equilibrium value at 60 minutes.

Results summarized in Table 5.1 show that in the absence of VPL there was an 8-fold difference in the efflux rates to equilibrium between the CQS and CQR strain. VPL had no significant effect on the efflux rates in the CQS strain under any conditions tested.

When VPL was present in the initial CQ accumulation phase and during the efflux phase there was a significant slowing of the efflux rate in the CQR strain. CQ efflux under these conditions was nevertheless still more rapid than the rate seen in the CQS strain. When VPL was present in the accumulation phase but absent in the efflux phase, there was a less pronounced slowing of the

efflux rate compared to when VPL is present in both phases. When VPL was present only in the efflux phase, there was no difference in T½ compared to when VPL is absent. The rapid initial efflux of CQ did not allow for differences to be measured within the first minute under the experimental conditions used. However it can be observed that VPL does start having an effect on slowing efflux down between 2-5 minutes (see Figure 5.5).

This demonstrates that VPL can slow the efflux of CQ from the CQR parasites. Also this confirms the rapid uptake of VPL seen in Figure 5.2. VPL is able to have a rapid effect on efflux of CQ (Figure 5.5) since it is rapidly equilibrated into the parasites. If VPL uptake was similar to CQ uptake in *P.falciparum* then one would not expect to see an effect within the short time to efflux.

Table 5.1 Efflux rates of ^{3}H-CQ for a CQS (D10) and CQR (K1) strain in the presence or absence of 5μM VPL. Values are expressed as the time taken to efflux 50% of the ^{3}H-CQ before achieving equilibrium (T½).

Strain	Experiment	T ½ (min)	N
D10	No VPL	6.5±1.1	3
	VPL in Accumulation and Efflux	6.0±1.3	3
	VPL in Accumulation only	7.0±0.9	5
	VPL in Efflux only	6.5±1.0	6
K1	No VPL	0.8±0.2	3
	VPL in Accumulation and Efflux	3.5±0.5	5
	VPL in Accumulation only	2.5±0.6	4
	VPL in Efflux only	0.8±0.3	3

N = the number of experiments performed in duplicate.

The biphasic nature of efflux confirms the observation of Bray et al (1992). The rapid efflux component is followed by a gradual slowing down phase where it is assumed there is an equilibration between CQ accumulation and efflux. This was a characteristic of both strains. Both

strains had lost a significant amount of ^{3}H-CQ after 10 minutes of efflux. Martiney et al (1995) stated there was no significant difference in the average CQ efflux between the two strains after 10min. They also used D10 as their CQS strain. Our results show that the significant efflux for both strains had occurred before 10 minutes had passed and to average the rates for comparison between the two strains after this time would not yield a significant difference. As with the accumulation rates the first few minutes appear to be critical in defining the resistance phenotype.

The rapid efflux in our CQR strain is confirmation of previous results (Krogstad et al, 1987; Krogstad et al, 1992; Wellems et al, 1990). However these efflux rates differed from those reported by Sanchez et al (2003) who found an efflux half-time of 6 minutes in the CQR strain. A reason for this could be that their accumulation phase was conducted in the absence of glucose. If an energy-dependent pump is involved in CQ efflux it may be that the slow efflux rates can be explained by the delay in the parasite's attempts to restore the energy levels for the pump to operate maximally.

It was observed that the CQS strain used in this study was more rapid at CQ efflux than some previous reports (Krogstad et al, 1987; Krogstad et al, 1992; Wellems et al, 1990). The parasites were not subjected to two washing steps before efflux was measured. This may account for the differences found in this study when compared to previously published results. Although prior to the initiation of the efflux measurements the amount of ^{3}H-CQ accumulated in parasites used in this study differed (depending on the strain or the pre-exposure to VPL), it has previously been show that the rapid efflux phenotype is maintained even when equal amounts of CQ are present in different strains (Krogstad et al, 1992). Thus the rapid efflux phenomenon is independent of the initial CQ load.

This chapter described how VPL operates to both reduce the rate of uptake of CQ to steady state as well as reduce the rate of efflux in CQR *Plasmodium falciparum*. It demonstrated the more rapid uptake of ^{3}H-VPL when compared to ^{3}H-CQ in both CQR and CQS strains. A comparison was made between the time-dependent ^{3}H-VPL uptake into mammalian cell lines and the equilibration of ^{3}H-VPL into plasmodial strains. The results explain how VPL is able to reduce the rate of CQ efflux very quickly because of the rapid equilibration of VPL.

Chapter 6

The Effect of Verapamil on Haem Crystallization *In Vitro*

6.1 Introduction

The binding of quinoline-based antimalarials to free haem (ferriprotoporphyrin IX; FPIX) has been well characterized (Chou et al, 1980; Egan et al, 1999).

However the interaction of resistance reversers with the haem system has not been well established. In recent publications it has been shown that certain chemosensitisers are able to bind to haem and/or inhibit haem crystallization (Loria et al, 1999; Kalkinidis et al, 2002; Agrawal et al, 2002). Loria et al (1999) suggest that some resistance reversers may act like the 4-aminoquinolines in inhibiting parasite growth. The authors demonstrated that chlorpromazine was particularly effective at inhibiting haem decomposition while desipramine and VPL were less so (Loria et al, 1999).

Agrawal et al (2002) demonstrated that cyproheptadine was able to inhibit haem crystallization. VPL and desipramine were 10 and 2.8 times less effective at inhibiting crystallization respectively. Cyproheptadine interfered with haem detoxification *in vivo* (Agrawal et al, 2002). Chong and Sullivan (2003) could not find any activity for cyproheptadine using their heme crystal growth assay.

When the phenothiazine-based chemosensitisers were modified to make them more effective at inhibiting haem crystallization, they appeared to lose their ability to reverse CQ resistance (Kalkinidis et al, 2002). This may imply that the phenomena of antimalarial action and resistance reversal are two distinct functions.

Some recently reported Brazilian strains of *P.falciparum* exhibit hypersensitivity to a range of chemosensitisers (Menezes et al, 2002; Menezes et al, 2003). It was proposed that these chemosensitisers may be inhibiting falcipain activity or inhibiting haem crystallization (Menezes et al, 2002). In this chapter a range of chemosensitisers will be examined for their activity in inhibiting haem crystallization to explain a possible role for the haem inhibition system in the hypersensitivity to these compounds.

A newly developed assay will be used to demonstrate the effect of VPL on haem crystal inhibition and compare its activity to that of several other chemosensitisers. The activity of VPL will then be examined in combination with CQ or quinine on haem crystal inhibition *in vitro* to establish its possible interaction with the drug receptor.

6.2 Results and Discussion

In order to examine the haem crystallization, 1mM haemin chloride was incubated with various concentrations of the compounds to be tested ranging from 1mM to 20mM (i.e. 1 equivalent to 20 equivalents). The mixture was made up in 4.5M acetate buffer at pH 4.5 and the reaction was run at 60°C for 1 hour. Thereafter a pyridine buffer was added to stop the reaction and visualize the β-haematin product (see Section 10.20 for detailed methodology).

6.2.1 The activity of chemosensitisers on inhibition of haem crystallization in vitro

Most of the conventional quinoline antimalarials used inhibit haem crystallization below 5 equivalents (i.e. 5mM, personal communication, T.J. Egan). In this study the drug activity is measured using the HI_{50}. This represents the number of drug equivalents required to inhibit 50% of the β-haematin (haemozoin) formation.

Table 6.1. Effect of the chemosensitisers on the inhibition of haem crystallization *in vitro*.

Chemosensitiser	Abbreviations	HI_{50} for haem crystallization
Verapamil	VPL	13.41±0.261
Desipramine	DES	9.895±0.035
Cyproheptadine	CYP	N.D.
Chlorpheniramine	CHF	N.D.
Phenothiazine	PHE	N.D.
2-Chlorophenothiazine	C-PHE	N.D.
Promethazine	PRO	10.37±0.078
Chlorpromazine	CHP	N.D.
Trifluoperazine	TRIF	N.D.

The HI_{50} is a measure of the number of equivalents of drug required to inhibit 50% of the β-haematin crystal formation.
N.D. - Not determinable (i.e. there was no inhibitory effect even at the highest drug concentration used = 20 equivalents)

Table 6.1 shows that chlorpromazine and trifluoperazine were not able to inhibit crystallization even at 20mM (20 equivalents). This contradicts previous reports for chlorpromazine (Kalkinidis et al, 2002). Spectroscopic analysis of the reaction products failed to reveal any changes the soret band indicating β-haematin (haemozoin) formation at 10 drug equivalents (Personal communication, T.J. Egan). Promethazine, a phenothiazine similar in structure to the both chlorpromazine and trifluoperazine, had a weak activity (see Figure 6.1 for structures). Promethazine's weak activity has been previously reported (Warhurst et al, 2003).

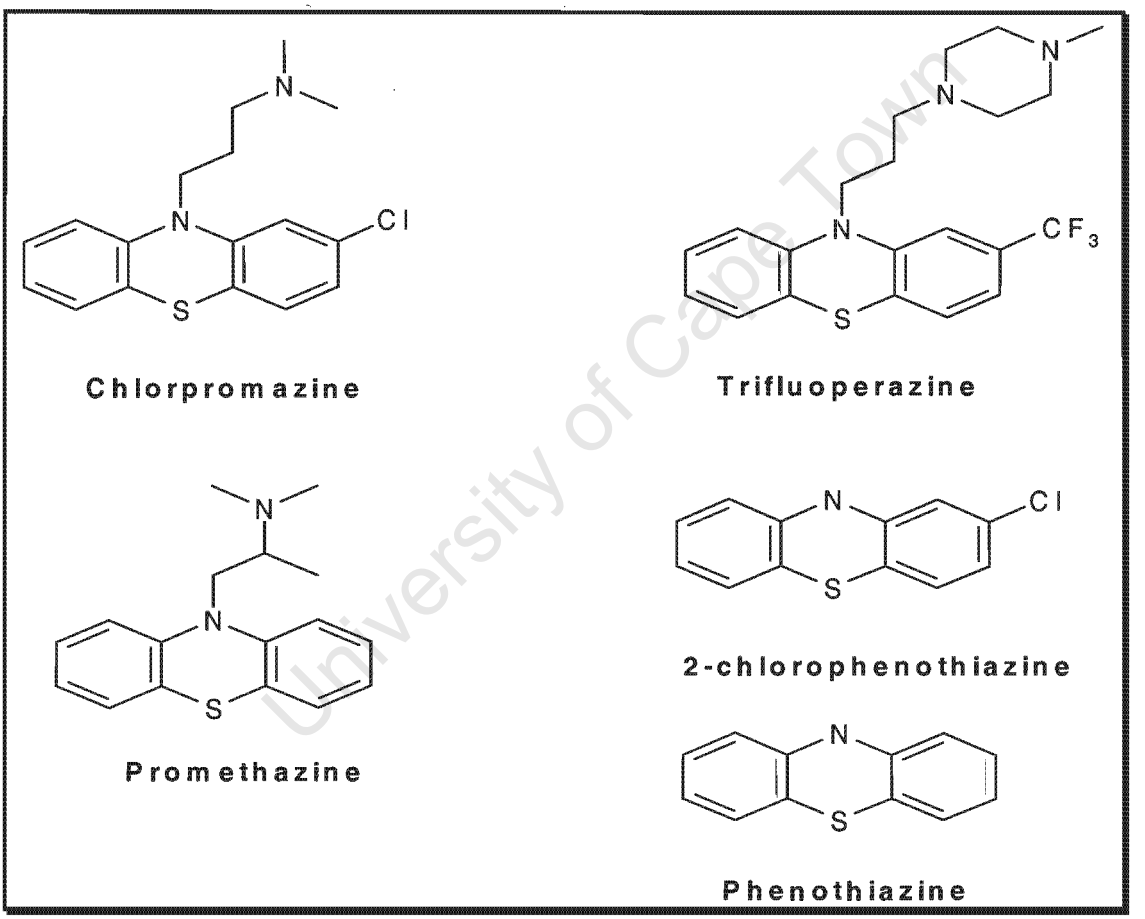


Figure 6.1 The chemical structures of the phenothiazines used to inhibit haem crystallization.

Promethazine possesses a side chain but lacks either a chlorine atom or a trifluoromethane group on the planar ring (see Figure 6.1). This may explain its activity at inhibiting crystallization when compared to chlorpromazine or trifluoperazine. There may be some steric hindrance by the groups attached to the planar phenothiazine ring that inhibit interaction with haem.
Given that phenothiazine and 2-chlorophenothiazine do not inhibit haem crystallization within 20 equivalents and that both lack the side chains of the other phenothiazine-based compounds, the presence of a side chain is possibly important in the drug activity on haem crystal inhibition.

VPL, like promethazine, weakly inhibits activity haem crystal inhibition. Warhurst et al (2003) could not demonstrate any activity for VPL but they only used 10 equivalents relative to haem. Structurally VPL is very different to most of the chemosensitisers since its two planar rings are not fused but instead are separated by an alkyl chain. Since the aminoquinolines and phenothiazines both have fused rings, it may be surprising that VPL has any effect at all. However the same could be said for the properties of VPL as a resistance reverser. Most chemosensitisers have fused rings also yet VPL is as effective as they are at reversing CQ resistance.

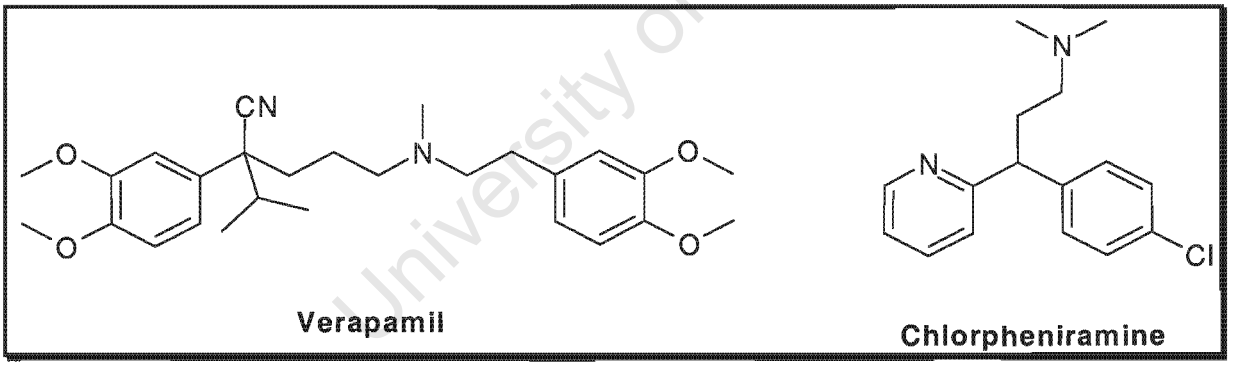


Figure 6.2 The chemical structures of the verapamil and chlorpheniramine.

Chlorpheniramine showed no inhibition of haem crystallization even at 20 equivalents. Like VPL its two planar rings are not fused but are separated by an alkyl chain (see Figure 6.2). Yet it is just as effective at the same concentrations as the other chemosensitisers at increasing ^{3}H-CQ accumulation in CQR strains (see Figure 3.4). Also it has shown promise in a couple of studies in humans in reversing resistance to CQ (Abok, 1997; Sowunmi, 1997). Thus the interaction with

haem is clearly not important in defining the efficacy of a resistance reverser in CQR *Plasmodium falciparum*. Chong and Sullivan (2003) were also unable to detect any heme crystal growth inhibition by chlorpheniramine.

Desipramine and cyproheptadine are similar in structure (see Figure 6.3) yet only desipramine could inhibit haem crystallization (see table 6.1). The lack of inhibition by cyproheptadine supports the results of Chong and Sullivan (2003). These compounds also lack any chlorine atoms or trifluoromethane groups on their planar rings.

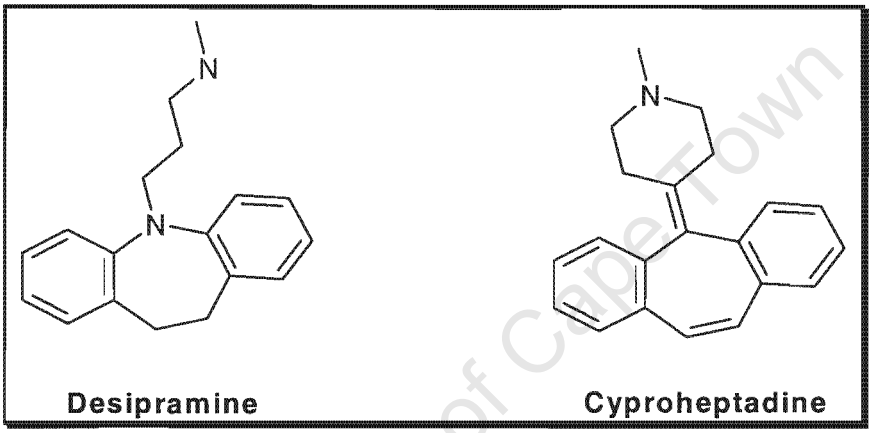


Figure 6.3 The chemical structures of the desipramine and cyproheptadine.

While the structural characteristics that make a chemosensitiser effective at inhibiting haem crystallization have not been thoroughly examined, the common structural features among chemosensitisers have been modeled and compared with *in vitro* resistance reversal activity (Bhattercharjee et al, 2002). Given that the inhibition by CQ of haem crystallization is very strongly determined by its structural features and that chemosensitisers share many structural similarities with CQ (tertiary nitrogen, 2 planar rings), it should not be surprising that some chemosensitisers also inhibit haem crystallization.

6.2.2 The activity of chemosensitisers in combination with antimalarials on the inhibition of haem crystallization in vitro

All the chemosensitisers tested in this study possess very low intrinsic antimalarial activity relative to CQ (between 5-20μM) and the IC_{50}s are not significantly different between strains (see Table 6.2). Since all of the chemosensitisers can reverse CQR yet only some of the chemosensitisers can inhibit haem crystallization, it was of interest to examine the possible role of chemosensitisers, if any, on CQ action at the level of haem crystallization *in vitro*. For this purpose a dose response curve was run for both CQ and quinine and a fixed amount of the chemosensitiser was added to each well similarly to the way the resistance reversal is performed using the parasite lactate dehydrogenase assay.

