



CHAPTER 1

INTRODUCTION

Malaria remains the major cause of morbidity and mortality in many tropical areas of the world. The World Health Organization (WHO) statistics indicate a yearly occurrence of 110 million clinical cases of malaria, within the order of 270 million people being infected and 1-2 million dying from the disease (Krogstad *et.al*, 1992 ; Schapira *et.al*, 1993). More than 80% of the world's malaria casualties occur in Africa, with around a million children under the age of five dying annually (Chen *et.al*, 1994 ; Kreamsner *et.al*, 1995). The global expansion of the disease has been attributed mainly to the failure of vector control programmes and the high occurrence and spread of antimalarial drug resistance, necessitating the development of novel, safe and effective chemoprophylactic and therapeutic strategies (Cabantchik, 1989 ; Kirk and Horner, 1995). Economic and political factors may retard progress in the fight against this reemerging scourge, the devastating health effects of this disease mandate a continued and vigorous effort to develop new agents (Ward *et.al*, 1994). In an effort to contribute towards finding solutions to this ongoing crisis, the laboratory research in this thesis was directed at characterising the *in vitro* and *in vivo* antimalarial potential of novel riminophenazine compounds, as well as elucidating their biochemical mode of action.

LITERATURE REVIEW

1.1. THE MALARIA PARASITE

1.1.1 Life cycle

Malaria is caused by protozoan parasites of the genus *Plasmodium*. The four malaria parasites known to cause infection in man are *Plasmodium falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *Plasmodium falciparum* is especially important as a cause of overwhelming morbidity and mortality in tropical areas of the world (Krogstad, *et.al*, 1992).

The malaria parasite completes its life cycle in two hosts, a vertebrate (man) and an invertebrate (female *Anopheles* mosquito). Infection in man begins with a mosquito bite and inoculation of threadlike sporozoites. The cardiovascular system carries the introduced sporozoites to various tissues and organs of the body; the liver parenchyma in mammals and endothelial cells in birds,

where they asexually develop into merozoites, while some remain dormant in liver cells as hypnozoites. The merozoites are ultimately released into the circulation and are located inside red cells as ring forms (Sherman, 1979 ; Matsumoto *et.al*, 1987).

The erythrocytic cycle is characterised by a synchronous asexual development (schizogony) of rings (early stages) through to the trophozoite and schizont stages (mature forms) in a 48 hour period. The cycle culminates in schizonts rupturing from the erythrocytes as 16 daughter merozoites and reinfection of red cells. Parasite development and rupturing of the erythrocytes is marked by periodic fever-chills cycles which are the hallmark of malaria infections (Knell, 1991; Haldar, 1992 ; White *et.al*, 1992).

Some merozoites continue reinvading new erythrocytes and developing asexually, while others differentiate into sexual stages called gametocytes. A sexual cycle (sporogony) is initiated by a mosquito feeding on the carrier vertebrate host. In the mosquito stomach the gametocytes transform into gametes and after fertilization, the resulting worm-like zygotes penetrate the stomach wall and reside on the outside. The zygote forms a cyst-like body called an oocyst within which thousands of sporozoites form asexually. The cyst bursts releasing the mature sporozoites into salivary glands of the host mosquito and upon feeding, the sporozoites are injected into the vertebrate host completing the life cycle (Sherman, 1979 ; Knell, 1991). The life cycle of the malaria parasite *Plasmodium falciparum* is shown in Figure 1 overleaf.

1.1.2 Morphology and growth of blood stages

The infective or invasive form of *Plasmodium* (the merozoite) is covered by a filamentous and membranous pellicle and contains a nucleus, mitochondria, endoplasmic reticulum, a cytosome and apical organelles called rhoptries and micronemes (Aikawa, 1971 ; Aikawa, 1977). After invading the red cell, the merozoite loses its apical organelles and pellicular membranes resulting in a metabolically quiescent, uninucleated ring form that develops into an amoeboid trophozoite. The erythrocytic stages are encapsulated by a parasitophorous vascular membrane (PVM) and it is within this structure that the trophozoite begins ingesting the host cell cytoplasm via the cytosome (Sam-Yellowe *et.al*, 1988). Vesicles, containing mostly host cell haemoglobin, pinch off from the base of the cytosome and fuse with the parasite food vacuole wherein the

haemoglobin is digested .

The trophozoite contains limited amounts of endoplasmic reticulum, numerous ribosomes, mitochondria, Golgi bodies, primary lysosomes and membrane-bound vesicles (Sherman, 1979). The feeding trophozoite grows in size and its nucleus enlarges. The trophozoite develops into a schizont that undergoes nuclear division (schizogony) to yield merozoites (Figure 1).

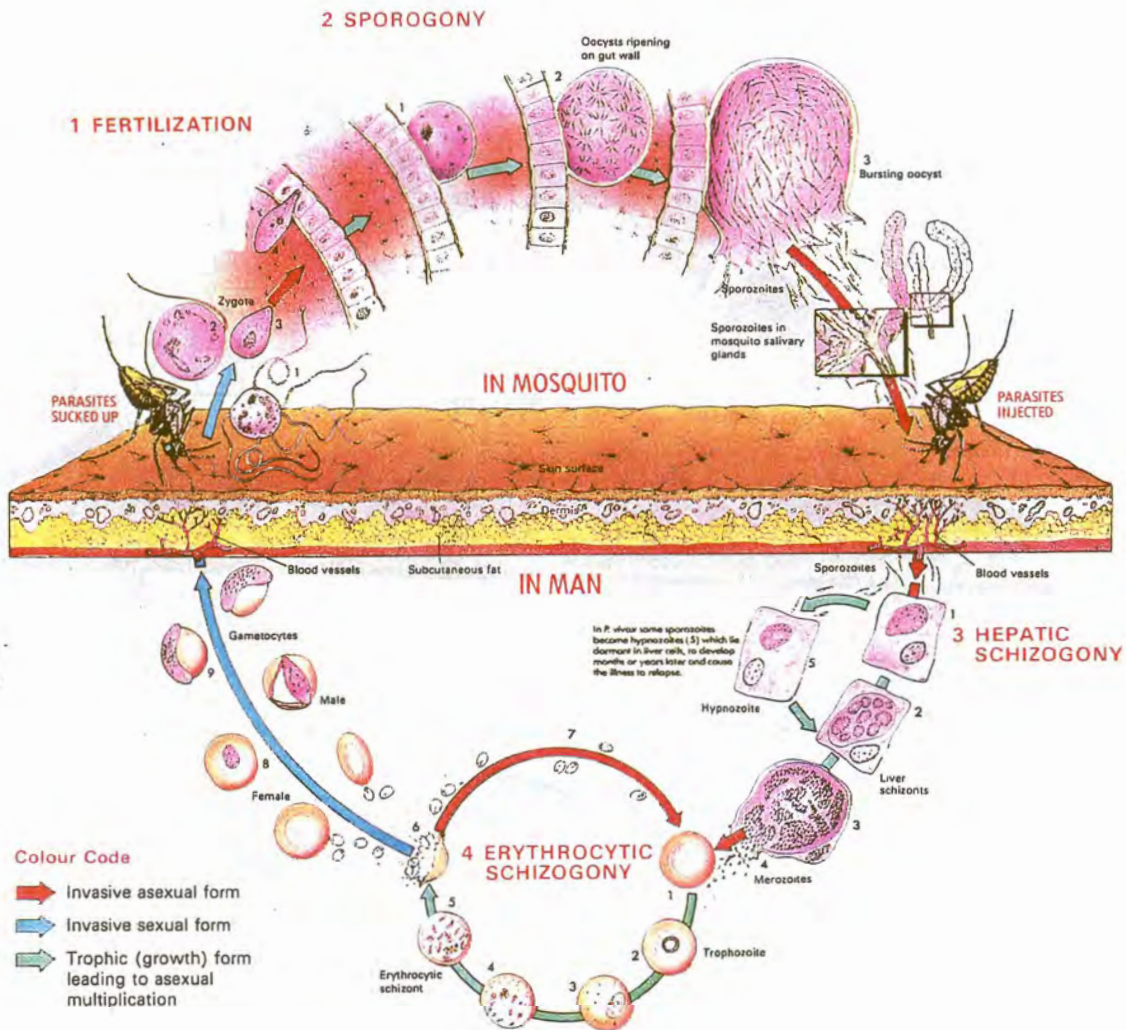


Figure 1 : The life cycle of *Plasmodium falciparum* (Kneel, 1991).

During schizogony, there is formation of intranuclear mitotic spindles, nuclear division, laying down of pellicular structures, development of rhoptry-microneme organelles and the nuclei and mitochondria are incorporated into the divided cytoplasm (Dluzewski *et.al*, 1995). The food vacuole, endoplasmic reticulum and membrane-bound vesicles form a structure called the residual body. The merozoite acquires its surface coat within the confines of the PVM and, when released, only the remnant of the host cell containing the residual body and PVM is left, ultimately being eliminated from the circulation (Sherman, 1979 ; Barnwell, 1990).

1.1.3 Host response to infection

The infection of humans by malaria parasites elicits an acute or initial immune response, resulting in generation of specific antigen-sensitized T cells and high antibody titers, which are generally unable to control infection (Khusmith and Druilhe, 1983). Individuals resident in areas with intense malaria transmission (endemic) develop clinical immunity after repeated exposure to *Plasmodium falciparum*. The parasitemia is low and antibodies fail to neutralise and eliminate the parasites. Parasite carriers remain symptomless (Cohen *et.al*, 1961; Bouharoun-Tayoun *et.al*, 1995).

Protective immunological mechanisms probably require cell-mediated responses and production of antibodies against asexual blood forms and soluble antigens that are released transiently during the invasion process (Van Heyde *et.al*, 1994 ; Good, 1995). Immunoglobulin G (IgG) has been found to be the major antibody class that drives humoral immunity responses against malaria parasites (Cohen *et.al*, 1961). The IgG antibodies protect humans against the malaria parasite blood stages, not on their own, but by acting in cooperation with monocytes. They are cytophilic and act by binding to monocytes via Fc receptors to promote antibody-dependent cellular immunity (ADCI) (Khusmith and Druilhe, 1983 ; Bouharoun-Tayoun *et.al*, 1990). A non-cytophilic antibody, immunoglobulin M (IgM), on the other hand inhibits invasion of merozoites into normal erythrocytes (Lunel and Druilhe, 1989).

During acute infection with the malarial parasite, gamma-delta T cells display marked increases in both proportion and absolute numbers which persist for 3 - 4 months (Langhorne *et.al*, 1994). Cytokine profiles of this class of T-cells are similar to that produced by the T-helper 1 (Th-1) cells

of the CD3⁺ T-lymphocytes, examples being interferon-gamma and tumour necrosis factor alpha (Clark and Rockett, 1994).

These cells also have co-stimulatory molecules that induce immunoglobulin switching in B cells and can help B cells in antibody production (Langhorne, 1996). They are localised within the spleen where it has been proposed that they have direct contact with the blood stage parasite leading to a direct cytolytic activity (Kumaratilake and Ferrante, 1994).

1.2 ERYTHROCYTE MEMBRANE STRUCTURE AND FUNCTION

The red cell membrane is undoubtedly one of the best examples of a biological membrane which has been studied in detail. The human erythrocyte provides a simple, experimentally accessible system for the study of membrane-cytoskeletal interactions at a biochemical level. This cell exhibits unusual durability as it survives thousands of passes through the circulation during its 120 days lifespan (Bennet, 1985). The durability derives from the mechanical properties of its plasma membrane. Structurally, the erythrocyte consists of an outer lipid bilayer, as well as integral and skeletal membrane proteins (Tanner, 1993). The structure of the red cell membrane is depicted in Figure 2.

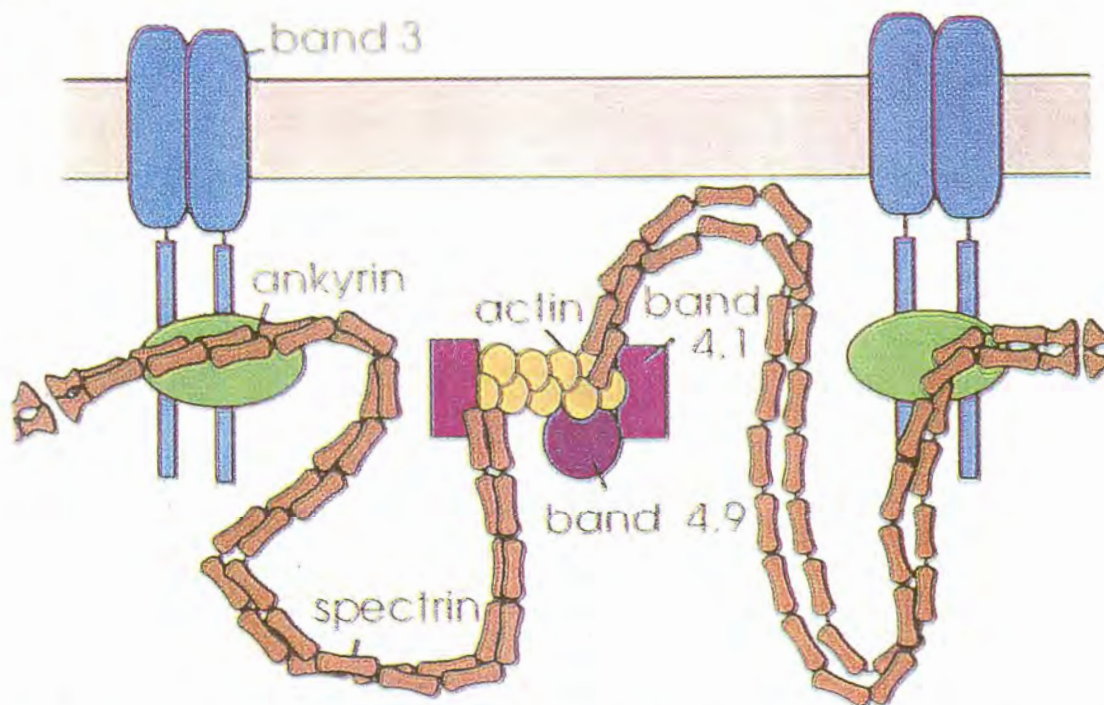


Figure 2 : Schematic presentation of the erythrocyte membrane and organization of its proteins.

1.2.1 Lipid bilayer

The bilipid layer of red cells is similar to that of other cells in that it is made of two leaflets of phospholipids with their hydrophilic heads interacting with either the external or internal environment. The hydrophobic tails form the mid-part of the leaflets. The red cell bilayer is distinguishable from that of other cells because its outer leaflet contains high amounts of phosphatidylcholine and the inner leaflet is rich in phosphatidylserine and -ethanolamine. The cholesterol of the membrane is found sandwiched between the two leaflets (Pasvol *et.al*, 1993).

1.2.2 Transmembrane proteins

The red cell membrane contains two major proteins, band 3 and glycophorin. The two proteins span the phospholipid bilayer with distinct domains expressed on the outer and cytoplasmic surfaces (Bennet, 1985).

Band 3 : This is a glycoprotein found in about 3 million copies in those cells which express the AB blood group. Band 3 consists of 911 amino acids, with the 360 which form the N-terminal being found on the cytoplasmic side, and with the 550 which form the C-terminal are associated with the transmembrane region of the phospholipid bilayer (Pasvol *et.al*, 1993). The N-terminus (cytoplasmic portion) is anchored to the red cell cytoskeleton via attachments to ankyrin, protein-4.1 and -4.2. The cytoplasmic portion also binds to the glycolytic enzymes aldolase, phosphofructokinase and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and also haemoglobin. The C-termini (transmembrane portion) are involved with anion transport (Bennet, 1985).

