The Effect of Quinoline Anti-Malarial Drugs on the Endolysosomal and Secretory Pathways of *Plasmodium falciparum* Strain 3D7, *Dictyostelium discoideum* and Mammalian A549 Cells

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Thesis presented for the degree of

MASTER OF MEDICINE

in the Division of Pharmacology
Department of Medicine

UNIVERSITY OF CAPE TOWN

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February 2007

DECLARATION

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

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ABSTRACT

The Effect of Quinoline Anti-Malarial Drugs on the Endolysosomal and Secretory Pathways of *Plasmodium falciparum* Strain 3D7, *Dictyostelium discoideum* and Mammalian A549 Cells

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Malaria is the most serious and widespread human parasitic disease, with almost half the world's population at risk. The increasing prevalence of parasite drug resistance and vector insecticide resistance has made the development of new anti-malarial strategies vitally important. A better understanding of parasite metabolism as well as the action of existing anti-malarial drugs may prove critical in this process. The precise mechanisms of action of the quinoline anti-malarial drugs are uncertain, although they have been found to influence endocytosis, vesicular processing and secretion in malarial parasites and mammalian cells. In this study, the effects of chloroquine, amodiaquine, halofantrine, mefloquine and quinine on the endolysosomal systems in *Plasmodium falciparum* 3D7, *Dictyostelium discoideum* and A549 pulmonary cancer cells were examined.

Treatment with chloroquine was found to cause hemoglobin accumulation in 3D7 parasites by either disrupting enzymatic function or inhibiting vesicular processing and resulting in the inhibition of hemoglobin digestion, and/or by disrupting the secretory pathway. Additionally, it was found that chloroquine may inhibit endocytosis to a small extent following a long incubation period. Furthermore, the drug appears to disrupt vesicular processing and/or secretion in *D. discoideum*. Amodiaquine may non-specifically disrupt membrane function in *P. falciparum*, *D. discoideum* and A549 cells, inhibiting endocytosis, vesicular docking and/or exocytosis concurrently. Mefloquine and halofantrine were found to largely inhibit endocytosis and/or possibly slightly stimulate exocytosis in *P. falciparum*, *D. discoideum* and A549 cells. Quinine inhibited endocytosis and/or stimulated exocytosis in *P. falciparum* and A549 cells. However, the drug appeared to affect exocytosis alone in *D. discoideum*. Mefloquine and halofantrine may thus be useful for cell biological studies where the specific inhibition of endocytosis is required.

ACKNOWLEDGEMENTS

Doctor Heinrich C. Hoppe, for your invaluable guidance and supervision.

Wynand Smythe, for all of your assistance in the lab. Thank you for always being so willing to help me or listen to one of my many tales of woe!

Sandy Meredith, Dale Taylor and Susan Yeh, for your assistance in the lab and office.

My parents, for supporting me financially and emotionally. I could not begin to imagine accomplishing this without you.

Scott Masson, for your endless support and love. And especially for accompanying me to the lab at all hours of the night, thank you!

Lynne and Ian Masson, for all of your support and encouragement and for allowing me to use your computer for hours on end.

The University of Cape Town, Department of Pharmacology, for their financial support.

The Medical Research Council of South Africa, for their financial support during my degree.

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LIST OF ABBREVIATIONS

AQ	Amodiaquine
BSA	Bovine Serum Albumin
CI	Confidence Interval
CQ	Chloroquine
DAPI	4'6-Diamidino-2-phenylindole Dihydrochloride
D. discoideum	*Dictystelium discoideum*
DMEM	Dulbecco's Modified Eagles Medium
ECL	Enhanced Chemiluminescence
EDTA	Ethylene diamine tetra-acetic acid
FCS	Fetal Calf Serum
FITC-Dextran	Fluorescein isothiocyanate-dextran
FP	Ferriprotoporphyrin IX
H	Halofantrine
Hb	Hemoglobin
HEPES	Hydroxyethane piperazine sulphonic acid
HRP	Horseradish Peroxidase
IC_{50}	50 % Inhibitory concentration
IgG	Immunoglobulin G
m	Milli
µM	Micromolar
M	Molar
ml(s)	Millilitres
MQ	Mefloquine
nM	Nanomolar
NBT	Nitroblue tetrazolium
OPD	O-phenylenediamine
PBS	Phosphate buffered saline
PES	Phenazine ethosulfate
PFA	Paraformaldehyde
P. falciparum	*Plasmodium falciparum*
PI(s)	Protease Inhibitor(s)

pRBC(s)	Parasitized red blood cells
PVDF	Polyvinylidene difluoride
Q	Quinine
RBC(s)	Red blood cell(s)
rcf	Relative centrifugal force
rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
Tris	Tris(hydroxymethyl)aminomethane

Chapter 1

Introduction

1.1 Perspective

Malaria is an infectious disease that is caused by a protozoan parasite of the *Plasmodium* genus. There are four species of *Plasmodium* that cause human malaria, of these, *Plasmodium falciparum* is the most dangerous and infection often results in death (Hyde, 2002). Malaria is the most serious and widespread human parasitic disease (Abath et al, 1998), with almost half of the world's population at risk (Foley and Tilley, 1997). The disease is most prevalent in Africa (Winstanely et al, 2002) where 90% of all deaths from *falciparum* malaria occur (Hyde, 2002).

Malaria has resurged in many parts of the world (Bryson and Goa, 1992), mainly due to parasite drug resistance and vector insecticide resistance (Abath et al, 1998). As a result, the development of new anti-malarial drugs has become increasingly important. A better understanding of the parasite's metabolism as well as the action of existing drugs may lead to the development of new therapeutic strategies and new mechanisms of drug resistance-reversal. The quinoline antimalarial drugs have previously been found to influence the endolysosomal system in malarial parasites (Macomber et al, 1967; Warhurst and Hockley, 1967; Yayon et al, 1984; Fitch et al, 2003a; Hoppe et al, 2004) and the phagocytic (Labro and Babin-Chevaye, 1988) and secretory pathways in mammalian cells (Smith et al, 1982; Moore et al, 1983). This project involves the further characterization of the influence of these drugs on this pathway in *Plasmodium falciparum*, *Dictyostelium discoideum*, an endocytosis model organism, and mammalian A549 cells.

1.2 Endolysosomal and Secretory Pathways in Mammalian Cells

Endocytosis occurs by a number of different mechanisms that fall into two broad categories, phagocytosis and pinocytosis (Figure 1.1). Phagocytosis involves the uptake of large particles, whilst pinocytosis occurs when cells take up fluid and solutes (Conner and Schmid, 2003). There are at least four mechanisms of pinocytosis: macropinocytosis (reviewed by Swanson and Watts, 1995), clathrin-mediated endocytosis (reviewed by Mousavi et al, 2004), caveolae mediated endocytosis (reviewed by Pelkmans and Helenius, 2002), and clathrin- and caveolae-independent endocytosis (Kirkman and Parton, 2005).

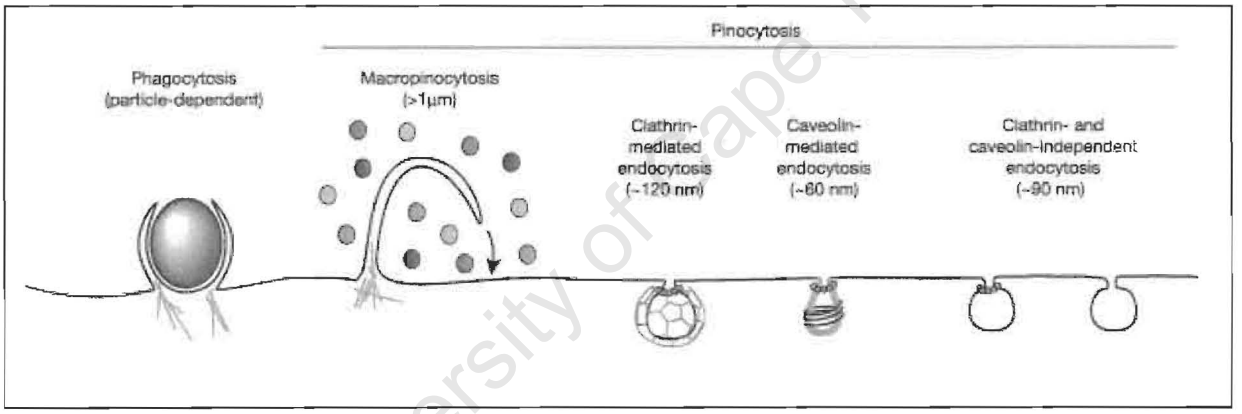


Fig 1.1. The endocytic pathways in mammalian cells. (Conner and Schmid, 2003)

1.2.1 Phagocytosis

Phagocytosis in mammalian cells is generally restricted to specialized cells, including macrophages, monocytes and neutrophils (Conner and Schmid, 2003). The function of these cells is to ingest and clear large pathogens (Allen and Aderem, 1996). Internalization begins when specific surface receptors on a phagocyte interact with ligands on the surface of a particle. Actin then polymerizes at the site of ingestion,

forming pseudopodial extensions, and the particle is taken up into a phagosome (Allen and Aderem, 1996; Aderem and Underhill, 1999).

1.2.2 Macropinocytosis

During macropinocytosis, actin-rich membrane protrusions form (Swanson and Baer, 1995), primarily at sites of ruffling (Swanson, 1989). These extensions collapse onto and fuse with the plasma membrane, generating endocytic vesicles which are heterogeneous in size. Vesicles may have diameters of up to 5 μm (Hewlett et al, 1994) and they may carry large volumes of fluid-phase (Swanson and Watts, 1995; Conner and Schmid, 2003).

1.2.3 Clathrin-Mediated Endocytosis

Clathrin-mediated endocytosis occurs constitutively or in response to certain stimuli (Hannah et al, 1999; Brodin et al, 2000). This type of endocytosis occurs in all mammalian cells, and facilitates the continuous uptake of essential nutrients (Conner and Schmid, 2003). It occurs at specialized sites where complex clathrin-coated pits form, concentrating surface proteins and lipids for internalization (Kirchhausen, 2000 a; Kirchhausen, 2000 b). Clathrin forms a distinct polygonal lattice on the cytoplasmic face of the plasma membrane (Takei and Volker, 2001). AP-2 (adaptor or assembly protein-2) links clathrin to the plasma membrane and coordinates the structural assembly of the lattice (Hirst and Robinson, 1998; Marsh and McMahon, 1999; Kirchhausen, 2000 a; Kirchhausen, 2000 b). Clathrin-coated vesicle formation is also dependent on several accessory proteins (Brodin et al, 2000; Slepnev and De Camilli, 2000). Lattice-formation progressively causes membrane curvature until a deep coated-pit has formed (Takei and Volker, 2001). Dynamin is then required for vesicular fission (Hinshaw, 2000), freeing clathrin vesicles which are then uncoated by an energy-dependent mechanism (Chappell et al, 1986; Ungewickell et al, 1995).

1.2.4 Caveolae-Mediated Endocytosis

Caveolae are typically rounded plasma membrane invaginations that are 50-80 nm in diameter (Pelkmans and Helenius, 2002). Their small size, in addition to their slow rate of internalization, indicates that they are unlikely to contribute significantly to the bulk of fluid-phase uptake (Conner and Schmid, 2003). The composition, appearance and function of caveolae are dependent on cell type (Pelkmans and Helenius, 2002). Caveolae are microdomains (lipid-rafts) that are rich in cholesterol and sphingolipids which are also essential for the formation and stability of these vesicles (Rothberg et al, 1992; Conner and Schmid, 2003). Caveolae have a protein coat that is primarily composed of caveolin (Rothberg et al, 1992). This protein is dimeric (Monier et al, 1995), consisting of cytosolic C- and N-terminal domains linked by a hydrophobic segment that is inserted in the plasma membrane (Dupree et al, 1993; Monier et al, 1995). Dynamin has been found to localize to the neck of caveolae (Henley et al, 1998; Oh et al, 1998) and is thus probably involved in pinching-off of the vesicle (De Camilli et al, 1995; Sever et al, 2000).

1.2.5 Clathrin and Caveolae-Independent Endocytosis

Although clathrin and caveolae-independent mechanisms of pinocytosis are poorly understood (Conner and Schmid, 2003), it has been found that lipid-rafts can be internalized within endocytic vesicles in the absence of clathrin and caveolae (Lamaze et al, 2001; Nichols and Lippincott-Schwartz, 2001; Conner and Schmid, 2003; Kirkham and Parton, 2005). These rafts are 40-50 nm in diameter (Edidin, 2001) and have a composition that provides a physical basis for membrane protein and glycolipid sorting (Edidin, 2001; Anderson and Jacobson, 2002).

1.2.6 Endosomal Trafficking

The basic organization of the endolysosomal pathway in mammalian cells includes early endosomes, recycling endosomes, late endosomes, lysosomes and the *trans*-Golgi

network (Mellman, 1996). Molecules that are internalized by endocytosis are extensively sorted in the early endosome. This organelle is responsible for the distribution of cargo to either be recycled to the cell surface via the recycling endosome, transported to the lysosome via the late endosome or to the *trans*-Golgi network (Vliet et al, 2003). Early endosomes mature to form late endosomes which fuse with lysosomes, where the digestion of internalized material is carried out. Lysosomes would then reform by the concentration of lysosomal enzymes and the formation of a new lysosomal compartment. (Luzio et al, 2000) The *trans*-Golgi network sorts and packages proteins into membrane carriers destined for the plasma membrane, endosomes and regulated secretory granules (Keller and Simons, 1997; Traub and Kornfeld, 1997).

1.2.7 Protein Secretion

Following translation in the cytosol, proteins to be secreted are targeted to the endoplasmic reticulum (Walter and Johnson, 1994). Proteins are exported from here via transitional sites (Palade, 1975) which are specialized domains for the production of transport vesicles. These vesicles subsequently fuse to form vesicular tubular clusters (Bannykh et al., 1996) known as pre-Golgi intermediates (Saraste and Kuismanen, 1992) or the endoplasmic reticulum-Golgi intermediate (Hauri and Schweizer, 1992). This compartment sorts proteins for recycling and delivers proteins to be secreted to the *cis*-Golgi (Warren and Mellman, 1999). Secretory cargo is then transported through the Golgi Stack to the *trans*-Golgi network. As mentioned above, the *trans*-Golgi network sorts proteins into membrane carriers destined for the plasma membrane, endosomes and regulated secretory granules (Keller and Simons, 1997; Traub and Kornfeld, 1997).

1.3 *Dictyostelium discoideum*

1.3.1 D. discoideum as an Endocytosis Model

D. discoideum is a simple eukaryotic organism that grows in the environment as a unicellular amoeba, feeding on bacteria by phagocytosis. These cells aggregate and differentiate to form a mature fruiting body during periods of starvation. This structure consists of stalk cells and spore cells that germinate to form amoebae under favorable conditions (Sharma et al, 2002).

Strains of this cellular slime mould have been isolated that grow axenically in nutrient rich media (Watts and Ashworth, 1970; Sharma et al, 2002). These cells pinocytose particles and fluid with high efficiency while retaining their full phagocytic capacity (Clarke and Kayman, 1987). Laboratory strains have pinocytic rates that are 2-10-fold higher than those observed in macrophages or neutrophils (Thilo, 1985). Additionally, like animal cells, *D. discoideum* is dependent on endocytosis for nutrition (Maniak, 2003). This organism is therefore widely used as a model organism for the study of endocytosis (Watts and Ashworth, 1970; Rupper and Cardelli, 2001).

1.3.2 Mechanisms of Endocytosis

Vesicular trafficking in *Dictyostelium* can be divided into four steps: uptake at the plasma membrane, formation of an early acidic endosome, followed by a late neutral compartment, and finally exocytosis (Aubry et al, 1993; Padh et al, 1993). Pinocytosis in *Dictyostelium* is not linear over time but reaches a plateau that indicates that an equilibrium between endocytosis and exocytosis is reached (Klein and Satre, 1986). The molecular mechanisms of the endocytic pathway in *Dictyostelium* have been found to show striking similarity to higher eukaryotic cells (Neuhaus and Soldati, 1999; Rupper and Cardelli, 2001).

It appears that *D. discoideum* has three different endocytic routes: macropinocytosis, micropinocytosis and phagocytosis (Neuhaus et al, 2002). However the precise contribution of each pinocytic uptake mechanism as well as the role of clathrin remains disputed.

It has previously been suggested that fluid-phase uptake occurs mainly via a clathrin-dependent mechanism, typically forming 0.1 μm vesicles (Aubry et al, 1997). These vesicular structures were characterized and numbered by Swanson et al (1981). Additionally, Ruscetti et al (1994) found that fluid-phase uptake was severely affected in mutant strains deficient in clathrin heavy chain. Damer and O'Halloran (2000) found that clathrin heavy chain expressed in *Dictyostelium* was functional and retained its native properties. Additionally, clathrin expression was able to rescue the phenotypic defects of clathrin null cells. It was also found that clathrin distributed into punctate loci found in the cytoplasm, forming clathrin-coated structures that were motile and capable of rapid and bidirectional transport across the cell.

Other studies have shown that fluid is mainly taken up by macropinocytosis, a process that is dependent on actin, coronin and other actin-binding proteins (Neuhaus et al, 2002). Hacker et al (1997) found that the observed rate of macropinosome formation could account for all measured fluid phase uptake. Thilo and Vogel (1980) recorded primary vesicles to be roughly 0.6 μm in size, whilst Hacker et al (1997) found that vesicles were mostly between 1 and 2 μm. Clathrin is thought by some to be mostly involved in membrane restructuring during cytokinesis and cell motility (Niswonger and O'Halloran, 1997; Damer and O'Halloran, 2000), as apposed to endocytosis as described in the previous paragraph. Macropinocytosis is morphologically and functionally related to phagocytosis. Therefore, the finding that mutants that are unable to grow on bacteria are also unable to grow in axenic media (Vogel, 1983) further implicates macropinocytosis as the main process of fluid-phase uptake. Clathrin-independent micropinocytosis was found to supplement macropinocytosis, accounting for roughly half of the membrane uptake but only a small part of fluid-phase uptake (Neuhaus et al, 2002). Nolta et al (1994) found that vesicles increased from 0.1-0.2 μm after 3 minutes of feeding to 2 μm

after 15 minutes.

The presence of different mechanisms of endocytosis may be essential to the survival of *Dictyostelium*, as these cells are dependent on this process for nutrition. Various mechanisms of internalization would confer greater flexibility, adaptability and different mechanisms of regulation (Neuhaus et al, 2002).

1.4 Endocytosis in Malarial Parasites

The malarial parasite has a life cycle that is divided into three overall stages: mosquito, liver and blood stages. The blood stage consists of the ring, trophozoite and schizont phases. During the blood stage malarial merozoites enter erythrocyte cells, differentiate and undergo asexual reproduction (Prescott et al, 1999). At this time, parasites utilize host erythrocyte hemoglobin as an amino acid source (Krugliak et al, 2002) and possibly for osmotic pressure regulation. Additionally, it has been suggested that uptake of host cell cytoplasm may provide space for parasite growth (Lew et al, 2003). The parasite takes up erythrocyte cytoplasm by endocytosis (Rosenthal and Meshnick, 1996). Little is known about this process, although it is presumably similar to endocytosis in the cells of other eukaryotes. In support of this, it has been found that the malarial parasite has an acidic calcium store that may be involved in regulating the endolysosomal system, as found in other eukaryotic cells. (Passos and Garcia, 1998; Marchesini et al, 2000; Alleva and Kirk, 2001; Biagini et al, 2003) Additionally, mefloquine, an inhibitor of strain D10 *P. falciparum* endocytosis (Hoppe et al, 2004), has been found to inhibit phagocytosis in immune cells (Labro and Babin-Chevaye et al, 1988), suggesting that these two processes may occur by similar mechanisms.

During the intraerythrocytic stage of the parasite's life cycle, a specialized organelle called the cytostome forms by localized invagination of the parasitophorous vacuolar membrane (PVM) and the parasite plasma membrane (PPM) (Goodyer et al, 1997; Foley and Tilley, 1998). Host cell cytoplasm is taken up when a double-membrane vesicle pinches off from this area (Goldberg, 1993; Goodyer et al, 1997; Foley and Tilley, 1998; Fitch, 2004). The inner membrane of the vesicle is formed by the PVM and the PPM forms the outer membrane (Goodyer et al, 1997; Fitch, 2004). The vesicles then mature (Bannister et al, 2000) by becoming acidic (Krogstad et al, 1985) and acquiring proteolytic enzymes (Levy et al, 1974; Goldberg et al, 1990; Banerjee et al, 2002). Parasite food vacuoles are initially formed either when endosomes dock and fuse with each other or when endosomes dock fuse with lysosomes (Bannister et al, 2000). Once the food vacuole has formed, transport vesicles fuse with

this organelle, lose their inner membranes, and hemoglobin is digested (Goodyer et al, 1997) by the action of parasite aspartic proteinase enzymes called plasmepsins I (Goldberg et al, 1991) and II (Gluzman et al, 1994) and a cysteine proteinase enzyme called falcipain (Francis et al, 1996). Ferriprotoporphyrin IX (FP) is released from hemoglobin and is detoxified by conversion to ß-hematin dimers. ß-hematin is then incorporated into hemazoin crystals (malaria pigment) (Fitch and Kanjananggulpan, 1987; Slater et al, 1991). FP dimerization is thought to be promoted by lipid (Fitch et al, 1999; Fitch et al, 2000) that may be derived from the inner membranes of the endocytic vesicles (Fitch et al, 2000; Hempelman et al 2003). The action of a neutral aminopeptidase may be required to expose the lipid (Fitch et al, 2003a). It has been suggested that only 30 % of FP is converted to hemazoin and that the majority is exported into the cytoplasm for degradation by glutathione (Ginsburg et al, 1998; Loria et al, 1999). However, Egan et al (2002) found that 90 % of intraparasitic iron is in the food vacuole, 88.9 % being in the form of hemozoin, suggesting that hemozoin formation is the primary mechanism of FP detoxification. In addition to incorporation into hemozoin, it has been suggested that FP may be peroxidatively degraded in the food vacuole (Loria et al, 1999), degraded by a glutathione dependent pathway in the parasite cytosol (Ginsburg et al, 1998) or it may exit the parasitized red blood cell and bind to serum albumin or hemopexin (Solar et al, 1989).

1.5 Quinoline Anti-malarial Drugs

There are two subclasses of quinoline anti-malarial drugs (Figure 1.3), which appear to act on the malarial endolysosomal system in a different way. The 4-aminoquinoline subclass includes chloroquine (CQ) and amodiaquine (AQ). The other subclass consists of the quinoline-4-methanol drugs such as quinine (Q) and mefloquine (MQ). Halofantrine (H) is a 9-phenathrenemethanol that was synthesized by the alteration of the quinoline-4-methanol structure (Foley and Tilley, 1998). These drugs are known to have pleiotropic effects, and the precise intracellular mechanisms of action are generally uncertain (Famin and Ginsburg, 2002). Additionally, the action of anti-malarial drugs in malarial parasites may be strain dependent (Hoppe et al, 2004), making elucidation of the modes of action more difficult.

