A Study of The Antimalarial Action of Pyronaridine and its Accumulation in *Plasmodium falciparum*

Chikumbusko J. Mtegha

Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY
in the Department of Pharmacology
UNIVERSITY OF CAPE TOWN

Supervisor
Professor Peter Folb

February 2004

This is in memory of Violet Mtegha

Research is essentially a dialogue with nature. The important thing is not to wonder about nature's answer, for she is always honest, but closely examine your questions to her...

Albert Szent-Gyorgi

ACKNOWLEDGEMENTS

I would like to acknowledge the following people for their contribution to this thesis:

My supervisor Professor PI Folb, for welcoming me to the department, giving me this opportunity and for his dedicated supervision.

Associate Professor TJ Egan and Kanyile from the chemistry department for guidance and advice on the heme binding work.

Dr Cridland and Associate Professor PJ Smith for their assistance throughout the course and their advice on the final draft.

Colleagues (past and present) – Fraction, Waako, Don, Jason, Dale, Denise, Siya, Malefa, Cailean, Carmen, Justin, Rudi, Gary, Heinrich, Grant, Sandy, Noluntu, Gilbert, Benni, Jenny, Helen, Karen, James, Susan, Tebogo, Eliya, Natalie, Quinton, Ushi, Brandon, Magula, Theo, Lazarus.

Staff members - Jessica, Sumaya, Noor, Noel, Alicia, Sherlayne, Sindiswa, Faldiela, Afia, Ludwig, Jean, Marion, Linda, Colleen, Diane, Nanette, Dr Barnes, Dr Robbins, Irene, Marc, Joe, Ushma, Bill, Carol.

Special thanks – My father for his support throughout this period. Extra special thanks to my brothers Tawonga and Marumbo and my good friends Salie, Kitso, and Bongi for their constant encouragements. I also thank the following for their thoughts and prayers: Prayer warriors (Abidjan), Zilanie, Miss Browne-Marke, Auntie Loveness, Taweni & Reggie, Patience & Victor, Miriam, Mkhuzi, Tamara, Faith, Hawa & Zwai Mahoi, Robert, Sonia, Carlos.

All praises to JAH who gives wisdom and understanding to all who humble themselves and seek Him.

ABSTRACT

A Study of the Antimalarial Action of Pyronaridine and its Accumulation into *Plasmodium falciparum*.

Pyronaridine (PND), a hydroxyanilino-benzonaphthyridine derivative and also a structural analogue of chloroquine, was synthesized in China in the 1970s as a result of a search for alternative drugs to treat chloroquine-resistant (CQR) *Plasmodium falciparum* malaria. It is a potent schizonticidal agent active against the erythrocytic stages of malarial parasites. It is efficacious against CQR falciparum malaria both *in vivo* and *in vitro*. In contrast to the wealth of data available about CQ accumulation and its relationship to activity, little is known about the uptake characteristics and mechanism of action of PND. In this study, the time, dose and stage specific actions of PND, the accumulation of PND in *Plasmodium falciparum*, and the correlation, if any, to activity were investigated in order to gain a better understanding of the mechanism of action of PND and to better understand the reasons for its superior activity over CQ. A comparison of the ability of PND and CQ to bind heme and inhibit its crystallization was done.

PND was shown to have high *in vitro* antimalarial activity against chloroquine-sensitive (CQS) and CQR strains of *Plasmodium falciparum*, with IC_{50} values comparable to values quoted in literature.

CQR parasites had larger IC_{50} values than CQS parasites, which indicates that loss of sensitivity to CQ may be associated with an altered sensitivity to PND. Schizonts and trophozoites were more sensitive to PND than the ring-stage of the parasite, and irreversible damage is done to the parasites within 1 hour of exposure to PND. PND accumulation, in parasitized red blood cells, was demonstrated to be saturable, temperature-, glucose- and pH-dependent. Evidence is presented for an intraparasitic receptor, which PND binds with an affinity in the order of 10^{-8}M. Competition studies showed that CQ and AMQ inhibited PND accumulation, suggesting that PND may share the same binding site as the 4-aminoquinolines. There was no significant difference in the amount of PND accumulated by either CQS- or CQR-*Plasmodium falciparum*. In CQS parasites, PND accumulated 14 times less than CQ, while in CQR parasites there was no significant difference in accumulation levels between the two drugs. The use of bafilomycin A1 in combination with PND in an *in vitro* sensitivity assay produced an apparent reduction in sensitivity of a CQS strain to PND, suggesting that the pH-dependent component of PND uptake is important for its antimalarial action. These results suggest that PND might have a similar mechanism of action to 4-aminoquinolines. PND was shown to bind heme with greater affinity than CQ and to be a more potent inhibitor of beta-hematin formation than CQ. This might, in part, explain the greater in vitro activity of PND compared with CQ.

Table of Contents

LIST OF FIGURES & TABLES

1

2

3

4

LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
ADP	adenosine diphosphate
AMQ	amodiaquine
APADH	reduced 3-acetylpyridine adenine dinucleotide
AR	accumulation ratio
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
BfA1	bafilomycin A1
BHIA	beta-hematin inhibitory activity
B_{max}	maximum binding capacity
°C	degrees Celsius
CQ	chloroquine
CQR	chloroquine-resistant
CQS	chloroquine-sensitive
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
f	femto
FPIX	ferriprotoporphyrin IX
g	gram
HEPES	N-[2-hydroxyethyl]piperazine-N'-[2-ethansulphonic acid]
hr	hour
IC_{50}	concentration to achieve 50% inhibition
K_D	association/dissociation constant
LDH assay	lactate dehydrogenase assay
M	molar
m	milli
μ	micro
MDR	multidrug resistant
min	minute(s)

ml	milliliter
µl	micro liter
MQ	mefloquine
nM	nanomolar
n	number of replicates
NBT	nitro blue tetrazolium
P.	Plasmodium
PBS	phosphate buffered saline
PES	phenazine ethosulfate
P. falciparum	*Plasmodium falciparum*
Pfmdr 1	*Plasmodium falciparum* multidrug resistance gene 1
Pgh1	P-glycoprotein homologue 1
pH	negative logarithm of the hydrogen concentration ion
PND	pyronaridine
pRBC(s)	parasitized red blood cell(s)
pLDH	parasite lactate dehydrogenase
RBC(s)	red blood cell(s)
SEM	standard error of the mean

1

BACKGROUND

Malaria, a disease caused by the protozoan parasites of the genus *Plasmodium*, makes ill and kills more people than any other infectious disease (Wernsdorfer and McGregor 1988). In humans, malaria is caused by four species of *Plasmodium;* namely, *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *P. falciparum* is the most troublesome as it is the species that causes life-threatening illness through its invasion and destruction of the red blood cells, and the consequent release of cytokines.

1.1 Parasite biology

The life cycle of the *Plasmodium* parasite, which requires both a mosquito and human host, is illustrated in figure 1. Mosquitoes inject parasites (in the form of sporozoites) into the subcutaneous tissue of humans, from where the sporozoites travel to the liver. Inside the liver cells each sporozoite develops into tens of thousands of

merozoites that can each invade a red blood cell (RBC) after release from the liver. The disease begins only once the asexual parasite multiplies in RBCs. *Plasmodium falciparum* develops over 48 hours in RBCs, producing approximately 20 merozoites per mature parasite, with each merozoite able to invade other RBCs. A small proportion of asexual parasites convert into gametocytes that are essential for transmitting the infection to others through female *Anopheline* mosquitoes. RBCs infected with *P. falciparum* (pRBCs) bind to vascular endothelial cells in various organs including the heart, lungs, brain, liver, kidneys, subcutaneous tissues and the placenta, which leads to much of the pathology of the disease (Sherman IW 1998).

As the malaria parasite matures from the ring to the schizont stage of its erythrocytic cycle it ingests more than half of the host cell hemoglobin, which it uses as a food source (Goldberg and Slater 1992). The hemoglobin is digested by specialized enzymes, plasmepsins I and II and falcipain, in the acidic digestive food vacuole into small fragments that are transported to the parasite cytosol for final degradation to amino acids utilized by the growing parasite (Rosenthal *et al* 1988, Goldberg *et al* 1990, Rosenthal and Nelson 1992, Francis *et al* 1994, Gluzman *et al* 1994). In the process of hemoglobin catabolism a highly reactive heme species is released in the form of ferriprotoporphyrin IX (FPIX) that is potentially toxic to the parasite (Orjih *et al* 1981, Chou and Fitch 1981, Choi and Mego 1987). The malaria parasite is well adapted to keep the steady-state

concentration of the transient, toxic form of FPIX at tolerable concentrations; otherwise, it would commit suicide by degrading hemoglobin. The parasite deals with FPIX by converting it into an insoluble crystalline material called hemozoin or malaria pigment (Zhang *et al* 1999, Sullivan 2002, Egan *et al* 2002,). Hemozoin pigment is a complex of heme dimers linked through an iron-carboxylate bond, formed by the linking of the central ferric iron of one heme dimer with the propionate side chain of another heme (Slater *et al* 1991, Bohle *et al* 1997, Pagola *et al* 2000). The exact mechanism of hemozoin formation is not fully known but the process resembles a type of biomineralisation process that may require scaffold proteins and lipids as a nucleation center or a template to initiate the process (Sullivan *et al* 1996, Fitch *et al* 1999, Lynn *et al* 1999, Egan *et al* 2001, Papalexis *et al* 2001). Hemozoin levels are found to correlate well with hemoglobin digestion and the efficacy of hemozoin production is stage-dependent, being less effective at the ring stage and becoming more adept as the parasite matures (Orjih *et al* 1994, Zhang *et al* 1999). The parasite may have alternative pathways for the neutralization of the highly reactive FPIX. It has been suggested that not all FPIX generated by the degradation of hemoglobin is incorporated in hemozoin (Wood and Eaton 1993, Ginsburg *et al* 1998, Zhang *et al* 1999). Because FPIX is an amphipathic compound with a great tendency to bind non-specifically to proteins and to dissolve in membranes (Cannon *et al* 1984, Rose *et al* 1985), it has been argued that FPIX should be able to dissolve into

and translocate across the food vacuole membrane into the parasite cytosol (Ginsburg *et al* 1999), where it is believed to be decomposed by reduced glutathione in *P. falciparum* or heme-oxygenase in *P. knowlesi* (Atamna and Ginsburg 1995, Ginsburg *et al* 1998, Zhang *et al* 1999). The mechanism of FPIX destruction by glutathione has not been fully described. It has been suggested that hydrogen peroxide may also be involved in the degradation of FPIX inside the food vacuole (Orjih *et al* 1988, Loria *et al* 1999). These two alternative pathways for heme detoxification have been disputed on the grounds that hemozoin is the only detectable iron species in trophozoites, and that direct evidence for the iron that would be released during glutathione and hydrogen peroxide degradation is lacking (Egan 2001, Egan *et al* 2002). Hemoglobin digestion and FPIX detoxification are crucial processes for the malaria parasite and, as will be discussed later, they play important roles in the mechanism of action of antimalarial drugs.

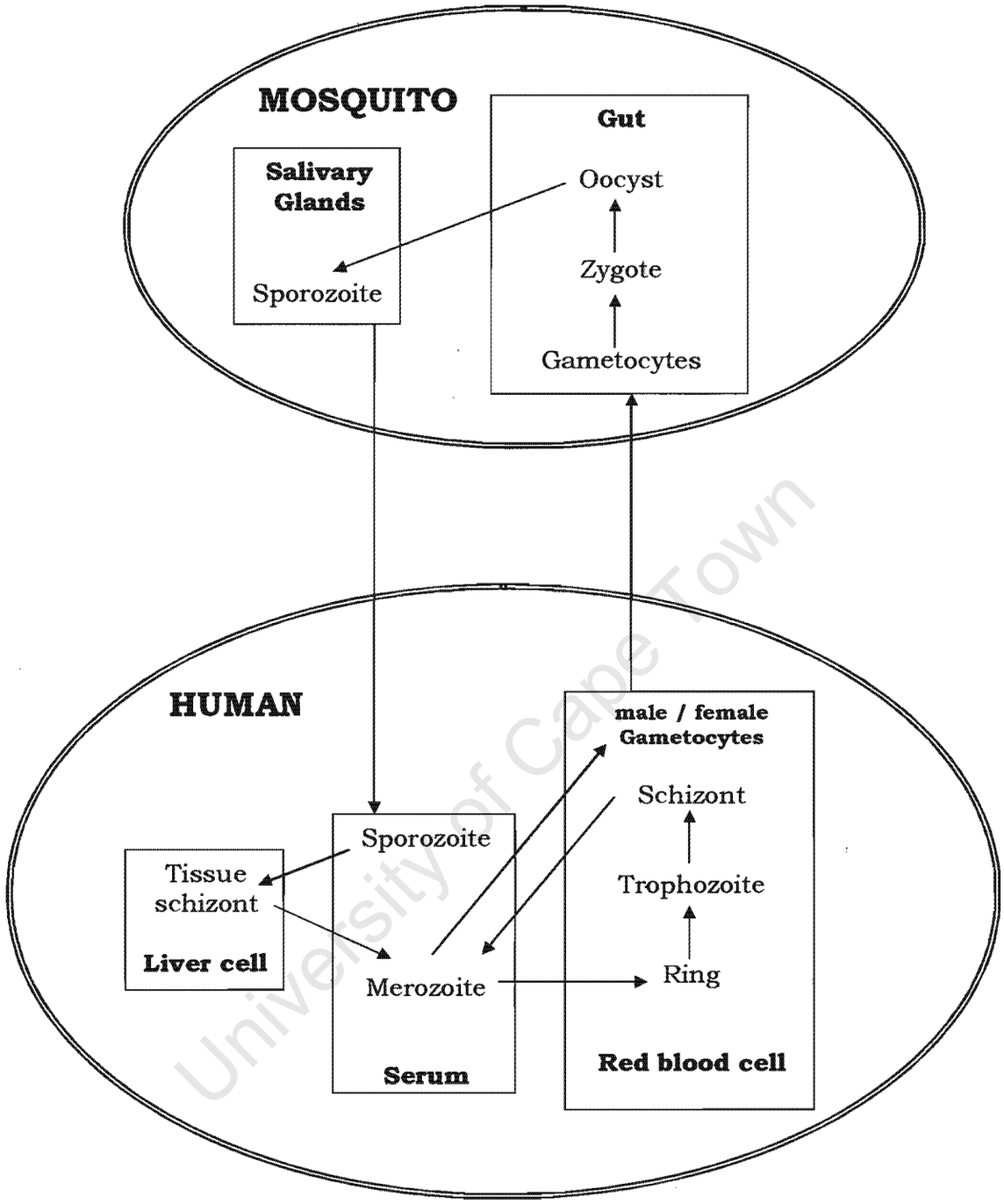


Figure 1.0: Life cycle of *Plasmodium falciparum*

1.2 Antimalarial drugs

Antimalarial drugs are usually classified according to their action against the different stages of the life cycle of the parasite (Wernsdorfer and McGregor 1990, Munson 1996, Winstanley 2000). Schizonticidal drugs are used to treat the acute attack. They are effective against the erythrocytic forms of the parasite and they include the 4-aminoquinolines (e.g. chloroquine, amodiaquine), the quinoline-methanols (e.g. quinine, mefloquine), the phenanthrene halofantrine, and drugs which interfere either with the synthesis of folate (e.g. sulphones) or with its utilization (e.g. pyrimethamine). Combinations of these drugs are frequently used. Antibiotics such as tetracycline and doxycycline have proved useful when combined with the above agents. Compounds derived from quinhaosu such as artemether, artemisinin and artesunate are effective antimalarials. The 8-aminoquinolines (e.g. primaquine) are used for radical cure and are effective against the parasites in the liver. These drugs also destroy gametocytes and thus reduce the spread of infection.

Of all the antimalarial drugs chloroquine has been the most successful and most widely used, as it is affordable, highly effective and well tolerated (Winstanley 2000). However, during the past 40 years malarial parasites, especially *Plasmodium falciparum*, have progressively developed resistance to chloroquine (Winstanley 2000). Resistance to other drugs has been reported in many parts of the

world, however, resistance to artemisinin-based antimalarial drugs has so far not been reported. Drug resistance has resulted in diminishing efficacy of antimalarial drugs and has made prophylaxis and treatment of malaria a therapeutic challenge (Winstanley 2000). In some parts of the world CQ has become virtually ineffective, making the need for new chemotherapeutic agents imperative (Winstanley 2000).

1.2.1 Pyronaridine

Pyronaridine was synthesized in 1970 at the Institute of Parasitic Diseases, at the Chinese Academy of Medical Sciences, as part of an extensive synthesis program in search of an alternative for chloroquine-resistant malaria (Shao-Bao 1990, Chen Chang and Zheng Xianyu 1992). The relationship between the structure-activity of chloroquine, amodiaquine, cycloquine and amopyroquine was used as a reference for the synthesis of pyronaridine (Chen Chang and Zheng Xianyu 1992). PND has the following chemical name: 2-methoxy-7-chloro-10- [3', 5'-bis(pyrrolidinyl-1-methyl)-4'-hydroxyphenyl] aminobenzo (b)-1,5-naphthyridine (figure 1.1).

Pyronaridine is highly active against the erythrocytic stages of the malaria parasite. *In vitro* studies have shown that PND is active against chloroquine-sensitive and chloroquine-resistant strains of *Plasmodium falciparum* (Childs *et al* 1988, Hassan Alin *et al* 1990,

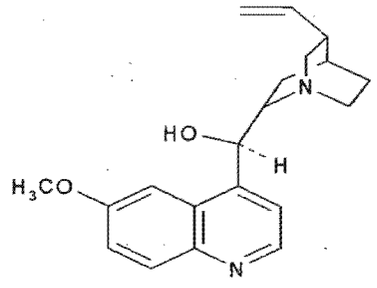
Basco and Le Bras 1992, Basco and Le Bras 1994, Elueze *et al* 1996, Ringwald *et al* 1996, Pradines *et al* 1998, Pradines *et al* 1999). PND has been tested in animal models of malaria; the results reveal activity in treating mice infected with CQS- and CQR-strains of *Plasmodium berghei* (Shao-Bao 1990, Chen Chang *et al* 1992, Peters and Robinson 1992).

PND has been used clinically in China for the treatment of malaria patients in CQR-endemic areas for more than a decade (Fu and Xiao 1991, Chen Chang *et al* 1992, Chen Chang and Zheng Xianyu 1992). Clinical trials in Cameroon, in areas endemic with CQR malaria, have been promising (Ringwald *et al* 1996, Ringwald *et al* 1998). In fact, The World Health Organization plans to complete the preclinical and clinical trials with the aim of registering PND in malaria-endemic areas with a view to replacing CQ with PND as the first-line treatment of malaria in Africa (Ringwald *et al* 1999).

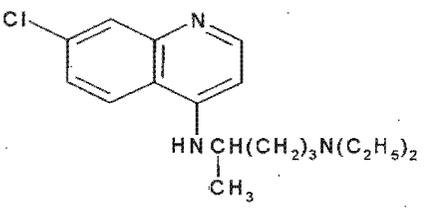
There have been reports of reduced sensitivity to PND in parts of China where PND has been in use for almost 15 years (Fan *et al* 1998, Yang *et al* 1998). Moreover, resistance to PND can be readily produced in mice models (Shao-Bao 1990, Peters and Robinson 1992). In seeking ways to avoid rapid development of resistance to PND, several studies have been carried out clinically and *in vitro* to determine any interaction of PND and other antimalarial compounds, with the aim of identifying a suitable drug for use in combination with

PND so as to prolong the life of the latter drug (Ringwald *et al* 1999, Yang *et al* 2000).

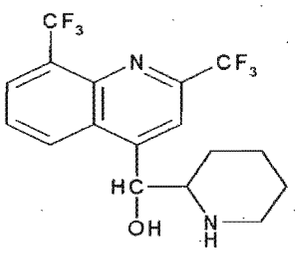
PND has gametocytocidal activity against *Plasmodium falciparum* (Chavalitshewinkoon-Petmir *et al*, 2000). The dual action of PND as both schizonticidal and gametocytocidal drug make it attractive for clinical application.



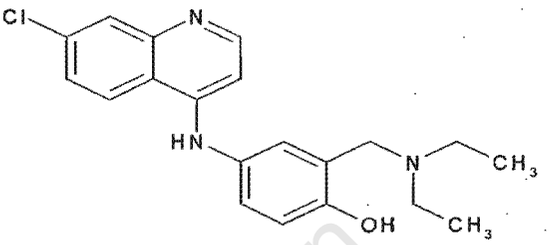
Quinine



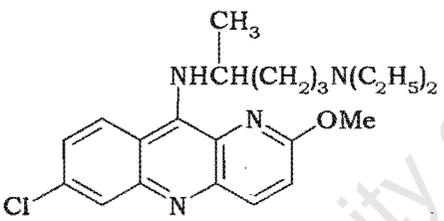
Chloroquine



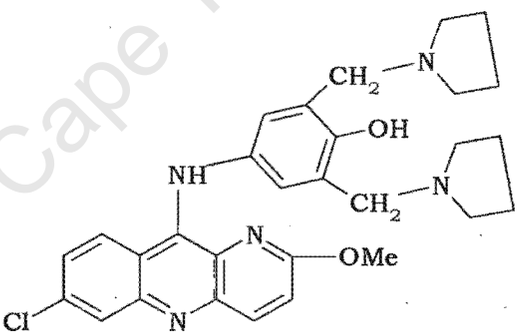
Mefloquine



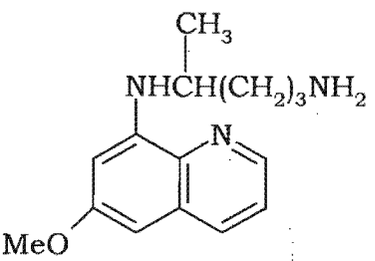
Amodiaquine



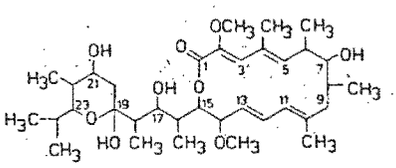
Mepacrine



Pyronaridine



Primaquine



Bafilomycin A1

Figure 1.1: Quinolines and Bafilomycin A1.

1.3 Mechanisms of action of chloroquine and pyronaridine

Although the mechanism of action of CQ and other 4-aminoquinolines is not yet fully understood, the weight of evidence points to the drugs acting by inhibiting hemozoin formation as a direct interaction between the drug and heme released from hemoglobin digestion. Hemoglobin catabolism plays a major part in the action of CQ, the drug being most active against the parasite stages that actively degrade hemoglobin; namely late rings forms and trophozoites (Yayon *et al* 1983, Geary *et al* 1989, Orjih *et al* 1994). Studies of the antimalarial effect of CQ on the morphology of the parasite have demonstrated initial damage to food vacuoles with alteration in the parasitic digestive system, which include digestive food vacuole swelling, pigment clumping and accumulation of undigested hemoglobin (Macomber *et al* 1967, Aikawa and Beaudon 1969, Aikawa 1972, Yayon *et al* 1984). The major role hemoglobin catabolism plays in CQ action is further demonstrated by the fact that reversible inhibitors of the enzymes responsible for the degradation of hemoglobin antagonize the actions of CQ (Moon *et al* 1997, Mungthin *et al* 1998).

The antimalarial action of CQ is specifically linked to its ability to accumulate selectively to millimolar levels in the parasite digestive food vacuole (Homewood *et al* 1972, Krogstad *et al* 1985, Yayon *et al* 1984, Ginsburg *et al* 1989). As a lipophilic weak base, with two

protonation sites (pKa_1 = 8.1, pKa_2 = 10.2), CQ is able to enter the malaria parasite by passive diffusion, primarily as the uncharged species down the pH gradient into the acidic digestive food vacuole. Once inside it becomes trapped, since the protonated form is membrane-impermeable. Based on this weak base model, the extent of CQ accumulation would depend on the difference in pH between the parasite food vacuole and the extracellular environment (Homewood *et al* 1972, Krogstad *et al* 1985, Yayon *et al* 1984, Ginsburg *et al* 1989). However, the extent of CQ accumulation and of other 4-aminoquinolines cannot be accounted for purely according to this model, as the actual levels of drug accumulation are considerably higher than those predicted by this model (Ferrari and Cutler 1991a, Ferrari and Cutler 1991b, Krogstad *et al* 1992, Hawley *et al* 1996a, Hawley *et al* 1996b). It was first demonstrated by Fitch *et al* that CQ accumulation in pRBCs is according to both saturable and non-saturable components. The saturable component occurs at pharmacological concentrations of CQ and this is considered responsible for the drug's antimalarial activity (Fitch 1969, Fitch 1970, Fitch *et al* 1974, Fitch 1989). The saturable uptake is thought to be due to CQ binding to an intracellular receptor, proposed to be heme released from hemoglobin digestion (Chou *et al* 1980, Fitch 1983). Comparison of affinities and specificities reveal close correspondence between FPIX in aqueous solution and the CQ receptor of malaria parasites (Chou *et al* 1980). The dissociation constant is estimated to be 10^{-8} M for the CQ-receptor complex in

infected erythrocytes (Fitch 1969) and 3.5 X 10^{-9} M for the CQ-receptor complex. In the presence of biological membranes the dissociation constant is estimated to be 2.3 X 10^{-7} M, indicating that FPIX has an affinity for CQ in the concentration range expected from studies of the CQ receptor (Chou *et al* 1980). Experiments by Bray and others provided conclusive evidence that CQ uptake into the parasite is mediated by high-affinity binding to heme released during hemoglobin degradation in the food vacuole (Bray *et al* 1998, Bray *et al* 1999, Raynes *et al* 1999). Using specific proteinase inhibitors, which reversibly inhibit hemoglobin digestion, thus stopping heme release, Bray *et al* (1998, 1999) were able to block specific and saturable CQ uptake. In cell-free systems the capacity of saturable CQ binding is dependent on FPIX concentration and the binding affinity of 25nM is similar to the apparent binding affinity of saturable CQ uptake in CQS *P. falciparum* (Bray *et al* 1998, 1999). These experiments show that the inhibitor specificity profile is the same in cell-free systems as in intact parasites (Bray *et al* 1999). Furthermore, CQ is associated with hemozoin *in vivo* and it has been shown to bind beta-hematin during *in vitro* synthesis in a specific and time-dependent manner (Sullivan *et al* 1996, Sullivan *et al* 1998). The proteinase inhibitors block incorporation of CQ into hemozoin (Mungthin *et al* 1998). The effect of the binding of CQ to heme or to heme crystals would be to remove CQ from solution chemistry equilibrium and thus to account for its excessive accumulation in the digestive food vacuole (Sullivan 2003). The pH gradient plays a role in

the uptake of CQ into the parasite as increasing or decreasing the gradient greatly influences CQ accumulation and antimalarial activity (Yayon *et al* 1984, Krogstad *et al* 1985, Yayon *et al* 1985, Geary *et al* 1990, Bray *et al* 1992). The pH gradient is possibly the initial driving force for CQ uptake by the parasite.