Table 6.2. A comparison of the intrinsic antimalarial effect of the chemosensitisers used in this study on a CQS (D10) and CQR strain (RSA11) of *Plasmodium falciparum*.

Compound Tested	IC_{50} in (μM)	
	D10	RSA11
Chloroquine[a]	0.026±0.003	0.354±0.039
Verapamil[a]	17.72±3.020	14.39±3.354
Chlorpromazine[c]	6.751±0.312	7.840±0.685
Trifluoperazine[b]	5.660±1.045	5.901±1.794
Chlorpheniramine[c]	5.290±0.423	4.800±0.260
Desipramine[b]	13.90±0.007	11.74±4.244
Promethazine[c]	10.94±0.535	12.06±0.550

[a] taken from Table 2.2.
[b] taken from Van Schalkwyk et al (2001).
[c] IC_{50} values provided by D.Taylor.
All values are the mean of at least 3 duplicate experiments performed on separate occasions. All the values obtained for individual drugs do not differ significantly between strains ($p > 0.05$) except for CQ ($p < 0.0001$).

Table 6.3 illustrates the effect of VPL at two concentrations on the inhibition of haem crystallization by both CQ and QUI. The concentration of VPL in the wells (1mM or 5mM) is well above the VPL concentration used to reverse CQ resistance (1μM). At 1mM (1 equivalent

relative to haem) VPL has no significant effect on changing the HI_{50} for the inhibition of haem crystallization by either of the antimalarials (See Table 6.3). At 5mM VPL antagonizes the haem crystal inhibition for both CQ and quinine significantly ($p > 0.05$).

VPL does not inhibit haem crystallization by itself at a 5mM concentration. Thus it may appear strange that it can antagonize CQ's action on haem. However it has been previously reported that CQ has two important components in inhibiting haem crystallization;

1) Binding to/complexing with haem and
2) Inhibiting crystallization (Egan et al, 2000).

The planar rings are important in the complexing with haem and the chlorine atom is important in inhibition of crystallization.

It may thus be that VPL is able to complex with haem at these concentrations even though it does not inhibit crystallization. This complexing would compete for/prevent CQ from binding to the haem. This could therefore antagonize CQ action on haem crystal inhibition. It is highly unlikely that VPL will reach such high concentrations in the parasite vacuole especially at concentrations at which resistance reversal is achieved.

Table 6.3. Effect of the antimalarials on the inhibition of haem crystallization either alone or in combination with 5mM VPL or 5mM chlorpromazine *in vitro*.

Antimalarial	HI_{50}		
	Alone	With 1mM VPL	With 5mM VPL
Chloroquine	1.956±0.263	2.160±0.032 (0.383)	7.757±0.339 (0.003)
Quinine	3.324±0.349	3.458±0.020 (0.642)	8.391±0.112 (0.003)

The HI_{50} is a measure of the number of equivalents of drug required to inhibit 50% of the β-haematin crystal formation. The p-value for the combination when compared to the single antimalarial agent is provided in parentheses.

A different effect is observed with CHP when combined with the antimalarials. At 1mM (1 equivalent relative to haem) CHP antagonizes CQ action but not that of quinine. Although

quinine is less effective than CQ at inhibiting haem crystallization, it is also less sensitive to the antagonism of the chemosensitiser. CHP antagonizes CQ action at both concentrations but only affects quinine at the higher concentration (5mM) similarly to the effect observed with VPL.

CQ's action of inhibiting haem crystallization has been shown to be reversible while quinine's effect is not (personal communication T.J. Egan). This suggests that the interaction of CQ with haem differs from that of quinine with haem. The nature of this interaction is not understood but may explain why CHP would affect CQ to a greater degree than quinine.

Table 6.4. Effect of the antimalarials on the inhibition of haem crystallization either alone or in combination with 5mM VPL or 5mM chlorpromazine *in vitro*.

Antimalarial	HI_{50}		
	Alone	With 1mM CHP	With 5mM CHP
Chloroquine	1.956±0.263	4.047±0.388 (0.024)	8.805±0.282 (0.002)
Quinine	3.324±0.349	3.354±0.617 (0.958)	8.214±0.104 (0.028)

The HI_{50} is a measure of the number of equivalents of drug required to inhibit 50% of the β-haematin crystal formation. The p-value for the combination when compared to the single antimalarial agent is provided in parentheses.

Agrawal et al (2002) also found that combining CQ with cyproheptadine had no greater effect on inhibiting haem crystallization than using either agent alone. Thus it would seem to confirm that the combination of a resistance reverser with chloroquine is not synergistic or additive at the level of haem crystallization.

It has been suggested also that the weak antimalarial activity of resistance reversers (VPL, DES and CHP) might be due to the interaction with haem (Loria et al, 1999). Ultrastructural studies of *Plasmodium* parasites under drug pressure with VPL show that the effects on the parasite morphology are quite distinct from effects seen with CQ even at high (100μM) concentrations of VPL (Jacobs et al, 1988). Thus it would be unlikely that VPL would be acting at the same target or in the same manner as CQ. It remains to be demonstrated whether this applies to other chemosensitisers. In addition, not all the chemosensitisers inhibit haem crystallization at similar

concentrations yet they all have IC_{50}s in *Plasmodium falciparum* of between 5-20μM. This would suggest that their toxicity to the parasites is not related to their interaction with haem.

CQ is also highly concentrated in the vacuole by means of the weak base effect and its binding to haem. It is unlikely that the chemosensitisers will be as concentrated as most are monoprotic (see Table 10.1) and do not interact with haem as strongly as does CQ. It was hypothesized that the reason for the lack of strong antimalarial activity of especially chlorpromazine (in micromolar range) against *P.falciparum* was due to its poor accumulation relative to the diprotic CQ (Kalkinidis et al, 2002). Indeed if chemosensitisers like chlorpromazine did reach the same levels of accumulation as CQ does and inhibited haem crystallisation then it might be expected to compete for ^{3}H-CQ accumulation in the CQS strain also. From Figure 3.3 it is evident that neither CHP nor any other chemosensitiser has an effect on ^{3}H-CQ accumulation in the CQS strain at the high molar excess of 1μM.

Recent studies on some Brazilian strains of *P.falciparum* have shown that resistance reversers lack the ability to modulate CQ action in resistant strains and themselves have intrinsic antimalarial activity in the concentration range associated with quinoline antimalarials (Menezes et al, 2002; Menezes et al, 2003). These include VPL, chlorpromazine and trifluoperazine. The activity of some of these chemosensitisers has been attributed to the inhibition of falcipain activity or the inhibition of haem crystallization (Menezes et al, 2002). Since chlorpromazine does not inhibit haem crystallization *in vitro* and yet has a higher activity than CQ in these new strains, it is unlikely that the interaction with haem would be sufficient to explain its action. Given that chlorpromazine is monoprotic, increased accumulation is also unlikely to explain its increased activity unless there is an active importer for CHP. The concentrations required to inhibit falcipain activity are unlikely to be as low as those reported for its antimalarial activity. Thus it would have to be a combination of factors that contribute to the intrinsic activity of these compounds against the new Brazilian strains of *P.falciparum*.

Fitch (1989) showed that VPL had no effect on the accumulation of ^{3}H-CQ in CQR strains of *Plasmodium berghei* that lacked ferriprotoporphyrin IX (FPIX or haem). The presence of haem is proposed to be necessary for VPL to reverse CQR. However VPL does not mediate a direct

interaction of CQ with the FPIX. Instead it increases the access of CQ to the FPIX by probably inhibiting the efflux of CQ away from the FPIX in the vacuole (Bray et al, 1998; Sanchez et al, 2004).

If the accumulation of VPL was also linked to its binding to haem, then one might expect VPL to be accumulated in a similar manner to CQ or possibly to compete for CQ binding to haem. The ^{3}H-VPL accumulation studies show that VPL accumulation is not saturable (unlike CQ accumulation) and this would argue against the binding of VPL to haem as a method of accumulation (see Figures 4.3 and 4.4). CQ analogues that are equally effective at inhibiting haem crystallization but lack the tertiary amines of CQ itself have poor antimalarial activity (Egan et al, 2000). Thus the action of CQ is highly dependent on both the interaction with haem and its pH-driven accumulation into the vacuole. This might explain why VPL has such a low intrinsic antimalarial activity despite the fact that it can inhibit haem crystallization.

It is demonstrated here that although some resistance reversers are able to interact with haem directly and inhibit its crystallization into haemozoin, this is not the primary action of these chemosensitisers. Many chemosensitisers tested here show very little or no effect on haem crystallization relative to antimalarials yet are equally as effective at reversing resistance to CQ as those that inhibit haem crystallization more strongly. Preventing the rapid efflux of CQ from the parasite is probably the most important determinant in the action of various chemosensitisers (Krogstad et al, 1987; Sanchez et al, 2004).

Chapter 7

The Effect of Drug Pressure with Verapamil on the Haemoglobin Content of Chloroquine-Resistant and Chloroquine-Sensitive *Plasmodium* parasites

7.1 Introduction

The ultrastructural effects of high and low concentrations of verapamil on have been examined previously in *P.falciparum* (Jacobs et al, 1989). At concentrations that reverse resistance (1.8μM) there was little effect on parasite morphology of either the CQS or CQR parasites of *Plasmodium falciparum*. Mild food vacuolar swelling and increased granular matrix was observed in a few parasites. At higher, toxic concentrations (100μM) there were significant changes in the parasites of both CQS and CQR strains. These effects were dissimilar to those observed with CQ in that there was no disintegration of the food vacuolar membrane.

Recently it was demonstrated that different quinoline antimalarials affect the parasite ability to take up haemoglobin (Hb) in different ways (Famin & Ginsburg, 2002; Hoppe et al, 2004). CQ and amodiaquine inhibited Hb degradation and this inhibition was related to parasite death. Mefloquine and quinine did not exhibit the same effects on Hb (Famin and Ginsburg, 2002).

Ammonium chloride and CQ have both been demonstrated to raise acidic vesicle pH (Krogstad et al, 1985). Both these agents can cause a buildup of endocytosed vesicles in the parasites (Hoppe et al, 2004).

Verapamil has been reported to increase the pH in the food vacuole of CQR parasites to a value near that seen in the CQS parasites (Ursos et al, 2000). However VPL does not alter the pH in the CQS parasite. The increase in the pH of the CQR parasites (0.23 units; Ursos et al, 2000) in the

presence of VPL should not be so large as to induce significant alterations in the parasite morphology or enzymatic processes.

Since the action of CQ is related to the inhibition of haemoglobin degradation in the CQS parasites, it was decided to investigate the effect of similar CQ concentrations on the haemoglobin levels in CQR parasites. The effect of VPL on haemoglobin degradation, either alone or in the presence of CQ, is not known.

This chapter examines the effect of verapamil on changing the amount of haemoglobin present in both the CQS and CQR parasites, individually and in combination with CQ at concentrations associated with resistance reversal. The concentration-dependent effect of VPL on haemoglobin buildup in CQS and CQR strains will be investigated. Lastly a comparison will be made between the action VPL and that of other chemosensitisers on changing the amount of haemoglobin in *Plasmodium falciparum* strains.

7.2 Results and Discussion

Parasitised erythrocytes were incubated in the presence of the particular drug or drug combination for 12 hours starting in the late ring/early trophozoite stage of growth. Thereafter the trophozoites were harvested by saponin lysis and run on a gel using sodium dodecyl polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane and probed using an anti-haemoglobin antibody (See sections 10.15-10.18 for detailed methodology).

7.2.1 The effect of verapamil and chloroquine alone and in combination on the haemoglobin buildup in CQS strains of Plasmodium falciparum

The 100nM concentration used to examine CQ action was selected because it is approximately 4 times the IC_{50} for the CQS strain D10 and thus should produce an effect within the time of exposure to the drug.

Plate 7.1 demonstrates how 100nM CQ caused a buildup of Hb relative to the control in the CQS D10 strain. This confirmed the previous findings on the action of CQ on the feeding of the parasite (Famin and Ginsburg, 2002; Hoppe et al, 2004). When combined with VPL, the effect of CQ on Hb remained the same as when CQ was used on its own. This confirmed that verapamil had no effect on changing CQ action in CQS strains of *P.falciparum*.

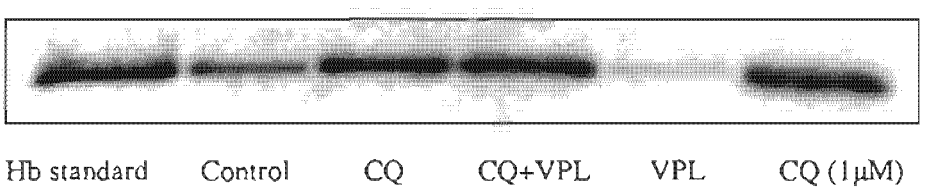


Plate 7.1 The effect of drug pressure of CQ and VPL on the chloroquine sensitive strain, D10, over 12 hours from the late ring stage. Bands from left to right: Haemoglobin standard, Control (no drug), 100nM CQ alone, 100nM CQ + 1μM VPL, 1μM VPL alone, 1μM CQ alone.

When used alone at 1μM, VPL appeared to cause a decrease in the amount of Hb in CQS strains. This concentration should not be cytotoxic as it is well within the IC_{50} for this strain. The last band represents parasites exposed to a very high concentration of CQ (1μM). This band was quite intense as growth was highly inhibited.

Table 7.1 shows that both the D10 and 3D7 strains responded similarly to all the drug combinations tested. Both contained more than twice the amount haemoglobin over the control when exposed to CQ alone or if CQ is combined with VPL. Both experienced a drop in the amount of haemoglobin compared to the control when exposed to 1μM VPL alone.

Table 7.1 The relative change in haemoglobin buildup in the presence of various drugs in the CQS *Plasmodium falciparum* strains, D10 and 3D7.

Sample	Relative change in Hb in D10	Relative change in Hb in 3D7
Control (No drug)	1.000	1.000
100nM CQ	2.097±0.383	2.490±0.304
100nM CQ + 1μM VPL	2.147±0.514	2.157±0.088
1μM VPL	0.837±0.566	0.829±0.078
1μM CQ	3.180±1.811	2.305±0.815

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

The increase in the amount of haemoglobin observed with CQ-treated CQS parasites confirmed recently published observations (Famin and Ginsburg, 2002; Hoppe et al, 2004). The lack of effect of VPL on CQ-treated parasites is expected since VPL neither changed the IC_{50} of CQS parasites (See Table 2.2) nor did it alter the ability of CQS parasites to accumulate ^{3}H-CQ (See Figure 3.3). Used on its own 1μM VPL did cause a decrease in the amount of haemoglobin relative to the control. Mefloquine also causes a decrease in the amount of haemoglobin in CQS parasites and this has been attributed to it inhibiting the process of endocytosis (Hoppe et al, 2004). These mefloquine concentrations are well above the IC_{50} so this effect is related to its

cytotoxicity whereas the effect of VPL occurs at a concentration which is sub-lethal to the parasites. Thus the mechanism of action of these two compounds may be different.

7.2.2 The effect of verapamil and chloroquine alone and in combination on the haemoglobin buildup in CQR strains of Plasmodium falciparum.

In the CQR strains there was no increase in the amount of haemoglobin over the control in the presence of 100nM CQ. When CQ was combined with 1μM VPL, there was an increase in the amount of haemoglobin similar to those found for CQ alone in the CQS strain. However when the parasites were exposed to VPL alone at 1μM there was also an increase in the amount of haemoglobin. The last band showed an increase in the amount of haemoglobin at the high CQ concentration. This was expected as the concentration is well above the IC_{50} for CQ in the CQR strain.

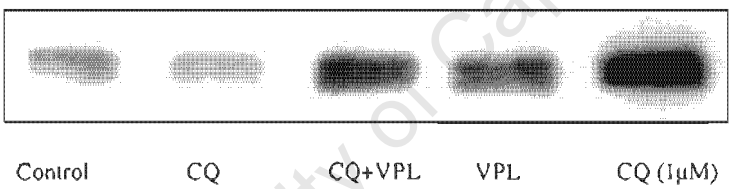


Plate 7.2 The effect of drug pressure of CQ and VPL on the chloroquine resistant strain, FAC8, over 12 hours from the late ring stage. Bands from left to right: Control (no drug), 100nM CQ alone, 100nM CQ + 1μM VPL, 1μM VPL alone, 1μM CQ alone.

Table 7.2 shows the same trend for all three CQR strains tested. All three showed a small decrease in the amount of haemoglobin relative to the control when exposed to 100nM CQ. All showed a greater than two-fold increase in haemoglobin content when CQ is combined with 1μM VPL. However all three strains also demonstrated a two-fold increase in haemoglobin content when exposed to 1μM VPL on its own. The high CQ concentrations all had the effect of increasing haemoglobin content in the CQR parasites.

Table 7.2 The relative change in haemoglobin buildup in the presence of various drugs in the CQR *Plasmodium falciparum* strains, K1, RSA11 and FAC8.

Sample	Relative change in Hb in K1	Relative change in Hb in RSA11	Relative change in Hb in FAC8
Control (No drug)	1.000	1.000	1.000
100nM CQ	0.755±0.144	0.963±0.002	0.707±0.042
100nM CQ + 1μM VPL	2.324±0.129	2.172±0.659	2.427±0.700
1μM VPL	2.325±0.968	2.062±0.496	2.054±0.564
1μM CQ	3.938±1.307	2.621±0.689	4.494±1.459

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

In the CQR strains, 100nM CQ has no effect on the buildup of Hb. This result is in agreement with the observation of Jacobs et al (1989) that CQ concentrations which kill CQS strains but are sub-lethal to CQR strains have no effect on the morphology of the CQR parasites.

When 1μM VPL is added to the 100nM CQ there is an increase in Hb similar to that seen with CQ alone in the CQS strains. This effect is expected as VPL alters CQ IC_{50}s (See Table 2.2) and increases the accumulation of ^{3}H-CQ (See Figure 3.4) under these conditions. This suggests that the changes in the amount of haemoglobin observed are related to the reversal of CQ resistance by VPL.