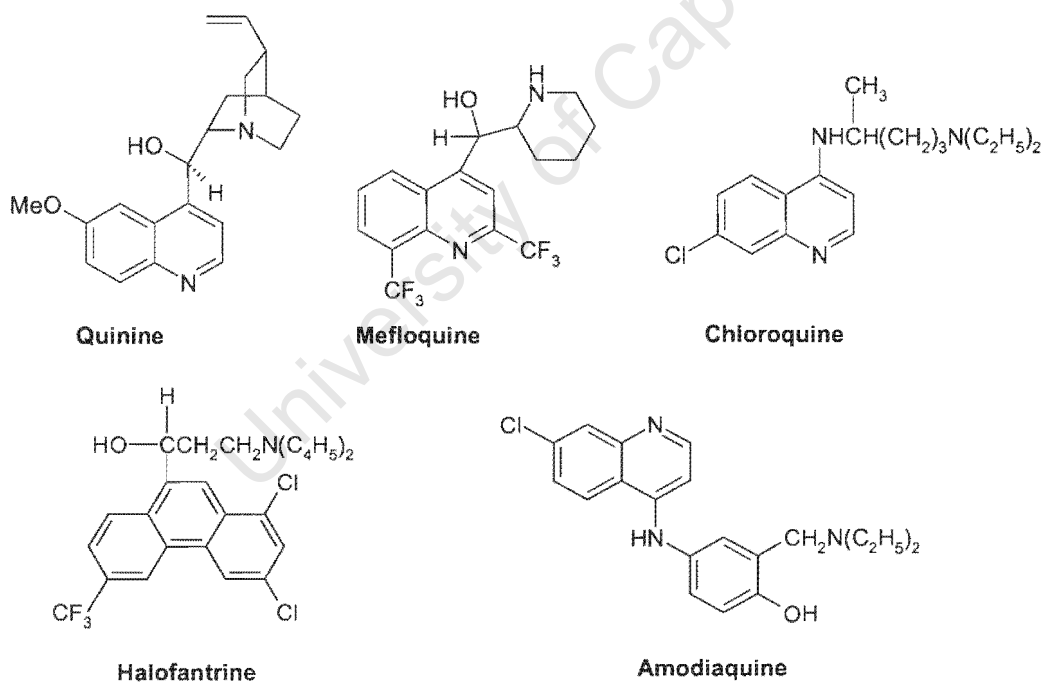


Fig 1.3. Structures of quinine, mefloquine, chloroquine, halofantrine and amodiaquine.

1.5.1 Chloroquine (CQ)

CQ was introduced shortly after World War II (Foley and Tilley, 1998). It became the drug of choice for the next several decades (Hyde, 2002), however, resistance to this drug is now so widespread that it is virtually useless in many parts of the world (Foley and Tilley, 1998).

The action of CQ has been widely studied and many pleiotropic effects have been identified. It has additionally been found that various strains of malarial parasites show differing responses to the drug, thus making it difficult to establish the primary mechanism of action of CQ. It is probable that CQ-treatment causes the death of parasites by a number of routes that have a cumulative effect.

1.5.1.1 Interaction with FP

CQ is a diprotic base that can accumulate several thousand-fold in the acidic food vacuole by an ion-trapping mechanism and by its high-affinity binding to FP and FP dimers (Aikawa, 1972; Hawley et al, 1996; Fitch et al, 1974; Krogstad et al, 1985). CQ accumulation has been found to correlate with the amount of free FP available in malarial parasites (Bray et al, 1999). The drug binds tightly to undimerized FP and disrupts dimerization at concentrations found in the food vacuole (Chou and Fitch, 1993; Egan et al, 1994; Dorn et al, 1995; Raynes et al, 1996). It has been shown that CQ slows the rate of ß-hematin formation rather than irreversibly blocking it (Egan and Ncokazi, 2005). As a result, toxic FP accumulates (Chou and Fitch, 1993; Zhang et al, 1999; Famin and Ginsburg, 2002). There is evidence suggesting that CQ can also prevent the formation of hemozoin (Sullivan et al, 1996) and disassemble it (Pandey and Tekwani, 1997). FP has been shown to have catalase and peroxidase activity (Green et al, 1996). CQ inhibits the catalase activity (Ribeiro et al, 1997) and enhances the peroxidase activity (Sugioka and Suzuki, 1991), resulting in the deregulation of the host-parasite redox balance (Ribeiro et al, 1997).

CQ inhibition of FP dimerization is thought by many to be the primary mode of CQ action. The presence of high levels of toxic CQ-FP complexes, undimerized FP or H_2O_2 have been shown to cause many secondary effects including peroxidative damage to lipids and proteins (Tappel, 1955; Sugioka and Suzuki, 1991; Foley and Tilley, 1998; Ginsburg et al, 1998), masking of lipids that promote FP dimerization (Fitch et al, 2003b) and loss of membrane function (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983). In support of the latter, CQ has been found to moderately inhibit hemoglobin endocytosis following long incubation periods (Hoppe et al, 2004). Additionally, high levels of FP or FP-CQ complexes would result in the inhibition of proteolytic enzymes (Van der Jagt et al, 1986), potentially inhibiting hemoglobin digestion (Famin and Ginsburg, 2002).

1.5.1.2 Inhibition of FP Detoxification

Inhibition of FP dimerization by CQ would result in an increase in efflux of FP out of the food vacuole. CQ has been shown to inhibit glutathione-dependent degradation of FP, presumably by forming complexes with it (Ginsburg et al, 1998; Famin et al, 1999; Loria et al, 1999; Zhang et al, 1999). CQ has additionally been shown to inhibit peroxidative degradation of FP (Loria et al, 1999), eliminating another method of detoxification.

1.5.1.3 Alkalinization of the Food Vacuole

CQ has been found to accumulate in the food vacuole until buffering capacity is reached. At this point the pH of the vacuole increases (Krogstad et al, 1985), potentially disrupting enzymatic function and vesicle-vacuole fusion.

1.5.1.4 Inhibition of Transport Vesicle Processing

CQ had been found to inhibit the processing of hemoglobin transport vesicles (Macomber et al, 1967; Warhurst and Hockley, 1967, Fitch et al, 2003a), either causing their accumulation in the parasitic food vacuole (Yayon et al, 1984) or alternatively, as found

in other strains, in the parasite cytoplasm (Fitch et al, 2003a; Hoppe et al, 2004). It has been suggested that the primary mechanism of CQ action may be inhibition of vesicle-vacuole or vesicle-vesicle fusion, or promotion of premature docking. However, these effects are likely to be secondary to primary effects such as CQ-induced alkalinization of the food vacuole or accumulation of FP. CQ may impair membrane function by interacting with phospholipid targets through an FP bridge (Fitch, 2004; Hoppe et al, 2004). Although CQ does not bind to phospholipids with high affinity (Chevli and Fitch, 1982), FP does (Shviro et al, 1982; Ginsburg and Demel, 1983). However, CQ is also able to disrupt the function of lysosomes and trafficking in secretory pathways in mammalian cells (Moore et al, 1983; Smith and Jarret, 1982), suggesting that loss of membrane function observed in parasites following CQ-treatment can occur independently of FP accumulation.

Transport vesicles that have not been properly processed, would probably fail to degrade hemoglobin properly, possibly only denaturing it and releasing FP. These vesicles may also fail to produce, import or activate a substance that is needed to unmask the unsaturated lipid that is involved in FP dimerization. This would result in the accumulation of hemoglobin (Famin and Ginsburg, 2002; Fitch et al, 2003a) and further accumulation of undimerized FP (Chou and Fitch, 1993; Zhang et al, 1999; Famin and Ginsburg, 2002).

1.5.1.5 Summary

CQ thus exerts a number of effects simultaneously. It appears that the build-up of toxic FP is likely to be the most important and effective method of CQ action. Accumulation of FP results from CQ inhibition of FP dimerization, glutathione and peroxidative degradation, CQ-induced food vacuole alkalinization and transport vesicle accumulation. Therefore, CQ-treatment causes primary and secondary effects that act cumulatively, resulting in a large increase in the concentration of FP that causes extensive damage to lipids and proteins, eventually disrupting membrane and enzymatic function, with consequent effects on the endolysosomal pathway.

1.5.2 Amodiaquine (AQ)

AQ was also developed shortly after the Second World War (Foley and Tilley, 1998), but use was restricted due to the success of CQ. With the emergence of widespread CQ resistance, a renewed interest in AQ has been stimulated, despite concerns of the toxicity of this drug when used as a prophylactic agent. (Hawley et al, 1996)

AQ is a diprotic base that only differs from CQ by having a *p*-hydroxyanilino aromatic ring. The drug has been found to act similarly to CQ (Foley and Tilley, 1998), but is a better inhibitor of *P. falciparum* growth (Hawley et al, 1996). AQ and CQ probably share a similar mechanism of accumulation, as AQ has been shown to competitively inhibit CQ build-up (Fitch, 1973). AQ accumulates inside the parasite's food vacuole more efficiently than CQ, possibly due to an enhanced affinity for an intraparasitic binding site (Hawley et al, 1996). It has been suggested that AQ inhibits hemoglobin degradation (Famin and Ginsburg, 2002). It also inhibits ß-hematin formation (Chou and Fitch, 1993; Egan et al, 1994) by forming complexes with it and slowing the rate of dimerization (Egan and Ncokazi, 2005). AQ has additionally been shown to competitively inhibit the degradation of FP by glutathione. (Ginsburg et al, 1998 ; Famin et al, 1999; Zhang et al, 1999)

1.5.3 Mefloquine (MQ)

The development of widespread CQ resistance led to the initiation of screening programmes in the 1960s. MQ was introduced and used effectively for the next 30 years (Foley and Tilley, 1998). Despite current concerns about resistance and the toxicity of this drug, it is still used due to a lack of effective alternatives (Croft and Garner, 1997; Foley and Tilley, 1998).

Quinoline anti-malarials are known to have pleiotropic effects (Foley and Tilley, 1998). MQ primarily acts on the intraerythrocytic asexual stages of the malarial parasite (Schmidt et al, 1978a, Schmidt et al 1978c). It is a lipophilic drug that binds tightly to serum components (Desneves et al, 1996), perhaps facilitating delivery to the parasite

(Grellier et al, 1991). It has been suggested that the mechanism of action of the quinoline-4-methanol drugs may be similar to that of CQ (Foley and Tilley, 1998). MQ, however, is not extensively concentrated in the food vacuole, although it does cause morphological changes in this organelle (Foley and Tilley, 1998). It interacts weakly with FP (Chou et al, 1980; Chevli and Fitch, 1982), and inhibits the formation of FP dimer *in vitro* (Slater and Cerami, 1992). However, it does not inhibit hemozoin formation *in vivo* (Chou and Fitch, 1993), probably because it does not reach a high enough concentration inside the food vacuole (Foley and Tilley, 1998). Additionally, MQ does not affect hemoglobin degradation (Famin and Ginsburg, 2002) and is a much less potent enhancer of FP peroxidase activity than CQ (Sugioka and Suzuki, 1991). It is thus likely that FP is not the major target of MQ.

MQ has been shown to bind to membranes and purified phospholipids with high affinity (Chevli and Fitch, 1982; San George et al, 1984). The drug additionally inhibits the phagocytic activity of human polymorphonuclear neutrophils (Labro and Babin-Chevaye, 1988). Furthermore, it has recently been demonstrated that MQ inhibits endocytosis in strain D10 of *P. falciparum* (Hoppe et al, 2004), providing a possible explanation for the reduction in hemoglobin levels in MQ-treated FCR3 *P. falciparum* (Famin and Ginsburg, 2002).

1.5.4 Quinine (Q)

Q was one of the first anti-malarial alkaloids to be extracted from the cinchona tree (Foley and Tilley, 1998; Berman, 2004). Q and MQ have similar structures, and thus probably share similar mechanisms of action. Q also primarily acts on the intraerythrocytic asexual stages of the malarial parasite (Schmidt et al 1978a, c). Like MQ, Q is lipophilic and binds tightly to serum components, possibly facilitating delivery of this drug to the parasite (Desneves et al, 1996). Additionally, Q is also a monoprotic base and is thus not extensively concentrated in the food vacuole (Foley and Tilley, 1998), although it also causes morphological changes in this organelle. It does not result in hemoglobin or FP accumulation (Chou and Fitch, 1993), although it interacts weakly

with FP (Chou et al, 1980), inhibiting ß-hematin formation (Slater et al, 1992; Chou and Fitch, 1993; Egan et al, 1994; Portela et al, 2003) by decreasing the rate of dimerization (Egan and Ncokazi, 2005). Q has additionally been found to inhibit FP catalase activity (Ribeiro et al, 1997).

In addition to FP, phospholipids may act as a target for Q. The drug has been found to antagonistically affect hemoglobin accumulation and may inhibit hemoglobin ingestion by endocytosis and vesicular docking by interacting with membrane phospholipids (Famin et al, 1999; Famin and Ginsburg, 2002). It may also impair membrane recycling, possibly by inhibiting calcium release from the acidic store. This would result in the accumulation of excess membrane and parasite death (Fitch, 2004).

1.5.5 Halofantrine (H)

H was first identified as a potential anti-malarial agent by the World War II Chemotherapy Program. Chloroquine resistance prompted its introduction in the 1960s (Bryson and Goa, 1992). Use of this drug has, however, been restricted due to reports of serious cardiotoxicity (Monlun et al, 1993; Berman, 2004). H was synthesized by replacing the quinoline portion of the quinoline-4-methanols with a different aromatic ring system, forming the aryl(amino)carbinols (Schmidt et al, 1978b). The 9-Phenanthrenemethanol subclass, which included H, was found to be most effective (Bryson and Goa, 1992; Foley and Tilley, 1998).

The mechanism of action of H is largely unknown. H is however likely to act similarly to Q and MQ as these drugs are structurally related. H has been found to cause efflux of CQ from malarial parasites (Bray et al, 2006). The drug is a blood schizonticide, which acts selectively against the intraerythrocytic stages of *Plasmodium* spp. H inhibits the activity of more mature parasite stages, and has no activity against gametocytes or against pre-erythrocytic hepatic stages (Bryson and Goa, 1992). Studies have shown that H binds to FP and

inhibits its dimerization (Egan et al, 1994; Basilico et al, 1997; Hawley et al, 1998; Egan et al, 1999; Ursos et al, 2001).

1.6 Purpose of this Study

The precise mechanisms of action of the quinoline anti-malarials are as yet unclear. A better understanding of the parasite's metabolism as well as the action of these drugs may prove useful in the development of new therapeutic strategies. The quinoline anti-malarials are thought to influence the endolysosomal pathway in malarial parasites. CQ had been found to inhibit the processing of hemoglobin transport vesicles (Macomber et al, 1967; Warhurst and Hockley, 1967, Fitch et al, 2003a), and moderately inhibit hemoglobin endocytosis following long incubation periods (Hoppe et al, 2004). Additionally, the drug is able to disrupt the function of lysosomes and trafficking in mammalian secretory pathways (Smith et al, 1982; Moore et al, 1983). MQ has been shown to bind to membranes and purified phospholipids with high affinity (Chevli and Fitch, 1982; San George et al, 1984), and has been found to inhibit the phagocytic activity of human polymorphonuclear neutrophils (Labro and Babin-Chevaye, 1988). Additionally, it has recently been demonstrated that MQ inhibits endocytosis in strain D10 of *P. falciparum* (Hoppe et al, 2004). AQ is structurally related to CQ and has been found to act similarly (Foley and Tilley, 1998). H and Q may act similarly to MQ, as the drugs have similar properties and are structurally related.

Therefore, in order to confirm the ability of CQ and MQ to influence the endolysosomal pathway in malarial parasites and to compare this to the action of AQ, H and Q, the effects of the drugs on endocytosis and vesicular trafficking in *Plasmodium falciparum* strain 3D7 were determined. Additionally, in order to shed further light on the mechanisms of action of these drugs, endocytosis and/or exocytosis assays were conducted in *Dictostelium discoideum* and mammalian A549 cells.

Chapter 2

The Sensitivity of *Plasmodium falciparum* 3D7 to Quinoline Anti-Malarial Drugs and Their Effect on General Parasite Morphology

2.1 Introduction

In order to determine the sensitivity of strain 3D7 *P. falciparum* to the quinoline anti-malarials, AQ, CQ, H, MQ and Q, the 50 % inhibitory concentrations (IC_{50}) for the drugs were determined. Parasites were exposed to a wide range of drug concentrations and viability was assessed by measuring parasite lactate dehydrogenase activity. The IC_{50} values were then used to obtain the concentrations of each drug that would be used for subsequent drug-treatments. It was decided that parasites would be treated with drugs at approximately 5 times the IC_{50} values.

CQ has previously been found to cause parasite morphology changes (Macomber and Sprinz, 1967; Warhurst and Hockley, 1967; Aikawa, 1972). Therefore, the morphological changes in parasites following drug-treatment were determined in order to shed light on how the drugs compare in their mode of action. The effects of treatment with 102 nM AQ, 137 nM CQ, 27 nM H, 156 nM MQ and 665 nM Q on the general morphology of *P. falciparum* 3D7 were determined by examining Giemsa-stained thin blood smears from treated cultures by light microscopy.

2.2 Results

2.2.1 Sensitivity of strain 3D7 P. falciparum to Quinoline Anti-Malarial Drugs

Table 2.1 shows the IC_{50} values that were obtained for the drugs, derived from residual lactate dehydrogenase activity. Parasite viability versus drug concentration is graphically depicted in Figure 2.1.

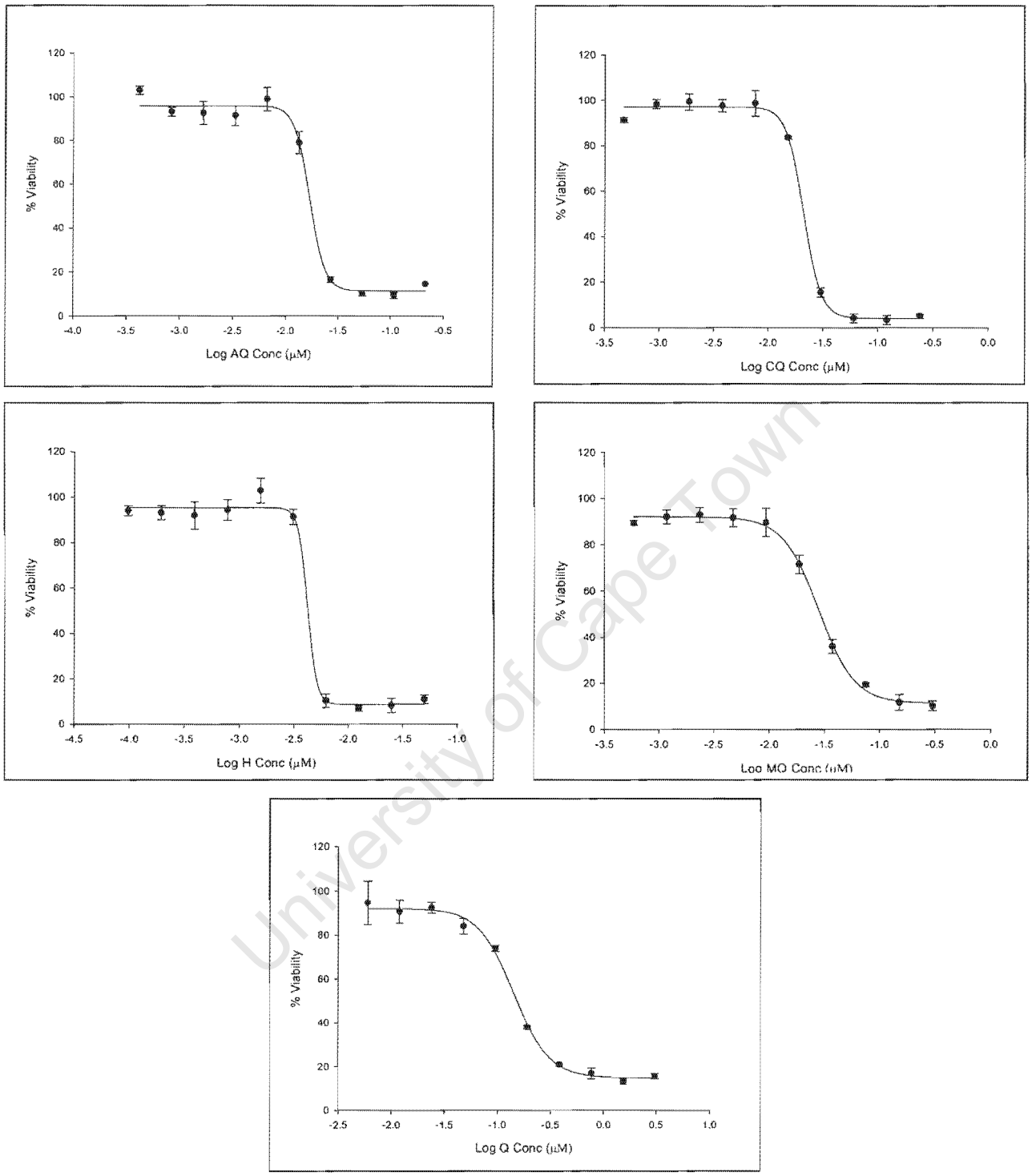


Fig 2.1. Anti-malarial activity of amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) and quinine (Q) on *in vitro* cultures of strain 3D7 of *Plasmodium falciparum*. The experiment was carried out in triplicate. The results are plotted as the percentage viability of the parasites versus the logarithm of the drug concentration in µM.

Drug	3D7 IC_{50} (nM)
AQ	17.78
CQ	22.01
H	4.47
MQ	31.62
Q	173.78

Table 2.1. IC_{50} values for strain 3D7 of *Plasmodium falciparum.* Parasites were incubated with increasing concentrations of amodiaquine (AQ), mefloquine (MQ), chloroquine (CQ), quinine (Q) or halofantrine (H) in triplicate. Cultures were then assayed for residual lactate dehydrogenase activity.

2.2.2 The Effect of Quinoline Anti-Malarials on the Morphology of 3D7 P. falciparum

In order to prepare Giemsa stains, parasites were incubated with each drug at a concentration approximately 5 times the IC_{50} value for 8 hours. Thin blood smears were prepared, fixed with methanol and stained with Giemsa. Slides were then viewed under a light microscope (Figure 2.2).

Figure 2.2 shows representative images of malarial parasites treated with each quinoline anti-malarial drug and stained with Giemsa. Red blood cells (RBCs) were left intact and are therefore generally visible as roughly circular grey bodies surrounding the parasites. At this stage in growth, untreated control parasites are approximately a third of the size of a RBC. Healthy trophozoite parasites are generally roughly circular and contain hemazoin crystals in their food vacuoles that are visible as small dark or shiny white areas or both (indicated by arrows). The presence of these crystals allows for the viewing of parasite food vacuoles with ease.