The precise mechanism of parasite killing by CQ is not fully understood, and it is likely that CQ inhibits parasite growth by a number of additive or synergistic effects. With the evidence that FPIX is the high affinity CQ target in the malaria parasite, it is probable that the interaction between CQ and FPIX mediates the antimalarial action of CQ. The binding of CQ to FPIX prevents incorporation of FPIX into hemozoin (Slater and Cerami 1992, Chou and Fitch 1993, Egan *et al* 1994, Dorn *et al* 1995). The inhibition of heme polymerization by CQ and related compounds correlates closely with the drug's antimalarial activity (Dorn *et al* 1998, Hawley *et al* 1998). The result of inhibition of heme polymerization would be a build up of toxic heme or heme-CQ complexes in the parasite food vacuole, ultimately causing irreversible damage to the parasite. The lytic action of FPIX is thought to be permeabilizing of membranes to cations through a detergent effect, causing hemolysis by a colloid-osmotic mechanism (Chou and Fitch 1981, Orjih *et al* 1981, Fitch *et al* 1982) and peroxidative damage to membranes or enzymes such as vacuolar proteases (Vander Jagt *et al* 1986, Sugioka and Suzuki 1991, Gluzman *et al* 1994). It has been argued that the lytic effects of FPIX

or FPIX-CQ complex would have to occur outside the food vacuole (Slater 1993, Ginsburg *et al* 1999) as the membrane integrity of the food vacuole is maintained for a long period after exposure to lethal doses of CQ (Yayon *et al* 1984b).

Ginsburg and co-workers have suggested that the main mechanism of antimalarial effect of CQ is inhibition of glutathione-dependent heme degradation (Ginsburg and Krugliak 1999, Ginsburg *et al* 1999). They argue that only 25 – 30% of FPIX generated during hemoglobin digestion is converted to hemozoin and that most of the FPIX exits the food vacuole into the cytosol where it is degraded by glutathione (Ginsburg *et al* 1998). They suggest that inhibition of hemozoin formation by CQ would not alone account for drug action as such inhibition would add at most 50% to the FPIX load on the glutathione –dependent detoxification mechanism, with which the parasite would cope (Ginsburg and Krugliak 1999). Inhibition of glutathione-dependent degradation of FPIX by CQ has been demonstrated *in vitro*, wherein treatment of intact infected cells with CQ resulted in dose- and time-dependent accumulation of FPIX in the membrane fraction of infected cells (Ginsburg *et al* 1998, Famin *et al* 1999, Zhang *et al* 1999). This accumulation is proportional to parasite killing, attributed to FPIX-dependent permeabilization of membranes to sodium and potassium (Ginsburg *et al* 1998).

It has been suggested that the interaction of CQ with FPIX blocks hydrogen peroxide-dependent degradation of FPIX in the food vacuole (Loria *et al* 1999). This is based on the observation that only one-third of FPIX is incorporated in hemozoin (Loria *et al* 1999), and therefore the parasite must have another means of getting rid of the toxic heme. Loria *et al* suggest that FPIX is degraded by reacting with the hydrogen peroxide formed when FPIX is oxidized from the Fe(II) to the Fe(III) state on its release from hemoglobin (Atamna and Ginsburg 1993). CQ is an efficient inhibitor of this process (Loria *et al* 1999). The inhibition of this reaction could prolong the half-life of hydrogen peroxide, and the build-up of hydrogen peroxide may cause peroxidative damage to proteins and lipids, which would explain the irreversible action of CQ against the parasite (Foley and Tilley 1998).

Another theory for the mechanisms of action of CQ is that accumulation of CQ in the parasite food vacuole causes an increase in the vacuolar pH; in doing so, metabolic processes such as receptor-mediated endocytosis, lysosomal enzyme targeting and enzyme-mediated hemoglobin degradation (Krogstad *et al* 1985, Krogstad and Schlesinger 1986,1987) are disturbed. As a weak base, CQ is expected to become protonated in the acidic environment of the food vacuole and since CQ is accumulated to a high concentration it is thought that vacuolar protons would be depleted thus raising the intra-vacuolar pH. Experimental evidence for this is inconsistent, however; some studies have shown an increase in vacuolar pH

(Krogstad *et al* 1985, Krogstad and Schlesinger 1986,1987) and others have not demonstrated a change in vacuolar pH (Yayon *et al* 1985, Ginsburg *et al* 1989). These inconsistencies might be due to the technical difficulties associated in trying to measure the vacuolar pH.

Other suggestions explaining the mechanism of action of CQ include direct inhibition of vacuolar phospholipase (Ginsburg and Krugliak 1992, Zidovetzki *et al* 1993,1994), inhibition of hemoglobin proteases (Gyang *et al* 1982, Vander Jagt *et al* 1986, Gluzman *et al* 1994), and inhibition of heme-dependent protein synthesis (Suriola and Padmanaban 1991). These ideas are derived from *in vitro* data however, the CQ concentrations required for inhibition are orders of magnitude higher than those necessary for inhibition of parasite growth.

The exact mechanism of action of PND is not known. It has been shown that PND is able to inhibit the DNA topoisomerase II enzyme of *P. falciparum* (Chavalitshewinkoon *et al*, 1993). Topoisomerase enzymes resolve the complex topological situations arising from DNA metabolism and they are required for nucleic acid synthesis, genome stability, chromosome condensation and decondensation and for segregation of replicated chromosomes (Cheesman, 2000). However, the concentrations required to inhibit *P. falciparum* DNA topoisomerase II activity are greatly in excess of those required to kill the parasite (IC_{50} values were 11μM for DNA topoisomerase inhibition

and 2.7nM for inhibition of parasite growth). It is unlikely that the antimalarial activity of PND is explained by this mechanism.

Studies of the effect of PND on *P. falciparum* and *P. berghei* ultrastructure revealed that the earliest and most distinct changes induced by the drug occur in the digestive food vacuole (Li-Ju Wu *et al* 1988, Kawai *et al* 1996). These include swelling of the food vacuole, formation of membrane-bound vesicles containing undigested hemoglobin, and decreased malarial pigment, suggesting that the parasite feeding mechanism is affected by PND. PND interacts with heme *in vitro* and inhibits polymerization of heme into beta-hematin, a synthetic polymer identical to hemozoin (Dorn *et al* 1998, Hawley *et al* 1998). PND inhibits the degradation of heme by glutathione (Famin *et al*, 1999). Based on these conclusions, it is possible that the mechanism of action of PND is similar to that of CQ and other 4-aminoquinolines.

1.4 Mechanisms of drug resistance

The mechanism of resistance to CQ and other related drugs is still unknown and is the subject of much debate, but it is apparent that CQR parasites accumulate significantly less CQ than CQS parasites (Macomber *et al* 1966, Fitch 1969, Fitch 1970, Fitch 1973, Fitch *et al* 1974, Fitch *et al* 1975, Diribe and Warhurst 1985, Krogstad *et al* 1992, Bray *et al* 1996). CQ accumulation is less compared to CQS

parasites, in isolated food vacuoles of CQR *P. falciparum* (Saliba *et al* 1998), suggesting that this might be the site for resistance development. The physiological basis of the reduced uptake is not clear, but it has been attributed either to a lower rate of influx of CQ into the parasite, to a higher rate of efflux of the drug from the parasite, or to a reduced binding affinity or access of CQ to its ultimate intracellular target, heme.

The observation that verapamil, a calcium channel blocker and inhibitor of multidrug resistance in cancer cells, as well as other compounds, is able to reverse chloroquine resistance in *P. falciparum* added to the observation that CQR parasites release pre-accumulated CQ 50 times faster than CQS parasites, suggest that the mechanism of CQ resistance in the malaria parasite might be same as that of the multidrug resistant phenotype in cancer cells (Krogstad *et al* 1987, Martin *et al* 1987, Bitonti *et al* 1988). In a number of cancer cells, multidrug resistance is mediated by an over-expressed multidrug-resistance protein that actively pumps out a wide spectrum of drugs from the cells, a function of that can be reversed by verapamil (Chen and Simon, 2000). Although the protein, P-glycoprotein 1 (a member of the 'ABC transporter' family), has been found located on the food vacuolar membrane in *P. falciparum* (Cowman *et al*, 1991), other studies have not shown an increased level of drug efflux in CQR parasites (Bray *et al*, 1992, Ginsburg and Krugliak, 1992, Bray *et al*, 1996) and others have shown that diminished accumulation, rather,

is responsible for the decreased steady–state levels of CQ in CQR parasites (Geary *et al*, 1990, Ginsburg and Stein 1991, Bray *et al* 1992a, Bray *et al* 1996). Theories to explain this diminished level of CQ accumulation in CQR parasites include: 1) a weakened vacuolar proton pump which leads to a raised vacuolar pH (Ginsburg and Stein 1991, Bray *et al* 1992b, Ginsburg and Krugliak 1992), 2) decreased activity of a proposed amiloride-sensitive Na^+/H^+ exchanger which actively transports CQ into the food vacuole (Sanchez *et al* 1997, Wunsch *et al* 1998), 3) alteration of an ion pump (stilbene-sensitive Cl^-/HCO_3^- antiporter) that is responsible for maintaining pH gradients, resulting in changed intracellular pH or membrane potential (Martiney *et al* 1999). Studies that have attempted to compare the vacuolar pH of CQS and CQR parasites have given inconsistent results and there is no consensus as to whether there is a consistent difference in the vacuolar pH of CQR and CQS parasites (Kirk and Saliba 2001). Besides, it has been argued that mechanisms of resistance which involve general changes in pH in the food vacuole should show cross-resistance between all weak base antimalarials (Egan 2001). This is not the case as numerous structural analogs of CQ are highly active against CQR parasites (Hawley *et al* 1996, Kotecka *et al* 1997, Kaschula *et al* 2002). Rather, the mechanism of CQ resistance is likely to involve some specific molecular recognition site that prevents the net build up of CQ in the food vacuole (Egan 2001). A compelling proposition is offered by Bray and co-workers who have suggested that CQ resistance results from a selective change in vesicular

function within relevant hemoglobin processing compartments, that reduces the affinity of CQ-FPIX binding (Raynes *et al* 1999). This idea builds on earlier work of Fitch (1969,1970) and Diribe and Warhurst (1985) which showed that erythrocytes infected with CQR parasites are deficient in CQ high-affinity binding. Bray *et al* (1998) showed that reduced CQ accumulation does not result from a decrease in the number of CQ binding sites but rather from a reduced affinity of CQ for FPIX. The apparent affinity of CQ in various strains of *P. falciparum* was correlated well with parasite sensitivity to CQ (Bray *et al* 1998, Raynes *et al* 1999).

Genetically, CQ resistance has been linked to two genes, *pfmdr1* and *pfcrt,* located on chromosomes 5 and 7 respectively. Polymorphisms that strongly link CQ resistance to the *pfmdr1* gene were identified in a number of *P. falciparum* samples after isolation of the gene (Foote *et al* 1990, Basco *et al* 1995, Cox Singh *et al* 1995, Adagu *et al* 1996). However, genetic cross experiments between CQS and CQR parasites have shown that mutations in *pfmdr1* are not necessarily associated with resistance in some strains of CQR parasites (Wellems *et al,* 1990). Wellems *et al* (1990) mapped CQ resistance to complex polymorphisms on chromosome 7 and the *cg1* and *cg2* genes in this segment were identified as the CQ resistance genes. Subsequent studies revealed that these are not in fact related to resistance, but rather the *pfcrt* gene, located closely to the cg genes, is responsible for CQ resistance (Fidock *et al* 2000a). The *pfcrt* gene codes for a new

integral lysosomal membrane protein PfCRT (Fidock *et al* 2000b). A K76T point mutation in PfCRT is 100% associated with CQR *in vitro* (Fidock *et al* 2000b).

As with P-glycoprotein1, PfCRT is located on the digestive food vacuole membrane of the malaria parasite and also possesses the multiple membrane-spanning configuration that is characteristic of membrane transport proteins (Fidock *et al* 2000b). The PfCRT protein resembles other transmembrane domain proteins (but is not a member of a known family) that facilitate transport of organic cations. It is possible that PfCRT may normally facilitate efflux of basic amino acids or short basic peptides (with hydrogen ion) that otherwise would accumulate in the lysosome (Warhurst 2001). The physiological roles that these proteins play in the mechanism of CQ resistance still remain to be elucidated and theories put forward to explain their functions generally include involvement of these proteins in directly influencing the level of CQ accumulated by acting as drug pumps or indirectly by modulating the pH of the digestive food vacuole (Foley and Tilley 1998, Ginsburg and Krugliak 1999, Reed *et al* 2000, Cooper *et al* 2002, Warhurst *et al* 2002, Hyde 2002). Dzekunov *et al* (2000) have recently demonstrated that the vacuolar pH is lower in CQR parasites than in CQS parasites and in lending support to the proposal that CQ resistance is due to a decreased affinity for FPIX, they have provided evidence that CQ binds FPIX less at the lower pH as a result of aggregation of the FPIX molecules.

1.5 General objectives

The superiority of PND antimalarial activity over CQ and the activity of PND against CQR parasites is of great interest because these two structurally related drugs seem to have the similar mechanisms of action. However, there are enough structural differences between the two drugs to suggest that their interactions with the malaria parasite might differ. The main focus of this study was to investigate how similar the antimalarial mode of action of PND is to that of CQ, and to try and get an insight into the reasons for the greater activity of PND over CQ against CQS and CQR *P. falciparum* strains. The following objectives were set to achieve this: a) To assess the *in vitro* antimalarial activity of PND against various strains of CQS and CQR *Plasmodium falciparum.* b) To characterize the *in vitro* stage-, time- and dose-dependence of PND antimalarial action against the erythrocytic forms of *Plasmodium falciparum.* c) To use radioligand binding studies to characterize the factors that control the uptake of PND into parasitized erythrocytes and to investigate the relationship between PND accumulation and PND antimalarial activity. d) To assess the heme-binding affinities and beta-hematin inhibitory activities of PND and CQ.

2

CHARACTERIZATION OF THE *IN VITRO* SENSITIVITY OF *PLASMODIUM FALCIPARUM* TO PYRONARIDINE

2.1 Introduction

The aim of this aspect of the research was to assess the *in vitro* activity of PND and to compare this activity with that of CQ against several strains of *Plasmodium falciparum* that have been maintained in our laboratory, either in continuous culture or by cryo-preservation. These strains, which have been characterized by their sensitivity to CQ, are 3D7, D10, K1, W2, Fac8 and RSA11. 3D7 is a CQS clone derived from the isolate NFS, which was obtained from an airport worker in Amsterdam, but may have its origins in Africa (Cowman *et al* 1991). D10 is a CQS clone derived from isolate FCQ-27 from Papua New Guinea (Ekong *et al* 1993). K1 is a CQR clone derived from an isolate from Kanchanaburi, Thailand (Thaitheng and Beale 1981). W2 is a CQR clone derived from a mixture of the Sierra

Leone I/CDC and Indochina III/CDC lines (Oduola *et al* 1988). Fac8 is a CQR clone derived from a Brazilian clone line, ITG2F6 (Cowman *et al* 1991). Finally, RSA11 is a CQR isolate from the province of KwaZulu Natal, South Africa (Freese *et al* 1991).

2.2 Methods

2.2.1 Drug-sensitivity Assay

Chloroquine diphosphate (Sigma-Aldrich Co, Poole, England) and pyronaridine diphosphate (a kind donation from Tom Kanyok, WHO, Geneva) were initially dissolved in water. Further dilutions were carried out in culture medium. Parasitized erythrocytes, synchronized at the trophozoite stage, set at 2% parasitemia and suspended in culture medium at a 1% hematocrit were used for the drug sensitivity assay. Exposure of pRBCs to drug was carried out in 96-flat bottom well microplates, arranged in a matrix of 8 rows (A through H) and 12 columns (1 through 12). Column 1 was used as blank (RBCs only). Column 2 was used as control wells (pRBCs in drug-free medium) and columns 3 through 12 were set as the test wells. Experiments were done in quadruplicate. 100µl of drug-free culture medium were aliquoted into each well except in column 3. 200µl of drug-containing medium at twice the final concentration of drug was then added to column 3. Using a multi-channel pipette dispenser, 10 two-fold serial

dilutions were done from columns 4 through to 12 starting by taking 100µl of drug-containing medium from column 3 and finally leaving a volume of 100µl in each well. 100µl of pRBC suspension was then added to columns 2 through to 12 with 100µl of RBC suspension being added to column 1, ending up with a final hematocrit of 1% in all wells. The microplate was then covered loosely with a lid, placed in a desiccator cabinet gassed with a gas mixture consisting of 93% N_2, 4% CO_2 and 3% O_2. The cabinet was sealed and incubated for 48 hours at 37°C.

At the end of the incubation period the cells that had settled at the bottom of the wells were resuspended. A 15µl sample of each well was transferred to corresponding wells on a new 96-well flat bottom microplate containing the reagents for detection of parasite viability using the LDH assay (see appendix A2).

Parasite survival in test wells was expressed as a percentage of the survival of the corresponding control well. Concentration-response analyses were done using non-linear regression methods with the Prism computer software program (version 3.0, GraphPad Software).

2.3 Results

The *in vitro* sensitivities of the various strains of *P. falciparum* to CQ and PND are summarized in Table 2.1. PND showed antimalarial activity against both CQS and CQR strains, with IC_{50} values ranging from 8.87nM to 18.26nM. The results also show that PND was less potent against the CQR strains with a mean IC_{50} value of 15.5nM ± 1.2 for the CQR strains (Fac8, K1, RSA11, W2) and 8.15nM ± 0.7 for the CQS strains (D10, 3D7). There was an average 1.9-fold difference in the activity of PND against CQR and CQS strains, while there was an average 9.6-fold difference in the activity of CQ against CQR and CQS parasites, indicating greater activity of PND against CQR parasites. The mean IC_{50} values for CQ against CQR and CQS strains were 197.7nM ± 20.1 and 20.6nM ± 0.8, respectively.

	Chloroquine			Pyronaridine		
Clone	*IC_{50}*	*SEM*	*n*	*IC_{50}*	*SEM*	*n*
3D7	19.84	6.41	3	8.87	2.10	3
D10	21.35	2.24	9	7.44	1.24	12
Fac8	162.60	33.27	4	13.69	5.67	4
K1	177.40	29.47	8	13.27	1.78	12
RSA11	196.40	110.30	2	18.26	4.00	4
W2	254.40	46.21	4	16.73	1.48	4

Table 2.1: In vitro sensitivity of various strains of *P. falciparum* to CQ and PND. Mean IC_{50} values are in nM units.

The Pearson correlation (r) and the coefficient of determination (r^2) were used to assess cross-resistance between CQ and PND. There was a significant positive correlation between responses to CQ and PND, with a Pearson r-value of 0.93, an r^2 value of 0.86 and a P value of 0.0074.

2.4 Discussion

The *in vitro* antimalarial activity of PND that has been reported previously, (Ringwald *et al* 1999, Childs *et al* 1988, Basco and Le Bras 1994, Pradines *et al* 1999, Basco and Le Bras 1992, Ringwald *et al* 1996, Elueze *et al* 1996, Pradines *et al* 1998, Chen Chang and Zheng Xianyu 1992, Chen Chang *et al* 1992, Shao Bao 1990) has been confirmed in this study. In this study, PND showed activity against both CQS and CQR strains of *P. falciparum* originating from several parts of the world, with IC_{50} values ranging from 8.87nM to 18.26nM. These values are comparable to values quoted in the previous studies.

The results presented here also demonstrate that the PND IC_{50} values for CQR strains were higher than those for the CQS strains, indicating that PND is less potent against CQR strains. There have been conflicting reports of PND activity against CQS and CQR isolates of *P falciparum*. The study by Childs *et al* (1988) showed that PND was equally effective *in vitro* against several isolates from Thailand with

varying CQ resistance levels. Basco and Le Bras (1992) showed no correlation between resistance to CQ and PND activity in 31 isolates from Central and West Africa. On the other hand, studies by Pradines *et al* (1998), Elueze *et al* (1996), Pradines *et al* (1999), Ringwald *et al* (1999) and Warsame *et al* (1991) have shown a significant positive correlation between the *in vitro* response to CQ and PND. Although the sample size used in this current study is smaller than in the aforementioned studies, a significant positive correlation was also found for the *in vitro* responses to PND and CQ.

Although the mechanisms of action of CQ and PND, and of CQ resistance are not fully known a positive correlation of the activities of CQ and PND suggests that resistance to CQ may facilitate the loss of sensitivity to PND. CQ and PND share similar features in their chemical structures and may even act on the same molecular target in the parasite, so it is conceivable that their antimalarial actions are similar. Indeed, studies have shown that antimalarial drugs that share chemical features (e.g. mefloquine and halofantrine, chloroquine and amodiaquine) usually have high correlation coefficients for their antimalarial activity against the malaria parasite and show close cross-resistant patterns (Churchill *et al* 1985, Bray *et al* 1996, Basco and Le Bras 1993, Peel *et al* 1994, Rojas-Rivero *et al* 1992). Peters and Robinson (1992) developed a PND resistant line of *Plasmodium berghei* that showed cross-resistance with CQ and other structurally similar compounds such as amodiaquine. Shao Bao-ru (1990) also

reports a PND-resistant line of *P. berghei*, which showed cross-resistance to CQ, piperaquine, amopyroquine, bispyroquine, mepacrine and quinine.

In conclusion, the *in vitro* sensitivity profile to PND of a number of *P. falciparum* strains has been established. PND showed a high antimalarial activity against all the strains tested. Although, CQR strains may be less sensitive to PND than CQS strains, this difference is not great.

3

TIME- AND CONCENTRATION-DEPENDENCE OF PYRONARIDINE ACTIVITY AGAINST DIFFERENT STAGES OF *PLASMODIUM FALCIPARUM*.

3.1 Introduction

The developmental cycle of *Plasmodium falciparum* in red blood cells lasts 48 hours, gradually progressing from early to late ring stages, small pigmented to large uninuclear trophozoites, and binucleate to segmented schizonts (Bannister and Mitchell 2003). Studies on several species of *Plasmodium* have demonstrated considerable stage dependence in various aspects of cellular metabolism, including DNA, RNA and protein synthesis, and phospholipid turnover (Vial *et al* 1982, Allred and Sherman 1983, Dean *et al* 1983, Gritzmacher and Reese 1984, Inselburg and Banyal 1984, de Rojas and Wassaman 1985, Ben Mamoun *et al* 2001, Mitamura and Palacpac 2003). Little is known about any differential sensitivity of these morphologically distinguishable stages to PND. Information on stage-selective effects

could provide important clues about the mechanism of action of PND. Any stage specificity of PND action might also be of clinical importance in parasite clearance kinetics and recrudescence rates. The studies in this chapter were designed to characterize the *in vitro* stage-, time- and concentration-dependence of PND antimalarial action against synchronous cultures of *Plasmodium falciparum*.

3.2 Methods

3.2.1 Time-dependence of PND action

The ring-, trophozoite- and schizont-infected pRBCs were exposed to a concentration of 10ng/ml of PND for 1, 2, 4, 6, 8 and 10 hours and to observe the onset of the effects of the drug in terms of inhibition of parasite growth. The selected concentration of 10ng/ml is close to the known IC_{50} of PND for the CQS clone, D10, used for this investigation. The parasitemia and hematocrit were set at 2% and 1%, respectively.

Synchronous cultures of pRBCs at the ring (0 hours), trophozoite (24 hours) or schizont (32 hours) stage were exposed to culture medium containing PND for the time durations indicated above, in 200ml sealed culture flasks, flushed with a gas mixture of 93% N_2, 4% CO_2 and 3% O_2, and incubated at 37°C. At the designated time points a 10ml sample of culture was removed from the flask. The flask was re-

flushed with gas mixture, sealed and re-incubated at 37°C. The 10ml sample was centrifuged at 700g for 3 minutes, the supernatant was discarded, and the pRBC pellet washed three times with drug-free culture medium and finally resuspended in drug-free culture medium at 1% hematocrit. 200µl of this culture was then seeded onto a 96-flat bottom well microplate. The plate was set up as follows: the 1st column was designated for the blank (RBCs alone), the 2nd column was for the control culture (pRBCs not exposed to PND) and columns 3 through to 8 were designated for pRBCs exposed to PND for 1 hour through to 10 hours. The experiment was set up in replicates of 8 (rows A through H). The microplate was then covered with a lid, placed in a desiccator cabinet, gassed and incubated at 37°C. The plates were incubated until the next trophozoite stage; i.e. the pRBCs were allowed to go through one cycle of multiplication. Therefore, rings were incubated for 72 hours, trophozoites for 48 hours and schizonts for 36 hours.

After the incubation period, 15µl samples from each well were transferred to corresponding wells on a new 96-flat bottom well microplate prepared for parasite viability testing using the LDH assay (see Appendix A3). Parasite viability in each test well was expressed as a percentage of the corresponding control well.

3.2.2 Concentration-dependence of PND action

The concentration dependence of PND action against the three RBC stages of the CQS clone, D10, was investigated. Highly synchronized pRBC cultures were exposed to PND as rings (0 hours), trophozoites (24 hours) and schizonts (32 hours). 100µl of PND drug solution (in water), at concentrations of 200ng/ml, 500ng/ml, 1µg/ml, 2µg/ml, 5µg/ml and 10µg/ml were aliquoted into sterile plastic 10ml centrifuge tubes. A 10ml pRBC culture at 1% parasitemia and 1% hematocrit was then added to the tubes, making final PND concentrations of approximately 2ng/ml, 5ng/ml, 10ng/ml, 20ng/ml, 50ng/ml and 100ng/ml. The tubes were then sealed and incubated at 37°C for 1 hour. The tubes were shaken gently at three time points during the incubation period to keep cells suspended. At the end of the incubation period the pRBCs were spun at 700g for 3 minutes. The supernatant was aspirated and discarded and the pRBC pellet was washed three times in drug-free culture medium. The pRBC was finally suspended in 10ml of drug-free culture medium.