Glycophorin : There are five different types of glycophorins, A, -B, -C, -D and -E in close association with Band 3. Each erythrocyte contains about a million copies of these glycoproteins with 250 kDa copies contributed by glycophorin-A and -B (Pasvol *et.al*, 1993). Glycophorins are highly glycosylated and rich in sialic-acid containing O-linked tetrasaccharides (Bennet, 1985). The portion protruding externally serves as a binding site for lectins and viruses, and the sialic acid-rich portion is a receptor for *Plasmodium falciparum* during red cell invasion by the malaria parasite. Various blood group antigens are expressed on this glycoprotein. The cytoplasmic portion is short and binds protein 4.1 (Gratzer, 1981 ; Pasvol *et.al*, 1982).

1.2.3 Membrane skeleton

Underlying the erythrocyte's phospholipid bilayer is a continuous reticulum of "scaffolding" protein structures known as the membrane skeleton. The cytoskeleton provides an elastic structure essential for the proper functioning of this highly deformable cell (Bennet, 1985). The membrane skeletons consist of a cross-linked meshwork of peripheral proteins including spectrin, actin, band 4.1, adducing and other associated proteins. Spectrin molecules constitute most of the skeleton and are arranged as dimers horizontal to the lipid bilayer, interacting in horizontal contacts at localities rich in actin, protein 4.1 and adducing and, at the extreme ends, with each other. Ankyrin or protein 2.1 form vertical links between spectrin molecules and Band 3 (Byers and Branton, 1985 and Pasvol *et.al*, 1993). Intermolecular relationships between spectrin, adducing, band 3, ankyrin, actin and protein 4.1 and the individual interactive forces are dependent on the phosphorylation state of these molecules (Pasvol *et.al*, 1993). The intact red cell barrier formed by a combination of the phospholipid bilayer, integral membrane proteins and membrane skeleton makes the erythrocyte impenetrable to most infectious agents. However, pathogens like *Plasmodium* and *Babesia* do invade these cells (Chishti *et.al*, 1994).

1.3 THE ERYTHROCYTE AND MALARIA PARASITE INVASION

Merozoites released from the hepatic (pre-erythrocytic) or erythrocytic schizonts enter the circulation where they come in contact with membranes of uninfected erythrocytes. Invasion of the red cell is a highly specific and sequential process in which the merozoite attaches to a susceptible red cell and orientates itself such that its apical end is in close contact with the red cell membrane and slowly invaginates into the cell (Ward *et. al*, 1994 ; Pasvol *et.al*, 1982). The invasion of red cells by *Plasmodium falciparum* involves a number of steps including; **a.** recognition, **b.** reorientation, **c.** junction formation and **d.** entry. The merozoite and invasion processes are presented diagrammatically overleaf in Figures 3 and 4 respectively.

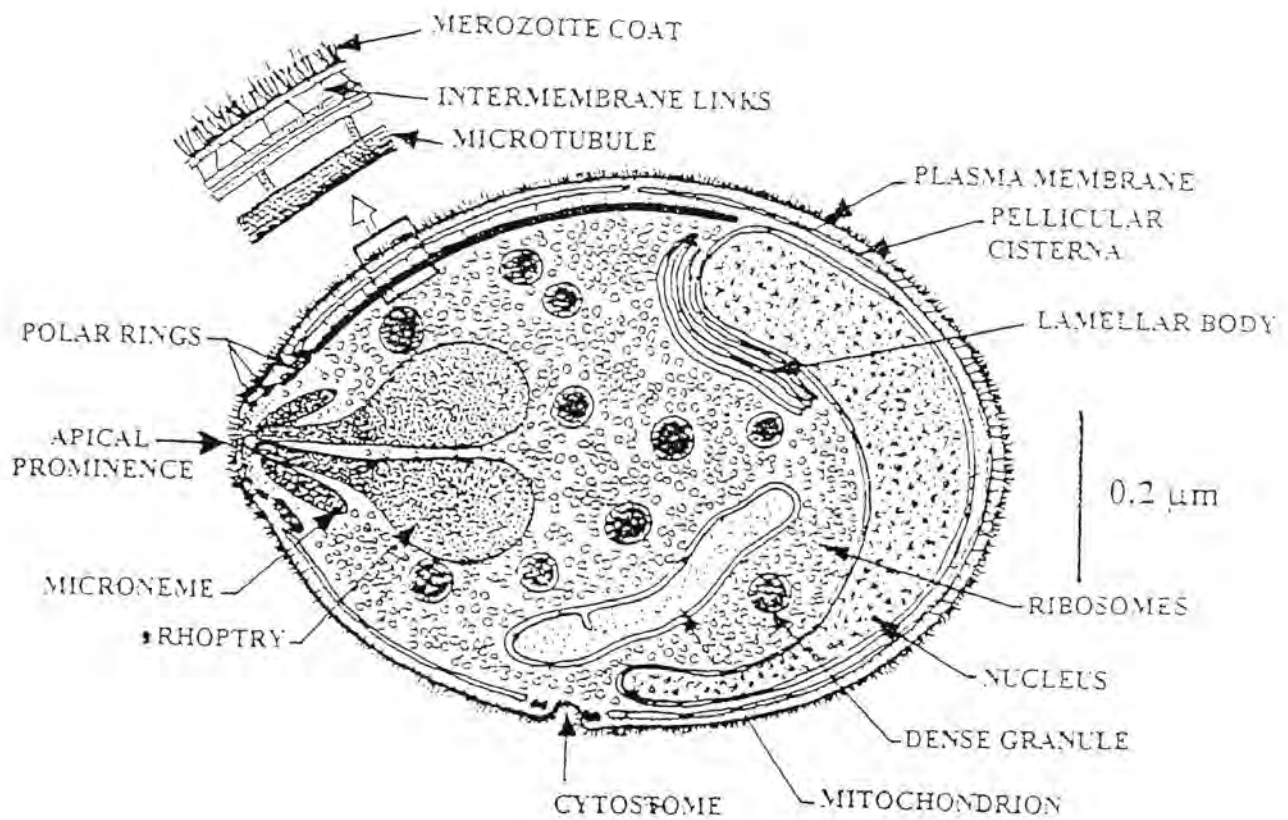


Figure 3: The structure of *Plasmodium falciparum* merozoite.

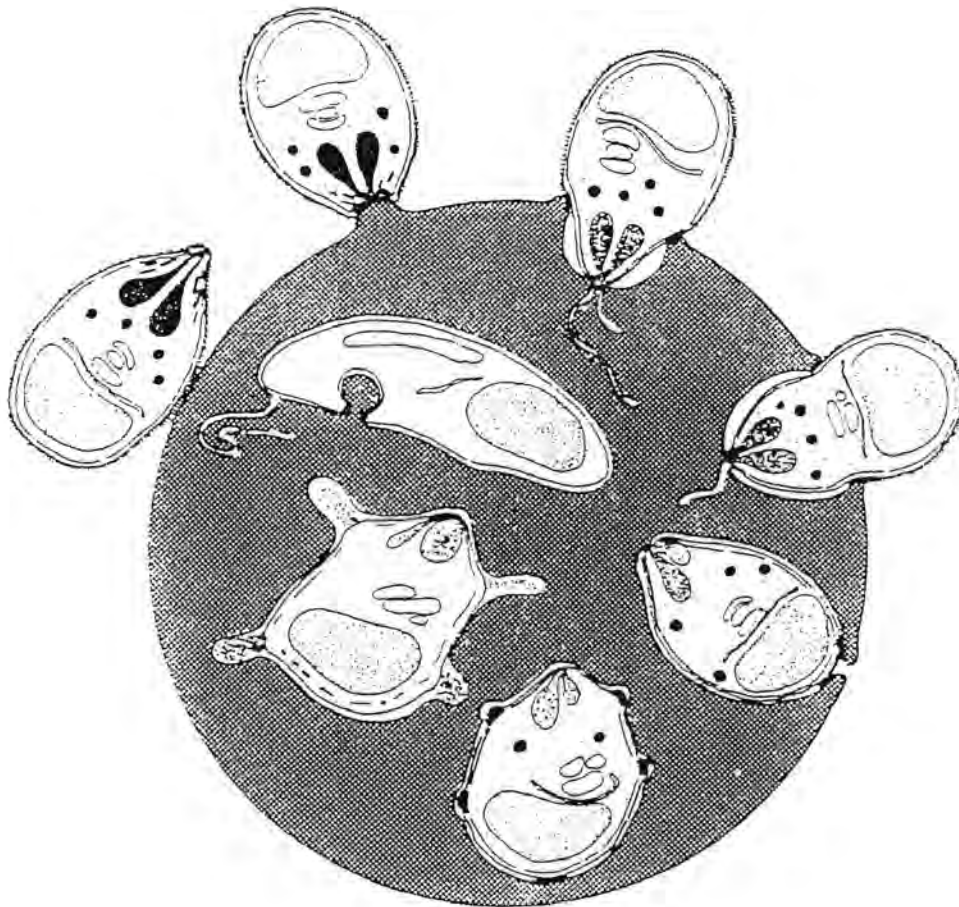


Figure 4 : Schematic presentation of the invasion of erythrocytes by merozoites.

Recognition: This is the first step which occurs when the merozoite collides with the red blood cell (RBC) and attaches loosely to the cell on any part of its surface. This initial encounter is mediated by interactions between the filamentous coat of the merozoite with the anionic glycocalyx of the RBC (Gratzer and Dluzewski, 1993 ; Bannister and Mitchell, 1995). The glycocalyx of the red cell is derived from sialoglycoproteins or glycoporphins and glycolipids. Glycophorin-A or -B are the major red cell receptors for the parasite's ligands, EBA-175 (erythrocyte binding protein) and MSP-1 (major surface protein), found in the late schizont stage and merozoites (Dluzewski *et.al*, 1983 ; Pasvol and Jungery, 1983 ; DeLuca *et.al*, 1996). Invasion through the glycoporphins is sialic acid-dependent. Neuraminidase and trypsin cleave part of the glycoporphin and reduce parasite invasion efficiency (Pasvol *et.al*, 1982 ; Dolan *et.al*, 1994; Sim *et.al*, 1994 ; Ward *et.al*, 1994 ; Sim, 1995).

Reorientation : The merozoite that has weakly attached to the RBC membrane brings its apical region in apposition with the red cell surface. A prominent pair of internal organelles are located at the apical region; (a) "tear drop-shaped" rhoptry and (b) elongated vesicular micronemes. Reorientation is a motile process as data confirms that the merozoite may pucker the RBC membrane at the contact zone (Gratzer and Dluzewski, 1993). The merozoite also has contractile proteins, actin and myosin filaments, in its cytoskeleton that assist in parasite motility (Bannister and Mitchell, 1995 ; Webb *et.al*, 1996 ; Bejon *et.al*, 1997).

Junction formation and entry : Receptor cross-linking due to interactions between parasite and red cell opens calcium channels and causes an increase in cAMP (cyclic adenosine monophosphate) and turnover of phosphatidylinositol within the merozoite. These biochemical events stimulate exocytosis of the contents of the rhoptry onto the red cell membrane (McCallum-Deighton and Horner, 1992).

A tight, undissociable junction forms between the merozoite and red cell causing convulsive disturbances of the red cell contour. The zone of contact is defined by an electron-dense junction. The area of the host cell membrane in contact with the junction is devoid of intra-membrane particles, (Matsumoto *et.al*, 1987 ; Wilson, 1990). Rhoptry contents e.g proteases, displace glycoproteins (band 3) and glycolipids of the RBC membrane as invagination continues.

As the invasion proceeds, smaller dense granules at the merozoite apical region are displaced to the apex, the parasite travels deeper into the interior and the junction or annulus moves backwards along its surface, while the filamentous coat of the merozoite is shed (Brenton *et.al*, 1992 ; Gratzer and Dluzewski, 1993 ; Foley and Tilley, 1995). Superficially, the parasite appears to encapsulate itself in the invaginated red cell. After full encapsulation, the RBC membrane closes leaving a vestigial plug attached at its distal end. The parasite is at this stage fully enclosed in a parasitophorous vacuole membrane (Bannister and Dluzewski , 1990 ; Wilson, 1990).

1.4 THE MALARIA-INFECTED ERYTHROCYTE

A mature normal red cell can be compared to a “sack of haemoglobin” designed for transport of O₂ and CQ during respiration. It contains no organelles and lacks synthetic and transport apparatus for proteins and lipids. It mainly acquires its nutrients from glycolysis and haemoglobin degradation from which essential sugars and amino acids respectively, are derived. Contamination of this cell by malaria parasites converts it into a fully operational eukaryotic cell (Foley and Tiley, 1995).

The parasitized cell is restructured both internally and externally enabling the parasite to survive within the hostile environment of the host cell. The membrane is modified so that new transport channels are attained and the metabolic activity of most macromolecules enhanced. The infected red blood cell (*i*RBC) also develops “bumps” on its surface due to newly expressed antigenic variants (Deitsch and Wellems, 1996).

These alterations are directed at solving two major “perils” or pitfalls faced by the intra-erythrocytic parasite:

- i trafficking of biosynthetic products to and waste products from the host cell so as to avoid starvation and toxicity and
- ii immune evasion by (a) acquiring adhesive surface properties to sequester and avoid splenic destruction and (b) evade immune recognition by rapidly varying exposed antigenic determinants (Atkinson and Aikawa, 1990 ; Berendt *et.al*, 1994). The factors indispensable for parasite survival will be detailed under membranous modifications and metabolic and transport properties.

1.4.1 Membrane modifications

The surface of a *Plasmodium*-infected red cell differs markedly from that of the normal red cell and Figure 5 overleaf shows the differences in membrane composition between the two. The new surface molecules that are expressed on the *i*RBC membrane serve various functions including: adhesion to other cells and extracellular matrix, protection against a variety of unfavourable biological, chemical and physical factors and the transduction of signals. The most detrimental environment confronting the circulating intra-erythrocytic parasite is an array of the host's immunologic machinery including bloodstream cytokines and antibodies and the spleen (Lingelbachi, 1993 ; Berendt *et.al*, 1994).

Falciparum malaria red cell membrane alterations become pronounced as the parasite develops from the ring to the mature stages i.e trophozoite and schizont stages. The alterations are mediated by proteins synthesised by the parasite and exported into the host RBC as well as by host-derived proteins (Leech *et.al*, 1984 ; Johnson *et.al*, 1994). The ring stage of infection is characterised by minimal alterations or deformations in the gross anatomy of the erythrocyte membrane. The ring-infected erythrocyte surface antigen (RESA) is the only protein that is anchored into the membrane at this stage of development (Sherman *et.al*, 1995). RESA is synthesized by mature stages and after merozoite invasion it is transported to the membrane surface where it interacts with spectrin and becomes phosphorylated by endogenous host kinases. It is suggested that RESA might be important in repairing the membrane skeleton following merozoite invasion and thus strengthening the host to allow continued parasite development (Foley and Tiley, 1995).