Parasites treated with 102 nM AQ for 8 hours varied between being swollen larger than the controls and being reduced in size (Figure 2.2). Parasites treated with 137 nM CQ were large and swollen to a greater extent than untreated or AQ-treated parasites. The food vacuoles (arrows) of CQ-treated parasites appeared to be normal in size. Parasites treated with 27 nM H, 156 nM MQ or 665 nM Q were all smaller than untreated

parasites. Additionally, their food vacuoles appeared reduced in size and hemozoin crystals were barely visible.

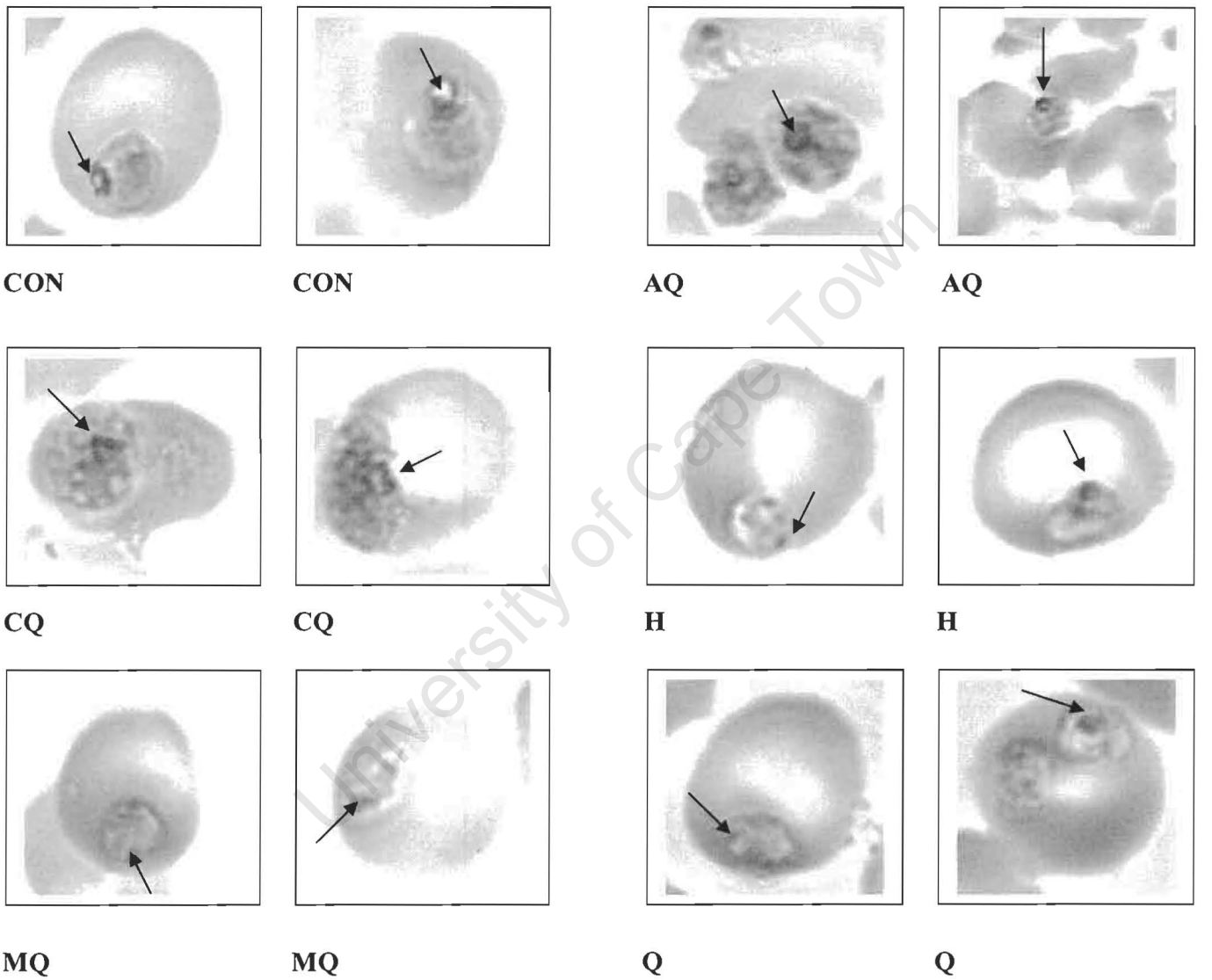


Fig 2.2. Giemsa stains of 3D7 *P. falciparum* parasites. Parasites were untreated (CON), incubated with amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) at a concentration 5 times the IC_{50} value for each drug for 8 hours. Thin blood smears were then prepared and stained with Giemsa. Slides were viewed under a light microscope. Arrows indicate parasite food vacuoles.

2.3 Discussion

2.3.1 Sensitivity of strain 3D7 P. falciparum to Quinoline Anti-Malarials

A previous study (Hawley et al, 1998) of the potencies of various anti-malarial drugs found that the 50% inhibitory concentrations of AQ, CQ, H, MQ and Q on strain 3D7 *P. falciparum* were 7.8 nM, 14.0 nM, 5.8 nM, 23.4 nM, and 34.2 nM respectively. Apart from the IC_{50} of H, the values that were determined in this study (Table 2.1, Figure 2.1) were higher than those observed by Hawley et al (1998). Although the differences in IC_{50} values may be due to the use of a different inoculum size, it is a common finding that the sensitivity of living organisms to drug-treatment may vary widely at different times. Therefore these findings are likely to be merely indicative of small changes in general parasite wellbeing. Alternatively, it is possible that a lengthy period of sub-culturing may be accompanied by a certain degree of genetic drift, resulting in small changes to parasite genetic information. This may account for larger variations in parasite viability.

2.3.2 The Effect of Quinoline Anti-Malarials on the Morphology of P. falciparum 3D7

Parasites treated with 27 nM H, 156 nM MQ or 665 nM Q were smaller in size relative to untreated parasites. Additionally, the food vacuoles of these parasites were reduced in size and less hemozoin appeared to be present. The similarities in the morphologies of parasites treated with these drugs, indicates that they may share similar mechanisms of action. The small size of the hemazoin crystal may indicate that hemoglobin digestion was reduced, however it has previously been found that MQ does not affect this process (Famin and Ginsburg, 2002). Alternatively, the uptake of hemoglobin by endocytosis may be inhibited. In support of this suggestion, MQ has previously been found to inhibit endocytosis in strain D10 *P. falciparum* (Hoppe et al, 2004). Another possible explanation is that the detoxification of FP by conversion to ß-hematin and subsequently hemazoin may be disrupted, causing less hemazoin to be present in food vacuoles.

Parasites treated with 137 nM CQ, however, were larger than untreated parasites,

indicating that CQ is likely to have a different mode of action. CQ may block the endolysosomal pathway or the digestion of hemoglobin, leading to the accumulation of internalized material. In support of the former suggestion, it has been found that treatment with CQ results in the loss of membrane function in malarial parasites (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983). CQ-treatment has additionally previously been suggested to cause hemoglobin accumulation (Famin and Ginsburg, 2002; Fitch et al, 2003a; Hoppe et al, 2004) by inhibiting the digestion of this protein (Famin and Ginsburg, 2002). In this assay, parasites that were treated with CQ had food vacuoles that appeared to be normal in size. Contrary to this, it has previously been found that CQ causes swelling of parasite food vacuoles (Macomber and Sprinz, 1967; Warhurst and Hockley, 1967; Aikawa, 1972), therefore suggesting that the effects of CQ are largely strain-dependent.

Parasites treated with 102 nM AQ were variably larger or smaller in size relative to untreated parasites. The effects of AQ thus appear to be intermediate, or a combination of MQ and CQ-type modalities. The drug may affect *P. falciparum* parasites in a manner that is stage-specific. Cultures were synchronized using the sorbitol method (Lambros and Vanderberg, 1979) in order to obtain predominantly ring-stage parasites. However both early and late ring-stage parasites would most likely be present. Therefore, at the time of drug addition, cultures most probably contained both early and late trophozoite-stage parasites. The effects of AQ on early trophozoite parasites may differ from the effects of the drug on late trophozoite parasites, resulting in the presence of parasites with variable morphology.

Chapter 3

The Effect of Quinoline Anti-Malarials on Hemoglobin Uptake and Accumulation in *P. falciparum* 3D7

3.1 Introduction

Blood stage malarial parasites endocytose and digest large amounts of hemoglobin (Ginsburg et al, 1998). Intracellular levels of this protein are thus an indirect measure of endocytosis and hemoglobin degradation. CQ-treatment has previously been found to result in the accumulation of undigested hemoglobin in malarial parasites (Famin and Ginsburg, 2002; Fitch et al, 2003a; Hoppe et al, 2004). Famin and Ginsburg (2002) suggested that CQ and AQ inhibit hemoglobin degradation. Although MQ was shown to have no influence on the digestion of this protein, treatment with this drug resulted in a reduction in hemoglobin levels in *P. falciparum* FCR3 (Famin and Ginsburg, 2002). This may be explained by an inhibition of endocytosis as found in strain D10 (Hoppe et al, 2004). It has been demonstrated that treatment with Q antagonistically affects hemoglobin accumulation and it has been suggested that this drug may, like MQ, inhibit hemoglobin uptake by endocytosis (Famin et al, 1999; Famin and Ginsburg, 2002). H is structurally related to MQ and Q, these drugs are therefore likely to act similarly. In order to determine the effects of treatment with quinoline anti-malarials on hemoglobin levels in 3D7 *P. falciparum*, Western Blotting assays were conducted.

3.2 Results

3.2.1 Hemoglobin Levels in 3D7 P. falciparum Treated with Quinoline Anti-Malarials for 6 Hours

Trophozoite-stage *P. falciparum* parasites were incubated with 102 nM AQ, 137 nM CQ, 27 nM H, 156 nM MQ and 665 nM Q for 6 hours. Hoppe et al (2004) found that MQ inhibited endocytosis in *P. falciparum* following a 5 hour incubation period. CQ however was only found to influence the endocytic pathway following a 12 hour

incubation period. Therefore the effects of the drugs were initially examined following a 6 hour incubation period. After incubation, parasites were released from RBCs by saponin treatment and washed to remove extraparasitic hemoglobin. Parasite lysates were run on SDS-polyacrylamide gels and transferred to membranes. Membranes were incubated with anti-hemoglobin antiserum and secondary antibody conjugated to peroxidase. Membranes were soaked in ECL Western blotting detection reagent and exposed to autoradiography film. Autoradiography films were developed and the net intensities of hemoglobin bands were determined using the histogram function of Adobe Photoshop. Readings were normalized relative to the controls (100 %).

The results show that treatment with CQ for 6 hours led to an increase in hemoglobin levels in the parasites (Table 3.1, Figure 3.2 A) (P = 0.0070).

Sample	Concentration (nM)	Mean Band Intensity Relative to Control (%) ± SD	*N*
Control	-	100.0 ± 10.6	10
AQ	102	84.0 ± 5.6	4
CQ	137	121.6 ± 13.0	4
H	27	77.8 ± 8.2	4
MQ	156	59.6 ± 12.7	4
Q	665	59.7 ± 7.5	4

Table 3.1. Western Blot hemoglobin band intensities after 6 hours of drug treatment. Parasite cultures were untreated (Control), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q). Intensities of hemoglobin bands were determined using the histogram function of Adobe Photoshop (version 7.0) software. The intensities were normalized to control intensities. *N* = sample size.

Treatment with AQ, H, MQ or Q for 6 hours led to a decrease in hemoglobin levels in comparison to the controls (Table 3.1, Figure 3.2 A) (P = 0.0150, P = 0.0028, P<0.0001, P<0.0001 for AQ, H, MQ and Q-treated parasites respectively).

3.2.2 Hemoglobin Levels in 3D7 P. falciparum Treated with Quinoline Anti-Malarials for 8 Hours

The incubation time of *P. falciparum* parasites with 102 nM AQ, 137 nM CQ, 27 nM H, 156 nM MQ and 665 nM Q, was expanded to 8 hours. Apart from AQ-treated parasites, this had the effect of amplifying the results obtained following a 6 hour incubation period.

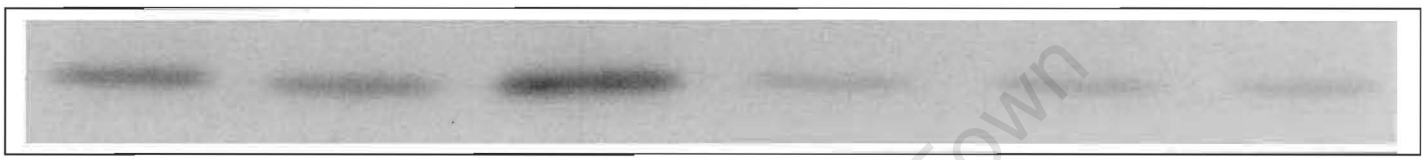


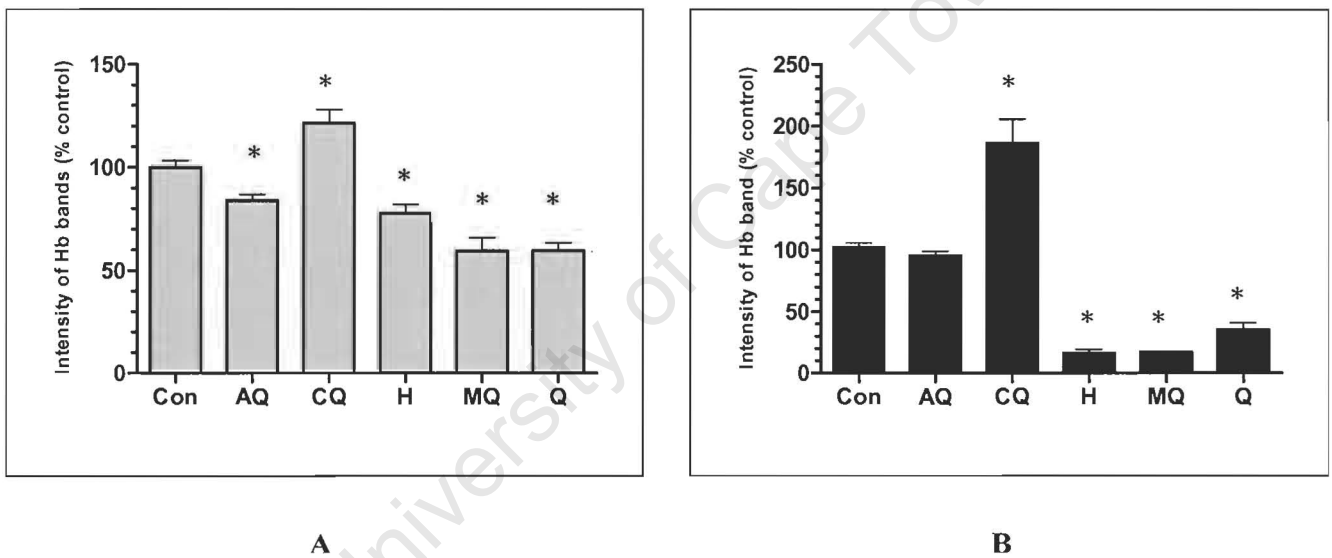
Fig 3.1. Representative Western Blot image showing hemoglobin levels in parasites treated with quinoline anti-malarials for 8 hours. Parasite cultures were untreated (control, Con), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q)..

Sample	Concentration (nM)	Mean Band Intensity Relative to Control (%) ± SD	*N*
Control	-	100.0 ± 9.0	6
AQ	102	95.2 ± 9.4	6
CQ	137	185.9 ± 48.4	6
H	27	16.2 ± 5.2	3
MQ	156	16.9 ± 2.1	3
Q	665	35.6 ±10.2	3

Table 3.2. Western Blot hemoglobin intensities of parasite lysate bands following drug-treatment of parasites for 8 hours. Parasite cultures were untreated (Control), incubated 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q). Intensities of hemoglobin bands were determined using the histogram function of Adobe Photoshop (version 7.0) software. The intensities were normalized to control intensities. *N* = sample size.

Treatment with CQ for 8 hours resulted in an increase in hemoglobin levels (P = 0.0212). Treatment with H, MQ or Q for 8 hours resulted in a further decrease in hemoglobin levels relative to the controls (P = 0.0001, P <0.0001, P = 0.001 for H, MQ and Q-treated parasites respectively) (Table 3.2, Figures 3.1 and 3.2 B).

Although treatment with AQ for 8 hours caused a slight reduction in hemoglobin levels relative to those in control parasites (Table 3.2, Figures 3.1 and 3.2 B), the change was however not statistically significant at the 95 % CI (P = 0.4780).

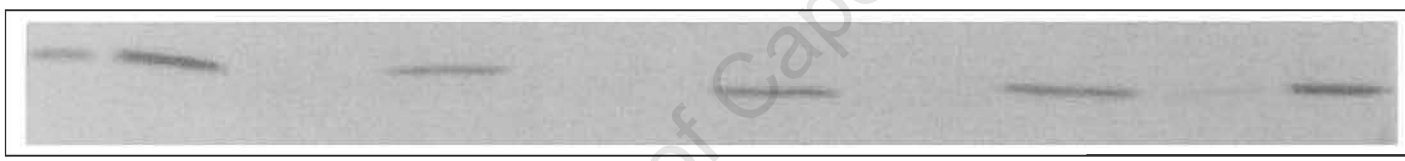


A B

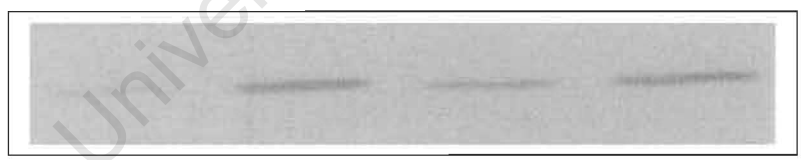
Fig 3.2 Hemoglobin levels in drug-treated parasites. Parasite cultures were untreated (control, Con), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q) for 6 hours **(A)** or 8 hours **(B)**. Intensities of hemoglobin bands on Western Blots were determined using the histogram function of Adobe Photoshop (version 7.0) software. The intensities were normalized to control intensities. Error bars indicate standard error. * Statistically significant change from the controls (95% CI).

3.2.3 Hemoglobin Levels in 3D7 P. falciparum Treated with Quinoline Anti-Malarials and Protease Inhibitors

Using hemoglobin levels in parasites as a measure of endocytosis is confounded by the fact that hemoglobin is rapidly digested in the food vacuole. Therefore in order to eliminate the contribution of digestion and to obtain a more accurate measure of the effects of the anti-malarial drugs on hemoglobin endocytosis and vesicular trafficking alone, protease inhibitors (PIs) ALLN and E64 were included during drug treatments. These inhibitors have previously been found to inhibit hemoglobin digestion in malarial parasites (Francis et al, 1997). Western Blotting Assays were conducted using cultures treated with the quinoline anti-malarial drugs at concentrations 5 times their IC_{50} values or with drug and PIs.



Con **Con (PI)** **MQ** **MQ (PI)** **Q** **Q (PI)** **H** **H (PI)** **AQ** **AQ (PI)**



Con **Con (PI)** **CQ** **CQ (PI)**

Fig 3.3. Representative Western Blot images showing hemoglobin levels in parasites. Parasite cultures were untreated (control, Con), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ), or 665 nM quinine (Q) for 8 hours. Cultures were treated with drug alone, or drug and protease inhibitors, ALLN and E64 (PI). 40 μM of ALLN and E64 were added.

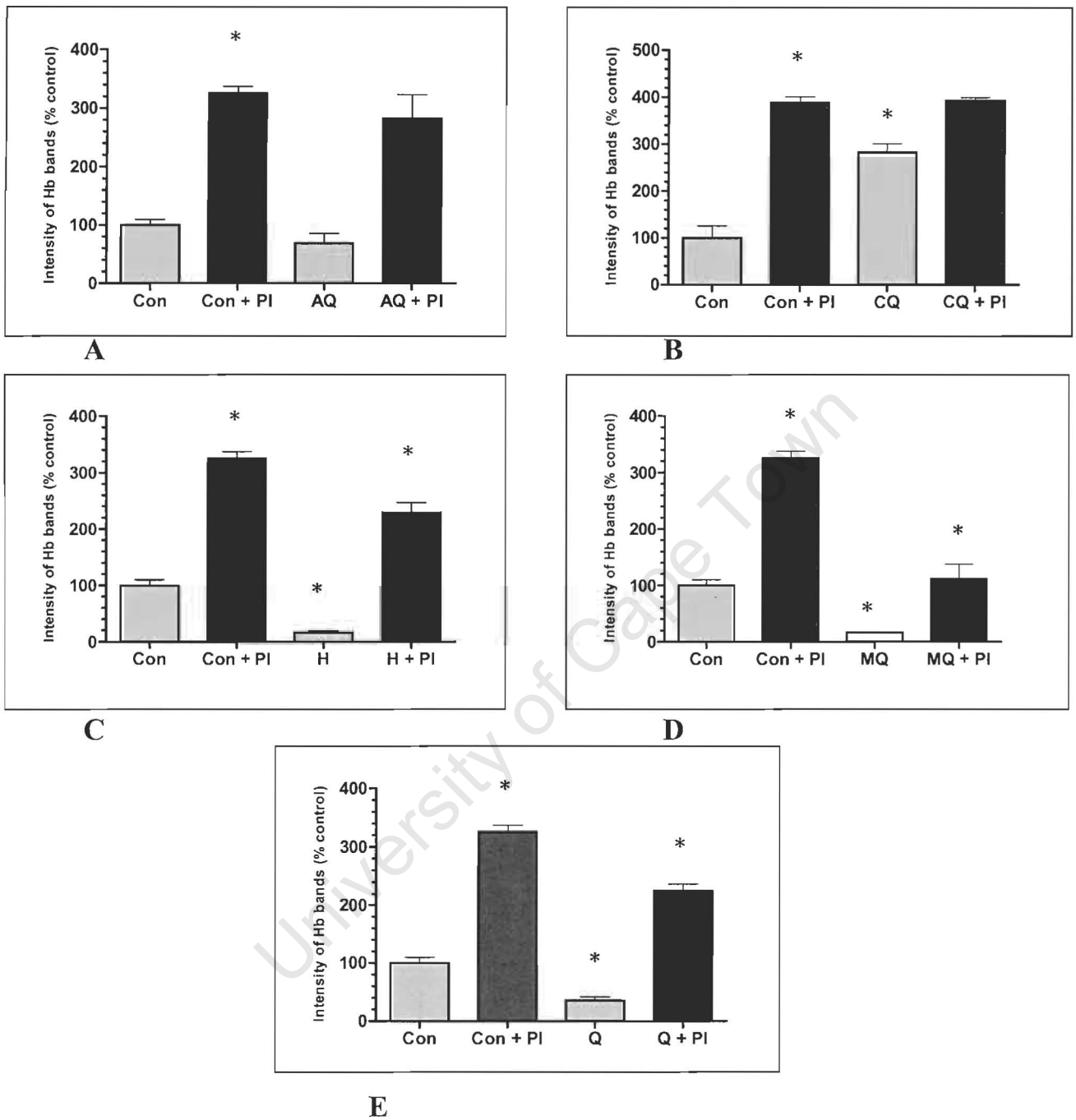


Fig 3.4. Hemoglobin levels in parasites. Parasite cultures were untreated (control, Con), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ), or 665 nM quinine (Q) (figures A to E respectively). Cultures were treated with drug alone, or drug and protease inhibitors, 40 μM ALLN and E64 (PI). Error bars indicate standard error. * Statistically significant change from controls (95% CI), either untreated or treated with PIs alone.