A 200µl sample from each tube was then seeded in each well on a 96-flat bottom well microplate. The first column on the microplate was set for the blank (RBCs alone), the second was set as the control wells (pRBCs not exposed to PND). Columns 3 through 8 were set as the test wells (pRBCs exposed to PND). Rows A through H were set for replicate samples (n = 8). The microplates were covered loosely with a

lid, placed in a desiccator cabinet, gassed and incubated at 37°C. Rings were incubated for 72 hours, trophozoites for 48 hours and schizonts for 36 hours to allow parasite development to the next trophozoite stage. After incubation, 15µl samples from each well were transferred to corresponding wells on a new microplate prepared for parasite viability testing using the LDH assay (see Appendix A3). Parasite viability in the test wells was compared to the parasite viability in corresponding control wells.

3.3 Results

3.3.1 Time dependence of PND action

Exposure of pRBCs to PND for various time lengths resulted in inhibition of parasite growth. In all the RBC stages of the D10 parasite that were tested, the inhibitory effects of 10ng/ml PND were seen within 1 hour of exposure to the drug, with growth inhibition at 34.3% (±10), 48.0% (±13.2) and 89.0% (±10.5) for rings, trophozoites and schizonts, respectively (figure 3.1). Schizonts were the most rapidly sensitive to PND, maximal inhibition being achieved within 1 hour of exposure, followed by trophozoites with maximal inhibition within 2 hours; the ring stage parasites were the least sensitive, with maximal inhibition obtained only after 4 hours exposure to PND.

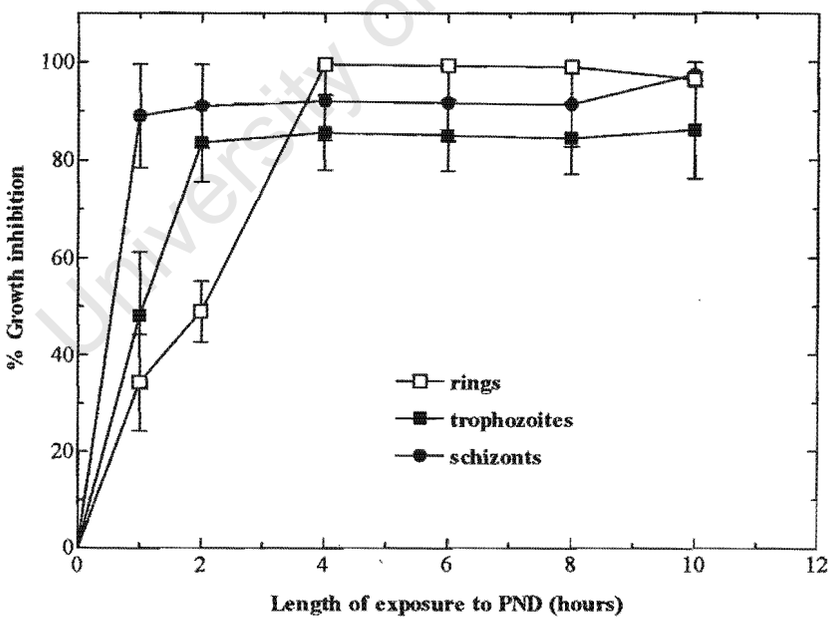


Figure 3.1: Time-dependent response of different stages of D10 parasites to PND. PND concentration = 10ng/ml. Each datum point represents a mean value, with the error bars representing the SEM. For rings n = 4, for trophozoites n = 5 and for schizonts n = 3 readings.

3.3.2 *Concentration dependence of PND action*

Figure 3.2 shows the stage-specific effects of PND against the D10 clone of *Plasmodium falciparum*. The estimated concentrations of PND required for 50% inhibition of parasite viability were 5.0ng/ml and 10.5ng/ml for schizonts and trophozoites, respectively. A 50% inhibitory concentration for ring stage parasites could not be estimated, as 50% parasite inhibition was not achieved with the concentration range employed here. The results indicate that the later stage parasites are more susceptible to PND that the earlier ring parasites with schizonts being more sensitive than trophozoites.

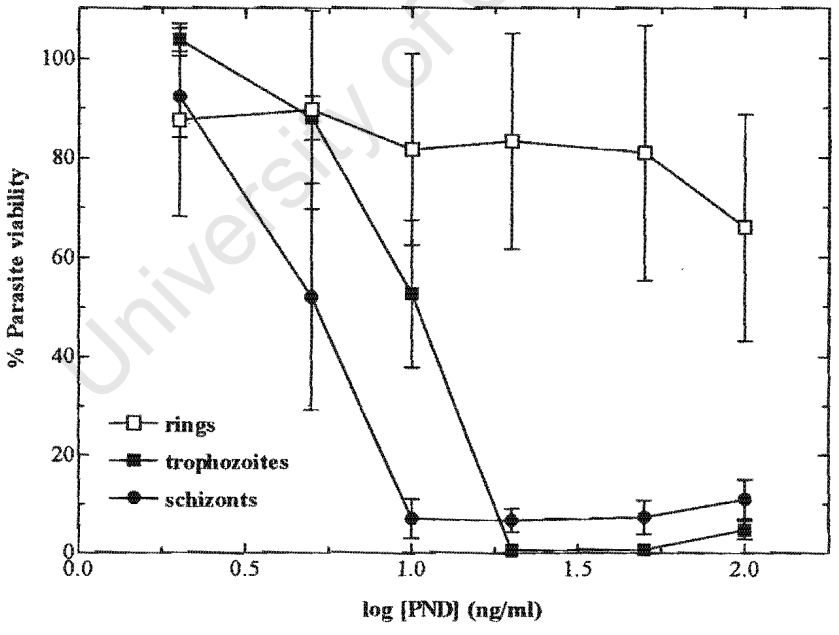


Figure 3.2: Concentration dependent effects of PND on the different stages of D10 parasite viability. Parasites were exposure to PND for 1 hour. Each datum point represents a mean value, with the error bars representing the SEM. For rings n = 3, for trophozoites n = 4 and for schizonts n = 3 readings.

3.4 Discussion

The intra-erythrocytic development of the malarial parasite is characterized morphologically by three stages: ring, trophozoite and schizont. Investigation of the response of the malarial parasite to PND would contribute towards the understanding of the mechanism of action of the drug. In the design of the experiments to investigate the stage- and time- dependence of PND action, PND was removed from the culture medium at a specified time after exposure; therefore, the effects on parasite growth later in the life cycle can be seen as a function of the sensitivity of the stages exposed to PND.

The degree of antimalarial activity of PND was dependent upon the time of exposure in all three stages (figure 3.1). Incubation of 1 hour was sufficient to cause significant effects on parasite viability. Longer exposure times caused a greater antimalarial effect. Washing and returning pRBCs to drug-free culture medium did not revive the parasites, indicating that the pharmacological effect is irreversible. The results suggest that the process(es) affected by PND is (are) critical for parasite survival, the interruption of which is eventually lethal. The increase in the antimalarial effects observed at longer exposure times may not be due to greater accumulation as steady state concentrations of PND within the parasite are reached within 30 minutes of exposure to the drug (see chapter 4, figure 4.1). Longer washing times of the pRBCs after exposure to PND did not alter

parasite viability (data not shown), meaning that the irreversible effects are not likely to be due to the retention of the drug in the pRBCs after washing. The rapid effects of PND seen here are consistent with morphological observations of the malarial parasite after exposure to PND. Wu *et al* (1988) and Kawai *et al* (1996), using electron microscopy, showed that the earliest morphological changes seen in *Plasmodium falciparum* exposed to PND are seen within 30 minutes. These include significant ultrastructural changes to the digestive food vacuole and the pellicular complexes. The irreversible effects of PND were also seen to be concentration dependent as exposure of pRBCs to larger concentrations for just 1 hour produced to same effect as longer exposure times at a lower single concentration of PND (figures 3.1 and 3.2). The exception was with rings, where 100% inhibition of parasite growth was not obtained at the concentration range used.

The different stages of the intra-erythrocytic parasite show clearly different sensitivities to PND. The schizonts are the most susceptible, followed by trophozoites and then rings. This sensitivity profile was seen in a time- and concentration-dependent manner in all three stages. Although the difference in sensitivity profile between schizonts and trophozoites is not great, what is clear is that ring stage parasites are more resistant to PND than the more mature parasites. This might be accounted for by differences in the rate of uptake of the drug by the parasite, differences in intracellular factors or components

necessary to accumulate the drug inside the parasite or changes in the activity or importance of a metabolic process that is specifically inhibited by PND. It appears that general metabolic activation occurs as ring stages become morphologically distinguishable as trophozoites, and this activation continues for various lengths of time for different physiological processes such as DNA and protein synthesis and hemoglobin catabolism (Allred and Sherman 1983, Inselburg and Banyal 1984, Gritzmacher and Reese 1984, de Rojas and Wasserman 1985, Orjih *et al* 1994). Of the possible targets of PND action, DNA synthesis (Chavalitshewinkoon *et al* 1993) and hemoglobin catabolism (Li-Ju Wu *et al* 1988, Kawai *et al* 1996) parallel closely the parasite's stage sensitivity to PND. Hemoglobin catabolism was assessed as hemozoin production (Orjih *et al* 1994) and results showed that at the early ring stage there is little or no hemoglobin catabolism. There is an abrupt switch to a high rate of catabolism with hemozoin production reaching a peak plateau between 30 and 48 hours. DNA synthesis occurs from early trophozoites to schizonts, with peak synthesis occurring just before schizogony (Gritzmacher and Reese 1984, de Rojas and Wassaman 1985, Ben Mamoun *et al* 2001). How PND is proposed to affect these processes has been discussed in chapter 1. Briefly, PND is proposed to bind heme released from hemoglobin digestion and inhibit its sequestration into hemozoin, causing a build up of toxic heme. In addition, as the parasite rapidly divides during schizogeny, interferences of DNA replication would be expected to inhibit asexual

development of the parasite. Results presented in chapter 4 (figure 4.2) show that PND accumulation into the parasite is greatest between 18 and 42 hours after merozoite invasion, corresponding to the trophozoite and schizont stages, and this would explain the greater sensitivity of these stages to PND. They accumulate more PND.

Of interest is that the stage- and time- specific actions of PND observed in this investigation are similar to CQ actions against *Plasmodium falciparum*. CQ is more active against parasites at the later stages (Yayon *et al* 1983, Geary *et al* 1989), and CQ also exerts an irreversible inhibitory effect on red blood cell stage parasites within one hour of exposure (Krugliak and Ginsburg 1991).

Now that the *in vitro* stage specificity of PND antimalarial activity against *Plasmodium falciparum* is better defined, it is not clear whether or not clinical benefits would be derived from combinations with other antimalarials that target different stages of the parasite intra-erythrocytic cycle (e.g. ring-stage parasites or merozoite invasion). The issues of pharmacokinetics would have to be taken into account.

What clues about PND mechanism of action have been revealed by the investigation of the stage-specific actions of PND? PND is more effective against the more mature parasites, and these are the stages that are actively degrading hemoglobin and in which hemozoin

production is at its maximum. This suggests a mode of action of PND similar to CQ, in which hemoglobin catabolism is critical.

4

CHARACTERIZATION OF PYRONARIDINE ACCUMULATION BY ERYTHROCYTES INFECTED WITH *PLASMODIUM FALCIPARUM*.

4.1 Introduction

The relevance of the process of PND uptake to the biological activity and mode of action of the drug is clear. The antimalarial activity, potency and mode of action of the 4-aminoquinolines (Diribe and Warhurst 1985, Krogstad *et al* 1988, Hawley *et al* 1996a, Hawley *et al* 1996b), and of other classes of antimalarials (San George *et al* 1984, Loyevsky *et al* 1993, Stead *et al* 2001, Vyas *et al* 2002) are linked to their intracellular accumulation in the infected erythrocyte. The specificity of the antimalarial activity of CQ stems from the ability of malaria parasites to accumulate more CQ than any other eukaryotic cell (Raynes *et al* 1999). Details of the accumulation of PND in the malaria parasite are not known. Although closely related structurally, the actions of CQ and PND in the parasite appear to differ. PND

shows greater anti-malarial potency than CQ against both CQR and CQS *Plasmodium falciparum.* Assuming that PND and CQ have a common primary pharmacological target in the parasite, the greater antimalarial activity of PND might be due to its superior accumulation into the parasite food vacuole or the target site could be more sensitive to PND. As with CQ, PND is a weak base with pKa values of 5.9 and 8.9 for ring and side chain nitrogens, respectively (Chavalitshewinkoon *et al* 1993), compared with 8.1 and 10.2 for CQ, and, like CQ, would be expected to accumulate in acidic compartments such as the parasitic food vacuole due to the lysosomotropic effect. PND should enter the parasite primarily by passive diffusion as an uncharged species. Since the protonated forms of the drug would be membrane-impermeable, PND might become trapped inside the acidic food vacuole. Having lower pKa values suggests that PND should accumulate to a lesser extent than CQ. However, it has been demonstrated that AMQ, which also has lower pKa values, accumulates to a greater extent than CQ in parasitized erythrocytes (Fitch *et al* 1974, Hawley *et al* 1996a). Accumulation studies might also highlight potential differences in pharmacological targets for CQ and PND.

The availability of radiolabelled drugs that retain their biological activity has been useful in identifying binding sites for these drugs and in obtaining information about the manner in which drugs interact with receptors (Limbird 1985, Kenakin 1997). Radioligand

binding studies was the approach taken to characterize the accumulation of PND in *Plasmodium falciparum*-infected erythrocytes.

4.2 Methods

4.2.1 Radiolabelled Drugs

The radiolabelled PND-[^{3}H] used in this study was obtained from Moravek Biochemicals Inc. The specific activity was 11.7 Ci/mmol. CQ-[^{3}H] with a specific activity of 7 Ci/mmol was obtained from Amersham Life Science, Buckinghamshire, England. PND-[^{3}H] and CQ-[^{3}H] were obtained dissolved in methanol and ethanol respectively, and subsequent dilutions were carried out in water.

4.2.2 Measurement of drug accumulation

Parasite cultures synchronized at the trophozoite stage (24 – 30 hours post-invasion) and set at a parasitemia of 10% were used for drug accumulation studies. All reactions were carried out at 37°C (water bath) in 1.5ml plastic microcentrifuge tubes for 60 minutes, unless otherwise noted. At the start of the experiment 20µl of labeled drug were aliquoted into the microcentrifuge tubes and the reaction was started by adding a 1000µl suspension of infected or uninfected erythrocytes in complete culture at a hematocrit of 0.5%. The tubes

were gently shaken at regular intervals during the incubation period. After 60 minutes the reaction was stopped by spinning down the cells through a layer of 100µl of dibutyl phthalate oil (Sigma-Aldrich Co, Poole, England) at 13000g for 60 seconds. When needed, a 100µl sample of the reaction medium was removed and placed in a scintillation vial, and the rest of the medium was aspirated together with the layer of dibutyl phthalate. The tip of the microcentrifuge tube containing the pellet was cut off and placed in a scintillation vial and processed for scintillation counting. For medium samples, 100µl of H_2O_2 (30% w/w H_2O) was added to the vial to bleach any colour, and 2ml of scintillation fluid (Ultima Gold, Packard Bioscience, Groningen, The Netherlands) was added. For cell samples, 200µl of Solvable (0.5 molar tissue and gel solubilizer, Packard Bioscience) was added to the vials. After incubation for 15 minutes at 70°C (heating block), 50µl of 0.1M EDTA was added to the vials. The samples were then decolorized with 100µl H_2O_2 before adding 2ml of scintillation fluid. Samples were then shaken vigorously and allowed to sit for at least 2 hours before scintillation counting in a Packard Tri-Carb 4640 liquid scintillation spectrophotometer. Drug accumulation was expressed either as fmoles taken up per million trophozoites or as the accumulation ratio (AR) calculated from the amount of labeled drug in the infected cells compared with the amount of labeled drug in a similar volume of medium after incubation. The amount of labeled

drug taken up by uninfected erythrocytes was subtracted from total uptake.

4.2.3 Stage specificity of PND accumulation

Parasite cultures were synchronized at the early ring stages as close as possible to merozoite invasion. The maximum age difference between parasites was 6 hours. The culture was washed three times in complete medium after sorbitol treatment, the parasitemia was set at 10%, and the culture was permitted to grow under normal culture conditions. At selected time intervals, aliquots of culture were removed to measure PND accumulation.

4.2.4 Glucose and temperature dependence of PND accumulation

Infected and uninfected erythrocytes, were incubated at the indicated temperature for 15 minutes before being exposed to PND- [^{3}H], after which the experiment was carried out at the indicated temperature. Where glucose levels were manipulated, the reaction was carried out in Phosphate Buffered Saline (PBS, pH 7.3). First, the cells were washed three times in glucose-free PBS to remove culture medium and then re-suspended in PBS supplemented with various glucose concentrations.

4.2.5 Dependence of parasite transmembrane proton gradient

The parasite transmembrane pH gradient was altered by changing the external medium pH with HCl and NaOH. The medium used was PBS, supplemented with 10mM glucose. The pH of the external medium was measured before and after the experiment period and was found not to be significantly different. The parasite transmembrane pH gradient was also perturbed with the proton ionophore nigericin (1μM) and the specific vacuolar ATPase inhibitor bafilomycin A1 (1μM). Bafilomycin A1 is a macrolide antibiotic with a 16-membered lactone ring, isolated from *Streptomyces.* It is an extremely potent inhibitor of vacuolar ATPases and its ultimate effect is inhibition of the acidification of lysosomes (Bowman *et al* 1988). Bafilomycin A1 has been used to alkalinize acidic compartments including the digestive food vacuole in *Plasmodium falciparum* (van Es *et al* 1994a, Yoshimori *et al* 1991, Marchesini *et al* 2000, Saliba *et al* 2002). Nigericin is a carboxylic ionophore that intercalates into membranes and promotes the exchange of monovalent cations through the membranes. Nigericin equilibrates both K^+ and H^+ across the lysosomal membrane, which results in the decrease of the pH gradient across the membrane (Adovelande and Schrevel 1996, Gumila *et al* 1996). The concentrations of bafilomycin A1 and nigericin in this investigation have previously been shown to achieve a significant change in the pH gradient across the food vacuole membrane without affecting parasite viability (Hawley *et al* 1996a).

The parasite cultures were incubated with nigericin and bafilomycin A1 for 15 minutes prior to initiation of the accumulation reaction.

4.2.6 Effect of external PND concentration on accumulation

The PND-[^{3}H] was mixed with non-radioactive PND to get a series of PND-[^{3}H] preparations with lower specific activities. PBS supplemented with 10mM glucose was used as the reaction medium.

4.2.7 Competitive binding experiments

The accumulation of a fixed concentration of radiolabelled drug was determined in the presence and absence of increasing concentrations of unlabelled drug. A final volume of 10µl of unlabelled drug was aliquoted into the microcentrifuge tubes containing the radiolabelled drug. The concentration of unlabelled drug was adjusted so as to get the desired final concentration when the erythrocyte suspension was added. These experiments were carried out in PBS supplemented with 10mM glucose.

4.2.8 Analysis of Radioligand binding experiments

The analyses of the radioligand binding experiments are based on the law of mass action (Limbird 1985, Kenakin 1997):

$$\text{DRUG} + \text{RECEPTOR} \leftrightarrows \text{DRUG-RECEPTOR}$$

At equilibrium, DRUG-RECEPTOR complexes form at the same rate as they dissociate:

$$[\text{DRUG}] \times [\text{RECEPTOR}] \times K_{ON} = [\text{DRUG-RECEPTOR}] \times K_{OFF}$$

where K_{ON} and K_{OFF} are the association and dissociation constants, respectively. The equation is rearranged to define the equilibrium dissociation constant K_D:

$$[\text{DRUG}] \times [\text{RECEPTOR}] / [\text{DRUG} \times \text{RECEPTOR}] = K_{OFF} / K_{ON} = K_D$$

K_D, expressed in units of moles / liter or molar, is the concentration of drug which occupies half the receptors at equilibrium.

4.2.9 Statistical analyses

Statistical analyses were carried out using the Prism, GraphPad software. With the t tests, a P value less than 0.05 was considered significant.

4.3 Results

4.3.1 Time course of PND accumulation

The time course of PND uptake into the CQS strain D10 at an extracellular concentration of 10nM is shown in figure 4.1. The uptake of PND was measured over 1 to 120 minutes and steady state or maximal accumulation appears to be achieved within 30 minutes, with no significant increase after that. The methods employed did not permit accurate measurement of uptake at incubation times less than 1 minute.

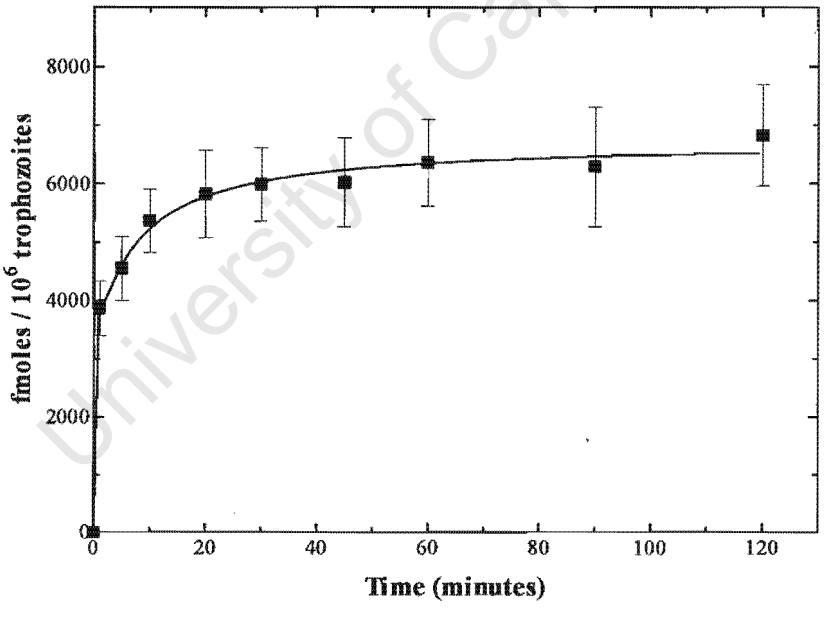


Figure 4.1: Time course of PND accumulation into pRBCs infected with D10, expressed as fmoles / 10^6 trophozoites. Each datum point represents the mean of 4 separate experiments, with error bars representing the SEM.

4.3.2 Stage specificity of PND accumulation

PND accumulation was measured at selected time intervals along the 48-hour growth period of the D10 strain of *Plasmodium falciparum*, at an extracellular concentration of the drug of 10nM. The 48-hour life cycle was divided up into 6-hour segments, as there were always a few hours difference in the ages of individual ring stage parasites after synchronization. This was the estimated maximal age difference between parasites. Figure 4.2 shows that PND uptake into the parasite increased with parasite maturation, reaching a plateau between 18 – 42 hours after invasion, a time period that is synonymous with early trophozoites and schizonts. Less mature parasites accumulated less drug. Time points after 42 hours were not taken as a significant proportion of the parasites had begun to erupt from the red blood cells (determined by visual inspection).

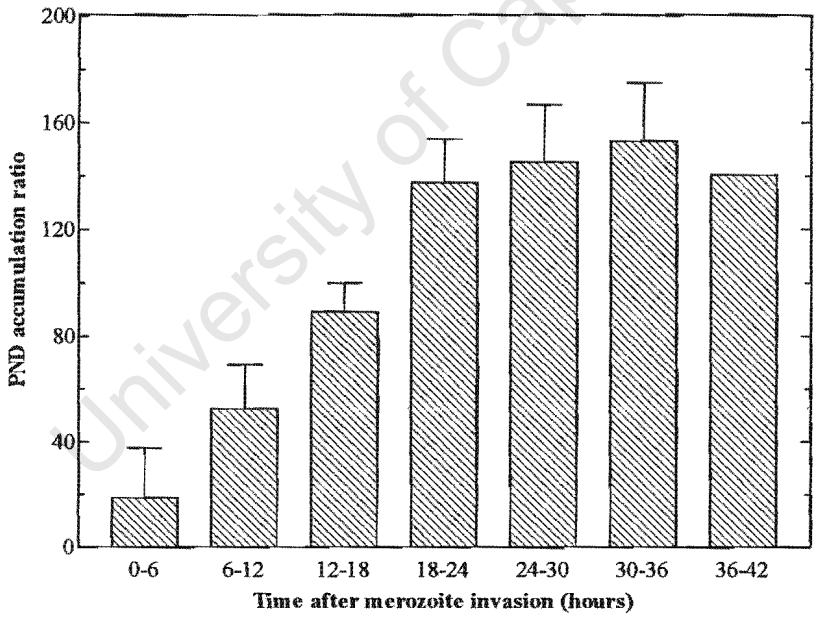


Figure 4.2: PND accumulation at different time points in the erythrocytic cycle of D10. Each column represents a mean of two separate experiments, each done in triplicate, with the SEM.

4.2.3 Effect of increasing [PND]$_{external}$ on PND uptake

PND accumulation into the D10 strain of *P. falciparum* was measured as a function of increasing external medium PND concentration over the 5nM to 500nM. Figure 4.3 shows that PND uptake occurs in a biphasic manner as the external concentration increases, with the first rapid (steeper slope) phase ending at an external concentration of 50nM and a second slower (less steep slope) phase taking over after that.

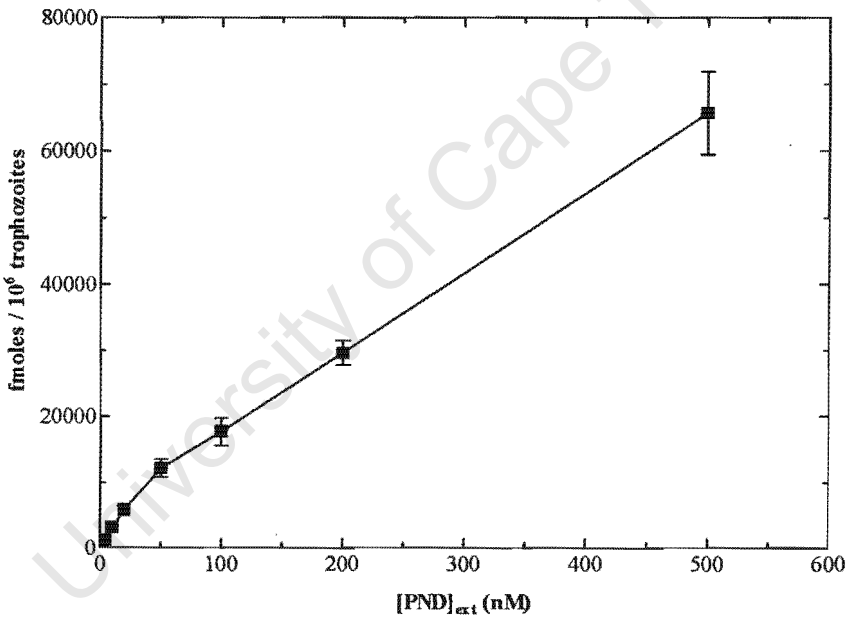


Figure 4.3: Effect of increasing [PND] $_{external}$ on PND uptake into the D10 strain of *P falciparum*. Each datum point represents the mean of 7 separate experiments each done in duplicate, with SEM.