However 1μM VPL is able to induce haemoglobin buildup in the absence of CQ in all the CQR strains. It is unlikely that this is due to toxicity since the VPL concentration is sub-lethal. CQ causes a buildup of haemoglobin in endocytosed vesicles (Hoppe et al, 2004; Yayon et al, 1984). It remains to be established where this haemoglobin is accumulating in CQR strains exposed to VPL alone.

The high CQ concentrations (1μM) all showed an intense band illustrating that at this concentration (well above the IC_{50}) the parasites' digestion of haemoglobin was significantly inhibited even in the absence of VPL.

7.2.3 The effect of increasing concentrations of verapamil on the haemoglobin buildup in CQS and CQR strains of Plasmodium falciparum.

To investigate the effect of VPL even further, the CQS and CQR strains were exposed to increasing concentrations of VPL in the absence of CQ and probed for Hb. The concentrations used were similar as those used in the ^{3}H-CQ accumulation experiments (Figures 3.1 and 3.2) and cover a large range.

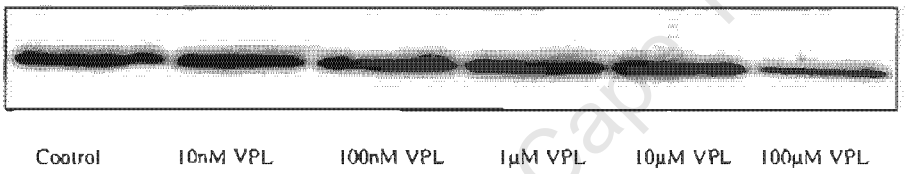


Plate 7.3 The effect of increasing amounts of VPL on the buildup of Hb in the chloroquine sensitive parasite, D10, after exposure for 12 hours from the late ring stage. Bands from left to right: Control (No drug), 10nM VPL, 100nM VPL, 1μM VPL, 10μM VPL and 100μM VPL.

In the CQS D10 strain, there is no change in Hb levels relative to the control between 10nM VPL and 10μM VPL. At the high concentration of 100μ, there is a significant decrease in the Hb levels detected. This is probably a result of the toxic effects of the drug on the parasites.

Table 7.3 shows the two CQS strains behaved similarly in their response to increasing VPL concentrations. Both had no significant change in the amount of haemoglobin over the control between 10nM and 10μM. Both show a decrease in haemoglobin at 100μM to almost half that of the control. At 1mM the haemoglobin band intensity is again close to the control value.

Table 7.3 The effect of increasing concentrations of VPL on the buildup of haemoglobin in the chloroquine-sensitive parasites, D10 and 3D7.

Sample	Relative change in Hb in D10	Relative change in Hb in 3D7
Control (No Drug)	1.000	1.000
10nM VPL	0.900±0.140	0.971±0.240
100nM VPL	1.056±0.281	0.845±0.018
1μM VPL	1.004±0.018	0.931±0.121
10μM VPL	1.030±0.106	0.845±0.368
100μM VPL	0.666±0.318	0.643±0.351

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

In the CQR strains, the pattern of haemoglobin content was very different. There was a dose dependent increase in the amount of haemoglobin between VPL concentrations of 10nM and 10μM. At 100μM VPL there was also a decrease in haemoglobin relative to the control as seen in the CQS strains.

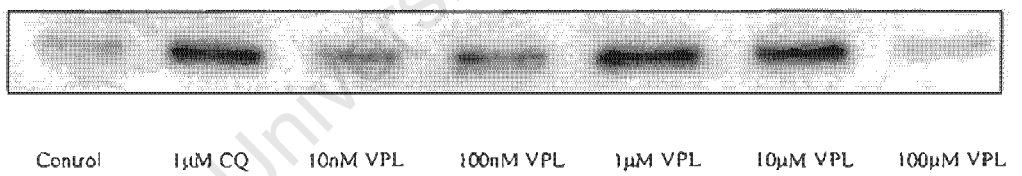


Plate 7.4 The effect of increasing amounts of VPL on the buildup of Hb in the chloroquine resistant parasite, FAC8, after exposure for 12 hours from the late ring stage. Bands from left to right: Control (No drug), 1μM CQ, 10nM VPL, 100nM VPL, 1μM VPL, 10μM VPL and 100μM VPL.

The same trend was observed in Table 7.4 for the K1 strain. There was little effect for 10nM VPL but at 100nM VPL there was an increase in the haemoglobin over the control. At 1μM and 10μM

the amount of haemoglobin found was greater than twice that of the control. At 100μM there was a drop in haemoglobin present.

Table 7.4 The effect of increasing concentrations of VPL on the buildup of haemoglobin in the chloroquine-resistant parasites, FAC8 and K1.

Sample	Relative change in Hb in FAC8	Relative change in Hb in K1
Control (No Drug)	1.000	1.000
10nM VPL	1.153±0.095	1.256±0.474
100nM VPL	1.832±0.825	1.564±0.015
1μM VPL	2.716±0.805	2.599±0.376
10μM VPL	2.165±0.398	2.353±1.418
100μM VPL	1.068±0.152	0.846±0.436

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

The lack of effect of VPL on the haemoglobin content in CQS parasites between 10nM and 10μM resembled the pattern observed for the concentration-dependent effect of VPL on ^{3}H-CQ accumulation in Chapter 3 (see Figure 3.1). These concentrations were all below the IC_{50} for VPL in both CQS strains and thus one would not expect to see any effect owing to VPL. At the high concentration of 100μM there was a drop in CQ accumulated presumably as VPL effects become toxic to the parasites and this was also observed in the amount of haemoglobin detected.

In the CQR strains there was a dose-dependent increase in the amount of haemoglobin found in the parasites due to VPL. This pattern mirrored the effect of VPL on increasing ^{3}H-CQ accumulation (See Figure 3.2). This suggests that the increase in the amount of haemoglobin observed in CQR strains due to VPL is in some way related to the ability of VPL to increase ^{3}H-CQ accumulation and thereby reverse CQ resistance. These effects are all observed at concentrations that are below the IC_{50} for VPL in the CQR strains.

At 100μM VPL causes a decrease in the amount of haemoglobin when compared to the effects observed at lower concentrations. This is probably due to toxic effects since this concentration is significantly above the IC_{50} of VPL for these strains.

Ursos et al (2000) showed that VPL (1-5μM) can increase the pH of the vacuole to levels seen in the CQS strain and it is this change in pH that may be responsible for the increase in haemoglobin. Any change in the pH of the vacuole may slow the activity of proteins/enzymes that may break down the haemoglobin. Since this pH change is not observed in the CQS strain when exposed to verapamil alone, it would not be expected to cause buildup of haemoglobin. This is indeed the case.

7.2.4 *The effect of different chemosensitisers on the haemoglobin buildup in CQS and CQR strains of* Plasmodium falciparum.

Other agents that reverse resistance to CQ were then tested to see if they exerted similar effects on the parasites' ability to take up haemoglobin. These agents were all tested at 1μM to allow for comparison of their effects relative to VPL. The chemosensitisers used were the same as those tested for comparison of their effects on ^{3}H-CQ accumulation in Chapter 3 (Figures 3.3 and 3.4). They were all tested in the absence of CQ on both the CQS and CQR strain.

None of the chemosensitisers had any effect at 1μM in the absence of CQ in causing a buildup of Hb in these CQS strains. This demonstrates that all these compounds behave similarly to verapamil.

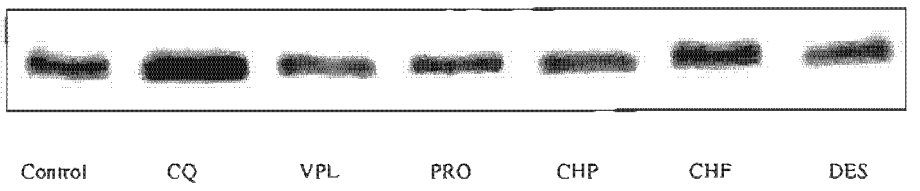


Plate 7.5 The effect of various chemosensitisers at a sub-lethal concentration (1μM) on the buildup of Hb in the chloroquine-sensitive strain, 3D7, after exposure for 12 hours. Bands from left to right: Control (No drug), CQ, VPL, Promethazine, Chlorpromazine, Chlorpheniramine, and Desipramine.

This lack of effect of the chemosensitisers on the amount of haemoglobin in the CQS strain was confirmed for both D10 and 3D7 (see Table 7.5). CQ at the same concentration caused a large increase in haemoglobin as expected.

Table 7.5 The effect of equimolar concentrations of chemosensitisers and CQ on the amount of haemoglobin present in CQS *Plasmodium falciparum* trophozoites.

Sample	Relative change in Hb in D10	Relative change in Hb in 3D7
Control (No Drug)	1.000	1.000
1μM CQ	3.180±1.811	2.305±0.815
1μM VPL	0.837±0.566	0.826±0.030
1μM PRO	0.667±0.191	0.758±0.062
1μM CHP	0.714±0.136	0.720±0.190
1μM CHF	0.904±0.454	1.128±0.062
1μM DES	0.755±0.188	0.905±0.023

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

All the chemosensitisers tested had no effect on the changing the amount of haemoglobin in the CQS parasites similarly to VPL. They also lack any effect on altering ^{3}H-CQ accumulation (See Figure 3.3). Clearly their varying ability to inhibit haem crystallization has no bearing on their effect on parasites at this concentration.

The CQR strains showed an increase in the amount of haemoglobin over the control for all of the chemosensitisers tested. Again CQ showed the largest increase but this concentration is well above its IC_{50}. All the chemosensitisers showed a buildup of haemoglobin at concentrations below their IC_{50}s (See Table 6.2).

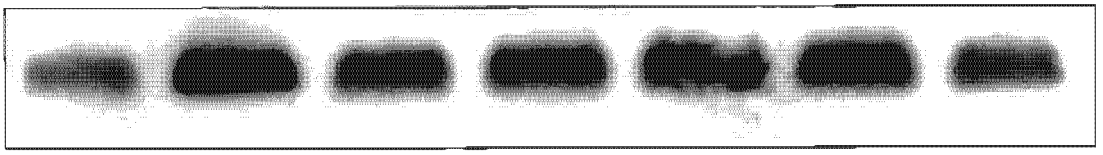


Plate 7.6 The effect of various chemosensitisers at a sub-lethal concentration (1μM) on the buildup of Hb in the chloroquine resistant strain, RSA11, after exposure for 12 hours. Bands from left to right: Control (No drug), CQ, VPL, Promethazine, Chlorpromazine, Chlorpheniramine, and Desipramine.

Table 7.6 confirmed that the buildup of Hb in the absence of CQ was specific to the CQR strain and that it was a characteristic of all chemosensitisers tested.

Table 7.6 The effect of equimolar concentrations of chemosensitisers and CQ on the amount of haemoglobin present in CQR *Plasmodium falciparum* trophozoites.

Sample	Relative change in Hb in K1	Relative change in Hb in RSA11
Control (No Drug)	1.000	1.000
1μM CQ	3.938±1.307	3.127±0.793
1μM VPL	2.325±0.968	2.062±0.496
1μM PRO	2.308±1.118	2.178±0.350
1μM CHP	2.133±0.407	2.045±0.190
1μM CHF	2.056±0.045	2.385±0.103
1μM DES	1.768±0.211	1.727±0.322

The relative change in haemoglobin content is determined by dividing the drug-pressured sample by the control (no drug). The parasites were exposed to the drug for 12 hours starting in the late ring stage. All experiments were performed twice in duplicate.

All the chemosensitisers increased the amount of haemoglobin in the CQR parasites at sub-lethal concentrations in the absence of CQ and all increased the accumulation of ^{3}H-CQ (See Figure

3.4). The ability of VPL to increase the amount of haemoglobin may be linked to its ability to increase CQ accumulation.

It has been proposed that VPL can increase the pH of the parasite food vacuole in CQR *Plasmodium falciparum* (Ursos et al, 2000). Similarly to VPL (1μM) the other chemosensitisers increase ^{3}H-CQ and cause an inhibition of haemoglobin breakdown. It is likely that they too increase the vacuolar pH. Given that the function of enzymes within the vacuole is pH dependent, this increase in pH induced by VPL may alter the CQR parasites' ability to degrade haemoglobin. Since the concentrations of VPL needed to cause this effect are sublethal to the parasites, this inhibition of haemoglobin breakdown is not detrimental to parasite survival.

Chapter 8

The Effect of Verapamil on the Localization of the Haemoglobin in Parasitized Erythrocytes of *Plasmodium falciparum* using Immunofluorescence Techniques

8.1 Introduction

Using several novel techniques, Hoppe et al (2004) were able to demonstrate that MQ inhibits the process of endocytosis while CQ inhibits the fusion of the endocytosed vesicles with the food vacuole. This inhibition of fusion by CQ leads to a buildup of Hb containing vesicles in the cytoplasm of the parasites causing parasite starvation. Artemisinin appears to have a dual action of both inhibiting endocytosis and inducing a Hb buildup. Using electron microscopy, fluorescent tracers or anti-Hb antibodies they were able to demonstrate that there is a significant accumulation of endocytosed vesicles in the parasite cytoplasm containing Hb in the presence of CQ. These experiments were all performed in the CQS strain, D10, and the effects on a CQR strain are not known. Moreover it is unknown what the effect of verapamil is on these processes when used either alone or in combination with CQ.

In Chapter 7 it was demonstrated that VPL, in the absence of CQ, causes an increase in Hb buildup in CQR parasites at concentrations used to reverse resistance (1μM). The Hb increase owing to the toxic effect of CQ is located in endocytosed vesicles accumulated within the parasite cytoplasm (Hoppe et al, 2004). The localization of the haemoglobin accumulated due to the VPL effect is not known.

In this chapter the effect of VPL is investigated on the uptake of haemoglobin by both CQS and CQR strains of *Plasmodium falciparum*. We will examine the VPL effect both alone (1μM) and in the presence of CQ to identify any differences in the drug response. The presence of haemoglobin in the parasites is identified using anti-haemoglobin antibodies and fluorescence microscopy. Endosome counts are used to compare the effect of VPL with that of CQ. A comparison is made with the results obtained for haemoglobin with the Western blots (see Chapter 7).

8.2 Results and Discussion

To explore the effect of VPL alone or in combination with CQ, the parasites were exposed to the compounds for 12 hours beginning in the late ring/early trophozoite phase (see section 10.18). Thereafter the parasites were harvested by saponin lysis and fixed to glass coverslips. The trophozoites were permeabilised with Triton X-100 and the primary anti-haemoglobin antibody was added. Thereafter the secondary antibody, linked to a fluorescent rhodamine label, was added and the slides were viewed microscopically (See section 10.19 for detailed methodology).

8.2.1 The effect of verapamil and chloroquine on the localization of haemoglobin in the parasitized erythrocytes of CQS Plasmodium falciparum

There is an increase in the amount of endosomes found in the CQ pressured parasites at both concentrations relative to the control (See Table 8.1). There is an almost 2.5 fold increase with the lower CQ concentration (100nM) and a greater than 6.5 fold increase in the highly CQ pressured parasites (1μM). Both these concentrations are well above the IC_{50} for CQ on D10 (See Table 2.2). This result confirms previously published results for CQ on D10 (Hoppe et al, 2004). When VPL is used in combination with CQ there is a slight decrease in the number of endosomes when compared to CQ alone (100nM) but there is still a greater than 2-fold increase in endosomes over the control. VPL used singly at 1μM did not induce a markedly higher number of endosomes than the control.

Table 8.1 The average number of endosomes counted per cell after 6 hours of drug pressure in the chloroquine-sensitive strain, D10.

Drug concentration Used	Mean ± Standard Deviation	Fold Increase
Control (No Drug)	1.070±1.350	1.000
100nM CQ	2.580±2.648	2.411
100nM CQ + 1μM VPL	2.300±2.584	2.150
1μM VPL	1.090±1.342	1.019
1μM CQ	7.090±2.598	6.626

The mean is taken from 100 individual randomly selected cells from two separate samples.

The images presented below show the increased number of endosomes present in all the CQ-treated cells when compared to the untreated control and the VPL-treated parasites. The DAPI-stained image demonstrates that the cells are still in the trophozoite phase (a single nucleus) and have not progressed to the schizont stage (multiple nuclei).