A representative Western Blot is shown in Figure 3.3. Results are summarized in Table 3.3 and shown graphically in Figure 3.4. Hemoglobin levels in control parasites treated with PIs increased significantly relative to untreated controls (P = 0.0001 for AQ, H, MQ and Q assays; P = 0.0005 for CQ assay).

Treatment with AQ and PIs caused a slight reduction in hemoglobin levels relative to control parasites that were treated with PIs alone (Figure 3.4 A). The change was however not statistically significant at the 95 % CI (P = 0.3696).

Sample	Anti-malarial Concentration (nM)	Mean Band Intensity Relative to Control (%) ± SD	*N*
Control	-	100.0 ± 17.1	3
Control+ PI	-	325.7 ± 20.5	3
AQ	102	68.8 ± 29.0	3
AQ + PI	102	282.9 ± 70.6	3
H	27	16.2 ± 5.2	3
H + PI	27	229.3 ± 31.3	3
MQ	156	16.9 ± 2.1	3
MQ + PI	156	111.8 ± 44.3	3
Q	665	35.6 ± 10.2	3
Q + PI	665	225.3 ± 19.1	3
Control	-	100.0 ± 43.4	3
Control+ PI	-	388.5 ± 21.5	3
CQ	137	283.1 ± 31.5	3
CQ + PI	137	393.0 ± 10.4	3

Table 3.3. Western Blot hemoglobin intensities of parasite lysate bands following treated of parasites with drug alone or with drug and PIs for 8 hours. Parasite cultures were untreated (Control), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q). Parasites were protease inhibitors, ALLN and E64 (PIs) at 40 μM concentrations. Intensities of hemoglobin bands were determined using the histogram function of Adobe Photoshop (version 7.0) software. The intensities were normalized to control intensities. *N* = sample size.

Treatment with H, MQ or Q and PIs resulted in a decrease in hemoglobin levels relative to control parasites treated with PIs alone (P = 0.011, P = 0.0016 and P = 0.0034 for H, MQ and Q respectively) (Figure 3.4 C, D and E). Treatment with CQ and PIs did not result in a statistically significant change in hemoglobin levels relative to control parasites that were treated with PIs alone (P = 0.7651) (Figure 3.4 B).

In summary, MQ, Q and H cause a significant decrease in hemoglobin levels, both with and without PIs. AQ caused a slight decrease in hemoglobin levels in both instances, but this only reached statistical significance at the 95 % CI in the 6 hour experiment. By contrast, in the presence of PIs, CQ-treatment did not cause a change in hemoglobin levels, compared to significant increases obtained without PIs.

3.3 Discussion

3.3.1 Chloroquine

Treatment with 137 nM CQ for 6 or 8 hours was shown to result in the accumulation of hemoglobin in *P. falciparum* 3D7 parasites, as previously found in other strains (Famin and Ginsburg, 2002; Fitch et al, 2003a; Hoppe et al, 2004). This suggests that either uptake of hemoglobin is stimulated by CQ-treatment or that hemoglobin digestion is inhibited. Treatment with PIs was found to extensively block hemoglobin digestion in control parasites, causing it to accumulate. Levels of hemoglobin in parasites treated with CQ and PIs and parasites treated with PIs alone were found to be similar, indicating that CQ-treatment is not likely to result in the stimulation of hemoglobin uptake. If this were the case, one would expect that the addition of CQ to parasites treated with PIs would further raise hemoglobin levels, provided that a threshold concentration of intracellular hemoglobin had not already been reached.

CQ has been found to accumulate in the parasite food vacuole (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996) and cause the pH to rise in this organelle (Krogstad et al, 1985). As a result, proteolytic enzymes that are involved in hemoglobin digestion may be disrupted. Additionally, as CQ has been found to slow the rate of FP

dimerization (Egan and Ncokazi, 2005) and cause it to accumulate (Chou and Fitch, 1993; Zhang et al, 1999; Famin and Ginsburg, 2002), this toxic molecule may impede enzymatic function and thus inhibit hemoglobin digestion. Alternatively, treatment with CQ may disrupt membrane function, inhibiting vesicular docking and fusion and in this way inhibiting hemoglobin digestion. This may be caused by CQ-mediated alkalinization of the food vacuole, CQ interaction with membrane through an FP bridge [as previously suggested by Hoppe et al (2004) and Fitch (2004)], or the presence of high levels of toxic FP (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983). Famin and Ginsburg (2002) suggested that treatment with CQ caused hemoglobin accumulation by inhibiting the digestion of this protein. Furthermore, it has previously been found that CQ-treatment inhibited the processing of hemoglobin transport vesicles in malarial parasites (Macomber et al, 1967; Warhurst and Hockley, 1967, Yayon et al, 1984; Fitch et al, 2003a; Hoppe et al, 2004).

Hemoglobin levels in CQ-treated parasites were lower than in parasites treated with PIs. One can deduce that treatment with CQ either does not cause a complete inhibition of hemoglobin digestion or that CQ also moderately inhibits endocytosis. In support of the latter, CQ-treatment has previously been found to result in the disruption of membrane function (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983), and an inhibition of endocytosis following long incubation periods (Hoppe et al, 2004). However, hemoglobin levels in parasites treated with CQ and PIs were similar to those in parasites treated with PIs alone, suggesting that CQ does not inhibit endocytosis following an 8 hour incubation period. For the same reason, it is unlikely that lower levels of hemoglobin in CQ-treated parasites relative to PI-treated parasites are due to decreased parasite viability following drug-treatment. Therefore, it is possible that treatment with CQ results in lower levels of parasite hemoglobin accumulation than treatment with PIs alone as hemoglobin digestion is not entirely inhibited.

3.3.2 Mefloquine, Halofantrine and Quinine

MQ was previously found to reduce hemoglobin levels in *P. falciparum* FCR3 (Famin and Ginsburg, 2002) and inhibit endocytosis in strain D10 (Hoppe et al, 2004), while Q has been found to antagonistically affect hemoglobin accumulation in malarial parasites (Famin et al, 1999; Famin and Ginsburg, 2002). It has been suggested that H actssimilarly to MQ and Q. The results of this study show that treatment with 156 nM MQ, 665 nM Q or 27 nM H for 6 or 8 hours resulted in a reduction in hemoglobin levels in strain 3D7, suggesting that either hemoglobin digestion is stimulated or that endocytosis is inhibited. Parasites treated with these drugs in combination with PIs for 8 hours showed reduced levels of hemoglobin relative to parasites treated with PIs alone. This indicates that the reduction in hemoglobin levels observed in MQ, H and Q-treated parasites is not due to the stimulation of hemoglobin digestion. In support, Famin and Ginsburg (2002) found that MQ does not affect hemoglobin digestion. Treatment with these drugs may thus result in inhibition of hemoglobin endocytosis in 3D7 *P. falciparum* parasites.

3.3.3 Amodiaquine

Famin and Ginsburg (2002) suggested that AQ, like CQ, inhibits hemoglobin digestion. However, the results of this assay showed that treatment with 102 nM AQ resulted in a slight reduction in the levels of this protein in *P. falciparum* 3D7 parasites after a 6 hour incubation period. Following an 8 hour incubation period, hemoglobin levels were also reduced, although statistical significance was not reached. Similarly, hemoglobin levels were slightly reduced in parasites treated with AQ and PIs for 8 hours in comparison to levels in parasites treated with PIs alone, although the results again were fairly variable and thus not statistically significant. It is commonplace to see variations in cellular responses to drug treatments, making it difficult to interpret results showing small changes. One could conclude that AQ does not significantly affect hemoglobin accumulation and that the small reduction in hemoglobin levels observed is a result of decreased parasite viability following drug-treatment. Alternatively one may hypothesize

that treatment with AQ results in the non-specific disruption of membrane function, concurrently inhibiting endocytosis and disrupting vesicular processing and hence hemoglobin digestion to variable degrees. Treatment with AQ may result in the inhibition of hemoglobin digestion, as previously suggested by Famin and Ginsburg (2002), either by inhibiting enzymatic function or disrupting vesicular docking (as proposed for CQ). One would expect to see an accumulation of hemoglobin if this were the case, however, AQ may simultaneously inhibit endocytosis (as proposed for MQ, H and Q), resulting in little change in the overall level of this protein inside parasites. This would suggest that AQ action is a combination of that of CQ and MQ, as concluded following the examination of thin blood smears in the previous chapter (Figure 2.2).

Chapter 4

The Effect of Quinoline Anti-Malarials on Horseradish Peroxidase Internalization by *P. falciparum* 3D7

4.1 Introduction

Hemoglobin levels in parasites are not only influenced by endocytosis, but also by the digestion of this protein, hence the inclusion of PIs in the hemoglobin Western Blotting experiments described in the previous chapter. Western Blotting assays (Chapter 3) demonstrated that, as well as inhibiting hemoglobin digestion by causing the disruption of enzymatic function or of vesicular processing, CQ may moderately inhibit endocytosis in strain 3D7 parasites. AQ may also inhibit hemoglobin digestion and endocytosis concurrently. MQ, however, has previously been found to have no influence on hemoglobin digestion (Famin and Ginsburg, 2002), and the results of Western Blotting assays with PIs (Chapter 3) suggest that the effects of MQ, H and Q on 3D7 parasites are independent of hemoglobin digestion. These drugs thus appear to cause a reduction in parasite hemoglobin levels in strain 3D7 parasites by inhibiting endocytosis. Therefore, in order further clarify the effects of the drugs on endocytosis in the absence of hemoglobin digestion, a novel assay was developed involving the use of an exogenous endocytic tracer, Horseradish Peroxidase (HRP) (Clague et al, 1995; Rode et al, 1997; D'Souza et al, 2006; Finnegan and Blumenthal, 2006) and an alternative method of quantification (HRP enzyme activity).

4.2 Results

For these assays, RBCs were preloaded with HRP and incubated with enriched trophozoite-containing RBCs. Over the next 24 hours, parasites were allowed to mature, invade the HRP-filled RBCs and grow to the late ring/early trophozoite stage. 102 nM AQ, 137 nM CQ, 27 nM H, 156 nM MQ or 665 nM Q were added and the parasites were incubated for a further 10 hours. The incubation period for this assay was increased

relative to times used during Western Blot assays as a longer period of time is required for parasites to endocytose sufficient HRP to be detectable. Following incubation, parasites were released from RBCs by saponin treatment and washed in order to remove extraparasitic HRP. Parasites were lysed and o-phenylenediamine (OPD, colorimetric HRP substrate) was added. The absorbance was then measured at 450 nm using a spectrophotometer.

All of the drugs caused a reduction in HRP internalization (Figure 4.1, Table 4.1). The changes were statistically significant at the 95 % CI (AQ: P<0.0001, CQ: P = 0.0055, H: P = 0.0002, MQ: P< 0.0001, Q: P<0.0001).

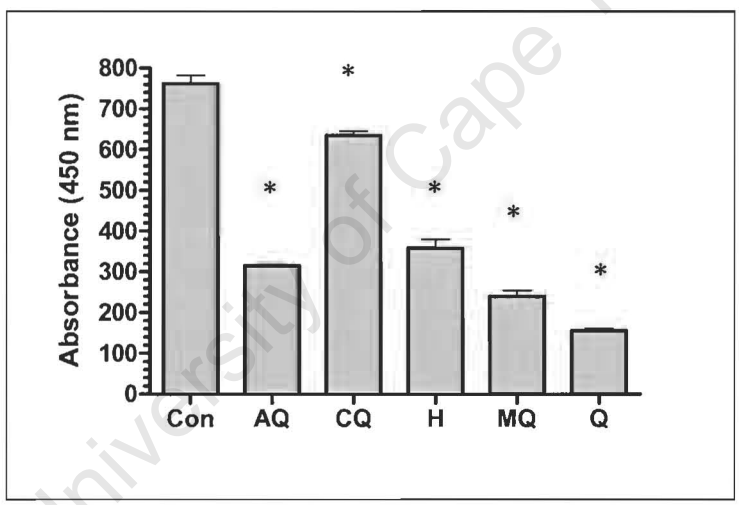


Fig 4.1. HRP levels in *P. falciparum* 3D7, measured as Absorbance at 450 nm after OPD (HRP substrate) addition. Parasites were allowed to invade RBCs preloaded with HRP. They were then left untreated (control), or treated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q) for 10 hours. Following incubation, parasites were released from RBCs, washed repeatedly and lysed. Error bars indicate standard error. * Statistically significant change from the control (95% CI).

Sample	Concentration (nM)	Absorbance (450 nm) ± SD	*N*
Control	-	762.0 ± 36.0	3
AQ	102	315.3 ± 6.8	3
CQ	137	635.3 ± 17.8	3
H	27	359.0 ± 37.3	3
MQ	156	240.3 ± 24.8	3
Q	665	159.0 ± 7.9	3

Table 4.1. HRP levels in *P. falciparum* 3D7, measured as Absorbance at 450 nm ± standard deviation (SD) after OPD (HRP substrate) addition. Parasites were untreated (control), or treated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ), or 665 nM quinine (Q) for 10 hours. *N* = sample size.

4.3 Discussion

4.3.1 Chloroquine

HRP appears to be indigestible (Montgomery et al, 1991). The levels of this tracer molecule may thus be an almost true indication of endocytosis in malarial parasites. Treatment with 137 nM CQ for 10 hours was found to cause a reduction in HRP levels in *P. falciparum* 3D7. This could be a result of decreased parasite viability following drug-treatment for a long period of time. In addition, the results may indicate that treatment with CQ does not cause hemoglobin to accumulate by the stimulation of endocytosis, the drug may have the opposite effect and inhibit this process to a small extent following longer incubation periods.

Preloading of RBCs with HRP resulted in the dilution of RBC content, and thus of hemoglobin. As a result, lower concentrations of both hemoglobin and FP would be present in the parasites. CQ would thus accumulate in parasites to a lesser degree in the

absence of high levels of FP, as build-up of this drug is partially dependent on its high-affinity binding to FP and FP dimers (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996). It is possible that higher levels of FP may amplify the effect of CQ. Alternatively, as 90 % of iron is found in the parasite food vacuole (Egan et al, 2002), FP primarily enhances CQ accumulation in this organelle. The level of FP in the food vacuole may thus not influence CQ action at the parasite's plasma membrane and hence endocytosis. CQ may therefore disrupt endocytosis in a manner that is independent of FP, or the drug may indirectly impede this process as a result of a CQ-mediated effect elsewhere in the cell.

4.3.2 Mefloquine, Quinine and Halofantrine

Treatment with MQ, H or Q at concentrations approximately 5 times their IC_{50} values (156 nM, 27 nM and 665 nM respectively) for 10 hours antagonistically affected the accumulation of HRP in *P. falciparum* 3D7 parasites. As HRP levels in malarial parasites have been proposed to be almost entirely dependent on endocytosis, the results of this assay provide further support for the possibility that treatment with MQ, H or Q results in the inhibition of endocytosis. Additionally, the reduction in HRP levels observed in parasites treated with these drugs could be partially due to decreased parasite viability following drug-treatment.

4.3.3 Amodiaquine

HRP levels in parasites treated with 102 nM AQ for 10 hours were largely reduced in comparison to control parasites. The reduction in HRP levels observed in AQ-treated parasites could be partially due to decreased parasite viability following drug-treatment. Additionally, as HRP levels in malarial parasites have been proposed to be a nearly true reflection of endocytosis, the results of this assay indicate that treatment with AQ may, as has been suggested in the previous chapter, cause the inhibition of endocytosis. Furthermore, these results and the finding that AQ only caused a slight decrease in

hemoglobin accumulation in Western Blotting assays (Chapter 3) suggest that the drug concurrently inhibits hemoglobin degradation, resulting in hemoglobin levels in parasites that were generally similar to those of control parasites. AQ, like CQ, may inhibit hemoglobin digestion by disrupting proteolytic enzymatic function or by blocking vesicular docking at the food vacuole.

Levels of intracellular FP are likely to be low in parasites that had invaded RBCs preloaded with HRP. As AQ may, like CQ (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996), be partially dependent on FP for accumulation, it is possibly that lower levels of AQ may build-up in parasites during this assay. Therefore, as in the case of CQ-treated cells, the decrease in HRP accumulation observed following AQ-treatment may indicate that the drug disrupts endocytosis in a manner that is independent of FP. Alternatively, AQ may indirectly cause the disruption of endocytosis by an AQ-mediated effect elsewhere in the cell.

Chapter 5

The Effect of Quinoline Anti-Malarials on Transport Vesicle Accumulation and Hemoglobin Levels in the Food Vacuoles of *P. falciparum* 3D7

5.1 Introduction

The results of Western Blotting assays (Chapter 3) and HRP uptake assays (Chapter 4) conducted using 3D7 *P. falciparum* parasites suggest that CQ-treatment may inhibit hemoglobin digestion by causing the disruption of enzymatic function or by inhibiting vesicular processing. In support of the latter hypothesis, CQ has previously been shown to inhibit the processing of hemoglobin transport vesicles in malarial parasites (Macomber et al, 1967; Warhurst and Hockley, 1967; Yayon et al, 1984; Fitch et al, 2003a; Hoppe et al, 2004). CQ may additionally moderately inhibit endocytosis. Like CQ, treatment with AQ appears to concurrently inhibit endocytosis and hemoglobin digestion, either by disrupting enzymatic function or by inhibiting vesicular processing. MQ, H and Q-treatment may cause a reduction in hemoglobin and HRP levels by inhibiting endocytosis. Immunofluorescence assays were conducted in order to provide an alternative means to evaluate the effects of the drugs on endocytosis and vesicular processing in malarial parasites. Furthermore, the precise intracellular location of hemoglobin was determined by counting the numbers of hemoglobin transport vesicles and measuring the hemoglobin levels in the food vacuoles of 3D7 *P. falciparum*.

5.2 Results

Parasites were incubated with 102 nM AQ, 137 nM CQ, 27 nM H, 156 nM MQ or 665 nM Q. Following this, parasites were released from the RBCs by saponin-treatment and washed. Samples were fixed onto slides and incubated with anti-hemoglobin antiserum, followed by a fluorescently-labeled secondary antibody. The location of parasite nuclei were determined by staining with DAPI (images not shown). Using a fluorescence microscope, hemoglobin transport vesicles were counted and the levels of hemoglobin in the food vacuoles of the drug-treated parasites were determined by measuring the mean

intensity of fluorescence in these organelles using the histogram function in Adobe Photoshop (version 7.0).

5.2.1 Hemoglobin Transport Vesicles

It was found that CQ treatment caused an increase in hemoglobin transport vesicles in the parasite cytoplasm in comparison to the controls (P<0.0001) (Table 5.1, Figures 5.1 and 5.3).

AQ-treatment also caused an increase in hemoglobin transport vesicles (P = 0.0002), although to a lesser extent than CQ. Treatment with H, MQ or Q resulted in a decrease in transport vesicles in comparison to control parasites (P = 0.0012, P<0.0001 and P = 0.013 respectively). (Table 5.1, Figures 5.1 and 5.3)

Sample	Concentration (nM)	Hemoglobin Transport Vesicles ± SD	*N*
Control	-	1.2 ± 1.2	100
AQ	102	2.2 ± 2.3	100
CQ	137	6.1 ± 5.4	100
H	27	0.7 ± 0.9	100
MQ	156	0.3 ± 0.6	100
Q	665	0.7 ± 0.9	100

Table 5.1. Mean number of hemoglobin transport vesicles in parasites ± standard deviation (SD). Parasite cultures were left untreated (Control), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q). *N* = sample size. Parasites were fixed onto slides and incubated with anti-hemoglobin antiserum, followed by fluorescently-labeled secondary antibody. Transport vesicles were counted under a fluorescent microscope.

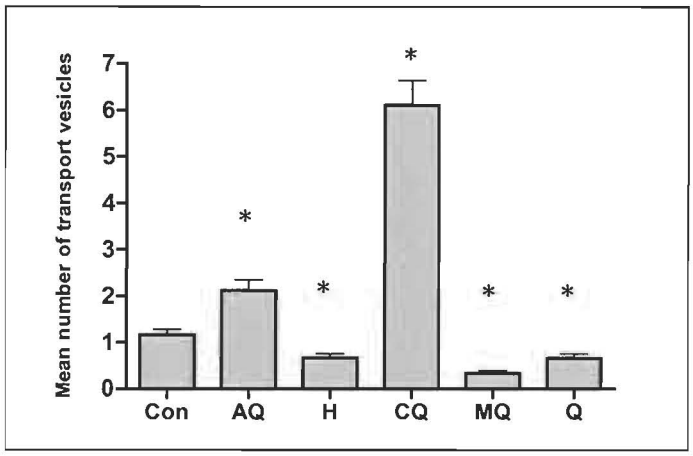


Fig 5.1. Hemoglobin transport vesicles in parasites. Parasite cultures were left untreated (control, Con), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q). Parasites were fixed onto slides and incubated with anti-hemoglobin antiserum, followed by fluorescently-labeled secondary antibody. Transport vesicles were counted under a fluorescent microscope. Error bars indicate standard error. * Statistically significant change from control (95% CI).

5.2.2 Hemoglobin Levels in Parasite Food Vacuoles

Sample	Concentration (nM)	Food Vacuole Fluorescent Intensity ± SD	*N*
Control	-	214.7 ± 37.3	100
AQ	102	217.1 ± 61.0	100
CQ	137	223.3 ± 41.1	100
H	27	172.4 ± 72.1	100
MQ	156	159.4 ± 66.0	100
Q	665	178.1 ± 68.2	100

Table 5.2. Mean fluorescent intensity (arbitrary units) in parasite food vacuoles ± standard deviation (SD). Parasites were untreated (control), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q) for 5-6 hours. Parasites were fixed onto slides and incubated with anti-hemoglobin antiserum, followed by fluorescently-labeled secondary antibody. Fluorescence intensity in parasite food vacuoles was determined using the histogram function of Adobe Photoshop (version 7.0). *N* = sample size.

It was found that treatment with AQ or CQ caused a slight increase in hemoglobin levels in parasite food vacuoles in comparison to control parasites (Table 5.2, Figures 5.2 and 5.3). The differences in hemoglobin levels in the food vacuoles of the AQ or CQ-treated parasites and the control parasites were, however, not statistically significant (P = 0.1214 and P = 0.7433 respectively) at the 95% CI.

Treatment with H, MQ and Q caused a decrease in the hemoglobin levels in parasite food vacuoles in comparison to the control parasites (Table 5.2, Figures 5.2 and 5.3) (P<0.0001).