The total uptake versus $[PND]_{external}$ data were fitted, by a computer program, using an iterative curve-fitting procedure (Graph Pad Prism version 3), to an equation that takes into consideration that the total uptake consists of two forms of uptake, saturable and a non-saturable (Motulsky, 1995). This equation also makes the assumption that the non-saturable (i.e. non-specific) component is directly proportional to the external drug concentration over the concentration range used:

$$[TD] = (([ED] \times B_{max}) / ([ED] + K_D)) + (m \times [ED])$$

Where [TD] is the total binding inside the parasite, [ED] is the external drug concentration, Bmax is the capacity or concentration of binding sites, K_D is the dissociation constant of PND at the binding site, and m is the slope of the non-saturable component of drug uptake. This equation describes a hybrid uptake curve that consists of a rectangular hyperbola superimposed on a straight line, indicative of a saturable and non-saturable component.

Figure 4.4 shows that the regression equation described above containing terms for a rectangular hyperbola and for a straight line fits the data better than the regression equation for a straight line. These data therefore support the existence of at least two components of PND accumulation, one that is saturable and the other which is non-saturable. The binding constant, K_D, was estimated to be 25.15 nM ± 6.47, with the B_{max} estimated to be 7749 ± 395.7 fmoles / 10^6 trophozoites.

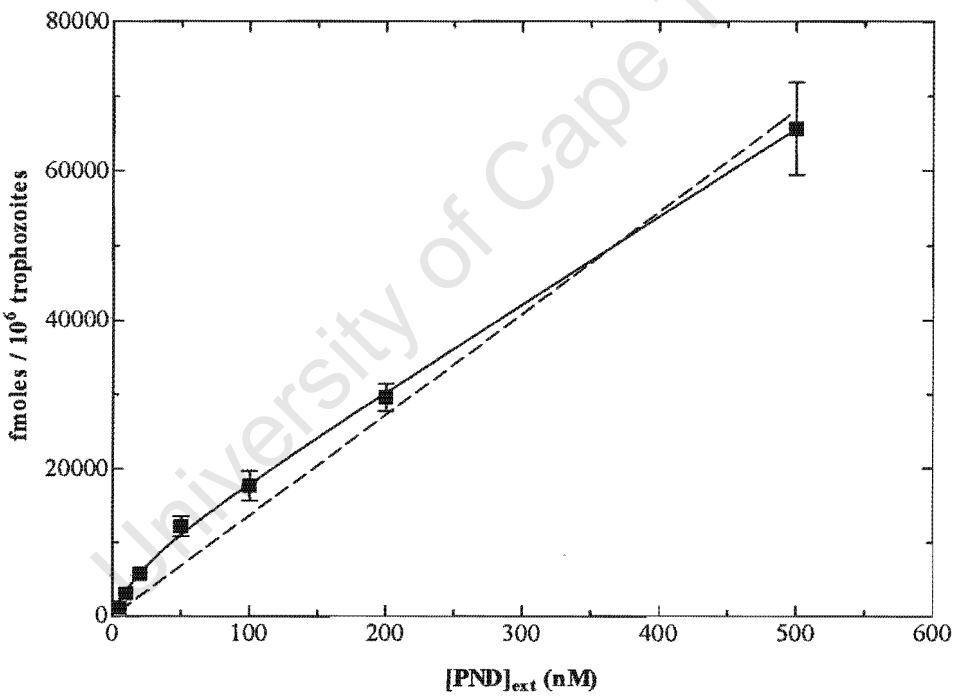


Figure 4.4: Effect of increasing $[PND]_{external}$ on PND uptake into the D10 strain of *P falciparum*. Each datum point represents the mean of 7 separate experiments each done in duplicate, with SEM. The solid line represents the curvilinear regression while the dashed line represents the linear regression.

When PND uptake is plotted as a function of increasing $[PND]_{external}$, and expressed as the accumulation ratio (figure 4.5), the saturable aspect of PND uptake is demonstrated more clearly. A steady decline of the accumulation ratio of PND uptake into pRBCs is seen as the external drug concentration increases from 5nM to 500nM. No significant difference was observed in PND uptake in uninfected RBCs across the drug concentration range used.

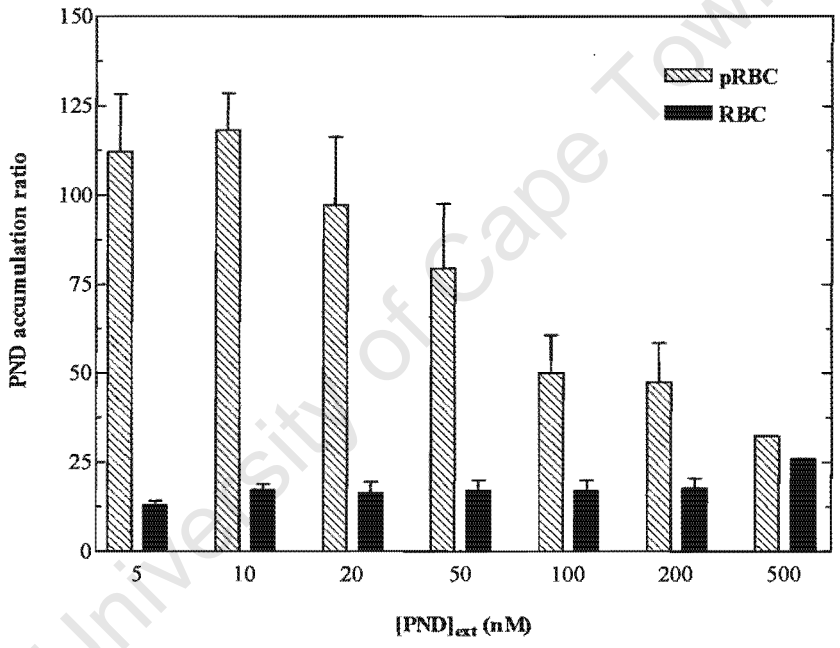


Figure 4.5: PND accumulation in parasitized red blood cells and unparasitized red blood cells (D10) at different concentrations of $[PND]_{external}$. Each column represents a mean value of 4 separate experiments each done in duplicate, with the SEM.

4.3.4 Homologous competitive binding experiments

Competitive binding experiments measure the binding of a single concentration of labeled drug in the presence of various concentrations of unlabeled drug. When using the same compound as the labeled and unlabeled drug, these competition curves can be used to determine the affinity the drug has for the intracellular receptor and also the receptor number i.e. the K_D and capacity (Motulsky, 1995). Homologous competitive binding is based on the assumptions that the receptor has an identical affinity for the labeled and unlabeled drug, that there is no cooperation in the uptake process, that there is minimal drug depletion (less than 10% of total) from the external medium, and that non-specific binding is proportional to the concentration of labeled drug.

The theory of homologous competition binding starts with the equation for equilibrium binding to a single class of receptors:

$$\text{Specific binding} = ([\text{ligand}] \times B_{max}) / ([\text{ligand}] + K_D)$$

[Ligand] is the sum of labeled and unlabeled drug, and specific binding of the labeled drug equals the specific binding of all drug times the fraction of the drug that is labeled (hot / (hot + cold)). Therefore specific binding of labeled drug is defined by the following equation:

Sp. binding of labeled drug = sp. binding of all drug x fraction of labeled drug

$$= (([Hot] + [cold]) \times B_{max}) / ([hot] + [cold] + K_D)) \times (([hot] / ([hot] + [cold]))$$

$$= (B_{max} \times [hot]) / ([hot] + [cold] + K_D)$$

Total binding equals specific binding plus non-specific binding. Since non-specific binding depends only on the hot drug (set at 10nM), it remains constant. Therefore:

Total binding = $((B_{max} \times [hot]) / ([hot] + [cold] + K_D))$ + non-specific binding

The GraphPad software program, Prism, fits this equation to a plot of total binding versus concentration of competing cold drug, and through an iterative procedure determines the values for B_{max}, log K_D and the non-specific binding.

Homologous competitive binding experiments were carried out for PND and CQ accumulation into pRBCs infected with D10, at an external drug concentration of 10nM. Under the experimental conditions used, depletion of radiolabelled drug from the medium was considered minimal (less than 10%). The homologous competitive binding curves

for PND and CQ are shown in figure 4.6. The curve described by the above equation for total binding fitted the data well with r^2 values of 0.9944 and 0.9994 for PND and CQ curves, respectively. The estimated K_D for PND and CQ were 68.2nM (95% confidence intervals = 18.36 - 253.5) and 202.8nM (95% confidence intervals = 106.9 - 384.6), respectively. The B_{max} values, at $[DRUG]_{EXTERNAL}$ = 10nM, were 11710 fmols/10^6 trophozoites ± 4122 and 160500 fmols/10^6 trophozoites ± 23970 for PND and CQ respectively. The B_{max} value for CQ was approximately 14 times greater than for PND. The percentage of the total binding that was non-specific was 45.28% ± 3.99 for PND and 27.68% ± 1.34 for CQ (figure 4.7).

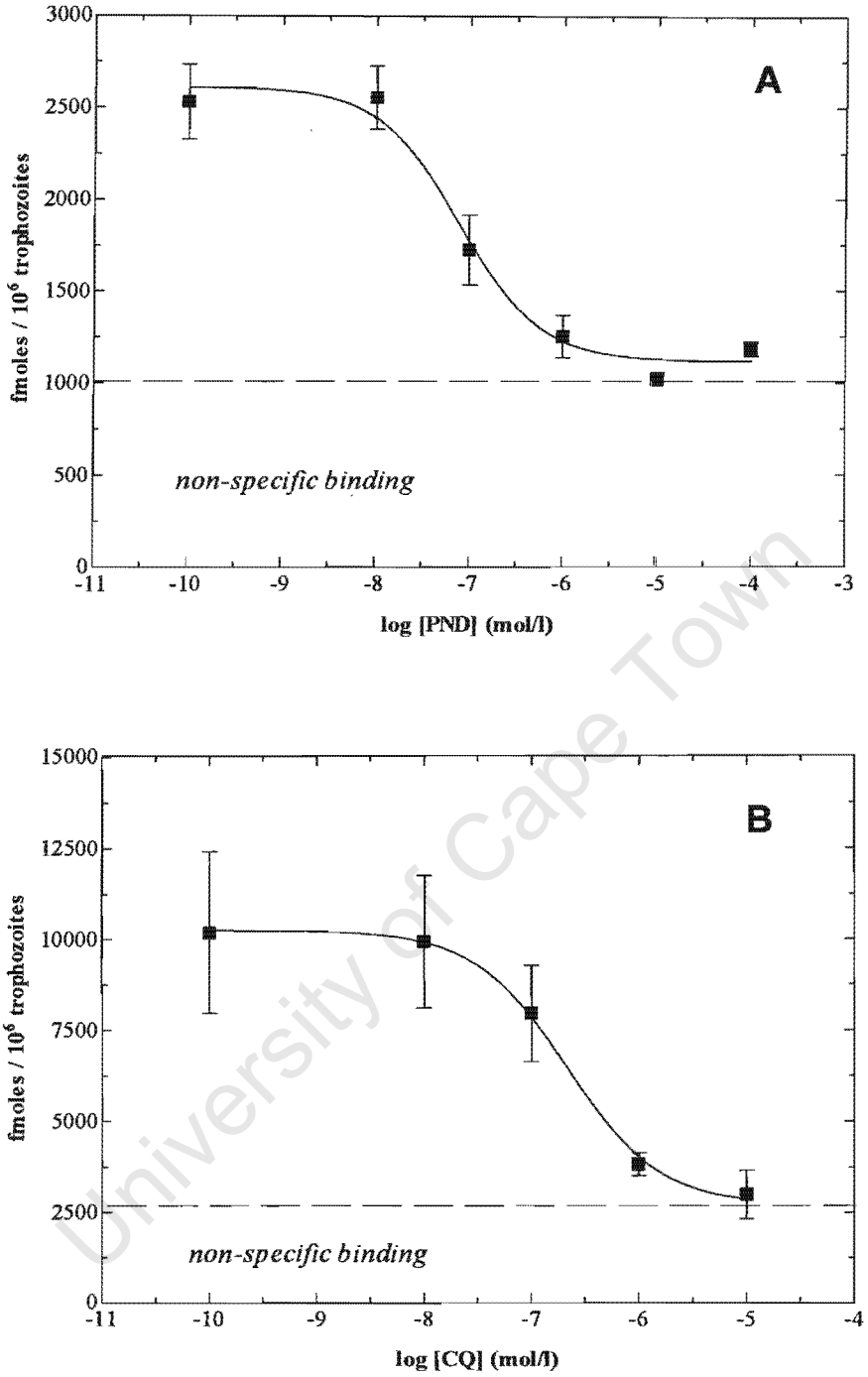


Figure 4.6: Homologous competition binding curves for PND (**A**) and CQ (**B**). Each datum point represents a mean of 3 separate experiments each done in triplicate, with the error bars representing the SEM.

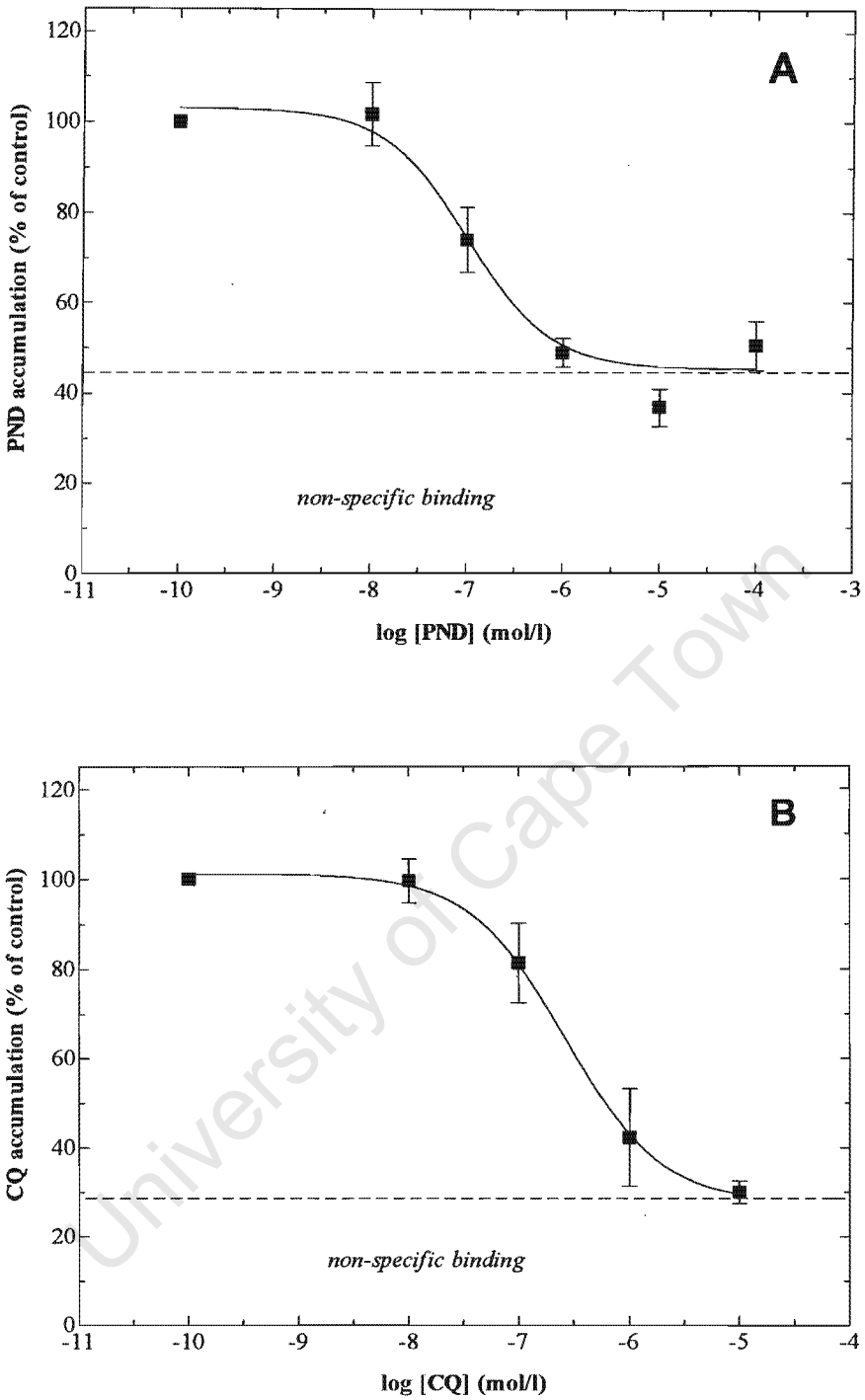


Figure 4.7: Homologous competition binding curves for PND (**A**) and CQ (**B**) with accumulation represented as percentage inhibition of control uptake. Each datum point represents a mean of 3 separate experiments each done in triplicate, with the error bars representing the SEM.

4.3.5 Effect of glucose on PND uptake

The influence of glucose on PND accumulation into D10-infected erythrocytes was investigated. PND uptake was measured in the absence of glucose and in the presence of 0.1mM, 1.0mM, 10.0mM and 100mM glucose (figure 4.8) at an external PND concentration of 10nM. Glucose did not affect PND uptake by unparasitized erythrocytes. Even in the absence of glucose pRBCs accumulated PND significantly more than RBCs with an AR of 70.0 ± 22.3 compared with 16.0 ± 1.5 for RBCs. Increasing the medium glucose concentration enhanced PND uptake into pRBCs, with a maximal uptake (AR = 143.7 ± 16.5) being achieved at a glucose concentration of 1.0mM. The higher glucose concentrations of 10mM and 100mM were less effective in stimulating PND uptake than a glucose concentration of 1.0mM.

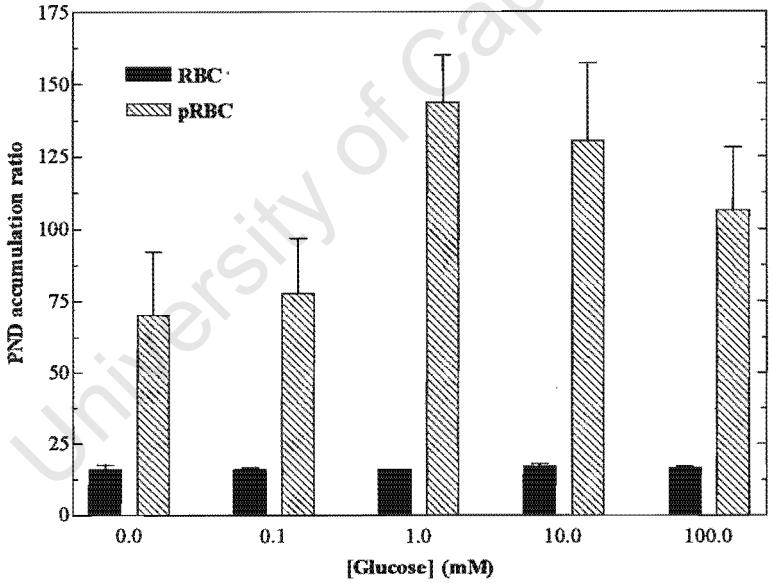


Figure 4.8: Effect of glucose concentration on PND accumulation into pRBCs and RBCs ($[PND]_{ext}$ = 10nM, parasite strain = D10). Each column represents a mean value of 3 separate experiments each done in triplicate, with the SEM.

4.3.6 Effect of temperature on PND uptake

The effect of temperature on the accumulation of PND by pRBCs (D10 strain) was measured at an external PND concentration of 5nM (this concentration is within the PND IC_{50} value for D10). Increasing the incubation temperature from 0°C to 37°C resulted in a progressive increase in PND accumulation (figure 4.9). 2553.5 ± 265.5 fmoles / 10^6 trophozoites of PND were accumulated at 37°C compared to 916.5 ± 252.0 fmoles / 10^6 trophozoites at 0°C.

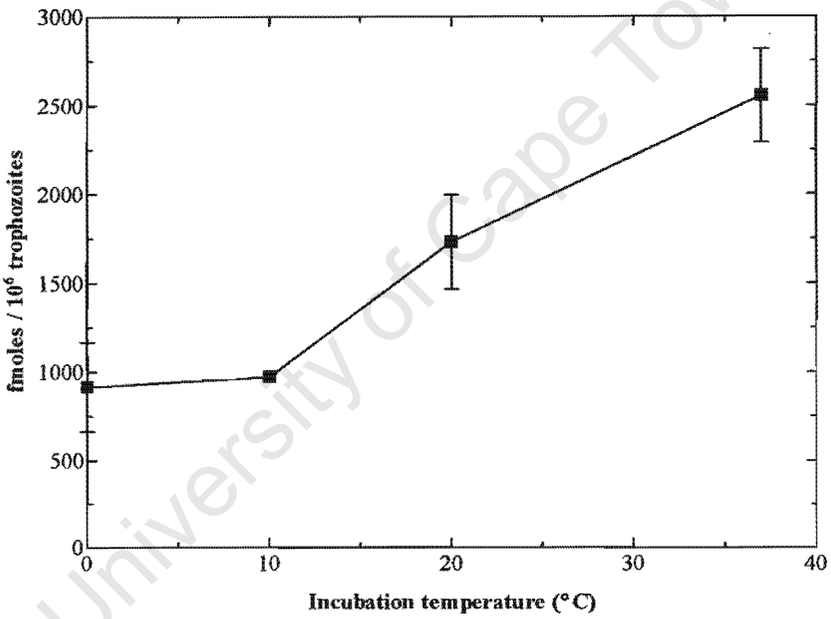


Figure 4.9: The effect of temperature on the accumulation of PND by D10. $[PND]_{ext}$ = 5nM. Each datum point represents the mean of 2 separate experiments each done in triplicate. The error bars represent the SEM.

4.3.7 The effect of external medium pH on PND uptake

Augmentation of the transmembrane pH gradient by increasing the external medium pH from 6.0 to 8.5 produced a remarkable increase in the amount of PND accumulated by pRBCs at an external PND concentration of 10nM (figure 4.10). The PND accumulation ratio increases from 40.0 ± 4.2 at pH 6.0 and peaks between pH 8.0 and 8.5 with accumulation ratios of 147.5 ± 8.8 and 135.3 ± 17.4, respectively. No appreciable difference was observed in the PND uptake by RBCs across this pH range (6.0 – 8.5).

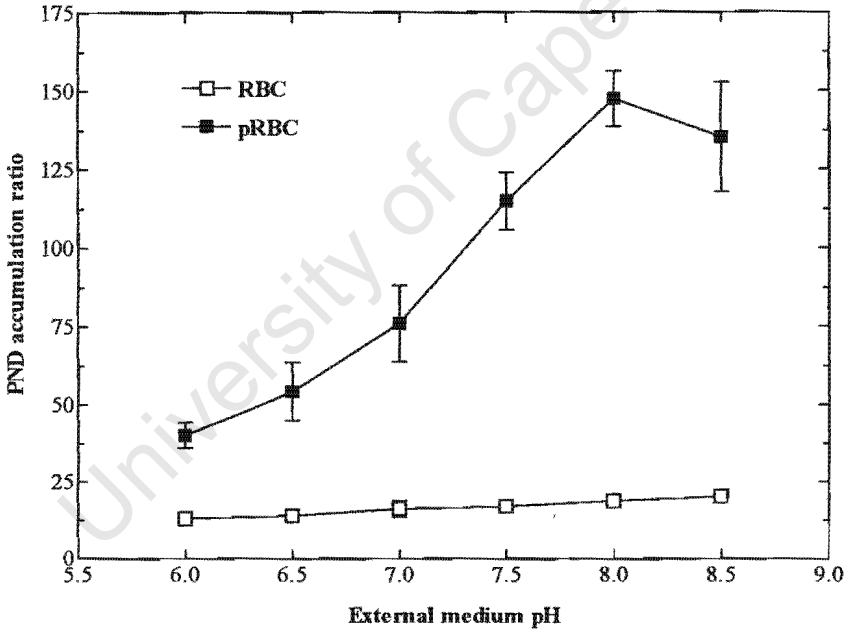


Figure 4.10: The effect of increasing external medium pH on PND accumulation by RBCs and pRBCs (infected with D10). Each datum point represents a mean value of 4 separate experiments each done in triplicate. The error bars represent the SEM.

4.3.8 Effect of Bafilomycin A1 and nigericin on PND accumulation

Figure 4.11 shows that the use of bafilomycin A1 (1μM) and nigericin (1μM), to reduce or collapse the transmembrane pH gradient, decreased PND accumulation by approximately 40% and 55%, respectively. No appreciable differences were observed in PND uptake by RBCs in the presence or absence of bafilomycin A1 and nigericin.

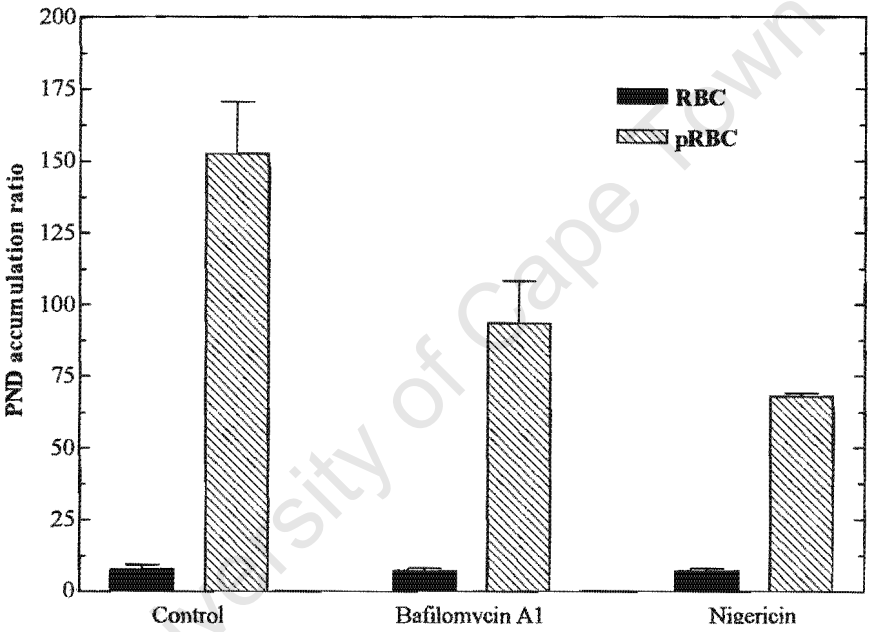


Figure 4.11: Effect of bafilomycin A1 and nigericin on PND uptake into RBCs and pRBCs (D10). $[PND]_{ext}$ = 10nM. Each column represents mean values of 2 separate experiments, each done in triplicate. Error bars represent the SEM.