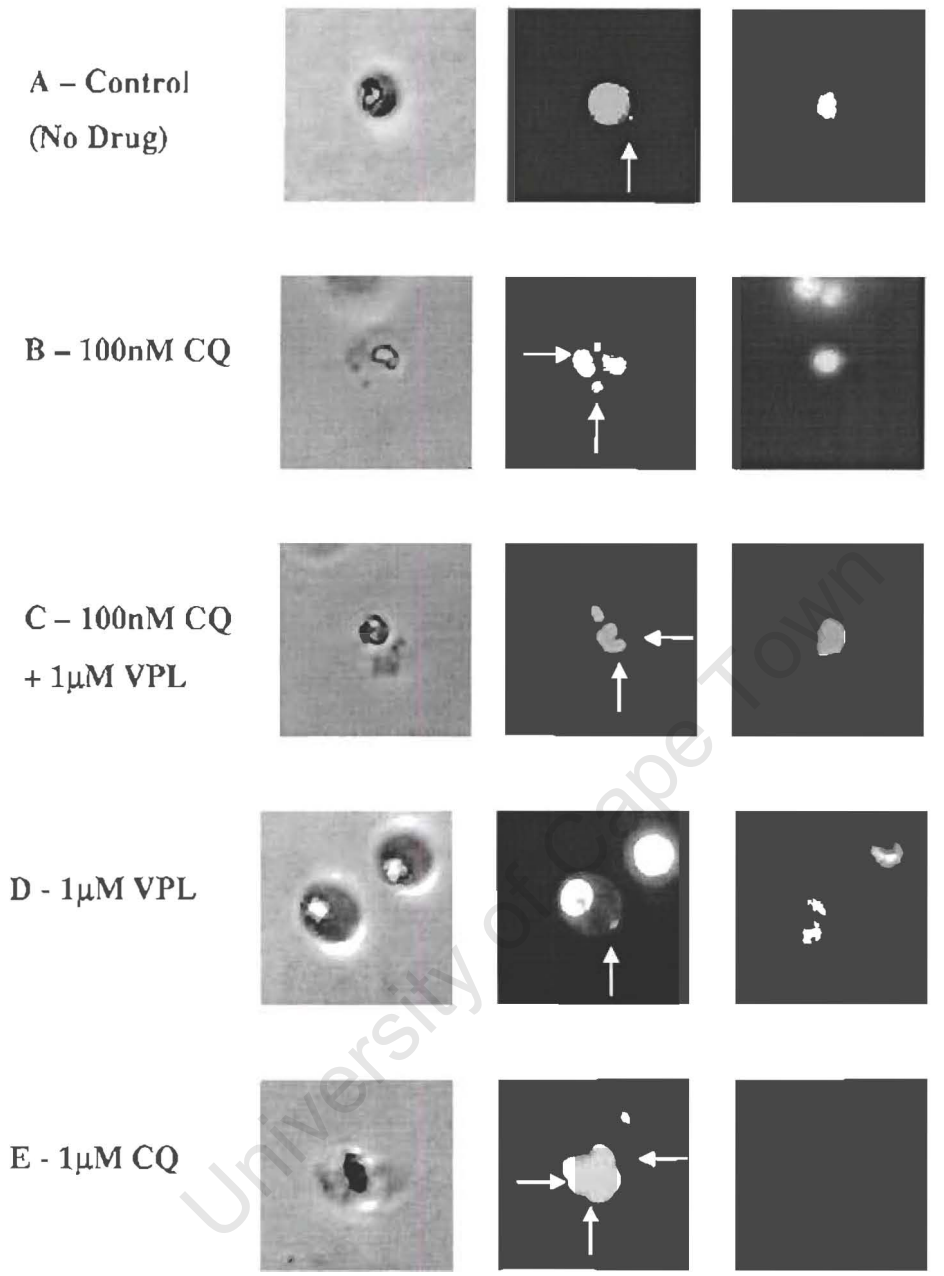


Plate 8.1 Microscope images of the effect of CQ and VPL on the chloroquine-sensitive strain, D10. The panels represent from left to right: phase-contrast image, TRITC-labeled anti-haemoglobin image, DAPI-stained nucleus image. The panels represent from top to bottom: (A) Control (No drug), (B) 100nM CQ-treated, (C) 100nM CQ + 1μM VPL treated, (D) 1μM VPL treated, (E) 1μM CQ treated.

The increase in the amount of haemoglobin found in CQS parasites exposed to either 100nM or 1μM CQ pressure (see Table 7.1) correlates with the buildup of endosomes in the cytosol of the parasites (see Plate 8.1 (B) and (E)). This confirms previous reports that CQ inhibits the fusion of the endosomes with the digestive vacuole leading to parasite starvation (Hoppe et al, 2004). VPL at 1μM has no marked effect on changing CQ action in the CQS strain as predicted from the IC_{50} (Table 2.2). 1μM VPL in the absence of CQ has no marked effect on the number of endosomes in the parasite cytosol. The decrease in haemoglobin relative to the control seen in the CQS strain (see Table 7.1) is probably due to haemoglobin digestion in the food vacuole given that there is no decrease in the number of endosomes. The VPL-treated CQS parasite may be breaking haemoglobin down more rapidly than in the control parasites.

8.2.2 The effect of verapamil and chloroquine on the localization of haemoglobin in the parasitized erythrocytes of CQR Plasmodium falciparum

From Table 8.2 it is evident that 100nM CQ produces a small increase in the number of endosomes in the parasite compared to the control. There is only a 1.3-fold increase compared with the 2.4-fold increase found at the same concentration in the CQS strain. This is not surprising since the concentration used is well below the IC_{50} for CQ in this CQR strain (354.0nM; see Table 2.2). When 1μM VPL is combined with the same concentration of CQ (100nM), there is a 2.5-fold increase in the number of endosomes accumulated relative to the control. This illustrates that VPL has restored CQ action. If VPL is used on its own at 1μM, there is also only a slight increase in endosome counts. As a result of the large variability in the number of endosomes counted per cell there is a large standard deviation in the value presented. Thus although 100 cells were counted there is no statistically significant difference that can be determined from comparing most of the samples.

Table 8.2 The average number of endosomes counted per cell after 6 hours of drug pressure in the chloroquine-resistant strain, RSA11.

Drug concentration Used	Mean ± Standard Deviation	Fold Increase
Control (No Drug)	1.010±1.425	1.000
100nM CQ	1.340±1.940	1.327
100nM CQ + 1μM VPL	2.644±2.952	2.618
1μM VPL	1.490±2.052	1.475
1μM CQ	6.520±2.213	6.455

The mean is taken from 100 individual randomly selected cells from two separate samples.

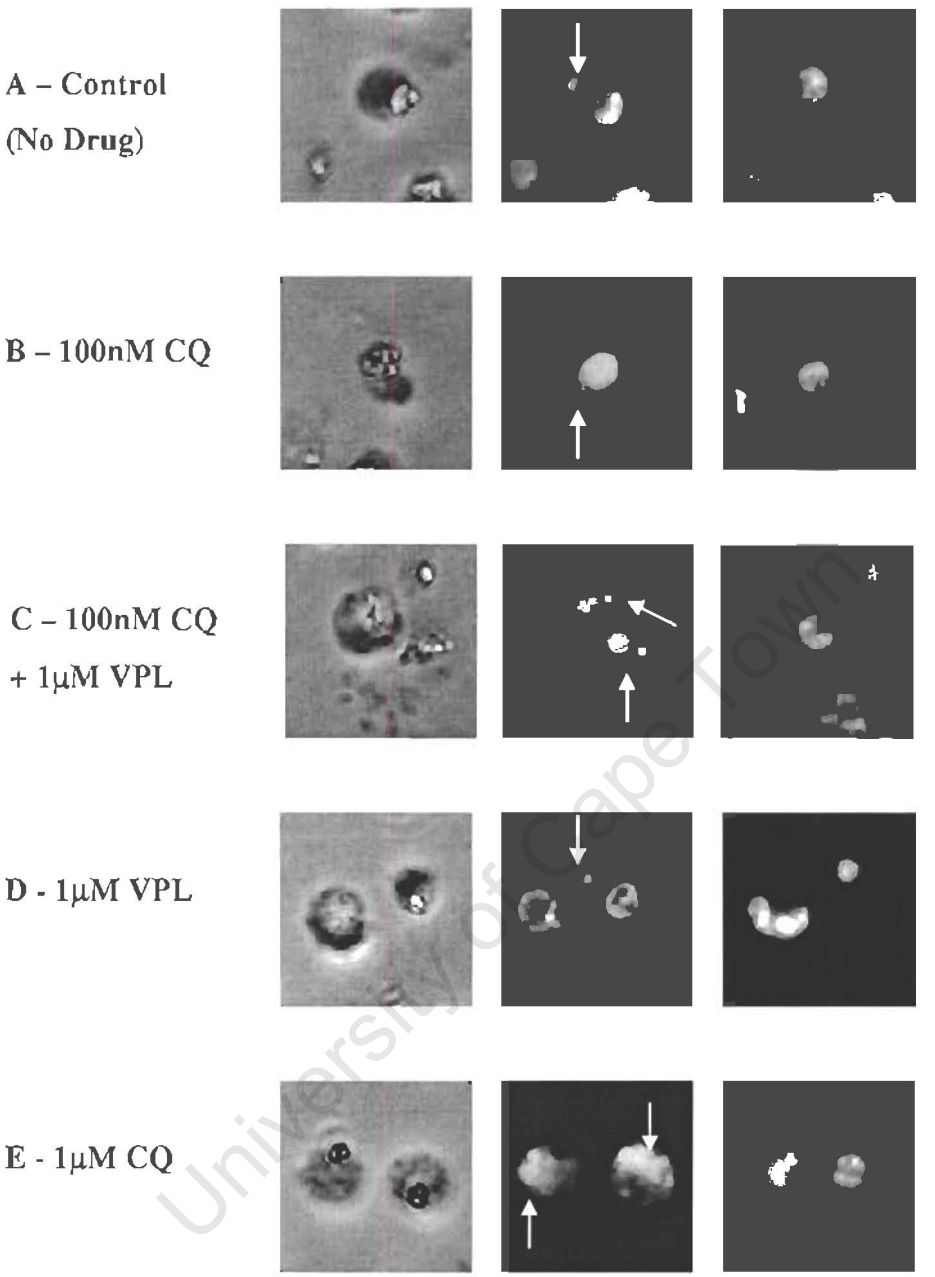


Plate 8.2 Microscope images of the effect of CQ and VPL on the chloroquine-resistant strain, RSA11. The panels represent from left to right: phase-contrast image, TRITC-labeled anti-haemoglobin image, DAPI-stained nucleus image. The panels represent from top to bottom: (A) Control (No drug), (B) 100nM CQ-treated, (C) 100nM CQ + 1μM VPL treated, (D) 1μM VPL treated, (E) 1μM CQ treated.

CQ at 100nM has no marked effect on the buildup of endosomes in the CQR strain. This would be expected given that the concentration used is below the IC_{50} for CQ in this strain (see Table 2.2). When CQ is combined with 1μM VPL, there is a significant increase in the accumulation of endocytosed vesicles. This supports the Western blot data which demonstrated that there is an increase in the buildup of haemoglobin in the CQR parasites which is most likely present in the endocytosed vesicles. The parasites will presumably starve since the endosomes are not being delivered to the digestive vacuole.

When 1μM VPL is used in the absence of CQ there is no large change in the buildup of haemoglobin-containing vesicles when compared to the control. This contrasts with the Western blot data as seen in Table 7.2. This implies that the increase in haemoglobin seen in the Western blots owing to a VPL effect (2-2.3-fold) may be confined to the digestive vacuole. Thus the effect of VPL is located in the vacuole. It also suggests then that the effect of VPL on CQ will also be manifest within the vacuole. Both PfCRT and Pgh1 are located in the vacuolar membrane. Since both have been implicated in the resistance of *Plasmodium falciparum* to CQ, VPL may be acting on either/both of these parasite proteins. It remains to be established whether VPL has any direct effect on these proteins and only recently has in been demonstrated that CQ binds directly to one of these proteins, PfCRT (Zhang et al, 2004).

How VPL may be altering the parasite physiology is also of interest. The evidence presented here (Chapter 5) and elsewhere suggests that VPL is inhibiting the efflux of CQ. The structural similarity between CQ and resistance reversers (at least 2 planar rings, a protonatable nitrogen, basic side chains of specific length) implies that these chemosensitisers may compete for a CQ target. Whatever this target may be, it may have an effect on the parasite's ability to digest haemoglobin given the VPL effects observed in this chapter and in Chapter 7. Clearly though the buildup of haemoglobin in the CQR strain and the drop in haemoglobin in the CQS strain in the presence of 1μM VPL (see Chapter 7 also) has little effect on parasite viability since the IC_{50}s for VPL are similar in both these strains.

It is known that the parasites do not require all the haemoglobin taken up from the erythrocyte (Krugliak et al, 2002). They can take up to 65% of the erythrocyte haemoglobin yet utilize only 16% for the production of parasite proteins. Thus if 1μM VPL is slowing down the breakdown of haemoglobin causing it to increase in the vacuole, this would not necessarily be fatal to the CQR parasites. It appears to be able to survive on the haemoglobin that it can break down. This differs from the action of CQ where the haemoglobin built up is not within the vacuole.

If VPL increased haemoglobin breakdown in the CQS parasites causing a decrease relative to the control it would just pump the excess amino acids out of the erythrocyte as it normally discards its excess. Alternatively VPL could be inhibiting the uptake of haemoglobin from the CQS parasite similarly to mefloquine by blocking endocytosis but this process would occur at the parasite plasma membrane and would probably affect the CQR strains also. Mefloquine action is antagonistic to CQ. One of the reasons given for this is that mefloquine inhibits the process of haemoglobin endocytosis and CQ action is dependent on the presence of free haem which is released when haemoglobin is broken down in the parasite digestive vacuole (Famin and Ginsburg, 2002). VPL either has no effect on CQ (CQS strains) or it is synergistic with CQ (CQR strains). So it is unlikely that VPL will be affecting the parasites similarly to mefloquine.

This chapter suggests that VPL restores the ability of CQ to induce parasite starvation in the CQR strain. Alternatively, VPL may be having an additive effect with CQ in causing a haemoglobin buildup in these CQR strains. It also shows that the effect of VPL in the absence of CQ in causing an increase in the amount of haemoglobin in the CQR strains (see Table 7.2) differs from the manner in which CQ increases the amount of haemoglobin. It suggests that VPL is exerting its action on the parasite within the digestive vacuole.

Chapter 9

Summary and Conclusion

With chloroquine resistance being prevalent to most regions where malaria is endemic, the need for new approaches to treating malaria is of vital importance. Development of new antimalarials has not yielded much success. Another approach involves restoring chloroquine action by combining it with chemosensitisers like verapamil. Owing to the toxicity of using these chemosensitisers at concentrations that reverse resistance optimally, this strategy has not yet been fully explored. Two potential strategies could be;

1) Using cocktails of chemosensitisers at concentrations that are non-toxic to humans to achieve optimal chloroquine resistance reversal (Adovelande et al, 1997; Van Schalkwyk et al, 2001)
2) Designing new single chemosensitisers that reverse chloroquine resistance optimally at concentrations that are non-toxic to humans.

The second approach requires a rational drug design based on an understanding of how these chemosensitisers interact with the *Plasmodium* parasites. While much is known of how these agents increase chloroquine accumulation, little is known of how chemosensitisers act in the absence of chloroquine.

This thesis describes the activity of verapamil on both chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* alone or in combination with chloroquine. Radiolabeled analogues of verapamil and chloroquine were extensively used to characterize the uptake of chemosensitisers under various conditions (temperature, concentration and energy-dependence) or to examine the action of chemosensitisers in altering chloroquine uptake. The observation that haemoglobin levels are altered in the

presence of chemosensitisers, at concentrations that reverse chloroquine resistance but are non-toxic to parasites, is also reported.

The intrinsic toxicity of verapamil was not significantly different among 6 strains of *Plasmodium falciparum* exhibiting varying sensitivity to chloroquine. The IC_{50} values of the other chemosensitisers tested on the parasites also did not differ significantly between a chloroquine resistant and a chloroquine sensitive strain. This would suggest that the action of these chemosensitisers in killing the parasites in the absence of chloroquine remains the same.

The intrinsic toxicity of these chemosensitisers is most likely not linked to any interaction with haem as is suggested for the action of chloroquine. Only verapamil, desipramine and promethazine could inhibit haem crystallization and this was achieved at very high concentrations relative to the activity of antimalarials. Chlorpheniramine and chlorpromazine did not inhibit crystallization within the concentration range tested. All these chemosensitisers have similar IC_{50} values (low micromolar concentrations) despite their differences in inhibiting haem crystallization. In addition all the chemosensitisers are monoprotic and would not be expected to accumulate to the same degree as chloroquine. The hypersensitivity of the new chloroquine resistant Brazilian strains to these chemosensitisers is therefore probably not related to any haem crystal inhibition. It is also unlikely that haem interaction has any impact on resistance reversal since all these chemosensitisers increase chloroquine accumulation to a similar degree at 1μM irrespective of whether they inhibit crystallization.

The overexpression of the P-glycoprotein homologue 1 (Pgh1) does not have any effect on the sensitivity of the parasites to verapamil. Pgh1 overexpression also does not influence the ability of verapamil to reverse chloroquine resistance. Verapamil reversed resistance to chloroquine in all 4 of the strains tested in this study. One African strain, RSA11, was more sensitive to verapamil reversal as indicated by its increased activity enhancement index (AEI) value. Unlike certain Brazilian strains that have lost their

ability to be sensitized to chloroquine in the presence of verapamil, all the strains in this study maintain their chemosensitization by verapamil despite their geographical diversity.

This thesis has described the differences in the uptake of verapamil compared to chloroquine. The differences in uptake necessitated the modification of the experimental conditions to compensate for the low affinity of verapamil binding within the parasites. The experiments were performed on enriched trophozoites to more clearly identify the parasite-specific binding from the uptake into unparasitized erythrocytes. In addition the parasites were spun through dibutyl phthalate to avoid any washing steps that would lead to loss of the radiolabel.

There was no significant difference in verapamil uptake between the parasitized and unparasitized erythrocytes. The uptake of verapamil into parasitized erythrocytes was non-saturable over a large concentration range. Unlike chloroquine uptake, verapamil uptake was not energy-dependent. Neither a large molar increase in verapamil nor chloroquine could compete for verapamil accumulation contrary to previous reports (Bray et al, 1992). This suggested that verapamil accumulation is more indicative of an equilibration of the compound from the external medium into the parasite.

Verapamil equilibration is rapid and complete within one minute. There is no difference between verapamil uptake in chloroquine resistant and chloroquine sensitive strains. This contrasts with chloroquine uptake which is time-dependent and where significant strain-dependent differences are evident. This rapid equilibration of verapamil allows the chemosensitiser to slow chloroquine efflux in resistant strains within the first minute from when efflux is measured. A comparison with verapamil accumulation within mammalian cell lines shows significant differences to plasmodial uptake. In mammalian cell lines verapamil uptake is time-dependent and reaches steady state only after an hour.

Verapamil both increases the amount of chloroquine accumulated as well as reduces the rate of chloroquine efflux in chloroquine resistant strains. The ability of verapamil to increase chloroquine accumulation is both temperature- and concentration-dependent.

None of the chemosensitisers tested had any effect on the chloroquine sensitive strains either in increasing chloroquine accumulation or in causing an increase of haemoglobin measured in the parasites.

However, all the chemosensitisers tested showed a similar capacity to increase chloroquine accumulation at 1μM in chloroquine resistant strains. In addition, all the chemosensitisers caused a similar increase in the amount of haemoglobin found in chloroquine resistant strains at 1μM. The 1μM chemosensitiser concentration is sublethal to the parasites so that, unlike with chloroquine action, the increase in haemoglobin does not result from a toxic effect. This would suggest that the increase in the amount of haemoglobin in the presence of the chemosensitisers is associated with their ability to increase chloroquine accumulation and thereby reverse chloroquine resistance.