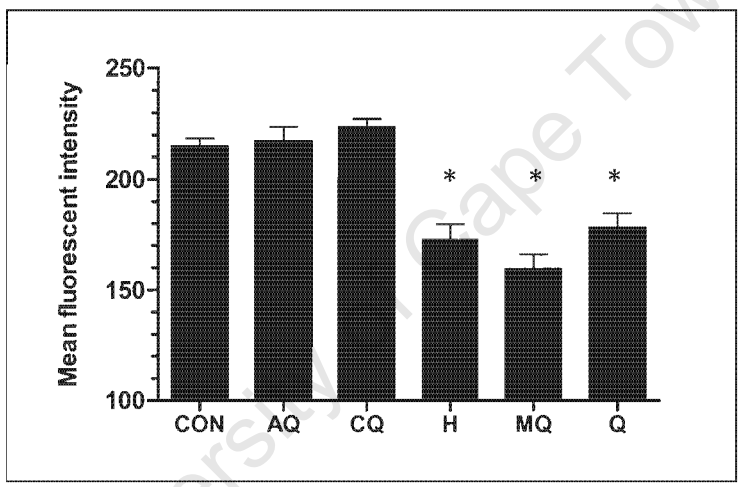


Fig 5.2. Fluorescence intensity (arbitrary units) in parasite food vacuoles. Parasite cultures were left untreated (control, CON), incubated with 102 nM amodiaquine (AQ), 137 nM chloroquine (CQ), 27 nM halofantrine (H), 156 nM mefloquine (MQ) or 665 nM quinine (Q) for 5-6 hours. Parasites were fixed onto slides and incubated with anti-hemoglobin antiserum, followed by a fluorescently-labeled secondary antibody. Parasites were then examined under a fluorescent microscope. Images were captured and the fluorescent intensities of parasite food vacuoles were determined using the histogram function of Adobe Photoshop. Error bars indicate standard error. * Statistically significant change from control (95% CI).

Representative Immunofluorescence images are shown in Figure 5.3. Parasites are visible as roughly circular structures in phase contrast images. RBCs were lysed prior to

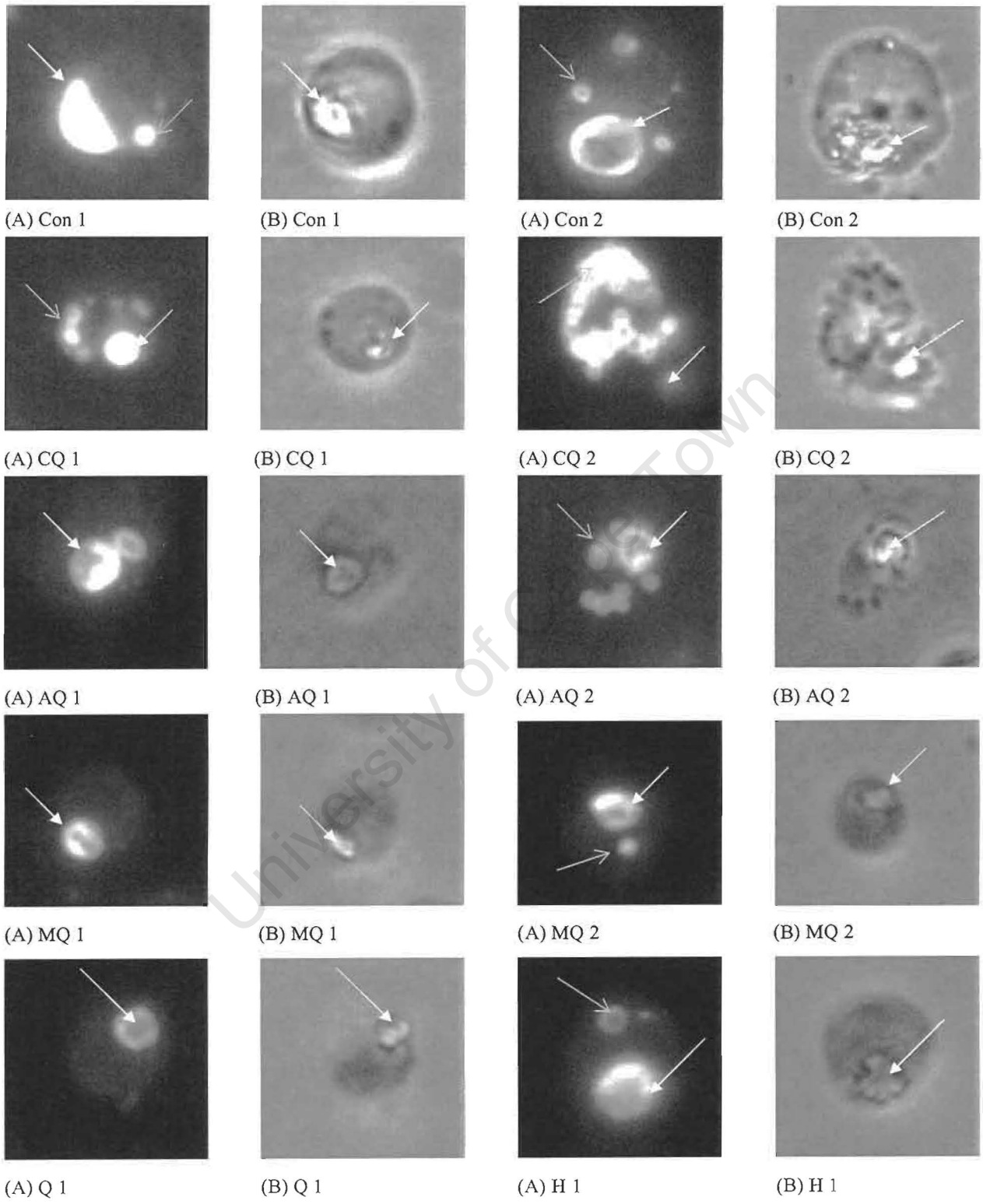


Fig. 5.3. Subcellular localization of hemoglobin by immunofluorescence. Parasites from control (Con), CQ-treated (CQ), AQ-treated (AQ), MQ-treated (MQ), Q-treated (Q), and H-treated (H) cultures. Left-handed panels (A), fluorescent images; right-handed panels (B), phase-contrast images. The hemazoin crystal marks the position of the food vacuole (white arrows). Transport vesicles (thin grey arrows).

following the treatment of strain NYU-2 of *Plasmodium berghei* with CQ. The same effect was observed by Hoppe et al (2004) following treatment of strain D10 of *P. falciparum* with CQ for 12 hours. In the latter study, accumulation of transport vesicles was, however, not observed following treatment with CQ for 6 hours. In this study of strain 3D7 of *P. falciparum,* it was found that transport vesicles accumulated after shorter periods of incubation with 137 nM CQ (5-6 hours) than observed by Hoppe et al (2004). The varying results of these studies suggest that the cell biological responses of malarial parasites to CQ-treatment may be species and strain dependent. Thus, in some strains (including 3D7, NYU-2 and D10), CQ may act by inhibiting vesicular docking and the delivery of hemoglobin from transport vesicles to the food vacuole, causing or contributing to the accumulation of hemoglobin seen in the Western blotting assay (Chapter 3).

Following CQ-treatment, hemoglobin levels in 3D7 *P. falciparum* food vacuoles did not differ from levels in untreated control parasites. This indicates that the increase in overall levels of hemoglobin observed in Western blotting assays (Chapter 3) is due to the accumulation of hemoglobin transport vesicles in the parasite cytoplasm. Additionally, these results further support the probability that the accumulation of transport vesicles is due to the inhibition of vesicular docking at the food vacuole, rather than a stimulation of endocytosis. If CQ-treatment had caused the stimulation of endocytosis, but not the inhibition of vacuole fusion, one would have expected to find an increase in transport vesicles as well as raised levels of hemoglobin in the food vacuole.

CQ accumulates in the parasite food vacuole by an ion-trapping mechanism or by its high affinity binding to FP (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996). The drug may then inhibit vesicular docking/fusion at the food vacuole by causing the pH to rise in this organelle (Krogstad et al, 1985). Alternatively, CQ may interact with membranes through an FP bridge and disrupt their function, as suggested by Hoppe et al (2004) and Fitch (2004). CQ has been found to slow the rate of FP dimerization (Egan and Ncokazi, 2005), causing it to accumulate (Chou and Fitch, 1993; Zhang et al, 1999; Famin and Ginsburg, 2002). Build-up of this toxic molecule may also

result in, or contribute to, the disruption of membrane function (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983). Additionally, high levels of FP may inhibit the proteolytic enzymes (Tappel, 1955; Sugioka and Suzuki, 1991; Foley and Tilley, 1998; Ginsburg et al, 1998) that are involved in hemoglobin digestion. CQ-mediated alkalinization of the food vacuole may alternatively, or concurrently, disrupt proteolytic enzymes and contribute to the inhibition of hemoglobin digestion. As a result, hemoglobin would remain undigested in the food vacuole, and vesicular docking/fusion and further delivery of hemoglobin would consequently be down-regulated.

5.3.2 Mefloquine, Quinine and Halofantrine

Treatment with 27 nM H, 156 nM MQ or 665 nM Q for 5 to 6 hours caused a reduction in both hemoglobin transport vesicles and hemoglobin levels in parasite food vacuoles relative to the controls. The results of this assay thus provide additional support for the hypothesis that these drugs inhibit endocytosis in strain 3D7 *P. falciparum*.

5.3.3 Amodiaquine

As found following CQ-treatment, it was observed that treatment with 102 nM AQ for 5 to 6 hours caused hemoglobin transport vesicles to accumulate in the parasite cytoplasm in comparison to the controls. It was also found that AQ-treatment did not cause a statistically significant change in hemoglobin levels in parasite food vacuoles in comparison to the controls. Thus, treatment with AQ may result in a build-up of hemoglobin transport vesicles by inhibiting vesicular docking/fusion at the food vacuole. AQ, like CQ, has been found to accumulate in this organelle (Hawley et al, 1996). AQ may also cause the pH to rise in the food vacuole, or may interact with FP to disrupt membrane function, consequently inhibiting vesicular docking and fusion. It has additionally been suggested that AQ inhibits hemoglobin digestion (Famin and Ginsburg, 2002), perhaps by causing the disruption of proteolytic enzymatic function. Hemoglobin would thus remain in the food vacuole, preventing further delivery of this protein via

vesicular docking/fusion and causing transport vesicles to accumulate. Fewer transport vesicles were found to accumulate in parasites treated with AQ relative to parasites treated with CQ. It is therefore possible that, in addition to disrupting vesicular docking and inhibiting hemoglobin digestion, AQ-treatment results in the more extensive inhibition of hemoglobin endocytosis than may be the case following CQ-treatment, as observed in HRP internalization assays (Chapter 4).

Chapter 6

The Effect of Quinoline Anti-Malarials on FITC-Dextran Uptake, Accumulation and Efflux from *Dictyostelium discoideum* Cells

6.1 Introduction

MQ has been found to bind to membranes with high affinity and disrupt their function (Chevli and Fitch, 1982; San George et al, 1984). Additionally, it has been demonstrated that MQ disrupts the phagocytic pathway in mammalian cells (Labro and Babin-Chevaye, 1988), whilst CQ has been shown to influence the secretory pathway (Moore et al, 1983; Smith and Jarret, 1982). Findings of this study (Chapters 3-5) demonstrate that AQ, CQ, H, MQ and Q influence the endolysosomal pathway in 3D7 *P. falciparum*. It is thus possible that the effects of these drugs are not parasite-specific, but may translate to other organisms as well.

Axenic strains of *D. discoideum* pinocytose fluid with high efficiency (Clarke and Kayman, 1987; Aubry et al, 1996; Rupper and Cardelli, 2001) and are therefore widely used as model organisms for the study of this process (Watts and Ashworth, 1970; Rupper and Cardelli, 2001). Additionally, as the pinocytic pathway is well understood in *Dictyostelium* relative to *Plasmodium*, the precise effect of these drugs on the endolysosomal system may be studied with greater clarity using *Dictyostelium* as a model organism, thus shedding further light on the modes of action in *P. falciparum*. Furthermore, *D. discoideum* cells are able to reach a point of equilibrium between rapid endocytosis and exocytosis, making *Dictyostelium* a useful organism for the study of cellular efflux. FITC-Dextran accumulation and efflux assays were therefore conducted using *D. discoideum* in order to determine the effect of the drugs on these processes.

6.2 Results

6.2.1 FITC-Dextran Uptake and Accumulation

Amoeboid *D. discoideum* cells were incubated with FITC-Dextran, a commonly used endocytosis tracer, and increasing concentrations of each quinoline anti-malarial drug for 90 minutes, in order to compare the activity of the drugs in *D. discoideum* versus *P. falciparum*. Cells were treated with drug for a shorter period of time relative to treatment of *P. falciparum* as *Dictyostelium* cells have been found to have a rapid rate of endocytosis (Thilo, 1985). Furthermore, it was previously demonstrated that a 90 minute incubation period was sufficient for a large degree of tracer molecule endocytosis to occur and that, at this time, equilibrium between between endocytosis and exocytosis had not yet been reached (Lim et al, 2005). Following incubation, cells were washed repeatedly and lysed to determine fluorescence levels using a fluorescence spectrophotometer, or fixed and mounted for viewing under a fluorescence microscope.

Figure 6.2 depicts representative images of amoeboid *D. discoideum* cells treated with 20 μM of each drug. Cells are irregular in shape, as apparent in phase-contrast images. Pinocytic vesicles are visible as small punctate cytoplasmic structures containing FITC-Dextran (white) in fluorescent images. Pinosomes are abundant in untreated cells, and in cells treated with AQ or CQ, however they are reduced in cells treated with MQ or H and slightly reduced in cells treated with Q. Treatment with the quinoline anti-malarials did not appear to influence the size of *Dictostelium* cells, indicating that drug-treatment was conducted at sub-toxic concentrations and incubation periods.

It was found that in order to influence tracer accumulation in *D. discoideum*, drug-treatment was required at significantly higher concentrations than in *P. falciparum*. Cells treated with 5 to 20 μM CQ for 90 minutes showed a small increase in fluorescence levels relative to the controls that was significant at the 95% CI (Figures 6.1 B and 6.2).

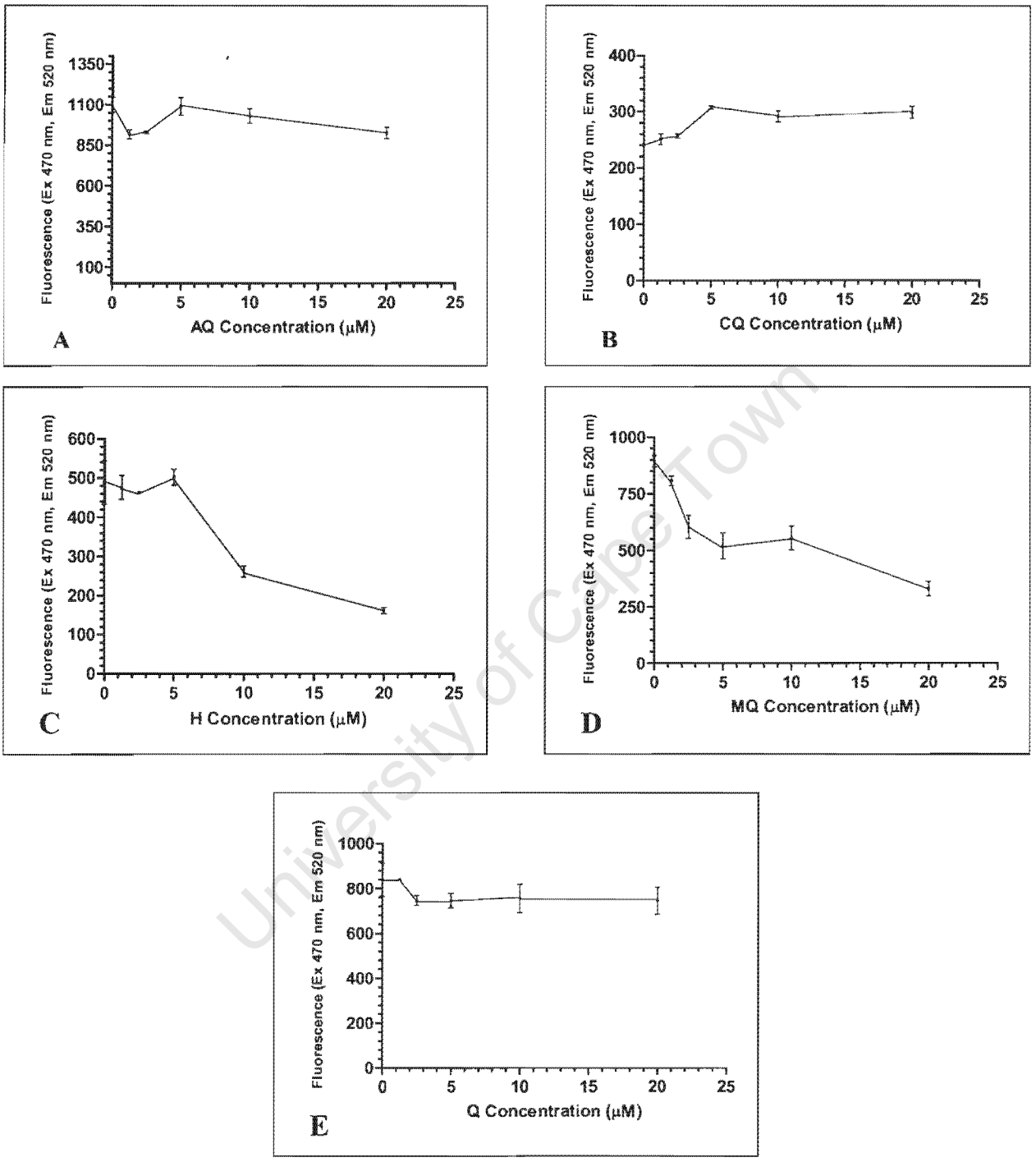


Fig 6.1. FITC-Dextran accumulation in *D. discoideum* amoeboid cells measured as fluorescence [excitation (ex) 470 nm, emission (em) 520 nm]. Cells were treated with 1.25-20 μM amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) (A-E respectively) for 90 minutes. Cells were then repeatedly washed, lysed and fluorescence levels were determined.

MQ and H-treated cells showed a large decrease in intracellular FITC-Dextran levels that was more pronounced with increasing concentrations of drug. The change was statistically significant at the 95% CI following treatment with 2.5 to 20 μM MQ or 10 to 20 μM H. (Figures 6.1C, D and 6.2) Although there was a small reduction in fluorescence levels in *D. discoideum*, following treatment with 2.5 to 20 μM Q for 90 minutes, the change however did not reach statistical significance at the 95 % CI (Figure 6.1 E).

Sample	Concentration (μM)	FITC-Dextran Uptake (% of control)	*N*
Control	-	100.0 ± 8.2	9
AQ 1	20	159.9 ± 12.2	3
AQ 2	20	82.9 ± 25.8	3
AQ 3	20	84.1 ± 4.4	3

Table 6.1. Levels of FITC-Dextran accumulation in amodiaquine (AQ)-treated *D. discoideum* cells ± standard deviation (SD). Cells were untreated (Control) or treated with 20 μM AQ on day 1 (AQ_1), day 2 (AQ_2) and day 3 (AQ_3) for 90 minutes. Cells were washed, lysed and fluorescence levels were determined using a fluorescence spectrophotometer (excitation 470 nm, emission 520 nm). Fluorescence readings were normalized to control readings. *N* = sample size.

AQ-treatment was found to cause variable changes in FITC-Dextran accumulation on different days (Table 6.1, Figure 6.3). On day 1, AQ-treated cells showed an increase in FITC-Dextran accumulation ($P<0.0001$). On day 2, AQ-treated cells did not show a statistically significant change in FITC-Dextran levels relative to the controls ($P = 0.1083$, 95% CI). On day 3, cells treated with AQ showed a decrease in tracer molecule accumulation ($P = 0.0328$).

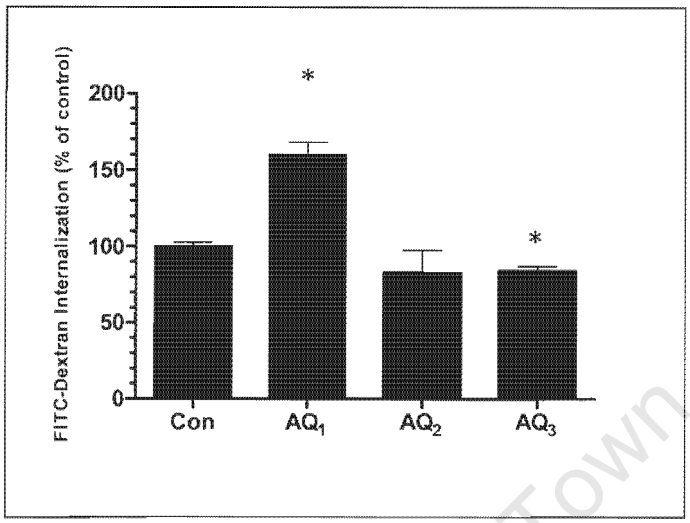


Fig 6.3. Levels of FITC-Dextran accumulation in *D. discoideum* cells treated with amodiaquine (AQ) for 90 minutes. Cells were untreated (Control, Con) or treated with 20 μM AQ on day 1 (AQ_3). Cells were washed, lysed and fluorescence levels were determined using a fluorescence spectrophotometer (excitation 470 nm, emission 520 nm). Readings were normalized to control readings. Error bars indicate standard error. * Statistically significant change from the control (95% CI).

6.2.2 FITC-Dextran Exocytosis Assay

As efflux has a large impact on intracellular FITC-Dextran levels in this organism, it is important to determine the contribution of this process to altered intracellular tracer molecule levels observed in drug-treated cells. Amoeboid *D. discoideum* cells were therefore pre-loaded with FITC-Dextran. Cells were washed and incubated with drug for 60 and 90 minute periods. The fluorescence in the supernatant of each sample was then determined using a fluorescence spectrophotometer.

Sample	Concentration (μM)	FITC-Dextran Efflux ± SD [Fluorescence (ex 470 nm, em 520 nm)]	*N*
60 min			
Control	-	745.0 ± 44.0	3
AQ	20	794.7 ± 4.0	3
CQ	20	794.7 ± 16.9	3
H	20	836.0 ± 2.7	3
MQ	20	839.3 ± 14.8	3
Q	20	833.7 ± 8.4	3
90 min			
Control	-	507.0 ± 22.6	3
AQ	20	497.7 ± 22.2	3
CQ	20	515.0 ± 69.4	3
H	20	618.0 ± 49.3	3
MQ	20	636.3 ± 68.2	3
Q	20	679.0 ± 27.8	3

Table 6.2. Efflux from *D. discoideum* cells preloaded with FITC-Dextran ± standard deviation (SD). Preloaded amoeboid cells were untreated (Control), or incubated with 20 μM amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) for 60 minutes or 90 minutes. Supernatant was retrieved and efflux was measured as fluorescence [excitation (ex) 470 nm, emission (em) 520 nm]. *N* = sample size.