4.3.9 The effect of bafilomycin A1 on pre-accumulated PND

The effect of bafilomycin A1 on PND pre-accumulated into pRBCs was investigated in order to determine the dependence of PND binding on vacuolar pH. Two parallel experiments were run. In the control experiment a normal time course of PND uptake over 1 hour was done, and in the other, bafilomycin A1 (1μM) was added to the parasite culture at 30 minutes after incubation with radiolabelled PND. Figure 4.12 shows that bafilomycin A1 caused a release of pre-accumulated PND from the pRBCs, the proportion of which is estimated to be approximately 20% of pre-accumulated PND. This release started within 5 minutes of bafilomycin A1 being added.

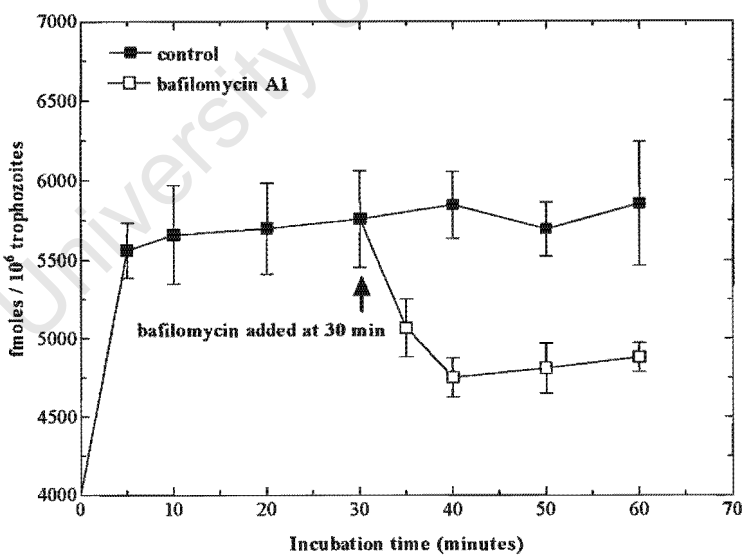


Figure 4.12: The effect of bafilomycin A1 on pre-accumulated PND into pRBCs (D10). $[PND]_{ext}$ = 10nM. Each datum point represents a mean value of 4 separate experiments, with the errors bars representing the SEM.

4.3.10 Heterologous competitive binding experiments

Competitive binding experiments were carried out to see what effect CQ, AMQ and MQ might have on PND accumulation in pRBCs (D10). This was to determine whether these drugs bind the same receptor in pRBCs. Figure 4.13(A) shows the competition curve for PND uptake with unlabelled CQ. The bottom of the curves appears to plateau at approximately 46% inhibition indicating that CQ does not completely exclude PND uptake. Figure 4.13(B) shows the competition curve for CQ uptake with unlabelled PND. CQ uptake was completely (100%) inhibited by unlabelled PND.

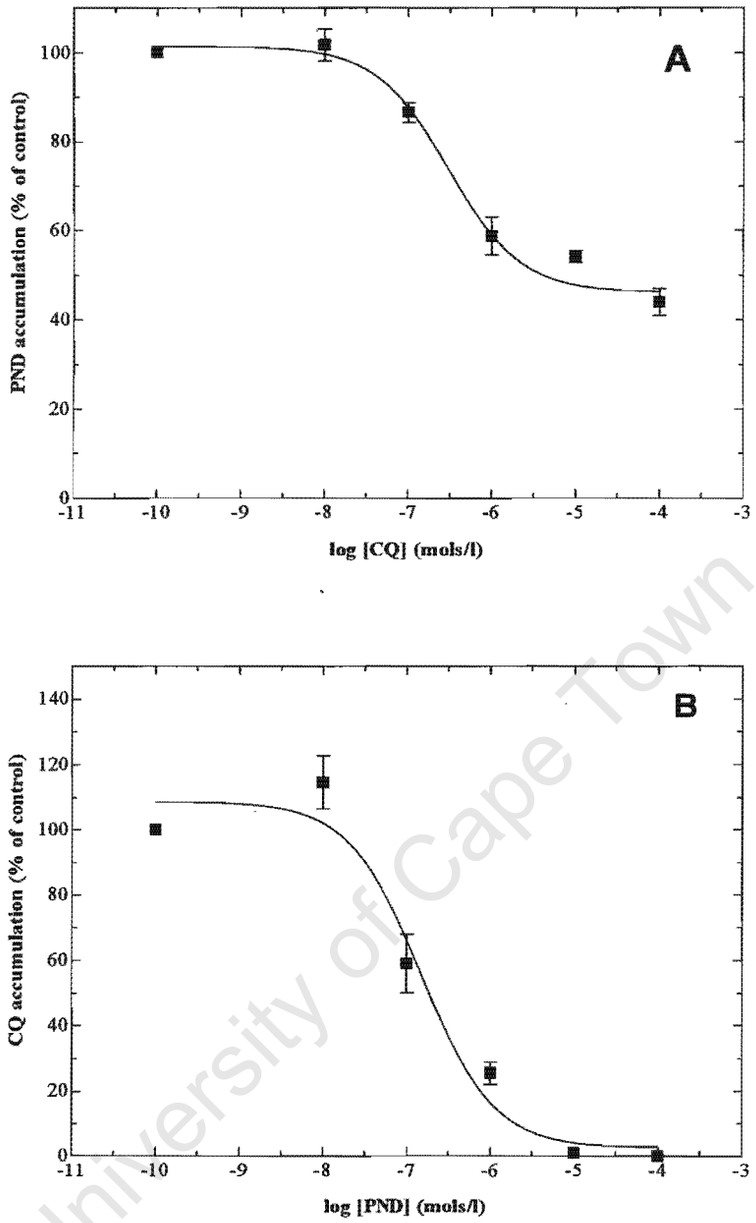


Figure 4.13: Competition binding curves for CQ and PND accumulation. **A** = PND accumulation as a function of increasing CQ concentration, $[PND\text{-}H^3]_{external}$ = 10nM (n = 5). **B** = CQ accumulation as a function of increasing PND concentration, $[CQ\text{-}H^3]_{external}$ = 10nM (n = 4). Error bars represent the SEM.

Increasing concentrations of AMQ inhibited PND uptake into pRBCs, with maximal inhibition of uptake (by approximately 20% compared to uptake into controls) occurring at [AMQ] of 250nM (Figure 4.14 A). MQ did not compete with PND uptake into pRBCs over the external concentration range of MQ tested (0 – 1000nM) (figure 4.14B).

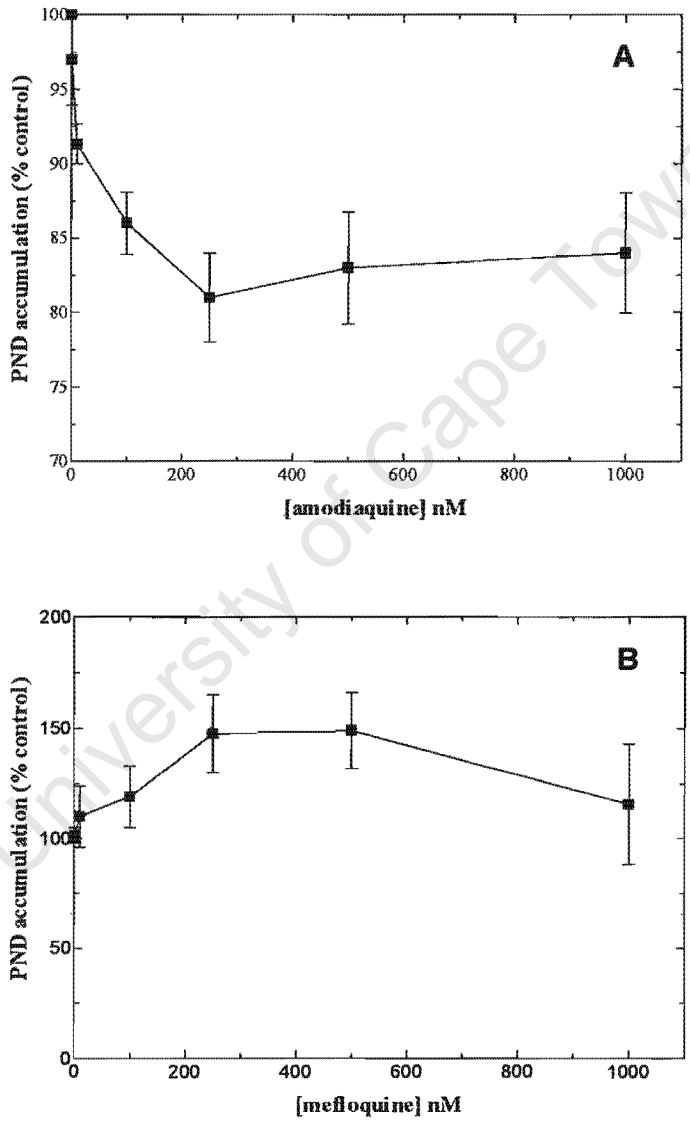


Figure 4.14: The percentage reduction of PND uptake in pRBCs (D10) by amodiaquine (A) (n = 3) and mefloquine (B) (n = 2). [PND-H^3] = 2nM. Error bars represent the SEM.

4.3.11 PND accumulation in various strains of Plasmodium falciparum

PND and CQ accumulation ratios were measured in various CQS and CQR strains of *Plasmodium falciparum* at external drug concentrations of 10nM. Table 4.1 summarizes the results obtained from these experiments. Uninfected erythrocytes accumulated approximately 2-times more PND than CQ; however, both drugs accumulated less in RBCs than in pRBCs. The mean CQ AR for CQS parasites (D10 and 3D7) (1857.0±376.0) was significantly greater (P = 0.0091) than the mean CQ AR for CQR parasites (Fac8, K1 and RSA11) (123.2±46.39). There was not a significant difference between the mean PND AR of CQS parasites (131.0±39.7) and the mean PND AR of CQR parasites (85.53±11.61), P = 0.26. In CQS parasites, the mean CQ AR was greater than the PND AR (P = 0.05), while in CQR parasites there was no difference between the mean AR of both drugs (P = 0.4749).

	Chloroquine			Pyronaridine		
Strain	AR	sem	n	AR	sem	n
D10	2233.0	160.7	3	170.7	2.8	3
3D7	1481.7	490.0	3	91.3	18.9	3
Fac8	213.8	21.9	4	64.3	5.0	4
K1	60.7	6.4	3	104.3	7.4	3
RSA11	95.0	3.0	2	88.0	24.0	2
RBCs	**3.4**	**0.2**	**15**	**7.9**	**0.5**	**15**

Table 4.1: Accumulation ratios (AR) for CQ and PND uptake into pRBCs infected with various strains of *Plasmodium falciparum*, and in uninfected red blood cells.

4 Discussion

Little is known about the uptake characteristics of PND into the parasitized erythrocyte. This is in contrast to what is known of CQ uptake. The results indicate that the mechanism of PND accumulation by the parasite is closely similar to that reported for CQ, but there are also some differences between the two drugs.

Increasing the external PND concentrations appears to result in a biphasic pattern of PND accumulation (figure 4.3). The first phase is more rapid due to the steeper slope and appears to saturate at an external concentration of 50nM. The second phase starts after 50nM and appears to be slower and not saturable. By analogy with CQ and AMQ, the first phase would represent the high-affinity low-capacity component of uptake and the second phase the low-affinity high-capacity component (Fitch 1970, Diribe and Warhurst 1985, Hawley *et al* 1996b). The high-affinity component would represent PND binding to a specific receptor, while the low-affinity component would represent non-specific binding. The low-affinity, high-capacity component represents non-specific van der Waal interactions with parasite components. The high-affinity component of PND uptake seems to have a lower capacity than CQ and AMQ, which saturated at an external concentration of 100nM (Hawley *et al* 1996b). Comparing the homologous binding curves for PND and CQ (figures 4.6 and 4.7), the uptake of PND involves greater non-specific binding than CQ.

This might be due to the greater lipophilicity of PND (Bray *et al* 1996a), and the presence of a hydroxyl group in the side chain of PND, which would encourage more non-specific hydrophobic interactions with parasite and RBC components. The estimated binding affinities for the intraparasitic receptor of PND and CQ were of the order of 10^{-8}M, which is similar to values reported previously for a CQ intraparasitic receptor (Fitch 1969). The binding affinities of PND and CQ were not significantly different (95% confidence intervals overlap considerably). The estimated binding capacities, at 10nM, for PND and CQ were different, with the binding capacity for CQ approximately 14 times greater than that of PND. This raises an intriguing question...if PND and CQ are interacting with the same receptor why are there more binding sites for CQ than for PND? A plausible explanation is that PND binds FPIX with a higher stoichiometry than CQ (i.e., a single molecule of PND binds more molecules of FPIX than CQ). It has been suggested that FPIX exist as a dimer in the environment of the food vacuole, and that drugs bind to FPIX in this form (Dorn *et al* 1998a). Dorn *et al* (1998a) suggest that the drug:FPIX-dimer stoichiometry is 1:2 for CQ and 1:3 for PND. On the other hand, Egan (2000) quotes a drug:heme stoichiometry of 1:4 and 1:7 for CQ and PND, respectively. It seems that less molecules of PND saturate all possible binding sites, in the form of FPIX, than CQ. This might explain why lower doses of PND, compared with CQ, are required to kill the parasite. Binding of drug to FPIX prevents incorporation of FPIX into hemozoin which eventually leads to a rise of

toxic free heme or drug:heme complex (Sullivan *et al* 1996b). Using the stoichiometry quoted by Dorn *et al* (1998a) or Egan (2000), a single molecule of PND would cause almost twice as much build-up of unpolymerized FPIX than CQ. In CQS strains of *P. falciparum* the IC_{50} values of PND for parasite killing are approximately two times lower than for CQ (table 2.1 in chapter 2). There could be other factors involved, but this is a reasonable explanation for the greater antimalarial activity of PND compared with CQ.

The stage-dependence of PND uptake into pRBCs indicates that as the parasite matures from rings to schizonts the capacity of the parasite to accumulate PND increases, reaching a plateau in the late trophozoites and schizonts (24 – 42 hours). Comparing the stage-dependent pattern of PND accumulation with that of CQ there is a major difference in that CQ uptake levels drop dramatically after 30 hours to uptake levels of ring stage parasites (Orjih *et al* 1994 and Saliba, 1997). This pattern of CQ uptake correlates well with the level of hemoglobin catabolism, which peaks at the trophozoite stage and switches off in the late trophozoite and early schizont stages (Orjih *et al* 1994). If it is accepted that the receptor for CQ is FPIX released from hemoglobin digestion (Chou *et al* 1980, Bray *et al* 1998a), the correlation of CQ uptake with hemoglobin catabolism is explained since the amount of FPIX is determined by the rate of hemoglobin digestion. PND might also be expected to bind to FPIX, and this is supported by the evidence that PND competes with CQ uptake (figure

4.13). PND uptake is not diminished after hemoglobin catabolism is switched off. The levels of PND uptake seem to follow hemozoin production levels (as measured by Orjih *et al* 1994), which peak and reach a plateau between 30 and 44 hours. Perhaps PND has affinity for hemozoin as well. However, this interpretation should be taken with caution, as the *P. falciparum* strain used by Orjih *et al* (1994) is different to the one used in these studies, and it has been shown that hemozoin production levels vary from strain to strain (Orjih and Fitch 1993). Moreover, estimation of parasite age can be different. It has recently been suggested that the drug affinity for heme crystals may be more important than binding to heme monomers (Sullivan 2002). This proposition is based on the evidence that quinolines bind to heme polymers in a saturable and specific manner (Sullivan *et al* 1998). PND may have a greater affinity for heme polymers than CQ. The structural differences (e.g. the presence of a hydroxyl group) on the PND molecule might contribute to this. The greater extent of PND accumulation achieved by the later stage parasites would explain the greater sensitivity of these parasites to PND (see chapter 3).

There is no significant difference in the uptake of PND in CQS compared to CQR parasites, whereas CQS parasites accumulate significantly more CQ than CQR parasites. Moreover, the mean CQ uptake in CQS parasites is approximately 14 times greater than the mean PND uptake in CQS parasites (this difference is similar to the difference in the binding capacities of the two drugs). On the other

hand, there is no significant difference in the mean AR of CQ and PND uptake in the CQR parasites. The significance of these results is that the superior antimalarial activity of PND over CQ is not due to greater accumulation of the former into pRBCs. This is in contrast to amodiaquine and other 4-aminoquinolines whose superior antimalarial activity over CQ is explained by their greater accumulation into pRBCs (Fitch 1973, Fitch *et al* 1974b, Hawley *et al* 1996a & b, Bray *et al* 1996b). The fact that PND accumulates less than CQ, but has greater activity, confirms that the intracellular target(s) for these drugs is (are) more sensitive to PND than CQ. Nonetheless, the undiminished accumulation of PND by erythrocytes infected with CQR P*lasmodium falciparum* provides a rational explanation for the high activity of this drug against CQR malaria.

The fact that CQ and AMQ compete with the uptake of radiolabelled PND suggests that these drugs might share the same intraparasitic binding site. Since the digestive food vacuole is the site of CQ accumulation (Aikawa 1972, Saliba *et al* 1998), it is assumed that this is also the site of PND accumulation. Unlabelled PND completely inhibited the uptake of radiolabelled CQ. Unlabelled CQ inhibited approximately 54% of the uptake of radiolabelled PND. The fact that CQ is unable to fully inhibit PND uptake whereas PND completely inhibits CQ uptake (100%) suggests one or more of the following: that PND has greater access and or penetrating ability to the intraparasitic receptor than CQ; that the site where the drugs recognize the receptor

accommodates PND more readily than CQ; or that PND has another receptor within the parasite to which CQ does not bind or have access. The structural differences between PND and CQ include an acridine ring system in PND instead of a quinoline ring, a phenolic group in the side chain of PND, to which 2 cyclic pyrrolidinyl groups are attached (see figure 1.2). These structural differences make PND a more lipophilic compound (Bray *et al* 1996a) and the presence of the hydroxyl group in the PND side chain would allow it to participate in inter-molecular interactions of which CQ is incapable. This structural difference might also explain the greater proportion of non-specific binding seen with PND uptake, compared with CQ uptake. Mefloquine did not compete with PND accumulation indicating that the drugs do not share a common receptor or alternatively that their mechanisms of uptake might be different.

The results show that PND accumulation in pRBCs is energy dependent, to an extent. Removal of glucose and lowering of the incubation temperature reduced accumulation of PND in pRBCs. A proportion of PND uptake occurs in the absence of glucose, and at 0°C. This would represent the non-specific uptake of PND, representing 48% and 36% of maximal uptake for no glucose and 0°C, respectively. These values are close to the 45% non-specific uptake obtained with homologous competition binding curves (figure 4.7).

Another aspect of PND uptake, demonstrated in this study, is its dependence on transmembrane pH gradient. Perturbation of the transmembrane proton gradient both externally (by altering the external medium pH) and internally (using agents to collapse proton gradients, bafilomycin A1 and nigericin) clearly affects levels of PND uptake (figures 4.10 & 4.11). The data also show that collapsing the proton gradient causes a rapid release of some of the bound PND from within the parasite (figure 4.12), suggesting a possible electrostatic interaction of PND with the receptor. As a weak base, PND is likely to be driven down the pH gradient into the parasite digestive food vacuole by passive diffusion. This would explain the influence of the transmembrane pH gradient on PND uptake by pRBCs. The pH gradient of the food vacuole in *P. falciparum* is maintained by two discrete, energy-dependent H^+ pumping mechanisms (Saliba *et al* 2002). One is a V-type H^+-ATPase and the other is a H^+-pyrophosphatase. Removal of glucose from the extracellular medium (a manoeuvre that results in a rapid depletion of parasite ATP) results in alkalinization of the food vacuole (Saliba *et al* 2002). This provides an explanation for the influence of energy on PND uptake into pRBCs; energy depletion indirectly affecting PND uptake by disturbing the food vacuole pH. The effects of glucose, temperature and the proton gradient are similar to the effects seen with CQ and AMQ accumulation (Fitch *et al* 1974b, Fitch *et al* 1975, Diribe and Warhurst 1985, Yayon *et al* 1985, Hawley *et al* 1996b).

In conclusion, the mechanisms that govern accumulation of PND and CQ in parasitized erythrocytes are similar. The main difference is how the two drugs interact with the putative receptor. PND has greater access to this receptor, which in turn seems more sensitive to PND binding. This might explain why PND displays greater activity against *P. falciparum* than CQ. The most likely candidate for the intraparasitic point of drug interaction is FPIX, a by-product of hemoglobin degradation that has been implicated as a high-affinity binding site for the quinolines (Chou *et al* 1980, Bray *et al* 1998a, 1999). The difference between PND and CQ, in their interactions with FPIX, might be explained by the physiochemical and structural differences between the two drugs. How this might influence the interaction of PND with FPIX is discussed in chapter 6.

5

EFFECT OF BAFILOMYCIN A1 ON THE *IN VITRO* SENSITIVITY *OF* *PLASMODIUM FALCIPARUM* TO PND AND CQ.

5.1 Introduction

In chapter 4 it was shown that PND accumulates in erythrocytes infected with *P. falciparum* in a specific manner. Evidence was provided that this uptake is energy dependent, and influenced by the parasite transmembrane pH gradient. Although there might be other possibilities, the results presented in chapter 4 point to the parasite digestive food vacuole as the main site of PND accumulation. In this chapter the relevance of PND uptake to its antimalarial activity is investigated. One of the most powerful techniques for determining the molecular mechanisms of drug / receptor function involves antagonism of the receptor response. Figure 4.11 shows that, at an external PND concentration of 10nM, bafilomycin A1 (1μM) reduces PND accumulation into pRBCs by approximately 40%. In the light of

this, it was assumed that bafilomycin A1 would have an antagonistic effect on PND action by limiting the uptake of PND in the digestive food vacuole, the proposed site of PND action. In view of this, the effect of bafilomycin A1 on the *in vitro* sensitivity of *P. falciparum* to PND was examined. A comparison between the effects of bafilomycin A1 on parasite sensitivity to PND and CQ, respectively, was made, in addition, as such comparison might highlight differences or similarities in the mechanism of action of the two drugs.

5.2 Methods

Chloroquine-sensitive D10 *P. falciparum* parasites were synchronized at the trophozoite stage, set at 2% parasitemia and suspended in complete medium at a hematocrit of 1%. Five separate parasite cultures were prepared, a control culture with pRBCs suspended in complete medium only, and four other cultures with pRBCs in complete medium were supplemented with four concentrations of bafilomycin A1 (10nM, 50nM, 100nM and 200nM). The pRBCs were allowed to stand in the medium containing bafilomycin A1 for about 10 minutes before being exposed to drug to allow bafilomycin A1 sufficient time diffuse to its site of action and act.

The concentration-response reaction was carried out in sterile 1.5ml microcentrifuge tubes. A final volume of 20µl of drug was aliquoted

into the microcentrifuge tubes. The drug concentrations were set so that when a final volume (1ml) of pRBC suspension was added to the tubes, the final drug concentration would be the desired concentration. The final PND concentration range was 2, 5, 10, 20, 50, 100, 200 and 500ng/ml, and the final concentration range for CQ was 5, 10, 20, 50, 100, 200, 500 and 1000ng/ml. Control tubes were also set, where pRBCs were suspended in drug-free medium at each concentration of bafilomycin A1.

The pRBCs were exposed to drug for 1 hour at 37°C. After this incubation period the pRBCs were spun down on a bench top centrifuge at 13000g for 30 seconds. The supernatant was aspirated and the pellet washed three times in drug-free complete medium. The pRBCs were finally suspended in 1ml of drug-free complete medium. Aliquots of 200µl of the pRBC culture were then put onto a 96-flat bottom well microplate and incubated at 37°C for 48 hours. After this incubation period the viability of the parasites was tested using the LDH assay (see appendix A.3). The viability of parasites exposed to drug alone is expressed as a percentage of the viability of parasites not exposed to any drug, while the viability of parasites exposed to a combination of drug and bafilomycin A1 is expressed as a percentage of the viability of parasites exposed to bafilomycin A1 alone.

5.3 Results

5.3.1 Effect of bafilomycin A1 on parasite viability

The effects of 1-hour exposure of the viability of the D10 strain of *P. falciparum* to various concentrations of bafilomycin A1 were examined. The results, summarized in figure 5.1, show that bafilomycin A1 had no effect on parasite viability over the concentration range tested.

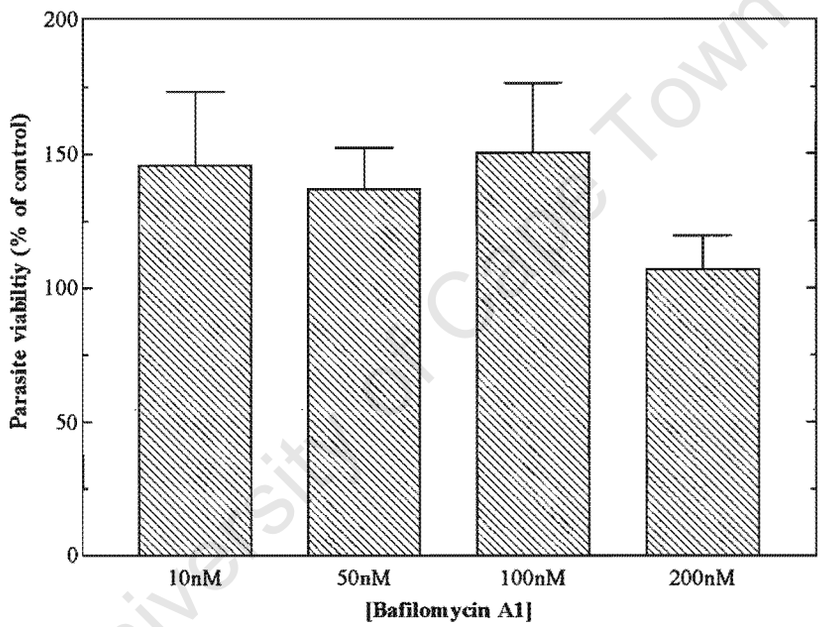


Figure 5.1: Bar chart showing the sensitivity of D10 to various concentrations of bafilomycin A1 over 1 hour. Each column represents the mean value with the error bars representing the SEM (for 10nM n = 31, for 50nM n = 32, for 100nM n = 28, and for 200nM n = 8).