The haemoglobin increase in the presence of verapamil was concentration-dependent and did not result in an increase in the number of endosomes found in the parasite cytoplasm. This shows that the verapamil effect differs from chloroquine effects and is not related to drug toxicity. It suggests also that verapamil is exerting its effect within the food vacuole where the increase in haemoglobin is observed.

In conclusion, this thesis demonstrates that verapamil equilibrates similarly into both chloroquine sensitive and chloroquine resistant *Plasmodium*-infected parasites. The main strain specific differences lie in the ability of these chemosensitisers to alter chloroquine accumulation and efflux. It describes for the first time the effect of verapamil on altering the amount of haemoglobin detected in chloroquine resistant parasites. This work suggests that any putative receptor for these chemosensitisers would have to be of low affinity.

Chapter 10

Materials and Methodology

10.1 *Plasmodium falciparum* Strains

3 chloroquine sensitive strains used:

1. D10 was derived from FQC-27 from Papua New Guinea (Ekong et al, 1993).
2. 3D7 is a clone from the NF54 strain, isolated from airport worker in Amsterdam.

4 chloroquine resistant strains used:

1. RSA11, is also a patient isolate in Kwa-Zulu Natal, (Freese at al, 1991).
2. K1 is a strain from Thailand (Thaithong and Beale, 1981).
3. FAC8 is a clone derived from the isolate ITG2F6 (Biggs et al, 1989).
4. W2 is a clone derived from the Indochina III strain (Oduola et al, 1988).

10.2 Drugs Used in This Study

All the compounds were dissolved in the solvents described in Table 10.1 and were stored at -20°C or -80°C until they were needed. Light sensitive compounds were stored in foil wrapped microcentrifuge tubes. For experiments performed on parasites the compounds that were dissolved in methanol or DMSO were diluted until the solvent concentration was less than 1% which is non-toxic to the parasites.

Table 10.1 A list of the compounds used in this study along with their molecular weights, dissociation constants (pKa's), solvents and the company from which they were purchased.

Compound Name	Molecular Weight	pKa's	Solvents	Source
Chloroquine diphosphate	515.9	8.4,10.8[b]	Water	Sigma
Verapamil hydrochloride	491.1	9.2[a]	Water	Sigma
Promethazine hydrochloride	320.9	9.1[b]	Water	Sigma
Chlorpromazine hydrochloride	355.3	9.3[b]	Water	Sigma
Chlorpheniramine maleate	274.8	9.1[b]	Water	Sigma
Desipramine hydrochloride	302.8	10.2[b]	Water	Sigma
Trifluoperazine dihydrochloride	480.4	3.9, 8.1[c]	Water	Sigma
Cyproheptadine hydrochloride	323.9		Methanol	Sigma
Quinine (Free Base)	324.4	4.1, 8.5[b]	Methanol	Sigma
Haemin chloride (Bovine)	652.0		0.1M NaOH	Sigma

[a] from Bray et al (1992)
[b] from Clarke (1986)
[c] from the Merck Index (eleventh edition)

10.3 *Plasmodium falciparum* Cell Culture *in vitro*

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

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

The parasitaemia was determined by counting the number of parasitised erythrocytes cells as a fraction of the total number of erythrocytes (parasitised + unparasitised).

10.4 Synchronization of *Plasmodium falciparum* Parasites

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

10.5 Parasite Lactate Dehydrogenase Assay for Determining Parasite Viability

The assay was performed in 96-well microtitre plates. The blank for the assay was the unparasitised erythrocytes without drug and the control was the parasitised erythrocytes without drug. The experiment involved incubating the parasites at a 1% haematocrit and a 2% parasitaemia in a volume of 200µl along with the particular drug at defined concentrations. Each drug concentration was measured in duplicate per experiment. The parasites were incubated for 48 hours in the plate in desiccator cabinets under the gas mixture as described previously.

The lactate dehydrogenase activity is used as a measure of parasite viability. The Malstat™ reagent contains lactate to initiate the reaction and APAD (3-acetyl pyridine adenine dinucleotide) which the parasites selectively use as a cofactor in the enzymatic reaction instead of NAD. The NBT/PES is a solution containing Nitro Blue Tetrazolium

(1.6mg/ml) and Phenazine Etho Sulphate (0.08mg/ml). As the reaction proceeds, the yellow tetrazolium ion is reduced to a purple formazan salt.

Once the 48 hour incubation is complete, 100µl of Malstat™ reagent and 25µl of NBT/PES solution were added to each well of a new flat bottomed 96-well microtitre plate. The samples in each of the wells of the original plate were resuspended and 15µl was taken from each well and added to the corresponding well of the Malstat plate, thus initiating the lactate dehydrogenase reaction. Colour development was monitored at 620nm as the reaction proceeded (Makler et al, 1993). The average absorbance of the blank (unparasitised erythrocytes) was subtracted from all the parasitized samples. The percentage viability was calculated by dividing the absorbance of the drug-exposed parasites by the absorbance of the control parasites (drug free) and multiplying this value by 100.

$$\text{Percentage Viability} = \frac{OD_{620}\text{ Drug Exposed parasites} - \text{Average blank } OD_{620}}{OD_{620}\text{ Control (No Drug) parasites} - \text{Average blank } OD_{620}} \times 100$$

10.6 Tritiated Chloroquine and Verapamil Accumulation in *Plasmodium falciparum*

The accumulation of tritiated chloroquine was carried out in 1ml of culture of *Plasmodium falciparum* (pst 5%, hc 1%) or with enriched trophozoites (>90% pst, 0.2% hc) at 37° over a 1-hour incubation period.

The parasites were incubated with either 1nM ^{3}H-CQ (7Ci/mmol; Moravek Biochemicals Inc.) or 1nM ^{3}H-VPL (85Ci/mmol; NEN Life Sciences Products, Inc.). The tubes were shaken after 30 minutes. After the 1-hour incubation was complete, the samples were processed for scintillation counting. Initially the cells were pelleted in a microcentrifuge at 13000rpm for 1 minute and the supernatant removed. The pellet was then washed by adding 1ml of ice cold PBS to the pellet before spinning it again. Thereafter the pellet

was processed as described below. When it was discovered that a large amount of ^{3}H-VPL counts were lost in this washing procedure, it was decided to use dibutyl phthalate to separate the supernatant from the pellet without losing the radioactivity in the parasitized or unparasitized erythrocytes.

Briefly, 100µl of dibutyl phthalate is added to each microcentrifuge tube containing the culture. The tubes were then centrifuged at 13000 rpm for 1 minute and the supernatant aspirated, leaving the pellet of parasitised red blood cells (Bray, 1992). The tips of the tubes are then cut off and placed into scintillation vials. 100µl of Solvable was then added to the pellet for 30 minutes to lyse the cells. Thereafter 100µl of hydrogen peroxide was added to the sample for 30 minutes to bleach the colour from the sample. Lastly, 2mls of scintillation fluid (Ultima Gold; Packard) was added and the samples were shaken vigorously. The vials are then left overnight and were counted in a β-scintillation counter (Packard Tri-Carb 2100TR).

When testing the effects of other compounds on the accumulation of the radiolabeled analogues, the compounds were first pre-incubated with the parasites for 15 minutes before the addition of the ^{3}H-CQ or ^{3}H-VPL.

10.6.1 Testing of Metabolic Deprivation or Temperature Dependence on Radioactive Accumulation

The temperature dependence of VPL's effect on ^{3}H-CQ accumulation was measured in complete medium at 37°C or 4°C for 1 hour. The parasites were then spun though dibutyl phthalate and processed as described above.

The effect of metabolic deprivation was examined in phosphate buffered saline (pH, 7.4) at 37°C for 1 hour. The control group of cells was incubated in PBS containing glucose (1mg/ml) whilst the energy-depleted cells were incubated in PBS that lacked glucose and contained sodium azide (10mM). A similar method was used by Bray et al (1992) for their metabolic inhibition experiments.

10.6.2 Measuring the Concentration Dependence of ^{3}H-VPL Accumulation

The concentration dependence of VPL on *Plasmodium falciparum* was measured between external concentrations of 1nM and 1mM. The VPL stocks were composed of 1μCi/ml ^{3}H-VPL (84Ci/mmol; NEN Life Sciences Products, Inc.) made up to the relevant concentrations with unlabelled VPL. The parasites (5% pst; 1% hc) were incubated with the ^{3}H-VPL for 1 hour before being spun through dibutyl phthalate and processed as described above.

10.7 Time Course experiments with Chloroquine and Verapamil in *Plasmodium falciparum*

10.7.1 Time Course for Accumulation of ^{3}H-CQ or ^{3}H-VPL

Parasites were loaded into a series of microcentrifuge tubes in 1ml of culture medium (5% pst; 1% hc). For competition experiments the parasites were first pre-incubated with 1μM CQ or 5μM VPL for 15 minutes. The tubes were all suspended in a waterbath at 37°C. Then 1nM ^{3}H-CQ or 1nM ^{3}H-VPL was added to each tube and the tubes shaken rapidly to mix the sample. At the relevant time point, the tubes were removed and 100µl of dibutyl phthalate added before centrifuging at 13000rpm for 30 seconds. At this stage the parasites were separated from the supernatant so no further accumulation took place. At the completion of the time course, the supernatant was aspirated off and the pellets were cut into scintillation vials for processing as described above. The time points used for the measurement of accumulation rates of both ^{3}H-CQ and ^{3}H-VPL were (in minutes): 1, 5, 10, 15, 25, 40 and 60.

10.7.2 Time Course for Efflux of ^{3}H-CQ

The effect of VPL on the efflux of ^{3}H-CQ was determined under various conditions. In all cases the ^{3}H-CQ was allowed to accumulate for 1 hour since this is the time required for CQ to reach steady state in the parasites. After 60 minutes, 1ml aliquots of the culture (5% pst; 1% hc) were spun down at 13000rpm for 1 minute in microcentrifuge tubes containing 100μl dibutyl phthalate. This step separated the pellet from the supernatant and was designated time point T=0. The supernatant was removed from each tube as well as much of the dibutyl phthalate thereby removing any non-accumulated radiolabel. At this stage 1ml of growth medium with or without 5μM VPL was added to the pellet in each tube and mixed rapidly. This initiated the efflux of CQ. At each time point another 100μl of dibutyl phthalate was added and the parasites were spun again at 13000rpm. The pellets were processed for scintillation counting as described above. The time points used for the measurement of efflux rates of ^{3}H-CQ were (in minutes): 0, 1, 2, 3, 5, 10, 30 and 60. After 60 minutes the efflux in both strains had reached equilibrium and no further decrease in radioactivity was observed. The rate of efflux was measured by dividing the amount of radioactivity remaining in the pellet at each time point by the amount of radioactivity present at T=0 and subtracting the equilibrium value. This represents the percent decrease before equilibrium.

$$\text{Percent decrease at equilibrium} = \frac{(\text{Counts at T = x}) - (\text{Counts at T = equilibrium})}{\text{Counts at T = 0}}$$

where 'x' represents a time point during the time course,
'equilibirium' represents 60 minutes after efflux was initiated and
'0' is the initiation of the efflux component on the experiment.

For the control there was no VPL present in either the ^{3}H-CQ accumulation stage or the efflux stage. In one sample 5μM VPL was present in both the initial accumulation phase and during the efflux phase. In the second sample 5μM VPL was present only in the initial 1 hour accumulation phase but omitted from the efflux phase. In the last sample

5μM VPL was omitted during the accumulation phase but included when efflux was initiated.

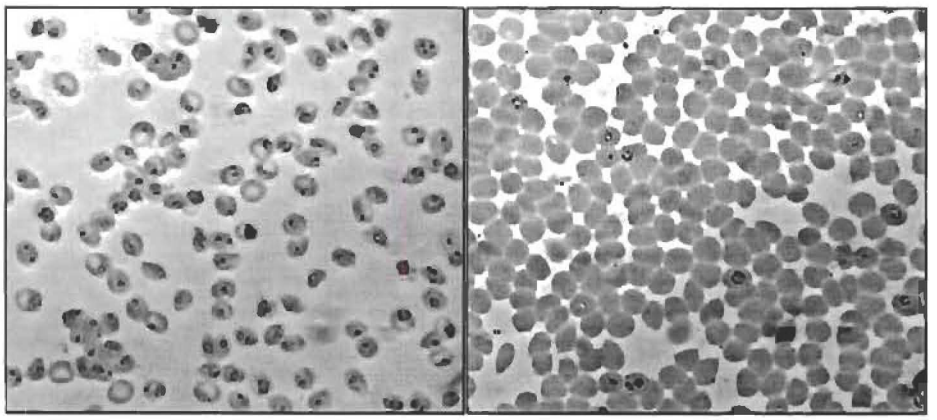
Table 10.2 Description of the experimental conditions used during the ^{3}H-CQ efflux experiments to examine the role of verapamil.

Sample	5μM VPL present during accumulation phase	5μM VPL present during efflux phase
Control	---	---
VPL in Acc and Efflux	+	+
VPL in Acc	+	---
VPL in Efflux	---	+

10.8 Enrichment of *Plasmodium* Cultures

This method is a modification of the method of Ginsburg et al (1999). A 90% and 60% Percoll solution was prepared in RPMI-1640 supplemented with 6g/L glucose and 25mM HEPES but without bicarbonate. 3% alanine was added to both solutions. 500μl of the 60% percoll solution was layered atop of 500μl of the 90% percoll in a microcentrifuge tube. Between 200-300μl of the parasitised erythrocyte pellet was then layered on the Percoll. This mixture was spun at 10000rpm for 20mins in a benchtop centrifuge. The enriched trophozoite layer is situated at the interface between the 60% Percoll and the 90% Percoll layers. This band of trophozoites was then removed and washed twice with warm 5X PBS.

The pellet was then resuspended in 10mls of complete medium to allow any residual alanine to equilibrate in the excess medium. After 30mins, the pellet was spun down at 600g for 5mins. This method regularly yields greater than 90% parasitaemia as confirmed using light microscopy (See Plate 10.1). The pellet is then aliquoted for accumulation experiments.



A B

Plate 10.1 Microscope images of Giemsa stained enriched trophozoites (Panel A) and non-enriched trophozoites (Panel B) from *Plasmodium falciparum*. Trophozoites are identified by the dark haemozoin crystals located in the food vacuole of the parasites (Parasite samples courtesy of U.I.M. Wiehart).

10.9 Trophozoite Isolation

Late trophozoites were isolated from the erythrocytes using saponin lysis. 2.5ml of a 1% saponin solution in water was added to 45ml of parasite pellet resuspended in PBS (final concentration of saponin: 0.05%). The parasites were left for between 2-5min and were then spun at 1500g for 10min to pellet the trophozoites. The parasite pellet was then transferred into microcentrifuge tubes and washed 4 times with PBS at 6500rpm to remove any excess haemoglobin.

If the parasitised erythrocytes were harvested in microcentrifuge tubes, 1ml of a 0.25% saponin solution was added and left for 2 minutes. Thereafter the parasite pellet was spun at 6500rpm for 3 minutes. The pellet was then washed 4 times in PBS to remove haemoglobin.

10.10 Vacuole Isolation

The isolation of purified and intact digestive vacuoles is based on the method of Saliba et al (1998). Trophozoites at a high parasitaemia (>10%) were isolated in centrifuge tubes using saponin (0.05%) as described above. The pelleted trophozoites were transferred into microcentrifuge tubes and washed twice with ice-cold PBS. The trophozoites were then resuspended in ice cold water (pH 4.5) and triturated 4-times through a 27-G 1.2cm needle. Thereafter the suspension was pelleted in a benchtop centrifuge at 13000rpm for 2 minutes. The pellet was exposed to 1ml DNase 1 (0.05mg/ml) for 5 minutes at 37°C before being spun at 13000 rpm for 2 minutes. This pellet was resuspended in 300μl uptake buffer and was further triturated twice. The uptake buffer contained 2mM $MgSO_4$, 100mM KCl, 10mM NaCl, 25mM HEPES, 25mM $NaHCO_3$ and 5mM Na_2HPO_4 at pH 7.4. This mixture was then layered atop a 1.2 ml Percoll solution containing 42% Percoll, 250mM sucrose and 2.5mM $MgCl_2$ (pH 7.4). This was then centrifuged at 13000rpm for 10 minutes at 4°C. The purified food vacuoles were located in the pellet below the density gradient. The debris was aspirated off and the vacuoles washed twice with ice cold uptake buffer to remove any residual Percoll.

The number of vacuoles was determined by microscope counts using a haematocytometer. The vacuoles were diluted and mounted onto a Bright Line Counting Chamber (Hausser Scientific Company) under a coverslip. No staining was necessary as the haemozoin crystals appear as dark spots.

10.11 Mammalian Cell Lines

Drug Sensitive Cell lines used:

1. The KB-3-1 cell line is a human cervix carcinoma line derived from a 1951 established HELA. This line was a gift from Dr. M. Gottesmann.

2. The NIH-3T3 murine fibroblasts were a gift from Dr. M. Gottesman.

10.12 Mammalian Cell Culture *in vitro*

All mammalian cells were maintained in DMEM supplemented with 10% foetal calf serum (FCS) and 50µg/l gentamycin sulphate. Cells were stored in 250ml vented cell culture flasks at 37°C in a humidified, CO_2 (5%) incubator. When cells achieved confluency, they were subcultured. The culture medium was removed and the cells washed twice with 10ml sterile phosphate buffered saline (PBS). Thereafter, 5ml of 0.25% trypsin/0.1% EDTA was added and the cells were left for two minutes at 37°C. After the cells had lifted from the flask, 5ml of the culture medium was added to inhibit the trypsin. The cells were transferred into a centrifuge tube and spun at 700rpm for 5 minutes. The supernatant was removed and the cells resuspended and diluted in fresh culture medium before being placed into a new culture flask and incubated at 37°C.