Following a 60 minute incubation period, *Dictyostelium* cells treated with 20 μM AQ or CQ did not show a statistically significant change in FITC-Dextran efflux in comparison to control cells (P = 0.1232 and P = 0.1417 respectively, 95 % CI) (Table 6.2, Figure 6.4 A). Cells treated with 20 μM MQ, H or Q for 60 minutes showed an increase in tracer molecule efflux in comparison to the controls (Table 6.2, Figure 6.4 A) (P = 0.0244, P = 0.0232 and P = 0.0265 for MQ, H and Q respectively).

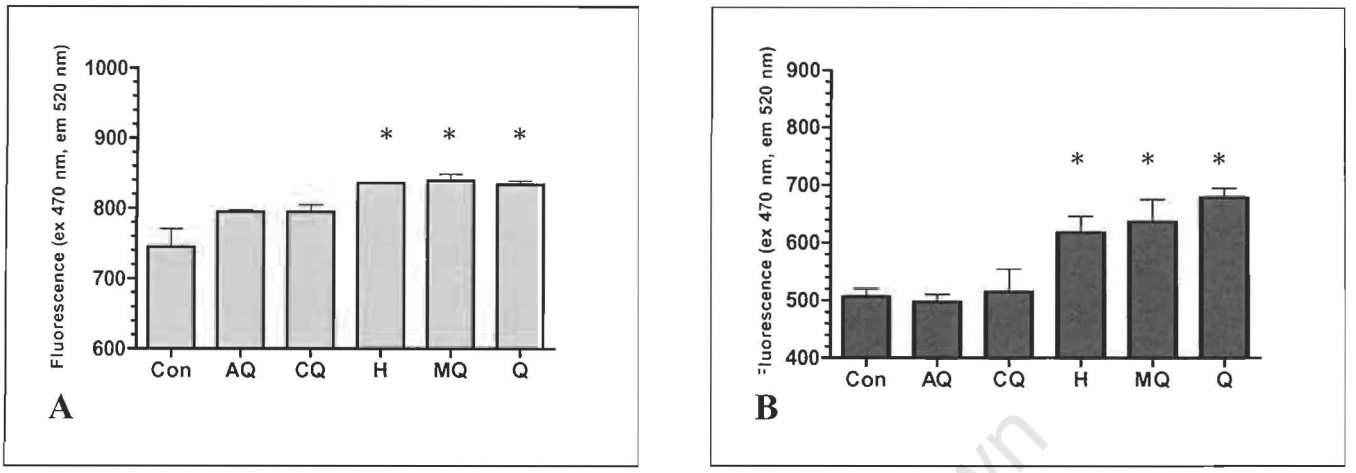


Fig 6.4. Efflux from *D. discoideum* cells preloaded with FITC-Dextran. Preloaded amoeboid cells were untreated (control, Con), or incubated with 20 μM amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) for 60 minutes (A) or 90 minutes (B). Supernatant was retrieved and efflux was measured as fluorescence [exitation (ex) 470 nm, emission (em) 520 nm]. Error bars indicate standard error. *Statistically significant change from control (95% CI).

Following a 90 minute incubation period, *Dictyostelium* cells treated with 20 μM AQ or CQ again did not show a statistically significant change in tracer molecule efflux relative to the controls (P = 0.6367 and P = 0.8587 respectively) (Table 6.2, Figure 6.4 B). Cells treated with MQ, H or Q for 90 minutes demonstrated further efflux of FITC-Dextran (Table 6.2, Figure 6.4 B) (P = 0.0357, P = 0.0239 and P = 0.0011 for MQ, H and Q respectively).

6.3 Discussion

6.3.1 Chloroquine

D. discoideum has an unusual endolysosomal pathway in that a state of equilibrium is reached between rapid endocytosis and exocytosis (Klein and Satre, 1986). Therefore,

indigestible tracer molecules such as FITC-Dextran would not accumulate in untreated cells (unlike HRP in malarial parasites) but would travel through the endolysosomal pathway and be shunted out of the cell. Intracellular FITC-Dextran levels in *D. discoideum* are thus reflective of endocytosis, vesicular trafficking, processing and docking, as well as of exocytosis. Treatment with 5 to 20 μM CQ for 90 minutes was found to cause a small increase in FITC-Dextran internalization and accumulation in amoeboid *D. discoideum* cells. CQ has previously been found to influence the secretory pathway in mammalian cells (Smith and Jarret, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998) from a concentration of 100 μM. Although CQ was found to disrupt secretory pathways in mammalian cells at higher concentrations, it is possible that treatment of *D. discoideum* with 20 μM CQ is sufficient to cause the drug to accumulate in intracellular acidic compartments. This may cause the pH to rise to a certain extent, disrupting exocytosis and resulting in pinosome accumulation and a small increase in intracellular tracer molecule levels. Alternatively, CQ may cause a slight increase in FITC-Dextran concentration by impeding pinosome processing. Disruption of vesicular trafficking or fusion events would cause a reduction in processes such as exocytosis that are further along the pathway, therefore, inhibition of pinosome processing may also cause pinosome build-up and thus tracer molecule accumulation in *D. discoideum* cells.

CQ accumulation in malarial parasites is partially dependent on its high affinity binding to FP (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996), therefore CQ would accumulate to a lesser degree in acidic compartments in the absence of large amounts of FP. CQ is thus likely to have greater toxicity at lower concentrations in malarial parasites than in other organisms due to the presence of large amounts of FP. Additionally, CQ may inhibit hemoglobin digestion by disrupting proteolytic enzymatic function in malarial parasites and in this way disrupt vesicular docking (Chapters 3-5). As hemoglobin is absent in *D. discoideum*, this potential mechanism of CQ disruption of vesicular processing does not apply in *Dictyostelium*. As a result, the effect of CQ-

treatment on *D. discoideum* is much less prominent than in strain 3D7 *P. falciparum* parasites.

An exocytosis assay demonstrated that CQ-treatment did not cause a statistically significant change in tracer molecule efflux. As a point of equilibrium is reached between endocytosis and exocytosis in *D. discoideum* (Klein and Satre, 1986), one would expect that changes in tracer molecule uptake and accumulation would also be apparent in the levels of efflux. It is possible, however, that the exocytosis assay is not sensitive enough to detect the slight changes that occurred in FITC-Dextran accumulation following CQ-treatment.

6.3.2 Mefloquine and Halofantrine

Treatment with 2.5 to 20 µM MQ or 10 to 20 µM H for 90 minutes caused an extensive concentration-dependent inhibition of FITC-Dextran uptake and accumulation in *D. discoideum* cells. MQ and H were found to inhibit pinocytosis at lower concentrations than drugs that are normally used to block this process in *Dictyostelium*. Treatment with 1 mM vanadate (Klein et al, 1989; Brenot et al, 1992) or 7.5 mM caffeine (Brenot et al, 1992) caused the full inhibition of tracer molecule uptake by pinocytosis in amoeboid *D. discoideum* cells. 100-200 µg/ml Cisplatin, a potential anti-cancer drug, has been found to inhibit pinocytosis in *D. discoideum* cells (Reddy and Chatterjee, 1997). Additionally, several protein synthesis inhibitors, including cycloheximide, anisomycin, puromycin (at millimolar concentrations) (Gonzalez and Satre, 1991), amino acid analogues such as hadacidin (at a concentration of 8 mg/ml) (Rossamando et al, 1980) have been found to inhibit this process in *D. discoideum*. As MQ and H inhibit pinocytosis in *D. discoideum* at lower concentrations, they may be more useful for cell biological assays which require the inhibition of this process. These drugs may target pinocytosis with greater specificity, inflicting less harm on general cellular metabolism. Additionally, the findings that MQ and H inhibit pinocytosis in *D. discoideum* and *P. falciparum* (Chapters 3-5) suggest that these drugs may inhibit this process in a number of organisms, possibly a wide range.

As mentioned in the previous section, a state of equilibrium is reached between rapid endocytosis and exocytosis in *D. discoideum*. Therefore, FITC-Dextran levels in these cells are likely to not only be indicative of endocytosis, but also of vesicular processing and exocytosis. Therefore, an exocytosis assay was conducted in order to determine the contribution of this process to reduced tracer molecule levels in MQ and H-treated cells. Treatment with 20 μM MQ or H was found to induce tracer molecule efflux following the addition of these drugs to *D. discoideum* cells that were preloaded with FITC-Dextran. This is not surprising given that the addition of 7.5 mM caffeine, also an effective endocytosis blocker, to *D. discoideum* cells preloaded with FITC-Dextran was found to cause efflux of 20-25 % of tracer molecules (Aubry et al, 1997).

In MQ and H-treated cells, efflux may partially contribute to the observed reduction in tracer molecule accumulation that was at first attributed to a block in pinocytosis. It is thus possible that drug-induced efflux is responsible for a small extent of the reduction in hemoglobin and HRP levels observed in *P. falciparum* 3D7 parasites treated with MQ or H (Chapters 4 and 5).

6.3.3 Quinine

Treatment with 2.5 to 20 μM Q was found to only cause a slight, but not statistically significant, reduction in FITC-Dextran internalization and accumulation following a 90 minute incubation period. It is thus possible that Q requires the presence of FP for its mechanism of action. Q was found to inhibit endocytosis in *P. falciparum* (Chapters 3-5), an organism in which FP is abundant. However, Q was found to effectively inhibit endocytosis in parasites that had invaded RBCs preloaded with HRP (Chapter 4), even though intracellular FP levels were probably reduced during this assay. Therefore, the action of Q may be enhanced by FP, but not dependent on the presence of this molecule. Additionally, Q may target endocytosis in *P. falciparum* with greater specificity. The

drug may interact with a molecule or process that is essential to endocytosis in *P. falciparum*, but is not present or is dissimilar in other organisms.

Treatment with 20 μM Q for 60 and 90 minute periods was found to induce tracer molecule efflux following the addition of the drug to *D. discoideum* cells preloaded with FITC-Dextran. The slight reduction observed in tracer molecule accumulation in cells following Q-treatment may therefore be due to efflux. This finding supports the hypothesis mentioned in the previous section that efflux may only be partially responsible for reduced intracellular levels of FITC-Dextran in *D. discoideum* cells and reduced levels of HRP and hemoglobin in *P. falciparum* cells following treatment with MQ or H. Intracellular levels of FITC-Dextran were only slightly reduced in Q-treated cells, but levels of efflux were similar in MQ, H and Q-treated cells. Therefore, in MQ and H-treated cells, efflux can at most account for the degree of the reduction in tracer molecule levels that was seen in Q-treated cells in the FITC-Dextran uptake and accumulation assays. It is furthermore possible that drug-induced efflux is responsible for a small extent of the reduction in hemoglobin and HRP levels observed in *P. falciparum* 3D7 parasites treated with Q (Chapters 4 and 5).

6.3.4 Amodiaquine

Treatment with 20 μM AQ for 90 minutes caused variable changes in FITC-Dextran accumulation in *D. discoideum* cells relative to the controls. Tracer molecule levels were sometimes raised but reduced at other times. AQ-treatment may result in the inhibition of pinocytosis and vesicular processing concurrently, inhibiting one or the other to varying degrees at different times. Additionally, as CQ has been found to disrupt the secretory pathway in mammalian cells (Smith and Jarret, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998) and possibly *D. disocideum*, AQ may have the same affect in these organisms. Treatment of *P. falciparum* 3D7 with AQ also resulted in variable changes in hemoglobin build-up (Chapter 3). AQ-treatment caused a reduction in HRP accumulation (Chapter 4), but resulted in increased numbers

of transport vesicles in the cytoplasm of *P. falciparum* (Chapter 5). These findings suggest that AQ inhibits endocytosis and vesicular docking and may disrupt exocytosis in *P. falciparum*, and therefore support the above hypothesis that AQ has the same effect in *D. discoideum* cells. Treatment with 20 μM AQ did not cause a significant change in FITC-Dextran efflux from *Dictyostelium* cells. One would not expect to see a reduction in FITC-Dextran efflux if endocytosis and vesicular docking and/or exocytosis were concurrently disrupted, as these cells would retain the tracer molecule.

Chapter 7

The Effect of Quinoline Anti-Malarials on Horseradish Peroxidase Uptake and Accumulation in A549 Cells

7.1 Introduction

It has been demonstrated that AQ, CQ, H, MQ and Q influence the endolysosomal pathway in *P. falciparum* 3D7 cells (Chapters 3-5). Additionally CQ, H, MQ, Q and possibly AQ were found to affect endocytosis, vesicular processing and/or exocytosis in *D. discoideum* (Chapter 6). Previous studies have found that MQ disrupts the phagocytic pathway in mammalian cells (Labro and Babin-Chevaye, 1988), whilst CQ influences the secretory pathway (Smith and Jarret, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998). It is thus possible that the quinoline anti-malarials influence endocytosis, vesicular processing and/or secretion in mammalian cells. As the endolysosomal and secretory pathways are well understood in mammalian cells, but poorly understood in *Plasmodium*, the precise effect of these drugs on these processes may be studied with greater clarity using mammalian cells. MQ and H have been found to inhibit endocytosis in *P. falciparum* and *D. discoideum*, therefore it is possible that these drugs inhibit endocytosis in a broad range of organisms. Knowledge of their effects may thus prove useful for cell biological studies in which the inhibition of endocytosis is specifically required. Additionally, side-effects may be revealed that could be of clinical relevance. Therefore, HRP internalization assays were conducted using human pulmonary cancer cells (A549) cells.

FP has previously been found to play a role in CQ accumulation (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996) and possibly CQ disruption of membrane function (Hoppe et al, 2004; Fitch, 2004) in malarial parasites. Therefore, as the action of one or more of the other drugs may be affected by the presence of FP, hemoglobin-containing RBC-lysate was added at subtoxic concentrations.

7.2 Results

A549 cells were allowed to attach to the surface of 24 well plates. They were then incubated in HRP-containing medium and 20 μM of each drug alone or drug and RBC lysate for 150 minutes. Drugs were added at this concentration as treatment with 20 μM MQ, H and CQ (as well as lower concentrations of each drug) were found to influence the endolysosmal pathway in *D. discoideum* (Chapter 6). The incubation time for this assay was longer than the times used during experimentation with *D. discoideum* as mammalian cells have previously been found to endocytose extracellular fluid and solutes at a slower rate than *Dictyostelium* cells (Thilo, 1985). Following incubation, cells were washed repeatedly and harvested with Trypsin/Versene. They were then lysed and OPD (a colorimetric HRP substrate) was added. The absorbance was measured at 450 nm on a spectrophotometer.

Sample	Concentration (μM)	CM Absorbance (450 nm) ± SD	*N*	25 % RBC lysate Absorbance (450 nm) ± SD	*N*
Control	-	1.1 ± 0.2	3	1.0 ± 0.0	3
AQ	20	1.2 ± 0.1	3	1.4 ± 0.1	3
CQ	20	1.2 ± 0.1	3	1.2 ± 0.1	3
H	20	0.6 ± 0.1	3	0.9 ± 0.1	3
MQ	20	0.1 ± 0.0	3	0.4 ± 0.0	3
Q	20	0.8 ± 0.1	3	0.6 ± 0.1	3

Table 7.1. The effect of anti-malarial drugs on HRP uptake and accumulation by A549 cells, measured as Absorbance (450 nm) ± standard deviation (SD) of the cell lysates following the addition of an HRP subtrate. Cells were untreated (Control), or treated with 20 μM amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) in complete medium (CM) or medium containing 25 % red blood cell (RBC) lysate for 150 minutes. *N* = sample size.

It was found that A549 cells treated with 20 μM CQ for 150 minutes did not show a statistically significant change in HRP internalization relative to untreated control cells (P = 0.5925, 95% CI) (Table 7.1, Figure 7.1). Cells treated with 20 μM H, MQ or Q showed

a reduction in HRP uptake and accumulation relative to untreated controls (P = 0.0101, P = 0.004 and P = 0.0408 for H, MQ and Q respectively) (Table 7.1, Figure 7.1). As shown in Figure 7.1, the addition of RBC lysate to untreated control A549 cells did not result in a statistically significant change in HRP levels following a 150 minute incubation period (P = 0.4957, 95% CI). Cells treated with RBC lysate and CQ did not show a statistically significant change in HRP levels relative to cells treated with CQ alone (P = 0.5282, 95% CI).

A549 cells that were treated with RBC lysate and H or MQ showed an increase in HRP accumulation relative to cells treated with H or MQ alone (P = 0.0178 and P = 0.0001 for H and MQ respectively). Cells treated with RBC lysate and Q showed a reduction in HRP levels relative to cells treated with Q alone (P = 0.0483).

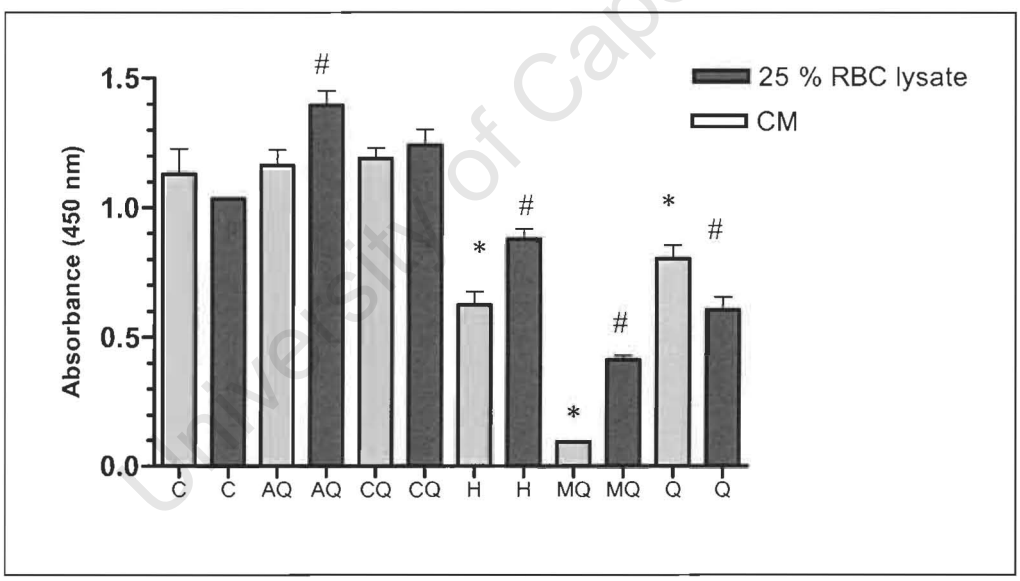


Fig 7.1. The effect of the quinoline anti-malarial drugs on HRP uptake and accumulation by A549 cells. Cells were incubated with HRP and treated with 20 μM amodiaquine (AQ), chloroquine (CQ), halofantrine (H), mefloquine (MQ) or quinine (Q) for 150 minutes. Cells were either incubated in complete medium (CM) or in medium containing 25 % red blood cell (RBC) lysate. Following incubation, cells were washed, lysed, a colorimetric HRP subtrate was added. Absorbance was measured at 450 nm. Error bars indicate standard error. * Statistically significant change from the control (95% CI). # Statistically significant change from cells treated with drug alone.

A549 cells that were treated with 20 μM AQ for 150 minutes showed variable changes in HRP uptake accumulation. Figure 7.2 shows the HRP levels in cells treated with AQ on 4 separate occasions. It was found that the addition of RBC lysate to untreated control cells did not result in a statistically significant change in HRP levels (P = 0.9994, 95 % CI). On day 1, AQ-treatment caused a reduction in HRP internalization relative to the untreated control cells (P = 0.0209). On day 2, cells treated with RBC lysate and AQ also showed a reduction in HRP levels relative to controls treated with RBC lysate alone (P = 0.0025). However, on day 3 cells treated with AQ did not show a statistically significant change in HRP uptake relative to untreated control cells (P = 0.7976). On the same day, cells treated with AQ and RBC lysate showed an statistically significant increase in HRP levels relative to cells treated with AQ alone (P = 0.0469). (Table 7.2)

Sample	Concentration (μM)	CM HRP Levels (% of Control ± SD)	*N*	25 % RBC lysate HRP Levels (% of Control ± SD)	*N*
Control	-	100.0 ± 17.6	3	100.0 ± 5.4	3
AQ 1	20	62.8 ± 7.1	3	-	-
AQ 2	20	-	-	51.5 ± 2.5	3
AQ 3	20	103.0 ± 9.2	3	123.4 ± 8.5	3
AQ 4	20	86.9 ± 15.6	3	-	-

Table 7.2. The effect of amodiaquine (AQ)-treatment on HRP uptake and accumulation by A549 cells. Cells were untreated (Control), or treated with 20 μM AQ on day 1 (AQ 1), day 2 (AQ 2), day 3 (AQ 3) and day 4 (AQ 4). Cells were either incubated in complete medium (CM) or medium containing 25 % red blood cell (RBC) lysate for 150 minutes. Cells were washed, lysed and a colorimetric HRP substrate was added. Absorbance readings (450 nm) were determined using a spectrophotometer and then normalized to control readings (100 %) ± standard deviation (SD). *N* = sample size.

On day 4 AQ-treated cells showed a reduction in HRP internalization relative to untreated control cells, however the change was not statistically significant at the 95 % CI (P = 0.3127). (Table 7.2)

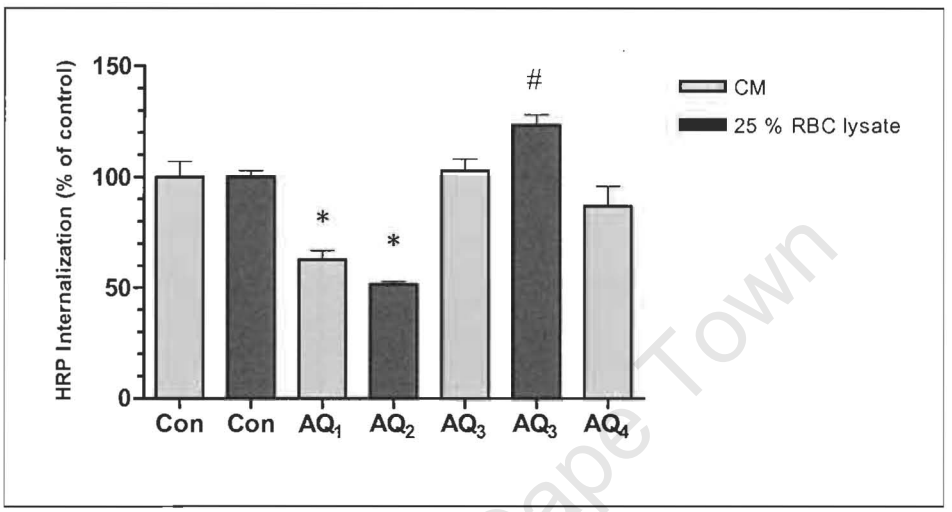


Fig 7.2. The effect of amodiaquine (AQ) on HRP uptake and accumulation by A549 cells, measured as Absorbance (450 nm) of the cell lysates following the addition of an HRP subtrate. Cells were untreated (Control, Con), or treated with 20 μM AQ on day 1 (AQ 1), day 2 (AQ 2), day 3 (AQ 3) and day 4 (AQ 4). Cells were either incubated in complete medium (CM) or medium containing 25 % red blood cell (RBC) lysate for 150 minutes. *N* = sample size. Absorbance readings were normalized to control readings. Error bars indicate standard error. *Statistically significant change from the control (95% CI). # Statistically significant change from cells treated with drug alone.