5.3.2 *Effect of bafilomycin A1 on parasite sensitivity to PND*

The effect of bafilomycin A1 on the concentration-response of PND on D10 is shown in figure 5.2. Bafilomycin A1 at concentrations of 10nM and 50nM had no significant effect on D10 sensitivity to PND. At 100nM bafilomycin A1 the PND concentration-response is shifted to the right and 100% inhibition of parasite growth is not achieved with the concentration range of PND used, indicating that the parasite is being protected from the effects of PND. At 200nM, bafilomycin A1 completely inhibited the action of PND on D10 except at the last dose of PND used (500ng/ml) where parasite viability was at 61%±16.

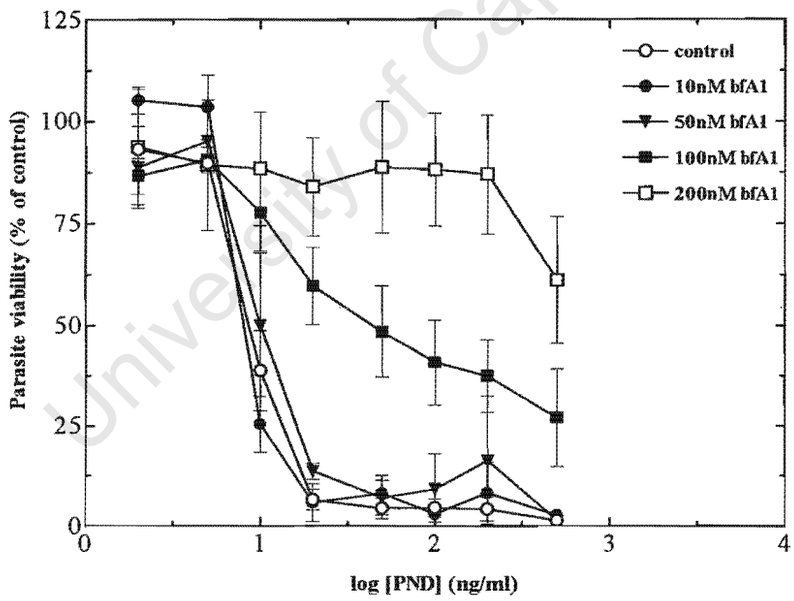


Figure 5.2: Effect of bafilomycin A1 on the dose-response of PND for D10. Each data point represents a mean value with the error bars representing the SEM, (for control n = 6, for 10nM n = 5, for 50nM n = 3, for 100nM n = 5, for 200nM n = 3).

5.3.3 Effect of bafilomycin A1 on parasite sensitivity to CQ

Figure 5.3 shows the effect of bafilomycin A1 on D10 sensitivity to CQ. After 1-hour exposure to CQ, 100% inhibition of parasite growth was not obtained, even in the control experiment. Perhaps CQ takes longer to exert its action than PND or a larger concentration range is needed for 100% inhibition. As the concentration of bafilomycin A1 is increased from 10nM to 100nM there is a progressive depression of the dose-response curve, with complete inhibition of CQ action at 100nM. The sensitivity of D10 to CQ in the presence of bafilomycin A1 is considerably reduced, in a dose dependent manner.

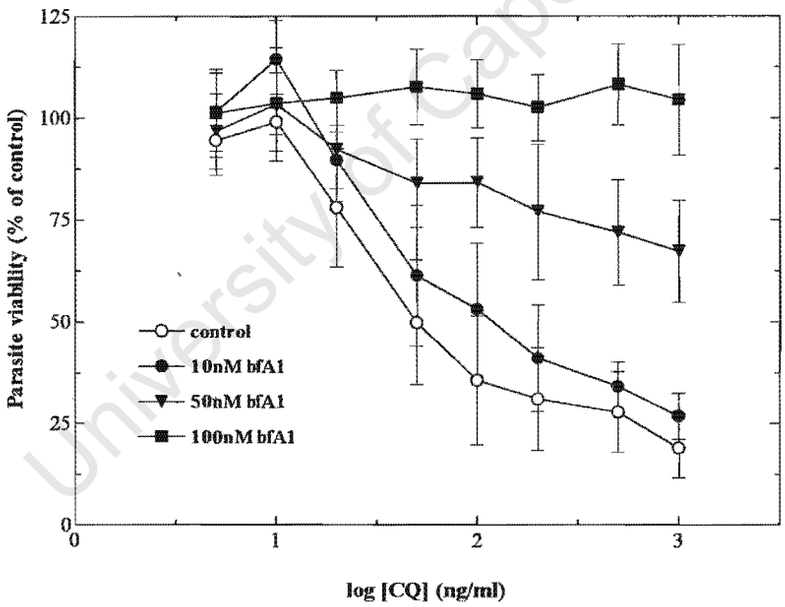


Figure 5.3: Effect of bafilomycin A1 on the concentration-response of CQ for D10. Each data point represents a mean value with the error bars representing the SEM, (for control n = 4, for 10nM n = 5, for 50nM n = 4, for 100nM n = 4).

5.4 Discussion

Following the observation that the proton gradient across the vacuolar membrane plays a part in determining the extent of PND accumulation in parasitized erythrocytes, the mode of action of PND was assessed by study of the role played by the transmembrane proton gradient in the susceptibility of parasites to PND. The vacuolar proton pump inhibitor, bafilomycin A1, has intrinsic antimalarial activity (Bray et al 1992b), but the concentrations and exposure times used in this study had no effect on parasite viability (figure 5.1). The *in vitro* sensitivity of the CQS strain D10 to PND and CQ, respectively, was measured in the presence and absence of bafilomycin A1. The sensitivity to both drugs was greatly reduced in the presence of bafilomycin A1. Although no attempt was made in this study to measure directly the effect of bafilomycin A1 on vacuolar pH, the reduction in parasite sensitivity might be a direct result of an increase in the vacuolar pH in the presence of bafilomycin A1. The effect of alkalinizing the food vacuole would be decreased uptake of the drugs into the food vacuole. These results demonstrate the importance of the pH of the parasite food vacuole in the antimalarial action of these drugs. The importance of the acidic parasite food vacuole has already been demonstrated for CQ action (Yayon et al 1985, Bray et al 1992b), but this is new evidence for PND, suggesting that the action of PND is related to its ability to concentrate inside the parasite food vacuole.

Comparing the effects of bafilomycin A1 on the concentration-response curves of PND and CQ, it is evident that bafilomycin A1 has a more pronounced effect on CQ than on PND action. 200nM bafilomycin A1 caused 100% inhibition of PND activity against the parasite, while this was achieved at 100nM bafilomycin A1 for CQ. Exact quantification of the difference in the effects of bafilomycin A1 on CQ and PND activity was not possible, as complete concentration-response curves were not obtained with the combination studies. In chapter 4 it was shown that 1µM bafilomycin A1 reduced PND uptake into pRBCs by approximately 40%, and another study carried out as part of this work the reduction in CQ uptake caused by the same concentration of bafilomycin A1 was approximately 80% (data not shown). Both CQ and PND are weak bases (with pKa values of 5.9 and 8.9 for ring and side chain nitrogens, respectively, for PND compared with 8.1 and 10.2 for CQ) and are expected to diffuse down the pH gradient into the acidic food vacuole primarily as uncharged species, where they are protonated and become trapped inside the vacuole because protonated species are not membrane-permeable (Yayon et al 1984a, Krogstad et al 1985). The lower pKa values for PND predict that its accumulation in the acidic food vacuole would be less affected by a change in the pH gradient than CQ accumulation, according to the Henderson-Hasselbach equation (Hawley et al 1996b).

The greater effect of bafilomycin A1 on CQ action might also be explained by the target in the food vacuole being more sensitive to PND. For CQ, the target in the food vacuole is thought to be heme released from hemoglobin digestion (Chou et al 1980, Bray et al 1998a, Bray et al 1999). This may also be the target for PND (Dorn et al 1998a). Competition studies described in chapter 4 indicate that PND and CQ may have a common receptor, and that PND has better access to this receptor than CQ. PND might be binding to heme with a greater stoichiometry than CQ (Dorn et al 1998a, Egan 2001), making heme more sensitive to the beta-hematin inhibitory activity of PND. According to this argument, the extent of drug accumulation plays a less important role than drug access to heme and heme sensitivity to drug binding, or the nature of the drug-heme interaction.

Another reason why bafilomycin A1 had less effect on parasite sensitivity to PND than CQ is that PND may have an additional site of action apart from the level of the food vacuole. Exposure of *P. berghei in vivo* to PND revealed that PND causes an early change in the parasites, apart from effects on the food vacuole, expressed ultrastructurally as the formation of multilamellate whorls in the pellicular complexes of trophozoites, schizonts and gametocytes (Wu et al 1988). An action of PND on both food vacuoles and pellicular complexes would to be somewhat different from that of CQ in which the pellicular complexes are not involved (Aikawa et al 1969). An

additional action of PND on the pellicular complexes might well contribute to the overall antimalarial action.

In conclusion, the data presented here indicate that an inhibitor specific to vacuolar ATPases, bafilomycin A1, influences the sensitivity of D10 parasites to PND. The findings support the involvement of the parasite digestive food vacuole in the antimalarial action of PND and they highlight the similarities and differences with CQ antimalarial action. Further investigations along this line could include studies on a CQR strain.

6

A COMPARISON OF THE HEME BINDING AFFINITIES AND BETA-HEMATIN INHIBITORY ACTIVITIES OF CHLOROQUINE AND PYRONARIDINE.

6.1 Introduction

Although the mechanism of action of chloroquine on the malaria parasite is still not fully understood, the importance of heme is clear. Free heme is generated inside the food vacuole of the parasite during digestion of ingested host cell hemoglobin (Chou and Fitch 1981, Orjih *et al* 1981, Fitch 1989). One of the ways by which the parasite avoids the toxicity of heme is to convert it to the harmless insoluble hemozoin (Slater *et al* 1991, Bohle *et al* 1997, Sullivan 2002). The action of CQ against malaria parasites relies on its ability to accumulate inside the parasite digestive food vacuole and to form complexes with heme, inhibiting the formation of hemozoin and in doing so, allowing free toxic FPIX to exert its cytotoxic effect. CQ affects hemozoin production *in vivo* (Chou and Fitch 1993, Orjih and Fitch 1993,

Asawamahasakda *et al* 1994, Orjih *et al* 1994, Orjih 1997). The heme polymerization reaction can be carried out *in vitro* using parasite extracts (Slater and Cerami 1992, Dorn *et al* 1998a), preformed hemozoin nucleation (Dorn *et al* 1995, 1998b), chemical synthesis (beta-hematin) (Egan *et al* 1994, Blauer and Akkawi 1995), synthetic lipids (Bendrat *et al* 1995), or *P. falciparum* histidine-rich proteins (Sullivan *et al* 1996a). CQ inhibits heme polymerization initiated by each of these methods.

There is evidence that PND acts in a similar manner to CQ and experimental results presented in previous sections seem to confirm this, although there could be various mechanisms of action for PND. It has previously been shown that PND interacts with heme and is able to inhibit polymerization of heme into beta-hematin (Dorn *et al* 1998a&b, Hawley *et al* 1998). In this chapter, the aim was to determine whether these actions might reveal why PND has greater antimalarial potency than CQ. Is the greater potency of PND explained by its stronger association with heme? Or is PND a superior inhibitor of heme polymerization to CQ?

Previous studies have given inconsistent results. Dorn *et al* (1998a) showed that PND bound to heme more weakly than CQ, with association constants of 3.0×10^{-5} M and 4.0×10^{-5} M, respectively, and that PND had a lower IC_{50} (30μM) than CQ (45μM) for inhibition of heme polymerization. Conversely, Hawley *et al* (1998) showed that

CQ had a lower IC_{50} (24.4μM) than PND (64.4μM) for the inhibition for heme polymerization. These studies were carried out in different laboratories although the same methods were used for measuring beta-hematin inhibitory activity. Under the experimental conditions used by Dorn *et al* 1998 (experiments were done in predominantly aqueous solution), heme exists as aggregates of μ-oxo dimers. Binding constants obtained for CQ and PND were for interactions with this species with a drug:heme μ-oxo dimer binding stoichiometry of 1:2 and 1:3 respectively. Under these conditions, results can be unreliable and difficult to interpret because when spectrophotometric titrations of heme with aminoquinolines are done in purely aqueous media, there is little difference between the Soret band of the porphyrin in the resulting complexes and that of the starting hematin dimers (Egan *et al* 1997, Kaschula *et al* 2002). The intention of this study was to measure the binding constants of CQ and PND with strictly monomeric heme to allow for direct comparison of the binding affinities of the two drugs. Heme was maintained in monomeric and unaggregated state in a minimum of 40% aqueous DMSO (Egan *et al* 1997, Kaschula *et al* 2002). A good indication of the trends in association constant likely to occur in the food vacuole is obtained using this method. This is supported by the fact that the free energies of association of heme with known quinoline antimalarials are similar using both methods (Egan and Marques 1999, Kaschula *et al* 2002).

The beta-hematin inhibitory activity of CQ and PND were measured using an assay different from that employed by Dorn *et al* (1998), one that has been optimized and standardized for assessing the beta-hematin inhibitory activity of antimalarial drugs (Parapini *et al* 2000).

6.2 Methods

6.2.1 Measurement of beta-hematin inhibitory activity

The assay used to measure the heme polymerization inhibitory activity of CQ and PND is the same as described by Parapini *et al* (2000). IT is a spectrophotometric assay that is based on the characteristic absorbance of hematin in NaOH at a wavelength of 405nm. The relative amounts of polymerized and unpolymerized hematin are determined, based on solubility in DMSO. This method has optimized and standardized the conditions for assessing the beta-hematin inhibitory activity of antimalarial drugs.

50µl of a 8mM solution of hemin chloride dissolved in DMSO was distributed in 96-well U-bottom microplates (0.4µmol/well). 50µl of either chloroquine diphosphate or pyronaridine diphosphate dissolved in water, in doses ranging from 3.91µM to 16mM was added to test wells in quadruplicate. In control wells, 50µl of water was added. Beta-hematin formation was initiated by the addition of 100µl of 8M

acetate buffer (pH 5) to the wells. The plates were then incubated at 37°C for at least 18 hours to allow for complete reaction and then centrifuged at 3300g for 15 minutes. The soluble fraction of unprecipitated material was aspirated. The remaining pellet was then resuspended with 200µl of DMSO to remove unreacted hematin, and the plates again were centrifuged at 3300g for 15 minutes. The DMSO-soluble fraction was aspirated and the remaining pellet, consisting of pure precipitated beta-hematin was dissolved in 200µl of 0.1M NaOH. 10µl of this fraction was then transferred to a new 96-well microplate, containing 200µl of 0.1M NaOH / well, for spectroscopic quantification. The amount of hematin in each well was determined by measuring the absorbance at a wavelength of 405nm, with NaOH a blank control, in a microplate reader. The amount of hematin in the test wells is expressed as a percentage of the amount of hematin in the control wells. The IC_{50} for inhibition of heme polymerization was determined by curve fitting non-linear regression analysis methods (Prism, GraphPad Software).

6.2.2 Measurement of heme-binding affinities

The method described by Egan *et al* (1997) was used to measure the binding affinity of CQ and PND to ferriprotoporphyrin IX (FPIX). FPIX-drug interactions are monitored spectrophotometrically by titrating a heme solution with a drug solution and measuring the characteristic Soret band at a wavelength of 405nm. A hemin stock solution was

made by dissolving 8.2mg of hemin chloride (Sigma) in 10ml DMSO. 4µl of the hemin stock solution was added to 2ml of a reaction mixture consisting of 800µl DMSO, 200µl of 0.2M HEPES buffer and 1000µl water, making a final concentration of hemin of 2.6µM. At this concentration hemin is monomeric in 40% DMSO solution. Chloroquine diphosphate and pyronaridine diphosphate solutions were dissolved in 4ml DMSO, 1ml 0.002M HEPES buffer (pH 7.4) and 5ml water to make drug solutions with final concentrations of 1.94mM and 1.93mM, respectively.

Spectrophotometric titrations were performed at 25°C in a glass cuvette, using a Beckman DV-68 spectrophotometer. FPIX-drug interactions were monitored by titrating the FPIX reaction mixture with either the CQ or the PND solution and measuring absorbance of the Soret band at 402nm. In each case, the absorbance data were corrected for dilution. PND diphosphate solution is orange in color and it exhibited significant absorbance at 402nm. A blank titration was performed with PND solution and the absorbance of PND was subtracted from the titration data. The resulting titration curves were analyzed using least square non-linear curve fitting. Analyses were performed using a 1:1 complexation model of drug:FPIX interaction. In this model the change in absorbance as a function of free drug concentration ($[DRUG]_{free}$) is given by the following equation:

$$A = (A_O + A_K \cdot [DRUG]_{free} / (1 + K \cdot [DRUG]_{free})$$

Where A_O is the absorbance without drug added, A_K is the absorbance for the FPIX-drug complex and K is the association constant.

6.3 Results

6.3.1 Beta-hematin inhibitory activity of CQ and PND

The beta-hematin inhibitory activity of CQ and PND is demonstrated in figure 6.1. Both CQ and PND inhibited the formation of beta-hematin, with PND showing greater inhibitory activity than CQ, with IC_{50} values of 98.45 ± 7.82 µM and 51.70 ± 3.14 µM for CQ and PND, respectively. An unpaired t test revealed that these mean IC_{50} values are significantly different (P = 0.0002).

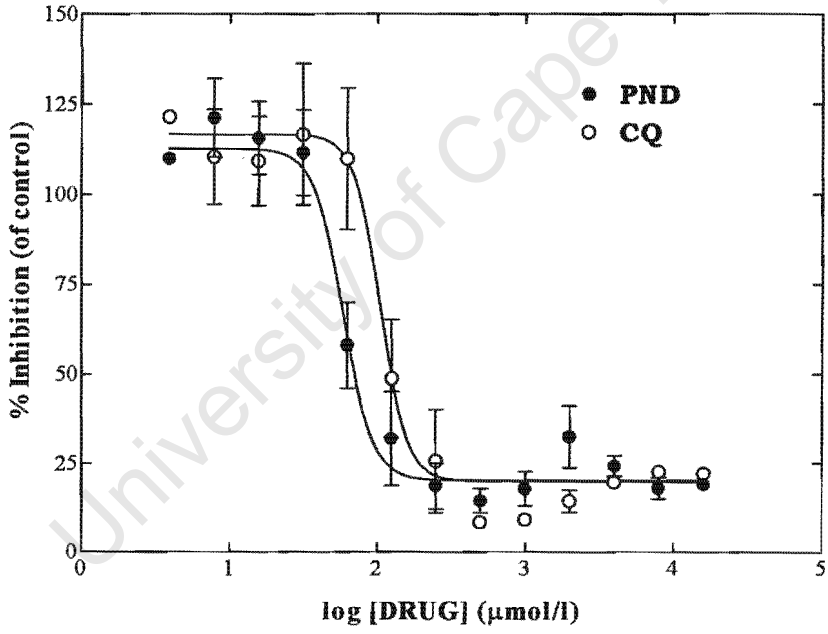


Figure 6.1: Beta-hematin inhibitory activity of CQ and PND. Each datum point represents a mean value of 6 separate experiments done in quadruplicate, with the error bars representing the SEM.

6.3.2 Heme Binding

Three individual experiments were done for each drug and the titration data shown in figure 6.2 is a sample of one experiment for each drug.

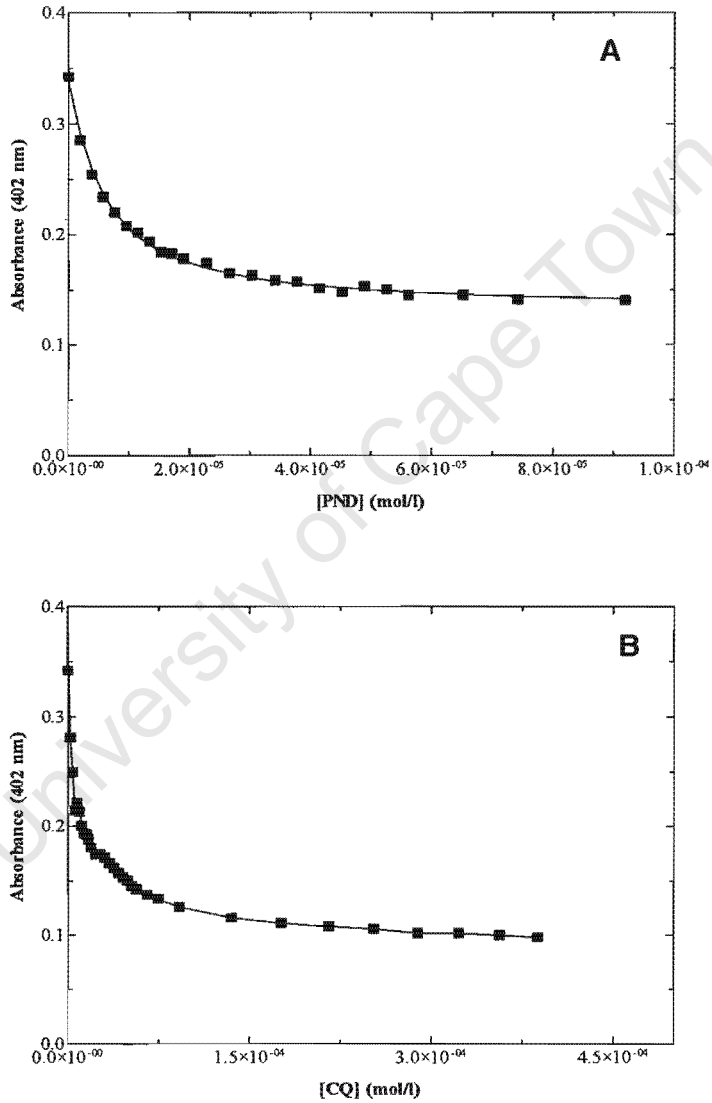


Figure 6.2: Variation in absorbance of FPIX at 402nm as a function of drug concentration. The solid lines are the best fits of the model described above to the data. A = titration curve for PND, B = titration curve for CQ.

The decrease in absorbance is an indication that both CQ and PND associate with FPIX. The data for both drugs fitted the 1:1 complexation model closely and the association constants (expressed as log K) obtained from this model were 4.963 ± 0.018 (n = 3) and 5.797 ± 0.389 (n = 3) for CQ and PND, respectively. The greater log K value for PND suggests that PND binds FPIX with greater affinity than CQ.

6.4 Discussion

In considering what role heme binding and inhibition of heme polymerization might play in the superior antimalarial action of PND over CQ it is necessary to understand the mechanisms of these processes.

The complexes formed between 4-aminoquinolines and other acridine-based drugs with heme, in neutral or weakly acidic aqueous medium where heme exists as a μ-oxo dimer, and in aqueous-DMSO where heme is monomeric, involve coplanar (π-π) interactions between the quinoline ring / acridine ring of the drug and the macrocyclic heme molecule (Dorn *et al* 1998a, Egan *et al* 1998, Egan 2001).

It was first suggested that quinoline drugs inhibit hemozoin production by acting at a putative heme-polymerase enzyme

responsible for hemozoin formation (Slater and Cerami 1992); however, subsequent investigations showed that these drugs inhibit heme polymerization in the absence of any protein, by directly interacting with heme (Egan *et al* 1994, Dorn *et al* 1995, Sullivan *et al* 1996b, Basilico *et al* 1998, Hawley *et al* 1998, Sullivan *et al* 1998). The current theories that explain how drugs inhibit heme polymerization include: a) binding of drug to heme prevents free heme from becoming incorporated into hemozoin; and b) the drug-heme complex confines the existing heme polymer and in doing so stops its extension. Since hemozoin is a crystal, the drugs are thought to block the fastest growing face of the crystal (Sullivan *et al* 1996b, Sullivan *et al* 1998, Egan *et al* 2001, Sullivan 2002).

The results presented here show that PND binds monomeric heme with greater affinity than CQ and that PND is the more potent inhibitor of beta-hematin formation. The greater affinity of PND for heme might be due to the greater number of drug-heme binding geometries accommodated by the larger acridine ring system in PND compared with the quinoline ring system in CQ (Dorn *et al* 1998a). Although other physiochemical properties such as size, shape and lipophilicity may also play a role, the results are consistent with the proposition that the strength of heme binding is important in determining the potency of a compound in inhibiting heme polymerization and parasite growth (Dorn *et al* 1998a, Egan *et al* 2000, Kaschula *et al* 2002). It can be concluded the greater

antimalarial activity of PND over CQ is in part due to the greater ability of the former to inhibit heme polymerization. Other factors such additional sites of action might contribute to the overall action of the drugs. As mentioned in chapter 1, histidine-rich proteins have been implicated in hemozoin formation in the malaria parasite (Sullivan *et al* 1996a, Choi *et al* 1999, Lynn *et al* 1999, Papalexis *et al* 2001, Pandey *et al* 2003); therefore, investigations into how PND and other quinoline antimalarials interact with these histidine-rich proteins in their effect on heme polymerization are required for a fuller understanding of how the drugs work. It has already been shown that CQ is able to inhibit beta-hematin formation in the presence of histidine-rich proteins.

7

CONCLUSION

One of the features of chloroquine resistance in *Plasmodium falciparum* is that the red blood cell stage parasites show cross-resistance with other 4-aminoquinolines, and with compounds such as mepacrine that share the alkylaminoalkyl side chain (Peters and Robinson 1992, Bray and Ward 1998). However, inter-group cross-resistance is not consistent. In some cases, clinical experience indicates that radical cure can be obtained in many infections with AMQ, which is a 4-aminoquinoline although it also has a Mannich base (Kotecka *et al* 1997). These findings have stimulated a search for other Mannich bases, among which PND was found to be highly effective in curing multiple drug-resistant falciparum infections in China and more recently in Africa (Fu and Xiao 1991, Ringwald *et al* 1998). Previously published data suggest that the mechanism of action of PND is similar to that of CQ and AMQ. However, analogous chemical structures do not necessarily mean identical modes of action, and further investigations are needed to fully determine the

details of the antimalarial mode of action of PND. Since the selective toxicity of CQ is related to its uptake in high concentration by parasitized red blood cells, the main focus of this thesis was to investigate the accumulation of PND in pRBCs infected with *Plasmodium falciparum* in order to gain a better understanding of the mechanism of action of PND. Radiolabelled PND was used to characterize the factors that control the uptake of PND into pRBCs and to investigate the relationship between PND accumulation and the antimalarial activity of the drug. The *in vitro* stage-, time- and dose-dependence of PND antimalarial action against the erythrocytic forms of *Plasmodium falciparum* was characterized. In addition, the heme-binding affinities and beta-hematin inhibitory activities of PND and CQ were assessed and compared.