10.13 Tritiated Verapamil Accumulation in Mammalian Cells

^{3}H-VPL accumulation was performed according to a modified method of Warr et al (1988). 2mls of cells were plated into each well of a 24-well plate (Nunc) at $1x10^5$ cells per ml approximately 3 days before the experiment was performed. The medium was aspirated off immediately before the experiment was performed. At time zero, 1nM ^{3}H-VPL (85Ci/mmol; NEN Life Sciences Products, Inc.) in DMEM without FCS was added to each well. The medium was aspirated off at the following time points (in minutes): 1, 2, 3, 5, 10, 15, 20, 25, 30, 45 and 50. The cells were then washed three times in the wells with ice-cold PBS. They were removed after exposure to 0.5mls of 0.25% trypsin/0.1% EDTA for 2 minutes and were placed into 20ml scintillation vials. The wells were washed twice with 0.5mls of 0.5M potassium hydroxide to harvest any remaining cells. These were also added to the scintillation vials. The cells were treated with an additional 1ml of 0.5M potassium hydroxide for 1 hour in the vials at 56°C. The samples were immersed in 4ml of liquid scintillation fluid (Ultima Gold; Packard) and shaken overnight. The results were read on the Packard liquid scintillation counter (Tri-Carb 2100TR).

10.14 Protein Determination Assay

Protein quantitation for western blotting was performed using the Bio-Rad Protein Assay. This assay was based on the method of Bradford et al (1976). A standard curve was run using bovine serum albumin (BSA). Briefly, 8 dilutions were made ranging between 5µg/ml and 50µg/ml from a stock solution (1mg/ml) of BSA in water in an 800µl volume. Thereafter 200µl of the Bio-Rad Protein Assay reagent was added up to a final volume of 1ml. Then 200µl of each sample was dispensed to each of four wells of a 96-well microtitre plate and read at 620nm in an Anthos Labtec HT2 spectrophotometer.

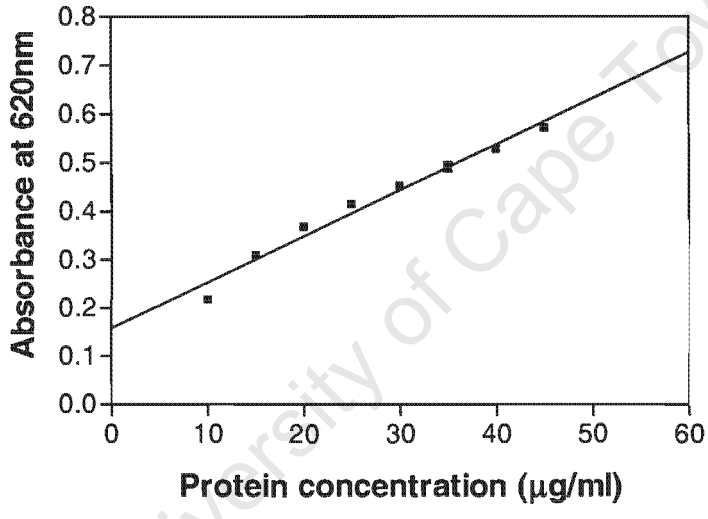


Figure 10.1 Standard curve for the protein determination assay performed using Bovine Serum Albumin. The concentrations covered in the linear range were between 10µg/ml and 45µg/ml. The linear correlation coefficient (r^2) was 0.9444.

To determine the amount of protein in the parasite samples, the original protein stocks were diluted until their absorbance fell within the linear range for the assay. The concentration of the original stock was then determined by multiplying the value obtained

by the dilution factor. The linear regression was performed using Prism 4.0 (Graphpad Software.).

10.15 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed according to the method of Laemmli (1970). 1mm thick gels were prepared in the Bio-Rad Mini PROTEAN® II electrophoresis cell and run at 20mA for a single gel or 40mA for two gels. The resolving gel (0.375M Tris-HCl, pH 8.8) was made up to between 8-10% acrylamide for Pgh1 gels and at 12% acrylamide for the haemoglobin gels. The stacking gel (0.125M Tris-HCl, pH 6.8) was made up to 3.5% acrylamide for all gels run. The reservoir buffer was composed of 0.025M Tris, 0.192M glycine and 0.1% (w/v) SDS at pH 8.3. The samples were run until the dye front had reached the bottom of the gel matrix. Thereafter the gels were removed and either stained in Coomassie or used for western blotting.

10.16 Sample Preparation

Isolated trophozoites or vacuoles were solubilized in sample application buffer composed of 1% (w/v) SDS, 0.25M Tris, 1% Mercaptoethanol, 10% glycerol and Bromophenol Blue (10mg/l). The samples were boiled for 5 minutes and then spun at 13000rpm for 5 minutes in a bench top centrifuge. This was done to pellet the haemozoin, which may cause "streaking" on the gels.

Haemoglobin controls were prepared as in internal standard in the western blotting experiments. Human haemoglobin (Sigma Chemical Co.) was diluted in water (1:1000). Sample application buffer was then added to the solution and the samples prepared as described above. The haemoglobin was then run on the SDS-PAGE gels simultaneously with the parasites samples for the western blotting experiments.

10.17 Immunoblotting (Western Blotting)

Proteins were transferred to 0.2μm Bio-Rad Immun-Blot Polyvinylidene difluoride (PVDF) membrane in the Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell. The gel was mounted against the membrane and flanked on both sides with filter paper and fiber pads before being loaded into the gel holder cassette. The cassette was placed inside the mini Trans-Blot module and loaded into the buffer chamber. The transfer was run at 100 volts for 1-1½ hours in running buffer containing 0.192M glycine and 0.025M Tris in 20% Methanol. The membrane was then removed and placed in blocking solution containing 5% fat-free milk powder and 0.1% Tween 20 in PBS for 1 hour. The primary antibody was then diluted to the blocking solution (1:200 for rabbit anti-Pgh1 and 1:2000 for rabbit anti-haemoglobin (Sigma)). The membrane was incubated in this solution for 1 hour after which it was washed 3 times with the blocking solution. The secondary antibody was then added (1:2000 for peroxidase linked goat anti-rabbit antibody; Sigma) for 1 hour. The membrane was then washed once with the blocking solution for 15 minutes followed by 3 washes in PBS each lasting 5 minutes. The membrane was then covered with the ECL™ Western Blotting System (Amersham Pharmacia Biotech) and left for 1 minute. Autoradiography was then performed with the membrane. The western blot images were captured using an EDAS 290 Gel Documentation System with a Kodak DC290 zoom digital camera. Images were analyzed using the Kodak 1D image analysis software (version 3.5).

10.18 Drug Pressure

Drug pressure in the *Plasmodium falciparum* parasites was initiated in the early trophozoite/ late ring stage. The parasites were exposed for 12 hours to allow for the effect to be observed. Thereafter the parasites were harvested by saponin lysis as described above and washed thoroughly in PBS to remove any excess haemoglobin.

Table 10.3 The drug concentrations used to treat the *Plasmodium* parasites for haemoglobin western blotting and for the haemoglobin endocytosis assay.

Drug/ Drug Combination Used	Drug concentration (nM)
Chloroquine (low)	100
Chloroquine (high)	1000
Chloroquine + Verapamil	100 + 1000
Verapamil	1000
Promethazine	1000
Chlorpromazine	1000
Chlorpheniramine	1000
Desipramine	1000

10.19 Haemoglobin Endocytosis Assay

Glass coverslips were exposed to 1mg/ml poly-L-lysine for 15 minutes. The coverslips were then placed in a 24-well microtitre plate and washed 4 times with PBS. All subsequent washing steps were performed 4 times in PBS. Parasitised erythrocytes were drug pressured as described above. The trophozoites were then harvested by saponin lysis and washed repeatedly in PBS to remove any excess hemoglobin. The free trophozoites were allowed to attach to the coverslips for 2 minutes before being spun at 700rpm for 2 minutes. Thereafter the slides were washed again to remove any unattached cells.

The cells were then fixed in 3% paraformaldehyde (PFA)/ 0.25% glutaraldehyde for 15 minutes after which they were washed. The membranes were permeabilised in 0.5mls Triton X-100 (0.5%) for 5 minutes and washed once more. Then 0.5ml glycine (0.15M) was added for 20 minutes to quench any aldehyde groups that may cause auto-fluorescence. The coverslips were washed once again.

In a moist chamber, the coverslips were placed in a blocking solution for 30 minutes. The blocking solution was composed of 50% Foetal calf serum, 1mM $MgCl_2$ and 1mM $CaCl_2$

in PBS. After the blocking step the rabbit anti-haemoglobin antibody (diluted 1:200 in the blocking solution) was added for 1 hour. Thereafter the coverslips were washed.

The Tetramethylrhodamine B Isothiocyanate (TRITC)-linked anti-rabbit antibody (diluted 1:250 in blocking solution) was then added to the coverslips for 1 hour in the dark. This was followed again by a washing step.

Lastly the coverslips were rinsed in 1μg/ml 4',6-Diamidino-2-phenylindole Dihydrochloride (DAPI) for 1 minute before being mounted onto a microscope slide.

The slides were viewed the following day by fluorescence microscopy with a Nikon Eclipse E600 fluorescence microscope under a 100X Apochromat objective. The images were captured with a Media Cybernetics CoolSNAP-Pro monochrome cooled charge-coupled device camera. Images were sharpened using Adobe Photoshop (version 7.0) software. Average endosome counts were determined from 100 individual randomly selected trophozoites for each sample.

10.20 Haematin Crystal Inhibition Assay

This assay is a modified method of Nkokazi et al, 2005. Stock solutions of sodium acetate trihydrate (12.9M, pH 5.0), sodium hydroxide (0.1M), HEPES buffer (2M, pH 8.2) and 1M Hydrochloric Acid (HCl) were prepared. A 11.36% pyridine stock was prepared by adding 11.36mls of pyridine to 10mls of the HEPES buffer and making it up to 100mls with distilled water (HP buffer). A haemin chloride solution of 1.68mM was prepared in the 0.1M sodium hydroxide solution within one hour of running the experiment. Drug solutions of 168mM were prepared in 1M HCl and various dilutions prepared also in the HCl. The assay is run in a 96 well round-bottomed plate. 2.01μl of the drug (1-20 equivalents) was added to 20.12μl of the haemin stock. To this, 11.74μl of the acetate buffer was added. The plate is left at 60°C for one hour to allow the reaction to proceed. The final concentrations of the reagents in the well are:

1) Haemin chloride - 1mM
2) Drugs - 1-20mM (1-20 equivalents)
3) Acetate buffer - 4.5M (pH 4.5)

After the reaction is complete, 250μl of the HP buffer is added to each well in the plate. Thereafter 10μl of the solution from each well is diluted in 200μl of the HP buffer in a duplicate 96-well flat-bottomed plate. The absorbance was read at 405nm in an Anthos Labtec HT2 microplate reader. The OD_{450} was plotted relative to the drug equivalents to obtain a dose response curve which was generated using a non-linear regression in Prism 4.0 (Graphpad Software).

10.21 Statistical Analyses

The percentage viabilities for the parasite lactate dehydrogenase assay were calculated using a SigmaPlot (Jandel Scientific) transformation. All dose response curves are generated using a non-linear regression (sigmoidal dose response function) in Prism 4.0 (Graphpad Software). Linear regressions were also performed using Prism 4.0. Statistical significances, presented as p-values, were determined using the unpaired t-test test. Differences were significant if $p \leq 0.05$. Statistical analyses were all performed using Prism 3.0.

References

Abok,K. (1997) Re: Malaria treatment in peri-urban area of Kisumu Town. East African Medical Journal. 757.

Adovelande,J., Bastide,B., Deleze,J., & Schrevel,J. (1993) Cytosolic free calcium in *Plasmodium falciparum*-infected erythrocytes and the effect of verapamil: a cytofluorimetric study. Experimental Parasitology, **76**, 247-258.

Adovelande,J., Deleze,J. & Schrevel,J. (1998) Synergy between two calcium channel blockers, verapamil and fantofarone (SR33557), in reversing chloroquine resistance in *Plasmodium falciparum*. Biochemical Pharmacology, **55**, 433-440.

Aikawa,M. (1972) High-resolution autoradiography of malarial parasites treated with ^{3}H-chloroquine. American Journal of Pathology, **67(2)**, 277-280.

Barnes,D.A., Foote,S.J., Galatis,D., Kemp,D.J. & Cowman,A.F. (1992) Selection for high-level chloroquine resistance results in deamplification of the *pfmdr1* gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. EMBO Journal, **11(8)**, 3067-3075.

Basco,L.K. & Le Bras,J. (1994) *In vitro* reversal of chloroquine resistance with chlorpheniramine against African isolates of *Plasmodium falciparum*. Japanese Journal of Medical Science and Biology, **47**, 59-63.

Bayoumi,R.A.L., Babiker,H.A. & Arnot,D.E. (1994) Uptake and efflux of chloroquine by chloroquine-resistant *Plasmodium falciparum* clones recently isolated in Africa. Acta Tropica, **58**, 141-149.

Bennett,T.N., Kosar,A.D., Ursos,L.M.B., Dzekunov,S., Sidhu,A.B.S., Fidock,D.A. & Roepe,P.D. (2004) Drug resistance-associated pfCRT mutations confer decreased *Plasmodium falciparum* digestive vacuolar pH. Molecular and Biochemical Parasitology, **133**, 99-114.

Bhattacharjee,A.K., Kyle,D.E. & Vennerstrom,J.L. (2001) Structural analysis of chloroquine resistance reversal by imipramine analogs. Antimicrobial Agents and Chemotherapy, **45(9)**, 2655-2657.

Bhattacharjee,A.K., Kyle,D.E., Vennerstrom,J.L. & Milhous,W.K. (2002) A 3D QSAR pharmacophore model and quantum chemical structure-activity analysis of

chloroquine (CQ)-resistance reversal. The Journal of Chemical Informatics and Computational Science, **42**, 1212-1220.

Biggs,B.A., Kemp,D.J. & Brown,G.V. (1989) Subtelomeric chromosome deletions in field isolates of *Plasmodium falciparum* and their relationship to loss of cytoadherence *in vitro*. Proceedings of the National Academy of Sciences of the USA, **86**, 2428-2432.

Bitonti,A.J., Sjoerdsma,A., McCann,P.P., Kyle,D.E., Oduola,A.M.J., Rossan,R.N., Milhous,W.K. & Davidson,D.E. Jr. (1988) Reversal of chloroquine resistance in malaria parasite *Plasmodium falciparum* by desipramine. Science, **242**, 1301-1303.

Boulter,M.K., Bray,P.G., Howells,R.E. & Ward,S.A. (1993) The potential of desipramine to reverse chloroquine resistance of *Plasmodium falciparum* is reduced by its binding to plasma protein. Transactions of the Royal Society of Tropical Medicine and Hygiene, **87**, 303.

Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, **72**, 248-54.

Bray,P.G., Howells,R.E., Richie,G.Y. & Ward,S.A. (1992a) Rapid chloroquine efflux phenotype in both chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. A correlation of chloroquine sensitivity with energy dependent drug accumulation. Biochemical Pharmacology, **44(7)**, 1317-24.

Bray.P.G., Howells,R.E. & Ward,S.A. (1992b) Vacuolar acidification and CQ sensitivity in *Plasmodium falciparum*. Biochemical Pharmacology, **43**, 1219-1227.

Bray,P.G., Boulter,M.K., Richie,G.Y., Howells,R.E. & Ward.S.A. (1994) Relationship of global chloroquine transport and reversal of resistance in *Plasmodium falciparum*. Molecular and Biochemical Parasitology, **63**, 87-94.

Bray,P.G., Hawley,S.R., Mungthin,M. & Ward,S.A. (1996) Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*. Molecular Pharmacology, **50**, 1559-1566.

Bray,P.G., Mungthin,M., Ridley,R.G. & Ward,S.A. (1998) Access to hematin: the basis of chloroquine resistance. Molecular Pharmacology, **54**, 170-179.

Bray,P.G., Janneh,O., Raynes,K.J., Mungthin,M., Ginsburg,H. & Ward,S.A. (1999) Cellular uptake of chloroquine is dependent on binding to ferriprotoporphyrin IX and is independent of NHE activity in *Plasmodium falciparum*. The Journal of Cell Biology, **145(2)**, 363-376.

Bray,P.G., Saliba,K.J., Davies,J.D., Spiller,D.G., White,M.R.H., Kirk,K. & Ward,S.A. (2002) Distribution of the acridine orange fluorescence in *Plasmodium falciparum*-infected erythrocytes and its implications for the evaluation of digestive vacuolar pH. Molecular and Biochemical Parasitology, **119**, 302-304.

Breman,J.G. (2001) The ears of the hippopotamus: manifestations, determinants, and estimates of the malaria burden. The American Journal of Tropical Medicine and Hygiene, **64(1,2)S**, 1-11.

Canu-Gauci,D.F. & Riordan,J.R. (1987) Action of calcium antagonists on multidrug resistant cells. Biochemical Pharmacology, **36(13)**, 2115-2123.

Carlton,J., Mackinnon,M. & Walliker,D. (1998) A chloroquine resistance locus in the rodent malaria parasite *Plasmodium chabaudi*. Molecular and Biochemical Parasitology, **93**, 57-72.

Chen,N., Kyle,D.E., Pasay,C., Fowler,E.V., Baker,J., Peters,J.M. & Cheng,Q. (2003) *pfcrt* allelic types with two novel amino acid mutations in chloroquine-resistant *Plasmodium falciparum* isolates from the Philippines. Antimicrobial Agents and Chemotherapy, **47(11)**, 3500-3505.

Chong,C.R. & Sullivan Jr.,D.J. (2003) Inhibition of heme crystal growth by antimalarials and other compounds: implications for drug discovery. Biochemical Pharmacology, **66**, 2201-2212.

Chou,I., Chevli,R. & Fitch,C.D. (1980) Ferriprotoporphyrin IX fulfills the criteria for the identification as the chloroquine receptor of malaria parasites. Biochemistry, **19**, 1543-1549.