It is during the mature stages of development that major renovations occur in the RBC membrane. The mature parasite-infected cell becomes more adhesive and less destructible. These properties may be induced by changes in membrane lipid composition (Sherman *et.al*, 1997) and the basic build-up of host membrane protein and saccharide composition (Sherman *et.al*, 1995). The *i*RBC with mature parasite stages develops knob-like protuberances on the plasma membrane exterior surface (Figure 5).

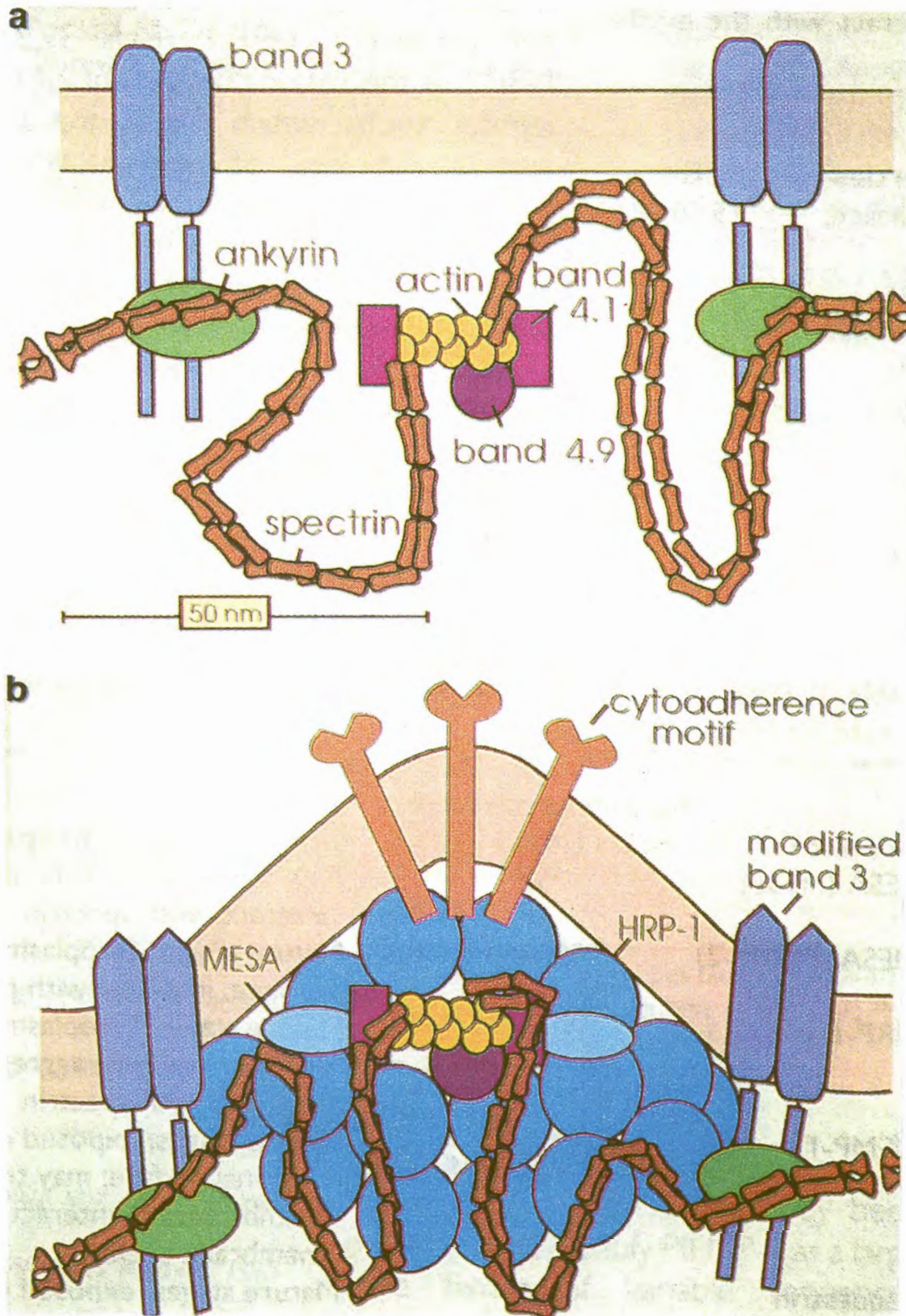


Figure 5 : Diagrammatic presentation of the membrane structural differences between normal (a) and parasite-infected (b) red blood cells.

The knobs are composed of parasite derived proteins. These are *Plasmodium falciparum* erythrocyte membrane protein-1 (PFEMP-1) and sequestrin, as well as host-derived protein, altered Band 3 protein (Pfalhesin) (Crandal and Sherman, 1991; Crandall and Sherman, 1994 ; Sherman *et. al*, 1995). Other parasite-derived proteins that are located sub-membranously include *Plasmodium falciparum* histidine rich protein-1 (PFHRP-1), *Plasmodium falciparum* erythrocyte membrane protein-2 and -3 (PFEMP-2 and -3). PFHRP-1 is the major constituent of the conical knobs and together with PFEMP-2 and -3 they stabilize the knob architecture (Sherman *et.al*, 1995).

The infected red cells adhere to endothelial cells through receptor-ligand associations between the iRBC's knob-constituting proteins (adhesions) and receptors on the endothelial cells in a phenomenon called sequestration (Udomsangpetch *et.al*, 1996). Sequestration is comprised of two processes, cytoadherence (adhesion of infected erythrocytes to post-capillary venular endothelial cells) and rosetting (adhesion of an infected red cell to two or more normal host erythrocytes) (Cooke and Coppel, 1995). The potential mediators of cytoadherence on the host endothelial cells identified to date are CD36, thrombospondin (TSP), intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and chondroitin sulphate A (CSA) (Gardner *et.al*, 1996 ; Baruch *et.al*, 1996 ; Rogerson and Brown, 1997).

Since endothelial receptors that mediate cytoadherence e.g VCAM-1 and CD36, are absent or rarely expressed on normal red cells then rosetting would be expected to be mediated through alternative mechanisms (Carlson *et.al*, 1990). The process of rosetting, in contrast to cytoadherence, is heparin-sensitive and highly dependent on calcium and magnesium ions (Udomsangpetch *et.al*, 1992). Infected erythrocytes exhibit fibrillar strands on their surfaces that have been implicated in mediating direct cell-to-cell interactions between iRBC and normal red cells. The major constituents of these strands are immunoglobulins of either the IgM and IgG classes, or a combination of both (Scholander *et.al*, 1996).

Sera from immune subjects and monoclonal antibodies to the abovementioned immunoglobulin classes has been shown to disrupt rosettes. Although the antibodies seem essential for the formation of the strands and rosetting of iRBC, other serum components may still play a vital role

in this process (Roberts *et.al*, 1992).

Red cells infected with mature parasites have also been shown to adhere *in vitro* to other cell types including platelets, monocytes, lymphocytes, umbilical vein endothelial cells, amelanomatic melanoma cells and other infected erythrocytes (Berendt *et.al*, 1990). Sequestered *i*RBC are responsible for most of the pathology following malaria infections which include vascular occlusion of the brain, causing cerebral malaria, as well as renal postcapillary venular endothelium, resulting in renal failure. Post mortem examinations of patients who died from cerebral malaria show increased rosettes and adhered infected red cells in the brain tissues (Carlson, 1990 ; Kaul *et.al*, 1991; Udomsangpetch *et.al*, 1996). In clinical infections, only the ring forms (without adhesive characters) are detected in the circulatory system, while the mature forms do not appear in the circulation as they have sequestered. Both cytoadherence and rosetting have never been correlated with disease severity, but can be described as being virulence determinants in malaria infections (Udomsangpetch *et.al*, 1992 ; Roberts *et.al*, 1993 ; Berendt *et.al*, 1994).

1.4.2 Transport and metabolic properties

After invasion of the red blood cell by malaria parasites, the infected cell exhibits increased membrane permeability to a variety of unrelated substrates including ions, amino acids, nucleosides and sugars (Kirk *et.al*, 1991). Enhanced membrane transport is accompanied by high levels of metabolic and biosynthetic activities. These activities become pronounced as the parasite develops from the metabolically inactive ring form to the mature stages i.e trophozoite and schizont stages, and they coincide with changes in the adhesive and antigenic properties of the *i*RBC (Elford and Pinches, 1992 ; Elford *et.al*, 1995). The new transport pathways facilitate the entry of metabolic and biosynthetic substrates into *i*RBC and export of toxic metabolites (Kirk *et.al*, 1994). The changes are represented diagrammatically in Figure 6 overleaf.

Transport in the *i*RBC is mediated by pathways or channels that have properties dissimilar to those known to operate in normal erythrocytes, but which show functional similarities to chloride channels in other cellular systems (Kirk *et.al*, 1993).

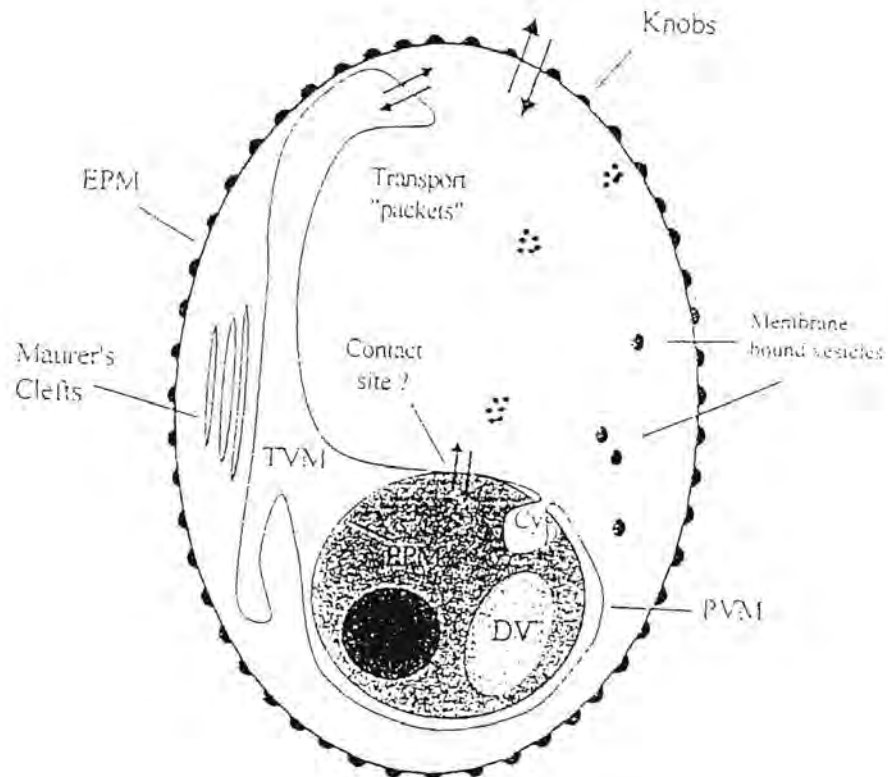


Figure 6 : Schematic presentation of the membranous structures and transport pathways of the infected erythrocyte. TVM, tubovesicular membrane network; PPM, parasite plasma membrane; PN, parasite nucleus; DV, digestive vacuole; EPM, erythrocyte plasma membrane; PVM, parasite vacuole membrane, $\uparrow \downarrow$, new trafficking sites introduced into the membranes.

Properties of the neotransporters include **(a)** anion selectivity with high permeability for chloride ions **(b)** high permeability for cations **(c)** non-discrimination between stereoisomers and other structurally unrelated molecules **(d)** non-saturability and **(e)** insensitivity to classical inhibitors of native transporters, but are inhibitable by most anion channel blockers e.g niflumate and furosemide (Kirk *et.al*, 1994 ; Kirk and Horner, 1995).

Ion metabolism: Ions are not really metabolized in *i*RBC, but the term is used as ions are highly implicated in various cellular metabolic activities (Tanabe and Cohen, 1990). Human erythrocytes maintain low levels of ions such as calcium (Ca^{2+}), potassium (K^+), hydrogen (H^+) and sodium (Na^+) (Desai *et.al*, 1991 ; Kirk and Horner, 1995). Upon invasion by the malaria parasite there is a marked decrease in the K^+ and Na^+ levels in the erythrocyte cytosol, while the parasite compartment has high K^+ and Ca^{2+} levels and low Na^+ levels. An increase in influx of calcium is evident at mature stages and the period after invasion (Tanabe and Cohen, 1990 ; Kirk *et.al*, 1994).

Malaria infection leads to changes in the host cell milieu, particularly an increase in Ca^{2+} concentration as the parasite matures and this enhanced Ca^{2+} influx is independent of Ca^{2+} channels (Garcia *et.al*, 1996). Within the *i*RBC, Ca^{2+} is located in the parasite and its fluxes are monitored by a Ca^{2+} -ATPase, an energy-dependent pump on membranes of infected cells (McCallum-Deighton and Holder, 1992). The parasitized red cell also has a Na^+ , K^+ ATPase, with decreased activity, that is partially sensitive to ouabain. This enzyme controls the movement of potassium ions into the parasite and sodium ions out to the red cell cytosol (Tanabe and Cohen, 1990 ; Gumila *et.al*, 1997).

Macromolecular metabolism: The malaria parasite derives most of its energy and nutritional requirements from glycolysis and catabolism of haemoglobin (Roth, 1990 ; Goldberg, 1994) and lacks the appropriate tools essential for the synthesis of proteins and lipids (Vial *et.al*, 1984 ; Elford *et.al*, 1995). The end-product of glycolysis in *Plasmodium falciparum*-infected red cells is lactate. This species of malaria parasite lacks the enzymatic capacities to pursue the citric acid cycle. Accumulation of lactate causes parasite acidification and death and the rates of glycolysis in the *i*RBC (100x more than normal cells) cause lactic acidosis and hypoglycaemia in patients

presenting with severe malaria (Kanaani and Ginsburg, 1991 ; Kirk *et. al.*, 1996). The parasite resides in the red cell that is rich in haemoglobin (Rosenthal and Meshnick, 1996). Haemoglobin is taken up into the parasite food vacuole via pinocytotic vesicles where it is degraded into globin and toxic heme (Slater *et.al.*, 1991 ; Meshnick, 1996). Globin is converted by aspartic protease (plasmepsins) and cysteine protease (falcipain) in the parasite's acidic digestive food vacuole into free amino acids and heme (Gluzman *et.al.*, 1994). The amino acids are necessary for protein synthesis, whilst heme is detoxified, after releasing essential iron, by polymerization into hemozoin (malaria pigment) (Goldberg, 1994 ; Francis *et.al.*, 1996 ; Rosenthal and Meshnick, 1996). The haemoglobin degradation pathway is shown in Figure 7.