The antimalarial activity of PND was shown against 6 strains of *Plasmodium falciparum*, with the CQR strains being less sensitive to PND than the CQS strains. The degree of antimalarial activity of PND against the red blood cell stages of *Plasmodium falciparum* is dependent upon the duration of exposure to the drug, longer exposure times having a greater effect on parasite viability. The more mature forms of the erythrocytic stages of the parasite (schizonts and trophozoites) are more susceptible to PND than the ring forms.

Pyronaridine accumulation into pRBCs is greatest in the trophozoite and schizont stages and is dependent on glucose, temperature and pH

gradients. The relationship between external PND concentration and PND accumulation in pRBCs follows an equation describing closely a rectangular hyperbola superimposed on a straight line, indicating that PND uptake consists of both a saturable and non-saturable components. Competitive homologous binding curves show that PND accumulation is specific, indicating that PND binds with an intraparasitic receptor, and that this binding site may also have affinity for CQ and AMQ. The latter is suggested by the competition for drug accumulation that has been demonstrated. In the CQS D10 parasite, there is no significant difference between PND- and CQ-binding affinities for the intraparasitic receptor; however, the binding site has a greater capacity for CQ than for PND. There is no significant difference in PND accumulation by CQS and CQR parasites, respectively. The pH-dependent component of PND uptake was found to play a critical role in the antimalarial activity of PND, as reduced PND uptake greatly reduced parasite sensitivity to PND. In this work it has been shown that PND binds heme with greater affinity and that it is a more potent inhibitor of heme crystallization than CQ.

Several conclusions might be drawn regarding the mode of action of PND against *Plasmodium falciparum,* drawing from published reports and the data provided in study. As a weak base, it was predicted that PND would diffuse into the digestive food vacuole of the malaria parasite, driven by an energy-dependent pH gradient across the food vacuole membrane and the external medium. Once inside the food

vacuole, PND would bind multiple molecules of heme released from degradation of hemoglobin and, in doing so, prevent heme incorporation into hemozoin crystals, thus causing a build up of toxic heme or PND:HEME complexes within the parasite, which eventually destroys the integrity of the parasite membranes or inhibits key metabolic processes.

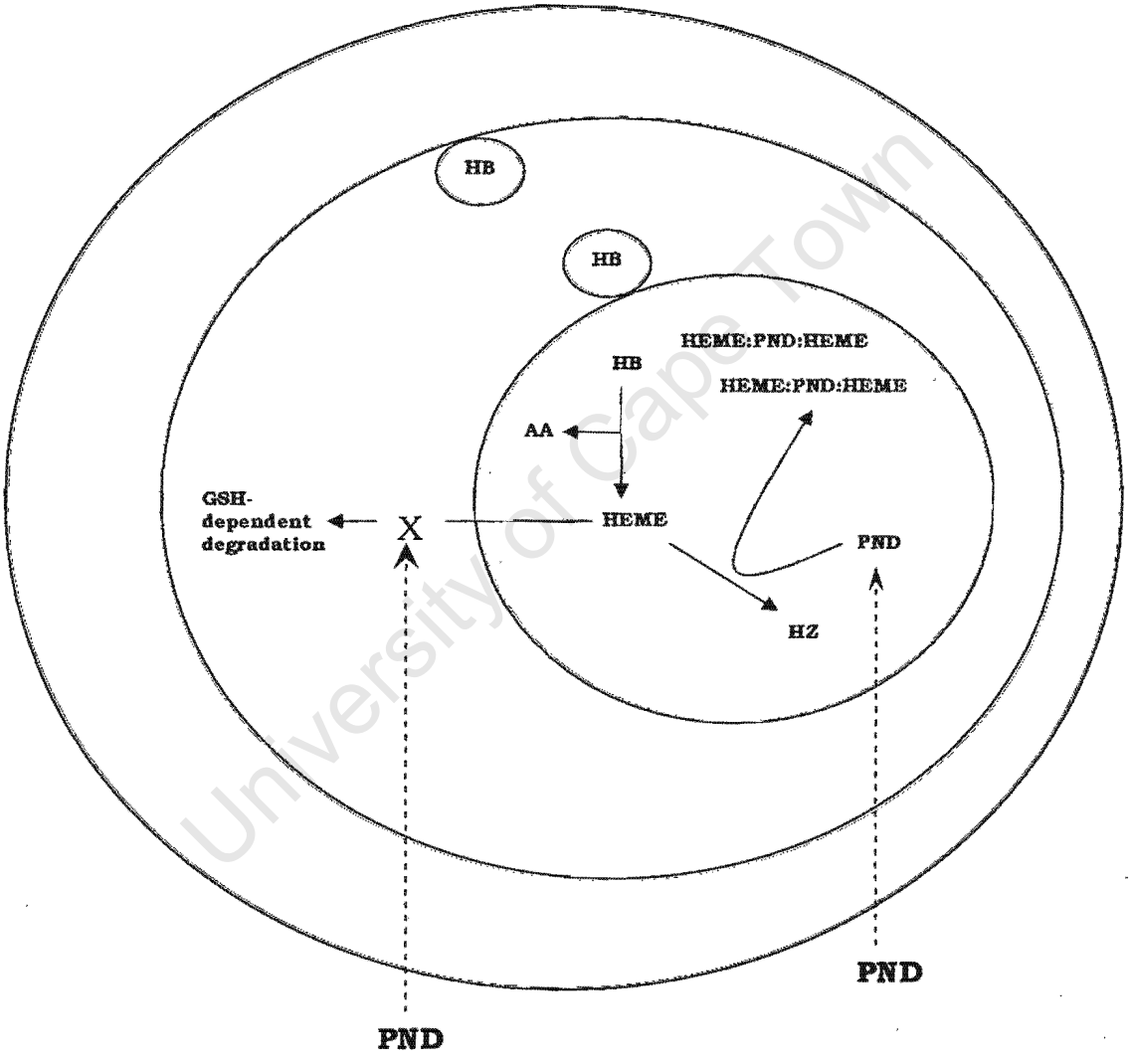


Figure 7.0: Proposed mechanism of action of PND. Color code: red - red blood cell cytoplasm, yellow - parasite cytoplasm, green - food vacuole. AA – amino acids, HB - hemoglobin, HZ - hemozoin, GSH – reduced glutathione.

PND might also inhibit glutathione-dependent degradation of heme in the parasite cytosol, causing accumulation within the parasite of toxic, membrane-associated, heme molecules. The entire drug action of PND might be the sum of PND acting on different targets, as additional mechanisms of action may exist, and the inhibition of heme crystallization could be the dominant mode of action of the drug.

The possibility that a single molecule of PND binds more molecules of heme than does an equivalent mass of CQ might be a significant factor when considering the superior activity of PND over CQ. A lesser concentration of PND would be required to cause an equivalent build up of free heme to lethal levels. The greater lipid solubility of PND over CQ might also be important, as greater lipophilicity would permit a significant increase in transmembrane movements of the drug and allow the drug better access to target sites. Structure-function relationship studies have revealed that greater lipophilicity is closely correlated with enhanced antimalarial activity (Chavalitshewinkoon *et al* 1993, Bray *et al* 1996a, Hawley *et al* 1996a).

The fact that PND accumulation is not different in either CQR or CQS parasites indicates that the CQ resistant mechanism (whatever it might be, and which is associated with reduced intraparasitic CQ accumulation) does not affect PND accumulation. This is in contrast to *in vitro* susceptibility studies, which show that PND is less effective against CQR parasites. Perhaps the techniques used to measure drug

accumulation were not sufficiently sensitive to detect a difference. The uptake studies measured drug uptake in various strains of *P. falciparum* at only one concentration. A comparison of PND accumulation in various strains of plasmodium with a range of external drug concentrations would have been more suitable for detecting different PND binding capacities in different strains. Another limitation to this study has been that the intraparasitic binding affinities of PND and CQ have not been compared in various CQS and CQR strains since CQ resistance might be due to reduced binding affinity to the intraparasitic receptor.

This study gives support to the suggestion that the mechanism of actions of PND and CQ, and other 4-aminoquinolines, are similar. Even in the presence of drug resistance, the CQ target in the parasite might be exploited by alterations in the chemical structure of related drugs. A better understanding of how these alterations might bypass the resistance mechanism is needed. This would include further investigation of PND action, including structure-function studies. Although the ability of the drug to concentrate inside the parasite digestive food vacuole is essential for antimalarial activity of 4-aminoquinoline related drugs, binding to heme with a greater stoichiometry might also be important in determining antimalarial potency. A study examining the correlation between heme binding stoichiometry and antimalarial activity would contribute to this

understanding, and provide a framework for the design of new quinoline drugs.

The radioligand binding studies reported in this thesis provide evidence that PND binds to an intraparasitic receptor. The likely candidate for this receptor is heme, released as a by-product of hemoglobin degradation in the digestive food vacuole. The stage-specificity of PND accumulation and its antimalarial action lends support to the suggestion that heme might be the receptor for PND. PND uptake and action are greatest in the mature forms of the red blood cell stage parasites when hemoglobin degradation is at its maximum. Further support is provided by the evidence that CQ and PND compete for intraparasitic binding, heme being the likely receptor for CQ in the malaria parasite (Chou *et al* 1980, Bray *et al* 1999). However, more direct evidence is needed unequivocally to identify heme as the receptor. Hemoglobin protease inhibitors would be useful in this regard. Investigations which measure parasite sensitivity to PND and PND accumulation in the presence of hemoglobin protease inhibitors might further clarify the importance of heme in the action of PND.

APPENDIX

A.1 Cultivation of Malaria Parasites

Stocks of *Plasmodium falciparum* parasite cultures were maintained essentially using the method described by Trager and Jensen (1994), but with a few modifications. The asexual form of the parasite was maintained in human O-positive erythrocytes suspended in culture medium, which consisted of RPMI-1640 with glutamine (GIBCO) supplemented with hypoxanthine (44mg/l), HEPES buffer (6g/l), glucose (4g/l), NaHCO3 (2.1g/l), gentamycin (50mg/l) and cell culture grade ALBUMAX II (5g/l). ALBUMAX II (GIBCO, Life Technologies) is lipid-enriched bovine serum albumin and was used as a replacement for human serum. Parasitized erythrocyte suspensions were maintained at parasitemias between 1% and 20% buy routine dilution with uninfected erythrocytes (washed twice with culture medium without ALBUMAX II to remove buffy coat). The pRBCs were incubated at 37°C in sealed 50ml culture flasks, flushed with a 93% N_2, 4% CO_2 and 3% O_2 gas mixture. Culture medium was changed daily and the parasites observed using thin blood smears fixed with methanol and stained with 10% Giemsa (BDH) in PBS and viewed under a light microscope with a 100X oil immersion objective.

A.2 Synchronization

Parasite cultures were synchronized using the method developed by Lambros and Vanderberg (1979). This method exploits the ability of D-sorbitol to selectively lyse erythrocytes infected with later stage parasites. Briefly, pRBCs containing mostly ring stage parasites were incubated with 5 volumes of 5% D-sorbitol at room temperature for 15 minutes. After the incubation period the pRBCs were spun down at 700g for 3 minutes and the supernatant was discarded. The pRBC pellet was then washed once in culture medium without ALBUMAX II before being returned to normal culture conditions.

A.3 Lactate Dehydrogenase Assay

The lactate dehydrogenase assay was developed by Makler and Hinrichs (1993) as a means to measure *Plasmodium falciparum* parasite viability. This assay exploits the fact that the *Plasmodium falciparum* lactate dehydrogenase enzyme can use 3-acetyl pyridine NAD (APAD) as a coenzyme, in the oxidation of lactate. This LDH activity is qualitatively dissimilar from that of the host erythrocyte and the increased enzymatic activity of infected erythrocytes is a result of the enzyme content of the growing and multiplying parasite added to that of the red blood cell (Sherman 1961, Makler and Hinrichs 1993). The pLDH assay has been used to measure drug susceptibility of

culture-adapted strains of *Plasmodium falciparum* with comparable results to other established methods (Makler *et al* 1993).

An optimized formulation (similar if not identical to the Malstat reagent, Flow Inc.) was made up for the detection of parasite LDH activity. The constituents were 400µl of Triton X100, 4g of lithium lactate, 1.32g TRIS buffer and 22mg APAD made up to 200ml with distilled water and the pH set to 9 using HCl. A 100µl solution combining NBT (160mg) and PES (8mg) was made up in Millipore water.

200µl/well of the pLDH detection formulation is aliquoted onto a 96-flat bottom well microplate in combination with 25µl/well of NBT/PES solution. 15µl of RBC or pRBC sample is then added to the appropriate wells containing the reagents. As APADH is formed, the NBT is reduced and forms a formazan product that is blue and can be detected visually and is an indication of parasite survival. The pLDH activity is quantitated by measuring the absorbance of this blue color in each well at 620 nm using a 7520 Microplate Reader (Cambridge Technologies, Inc.). The absorbance readings are automatically blanked on the wells containing RBCs only.

BIBLIOGRAPHY

Adagu IS, Dias F, Pinheiro L, Rombo L, Do Rosario V and Warhurst DC (1996) Guinea Bissau: association of chloroquine resistance of *Plasmodium falciparum* with the Tyr 86 allele of the multidrug resistance gene *pfmdr1*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **90,** 90-91.

Adovelande J and Schrevel J (1996) Carboxylic ionophores in malaria chemotherapy: the effects of monensin and nigericin on *Plasmodium falciparum* in vitro and *Plasmodium vinckei petteri* in vivo. *Life Sciences*, **59,** 309-315.

Aikawa M and Beaudoin R (1969) Effects of chloroquine on the morphology of the erythrocytic stages of *Plasmodium gallinaceum*. *American Journal of Tropical Medicine and Hygiene*, **18,** 166-181.

Aikawa M (1972) High-resolution autoradiography of malarial parasites treated with ^{3}H-chloroquine. *American Journal of Pathology*, **67,** 277-284.

Allred DR and Sherman IW (1983) Developmental modulation of protein synthetic patterns by the human malarial parasite *Plasmodium falciparum*. *Canadian Journal of Biochemical Cellular Biology*, **61,** 1304-1314.

Asawamahasakda W, Ittarat I, Chang C, McElroy P and Meshnick SR (1994) Effects of antimalarials and protease inhibitors on plasmodial hemozoin production. *Molecular and Biochemical Parasitology*, **67,** 183-191.

Atamna H and Ginsburg H (1993) Origin of reactive oxygen species in erythrocytes infected with *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **61,** 231-241.

Atamna H and Ginsburg H (1995) Heme degradation in the presence of glutathione. A proposed mechanism to account for the high levels of non-heme iron found in the membranes of hemoglobinopathic red blood cells. *Journal of Biological Chemistry*, **270,** 24876-24883.

Bannister LH and Mitchell GH (2003) The ins, outs and roundabouts of malaria. *Trends in Parasitology*, **19,** 209-213.

Basco LK and Le Bras J (1992) In vitro activity of pyronaridine against African strains of *Plasmodium falciparum*. *Annals of Tropical Medicine and Parasitology*, **86,** 447-454.

Basco LK and Le Bras J (1993) *In vitro* activity of monodesethylamodiaquine and amopyroquine against African isolates and clones of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene*, **48,** 120-125.

Basco LK and Le Bras J (1994) In activity susceptibility of Cambodian isolates of *P. falciparum* to halofantrine, pyronaridine and artemisinin derivatives. *Annals of Tropical Medicine and Parasitology*, **38,** 137-144.

Basco LK, Le Bras J, Rhoades Z and Wilson CM (1995) Analysis of *pfmdr1* and drug susceptibility in fresh isolates of *Plasmodium falciparum* from sub-Saharan Africa. *Molecular and Biochemical Parasitology*, **74,** 157-166.

Basilico N, Pagani E, Monti D, Olliaro P and Taramalli D (1998) A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. *Journal of Antimicrobial Chemotherapy*, **42,** 55-60.

Ben Mamoun C, Gluzman IY, Hott C, Macmillan SK, Amarakone AS, Anderson DL, Carlton JM, Dame JB, Chakrabarti D, Martin RK, Brownstein BH and Goldberg DE (2001) Co-ordinated program of gene expression during asexual intra-erythrocytic development of the human malaria parasite *Plasmodium falciparum* revealed by microarray analysis. *Molecular Microbiology*, **39,** 26-36.

Bitonti AJ, Sjoerdsma A and Mccann PP (1988) Reversal of chloroquine resistance in malaria parasite *Plasmodium falciparum* by desipramine. *Science*, **242,** 1301-1303.

Blauer G and Akkawi M (1995) B-hematin. *Biochemistry and Molecular Biology International*, **35,** 231-235.

Bohle DS, Dinnebier RE, Madsen SK and Stephens PW (1997) Characterization of the products of the heme detoxification pathway in malarial late trophozoites by X-ray diffraction. *The Journal of Biological Chemistry*, **272,** 713-716.

Bowman EJ, Siebers A and Altendorf K (1988) Bafilomycins: a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. *Proceedings of the National Academy of Sciences*, **85,** 7972-7976.

Bray PG, Howells RE, Ritchie GY and Ward SA (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,** 1317-1324.

Bray PG, Howells RE and Ward SA (1992b) Vacuolar acidification and chloroquine sensitivity in *Plasmodium falciparum*. *Biochemical Pharmacology*, **43,** 1219-1227.

Bray PG, Hawley SR, Mungthin M and Ward SA (1996a) Physicochemical properties correlated with drug resistance and the reversal of drug resistance in *Plasmodium falciparum*. *Molecular Pharmacology*, **50,** 1559-1566.

Bray PG, Hawley SR and Ward SA (1996b) 4-Aminoquinoline resistance of *Plasmodium falciparum*: insights from the study of amodiaquine uptake. *Molecular Pharmacology*, **50,** 1551-1558.

Bray PG, Mungthin M, Ridley RG and Ward SA (1998) Access to hematin: the basis of chloroquine resistance. *Molecular Pharmacology*, **54,** 170-179.

Bray PG and Ward SA (1998) A comparison of the phenomenology and genetics of multidrug resistance in cancer cells and quinoline resistance in *Plasmodium falciparum*. *Pharmacology and Therapeutics*, **77,** 1-28.

Bray PG, Janneh O, Raynes KJ, Mungthin M, Ginsburg H and Ward SA (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,** 363-376.

Brendat K, Berger BJ, Cerami A, Ridley RG, Dorn A, Matile H and Kansy M (1995) Haem polymerization in malaria. *Nature*, **378,** 138-139.

Cannon JB, Kuo FS, Pasternack RF, Wong NM and Muller-Eberhard U (1984) Kinetics of the interactions of hemin liposomes with heme binding proteins. *Biochemistry*, **23,** 3715-3721.

Chavalitshewinkoon P, Wilairat P, Gamage S, Denny W, Figgitt and Ralph R (1993) Structure-Activity relationships and modes of action of 9-anilinoacridines against chloroquine-resistant *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy*, **37,** 403-406.

Chavalitshewinkoon-Petmitr P, Pongvilairat G, Auparakkitanon and Wilairat P (2000) Gametocytocidal activity of pyronaridine and DNA topoisomerase II inhibitors against multidrug-resistant *Plasmodium falciparum* in vitro. *Parasitology International*, **48,** 275-280.

Cheesman SJ (2000) The Topoisomerase of Protozoan Parasites. *Parasitology Today*, **16,** 277-281.

Chen C and Zheng X (1992) Development of the New Antimalarial drug Pyronaridine: A Review. *Biomedical and Environmental Sciences*, **5,** 149-160.

Chen C, Tang L-H and Chun J (1992) Studies on a new antimalarial compound: pyronaridine. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **86,** 7-10.

Chen Y and Simon SM (2000) In situ biochemical demonstration that P-glycoprotein is a drug efflux pump with broad specificity. *The Journal of Cell Biology*, **148,** 863-870.

Childs GE, Hausler B, Milhous W, Chen C, Wimonwattrawatee T, Pooyindee N and Boudreau EF (1988) In vitro activity of pyronaridine against field isolates and reference clones of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene*, **38,** 24-29.

Choi CYH, Cerda JF, Chu HA, Babcock GT and Marletta MA (1999) Spectroscopic characterization of the heme-binding sites in *Plasmodium falciparum* histidine-rich protein 2. *Biochemistry*, **38,** 16916-16924.

Choi I and Mego JL (1987) Intravacuolar proteolysis in *Plasmodium falciparum* digestive vacuoles is similar to intra-lysosomal proteolysis in mammalian cells. *Biochimica et Biophysica Acta*, **926,** 170-176.

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

Chou AC and Fitch CD (1981) Mechanism of hemolysis induced by ferriprotoporphyrin IX. *Journal of Clinical Investigation*, **68,** 672-677.

Chou AC and Fitch CD (1993) Control of heme polymerase by chloroquine and other quinoline derivatives. *Biochemical and Biophysical Research Communications*, **195,** 422-427.

Churchill FC, Patchen LC, Campbell CC, Schwartz IK, Nguyen-Dinh P and Dickinson CM (1985) Amodiaquine a pro-drug: importance of metabolite(s) in the antimalarial effect of amodiaquine in humans. *Life Sciences*, **36,** 53-62.

Cooper RA, Ferdig MT and Su XZ (2002) Alternative mutations at position 76 of the vacuolar transmembrane protein PfCRT are associated with chloroquine resistance and unique stereo-specific quinine and quinidine responses in *Plasmodium falciparum*. *Molecular Pharmacology*, **61,** 35-42.

Cowman AF, Karcz SR, Galatis D and Culvenor JG (1991) A P-glycoprotein homologue of *Plasmodium falciparum* is localized on the digestive vacuole. *The Journal of Cell Biology*, **113,** 1033-1042.

Cox Singh J, Singh B, Alias A and Abdullah MS (1995) Assessment of the association between three pfmdr1 point mutations and chloroquine resistance in vitro of Malaysian *Plasmodium falciparum* isolates. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **89,** 436-437.

de Rojas MO and Wasserman M (1985) Temporal relationships on macromolecular synthesis during the asexual cell cycle of *Plasmodium falciparum*. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **79,** 792-796.

Deans JA, Thomas AW, Inge PM and Cohen S (1983) Stage-specific protein synthesis by asexual blood stage parasites of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **8,** 45-51.

Diribe CO and Warhurst DC (2001) A study of the uptake of chloroquine in malaria-infected erythrocytes. High and low affinity uptake and the influence of glucose and its analogues. *Biochemical Pharmacology*, **34,** 3019-3027.

Dorn A, Stoffel R, Matile H, Bubendorf A and Ridley RG (1995) Malarial haemozoin / beta-hematin supports haem polymerization in the absence of protein. *Nature*, **374,** 269-271.

Dorn A, Vippagunta SR, Matile H, Jaquet C, Vennerstrom JL and Ridley RG (1998a) An assessment of drug-haematin binding as a mechanism for inhibition of haematin polymerization by quinoline antimalarials. *Biochemical Pharmacology*, **55,** 727-736.

Dorn A, Vippagunta SR, Matile H, Bubendorf A, Vennerstrom JL and Ridley RG (1998b) A comparison and analysis of several ways to promote haematin (haem) polymerization and an assessment of its initiation in vitro. *Biochemical Pharmacology*, **55,** 737-747.

Dzekunov SM, Uros LMB and Roepe PD (2000) Digestive vacuolar pH of intact intra-erythrocytic *P. falciparum* either sensitive or resistant to chloroquine. *Molecular and Biochemical Parasitology*, 107-124.

Egan TJ, Ross DC and Adams PA (1994) Quinoline anti-malarial drugs inhibit spontaneous formation of beta-haematin (malaria pigment). *FEBS Letters*, **352,** 54-57.

Egan TJ, Mavuso WW, Ross DC and Marques HM (1997) Thermodynamic factors controlling the interaction of quinoline antimalarial drugs with ferriprotoporphyrin IX. *Journal of Inorganic Biochemistry*, **68,** 137-145.

Egan TJ, Hunter R, Kaschula CH, Marques HM and Mavuso WW (1998) Towards the rational design of antimalarial drugs. *South African Journal of Science*, **94,** 277-278.

Egan TJ and Marques HM (1999) The role of heme in the activity of chloroquine and related antimalarial drugs. *Coordination Chemistry Reviews*, 493-517.

Egan TJ, Hunter R, Kaschula CH, Marques HM, Misplon A and Walden J (2000) Structure-function relationships in aminoquinolines: effect of amino and chloro groups on quinoline-hematin complex formation, inhibition of beta-hematin formation, and antiplasmodial activity. *Journal of Medicinal Chemistry*, **43,** 283-291.

Egan TJ, Mavuso WW and Ncokazi KK (2001) The mechanism of beta-hematin formation in acetate solution. Parallels between hemozoin formation and biomineralization processes. *Biochemistry*, **40,** 204-213.

Egan TJ (2001) Quinoline antimalarials. *Expert Opinion on Therapeutic Patents*, **11,** 185-209.

Egan TJ, Combrick JM, Egan J, Hearne GR, Marques HM, Ntenteni S, Sewell BT, Smith PJ, Taylor D, van Schalkwyk D and Walden J (2002) Fate of haem iron in the malaria parasite *Plasmodium falciparum*. *Biochemical Journal*, **365,** 343-347.

Ekong RM, Robson KJH, Baker DA and Warhurst DC (1993) Transcripts of the multidrug resistance genes in chloroquine-sensitive and chloroquine -resistant *Plasmodium falciparum*. *Parasitology*, **106,** 107-115.

Elueze EI, Croft SL and Warhurst DC (1996) Activity of PND and mepacrine against twelve strains of *P. falciparum* in vitro. *Journal of Antimicrobial Chemotherapy*, **37,** 511-518.

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

Fan B, Zhao W, Ma X, Huang Z, Wen Y, Yang J and Yang Z (1998) In vitro sensitivity of *Plasmodium falciparum* to chloroquine, piperaquine, pyronaridine and artesunate in Yuxi prefecture of

Yunnan province. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi*, **16,** 460-462.

Ferrari V and Cutler DJ (1991a) Kinetics and thermodynamics of chloroquine and hydroxychloroquine transport across the human erythrocyte membrane. *Biochemical Pharmacology*, **41,** 23-30.