Chou,I. & Fitch,C.D. (1981) Mechanism of hemolysis induced by ferriprotoporphrin IX. Journal of Clinical Investigation, **68(3)**, 672-677.

Ciach,M., Zong,K., Kain,K.C. & Crandall,I. (2003) Reversal of mefloquine and quinine resistance in *Plasmodium falciparum* with NP30. Antimicrobial Agents and Chemotherapy, **47(8)**, 2393-2396.

Clarke E.C. Isolation and identification of drugs in pharmaceuticals, body fluids, and post-mortem material. 2nd Edition, Pharmaceutical Press, London, 1986. pgs. 1060-1061.

Cooper,R.A., Ferdig,M.T., Su,X-Z., Ursos,L.M.B., Mu,J., Nomura,T., Fujioka,H., Fidock,D.A., Roepe,P.D. & Wellems,T.E. (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereospecific quinine and quinidine responses in *Plasmodium falciparum*. Molecular Pharmacology, **61(1)**, 35-42.

Cornwell,M.M., Pastan,I. & Gottesman,M.M. (1987) Certain calcium channel blockers bind specifically to multidrug-resistant human KB carcinoma membrane vesicles and inhibit drug binding to P-glycoprotein. The Journal of Biological Chemistry, **262(5)**, 2166-2170.

Cowman,A.F., Karcz,S., Galatis,D. & Culvenor,J.G. (1991) A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. The Journal of Cell Biology, **113(5)**, 1033-1042.

Cowman,A.F., Galatis,D & Thompson,J.K. (1994) Selection of mefloquine resistance in *Plasmodium falciparum* is linked to the amplification of the *pfmdr1* gene and cross-resistance to halofantrine and quinine. Proceedings of the National Academy of Sciences of the USA, **91**, 1143-1147.

Crandall,I., Charuk,J. & Kain,K.C. (2000) Nonylphenolethoxylates as malarial chloroquine resistance reversal agents. Antimicrobial Agents and Chemotherapy, **44(9)**, 2431-2434.

Cremer,G., Basco,L.K., Le Bras,J., Camus,D. & Slomianny,C. (1995) *Plasmodium falciparum*: detection of P-glycoprotein in chloroquine-sensitive and chloroquine-resistant clones and isolates. Experimental Parasitology, **81**, 1-8.

Czejka,M.J., Zwoelfer,N. & Podesser,B. (1992) Red blood cell partitioning of gallopamil, verapamil and norverapamil. Il Farmaco, **47(3)**, 387-391.

De,D., Krogstad,F.M., Cogswell,F.B. & Krogstad,D.J. (1996) Aminoquinolines that circumvent resistance in *Plasmodium falciparum in vitro*. The American Journal of Tropical Medicine and Hygiene, **55(6)**, 579-583.

De Duve,C., De Barsy,T., Poole,B., Trouet,A., Tulkens,P. & Van Hoof,F. (1974) Commentary: Lysosomotropic agents. Biochemical Pharmacology, **23**, 2495-2531.

Deharo,E., Barkan,D., Krugliak,M., Golenser,J. & Ginsburg,H. (2003) Potentiation of the antimalarial action of chloroquine in rodent malaria by drugs known to reduce cellular glutathione levels. Biochemical Pharmacology, **66**, 809-817.

Desneves,J., Thorn,G., BermanA., Galatis,D., La Greca,N., Sinding,J., Foley,M., Deady,L.W., Cowman,A.F. & Tilley,L. (1996) Photoaffinity labeling of mefloquine-binding proteins in human serum, uninfected erythrocytes and *Plasmodium falciparum*-infected erythrocytes. Molecular and Biochemical Parasitology, **82**, 181-194.

Dorn,A., Stoffel,R., Matile,H., Bubendorf,A. & Ridley,R.G. (1995) Malarial haemozoin/β-haematin supports haem polymerization in the absence of protein. Nature, **374**, 269-271.

Drori,S., Eytan,G.D. & Assaraf,Y.G. (1995) Potentiation of anticancer-drug cytotoxicity by multidrug-resistance chemosensitisers involves alterations in membrane fluidity leading to increased membrane permeability. European Journal of Biochemistry, **228**, 1020-1029.

Dzekunov,S.M., Ursos,L.M. & Roepe,P.D. (2000) Digestive vacuolar pH of intact intraerythrocytic *P.falciparum* either sensitive or resistant to chloroquine. Molecular and Biochemical Parasitology, **110(1)**, 107-124.

Dzekunov,S.M., Ursos,L.M.B. & Roepe,P.D. (2002) Response to Bray et al. 'Distribution of acridine orange fluorescence in *P.falciparum*-infected erythrocytes and its implications for the evaluation of the digestive vacuole pH'. Molecular and Biochemical Parasitology, **119**, 307-309.

Egan,T.J., Ross,D.C. & Adams,P.A. (1994) Quinoline antimalarials inhibit spontaneous formation of β-haematin (malaria pigment). FEBS Letters, **352**, 54-57.

Egan,T.J., Hemplemann,E. & Mavuso,W.W. (1999) Characterisation of synthetic β-haematin and effects of the antimalarial drugs quinidine, halofantrine, desbutylhalofantrine and mefloquine on its formation. The Journal of Inorganic Biochemistry, **73**, 101-107.

Egan,T.J., Hunter,R., Kaschula,C.H., Marques,H.M., Misplon,A. & Walden,J. (2000) Structure-function relationships in aminoquinolines: effect of amino and chloro groups on quinoline-hematin complex formation, inhibition of β-hematin formation, and antiplasmodial activity. Journal of Medicinal Chemistry, 43, 283-291.

Egan,T.J., Combrinck,J.M., Egan,J. Hearne,G.R., Marques,H.M., Ntenteni,S, Sewell,B.T., Smith,P.J., Taylor,D., Van Schalkwyk,D.A. & Walden,J.C. (2002) Fate of haem iron in the malaria parasite *Plasmodium falciparum*. Biochemical Journal, **365**, 343-347.

Egan,T.J. (2002) Turning a chloroquine-resistance reverser into an antimalarial. Trends in Parasitology, **18(8)**, 344.

Ekong,R., Robson,K., Baker,D. & Warhurst,D. (1993) Transcripts of the multidrug resistance genes in chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. Parasitology, **106**, 107-115.

Famin,O., Krugliak,M. & Ginsburg,H. (1999) Kinetics of inhibition of glutathione-mediated degradation of ferriprotoporphyrin IX by antimalarial drugs. Biochemical Pharmacology, **58**, 59-68.

Famin,O. & Ginsburg,H. (2002) Differential effects of 4-aminoquinoline-containing antimalarial drugs on hemoglobin digestion in *Plasmodium falciparum*-infected erythrocytes. Biochemical Pharmacology, **63**, 393-398.

Ferrari,V. & Cutler,D.J. (1991) Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with *Plasmodium falciparum*. Biochemical Pharmacology, **42**, S167-S179.

Fidock,D.A., Nomura,T., Cooper,R.A., Su,X.-Z., Talley,A.K. Wellems,T.E. (2000a) Allelic modifications of the *cg2* and *cg1* genes do not alter the chloroquine response of drug-resistant *Plasmodium falciparum*. Molecular and Biochemical Parasitology, **111**, 1-10.

Fidock,D.A., Nomura,T., Talley,A.K., Cooper,R.A., Dzekunov,S.M., Ferdig,M.T., Ursos,L.M.B., Sidhu,A.B.S., Naude,B., Deitsch,K.W., Su,X-Z., Wootton,J.C., Roepe,P.D. & Wellems,T.E. (2000b) Mutations in the *P.falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. Molecular Cell, **6(4)**, 861-871.

Fitch,C.D. (1970) *Plasmodium falciparum* in Owl monkeys: drug resistance and chloroquine binding capacity. Science, **169**, 289-290.

Fitch,C.D. (1989) Ferriprotoporphyrin IX: role in chloroquine susceptibility and resistance in malaria. Malaria and the Red Cell 2, Alan R. Liss, Inc. 45-52.

Foley,M., Deady,L.W., Ng,K, Cowman,A.F. & Tilley,L. (1994) Photoaffinity labeling of chloroquine-binding proteins in *Plasmodium falciparum*. The Journal of Biological Chemistry, **269(9)**, 6955-6961.

Fojo,A., Akiyama,S-I., Gottesman,M.M. & Pastan,I. (1985) Reduced drug accumulation in multiply drug-resistant human KB carcinoma cell lines. Cancer Research, **45**, 3002-3007.

Foote,S.J., Thompson,J.K., Cowman,A.F. & Kemp,D.J. (1989) Amplification of the multidrug resistance gene in some chloroquine-resistant isolates of *P.falciparum*. Cell, **57**, 921-930.

Freese,J.A., Markus,M.B. & Golenser,J. (1991) *In vitro* sensitivity of southern African reference isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine. Bulletin of the World Health Organization, **69(6)**, 707-712.

Geary,T.G. & Jensen,J.B. (1983) Lack of cross-resistance to 4-aminoquinolines in chloroquine-resistant *Plasmodium falciparum in vitro*. The Journal of Parasitology, **69(1)**, 97-105.

Geary,T.G., Divo,A.A. & Jensen,J.B. (1989) Stage specific actions of antimalarial drugs on *Plasmodium falciparum* in culture. American Journal of Tropical Medicine and Hygiene, 40(3), 240-244.

Gerena,L., Bass,G.T., Kyle,D.E., Oduola,A.M.J., Milhous,W.K. & Martin,R.K. (1992) Fluoxetine hydrochloride enhances *in vitro* susceptibility to chloroquine in resistant *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy, **36(12)**, 2761-2765.

Ginsburg,H., Famin,O., Zhang,J-M. & Krugliak,M. (1998) Inhibition of glutathione-dependent degradation of heme by chloroquine and amodiaquine as a possible basis for their antimalarial mode of action. Biochemical Pharmacology, **56**, 1305-1313.

Ginsburg,H. & Stein,W.D. (1991) Kinetic modelling of chloroquine uptake by malaria-infected erythrocytes: assessment of factors that may determine drug resistance Biochemical Pharmacology, **41(10)**, 1463-1470.

Goldberg,D.E., Slater,A.F.G., Cerami,A. & Henderson,G.B. (1990) Haemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. Proceedings of the National Academy of Sciences of the USA, **87**, 2931-2935.

Grogl,M., Martin,R.K., Oduola,A.M.J., Milhous,W.K. & Kyle,D.E. (1991) Characteristics of multidrug resistance in *Plasmodium* and *Leishmania*: detection of P-glycoprotein-like components. The American Journal of Tropical Medicine and Hygiene, **45(1)**, 98-111.

Gross,A.S., Heuer,B. & Eichelbaum,M. (1988) Stereoselective protein binding of verapamil enantiomers. Biochemical Pharmacology, **37(24)**, 4623-4627.

Guan,J., Kyle,D.E., Gerena,L., Zhang,Q., Milhous,W.K. & Lin,A.J. (2002) Design, synthesis and evaluation of new chemosensitisers in multi-drug-resistant *Plasmodium falciparum*. Journal of Medicinal Chemistry, **45**, 2741-2748.

Haruki,K., Bray,P.G., Ono,M. & Ward,S.A. (2000) Potent enhancement of the sensitivity of *Plasmodium falciparum* to chloroquine by the bisbenzylisoquinoline alkaloid Cepharanthin. Antimicrobial Agents and Chemotherapy, **44(10)**, 2706-2708.

Hawley,S.R., Bray,P.G., O'Neill,P.M., Park,B.K. & Ward,S.A. (1996) The role of drug accumulation in 4-aminoquinoline antimalarial potency: the influence of structural substitution and physicochemical properties. Biochemical Pharmacology, **52**, 723-733.

Hawley,S.R., Bray,P.G., Mungthin,M., Atkinson,J.D., O'Neill,P.M. & Ward,S.A (1998) Relationship between antimalarial drug activity, accumulation and inhibition of heme polymerization in *Plasmodium falciparum in v*itro. Antimicrobial Agents and Chemotherapy, **42(3)**, 682-686.

Herwaldt,B.L., Schlesinger,P.H. & Krogstad,D.J. (1990) Accumulation of chloroquine by membrane preparations from *Plasmodium falciparum*. Molecular and Biochemical Parasitology, **42**, 257-268.

Hoppe,H.C., van Schalkwyk,D.A., Wiehart, U.I.M., Meredith,S.A., Egan,J. & Weber,B.W. (2004) Antimalarial quinolines and artemisinin inhibit endocytosis in *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy, **48(7)**, 2370-2378.

Hunt,P., Cravo,P.V.L., Donleavy,P., Carlton,J.M-R. & Walliker,D. (2004) Chloroquine resistance in *Plasmodium chabaudi*: are chloroquine-resistance transporter (*crt*) and multi-drug resistance (*mdr1*) orthologues involved? Molecular and Biochemical Parasitology, **133**, 27-35.

Jacobs,G.H., Oduola,A.M.J., Kyle,D.E., Milhous,W.M., Martin,S.K. & Aikawa,M. (1988) Ultrastructural Study of the effects of chloroquine and verapamil on *Plasmodium falciparum*. The American Journal of Tropical Medicine and Hygiene, **39(1)**, 15-20.

Juliano,R.L. & Ling,V. (1976) A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochimica et Biophysica Acta, **455**, 152-162.

Kalkinidis,M., Klonis,N., Tilley,L. & Deady,L.W. (2002) Novel phenothiazine antimalarials: synthesis, antimalarial activity and inhibition of the formation of β-haematin. Biochemical Pharmacology, **63**, 833-842.

Kaschula,C.H., Egan,T.J., Hunter,R., Basilico,N., Parapini,S., Taramelli,D., Pasini,E. & Monti,D. (2002) Structure-activity relationships in 4-aminoquinoline antiplasmodials. The role of the group at the 7-position. Journal of Medicinal Chemistry, **45**, 3531-3539.

Klokouzas,A., Tiffert,T., Van Schalkwyk,D.A., Wu,C-P., van Veen,H.W., Barrand,M.A. & Hladky,S.B. *Plasmodium falciparum* expresses a multidrug resistance associated protein. Biochemical and Biophysical Research Communications, **321**, 197-201.

Krogstad,D.J., Schlesinger,P.H. & Gluzman,I.Y. (1985) Antimalarials increase vesicle pH in *Plasmodium falciparum*. The Journal of Cell Biology, **101**, 2302-2309.

Krogstad,D.J., Gluzman,I.Y., Kyle,D.E., Oduola,A.M.J., Martin,S.K., Milhous,W.K. & Schlesinger,P.H. (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. Science, **238**, 1283-1285.

Krogstad,D.J., Gluzman,I.Y., Herwaldt,B.L., Schlesinger,P.H. & Wellems,T.E. (1992) Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. Biochemical Pharmacology, **43(1)**, 57-62.

Kyle,D.E., Oduola,A.M., Martin,S.K. & Milhous,W.K. (1990) *Plasmodium falciparum*: modulation by calcium antagonists of resistance to chloroquine, desethylchloroquine, quinine and quinidine *in vitro*. Transactions of the Royal Society of Tropical Medicine and Hygiene, **84(4)**, 474-478.

Kyle,D.E., Milhous,W.K. & Rossan,R.N. (1993) Reversal of *Plasmodium falciparum* resistance to chloroquine in Panamanian *Aotus* Monkeys. The American Journal of Tropical Medicine and Hygiene, **48(1)**, 126-133.

Laemmli,U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, **227(5259)**, 680-685.

Lambros,C. & Vanderberg,J.P. (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. Journal of Parasitology, **65(3)**, 418-420.

Lehnert,M., de Giuli,R., Kunke,K., Emerson,S., Dalton,W.S. & Salmon,S.E. (1996) Serum can inhibit reversal of multidrug resistance by chemosensitisers. European Journal of Cancer, **32A(5)**, 862-867.

Lelong,I.H., Guzikowski,A.P., Haugland,R.P., Pastan,I., Gottesman,M.M. & Willingham,M.C. (1991) Fluorescent verapamil derivative for monitoring activity of the multidrug transporter. Molecular Pharmacology, **40**, 490-494.

Li,G-D., Qu,F-Y., Chen,X. & Ni,C-R. (1993) A 54-kDa protein overexpressed by chloroquine-resistant *Plasmodium berghei* ANKA strain. Acta Pharmacologica Sinica, **14(1)**, 9-12.

Lim,A.S.Y., Galatis,D. & Cowman,A.F. (1996) *Plasmodium falciparum*: Amplification and overexpression of *pfmdr1* is not necessary for increased mefloquine resistance. Experimental Parasitology, **83**, 295-303.

Loria,P., Miller,S., Foley,M. & Tilley,L. (1999) Inhibition of the peroxidative degradation of haem as the basis of action of chloroquine and other antimalarials. Biochemical Journal, **339**, 363-370.

Makler,M.T., Ries,J.M., Williams,J.A., Bancroft,J.E., Piper,R.C., Gibbons,B.L. & Hinrichs,D.J. (1993) Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. The American Journal of Tropical Medicine and Hygiene, **48(6)**, 739-74

Martin,S.K., Oduola,A.M.J. and Milhous, W.K. (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. Science, **235**, 899-901.

Martiney,J.A., Cerami,A. & Slater,A.F.G. (1995) Verapamil reversal of chloroquine resistance in the malaria parasite *Plasmodium falciparum* is specific for resistant

parasites and independent of the weak base effect. The Journal of Biological Chemistry, **270(38)**, 22393-22398.

Mehlotra,R.K., Fujioka,H., Roepe,P.D., Janneh,O., Ursos,L.M.B., Jacobs-Lorena,V., McNamara,D.T., Bockarie,M.J., Kazura,J.W., Kyle,D.E., Fidock,D.A. & Zimmerman,P.A. (2001) Evolution of a unique *Plasmodium falciparum* chloroquine-resistance phenotype in association with *pfcrt* polymorphism in Papua New Guinea and South America. Proceedings of the National Academy of Sciences of the USA, **98(22)**, 12689-12694.

Menezes,C.M.S., Kirchgatter,K., Di Santi,S.M., Savalli,C., Monteiro,F.G., Paula,G.A. & Ferreira,E.I. (2002) *In vitro* chloroquine resistance modulation study on fresh isolates of Brazilian *Plasmodium falciparum*: intrinsic antimalarial activity of phenothiazine drugs. Memorias do Instituto Oswaldo Cruz, **97(7)**, 1033-1039.