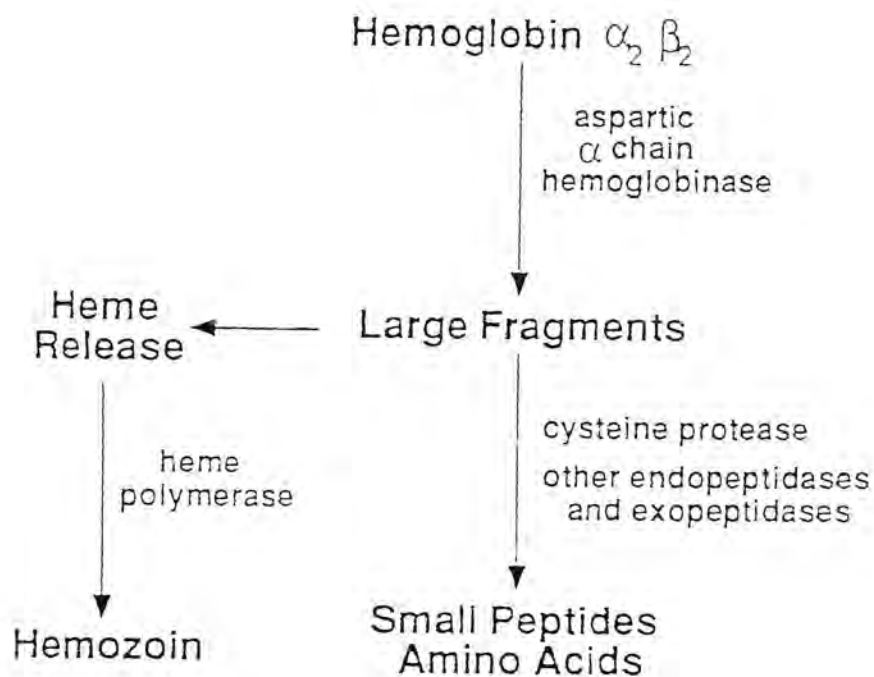


Figure 7 : Proposed haemoglobin degradation pathway in *Plasmodium falciparum*.



The parasite cannot synthesize proteins and phospholipids *de novo* and salvages most of the building blocks or precursors i.e fatty acids (e.g stearic acid), lipid polar heads (e.g ethanolamine) and essential amino acids (e.g glutamine) from the host cell's vascular compartment (Simoes *et.al*, 1992 ; Elford *et.al*, 1995).

1.5 TREATMENT AND PREVENTION OF MALARIA INFECTIONS

The mortality rate due to malaria infection is more than that of the recent Rwandan genocide with a child of under five years dying every 12 seconds in endemic areas (Butler, 1997). About one-third of the world's populace reside in areas where they risk contact with infectious mosquitoes (Hoessli *et.al*, 1996). Eighty percent of malaria cases occur in sub-Saharan Africa, while the remainder are confined to countries like India, Brazil, Sri Lanka, Vietnam, Colombia and the Solomon Islands (in decreasing order of prevalence) (Schrével *et.al*, 1996 ; Kharazmi *et.al*, 1997). The success in proper control and prevention of malaria depends on measures taken against both the parasite and the mosquito vector.

1.5.1 Antimalarial drugs

Quinoline-containing antimalarials still serve as the most useful for the prophylactic and chemotherapeutic treatment of falciparum malaria, although they have not been effective in every patient (White, 1992; Pussard and Verdier, 1994). Chloroquine, developed in the 1940s, is the most widely prescribed antimalarial in the world (White *et.al*, 1987) and is preferred for its safety, efficacy and low cost (Slater, 1993). Despite increasing resistance of *Plasmodium falciparum* to this drug in recent years, chloroquine remains the drug of choice for treating sensitive strains of this parasite and also for treating the other three human malarías (White, 1992; Foote and Cowman, 1994).

Drug research has led to the discovery of other antimalarials which are also now in clinical use. These antimalarial agents are divided into six classes (Gilles and Warrel, 1993):

1. Arylaminoalcohols : quinine, mefloquine, quinidine and halofantrine.
2. 4-aminoquinolines : chloroquine, amodiaquine, meprazine and pyronaridine.
3. Sulfones such as diaminodiphenyl sulphone and sulfadoxine and sulphonamides such as sulfene and cotrimoxazole.



4. Biquanides such as proquanil, triazine derivatives such as cycloquanil and chlorcycloquanil and diamine derivatives such as pyrimenthamine.
5. 8-aminoquinolines : primaquine.
6. Peroxide antimalarials : artemisinin derivatives including artemether, arteether and artesunate.
7. Antibiotics : tetracycline, doxycycline, clindamycin and fluoroquinolones.
8. Naphthoquinones : atovaquone.

1.5.2 Mechanisms of drug action

The precise mode of action of most antimalarials still remains to be elucidated, or is poorly understood, despite the global impact of the health and socio-economic problems caused by malaria (Ginsburg and Krugliak, 1992). This lack of progress in the field of malaria research might be due to insufficient funding, complexity of the subject or lack of scientific interest. The mechanisms of action of antimalarials in general use are as follows :

Quinine and quinidine.

These drugs are primarily blood schizonticides and exert their effects by increasing the intravesicular pH of the parasite thus inhibiting enzymatic systems crucial for parasite survival (Gilles and Warrel, 1993).

Chloroquine.

This agent is highly effective against asexual blood forms of all human plasmodial species except the chloroquine-resistant *Plasmodium falciparum*. Chloroquine also kills gametocytes of *P. vivax*, *P. ovale* and *P. malariae* and immature forms of *P. falciparum* (White and Ho, 1992 ; Gilles and Warrel, 1993). Chloroquine inhibits parasite growth through the following mechanisms :

*It binds to parasite's DNA thus inhibiting protein synthesis and causing parasite death (Meshnick, 1990 and Slater, 1993).

*Chloroquine binds to ferriprotoporphyrin IX, a product of infected red blood cell's (iRBC) haemoglobin catabolism, forming a complex toxic for the parasite (Ginsburg and Krugliak, 1992; Pussard and Verdier, 1994).



*Chloroquine accumulates to high concentrations within the parasite's food vacuole and inhibits heme polymerization with the free heme molecules being toxic for the parasite (Slater and Cerami, 1992).

Pyrimethamine/Sulfadoxine (Fansidar®)

Both drugs are blood schizonticides and pyrimethamine is active against all human plasmodium species, while sulfadoxine is used in the treatment of falciparum malaria only. The two drugs inhibit parasite proliferation through multipoint inhibition of the parasite's folate metabolism. Pyrimethamine and sulfadoxine inhibit the enzymatic activities of dihydrofolate reductase and dihydropteroate synthetases respectively. These two enzymes comprise the rate limiting step in the parasite's folic acid metabolism (White and Olliaro, 1996).

Primaquine

This drug is converted into active quinine metabolites in the liver where it acts on hypnozoites and gametocytes by inhibiting the parasite's mitochondrial respiration (White and Ho, 1992).

Artemisinin derivatives

These drugs exert their effects on asexual blood forms of the parasite. They are valuable for the treatment of chloroquine- and quinine-resistant parasite strains in severe and complicated forms of the disease. The mode of action appears to involve two distinct steps. In the first step, cleavage of the endoperoxide bridge of the drug is catalysed by intraparasitic iron and heme to generate unstable free radical intermediates. The resulting free radicals bind to oxidant-sensitive parasite proteins and as such alter their structure and activity which leads to parasite death (Meshnick *et.al*, 1996).

Antibiotics (doxycycline)

Doxycycline is active against exoerythrocytic parasite forms. It acts by inhibiting or interfering with the parasite's normal ribosomal protein synthesis (Gilles and Warrel, 1993).

Atovaquone

This agent is active against all stages of the malaria parasite and potentiates the activities of

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tetracycline and proquanil. It is structurally related to coenzyme Q and disrupts the parasite's electron mitochondrial electron transport chain (Srivastava *et.al*, 1997).

1.5.3 Treatment criteria of malaria infection

Plasmodium falciparum has the ability to resist treatment against most drugs developed against it to date, therefore the choice of an antimalarial drug depends on the knowledge of levels of drug sensitivity in the area of disease prevalence, as well as drug availability.

A mild, uncomplicated form of malaria is treated with the first line agent, chloroquine. Cases of uncomplicated chloroquine-resistant and severe malaria are curable with alternative drugs like mefloquine, quinine and halofantrine. Whenever possible, artemisinin analogues should be used in combination with mefloquine (Schelinger *et.al*, 1988 ; Meshnick, 1990).

Recommendations for malaria treatment in South Africa have been published (Baker *et.al*, 1993; Frean and Blumberg, 1993 ; Hansford, 1994). Chloroquine is still the drug of choice in South Africa, except in northern Natal, where doxycycline is used. For the rest of S. Africa and Africa, chloroquine in combination with mefloquine, proquanil or doxycycline remains the best option.

1.5.4 Pharmacokinetic and pharmacodynamic properties of antimalarials drugs

Most hospital deaths from severe malaria infections occur within 24 hours of admission, such that the typical treatment required to save lives should act rapidly before pathological processes become irreversible (WHO, 1990). The speed and effectiveness of antimalarial chemotherapy depend on many factors; including parasite biomass, development phase, intrinsic susceptibility to drugs as well as the status of the host immune response (White and Krishna, 1989).

Evaluation of treatment response depends on the clinical outcome i.e recovery from coma, fever clearance and mortality and parasitological outcome. Parasitological outcome is determined by a predictive index derived from (i) parasite clearance time (PCT), which is the time from drug administration until no asexual parasites are detectable in a peripheral smear and (ii) parasite reduction ratio (PRR) which measures differences between parasite count at initial time of treatment and 48 hours later. (White, 1997). In infections due to highly resistant strains, parasites

do not disappear from circulation after drug administration while in cases of mild resistance, parasites do disappear but recur at a later time with a return of symptoms whereas sensitive parasites are totally cleared from circulation (WHO, 1990).

In falciparum malaria, parasite stages in the first 24 hours of development appear in peripheral circulation while the mature forms are sequestered in deep vasculature. The pathological processes (fever, cerebral malaria and organ dysfunction) in malaria patients are related to the sequestered forms of the parasite and subsequent merogony (rupturing of merozoites to liberate new infective rings) and not the immature circulating forms (White and Ho, 1992). Large rings, trophozoites and schizonts are sometimes seen in the peripheral blood of severely ill patients and this may be due to an overspill from saturated vascular beds, or failure of splenic clearance. Occurrence of such an unusual phenomenon predicts a poor prognosis. Surges in parasitemia immediately after treatment are natural and must not be interpreted as reflecting some sort of drug resistance and the parasites are predominantly tiny rings (Silamut and White, 1993).

The stage and synchronicity of parasite development are important measurable parameters of immediate antimalarial efficacy, because drug sensitivity varies as the parasite develops. The dihydrofolate reductase inhibitor (DHFR) pyrimethamine and the quinolines quinine, mefloquine and quinidine do not significantly inhibit the asexual malaria parasites in the first half (24 hours) of the parasite life cycle (Geary *et.al*, 1989 ; Ter Kuile *et.al*, 1993). As a result, exposure of ring forms to these agents does not prevent sequestration and any decline in parasitemia after their administration would have occurred in their absence. Artemisinin, chloroquine and halofantrine increase the clearance of ring forms *in vivo* (Hassan *et.al*, 1992 ; Ter Kuile *et.al*, 1993). An early decline in parasitemia after treatment with these drugs is rapid as compared with treatment with those in the first category (White and Krishna, 1989). Due to its narrow time window of activity, pyrimethamine is only effective during the mature stages (short phase) of parasite development while artemisinin, with the broadest range, is effective at the early and longest phase of parasite development.

The ring and mature schizonts are the most drug-resistant forms during infection (Ter Kuile *et.al*, 1993). Most drugs that act on sequestered parasites inhibit trophozoite development with

consequent reduction of parasite multiplication rate and time for drug administration. Since sensitivity is stage-dependent, therapeutic response may be determined by timed drug administration such that appropriate synchronous stages of development are exposed to the drug (chronotherapy). This will serve to optimize anti-parasite treatment if peak drug levels coincide with the most susceptible stage of parasite maturation although this might be difficult in practice (Landau *et al.*, 1991). Advantages of chronotherapy remain to be further researched.

Semi-immune and immune patients respond better to treatment than non-immune patients. This last group of patients develop a potent strain specific immunity over a long period of time (about seven months) and in circumstances where they are infected by MDR strains they require more courses of antimalarial chemotherapy before they are cured. In endemic areas, the well-developed immune response will act together with administered drugs to ensure shorter treatment courses and better therapeutic responses even in cases when clinical cases are due to drug-resistant strains. In these instances, therapeutic response improves with age, coinciding with acquisition of immunity. Age is as such an important factor in endemic areas and should be considered during execution of clinical trials. During pregnancy the immune system is temporarily suppressed and these women are difficult to treat since the choice of drugs is limited.

1.5.5 Drug resistance

Drug resistance in malaria has been defined by the WHO as "the ability of the parasite to survive or multiply in the presence of drug concentrations that normally destroy parasites of the same species or prevent their multiplication". The resistance may be relative i.e. parasites destroyed by increased drug doses tolerated by the host, or complete i.e. parasites withstand maximum drug doses tolerated by the host (Wernsdorfer, 1991). The global resurgence of malaria is due mainly to the advent of drug-resistant parasites and insecticide-resistant mosquito vectors (Hansford, 1994).

The African crisis has triggered research into malaria infections. The parasite's resistance to chloroquine, the main prophylactic and chemotherapeutic agent in Africa, is spreading rapidly (Ridley *et al.*, 1996) and Fansidar[®] (cheap alternative to chloroquine) (White and Olliaro, 1996; Butler, 1997) seems destined to follow the same path of resistance as chloroquine. The absence

of efficient vaccines as well as cross-resistance, necessitates development of new antiparasitic measures (Schrével. *et.al*, 1996).

Responses of *Plasmodium falciparum* to drugs are graded according to the following categories (White and Ho, 1992 ; Gilles and Warrel, 1993) :

1 Sensitivity - clearance of blood parasitemia within seven days of the first day of treatment without recrudescence.

2 R1 resistance - clearance of parasitemia as in sensitivity but with delayed recrudescence.

3 R2 resistance - marked reduction of parasitemia without clearance.

4 R3 resistance - persistently high parasitemias.

1.5.5.1 Development and spread of drug resistance

The success of the malaria eradication campaign in the 1950s gave many countries a false sense of security and the sudden re-emergence of the disease has caught many unprepared. New control measures and treatments are desperately needed, particularly in sub-Saharan Africa, and by tourists and other people who visit the malarious areas (Vial, 1996 ; Butler, 1997).

Drug resistance was first described in Brazil in 1910 after treatment failure in malaria clinical cases with a quinine regimen (Wernsdorfer, 1991). Chloroquine-resistance in *Plasmodium falciparum* emerged in the 1950's in both South America and South-East Asia (Wernsdorfer, 1991) and it was first reported in Africa in 1979 from both Kenya and Tanzania (Foghs *et.al*, 1979 ; Kean, 1979). Drug resistance has been reported in all areas where malaria is endemic (Butler, 1997). In 1980, cases of chloroquine resistance were observed from the southern African states of Botswana, Mozambique, Angola, Namibia, Swaziland and Zimbabwe (Freese *et.al*, 1991 ; Freese *et.al*, 1993). Chloroquine resistance in South Africa was first reported in 1985 from Mpumalanga, Natal and Northern Province (Freese *et.al*, 1988 ; Soni *et.al*, 1993 ; Deacon *et.al*, 1994).