Ferrari V and Cutler DJ (1991b) Simulation of kinetic data on the influx and efflux of chloroquine by erythrocytes infected with *Plasmodium falciparum*. Evidence for a drug-importer in chloroquine-sensitive strains. *Biochemical Pharmacology*, **42,** S167-S179.

Fidock DA, Nomura T, Talley AK, Cooper RA, Dzekunov SM, Ferdig MT, Uros LMB, Sidhu ABS, Naude B, Deitsch KW, Su XZ, Wootton JC, Roepe PD and Wellems TE (2000a) Mutations in the *P. falciparum* digestive vacuole transmembrane protein PfCRT and evidence for their role in chloroquine resistance. *Molecular Cell*, **6,** 861-871.

Fidock DA, Nomura T, Cooper RA, Su XZ, Talley AK and Wellems TE (2000b) Allelic modifications of the *cg2* and *cg1* genes do not alter the chloroquine response of drug-resistant *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **110,** 1-10.

Fitch CD (1969) Chloroquine resistance in malaria: a deficiency of chloroquine binding. *Proceedings of the National Academy of Sciences*, **64,** 1181-1187.

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

Fitch CD (1973) Chloroquine-resistance *Plasmodium falciparum*: difference in the handling of ^{14}C-amodiaquin and ^{14}C-chloroquine. *Antimicrobial Agents and Chemotherapy*, **3,** 545-548.

Fitch CD, Yunis NG, Chevli R and Gonzalez Y (1974a) High-Affinity Accumulation of Chloroquine by Mouse Erythrocytes Infected with *Plasmodium berghei*. *The Journal of Clinical Investigation*, **54,** 24-33.

Fitch CD, Chevli R and Gonzalez Y (1974b) Chloroquine-resistant *Plasmodium falciparum*: effect of substrate on chloroquine and amodiaquine accumulation. *Antimicrobial Agents and Chemotherapy*, 757-762.

Fitch CD, Chevli R and Gonzalez Y (1975) Chloroquine resistance in malaria: variations of substrate-stimulated chloroquine accumulation. *The Journal of Pharmacology and Experimental Therapeutics*, **195,** 389-396.

Fitch CD, Chevli R, Banyal HS, Philips G, Krogstad DJ and Pfaller MA (1982) Lysis of *Plasmodium falciparum* by ferriprotoporphyrin IX and a chloroquine-ferriprotoporphyrin complex. *Antimicrobial Agents and Chemotherapy*, **21,** 819-822.

Fitch CD (1983) Mode of action of antimalarial drugs. *Ciba Foundation Symposium*, **94,** 222-232.

Fitch CD (1989) Ferriprotoporphyrin IX: role in chloroquine susceptibility and resistance in malaria. *Malaria and the Red Cell*, **2,** 45-52.

Fitch CD, Cai G, Chen Y-F and Shoemaker JD (1999) Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. *Biochimica et Biophysica Acta*, **1454,** 31-37.

Foley M and Tilley L (1998) Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacology and Therapeutics*, **79,** 55-87.

Foote SJ, Kyle D and Martin RK (1990) Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature*, **345,** 255-258.

Francis SE, Gluzman IY, Oksman A, Knickerbocker A, Mueller R, Bryant ML, Sherman DR, Russell DG and Goldberg DE (1994) Molecular characterization and inhibition of a *Plasmodium falciparum* aspartic hemoglobinase. The *EMBO Journal*, **13,** 306-317.

Freese JA, Markus MB and 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,** 707-712.

Fu S and Xiao S-H (1991) Pyronaridine: A New Antimalarial Drug. *Parasitology Today*, **7,** 310-313.

Geary TG, Divo AA and Jensen JB (1989) Stage specific actions of antimalarial drugs on *Plasmodium falciparum* in culture. *American Journal of Tropical Medicine and Hygiene*, **40,** 240-244.

Geary TG, Divo AA, Jensen JB, Zangwill M and Ginsburg H (1990) Kinetic modeling of the response of *Plasmodium falciparum* to

chloroquine and its experimental testing in vitro: Implications for mechanism of action of and resistance to the drug. *Biochemical Pharmacology*, **40,** 685-691.

Ginsburg H, Nissani E and Krugliak M (1989) Alkalinization of the food vacuole of malaria parasites by quinoline drugs and alkylamines is not correlated with their antimalarial activity. *Biochemical Pharmacology*, **38,** 2645-2654.

Ginsburg H and Stein WD (1991) Kinetic modeling of chloroquine uptake by malaria infected erythrocytes. Assessment of the factors that may determine drug resistance. *Biochemical Pharmacology*, **41,** 1463-1470.

Ginsburg H and Krugliak M (1992) Quinoline-containing antimalarials--mode of action, drug resistance and its reversal. An update with unresolved puzzles. *Biochemical Pharmacology*, **43,** 63-70.

Ginsburg H, Famin O, Zhang J and 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, Ward SA and Bray PG (1999) An integrated model of chloroquine action. *Parasitology Today*, **15,** 357-360.

Ginsburg H and Krugliak M (1999) Chloroquine - some open questions on its antimalarial mode of action and resistance. *Drug Resistance Updates*, **2,** 180-187.

Gluzman IY, Francis SE, Oksman A, Smith CE, Duffin KL and Goldberg DE (1994) Order and specificity of the *Plasmodium falciparum* hemoglobin degradation pathway. *Journal of Clinical Investigation*, **93,** 1602-1608.

Goldberg DE, Slater AFG, Cerami A and Henderson GB (1990) Hemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. *Proceedings of the National Academy of Sciences*, **87,** 2931-2935.

Goldberg DE and Slater AFG (1992) The pathway of hemoglobin degradation in malaria parasites. *Parasitology Today*, **8,** 280-283.

Gritzmacher CA and Reese RT (1984) Protein and nucleic acid synthesis during synchronized growth of *Plasmodium falciparum*. *Journal of Bacteriology*, **160,** 1165-1167.

Gumila C, Ancelin ML, Jeminet G, Delort A, Miquel G and Vial HJ (1996) Differential in vitro activities of ionophore compounds against *Plasmodium falciparum* and mammalian cells. *Antimicrobial Agents and Chemotherapy*, **40,** 602-608.

Gyang FN, Poole B and Trager W (1982) Peptidases from *Plasmodium falciparum* cultured in vitro. *Molecular and Biochemical Parasitology*, **5,** 263-273.

Hassan Alin M, Bjorkman A and Ashton M (1990) In vitro activity of artemisinin, its derivatives, and pyronaridine against different strains of *P falciparum. Transactions of the Royal Society of Tropical Medicine and Hygiene*, **84,** 635-637.

Hawley SR, Bray PG, Park BK and Ward SA (1996b) Amodiaquine accumulation in *Plasmodium falciparum* as a possible explanation for its superior antimalarial activity over chloroquine. *Molecular and Biochemical Parasitology*, **80,** 15-25.

Hawley SR, Bray PG, O'Neill PM, Park BK and Ward SA (1996a) The role of drug accumulation in 4-aminoquinoline antimalarial potency: The influence pf structural substitution and physiochemical properties. *Biochemical Pharmacology*, **52,** 723-733.

Hawley SR, Bray PG, Mungthin M, Atkinson JD, O'Neill PM and Ward SA (1998) Relationship between antimalarial drug activity, accumulation, and inhibition of heme polymerization in *Plasmodium falciparum* in vitro. *Antimicrobial Agents and Chemotherapy*, **42,** 682-686.

Homewood CA, Warhurst DC, Peters W and Baggaley VC (1972) Lysosomes, pH and the anti-malarial action of chloroquine. *Nature*, **235,** 50-52.

Hyde JE (2002) Mechanisms of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes and Infection*, **4,** 165-174.

Inselburg J and Banyal HS (1984) Synthesis of DNA during the asexual cycle of *Plasmodium falciparum* in culture. *Molecular and Biochemical Parasitology*, **10,** 79-87.

Kaschula CH, Egan TJ, Hunter R, Basilico N, Parapini S, Taramalli D, Pasini E and Monti D (2002) Structure-activity relationships in 4-aminoquinoline anti-plasmodials. The role of the group at the 7-position. *Journal of Medicinal Chemistry*, **45,** 3531-3539.

Kawai S, Kano S, Chen C and Suzuki M (1996) The effects of pyronaridine on the morphology of *Plasmodium falciparum* in

Aotus trivirgatus. *American Journal of Tropical Medicine and Hygiene*, **55,** 223-229.

Kenakin T (1997) *Molecular Pharmacology - A Short Course*. Blackwell Science.

Kirk K and Saliba KJ (2001) Chloroquine resistance and the pH of the malaria parasite's digestive vacuole. *Drug Resistance Updates*, **4,** 335-338.

Kotecka BM, Barlin GB, Edstein MD and Reickmann KH (1997) New quinoline di-Mannich base compounds with greater antimalarial activity than chloroquine, amodiaquine, or pyronaridine. *Antimicrobial Agents and Chemotherapy*, **41,** 1369-1374.

Krogstad DJ, Schlesinger PH and Gluzman IY (1985) Antimalarials increase vesicle pH in *Plasmodium falciparum*. *The Journal of Cell Biology*, **101,** 2302-2309.

Krogstad DJ and Schlesinger PH (1986) A perspective on antimalarial action: effects of weak bases on *Plasmodium falciparum*. *Biochemical Pharmacology*, **35,** 547-552.

Krogstad DJ, Gluzman IY, Kyle D, Oduola A, Martin SK, Milhous W and Schlesinger PH (1987) Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science*, **238,** 1283-1285.

Krogstad DJ and Schlesinger PH (1987) Acid-vesicle function intracellular pathogens and the action of chloroquine against *Plasmodium falciparum*. *New England Journal of Medicine*, **317,** 542-549.

Krogstad DJ, Schlesinger PH and Herwaldt BL (1988) Antimalarial agents: mechanism of chloroquine resistance. *Antimicrobial Agents and Chemotherapy*, **32,** 799-801.

Krogstad DJ, Gluzman IY, Herwaldt BL, Schlesinger PH and Wellems TE (1992) Energy dependence of chloroquine accumulation and chloroquine efflux in *Plasmodium falciparum*. *Biochemical Pharmacology*, **43,** 57-62.

Krugliak M and Ginsburg H (1991) Studies on the antimalarial mode of action of quinoline-containing drugs: time-dependence and irreversibility of drug action, and interactions with compounds that alter the function of the parasite's food vacuole. *Life Sciences*, **49,** 1213-1219.

Lambros C and Vanderberg JP (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *Journal of Parasitology*, **35,** 418-420.

Limbird LE (1985) *Cell Surface Receptors: A Short Course on Theory and Methods*. Boston: Martinus Nijhoff.

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

Loyevsky M, Lytton S, Mester B, Libman J, Shanzer A and Cabantchik ZI (1993) The antimalarial action of desferal involves a direct access route to erythrocytic (*Plasmodium falciparum*) parasites. *Journal of Clinical Investigation*, **91,** 218-224.

Lynn A, Chandra A, Malhotra P and Chauhan VS (1999) Heme binding and polymerization by *Plasmodium falciparum* histidine rich protein II: influence of pH on activity and conformation. *FEBS Letters*, **459,** 267-271.

Macomber PB, O'Brien RL and Hahn FE (1966) Chloroquine: Physiological basis of drug resistance in *Plasmodium berghei*. *Science*, **152,** 1374-1375.

Macomber PB and Sprinz H (1967) Morphological effects of chloroquine on *Plasmodium berghei* in mice. *Nature*, **214,** 937-939.

Makler MT and Hinrichs DJ (1993) Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *American Journal of Tropical Medicine and Hygiene*, **48,** 205-210.

Makler MT, Ries JM, Williams JA, Bancroft JE, Piper RC, Gibbins BL and Hinrichs DJ (1993) Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *American Journal of Tropical Medicine and Hygiene*, **48,** 739-741.

Marchesini N, Luo S, Rodrigues CO, Moreno SNJ and Docampo R (2000) Acidocalcisomes and a vacuolar H+-pyrophosphatase in malaria parasites. *Biochemical Journal*, **347,** 243-253.

Martin SK, Oduola AM and Milhous WK (1987) Reversal of chloroquine resistance in *Plasmodium falciparum* by verapamil. *Science*, **235,** 899-901.

Martiney JA, Ferrer AS, Cerami A, Dzekunov SM and Roepe PD (1999) Chloroquine uptake, altered partitioning and the basis of drug

resistance: evidence for iron chloride-dependent ionic regulation. *Novartis Foundation Symposium*, **226,** 265-280.

Mitamura T and Palacpac NMQ (2003) Lipid metabolism in *Plasmodium falciparum*-infected erythrocytes: possible new targets for malaria chemotherapy. *Microbes and Infection*, **5,** 545-552.

Moon RP, Tyas L and Certa U (1997) Expression and characterization of Plasmepsin I from *Plasmodium falciparum*. *European Journal of Biochemistry*, **244,** 552-560.

Motulsky H (1995) *The GraphPad Guide to Analyzing Radioligand Binding Data.* GraphPad Software.

Motulsky H (1999) *Analyzing Data With GraphPad Prism.* GraphPad Software, Inc.

Mungthin M, Bray PG, Ridley RG and Ward SA (1998) Central role of hemoglobin degradation in mechanisms of action of 4-aminoquinolines, quinoline methanols, and phenanthrene methanols. *Antimicrobial Agents and Chemotherapy*, **42,** 2973-2977.

Munson P (1996) *Principles of Pharmacology - Basic Concepts and Clinical Applications.* Chapman and Hall.

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

Orjih AU, Banyal HS, Chevli R and Fitch CD (1981) Hemin Lyses Malaria Parasites. *Science*, **214,** 667-669.

Orjih AU, Kanjananggulpan P and Fitch CD (1988) Ferriprotoporphyrin IX and cell lysis: A protective role for hydrogen peroxide. *Life Sciences*, **42,** 2603-2607.

Orjih AU and Fitch CD (1993) Hemozoin production by *Plasmodium falciparum*: variation with strain and exposure to chloroquine. *Biochimica et Biophysica Acta*, **1157,** 270-274.

Orjih AU, Ryerse JS and Fitch CD (1994) Hemoglobin catabolism and the killing of intraerythrocytic *Plasmodium falciparum* by chloroquine. *Experientia*, **50,** 34-39.

Orjih AU (1997) Heme polymerase activity and the stage specificity of antimalarial action of chloroquine. *The Journal of Pharmacology and Experimental Therapeutics*, **282,** 108-112.

Pagola S, Stephens PW, Bohle DS, Kosar AD and Masden SK (2000) The structure of malaria pigment beta-haematin. *Nature*, **404,** 307-310.

Pandey AV, Babbarwal VK, Okoyeh JN, Joshi RM, Puri SK, Singh RL and Chauhan VS (2003) Hemozoin formation in malaria: a two-step process involving histidine-rich proteins and lipids. *Biochemical and Biophysical Research Communications*, **308,** 736-743.

Papalexis V, Siomos MA, Campanale, Guo X, Kocak G, Foley M and Tilley L (2001) Histidine-rich protein 2 of the malaria parasite, *Plasmodium falciparum*, is involved in detoxification of the by-products of haemoglobin degradation. *Molecular and Biochemical Parasitology*, **115,** 77-86.

Parapini S, Basilico N, Pasini E, Egan TJ, Olliaro PL, Taramelli D and Monti D (2000) Standardization of the physicochemical parameters to assess in vitro the beta-hematin inhibitory of antimalarial drugs. *Experimental Parasitology*, **96,** 249-256.

Peel SA, Bright P, Yount B, Handy J and Baric RS (1994) A strong association between mefloquine and halofantrine resistance and amplification, over-expression, and mutation in the P-glycoprotein gene homolog (*pfmdr*) of *Plasmodium falciparum* in vitro. *American Journal of Tropical Medicine and Hygiene*, **51,** 648-658.

Peters W and Robinson BI (1992) The chemotherapy of rodent malaria. XLVII. Studies on pyronaridine and other Mannich base antimalarials. *Annals of Tropical Medicine and Parasitology*, **86,** 455-465.

Pradines B, Tall A, Parzy D, Spiegel A, Fusai T, Hienne R, Trape JF and Doury JC (1998) In vitro activity of pyronaridine and amodiaquine against African isolates (Senegal) of *Plasmodium falciparum* in comparison with standard antimalarials agents. *Journal of Antimicrobial Chemotherapy*, **42,** 333-339.

Pradines B, Mabika Mamfoumbi M, Parzy D, Owono Medang M, Lebeau C, Mourou Mbina JR, Doury JC and Kombila M (1999) In vitro susceptibility of African isolates of *Plasmodium falciparum* from Gabon to PND. *American Journal of Tropical Medicine and Hygiene*, **60,** 105-108.

Raynes KJ, Bray PG and Ward SA (1999) Altered binding of chloroquine to ferriprotoporphyrin IX is the basis for chloroquine resistance. *Drug Resistance Updates*, **2,** 97-103.

Reed MB, Saliba KJ, Caruana SR, Kirk K and Cowman AF (2000) Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature*, **403,** 906-909.

Ringwald P, Bickii J and Basco LK (1996a) In vitro activity of antimalarials against clinical isolates of *P. falciparum* in Yaounde, Cameroon. *American Journal of Tropical Medicine and Hygiene*, **55,** 254-258.

Ringwald P, Bickii J and Basco LK (1996b) Randomized trial of pyronaridine versus chloroquine for acute uncomplicated falciparum malaria in Africa. *Lancet*, **347,** 24-28.

Ringwald P, Bickii J and Basco LK (1998) Efficacy of oral pyronaridine for the treatment of acute uncomplicated falciparum malaria in African children. *Clinical Infectious Diseases*, **26,** 946-953.

Ringwald P, Eboumbou ECM, Bickii J and Basco LK (1999) In vitro activities of pyronaridine, alone and in combination with other antimalarial drugs, against *Plasmodium falciparum*. *Antimicrobial Agents and Chemotherapy*, **43,** 1525-1527.

Rojas-rivero L, Gay F, Bustos MDG, Ciceron L, Pichet C, Danis M and Gentilini M (1992) Mefloquine-halofantrine cross-resistance in *Plasmodium falciparum* induced by intermittent mefloquine pressure. *American Journal of Tropical Medicine and Hygiene*, **47,** 372-377.

Rose MY, Thompson RA, Light WR and Olson JS (1985) Heme transporter between phospholipid membranes and uptake by apohemoglobin. *Journal of Biological Chemistry*, **260,** 6632-6640.

Rosenthal PJ, Mckerrow JH, Aikawa M, Nagasawa H and Leech JH (1988) A malarial cysteine proteinase is necessary for hemoglobin degradation by *Plasmodium falciparum*. *Journal of Clinical Investigation*, **82,** 1560-1566.

Rosenthal PJ and Nelson RG (1992) Isolation and characterization of a cysteine protease gene of *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **51,** 143-152.

Saliba KJ, Folb PI and Smith PJ (1998) Role for the *Plasmodium falciparum* digestive vacuole in chloroquine resistance. *Biochemical Pharmacology*, **56,** 313-320.

Saliba KJ, Allen RJW, Zissis S, Bray PG, Ward SA and Kirk K (2003) Acidification of the malaria parasite's digestive vacuole by a H+-ATPase and a H+-pyrophosphatase. *The Journal of Biological Chemistry*, **278,** 5605-5612.

San George R, Nagel RL and Fabry ME (1984) On the mechanism for red cell accumulation of mefloquine, an antimalarial drug. *Biochimica et Biophysica Acta*, **803,** 174-181.

Sanchez CP, Wunsch S and Lanzer (1997) Identification of a chloroquine importer in *Plasmodium falciparum.* Differences in import kinetics are genetically linked with the chloroquine-resistant phenotype. *The Journal of Biological Chemistry*, **272,** 2652-2658.

Shao Bao-ruo (1990) A Review of Antimalarial Drug Pyronaridine. *Chinese Medical Journal*, **103,** 428-434.

Sherman IW (1961) Molecular heterogenicity of lactic dehydrogenase in avian malaria (*Plasmodium Lophurae*). *Journal of Experimental Medicine*, **114,** 1049-1062.

Sherman IW (1998) Malaria: Parasite biology, Pathogenesis, and Protection. *ASM Press, Washington DC.*

Slater AFG, Swiggard WJ, Orton BR, Flitter WD, Goldberg DE, Cerami A and Henderson GB (1991) An iron-carboxylate bond links the heme units of malaria pigment. *Proceedings of the National Academy of Sciences*, **88,** 325-329.

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

Slater AFG (1993) Chloroquine: mechanism of drug action and resistance in *Plasmodium falciparum. Pharmacology and Therapeutics*, **57,** 203-235.

Stead AMV, Bray PG, Edwards G, Dekoning HP, Elford BC, Stocks PA and Ward SA (2001) Diamidine compounds: selective uptake and targeting in *Plasmodium falciparum. Molecular Pharmacology*, **59,** 1298-1306.

Sugioka Y and Suzuki M (1991) The chemical basis for the ferriprotoporphyrin IX-chloroquine complex induced lipid peroxidation. *Biochimica et Biophysica Acta*, **1074,** 19-24.

Sullivan DJ, Gluzman IY and Goldberg DE (1996a) Plasmodium hemozoin formation mediated by histidine-rich proteins. *Science*, **271,** 219-221.

Sullivan DJ, Gluzman IY, Russell DG and Goldberg DE (1996b) On the molecular mechanism of chloroquine antimalarial action. *Proceedings of the National Academy of Sciences*, **93,** 11865-11870.

Sullivan DJ, Matile H, Ridley RG and Goldberg DE (1998) A common mechanism for blockade of heme polymerization by antimalarial quinolines. *The Journal of Biological Chemistry*, **273,** 31103-31107.

Sullivan DJ (2002) Theories on malarial pigment formation and quinoline action. *International Journal for Parasitology*, **32,** 1645-1653.

Surolia N and Padmanaban G (1991) Chloroquine inhibits heme-dependent protein synthesis in *Plasmodium falciparum*. *Proceedings of the National Academy of Sciences*, **88,** 4786-4790.

Trager W (1994) Cultivation of Malaria Parasites. *Methods in Cell Biology* pp. 7-24. Academic Press Inc.

van Es HHG, Renkena H, Aerts H and Schurr E (1994) Enhanced lysosomal acidification leads to increased chloroquine accumulation in CHO cells expressing the pfmdr1 gene. *Molecular and Biochemical Parasitology*, **68,** 209-219.

Vander Jagt DL, Hunsaker LA and Campos NM (1986) Characterization of a hemoglobin-degrading, low molecular weight protease from *Plasmodium falciparum*. *Molecular and Biochemical Parasitology*, **18,** 389-400.

Vial HJ, Thuet MJ and Philippot JR (1982) Phospholipid biosynthesis in synchronous *Plasmodium falciparum* cultures. *Journal of Protozoology*, **29,** 258-263.

Vyas N, Avery BA, Avery MA and Wyandt CM (2002) Carrier-mediated partitioning of artemisinin into *Plasmodium falciparum*-infected erythrocytes. *Antimicrobial Agents and Chemotherapy*, **46,** 105-109.

Warhurst DC (2001) New developments: Chloroquine-resistance in *Plasmodium falciparum*. *Drug Resistance Updates*, **4,** 141-144.

Warhurst DC, Craig JC and Adagu IS (2002) Lysosomes and drug resistance in malaria. *The Lancet*, **360,** 1527-1529.

Warsame M, Wernsdorfer WH, Payne D and Bjorkman A (1991) Positive relationship between the response of *P. falciparum* the CQ and PND. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, **85,** 570-571.

Wellems TE, Panton LJ and Gluzman IY (1990) Chloroquine resistance not linked to mdr-like genes in a *Plasmodium falciparum* cross. *Nature*, **345,** 253-255.

Wernsdorfer and McGregor (1988) *Principles and Practice of Malariology Volumes I and II.* Churchill Livingstone.

Winstanley PA (2000) Chemotherapy for falciparum malaria: the armory, the problems and the prospects. *Parasitology Today,* **16,** 146-153.

Wood PA and Eaton JW (1993) Hemoglobin catabolism and host-parasite heme balance in chloroquine-sensitive and chloroquine resistant *Plasmodium berghei* infections. *American Journal of Tropical Medicine and Hygiene,* **48,** 465-472.

Wu LJ, Rabbege JR, Nagasawa H, Jacobs G and Aikawa M (1988) Morphological effects of pyronaridine on malarial parasites. *American Journal of Tropical Medicine and Hygiene,* **38,** 30-36.

Wunsch S, Sanchez CP, Gekle M, Grossewortman L, Wiesner J and Lanzer M (1998) Differential stimulation of the Na^+/H^+ exchanger determines chloroquine uptake in *Plasmodium falciparum. Journal of Cellular Biology,* **140,** 335-345.

Yang H, Liu D, Huang K, Zhang C and Li C (1998) Longitudinal surveillance of sensitivity of *Plasmodium falciparum* to pyronaridine in south Yunnan. *Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi,* **16,** 81-93.

Yang HL, Gao BH and Huang KG (2000) Effect of pyronaridine, mefloquine and quinine on artesunate-sensitive and artesunate-resistant *Plasmodium falciparum. Zhongguo Ji Sheng Chong Xue Yu Ji Sheng Chong Bing Za Zhi,* **18,** 5-7.

Yayon A, Vande Waa JA, Yayon M, Geary TG and Jensen JB (1983) Stage-dependent effects of chloroquine on *Plasmodium falciparum* in vitro. *Journal of Protozoology,* **30,** 642-647.

Yayon A, Cabantchik ZI and Ginsburg H (1984a) Identification of the acidic compartment of *Plasmodium falciparum* infected human erythrocytes as the target of the antimalarial drug chloroquine. *The EMBO Journal,* **3,** 2695-2700.

Yayon A, Timberg R, Freidman S and Ginsburg H (1984b) Effects of chloroquine on the feeding mechanism of the intra-erythrocytic human malarial parasite *Plasmodium falciparum. Journal of Protozoology,* **31,** 367-372.

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

Yoshimori T, Yamamoto A, Moriyama Y, Futai M and Tashiro Y (1991) Bafilomycin A1, a specific inhibitor of vacuolar-type H(+)-ATPase, inhibits acidification and protein degradation in lysosomes of cultured cells. *The Journal of Biological Chemistry*, **266,** 17707-17712.

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

Zidovetski R, Sherman IW and O'Brien RL (1993) Inhibition of *Plasmodium falciparum* phospholipase A2 by chloroquine, quinine, and arteether. *Journal of Parasitology*, **79,** 565-570.

Zidovetski R, Sherman IW, Prudhomme J and Crawford J (1994) Inhibition of *Plasmodium falciparum* lysophospholipase by anti-malarial drugs and sulphydryl reagents. *Parasitology*, **108,** 249-255.