Menezes,C.M.S., Kirchgatter,K., Di Santi,S.M., Savalli,C., Monteiro,F.G., Paula,G.A. & Ferreira,E.I. (2003) *In vitro* evaluation of verapamil and other modulating agents in Brazilian chloroquine-resistant *Plasmodium falciparum* isolates. Revista da Sociedade Brasileira de Medicina Tropical, **36(1)**, 5-9.

Menting,J.G.T., Tilley,L., Deady,L.W., Ng,K., Simpson,R.J., Cowman,A.F. & Foley,M. (1997) The antimalarial drug, chloroquine, interacts with lactate dehydrogenase from *Plasmodium falciparum*. Molecular and Biochemical Parasitology, **88**, 215-224.

Miki,A., Tanabe,K., Nakayama,T., Kiryon,C. & Ohsawa,K. (1992) *Plasmodium chabaudi*: association of reversal of chloroquine resistance with increased accumulation of chloroquine in resistant parasites. Experimental Parasitology, **74**, 134-142.

Mosmann,T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, **65**, 55-63.

Ncokazi,K.K. & Egan,T.J. (2005) A colorimetric high-throughput β-haematin inhibition screening assay for use in the search for antimalarial compounds. Analytical Biochemistry, **338(2)**, 306-319.

Ndifor,A.M., Howells,R.E., Bray,P.G., Ngu,J.L. & Ward,S.A. (1993) Enhancement of drug usceptibility in *Plasmodium falciparum in vitro* and *Plasmodium berghei in vivo* by mixed-function oxidase inhibitors. Antimicrobial Agents and Chemotherapy, **37(6)**, 1318-1323.

Nzila,A., Mberu,E., Bray,P., Kokwaro,G., Winstanley,P., Marsh,K. & Ward,S. (2003) Chemosensitization of *Plasmodium falciparum* by probenecid *in vitro*. Antimicrobial Agents and Chemotherapy, **47(7)**, 2108-2112.

Oduola,A., Weatherly,N., Bowdre,J. & Desjardins,R. (1988) *Plasmodium falciparum*: cloning by single erythrocyte micromanipulation and heterogeneity *in vitro*. Experimental Parasitology, **66**, 86-95.

Oduola,A.M.J., Omitowoju,G.O., Gerena,L., Kyle,D.E., Milhous,W.K., Sowunmi,A. & Salako,L.A. (1993) Reversal of mefloquine resistance with penfluridol in isolates of *Plasmodium falciparum* from south-west Nigeria. Transactions of the Royal Society of Tropical Medicine and Hygiene, **87**, 81-83.

Oduola,A.M.J., Sowunmi,A., Milhous,W.K., Brewer,T.G., Kyle,D.E., Gerena,L., Rossan,R.N., Salako,L.A. & Schuster,B.G. (1998) *In vitro* and *in vivo* reversal of chloroquine resistance in *Plasmodium falciparum* with promethazine. The American Journal of Tropical Medicine and Hygiene, **58(5)**, 625-629.

Ohsawa,K., Tanabe,K., Kimata,I. & Miki,A. (1991) Ultrastructural changes associated with reversal of chloroquine resistance by verapamil in *Plasmodium falciparum*. Parasitology, **103**, 185-189.

Pagola,S., Stephens,P.W., Bohle,D.S., Kosar,A.D. & Madsen,S.K. (2000) The structure of malaria pigment (β-haematin). Nature, **404**, 307-310.

Payne,D. (1987) Spread of chloroquine resistance in *Plasmodium falciparum*. Parasitology Today, **3**, 241-246.

Peters,W., Ekong,R., Robinson,B.L., Warhurst,D.C. & Pan,X-Q. (1990) The chemotherapy of rodent malaria. XLV. Reversal of chloroquine resistance in rodent and human *Plasmodium* by antihistaminic agents. Annals of Tropical Medicine and Parasitology, **84(6)**, 541-551.

Peters,W. & Robinson,B.L. (1991) The chemotherapy of rodent malaria. XLVI. Reversal of mefloquine resistance in rodent *Plasmodium*. Annals of Tropical Medicine and Parasitology, **85(1)**, 5-10.

Platel,D.F., Mangou,F. & Tribouley-Duret,J. (1998) High-level chloroquine resistance of *Plasmodium berghei* is associated with multiple drug resistance and loss of reversal by calcium antagonists. International Journal of Parasitology, **28**, 641-651.

Pouliot,J-F., L'Heureux,F., Liu,Z., Prichard,R.K. & Georges,E. (1997) Reversal of P-glycoprotein-associated multidrug resistance by Ivermectin. Biochemical Pharmacology, **53**, 17-25.

Pradines,B., Alibert,S., Houdoin,C., Santelli-Rouvier,C., Mosnier,J., Fusai,T., Rogier,C., Barbe,J. & Parzy,D. (2002) *In vitro* increase in chloroquine accumulation induced by dihydroethano- and ethenoanthracene derivatives in *Plasmodium*

falciparum-parasitized erythrocytes. Antimicrobial Agents and Chemotherapy, **46(7)**, 2061-2068.

Prescott,L.M., Harley,J.P. & Klein,D.A. 1993. Microbiology Second Edition: Wm. C. Brown Publishers. pp 793-794.

Price,R.N., Uhlemann,A-C., Brockman,A., McGready,R., Ashley,E., Phaipun,L., Patel,R., Laing,K., Looareesuwan,S., White,N.J. & Krishna,S. (2004) Mefloquine resistance in *Plasmodium falciparum* and increased *pfmdr1* gene copy number. Lancet, **364**, 438-447.

Raynes,K.J., Bray,P.G. & Ward,S.A. (1999) Altered binding of chloroquine to ferriprotoporphyrin IX is the basis of chloroquine resistance. Drug Resistance Updates, **2**, 97-103.

Reed,M.B., Saliba,K,J., Caruana,S.R., Kirk,K. & Cowman,A.F. (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. Nature, **403**, 906-909.

Ridley,R.G., Hofheinz,W., Matile,H., Jaquet,C., Dorn,A., Masciadri,R., Jolidon,S., Richter,W.F., Guenzi,A., Girometta,M-A., Urwyler,H., Huber,W., Thaithong,S. & Peters,W. (1996) 4-Aminoquinoline analogs of chloroquine with shortened side chains retain activity against chloroquine-resistant *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy, **40(8)**, 1846-1854.

Ridley,R.G. (1998) Malaria: Dissecting chloroquine resistance. Current Biology, **8**, R346-R349.

Riffkin,C.D., Chung,R., Wall,D.M., Zalcberg,J.R., Cowman,A.F., Foley,M. & Tilley,L. (1996) Modulation of the function of human MDR1 P-glycoprotein by the antimalarial drug mefloquine. Biochemical Pharmacology, **52**, 1545-1552.

Rogan,A.M., Hamilton,T.C., Young,R.C., Klecker,R.W.Jr & Ozols,R.F. (1984) Reversal of adriamycin resistance by verapamil in human ovarian cancer. Science, **224**, 994-996.

Rubio,J.P. & Cowman,A.F. (1994) *Plasmodium falciparum*: the *pfmdr2* protein is not overexpressed in chloroquine-resistant isolates of the malaria parasite. Experimental Parasitology, **79**, 137-147.

Safa,A.R., Glover,C.J., Meyers,M.B., Biedler,J.L. & Felsted,R.L. (1986) Vinblastine photoaffinity labeling of a high molecular weight surface membrane glycoprotein specific for multidrug-resistant cells. The Journal of Biological Chemistry, **261(14)**, 6137-6140.

Safa,A.R., Glover,C.J., Sewell,J.L., Meyers,M.B., Biedler,J/L. & Felsted,R.L. (1987) Identification of the multidrug resistance-related membrane glycoprotein as an acceptor for calcium channel blockers. The Journal of Biological Chemistry, **262(16)**, 7884-7888.

Safa,A.R. (1988) Photoaffinity labeling of the multidrug-resistance-related P-glycoprotein with photoactive analogs of verapamil. Proceedings of the National Academy of Sciences of the USA, **85**, 7187-7191.

Safa,A.R. (1999) Photoaffinity analogs for multidrug resistance-related transporters and their use in identifying chemosensitizers. Drug Resistance Updates, **2**, 371-381.

Saliba,K, Folb,P.I. & Smith,P.J. (1998) Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance. Biochemical Pharmacology, **56**, 313-320.

Sanchez,C.P., Wunsch,S. & Lanzer,M. (1997) Identification of a chloroquine importer in *Plasmodium falciparum*. The Journal of Biological Chemistry, **272(5)**, 2652-2658.

Sanchez,C.P., Stein,W. & Lanzer,M. (2003) Trans stimulation provides evidence for a drug efflux carrier as the mechanism of chloroquine resistance in *Plasmodium falciparum*. Biochemistry, **42**, 9383-9394.

Scheibel,L.W., Colombani,P.M., Hess,A.D., Aikawa,M., Atkinson,C.T. & Milhous,W.K. (1987) Calcium and calmodulin antagonists inhibit human malaria parasites (*Plasmodium falciparum*): implications for drug design. Proceedings of the National Academy of Sciences of the USA, **84**, 7310-7314.

Sehested,M., Skovsgaard,T., Buhl Jensen,P., Demant,E.J.F., Friche,E. & Bindslev,N. (1990) Transport of the multidrug resistance modulators verapamil and azidopine in wild type and daunorubicin resistant Ehrlich ascites tumour cells. British Journal of Cancer, **62**, 37-41.

Sidhu,A.B.S, Vernier-Pinard,D. & Fidock,D.A. (2002) Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by *pfcrt* mutations. Science, **298**, 210-213.

Singh,N. & Puri,S.K. (2000) Interaction between chloroquine and diverse pharmacological agents in chloroquine resistant *Plasmodium yoelii nigeriensis*. Acta Tropica, **77**, 185-193.

Slater,A.F.G. & Cerami,A. (1992) Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. Nature, **355**, 167-169.

Sowunmi,A., Oduola,A.M.J., Ogundahunsi,O.A.T., Falade,C.O., Gbotosho,G.O. & Salako,L.A. (1997) Enhanced efficacy of chloroquine-chlorpheniramine combination

in acute uncomplicated *falciparum* malaria in children. Transactions of the Royal Society of Tropical Medicine and Hygiene, **91**, 63-67.

Su,X-Z, Kirkman,L.S. & Wellems,T.E. (1997) Complex polymorphisms in a ~330kDa protein are linked to chloroquine-resistant *P.falciparum* in Southeast Asia and Africa. Cell, **91**, 593-603.

Tanabe,K., Kato,M., Izumo,A., Hagiwara,A. & Doi,S. *Plasmodium chabaudi*: *in vivo* effects of Ca^{2+} antagonists on chloroquine-resistant and chloroquine-sensitive parasites. Experimental Parasitology, **70**, 419-426.

Taylor,D., Walden,J.C., Robins,A.H. & Smith,P.J. (2000) Role of the neurotransmitter reuptake-blocking activity of antidepressants in reversing chloroquine resistance in *Plasmodium falciparum*. Antimicrobial Agents and Chemotherapy, **44(10)**, 2689-2692.

Thaithong,S. & Beale,G.H. (1981) Resistance of ten Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. Transactions of the Royal Society of Tropical Medicine and Hygiene, **75**, 271-273.

Tran,C.V. & Saier,M.H.Jr. (2004) The principal chloroquine resistance protein of *Plasmodium falciparum* is a member of the drug/metabolite transporter superfamily. Microbiology Comments, 149-151.

Ursos,L.M.B., Dzekunov,S.M. & Roepe,P.D. (2000) The effects of chloroquine and verapamil on digestive vacuolar pH of *P.falciparum* either sensitive or resistant to chloroquine. Molecular and Biochemical Parasitology, **110**, 125-134.

Van Schalkwyk,D.A., Walden,J.C. & Smith,P.J. (2001) Reversal of chloroquine resistance in *Plasmodium falciparum* using combinations of chemosensitisers. Antimicrobial Agents and Chemotherapy, **45(11)**, 3171-3174.

Waller,K.L., Muhle,R.A., Ursos,L.M., Horrocks,P., Verdier-Pinard,D., Sidhu,A.B.S., Fujioka,H., Roepe,P.D. & Fidock,D.A. (2003) Chloroquine resistance modulated *in vitro* by expression levels of the *Plasmodium falciparum* chloroquine resistance transporter. The Journal of Biological Chemistry, **278(35)**, 33593-33601.

Ward,S.A. & Bray,P.G. (2001) Is reversal of chloroquine resistance ready for the clinic? The Lancet, **357**, 904.

Warhurst,D.C. (2003) Polymorphism in the *Plasmodium falciparum* chloroquine-resistance transporter protein links verapamil enhancement of chloroquine sensitivity with the clinical efficacy of amodiaquine.
http://www.malariajournal.com/content/2/1/31

Warhurst,D.C., Craig,J.C., Adagu,I.S., Meyer,D.J. & Lee,S.Y. (2003) The relationship of physico-chemical properties and structure to the differential antiplasmodial activity of the cinchona alkaloids. http://www.malariajournal.com/content/2/1/26

Warr,J.R., Anderson,M. & Fergusson,J. (1988) Properties of verapamil-hypersensitive multidrug-resistant Chinese hamster ovary cells. Cancer Research, **48**, 4477-4483.

Warsame M, Wernsdorfer WH, Bjorkman A (1992) Lack of effect of desipramine on the response to chloroquine of patients with chloroquine-resistant *falciparum* malaria. Transactions of the Royal Society Tropical Medicine and Hygiene, **86**, 235-236.

Watt,G., Long,G.W., Grogl,M. & Martin,S.K. (1990) Reversal of drug-resistant *falciparum* malaria by calcium antagonists: potential for host cell toxicity. Transactions of the Royal Society of Tropical Medicine and Hygiene, **84**, 187-190.

Wellems,T.E., Panton,L.J., Gluzman,I.Y., de Rosario,V.E., Gwadz,R.W., Walker-Jonah,A. & Krogstad,D.E. (1990) Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. Nature, **345**, 253-255.

Wellems,T.E., Walker-Jonah,A. & Panton,L.J. (1991) Genetic mapping of the chloroquine-resistance locus on *Plasmodium falciparum* chromosome 7. Proceedings of the National Academy of Sciences of the USA, **88**, 3382-3386.

Williams,H.L., Johnson,D.J., Okoye,V.C.N. and Martin,S.K. (1992) Treatment of chloroquine-resistant malaria in monkeys with a drug combination that reverses resistance *in vitro*. Annals of Tropical Medicine and Parasitology, **86(5)**, 467-473.

Wilson,C.M., Serrano,A.E., Wasley,A., Bogenschutz,M.P., Shankar,A.H. & Wirth,D.F. (1989) Amplification of a gene related to mammalian mdr genes in drug-resistant *Plasmodium falciparum*. Science, **244**, 1184-1186.

Wunsch,S., Sanchez,C.P., Gekle,M., GroBe-Wortmann,L., Wiesner,J. & Lanzer,M. (1998) Differential stimulation of the Na+/H+ exchanger determines chloroquine uptake in *Plasmodium falciparum*. The Journal of Biological Chemistry, **140(2)**, 335-345.

Yayon,A., Timberg,R., Friedman,S. & Ginsburg,H. (1984a) Effects of chloroquine on the feeding mechanism of the intraerythrocytic parasite *Plasmodium falciparum*. Journal of Protozoology, **31**, 367-372.

Yayon,A., Cabantchik,Z.I. & Ginsburg,H. (1984b) Identification of the acidic compartment of *Plasmodium falciparum*-infected erythrocytes as the target of the antimalarial drug chloroquine. The EMBO Journal, **3(11)**, 2695-2700.

Yayon,A., Cabantchik,I. & Ginsburg,H. (1985) Susceptibility of human malaria parasites to chloroquine is pH dependent. Proceedings of the National Academy of Sciences of the USA, **82**, 2784-2788.

Ye,Z. & Van Dyke,K. (1988) Reversal of chloroquine resistance in *falciparum* malaria independent of calcium channels. Biochemical and Biophysical Research Communications, **155(1)**, 476-481.

Ye,Z., Van Dyke,K., Spearman,T. & Safa,A.R. (1989) ^{3}H-Azidopine photoaffinity labeling of high molecular weight proteins in chloroquine resistant falciparum malaria. Biochemical and Biophysical Research Communications, **162(2)**, 809-813.

Yusa,K. & Tsuruo,T. (1989) Reversal mechanism of multidrug resistance by verapamil: direct binding of verapamil to P-glycoprotein on specific sites and transport of verapamil outward across the plasma membrane of K562/ADM cells. Cancer Research, **49**, 5002-5006.

Zacherl,J., Hamilton,G., Thalhammer,T., Riegler,M., Cosentini,E.P., Ellinger,A., Bischof,G., Schweitzer,M., Teleky,B., Koperna,T. & Wenzl,E. (1994) Inhibition of P-glycoprotein-mediated vinblastine transport across HCT-8 intestinal carcinoma monolayers by verapamil, cyclosporine A and SDZ PSC 833 in dependence on extracellular pH. Cancer Chemotherapy and Pharmacology, **34**, 125-132.

Zaman,G.J.R., Flens,M.J., Van Leusden,M.R., De Haas,M., Mulder,H.S., Lankelma,J., Pinedo,H.M., Scheper,R.J., Baas,F., Broxterman,H.J. & Borst,P. (1994) The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. Proceedings of the National Academy of Science of the USA, **91**, 8822-8826.

Zhang,J., Krugliak,M. & Ginsburg,H. (1999) The fate of ferriprotoporphyrin IX in malaria infected erythrocytes in conjunction with the mode of action of antimalarial drugs. Molecular and Biochemical Parasitology, **99**, 129-141.

Zhang,H., Paguio,M. & Roepe,P.D. (2004) The antimalarial drug resistance protein *Plasmodium falciparum* chloroquine resistance transporter binds chloroquine. Biochemistry, **43**, 8290-8296.