Mefloquine or Lariam® was first used as an alternative antimalarial for the treatment of chloroquine-resistant falciparum malaria in the early 1970's and resistance to this agent was first reported in Thailand in 1982 (Lambros and Notsch, 1984 ; Childs *et.al*, 1991 ; Mockenhaupt, 1995). This resistance pattern has since spread to South America (Brazil) and some African states

(Burkina Faso) at alarming rates (Bjorkman and Phillips-Howard, 1990). Mefloquine resistance is associated with halofantrine and quinine resistance but not chloroquine (Brasseur *et.al*, 1992; Cowman *et.al*, 1994). Multidrug resistance patterns involving quinine/mefloquine/halofantrine or Fansidar® have also been detected and this is a serious public health threat in Thailand, Indonesia, Papua New Guinea and Pakistan (Strickland *et.al*, 1986 ; Qilin *et.al*, 1988., Nateghpour *et.al*, 1993 ; Karbwang *et.al*, 1994). The geographical distribution of chloroquine resistance is shown in Figure 8 (Wernsdorfer, 1991). An inverse correlation between chloroquine and mefloquine resistance (Mockenhaupt, 1995) confirms reports of reduced chloroquine resistance in areas where mefloquine sensitivity is decreasing (Thaithong *et.al*, 1988). The positive correlations do not imply that resistance to one drug will automatically be expressed in the other, but this will eventually hasten expression of such a resistant trait as shown in other studies (Childs *et.al*, 1991).

Amazingly, quinine has been used for treating malaria for the past 350 years (Meshnick, 1997) and moderate resistance has only been mapped to limited areas (Pukrittayakamee *et.al*, 1994). There might be two possible factors contributing to this uniqueness : (i) the intraparasitic target of quinine might be such that specific mutations conferring resistance do so at a very slow rate and (ii) the infrequent use of quinine might have led to slow exertion of drug pressure and resistance development. Understanding quinine's longevity could have important implications, it could serve as a lesson to malariologists when forging plans to preserve the efficacy of antimalarials.

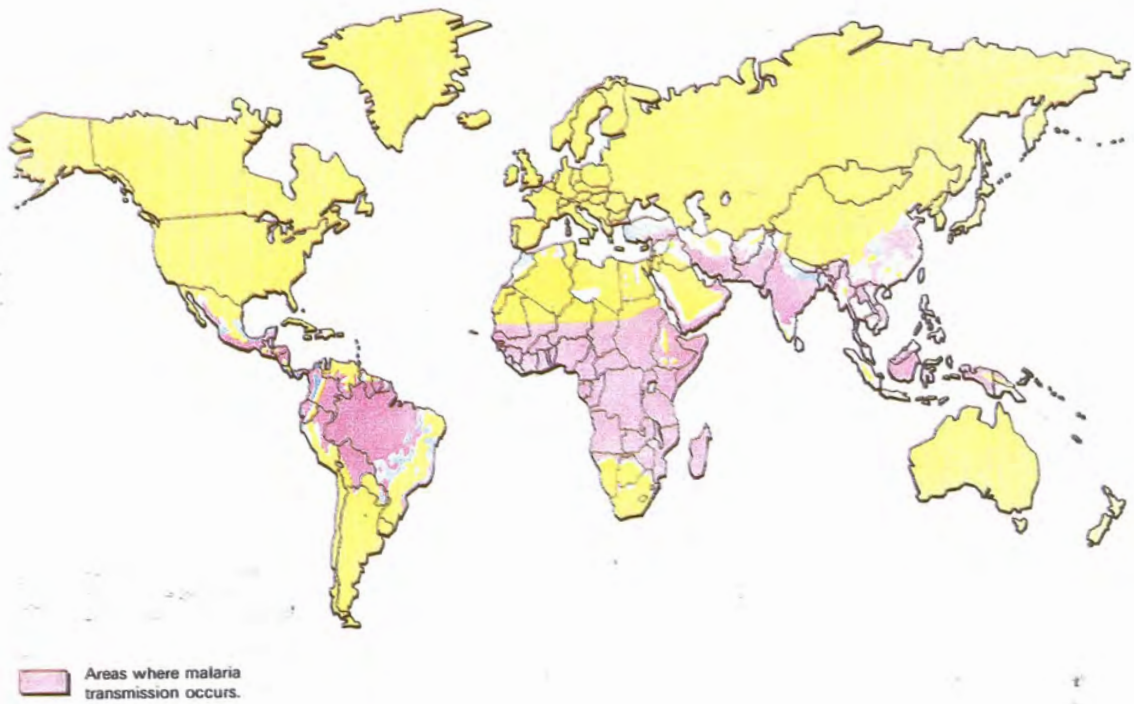


Figure 8 : Geographical distribution of chloroquine resistance.

1.5.5.2 Mechanisms of drug resistance

The mechanisms responsible for decreased drug sensitivity are important when one tries to determine the speed by which resistance develops. For instance, if resistance is conferred by a single point mutation, e.g a single base pair change in the dihydrofolate reductase gene is enough to confer resistance to sulfadoxine/pyrimethamine, then a switch from sensitive to highly resistant parasites occurs abruptly (Peterson *et.al*, 1990; Sirawaporn *et.al*, 1997). The typical drug will be vulnerable and have a short life span. The elimination kinetics of particular antimalarials also serve as determinants for the development of drug resistance (Watkins and Mosobo, 1993). Generally, drugs with long elimination half-lives are vulnerable to develop decreased sensitivity patterns because parasites are exposed to sub-therapeutic drug concentrations for long periods and this continued exposure to inhibitory, and not eliminatory, drug levels acts as a selective pressure for the development of drug resistance (White and Olliaro, 1996). Short-lived i.e rapidly eliminated, drugs like quinine and artemisinin require multiple administration and longer treatment courses which ultimately leads to treatment failure due to poor compliance. However, short-lived drugs are relatively safe since they are present at subtherapeutic concentrations for shorter periods such that the parasite acquires selective pressure for resistance development at a slow rate (White, 1992).

In the absence of resistance, drug pressure will occur when patients acquire a new infection while they still have sublethal levels of an antimalarial in their blood from treatment of a previous infection (White, 1987). The likelihood of resistance developing from the original infection during or after treatment depends on factors such as parasite biomass, drug blood concentration, stage specificity of the drug and synchronicity of the infecting parasites (Gassis and Rathod, 1996). Generally, parasites exhibiting resistance-conferring genes exist at low frequencies within an infecting population, hence the larger the biomass, the greater the chances that treatment will fail (White, 1992). Drugs with weak intraparasitic activity also provide a vessel for selecting resistant mutants because a higher number of parasites in each successive life cycle will be viable after the onset of treatment (Peters, 1987). New antimalarial agents that appear to be active *in vitro* may be of limited clinical use if the parasites develop resistance easily and the agents fail to clear parasites after a single course of treatment (Gassis and Rathod, 1996).

The exponential increase in antimalarial drug resistance might be blamed on three serious errors committed by researchers in this field of specialization : (i) malariologists were of the opinion that malaria parasites respond to drugs differently as compared to bacteria, (ii) persistent use of single drug treatment (monotherapy) even though treatment failure was apparent and (iii) use of empirical drug mixtures (sometimes commercially dictated) without any experimental information to support selection (Peters, 1987). Drugs used in combinations should have compatible pharmacokinetics and no additional toxicity. In drug combination chemotherapy, the possibilities of a resistant mutant developing are reduced because the parasite is not exposed to one drug and the second drug has a small biomass to eradicate whilst at maximum blood concentrations (White, 1997).

The declining efficacy of chloroquine and mefloquine as potent antimalarials and the resultant drug insensitivity can be attributed to factors such as increased drug demand, cross resistance with other antimalarials and the parasite's innate resistance. The mechanisms of drug resistance in falciparum malaria infection, especially chloroquine resistance, still remain unresolved despite accumulated scientific evidence documented thus far (Bray *et.al*, 1992 ; Ward *et.al*, 1995).

There are striking similarities between drug resistance in *Plasmodium falciparum* and the P-glycoprotein-mediated MDR phenomenon occurring in mammalian tumour cells (Cowman, 1991). MDR-typed tumour cells can expel a vast array of chemically and structurally unrelated antitumour agents, thus preventing accumulation of lethal doses of the drugs within the cells. The drug effluxing property is conferred by amplification of MDR genes and over expression of a 170kDa protein molecule, P-glycoprotein, that is located on membranes of drug-resistant tumour cells. This protein molecule derives energy from ATP to pump antitumour drugs out of target cells (Krogstad *et.al*, 1988 ; Van der Heyden *et.al*, 1995).

Chloroquine targets the intra-erythrocytic stages of Plasmodium parasites (Zhang *et.al*, 1986) . These parasite stages ingest the erythrocytic haemoglobin as the major nutrient source. The toxic heme moiety released during the catabolic process is polymerized into insoluble and inert hemozoin or malaria pigment (Slater, 1991). Chloroquine, a weak base, accumulates within the acidic parasite food vacuole to high concentrations, where it exerts its specific antimalarial effect

by inhibiting the polymerization of heme (Slater and Cerami, 1992). *In vitro* experiments demonstrated that chloroquine at millimolar concentrations inhibits hemepolymerization in extracts prepared from both chloroquine-sensitive (CQS) and chloroquine-resistant (CQR) parasite isolates (Slater and Cerami, 1992 ; Dorn *et.al*, 1995). *In vivo*, however, inhibitory levels of chloroquine are only attained in the vacuoles of CQS parasite lines, while CQR parasites accumulate significantly less chloroquine (Fitch, 1970 ; Fitch, 1983). Decreased chloroquine accumulation seems to be the basis of resistance suggesting that CQR lower their vacuolar chloroquine levels below that sufficient to inhibit heme polymerization.

Two different models have been proposed to clarify the differences in chloroquine accumulation as it occurs in CQS and CQR parasite clones. The first model, reminiscent of that of MDR in cancer cells, invokes that CQR parasites have a rapid chloroquine efflux mechanism that is conferred by amplification or mutation of MDR-like genes (Foote *et.al*, 1989 ; Foote *et.al*, 1990). This model has however been invalidated due to lack of linkages between levels of MDR expression and chloroquine resistance in a CQR phenotype (Wellems *et.al*, 1990). The second model proposes that CQR parasites have an elevated pH that would reduce acidotropic accumulation of the weak base chloroquine, a phenomenon termed "weak base hypothesis" (Ginsburg and Stein, 1991). This model or the "weak base hypothesis" is contradicted by the absence of detectable pH differences in the vacuoles of CQR and CQS parasite isolates (Krogstad *et.al*, 1985 ; Krogstad *et.al*, 1992). Both of these models were based on the assumption that chloroquine permeates the parasite by simple diffusion and not by an active uptake system.

Sanchez and colleagues (Sanchez *et.al*, 1997 ; Wunsch *et.al*, 1998) have recently proposed a third model that clarifies the differences in chloroquine accumulation between CQR and CQS parasite lines. Their work provides compelling evidence that a carrier-mediated transport system is responsible for chloroquine uptake in *Plasmodium falciparum* parasites emanating from observations that chloroquine uptake was found to be temperature dependent, saturable and inhibitable. These observations rule out the possibility of chloroquine being taken up by the parasite through passive diffusion pathways as it occurs in normal red blood cells (Ferrari and Cutler, 1990). Since the uptake of chloroquine was found to be inhibited by amiloride derivatives e.g 5-(N-ethyl-N-isopropyl) amiloride (EIPA), that specifically and reversibly inhibit eukaryotic

Na^+/H^+ exchangers (Kleyman and Cragoe, 1990), then a plasmodial Na^+/H^+ exchanger (NHE) was implicated as the chloroquine importer (Sanchez *et.al*, 1997). The plasmodial NHE is located within the parasite's plasma membrane and it helps in the maintenance of the parasite's cytoplasmic pH by expelling excess protons (H^+) generated during metabolism in exchange for sodium ions (Bosia *et.al*, 1993).

The proposed model for chloroquine uptake by NHE is as follows : chloroquine permeates the erythrocyte membrane by diffusion and activates NHE, as shown by transient increased efflux of protons and sodium ions influx. It is during this initial activation phase that chloroquine is taken up into the parasite cytoplasm. NHE reaches maximal activity (activation phase) and chloroquine import ceases. This model occurs only in CQS parasites. The CQR parasites seem to express an NHE that is constitutively activated that is incapable of concentrating chloroquine within the parasite (Sanchez *et.al*, 1997). It was based on these data that the inability of chloroquine to stimulate its own uptake by the constitutively activated NHE of CQR parasites form a minimal and important event in the generation of the CQR phenotype. A constitutively activated phenotype may result from mutations within the importer itself or factors regulating its activity, such as kinases and accessory binding proteins (Noel and Pouyssegur, 1995). Therefore a plasmodial NHE, or a factor regulating its activity, might reside within the chloroquine resistance gene locus as defined by the genetic cross between CQR and CQS parasites (Wellems *et.al*, 1991).

It should also be noted that extrapolation of data obtained from MDR cancer cell studies to chloroquine-resistant *Plasmodium falciparum* is undermined by some fundamental differences between these two entities, namely (Krogstad *et.al*, 1988 ; Ginsburg and Stein, 1991) :

- a Antitumour agents permeate into target cells more slowly than antimalarial drugs.
- b Resistant lines in both systems accumulate less drug than their sensitive counterparts, but upon metabolic deprivation i.e absence of glucose and subsequent depletion of ATP leading to insufficient energy supply for the P-glycoprotein efflux pump, drug levels in cancer cells revert to normal while this is not the case with malaria parasites.
- c The MDR reversal agents in cancer cells will totally restore drug sensitivity, while drug resistance modulators in malaria parasites lead only to partial restoration of drug sensitivity.

d The MDR pattern for cancer cells is not the same as that of plasmodium parasites i.e cancer cells can become resistant to various unrelated agents while this is not the case with malaria parasites, which only become resistant to specific related agents.

1.5.5.3 Overcoming drug resistance

Research into the development of new drugs and novel and effective vaccines, as well as new strategies for effective deployment of control measures is essential (Olliaro and Trigg, 1995). Regrettably, the recent, untimely withdrawal of most pharmaceutical companies from vaccine research and development of antimalarials has exacerbated this problem (Di Masi *et.al*, 1991 ; Butler, 1997).

ANTIMALARIAL DRUGS

New strategies for developing agents with novel or original mechanisms of action are crucial for the proper control, treatment and eradication of this endemic disease. General approaches followed in this research and development endeavour include:

(i) improving the structure-function properties of classical antimalarial drugs (chloroquine) aiming for enhanced antiparasite activity and reduced resistance (Ridley *et.al*, 1996 ; Sánchez-Delgado *et.al*, 1996).

(ii) developing agents with the ability to enhance the activity of, or reverse drug resistance to classical antimalarials (Bitoni *et.al*, 1988 ; Frappier *et. al*, 1996 ; Gumila *et. al*, 1997).

(iii) developing compounds with novel biochemical targets so as to avoid cross-resistance (Darkin-Rattray *et.al*, 1996).

(iv) develop pharmacological agents with less toxicity to the host compared to presently used drugs (Kirk and Horner, 1995 ; Adovelande and Shrevél, 1996 ; Vial, 1996).

(v) manufacture insecticides that can eliminate mosquito reservoirs but maintain environment friendly properties (Freaan, 1996 ; Vial, 1996 ; Butler, 1997) and

(vi) identify potent vaccine candidates with either anti-sexual or -asexual capabilities (Leach *et.al*, 1995 ; Scheller and Azad, 1995 ; Butler, 1997). The blood stages of the malaria parasites are the major cause of the clinical manifestations associated with the disease and as such most chemotherapeutic interventions are directed against this phase of parasite growth. An effective antimalarial should clear the parasite at all developmental stages within a short time period



(Cabantchik, 1989).