A549 cells were treated with lower concentrations of MQ in order to determine the minimum concentration of drug required to affect HRP uptake and accumulation and to compare the activity of MQ in these cells to *D. discoideum* and *P. falciparum*. It was found that MQ caused a significant reduction in levels of tracer enzyme following treatment with 10 μM drug for 150 minutes (Figure 7.3).

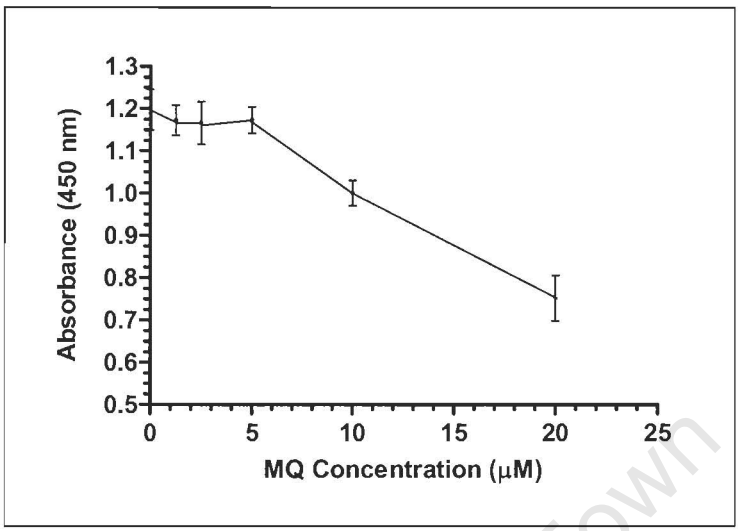


Fig 7.3. The effect of increasing concentrations of mefloquine (MQ) on HRP uptake and accumulation by A549 cells. Cells were treated with 1.25 – 20 μM MQ for 150 minutes. Cells were washed, lysed and OPD (colorimetric HRP substrate) was added. Absorbance at 450 nm was measured using a spectrophotometer. Error bars indicate standard error.

7.3 Discussion

7.3.1 Chloroquine

Although CQ has previously been shown to influence the secretory pathway in mammalian cells from concentrations of 100 μM (Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998), the effect of the drug on the endocytic pathway in these cells has not been evaluated. Therefore, as results obtained in Chapter 6 suggest that CQ may disrupt the endolysosomal pathway in *D. discoideum* at a concentration of 20 μM, A549 cells were treated the drug at this concentration. CQ was however not found to have a significant effect on HRP accumulation in A549 cells at this concentration.

The presence of FP in compartments along the endocytic and secretory pathways may enable CQ to accumulate to a greater degree, resulting in disruption of trafficking and processing at lower concentrations of the drug. Additionally, CQ-mediated disruption of

membrane function has been found to be dependent on the presence of FP (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983). Therefore, RBC lysate was added in order to determine the effect of the presence of FP and/or hemoglobin on the potency of CQ action. It was found that the addition of RBC lysate resulted in a slight increase in HRP levels, however the change was not statistically significant at the 95% CI. FP may thus be required at a higher concentration than used in this experiment in order to substantially affect CQ accumulation and action, however the toxicity of FP may then influence the assay.

7.3.2 Melfoquine and Halofantrine

Treatment with 20 μM H or MQ for 150 minutes resulted in a reduction in HRP uptake and accumulation in A549 cells relative to untreated control cells. These drugs are thus likely to disrupt endocytosis and/or stimulate efflux from mammalian cells, as well as *D. discoideum* cells (Chapter 6) and *P. falciparum* parasites (Chapters 3-5). Therefore, as these drugs have been found to inhibit endocytosis and/or stimulate efflux in the cells of three diverse types of organisms at relatively low concentrations, it is likely that they have the same effect in a wide range of other organisms. These drugs may thus prove useful for cellular biological studies where specific inhibition of endocytosis is required. On the other hand, MQ was previously shown to bind to membranes and purified phospholipids with high affinity (Chevli and Fitch, 1982; San George et al, 1984). Additionally, the drug has been found to inhibit the phagocytic activity of human polymorphonuclear neutrophils (Labro and Babin-Chevaye, 1988). These findings, along with the finding that MQ and H affect exocytosis in *D. discoideum* (Chapter 6), suggest that these drugs do not have a specific target, but rather that they disrupt membrane function non-specifically. Treatment of A549 cells with MQ was found to cause a reduction in HRP uptake and accumulation at concentrations of 10 μM or above. Therefore, the drug is unlikely to disrupt membrane function in mammalian cells at therapeutic or prophylactic concentrations.

FP has been found to play an important role in CQ accumulation (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996; Bray et al, 1999) and CQ- mediated

disruption of membrane function (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih etal, 1981; Fitch et al, 1982; Fitch et al, 1983). As CQ is structurally related to MQ and H, the presence of FP in malarial parasites may also influence the mechanism(s) of action of these drugs. Therefore, A549 cells were treated with 20 μM H or MQ and RBC lysate for 150 minutes. The addition of RBC lysate was found to result in the accumulation of HRP to a greater extent than in cells treated with drug alone. It is possible that MQ and H bind to hemoglobin or FP and are sequestered away from their site of action, thus reducing their inhibitory effects on endocytosis. In support, MQ (Chou et al, 1980; Chevli and Fitch, 1982) and H (Egan et al, 1994; Basilico et al, 1997; Hawley et al, 1998; Egan et al, 1999; Ursos et al, 2001) were previously found to interact weakly with FP.

7.3.3 Quinine

Treatment of A549 cells with 20 μM Q for 150 minutes also caused a reduction in HRP accumulation relative to the controls, although to a lesser extent than in MQ or H-treated cells. This is consistent with the findings of uptake and accumulation assays conducted in *D. discoideum,* where Q was found to have little or no effect (Chapter 6). Treatment with Q and RBC lysate resulted in a further reduction in HRP levels, suggesting that FP has an adjuvant effect on Q action and that Q may inteact with FP to inhibit endocytosis to a greater extent. In support, it has previously been found that Q does interact weakly with FP (Chou et al, 1980).

7.3.4 Amodiaquine

AQ-treatment was found to result in variable levels of hemoglobin accumulation in malarial parasites (Chapter 3). Additionally, treatment of *D. discoideum* with this drug resulted in inconsistent degrees of FITC-Dextran accumulation (Chapter 6). The same trend was observed in A549 cells following treatment with 20 μM AQ for 150 minutes. Levels of HRP accumulation were found to vary widely at different times. AQ-treatment is likely to result in the concurrent disruption of endocytosis, vesicular processing and docking in *P. falciparum* 3D7 (Chapters 3-5) and *D. discoideum* (Chapter 6). Therefore, it is possible that AQ-treatment has the same effect in A549 cells. As suggested in

Chapter 6, the possible complexity and pleiotropy of AQ's effects means that AQ-treatment may cause the inhibition of endocytosis and of vesicular processing and docking as well as exocytosis to variable degrees at different times, an effect that is not surprising given that living organisms are likely to respond to drug treatment in varying ways at different stages of growth or health. AQ may additionally influence the secretory pathway in these cells, as has previously been found in mammalian cells treated with CQ (Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998).

A549 cells were treated with AQ and RBC lysate in order to investigate the influence of the presence of hemoglobin and FP on AQ action. It was found on one occasion that cells treated with AQ and RBC lysate contained raised levels of HRP relative to cells treated with AQ alone. However, due to the variable changes in A549 cellular reponses to AQ-treatment, it is difficult to draw any conclusions regarding the effect of the treatment with AQ in the presence of RBC lysate. In support, on another occasion, it was found that treatment with AQ and RBC lysate caused a large reduction in intracellular HRP levels relative to cells treated with RBC lysate alone. Therefore, numerous additional assays would be required in order to draw any sound conclusions regarding the effect of the presence of RBC lysate on the mode of action of AQ in these cells.

Chapter 8

Summary and Conclusion

With the increasing prevalence of parasite drug resistance and vector insecticide resistance, the development of new anti-malarial strategies has become vitally important. A better understanding of parasite metabolism as well as the action of existing anti-malarial drugs may prove critical in this process. The precise mechanisms of action of the quinoline anti-malarials are still uncertain, even though these drugs have been indispensable to the treatment and prevention of malaria for many years. A number of previous studies have indicated that the quinoline drugs influence the endolysosomal pathway in malarial parasites.

This thesis describes the effects of amodiaquine, chloroquine, halofantrine, mefloquine and quinine on this pathway in *Plasmodium falciparum* 3D7 parasites. The effects of the drugs on endocytosis in *Dictyostelium discoideum* and A549 cells were also examined in the hope that additional light would be shed on the modes of action in *P. falciparum*. *D. discoideum* is a commonly used model organism for the study of endocytosis. Amoeboid cells of this organism endocytose extracellular medium with high efficiency. Additionally, these cells rapidly exocytose ingested material, making *Dictyostelium* a useful organism for the study of cellular efflux. Furthermore, the endolysosomal system in these cells is well understood in comparison to that of *Plasmodium* parasites. In mammalian A549 cells, the endolysosomal pathway is understood with even greater clarity than in *D. discoideum*. Therefore, these cells were also utilised for the further evaluation of the modes of action of the drugs.

8.1 Chloroquine

Treatment with CQ was found to result in hemoglobin accumulation in transport vesicles in *P. falciparum* strain 3D7 parasites. Parasites that were treated with CQ for 8 hours and observed under a light microscope following Giemsa staining were swollen relative to untreated parasites (Chapter 2), suggesting that the endolysosmal

pathway or hemoglobin digestion may be inhibited. Food vacuoles were however normal in size, in contradiction to previous reports (Macomber and Sprinz, 1967; Warhurst and Hockley, 1967; Aikawa, 1972). Hemoglobin levels were found to be largely increased in parasites treated with CQ in Western Blotting assays (Chapter 3), as previously found (Famin and Ginsburg, 2002; Fitch et al, 2003a; Hoppe et al, 2004). Additionally, in an immunofluorescence assay (Chapter 5) it was found that the numbers of hemoglobin transport vesicles in the parasite cytoplasm were raised in CQ-treated parasites relative to those in untreated parasites [as found by Hoppe et al (2004)], while parasite food vacuoles contained normal levels of hemoglobin. Treatment with CQ has previously been found to inhibit the processing of hemoglobin transport vesicles (Macomber et al, 1967; Warhurst and Hockley, 1967; Fitch et al, 2003a), causing their accumulation in either the food vacuole in some strains (Yayon et al, 1984), or the cytoplasm in other strains (Fitch et al, 2003a; Hoppe et al, 2004). The varying results of these studies suggest that the cell biological responses of malarial parasites to CQ-treatment may be species and strain dependent. The findings of this study as well as of previous studies, suggest that CQ may disrupt the endolysosomal pathway in malarial parasites, either causing or as a result of the inhibition of hemoglobin digestion, as previously suggested (Famin and Ginsburg, 2002).

CQ is a weak base that accumulates in the parasite food vacuole by an ion-trapping mechanism or by its high affinity binding to FP (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996). CQ may then inhibit vesicular docking and fusion at the food vacuole in strain 3D7 parasites by causing the pH to rise in this organelle (Krogstad et al, 1985) or by interacting with membranes through an FP bridge and disrupting their function [as suggested by Hoppe et al (2004) and Fitch (2004)]. CQ has been found to slow the rate of FP dimerization (Egan and Ncokazi, 2005), causing it to accumulate (Chou and Fitch, 1993; Zhang et al, 1999; Famin and Ginsburg, 2002). Build-up of this toxic molecule may cause the disruption of membrane function (Chou and Fitch, 1980; Chou and Fitch, 1981; Orjih et al, 1981; Fitch et al, 1982; Fitch et al, 1983), inhibiting vesicular docking as a consequence. Additionally, high levels of FP may inhibit the proteolytic enzymes (Tappel, 1955; Sugioka and Suzuki, 1991; Foley and Tilley, 1998; Ginsburg et al, 1998) that are

involved in hemoglobin digestion. CQ-mediated alkalinization of the food vacuole may alternatively, or concurrently, disrupt proteolytic enzymes (Krogstad et al, 1985), contributing to the inhibition of hemoglobin digestion. As a result, vesicular docking and further delivery of hemoglobin to the food vacuole may be inhibited due to excess undigested hemoglobin in this organelle and consequent down-regulation of endocytic cargo delivery.

It is possible that, in addition to CQ-mediated inhibition of vesicular docking and hemoglobin digestion, treatment with CQ may contribute to the accumulation of transport vesicles by disrupting the secretory pathway in malarial parasites. CQ has previously been found to influence the secretory pathway in mammalian cells (Smith and Jarret, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998). CQ disrupts the pH gradient along this pathway (Davis and Mecham, 1998) by accumulating in any acidic organelle or vesicle (Wibo and Poole, 1974). As a result, endosomes accumulate, receptor-ligand interactions are disrupted and acid proteases are inhibited (Mellman et al. 1986). The accumulation of endosomes in parasites may thus be partially due to the disruption of the recycling of endocytosed material from early endosomes to the plasma membrane, causing a build-up of recycling endosomes. Alternatively, recycling via a *trans*-Golgi-like network in malarial parasites may be disrupted. Treatment with CQ may thus lead to a build-up of transport vesicles by concurrently or alternatively inhibiting vesicular docking and/or by disrupting recycling along the parasite secretory pathway. The process of recycling via the secretory pathway in malarial parasites has not been identified or characterised. In order to further evaluate the effect of CQ-treatment on this process in malarial parasites, a novel assay along the following lines could be designed: Parasites would be released from red blood cells, allowed to take up a tracer molecule, washed and incubated in fresh medium containing the drug of interest. The levels of extraparasitic tracer molecule would then be determined over time.

Treatment with CQ was found to cause a moderate inhibition of hemoglobin endocytosis in 3D7 parasites following a long incubation period. In a Western Blot assay, although treatment of parasites with CQ resulted in the accumulation of less hemoglobin than in parasites treated with PIs, similar levels of hemoglobin were seen

in parasites treated with CQ and PIs relative to parasites treated with PIs alone. This suggests that CQ does not inhibit endocytosis following an 8 hour incubation period, but rather causes less hemoglobin to accumulate as hemoglobin digestion is not entirely inhibited. However, it was found in a Horseradish Peroxidase uptake assay (Chapter 4) that levels of HRP, an enzyme that is potentially indigestible in malarial parasites, were reduced in CQ-treated parasites relative to control parasites. This indicates that CQ may inhibit endocytosis following a 10 hour incubation period. In support, CQ has previously been found inhibit endocytosis following long incubation periods (Hoppe et al, 2004).

D. discoideum has an unusual endolysosomal pathway in that a state of equilibrium is reached between rapid endocytosis and exocytosis. Therefore, indigestible tracer molecules such as FITC-Dextran would not accumulate in untreated cells but would travel through the endolysosomal pathway and be shunted out of the cell. FITC-Dextran levels in *D. discoideum* are thus reflective of endocytosis, vesicular trafficking, processing and fusion, as well as exocytosis. Treatment with CQ was found to result in a moderate accumulation of the tracer molecule FITC-Dextran in *D. discoideum* during an uptake and accumulation assay (Chapter 6). As CQ has been found to disrupt the secretory pathway in mammalian cells (Smith et al, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998), and may influence this process in *P. falciparum*, the drug may cause tracer molecule accumulation in *D. discoideum* in the same way. Alternatively, as may occur in *P. falciparum*, CQ may disrupt pinosome processing in these cells, causing FITC-Dextran to accumulate in this way. Although an exocytosis assay did not demonstrate a change in tracer molecule secretion from *D. discoideum* cells, this assay may not be sensitive enough to detect the small changes that were seen in FITC-Dextran levels in the endocytosis assay.

In an HRP internalization assay with A549 cells, it was found that CQ-treatment did not affect HRP accumulation (Chapter 7). CQ has been found to disrupt the secretory pathway in mammalian cells from concentrations of 100 μM (Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998). In this study, A549 cells were treated with CQ at a concentration of 20 μM. The presence of FP in acidic compartments

along the endolysosomal pathway in malarial parasites enables CQ to accumulate to a higher concentration (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996) and disrupt vesicular processing when added at lower concentrations. Therefore, in mammalian cells (and in *D. discoideum* cells) in the absence of FP, treatment with CQ at higher concentrations appears to be necessary to effectively disrupt the endolysosomal system. A549 cells were incubated with CQ and RBC lysate which contained large amounts of hemoglobin in order to determine whether the presence of FP would amplify the effect of CQ. However, it was found that the addition of RBC lysate did not cause a significant change in HRP uptake and accumulation. It can thus be hypothesized that, either hemoglobin remains undigested in these cells and FP is thus not released, or that FP is not present at a high enough concentration to affect CQ accumulation and/or action. Conducting experiments using pure FP or RBC lysate at a higher concentration could prove inconclusive as the toxicity of FP may substantially influence the assay.

8.2 Mefloquine, Halofantrine and Quinine

Due to the structural similarity of MQ, H and Q, it has been hypothesized that the modes of action of these drugs are similar. MQ has previously been found to inhibit endocytosis in strain D10 *P. falciparum* (Hoppe et al, 2004), while it has been suggested that Q has the same effect (Famin et al, 1999; Famin and Ginsburg, 2002). MQ, H and Q were indeed found to act similarly and inhibit endocytosis in strain 3D7 *P. falciparum*. Parasites that were treated with these drugs for 8 hours and observed under a light microscope following Giemsa staining were reduced in size relative to untreated parasites (Chapter 2). Additionally, parasite food vacuoles and hemozoin crystals were barely visible, if at all, suggesting that either endocytosis is inhibited or that hemoglobin digestion is disrupted. In a Western Blotting assay (Chapter 3), it was found that treatment of parasites with MQ, H or Q each caused a reduction in hemoglobin levels relative to untreated parasites. Likewise, MQ (Famin and Ginsburg, 2002; Hoppe et al, 2004) and Q-treatment (Famin et al, 1999; Famin and Ginsburg, 2002) have previously been shown to have this effect on parasite hemoglobin levels. MQ, H and Q were additionally found to cause a reduction in hemoglobin levels following treatment with drug and PIs relative to parasites treated

with PIs alone (Chapter 3), suggesting that hemoglobin reduction in parasites treated with these drugs is independent of the digestion of this protein. In support, MQ has previously been found to have no effect on hemoglobin degradation (Famin and Ginsburg, 2002). In an HRP internalization assay (Chapter 4), it was found that treatment with MQ, H or Q caused a reduction in HRP uptake and accumulation. Furthermore, in an Immunofluorescence assay (Chapter 5) it was found that the numbers of hemoglobin transport vesicles in the parasite cytoplasm, as well as the levels of hemoglobin in parasite food vacuoles, were reduced in comparison to untreated parasites.

The effects of MQ, H and Q on endocytosis and exocytosis in *D. discoideum* were evaluated in an FITC-Dextran uptake assay (Chapter 6). It was found that treatment with increasing concentrations of MQ and H caused a large reduction in tracer molecule uptake and accumulation. Therefore, these drugs may also inhibit endocytosis in *D. discoideum*. MQ and H were additionally found to inhibit endocytosis at lower concentrations than drugs, such as vanadate, caffeine (Brenot et al, 1992) and cisplatin (Reddy and Chatterjee, 1997), which are normally used to inhibit this process in *Dictyostelium*. Treatment with Q, however, only resulted in a small decrease in tracer molecule levels relative to untreated cells. In order to determine the contribution of exocytosis to reduced FITC-Dextran levels in *D. discoideum* cells following treatment with these drugs, an exocytosis assay was conducted. It was found that treatment with 20 μM MQ, H or Q caused FITC-Dextran efflux from *Dictyostelium* cells. This suggests that the drugs may stimulate the secretory pathway in *D. discoideum* and that this may partially account for the reduction in intracellular tracer molecule levels observed in the previous assay, as well as the reduction in hemoglobin levels observed in 3D7 *P. falciparum* parasites following treatment with these drugs. Numbers of hemoglobin transport vesicles in parasites treated with MQ, H or Q were found to be reduced (Chapter 5). Therefore, the reduction in hemoglobin levels (Chapter 3) and HRP levels (Chapter 4) observed in 3D7 parasites treated with MQ, H or Q are likely to be primarily due to inhibition of endocytosis, rather than stimulation of hemoglobin efflux from the cell. One would expect to still see intracellular transport vesicles were the latter the primary reason for

reduced hemoglobin or HRP levels. Therefore protein efflux is likely to only partially (at most) contribute to reducing these levels in *P. falciparum*.

In order to determine whether MQ, H and perhaps Q have similar effects in the cells of other organisms, an HRP uptake assay was conducted using mammalian A549 cells (Chapter 7). It was found that treatment with 20 μM MQ, H and Q all caused a reduction in tracer molecule accumulation, although Q had a lesser effect than the other drugs. Therefore, MQ and H appear to inhibit endocytosis in mammalian cells, as well as in *P. falciparum* and *D. discoideum*. MQ and H may inhibit endocytosis in a wide range of organisms and may therefore prove valuable for cell biological studies in which the inhibition of this process is specifically required. However, as MQ has previously been found to bind to purified phospholipids with high affinity (Chevli and Fitch, 1982; San George et al, 1984), MQ and H may disrupt membrane function non-specifically. In support, MQ has been found to inhibit phagocytic activity in human immune cells (Labre and Babin-Chevaye, 1988). Additionally, MQ and H were found to influence exocytosis in *D. discoideum* cells (Chapter 6). In order to determine the sensitivity of A549 cells to MQ relative to *D. discoideum* and *P. falciparum* 3D7 and to evaluate the possibility of side effects during anti-malarial-treatment, A549 cells were incubated with lower concentrations of the drug. It was found that MQ inhibited endocytosis in A549 cells from a concentration of 10 μM. It is thus unlikely that these drugs influence membrane function in mammalian cells at malarial prophylactic or therapeutic concentrations.

FP has previously been found to play an important role in CQ accumulation (Aikawa, 1972; Fitch et al, 1974; Krogstad et al, 1985; Hawley et al, 1996) and possibly CQ disruption of membrane function (Fitch, 2004; Hoppe et al, 2004) in malarial parasites. Therefore, FP may influence the action of other quinoline anti-malarials, as these drugs are structurally related. A549 cells were incubated with MQ, H or Q and 25% red blood cell (RBC) lysate. Treatment with MQ or H in the presence of RBC lysate was found to result in less of a reduction in HRP accumulation than observed in cells treated with drug alone. It can thus be suggested that these drugs bind to hemoglobin or FP and are sequestered away from their site of action. In support, MQ (Chou et al, 1980; Chevli and Fitch, 1982) and H (Egan et al, 1994; Basilico et al,

1997; Hawley et al, 1998; Egan et al, 1999; Ursos et al, 2001) were previously found to interact weakly with FP. Treatment with Q and RBC lysate resulted in a further reduction in HRP levels relative to cells treated with Q alone. Therefore, the activity of Q appears to be enhanced by the presence of hemoglobin or FP. Q has previously been found to interact with FP (Chou et al, 1980).