Recently two groups in France have evaluated ionophores for their therapeutic efficacy in laboratory and animal settings of malaria infections (Adovelande and Schrevél, 1996 ; Gumila *et.al*, 1997). A variety of these compounds was tested and showed *in vitro* antimalarial activity at nano- and pico-molar concentrations i.e 25 to 30 000 fold more active than chloroquine against all stages of the parasite and some, such as nigericin and monensin, had synergistic activities, thus permitting usage of these ionophores at lower doses so as to prevent development of drug resistance. These agents also had impressive bioactivities in animal models of malaria infection (*Plasmodium chabaudi* and *Plasmodium vinckei petteri*).

Sánchez-Delgado and co-workers (Sánchez-Delgado *et.al*, 1996) have developed a new approach to drug development by changing drugs with known or potential antiparasite activity through addition transitional metals into their molecular structures. Their two compounds, chloroquine metal-based complexes, ruthenium- and rhodium-chloroquine are highly active against *Plasmodium falciparum* and *Plasmodium berghei* and they appear to be nontoxic.

Quinoline-containing antimalarials are among the most widely used drugs for treating malaria (Ginsburg and Krugliak, 1992). Modified forms of this class of antimalarials, bisbenzylisoquinolines (BBIQ) e.g fangchinoline, have been documented as displaying antiparasite activity against chloroquine-susceptible and -resistant lines of *Plasmodium falciparum* and synergise with chloroquine and artemisinin. Interestingly these BBIQ's also reverse resistance in multidrug-resistant tumour cells (Frappier *et.al*, 1996).

A number of agents that potentiate the activities of existing antimalarials have already been tested (Bray *et.al*, 1994). Calcium channel blockers (verapamil) and calcium channel antagonists of various chemical classes (Martin *et.al*, 1987), tricyclic antidepressants (desipramine) (Bitoni *et.al*, 1988 ; Basco and Le Bras, 1990), tricyclic antihistamines (cyproheptadine) (Peters *et.al*, 1989) phenothiazines and calmodulin inhibitors (Watt *et.al*, 1990) have been shown to reverse chloroquine resistance in *Plasmodium falciparum* *in vitro* and *in vivo* in *Aotus* monkeys and *P. berghei*-infected mice. Desipramine has also been the subject of clinical trials in Somalia, but failed

to resolve malaria infection symptoms in chloroquine-resistant infected patients. This ineffectiveness might be attributable to the plasma-binding properties of the agent (Warsame *et.al*, 1992 ; Boulter *et.al*, 1993). On the other hand, clinical studies with verapamil are complicated by the toxic side effects associated with this compound (Watt *et.al*, 1990).

A recent report provided by the Antimalarial Drugs Focus Group documents a general review of drugs currently available, drugs in clinical studies, drugs in transition from pre-clinical to clinical studies , drugs in preclinical studies and potential antimalarial drug targets. It was generally agreed in this meeting that the movement of new agents from basic to clinical research has gained momentum. The lead compounds under clinical scrutiny include, protease inhibitors, maleperox compounds, biguanides, acridine orange and ferrocene chloroquine.

Antitumour agents such as plant-derived taxoids, taxol and taxotere, have also shown potent antischizonticidal activity in both chloroquine-resistant and -susceptible laboratory strains of *Plasmodium falciparum*. A single injection of taxotere or docetaxel significantly inhibited growth of *Plasmodium vinckei* in mice 5 days after induction of experimental infection (Pouvelle *et.al*, 1994 ; Sinou *et.al*, 1996).

The new transport processes and metabolic activities acquired by infected red cells also serve as potential chemotherapeutic targets (Kirk and Horner, 1995). A number of selective inhibitors have been studied with arylaminobenzoates e.g (5-nitro-2-(3-phenylpropylamino)benzoic acid) (NPPB) being the most active inhibitor of parasite-induced transport of small ions and solutes like chloride ions and choline. Development of this class of agent is important since most of them do not interfere with transport or metabolic systems in host tissues (Cabantchik, 1989; Kirk and Horner, 1995).

The need for lipids for extensive membrane biogenesis accompanying parasite maturation in erythrocytes has also attracted major attention as a potential drug target. The agents lovastatin and simvastin have been evaluated for *in vitro* antimalarial activity and promising results have been derived from the studies (Grellier *et.al*, 1994 ; Schrével. *et.al*, 1996). They both retard parasite growth by inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

CoA), an enzyme used by the parasite to convert acetyl coenzyme A (acetyl CoA) to farsenyl pyrophosphate during the cholesterol metabolism.

VACCINES

Preliminary testing of a vaccine developed by SmithKline Beechman Biologicals and the Walter Reed Army Institute of Research in America has raised the hopes of malaria researchers since the vaccine was found to protect six out of seven volunteers exposed to repeated mosquito bites. The vaccine has been targeted against the sporozoite's major surface protein, circumsporozoite protein, and is administered with adjuvants that potentiate the immune response (Duffy and Kaslow, 1997). Sensitization of volunteers with sporozoites from irradiated mosquitoes also led to resistance to subsequent exposure to infective mosquitoes, although the execution of field trials is impractical. Another pre-erythrocytic vaccine showing positive results in rodents and primate models is directed against the merozoite surface protein, MSP-1 (Butler, 1997).

Another class of vaccine under investigation is the "transmission blocking vaccines" that interrupt fusion and development of parasitic sexual stages within the mosquito. These vaccines do not necessarily protect individuals, but reduce transmission rates by infected mosquitoes (Sinden, 1997). The highly publicised and controversial vaccine (SPf66) developed by Colombian biochemist Manuel Pattaroyo is being subjected to extensive field studies with the QS21 adjuvant.

VECTOR CONTROL

Recent research projects on vector control are focusing on developing transgenic mosquitoes incapable of transmitting infections. Release of these genetically-engineered insects in malaria-endemic areas would theoretically result in spreading the non-infective gene trait in wild types (Butler, 1997). The use of DDT, an agricultural pesticide has been abandoned due to the development of resistance to this agent by mosquitoes and its association with contamination of agricultural products destined for human consumption (Curtis et.al, 1987). A recent alternative has been to add pyrethroids to bed nets and this technique has proved a cost effective vector control measure compared to DDT spraying and is now widely termed insecticide-treated bed net (ITBN). Pyrethroids like deltamethrin and permethrin have already been employed in field trials (Zuzi *et.al*, 1989 ; Bozhao *et.al*, 1998). Over the years numerous approaches to counter malaria

infections have been devised without any proper or full implementation. An infrastructure is required for malaria control especially in Africa and governments as well as the pharmaceutical industry should play a pivotal role in coordinating and funding such initiatives.

1.6 RIMINOPHENAZINES

1.6.1 Classification and pharmacology

Riminophenazines were first discovered in the laboratories of the Irish Medical Research Council in Dublin (Barry *et.al*, 1957). The prototype structural formula, as shown in Figure 10, is modelled on anilinoaposafranine and consists of a phenazine nucleus with substituent side chains at position 2, 3 and 10 of the phenazine core. Oxidation of the *o*-phenylenediamine derivative with certain ketones results in a new phenazine type, glyoxalino-phenazine which, upon further catalytic hydrogenation, yields imino-substituted compounds, riminophenazines (Barry *et.al*, 1957; Van Rensburg *et.al*, 1997). The prototype riminophenazine, clofazimine [3-(*p*-chloroanilino)-10-(*p*-chlorophenyl)-2,10-dihydro-2-(isopropylimino)-phenazine] or B663 was first described as antituberculosis agent by Barry and colleagues (Barry *et.al*, 1957). Various analogues have been developed to date (O'Sullivan *et.al*, 1988 and ; Savage *et.al*, 1988).

Clofazimine is administered as a microcrystalline suspension in an oil-wax base so as to increase its absorption (Yawalkar and Fischer, 1978). Administration of 200mg of the drug with food results in peak plasma levels of 0.41ug/mL in 8 hours. However, when taken without food, much less drug is absorbed (Schaad-Lanyi *et.al*, 1987). Leprosy patients achieve serum levels of 0.7, 1 and 1.4ug/mL with daily oral administration of 100, 300 and 400mg of clofazimine (Yawalkar and Fischer, 1978) while leprosy patients receiving 600mg/day of oral clofazimine have average serum levels of 4ug/mL (Schaad-Lanyi *et.al*, 1987). Pharmacodynamic investigations of clofazimine have shown that the drug concentrates in tissues with high fat content, reticuloendothelial components and organs highly perforated by the vascular system such as the liver, with almost zero quantities detected in the brain. The half life of clofazimine in the tissues is 70 days and it is secreted mainly through the bile (McDougall *et.al*, 1980). The main side-effects associated with clinical use of clofazimine are reddish-brown pigmentation of skin, conjunctivae and urine and crystal drug deposition leading to abdominal discomfort and splenic infarction. These effects mostly disappear on cessation of treatment (Moore, 1983 ; Arbiser and

Moschella, 1995). Other uncommon side-effects include ichthyosis, pruritis, phototoxicity, acneform eruptions, exfoliative dermatitis and non-specific rash. Gastrointestinal complications such as diarrhoea, anorexia, constipation, nausea and weight loss have been reported (Hastings *et.al*, 1976; Moore, 1983).

1.6.2 Clinical applications

Clofazimine is presently used as a component of the anti-leprosy regimen and (World Health Organization, 1990) recommended in the treatment of *Mycobacterium avium* infections in AIDS patients (Van Rensburg *et.al*, 1997). A broad range of *in vitro* and *in vivo* biological activities have been attributed to clofazimine and its analogues. Studies by Savage and co-workers (Zeis *et.al*, 1987 ; Savage *et.al*, 1988) relating to the structural properties of clofazimine and its analogues indicate that the biological activities of these compounds are dependent on the substituent chemical group at position 2 of the phenazine nucleus and the chlorination or halogenation patterns at positions 3 and 10. Collaborative research work (Jagannath *et.al*, 1995; Reddy *et.al*, 1996) between doctors J.F O'Sullivan (Chemistry Department, University College Dublin, Ireland) and M.V Reddy (Mycobacterial Research Laboratories, University of Illinois, Chicago) has led to the development of analogues such as B746, B4101, B4154 and B4157 with improved bactericidal activity in experimental models of tuberculosis i.e *in vitro* and *in vivo* settings. Further collaborative initiatives between Dr. JF O'Sullivan (Chemistry Department, University College Dublin, Ireland) and members of the Department of Immunology, University of Pretoria, South Africa has established novel biological activities of riminophenazines distinct from their classical anti-mycobacterial properties (Van Rensburg *et.al*, 1997).

1.6.2.1 As anti-inflammatory and pro-oxidative agents

Various riminophenazines have been shown to have anti-inflammatory and immunosuppressive properties. These properties result from the ability of the agents to contribute to the production of immunosuppressive prostaglandins (PG), especially prostaglandin E₂ (PGE₂) by polymorphonuclear and mononuclear leukocytes (Zeis *et.al*, 1987). Anderson and colleagues (Anderson *et.al*, 1988) have also shown that the immunosuppressive and anti-inflammatory activities of clofazimine and B669 [3-anilino-10-phenyl-2,10-dihydro-2-(cyclohexylimino)-phenazine] are related to their anti-proliferative effects on lymphocytes. This inhibitory effect has

been shown to be exerted through inhibition of the lymphocyte Na^+/K^+ ATPase by a lysophospholipid-dependent mechanism. These activities make the agent clofazimine valuable for the treatment of immunological reactions such as erythema nodosum leprosum in leprosy patients (Imkamp, 1968). Various other non-mycobacterial chronic inflammatory diseases of the skin such as discoid lupus erythematosus (Mackey and Barnes, 1974 ; Krivanek *et.al*, 1976), pyoderma gangrenosum (Michaëlson *et.al*, 1976 ; Kaplan *et.al*, 1992) and pustular psoriasis (Landow, 1988) are also treatable by clofazimine.

Clofazimine and B669 also potentiate the antimicrobial activity of human phagocytes (neutrophils) by enhancing the production of lysosomal enzymes and the respiratory burst enzyme, NADPH oxidase, leading to increased production of the microbicidal reactive oxidant species such as superoxide anion and hydrogen peroxide (Krajewska and Anderson, 1993 and Anderson, 1995). NADPH oxidase is the electron-transporting membrane-bound enzyme of phagocytes that is sensitive to activation by leucoattractants, cytokines and opsonized particles (Baggiolini *et.al*, 1993). The production of reactive oxidants is mediated by a variety of signal transduction components of the phagocyte membrane including phospholipases that act in combination or individually depending on the binding signal on the membrane receptor. The secondary messengers generated by these transduction mechanisms serve to activate cytosolic protein kinase C (PKC) that, through phosphorylation-dependent pathways, initiates the pro-oxidative activities of NADPH oxidase that culminate in production of reactive oxidants (Anderson *et.al*, 1988 ; Krajewska and Anderson, 1993). These pro-oxidative interactions between phagocytes and clofazimine and B669 may contribute to the antimicrobial properties of riminophenazines.

1.6.2.2 As anti-tumor agents

Van Rensburg and co-workers (1993) have reported that clofazimine and its analogue, B669, possess anti-neoplastic activity *in vitro*, inhibiting the proliferation of various cancer cell lines including those with intrinsic (Van Rensburg *et.al*, 1996) or acquired multi-drug resistance (Van Rensburg *et.al*, 1994) mechanisms. The tumor cell lines sensitive to clofazimine and B669 include human hepatocellular carcinomas (HepG2 and Mahlavu), human colorectal carcinoma (CaCo2) and human cervix epithelioid carcinoma (HeLa). The anti-neoplastic potential of these agents has also been demonstrated in a murine model of experimental cancer chemotherapy whereby oral

administration of clofazimine and B669 at 30mg/kg/day delayed the development of carcinogen-induced squamous carcinomas in mice and mammary tumors in rats (Van Rensburg *et.al*, 1993). Durandt and co-workers (1996) have shown that novel riminophenazine compounds, with varying cycloalkylimino groups at position 2 of the phenazine ring instead of an isopropylimino group (as in clofazimine), exhibit superior anti-tumor properties to clofazimine.

The reported anti-proliferative properties of clofazimine and B669 against various tumor cell lines *in vitro* is mediated by a dual mechanism of action (Van Rensburg *et.al*, 1993 ; Van Rensburg *et.al*, 1994), involving phospholipaseA₂(PLA₂)-dependent oxidative and non-oxidative mechanisms as shown in Figure 9 overleaf. The oxidative pathway involves the production of tumoricidal oxidants by activated phagocytes upon exposure to riminophenazines whereas the non-oxidative pathway occurs in the absence of phagocytes, whereby drug treatment of tumor cells results in increased PLA₂ activity with subsequent inhibition of Na⁺/K⁺-ATPase, an enzyme that is essential for cellular metabolism and proliferation.

1.6.2.3 As antimicrobial agents

Van Rensburg and co-workers (1992) have shown that clofazimine and its more potent analogue B669 are microbicidal for Gram-positive, but not Gram-negative bacteria. This activity is achieved through activation of microbial PLA₂ causing generation of lysophospholipids that are selectively antimicrobial for Gram-positive microorganisms and mycobacteria. De Bruyn and co-workers (1996) have proposed that microbial potassium transport systems are lysophospholipid-sensitive targets of riminophenazines in Gram-positive bacteria, which are either resistant or inaccessible to these agents in Gram-negative bacteria.

Modifications of the riminophenazines by substitution of the isopropyl substituent on the imino functional at position 2 of the phenazine nucleus of clofazimine with a tetramethylpiperidine group (TMP) and variation in substituents on the aniline and phenyl rings attached to positions 3 and 10 of the phenazine nucleus respectively results in a new group of phenazines called TMP-substituted phenazines with agents B4090 and B3786 being the classical examples. These new agents were developed for activity against a clofazimine-resistant mycobacterium (Franzblau *et.al*, 1989).

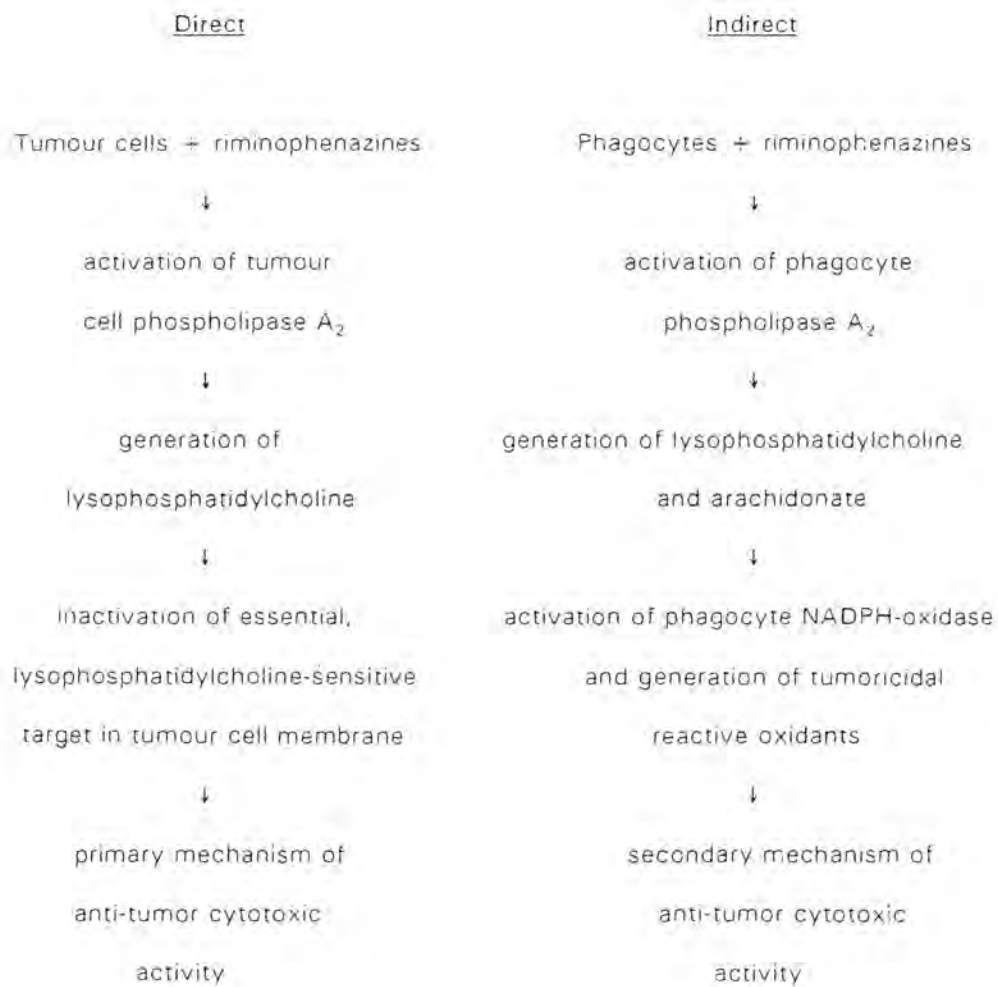


Figure 9 : Pathways of riminophenazine-mediated anti-tumor activity.



This class of compounds was the most active and activity increased with the degree of chlorination. TMP-substituted phenazines are impressive due to the following properties: (I) they accumulate to higher levels in tissues than clofazimine, (ii) they are not absorbed by body fat and as such will cause less skin colouration than clofazimine and (iii) they do not crystallize inside macrophages and as such will have a shorter half-life. Several other TMP-substituted phenazines studied in our laboratories have demonstrated impressive *in vitro* anti-mycobacterial activities (Matlola, 1996).

The studies with TMP-substituted phenazines were extended to investigate their activity against a laboratory strain of *Plasmodium falciparum* (Makgatho, 1996). In the antimalarial studies a modified flow cytometric procedure that is comparable to, with respect to sensitivity, but considerably less time-consuming than microscopic and radiometric assays was employed for large scale screening of the phenazine compounds for antiparasite activity (Makgatho, 1996; Schulze *et.al*, 1997). The results obtained from these studies showed that the TMP-substituted phenazines evaluated (structures shown in Figure 10) inhibited the growth of the plasmodial parasite at concentrations of 0.2µg/mL (1µM) to 2µg/mL (8µM) with the order of potency : B4158 < B4112 < B4103 < B4100 < B4121 < B4169. The effective *in vitro* concentrations of the TMP-substituted phenazines tested are similar to chloroquine. The antimalarial activity of the above compounds was compared to that of clofazimine (B633). Clofazimine showed insignificant activity.

1.7 AIMS AND OBJECTIVES

An antimalarial screening programme in our laboratories against a low grade chloroquine-resistant *Plasmodium falciparum* laboratory strain (RB1), has identified the TMP-substituted phenazines B4158 [3-(4-isopropylanilino)-10-(4-isopropylphenyl)-2,10-dihydro-2-(2,2,6,6-tetramethylpiper-4-ylimino)phenazine] (Makgatho, 1996) together with a newly acquired TMP-substituted phenazine, B4119 [3-(3-chloro-4-fluoroanilino)-10-(3-chloro-4-fluorophenyl)-2,10-dihydro-2-(2,2,6,6-tetramethyl piper-4-ylimino)phenazine], structures depicted in Figure 10, to possess the most impressive antiparasitic activity of a range of TMP-substituted phenazines tested to date.

I have intensively evaluated these two promising novel anti-plasmodial agents for the following:

1 *in vitro* activity against a chloroquine-sensitive, chloroquine-resistant, quinine-resistant and sulfadoxine-pyrimethamine (Fansidar®)-resistant laboratory isolates of *Plasmodium falciparum*.

2 antiplasmodial interaction with the conventional antimalarials, chloroquine and mefloquine *in vitro*.

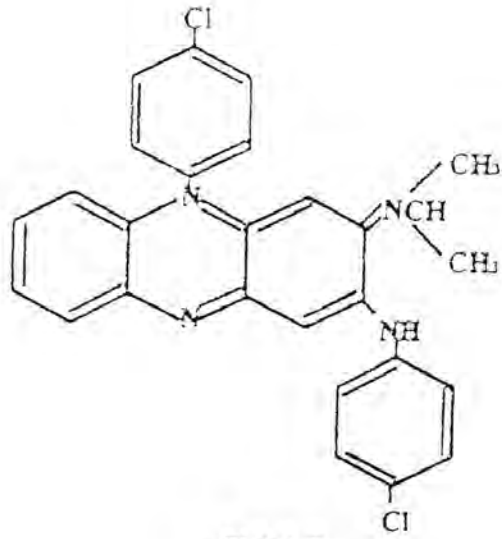
3 effects on the growth of the various stages of the parasite i.e stage-specific activity.

4 effects on merozoite invasion of erythrocytes i.e invasion inhibition potential.

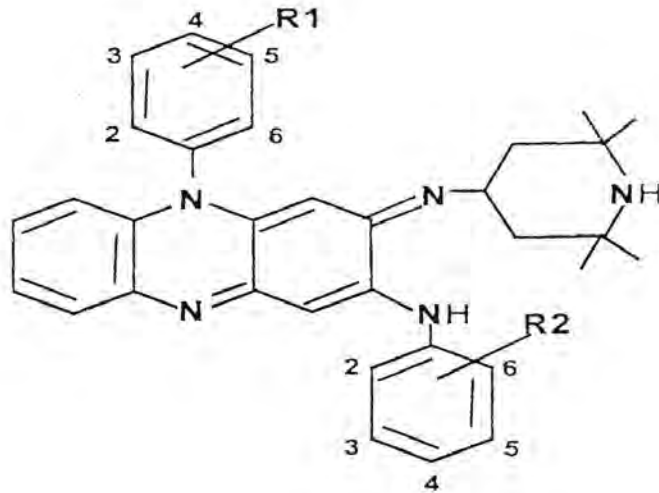
5 haem polymerization inhibitory activity (HPIA).

6 effects on membrane integrity (haemolytic potential), rubidium uptake as well as lactate and ATP levels in normal red blood cells.

7 *in vivo* antimalarial activity of B4119 alone and in combination with subtherapeutic concentrations of chloroquine against *Plasmodium berghei* in Balb/C mice.



B663



TMP-SUBSTITUTED PHENAZINES

COMPOUND	R ₁ AND R ₂
B4119	3-Cl, 4-F,
B4158	4-CH (CH ₃) ₂

Figure 10 : Molecular structures of clofazimine (B663) and TMP-substituted phenazines.



CHAPTER 2

EVALUATION OF PARASITEMIA IN MALARIA CULTURES

2.1 INTRODUCTION

Scientists in pharmaceutical companies as well as government and private research institutions are putting enormous efforts into the development of new chemotherapeutic agents and vaccines against fatal malaria infections. The success of this scientific venture depends to a large extent on techniques available to test and analyse the new agents *in vitro*. The historical assays used to assess the viability of malaria parasites in infected red blood cell cultures include microscopic evaluation of Giemsa-stained slides and radiometric measurement of the amount of radiolabelled nucleotide (hypoxanthine) incorporated into viable parasites. These methods, although still in use, are time-consuming, insensitive and subject to human error (Wyatt *et.al*, 1991).

To obviate the inherent difficulties with the above assays, recent studies have taken advantage of advances in instrument technology that have led to the development of high speed flow cytometers with a laser light source capable of analysing cells by the fluorescent properties of their protein or DNA content, light scattering properties or antigenic make up (Jackson *et.al*, 1977). The viability of intra-erythrocytic hemiparasites is assessed by intercalation of a fluorochrome into the DNA of viable parasites thus permitting the use of flow cytometry to distinguish infected erythrocytes containing viable organisms from those without or containing nonviable parasites. Numerous dyes or fluorochromes have been used for evaluating viability of parasites such as *P. falciparum*, *P. yoelii*, *P. berghei*, *B. bovis* and *T. gambiense*. A feature of the flow cytometric assay is simplicity, as all washing and centrifugation steps have been eliminated and the technique is capable of discriminating between parasite stages (Wyatt *et.al*, 1991).

Initial studies have employed the use of fluorescent dyes like propidium iodide, mithramycin, Feulgen-acriflavin, hydroethidine, acridine orange and Hoescht 33258 as tracers of parasite's DNA. Assays carried out using these dyes were not reproducible because they (i) required stringent incubation measures and complex culture medium formulations (ii) have irreversible effects on cell viability and growth and (iii) require prompt sample analysis (Howard *et.al*, 1979).

Hoescht 33258, a *bis*-benzimidazole dye, binds non-intercalatively to A-T rich DNA sequences that are abundant in the plasmodium genome and can be used to quantitate parasitemia in fixed malaria cultures, but conflicting results are still obtained due to brief incubation or storage period of fixed cultures and concentration of the dye used. The above drawback is also observed when using acridine orange (Whaun *et.al*, 1983 and Bianco *et.al*, 1986).

In 1987 Makler and colleagues (Makler *et.al*, 1987) first used thiazole orange, a membrane permeable compound that binds DNA and RNA of live reticulocytes and lymphocytes, to monitor the growth and multiplication of malaria parasites under *in vitro* conditions. They documented that (i) thiazole orange is excitable at 488nm, (ii) thiazole orange is soluble in phosphate buffered saline (PBS), (iii) the results obtained from the flow cytometric procedure correlated well with both microscopy and radiometry and (iv) flow cytometry allows parasite counting to be performed with speed, precision and sensitivity, and can consequently serve as a valuable tool for mass screening of potential antimalarial agents.

2.2 AIMS AND OBJECTIVES

The objectives of the experimental work described in this section were to standardise fluorescence flow cytometry with the inclusion of a fixation step as a novel procedure for measuring the percentage parasitemia in malaria cultures and document its effectiveness as compared to microscopy and radiometry.

2.3 MATERIALS AND METHODS

2.3.1 Media and Reagents

Unless indicated, all chemicals and reagents used were obtained from Sigma Chemical Co. (St. Louis, Mo.).

PARASITE CULTURE MEDIUM

RPMI-1640 medium [Highveld Biological Products, Kelvin, SA]; 500ml

Supplemented with :

22mg Hypoxanthine

150mg Glutamine



200 μ l Gentamicin (25mM)

50ml human serum from blood group O⁺ or A⁺ donors

WASH MEDIUM

Parasite culture medium without human serum

CRYOPRESERVATION MEDIUM

28g Glycerol

100ml wash medium

SYNCHRONIZATION SOLUTION

5g D-Sorbitol

100ml distilled water

FIXATION SOLUTION

TRIS-buffer :

10mM TRIS

10mM Na-Azide

150mM NaCl

dissolve in 500ml distilled water.

Reconstitute 10% formaldehyde and 4% glucose in TRIS-buffer at pH 7.3

15mM PHOSPHATE-BUFFERED SALINE (PBS)

9.23g FTA Hemagglutinin buffer [BBL Microbiology Systems, Cockeysville, USA]

1000ml distilled water

10mM CHLOROQUINE DIPHOSPHATE SALT

2.5mg chloroquine diphosphate salt

0.5ml distilled water

Further dilution was made in complete parasite culture medium



2.4Ci/mMol TRITIATED HYPOXANTHINE [Amersham Life Science International, England]

THIAZOLE ORANGE [Molecular Probes, Oregon]

1mg thiazole orange/ml ethanol

Kindly provided by Prof Braam Louw, Dept. of Biochemistry, University of Pretoria.

GIEMSA STAIN

0.2ml Giemsa Stain [MERCK, Germany]

2ml PBS

2.3.2 Parasite culture maintenance

A laboratory strain of low-grade chloroquine-resistant (RSA8) *P. falciparum* was kindly provided by Prof. B. Louw, Department of Biochemistry, University of Pretoria.

(A) Erythrocyte and plasma preparation

Erythrocytes : Venous blood from blood group O+ donors, without previous exposure to malaria and not under any antimalarial or anti-inflammatory medication, was drawn in 5ml EDTA vacutainer tubes. The blood was centrifuged three times in wash medium at 350g for 5min with subsequent aspiration of the leukocyte supernatant after every centrifugation step. The final red cell pellet was resuspended in the wash medium and stored at 4⁰C for not more than ten days. The wash medium was exchanged with fresh medium every second day for proper preservation of the erythrocytes.

Plasma : Human plasma samples [South African Blood Transfusion Services, Johannesburg] from five blood group O+ or A+ donors with no previous malaria exposure were pooled into a 1L flask and mixed thoroughly. The mixed plasma was aseptically collected into 50ml centrifuge tubes and stored at -20⁰C. The plasma was heat-inactivated at 56⁰C for 45min before use.

(B) Cryopreservation

Cultures containing more than 5% ring forms were suitable for storing in liquid nitrogen (cryopreservation). Cultures were collected into 15ml centrifuge tubes and centrifuged at 200g



for 5min. After removing the supernatant, 0.5ml freezing medium and red cells were added to the pellet and mixed. Aliquots of 1ml were placed into cryopreservation tubes and stored in liquid nitrogen.