8.3 Amodiaquine

AQ may inhibit endocytosis in 3D7 *P. falciparum* cells, while concurrently inhibiting vesicular processing and hemoglobin digestion. Parasites that were treated with AQ for 8 hours and stained with Giemsa were found to be variably smaller and larger than untreated parasites (Chapter 2), suggesting that the effects of the drug may be intermediate, or a combination of MQ and CQ-type modalities. The effects of AQ are furthermore likely to be stage-specific. In Western Blotting assays (Chapter 3), AQ-treated parasites either showed a slight reduction in hemoglobin levels or no change relative to the controls, while in an HRP internalization assay, parasites treated with AQ accumulated less HRP relative to untreated parasites (Chapter 4). The finding that AQ inhibited HRP internalization, but did not cause a large reduction in hemoglobin levels in parasites suggests that both endocytosis and hemoglobin digestion are inhibited by the drug. Hemoglobin levels in AQ-treated parasites were found to vary, implying that the predominant effect of AQ may fluctuate between the inhibition of endocytosis and the inhibition of hemoglobin digestion. This observation is not surprising given that living organisms are likely to respond to drug treatment in different ways at different stages of growth or health.

Immunofluorescence assays were conducted in order to shed further light on the precise nature of the effect of AQ on the endolysosomal pathway in malarial parasites (Chapter 5). The findings of these assays provided support for the hypothesis that AQ concurrently inhibits endocytosis, vesicular docking and hemoglobin digestion. As observed in CQ-treated parasites, hemoglobin transport vesicles were found to accumulate in parasites treated with AQ, although to a lesser extent. It can thus be deduced that treatment with AQ may result in a build-up of transport vesicles by inhibiting vesicular docking and fusion at the food vacuole. AQ, like CQ, has been

found to accumulate inside the parasite's food vacuole (Hawley et al, 1996). AQ may also cause the pH to rise in this organelle, or may interact with FP to disrupt membrane function. Additionally, FP may accumulate and impede membrane function, due to AQ-mediated disruption of ß-hematin formation (Chou and Fitch, 1993; Egan et al, 1994; Egan and Ncokazi, 2005). As a result, vesicular processing and docking may be inhibited, causing the inhibiton of hemoglobin digestion. It has additionally been suggested that AQ inhibits hemoglobin digestion in a more direct manner (Famin and Ginsburg, 2002), perhaps by causing the disruption of proteolytic enzymatic function. As a result, hemoglobin may remain in the food vacuole at a threshold level, preventing further delivery of this protein via vesicular docking and fusion and causing transport vesicles to accumulate in this way. As suggested above, as overall hemoglobin levels were not raised in AQ-treated parasites (Chapter 3), despite the accumulation of transport vesicles (Chapter 5), and HRP levels were reduced (Chapter 4), AQ is likely to inhibit endocytosis concurrently to vesicle docking/fusion. Furthermore, as fewer hemoglobin transport vesicles were observed to accumulate in AQ-treated parasites relative to parasites treated with CQ, AQ appears to cause a more extensive inhibition of endocytosis than CQ [as observed in HRP uptake assays (Chapter 4)]. Additionally, during the Immunofluorescence assays, it was observed that large amounts of transport vesicles accumulated in some parasites while other parasites appeared shrunken in size and contained no transport vesicles, further supporting the hypothesis that the predominant effect of AQ may fluctuate between the inhibition of vesicular docking, fusion and hemoglobin digestion and the disruption of endocytosis.

It is additionally possible that AQ, as may be the case for CQ, causes hemoglobin transport vesicle accumulation by disrupting secretion via recycling endosomes or a *trans*-Golgi-like network in malarial parasites. AQ, like CQ (Wibo and Poole, 1974), may accumulate in any organelle or vesicle with an acidic pH, as well as the food vacuole (Hawley et al, 1996) and disrupt membrane function. Therefore, AQ may concurrently inhibit endocytosis and inhibit vesicular docking and hemoglobin digestion as well as recycling via the secretory pathway.

AQ may have the same effect in *D. discoideum* amoeboid cells. AQ-treatment for 90 minutes was found to cause variable changes in FITC-Dextran uptake and accumulation (Chapter 6). Levels of tracer molecules were sometimes reduced, but raised at other times. Intracellular FITC-Dextran levels reflect endocytosis, vesicular trafficking, processing and docking, as well as exocytosis. Therefore, as may be thecase in *P. falciparum*, AQ may inhibit endocytosis and concurrently disrupt vesicular processing to variable degrees at different times. Additionally, as CQ has been hypothesized to disrupt the secretory pathway in *Dictyostelium* due to its effect in mammalian cells (Smith et al, 1982; Moore et al, 1983; Schrader-Fischer and Paganetti, 1996; Davis and Mecham, 1998), AQ may also influence this pathway in *D. discoideum* cells. It was found in an exocytosis assay that treatment with AQ did not cause a change in FITC-Dextran secretion from *D. discoideum* cells (Chapter 6). This finding supports the hypothesis that treatment with AQ results in the concurrent inhibition of endocytosis and vesicular docking and/or exocytosis, as these cells would retain the tracer molecule.

As the endolysosomal pathway is well understood in mammalian cells, HRP internalization assays were conducted using A549 cells in order to further evaluate the mode of action of AQ. The drug again caused variable changes in tracer molecule uptake and accumulation in these cells. This further implies that the predominant effect of AQ in drug-treated cells may fluctuate between the inhibition of endocytosis and the inhibition of vesicular processing and docking and/or secretion. The findings that the effects of AQ appear to be non-specific in the cells of individual organisms, but also that the drug affects three diverse types of organisms in a similar way, indicates that the drug is likely to non-specifically disrupt membrane function. Given the pleiotropy of A549 cellular reponses to AQ-treatment, it is difficult to draw any conclusions regarding the effect of treatment with AQ in the presence of RBC lysate. On one occasion, cells treated with AQ and RBC lysate were found to cause an increase in HRP accumulation relative to cells treated with AQ alone. However, on another occasion, cells that were treated with AQ and RBC lysate caused a large reduction in HRP levels relative to cells treated with RBC lysate alone. Therefore, numerous additional assays would be required in order to draw any sound conclusions regarding the effect of the presence of RBC lysate on the mechanism of action of AQ

in A549 cells, and hence the importance of the presence of FP and/or hemoglobin on the mode of action of this drug in *P. falciparum*.

8.4 Conclusion

The effects of the quinoline anti-malarials have been found to be pleiotropic in different strains of *P. falciparum* and in parasites at various stages of growth. It is thus difficult to distinguish between the secondary and primary mechanisms of action of these drugs. Quinoline anti-malarials have been found to influence the endolysosomal pathway in malarial parasites. As this process is poorly understood, the difficulty in determining their precise mode of action if further confounded. A number of conclusions can however be drawn using the data generated during this study and that of previous studies. Treatment with CQ caused hemoglobin accumulation in 3D7 parasites by either disrupting enzymatic function or inhibiting vesicular processing and resulting in the inhibition of hemoglobin digestion, and/or by disrupting the secretory pathway. Additionally, it was found that CQ may inhibit endocytosis to a small extent following a long incubation period. Furthermore, the drug appears to disrupt vesicular processing and/or secretion in *D. discoideum*. AQ may non-specifically disrupt membrane function in *P. falciparum*, *D. discoideum* and A549 cells, inhibiting endocytosis, vesicular docking and/or exocytosis concurrently. It was also found that the action of this drug may be largely stage-specific. MQ and H were found to inhibit endocytosis and/or possibly slightly stimulate exocytosis in *P. falciparum*, *D. discoideum* and A549 cells. It was shown that Q inhibited endocytosis and/or stimulated exocytosis in *P. falciparum* and A549 cells. However, the drug appeared to affect exocytosis alone in *D. discoideum*.

Therefore the quinoline anti-malarials, MQ and H in particular, are likely to prove useful for further study of the endocytic pathway in malarial parasites and other organisms. A better understanding of this essential process may prove useful for future drug discovery. Additionally, as the quinoline anti-malarials have been found to influence the endolysosomal pathway, these drugs may be used in combination with drugs that target other essential cellular processes in order to avoid drug-resistance.

Chapter 9

Materials and Methodology

9.1 Cell Culture and Maintenance

9.1.1 Plasmodium falciparum

The 3D7 strain of *P. falciparum* was maintained in 4 % (vol/vol) O^+ human red blood cells (RBCs) and RPMI 1640 medium supplemented with 50 mM glucose, 25 mM HEPES, 0.65 mM hypoxanthine, 0.048 mg/ml gentamycin, 0.5 % (wt/vol) Albumax II, and 0.2 % (wt/vol) Sodium Bicarbonate ($NaHCO_3$). The cultures were stored in 50 ml culture flasks and incubated at 37 °C under a gas mixture of 4 % CO_2, 3 % O_2, 93 % N_2.

The cultures were synchronized using the sorbitol method (Lambros and Vanderberg, 1979). The parasites were viewed by oil immersion light microscopy using Giemsa-stained blood smears. The parasitaemia was calculated by counting the number of parasites relative to the number of RBCs.

9.1.2 Dictyostelium discoideum

The axenic strain of *D. discoideum* (Ax2) was maintained in HL5 medium at 22 °C in suspension in flasks shaken at 150 rpm (Watts and Ashworth, 1970).

9.1.3 A549 Cells

A549 cells, established in permanent culture from a human lung adenocarcinoma (Lieber et al., 1976), were cultured in Dulbeco's modified Eagle medium (DMEM, 4.5 g/L Glucose and L-Glutamine; BioWhittaker), supplemented with 10 % heat inactivated fetal calf serum (FCS) and 1 % L-Glutamine, in the presence of 1 % Penicillin/Streptomycin/Fungizone. Cultures were incubated at 37 °C in a 5 % CO_2

atmosphere. Cells were harvested by addition of Trypsin/Versene (0.25 % trypsin, 0.1 % versene EDTA in PBS; Highveld Biological) and incubation for 10 minutes at 37 °C.

9.2 Parasite Lactate Dehydrogenase Assay

The assay was performed in 96-well microtitre plates. The blank for the assay was unparasitised RBCs without drug and the control was parasitized RBCs (pRBCs) without drug. The final haematocrit in each well was 1 % and the final parasitaemia was 2 %. Each drug was two-fold serially diluted in triplicate. The final volume in each well was 200 µl. Plates were incubated for 48 hours at 37 °C in desiccator cabinets under 4 % CO_2, 3 % O_2, 93 % N_2.

Following incubation, 100 µl Malstat reagent and 25 µl NBT/PES were added to the wells of a separate 96-well microtitre plate. Malstat reagent consists of lactate (substrate), $APAD^+$ (cofactor), Triton X-100 (membrane detergent), and HEPES (buffer). NBT/PES contains equal volumes of nitroblue tetrazolium (1.6 mg/ml) and phenazine ethosulfate (0.08 mg/ml). The parasite cultures were resuspended and 15 µl from each well was added to the corresponding well of the duplicate plate. The plates were read at a wavelength of 620 nm in a scanning multiwell spectrophotometer (ELISA reader).

9.3 *P. falciparum* Drug Treatments

9.3.1 Western Blot and Immunofluorescence Assays

Drugs were added to the parasite cultures at concentrations approximately 5 times the IC_{50} values of each drug: 137 nM CQ; 156 nM MQ; 665 nM Q; 102 nM AQ; 27 nM H. The drugs were added in triplicate to 2 ml cultures in 24-well plates. The cultures were used at 10-20 % parasitaemia, 1 % haematocrit, with parasites in the early trophozoite stage. The plates were placed in desiccator chambers and incubated for 6 to 8 hours at 37 °C under 4 % CO_2, 3 % O_2, 93 % N_2.

9.3.2 Protease Inhibitor Assay

40 µM of protease inhibitors E64 and ALLN (Sigma Aldrich) were added in triplicate to 2 ml cultures containing drug at the following concentrations: 137 nM CQ; 156 nM MQ; 665 nM Q; 102 nM AQ; 27 nM H. The cultures had a 10-20 % parasitaemia, a 1 % haematocrit, and contained parasites in the early trophozoite stage. The plates were placed in desiccator chambers and incubated for 8 hours at 37 °C under 4 % CO_2, 3 % O_2, 93 % N_2.

9.4 *P. falciparum* Western Blot Assay

pRBCs from 2ml cultures were pelleted by centrifugation at 2000 rcf for 3 minutes in a microcentrifuge. The pellets were resuspended in 1ml of 0.25 % saponin in phosphate buffered saline (PBS) to lyse the red blood cell membranes. The released parasites were pelleted by centrifugation at 3300 rcf for 3 minutes. The parasites were washed 4-5 times in cold PBS and solubilized in 60 µl distilled water and 20 µl reducing SDS-PAGE sample buffer. The samples were boiled for 5 minutes and aliquots were run on an SDS-10 % polyacrylamide gel. The proteins were then transblotted onto Immun-Blot PVDF membranes (Biorad Laboratories) or Hybond-ECL nitrocellulose membranes (Amersham Biosciences). The membranes were incubated in blocking buffer (PBS containing 2 % [wt/vol] fat-free milk powder) for 45 minutes. They were then incubated in blocking buffer containing rabbit anti-hemoglobin antiserum (1:2000) (Sigma Aldrich) for 1 hour. The membranes were washed and incubated in blocking buffer containing peroxidase-conjugated goat anti-rabbit IgG (1:5000) (KP4 Laboratories) for 1 hour. The membranes were washed again and then soaked with an enhanced chemiluminescence (ECL) Western blotting detection reagent (Amersham Pharmacia Biotech) and exposed to Kodak BioMax Light autoradiography film. A Kodak EDAS 290 gel documentation system with a Kodak DC290 digital camera was used to capture the images of the developed autoradiographs. The net intensities of individual bands were determined using the histogram function of Adobe Photoshop (version 7.0) software.

9.5 *P. falciparum* Immunofluorescence Assay

pRBCs from 2 ml cultures were pelleted by centrifugation at 2000 rcf for 3 minutes in a microcentrifuge. The pellets were resuspended in 1ml of 0.25 % saponin in PBS to lyse the RBC membranes. The released parasites were pelleted by centrifugation at 3300 rcf for 3 minutes. The parasites were washed 4-5 times in cold PBS and resuspended in 1 ml PBS. Glass coverslips were incubated in poly-L-lysine for 15 minutes and placed in a 24-well plate. They were washed 4 times with PBS and left in 1ml PBS. 500 µl aliquots of the parasite suspensions were added to the wells. The parasites were then pelleted onto the coverslips by centrifugation at 99 rcf for 2 minutes and the coverslips were washed 4 times with PBS. The coverslips were fixed with 500 µl 4 % (wt/vol) paraformaldehyde/ 0.25 % gluteraldehyde for 15 minutes. They were then washed 4 times with PBS and incubated in 500 µl 0.5 % triton for 5 minutes. The coverslips were washed 4 times with PBS and then incubated in 500 µl 0.15 M glycine for 20 minutes. They were washed 4 times in PBS and then incubated in blocking solution (PBS containing 1mM $CaCl_2$, 1mM $MgCl_2$, 2 % [wt/vol] bovine serum albumin [BSA], 10 % [vol/vol] fetal calf serum, and 0.1 % [vol/vol] Tween 20) for 30 minutes. The coverslips were then incubated in blocking solution containing rabbit anti-hemoglobin antiserum (1:200) for 1 hour. They were then washed 4 times with wash medium (PBS containing 0.1 % [wt/vol] BSA and 0.1 % [vol/vol] Tween 20) and incubated in blocking solution containing rhodamine-conjugated goat anti-rabbit IgG (1:250) (Invitrogen) for 1 hour in the dark. All subsequent steps were conducted in the dark. Following incubation, the coverslips were washed 4 times with wash medium and once with PBS. They were then dipped in PBS containing DAPI (1 mg/ml), rinsed in water and drained. The coverslips were mounted in Permafluor mounting medium (Becton-Dickinson) and examined by fluorescence microscopy.

9.6 *P. falciparum* HRP Endocytosis Assay

9.6.1 Hypotonic Preloading of RBCs with Endocytosis Tracers

Horseradish Peroxidase (HRP) from Sigma Aldrich was used as an endocytosis tracer. In order to preload RBCs with HRP, the cells were first heated to 30 °C for 5 minutes. Hypotonic solution (5 mM HEPES, 11 mM glucose, 2 mM $MgCl_2$, 2 mM Na_2ATP) containing 5 µg/ml HRP was added and rapidly mixed with the RBCs. The lysed cells were then incubated for 10 minutes at 30 °C. Hypertonic solution (280 mM NaCl, 40 mM KCl, 11 mM glucose) preheated to 37 °C was added and the cells were incubated at 37 °C for 5 minutes. The RBCs were then centrifuged at 1000 rcf for 3 minutes and washed with culture medium.

9.6.2 Trophozoite-Infected RBC Enrichment

RBCs containing trophozoites were enriched using MACS Separation Columns from Miltenyi Biotech. Iron column paticles were blocked with culture medium. 2 ml parasitized RBCs were then added to the column placed alongside a magnet. The column was washed with culture medium and trophozoite-infected RBCs were eluted by removing the column from the magnet.

9.6.3 Drug Treatment

Enriched trophozoite-infected RBCs were added to RBCs preloaded with HRP. Cultures were incubated at 37 °C in desiccator cabinets under 4 % CO_2, 3 % O_2, 93 % N_2 for 24 hours until the parasites reached the late ring/early trophozoite stage. 137 nM CQ, 156 nM MQ, 665 nM Q, 102 nM AQ, or 27 nM H were then added in triplicate to 2 ml cultures in 24-well plates. Cultures were used at 10-20 % parasitaemia, 1 % haematocrit. The plates were placed in desiccator chambers and incubated for 10 hours as above.

9.6.4 HRP Endocytosis Assay

pRBCs from 2 ml cultures were pelleted by centrifugation at 2000 rcf for 3 minutes in a microcentrifuge. The pellets were resuspended in 1 ml of 0.25 % saponin PBS to lyse the RBC membranes. The released parasites were pelleted by centrifugation at 3300 rcf for 3 minutes. The parasites were washed 4-5 times in cold PBS and lysed with 1 ml 1 % Triton in 0.1 M phosphate citrate buffer (pH 4.8). Samples were then centrifuged at 3300 rcf for 3 minutes in order to pellet hemazoin crystals. 100 µl aliquots of each sample were added to 100 µl aliquots of 6 mg/ml o-phenylenediamine (OPD, colorimetric HRP substrate; Sigma Aldrich) and 0.2 % H_2O_2 in phosphate citrate buffer. Absorbance was measured at a wavelength of 450 nm in a scanning multiwell spectrophotometer (ELISA reader).

9.7 *D. discoideum* Fluid-Phase Endocytosis Assay

FITC-Dextran (average molecular weight, 70 000) from Sigma Aldrich was used as a fluid-phase marker (Thilo and Vogal, 1980). Amoeboid cells were suspended at a concentration of 3 x 10^6 cells/ml in HL5 medium containing 2 mg/ml FITC-Dextran (Lim et al, 2005). Cells were incubated in triplicate with increasing concentrations of AQ, CQ, H, MQ and Q for 90 minutes at 22 °C. Cells were incubated for a short period of time relative to *P. falciparum* experiments due to the rapid rate of endocytosis in *D. discoideum*. 150 µl aliquots were then removed from all cultures. FITC-Dextran was added to control aliquots, followed immediately by 1ml ice-cold 20 mM potassium phosphate buffer (KH_2PO_4, pH 6.0), in order to stop the reaction (Sharma et al, 2002). KH_2PO_4 was added to the remaining aliquots and the samples were centrifuged at 2000 rcf for 3 minutes. The pellets were then washed three times. Cells were either were fixed with 4% paraformaldehyde (PFA) for 5 minutes (Ravanel et al, 2001) or lysed with 100 µl 6.25 % Triton X-100. Fixed cells were washed with KH_2PO_4 and mounted under a coverslip in anti-fading polyvinyl alcohol mounting medium for viewing under a fluorescence microscope. 900 µl 50 mM sodium phosphate buffer (Na_2HPO_4, pH 9.2) was added to lysed cells (Sharma et al, 2002). Samples were further diluted 1:10 in

Na_2HPO_4 and were read on a fluorescence spectrophotometer (excitation 470 nm; emission, 520 nm) (Reddy and Chatterjee, 1997).

9.8 *D. discoideum* Fluid-Phase Exocytosis Assay

FITC-Dextran was used as a fluid-phase marker (Thilo and Vogal, 1980). Amoeboid cells were suspended at a concentration of 3 x 10^6 cells/ml in HL5 medium containing 2 mg/ml FITC-Dextran (Lim et al, 2005), and allowed to take up tracer molecule for 90 minutes at 22 °C. The cells were then washed twice with HL5 medium and 20 µM of each drug was added. The cultures were incubated for a further 60 minutes and 90 minutes at 22 °C. 150 µl aliquots were then removed and centrifuged at 3300 rcf for 3 minutes. 100 µl aliquots of the supernatant was retrieved from each sample and added to 900 µl Na_2HPO_4. Samples were read on a fluorescence spectrophotometer (excitation 470 nm; emission, 520 nm).

9.9 A549 HRP Endocytosis Assay

Cells were allowed to attach to the surface of the wells in 24 well plates for 24 hours. Fresh medium containing 20 µM of each drug and 0.5 mg/ml HRP was added and cultures were incubated at 37 °C in a 5 % CO_2 atmosphere for 150 minutes. Cells were then washed 3 times with PBS and harvested with Trypsin/Versene. Samples were centrifuged at 2000 rcf for 3 minutes and again washed with PBS. Cells were lysed with 250 µl aliquots of 10 % triton in 0.1 M phosphate citrate buffer (pH 4.8). 100 µl aliquots of each sample were then added to 100 µl 6 mg/ml OPD (HRP colorimetric substrate) and 0.2 % H_2O_2 in phosphate citrate buffer. Absorbance was measured at a wavelength of 450 nm in a scanning multiwell spectrophotometer (ELISA reader).

9.10 Fluorescence Microscopy

A Nikon Eclipse E600 fluorescence microscope fitted with a ×100 Apochromat objective was used to examine the slides. The images were captured with a Media Cybernetics CoolSNAP-Pro monochrome charge couple device camera. The histogram function of Adobe Photoshop (version 7.0) software was used to determine the mean intensity of fluorescence in the parasite food vacuoles in the Immunofluorescence assay.

9.11 Statistical Analyses

Dose response curves of the parasite lactate dehydrogenase assay were generated using a non-linear regression (sigmoidal dose response function) in SigmaPlot version 8. The inhibitory concentrations at which 50 % of the parasites were killed (IC_{50}) were determined by extrapolation from the curves. Statistical significances were determined using unpaired t-tests in Prism 4.0. Differences were considered significant if $P = 0.05$.

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