(C) Thawing and establishment of parasite culture

Cultures were defrosted in a 37°C water bath and transferred to 15ml centrifuge tubes. One ml sterile 3.5% NaCl was added to each culture and centrifuged at 200g for 5min. This procedure was repeated three times. The cultures were maintained as described by Trager and Jensen (Trager and Jensen, 1976) with modifications. Briefly, the cell pellets were transferred to culture flasks containing 10ml complete culture medium and 0.1ml O+ human red cells. Cultures were gassed and incubated at 37°C. Medium and red cells were replaced on a daily basis.

(D) Gassing of cultures

A sterile gas mixture of 5% oxygen / 5% carbon dioxide / 90% nitrogen was introduced daily into the parasite culture flask by gassing for 3-4 mins after addition of fresh culture medium and red cells. The culture flasks were closed tightly and incubated at 37°C.

2.3.3 Synchronization of parasite cultures

A synchronization procedure was necessary to obtain only the ring-stage forms of the parasite for carrying out the assays. The procedure is only performed if the parasitemia levels in the cultures exceed 5%. Ring-stage synchronization was achieved by sorbitol lysis as first described by Lambros and Vanderberg (Lambros and Vanderberg, 1979) with minor modifications. Briefly, parasitized erythrocytes were centrifuged at 200g for 5min, the supernatant discarded and the parasite pellet resuspended in 9ml of aqueous 5% D-sorbitol for 10min at room temperature. After an additional centrifugation, the cultures were reestablished as described in section 2.3.2 © until ready for use.

2.3.4 Comparison of methods used to determine parasitemia in malaria cultures

2.3.4.1 *Exposure of parasite cultures to chloroquine*

The RSA8 laboratory strain of *Plasmodium falciparum* was used for all the assays performed in this section. Ring-stage-infected erythrocyte cultures (20 μ L) at 2% parasitaemia and 5% haematocrit were incubated with serial dilutions of chloroquine (20 μ L) ranging from 4nM to 250nM (final dilution) in 96-well microculture plates made up to 200 μ L with complete culture medium. Wells without chloroquine and with chloroquine at 1000nM served as negative and positive control systems respectively. The plates were placed in a modular gas chamber, gassed for 15min, sealed and incubated for 48 hours. Parasitemia was determined using microscopy, radiometry and flow cytometry.

2.3.4.2 *Microscopy*

At the end of the incubation period, thin smears were prepared on microscope slides and air-dried. The smears were stained with Giemsa solution (0.2mL Giemsa / 2ml PBS) for 5min. After staining, the slides were washed with running tap water, air-dried and investigated under a light microscope at a magnification of 787.5. The level of parasitemia was determined by documenting the number of infected red cells out of a total number of 300 cells.

2.3.4.3 *Radiometry*

Twenty-four hours before the end of the incubation period, 150 μ L of medium was replaced with hypoxanthine-free medium containing tritiated hypoxanthine (1 μ Ci). After 18 hours, the cultures were harvested on filter discs by a cell harvester and the radioactivity counted in a Tricarb 2100-TR scintillation counter [Parckard Industrial Company, USA]. All treatments were performed in quadruplicate.

2.3.4.4 *Flow cytometry*

At the end of the incubation period, 100 μ L of medium was removed from the wells and replaced with 100 μ L of the fixation solution. The plates were stored overnight at 4°C. The fixed parasite cultures (25 μ L) were stained with 0.5mL of a thiazole orange solution (0.26 μ g/mL), reconstituted in PBS, in test tubes. The tubes were incubated at room temperature for one hour in the dark and

the reaction stopped by placing the tubes on ice. The level of parasitemia was determined using an EPICS II flow cytometer [Coulter Electronics, USA].

2.3.5 Expression and statistical analysis of results

Results of each assay are expressed as percentage inhibition \pm SEM of parasite growth in drug-treated wells and IC_{50} concentrations were obtained using the Graphpad InStat2 programme. Statistical analysis of the data was performed using the Student's t-test for paired values and Bland and Altman measure of agreement (Bland and Altman, 1986).

2.4 RESULTS

The RSA8 laboratory strain of *Plasmodium falciparum* was incubated with chloroquine at concentrations ranging between 4nM to 250nM for 48 hours. The extent of inhibition of parasite growth was assessed using microscopy, radiometry ($[^3H]$ hypoxanthine uptake) and flow cytometry. As shown in Figure 11, a dose-related inhibition of parasite growth by chloroquine which was evident at concentrations of 62nM and statistically significant ($p < 0.05$) at 125nM and higher was observed with all three methods. The Bland and Altman measure of agreement revealed a good agreement between flow cytometry and microscopic methods as well as between the flow cytometric and radiometric methods with 95% confidence intervals of 8.643 to -11.11 and 12.407 to -6.893 respectively.

These limits of confidence are small enough for us to be confident that the flow cytometric method can be used in place of the standard methods to measure the total parasitemia in drug-treated parasite cultures. The IC_{50} values (the concentration necessary to inhibit 50% of parasite growth) of chloroquine for the RSA8 strain using flow cytometry, microscopy and hypoxanthine uptake assay (radiometry) were 76nM, 70nM and 74nM respectively.

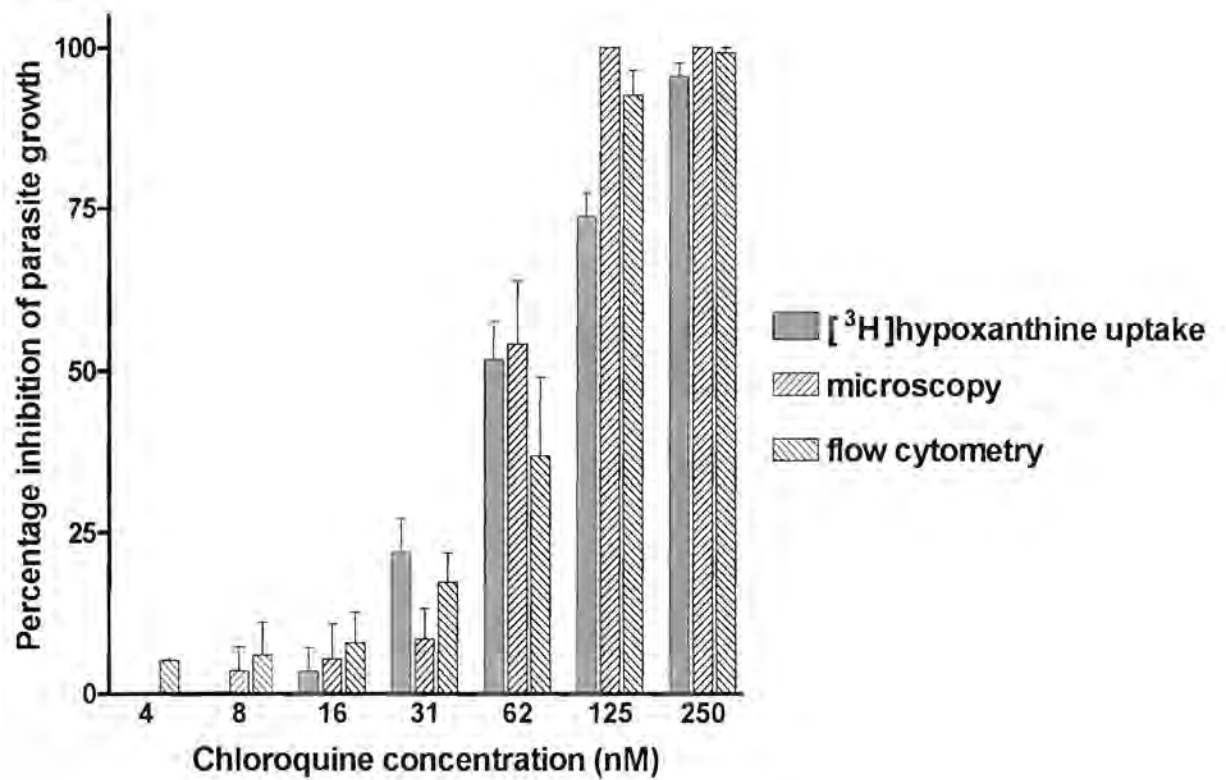


Figure 11: Evaluation of parasitemia in malaria parasite cultures using flow cytometric, radiometric and microscopic methods. Results are expressed as percentage inhibition of parasite growth and show mean values and SEM of five different experiments for each method

2.5 DISCUSSION

The rapid quantification of malaria parasitemia is essential for *in vitro* studies designed to evaluate the effects of new chemotherapeutic agents on the growth and development of the malaria parasite. In this study thiazole orange was compared with standard assays for tracing and quantitating *Plasmodium falciparum* parasites in infected human red blood cells. An accurate assay is most important when testing activities of new antimalarial agents in vitro as well as for studies identifying geographical areas where chloroquine-resistant strains prevail.

The 95% confidence intervals are small enough to indicate that the flow cytometric procedure agrees sufficiently with the two classical assays, microscopy and radiometry, to be used with confidence in assays testing new antimalarial drugs. Other fluorescent dyes have previously been used but with the disadvantage that samples had to be analysed immediately after termination of the experiment (Bianco *et.al*, 1986). However, the use of thiazole orange allows for a fixing procedure which permits analysis of samples at a later more convenient period. The results of all three methods suggest that the laboratory strain of *Plasmodium falciparum* used in this study, RSA8, is of low grade chloroquine resistance with an average chloroquine IC_{50} of 74nM.

The flow cytometric procedure yielded results comparable to those obtained by the microscopic and radiometric assays but the following advantages should however make flow cytometry the preferred method of analysis : (i) it is sensitive (antimalarial activity of chloroquine as low as 4nM was detectable) and lends itself to rapid evaluation of the effects of pharmacological agents on the growth of malaria parasite in erythrocyte cultures, (ii) the procedure offers the means to determine *P. falciparum* parasitaemia in large numbers of samples with speed and precision and (iii) the results obtained using this procedure are clearly quantitative since the intensity of the fluorescence is proportional to the amount of parasite nucleoprotein present in the red cell (Makler *et.al*, 1987).

CHAPTER 3

***IN VITRO* ANTIMALARIAL ACTIVITIES OF TMP-SUBSTITUTED PHENAZINES, B4119 AND B4158**

3.1 INTRODUCTION

The development of pharmacological agents for the treatment of human falciparum malaria is slow and expensive (Kolberg, 1994; Flowers and Melmon, 1997) and is complicated by a number of factors including : (i) ease by which parasite develops resistance to drugs (Yeo *et.al*, 1997) (ii) bio-physiological diversity and complexity of the parasite's life cycle (Sherman, 1979) and (iii) reduced funding for projects addressing the control and treatment of the disease (Di Masi *et. al*, 1991; Butler, 1997). There is clinical experience and experimental evidence pointing to the fact that however effective when first introduced, the continual usage of antimalarials is unavoidably curtailed by the emergence of drug-resistant parasites (White and Olliaro, 1996). As a result there is a need for new and novel chemotherapeutic compounds for treating malaria infections.

Screening programmes in our laboratories (Makgatho, 1996) have identified a series of TMP-substituted phenazines with antimalarial activity and preliminary results have shown that B4158 and a newly acquired agent, B4119, are the most potent representatives of this group tested to date.

3.2 AIMS AND OBJECTIVES

This study was designed to investigate the *in vitro* anti-plasmodial potential of the novel TMP-substituted phenazines, B4119 [3-(3-chloro-4-fluoroanilino)-10-(3-chloro-4-fluorophenyl)-2,10-dihydro-2-(2,2,6,6-tetramethylpiper-4-ylimino)phenazine] and B4158 [3-(4-isopropylanilino)-10-(4-isopropylphenyl)-2,10-dihydro-2-(2,2,6,6-tetramethylpiper-4-ylimino)phenazine], against various laboratory strains of *Plasmodium falciparum* in both the absence or presence of the standard chemotherapeutic agents, chloroquine and mefloquine. Laboratory research presented in this chapter was also designed to evaluate the stage-dependent effects of these TMP-substituted phenazines, B4119 and B4158, on the growth of the ring and trophozoite forms of the PfUP10 laboratory strain of *P. falciparum in vitro*. The effects of pre-treatment of the erythrocytes with

B4119 and B4158 on the invasive potential and growth of the parasite were also investigated.

3.3 MATERIALS AND METHODS

3.3.1 Media and reagents

See section 2.3.1

3.3.2 Parasite laboratory strains

Four culture-adapted strains of *P. falciparum* were used in this study. RSA9, RSA16 and RSA17 were kindly provided by Dr. Brian Sharp, National Malaria Research Programme (NMMP) of the South African Medical Research Council, Durban while PfUP10 was provided by Prof. B. Louw, Department of Biochemistry, University of Pretoria, South Africa. The cultures were all maintained as in sections 2.3.2 and 2.3.3.

3.3.3 Preparation of drugs

TMP-SUBSTITUTED PHENAZINES

Pure compounds of the two TMP-substituted phenazines, B4119 (MW 606) and B4158 (MW 585), were kindly provided by Dr. J.F. O'Sullivan, Department of Chemistry, University College Dublin, Ireland. The agents were dissolved in ethanol containing 10mM acetic acid resulting in 3mM (2mg/mL) stock solutions. Further dilutions were made in parasite culture medium.

10mM CHLOROQUINE

2.5mg chloroquine diphosphate salt

0.5mL distilled water

Further dilutions were made in complete parasite culture medium

2.6mM MEFLOQUINE [Hoffman-La-Roche; Basel, Switzerland]

1mg mefloquine hydrochloride dissolved in 70% ethanol (1mL)

Further dilutions were made in complete parasite culture medium.

3.3.4 Experimental procedures

3.3.4.1 *Direct anti-plasmodial activity of B4119 and B4158 in vitro*

The four laboratory strains of *Plasmodium falciparum* were cultured as described in section 2.3.2. Drug testing was carried out in 96-well microtiter plates. To each well was added 20 μ L ring-infected erythrocyte suspension (2% parasitemia and 0.5% final haematocrit), and 180 μ L of complete medium with B4119 and B4158 [(1-8 μ M) or (0.2-2 μ g/mL)] or without the drug (control). The total volume in all wells was 200 μ L. The plates were placed in a modular gas chamber, gassed for 15 min. with the gas cocktail, sealed and incubated for 48 hours. At the end of the incubation period, levels of parasitemia were determined using microscopy (single wells) and flow cytometry (duplicate wells) described in sections 2.3.4.2 and 2.3.4.4.

3.3.4.2 *Chloroquine- and mefloquine-sensitizing activities of B4119 and B4158*

For this part of the study the PfUP10 laboratory strain of *Plasmodium falciparum* was passaged for 2 months without cryopreserving. At this stage the IC₅₀ for chloroquine of this parasite strain increased from 0.075 to 0.38 μ M in the absence of drug pressure. To each well of a microtiter plate was added 20 μ L of a ring-infected red cell suspension. For the drug combination assays, 20 μ L of both chloroquine diphosphate (0.044 to 0.50 μ M) or mefloquine hydrochloride (0.001 to 0.065 μ M) were added to the wells, either individually or in combination with 20 μ L of either B4119 (0.4 μ M) or B4158 (0.8 μ M). The suspension was made up to 200 μ L with culture medium. The plates were placed in a modular gas chamber, gassed for 15 min, sealed and incubated for 48 hours. At the end of the incubation period, levels of parasitemia were determined using flow cytometry as described in section 2.3.4.4.

3.3.4.3 *Stage-dependent effects of B4119 and B4158 on the growth of P. falciparum in vitro*

The PfUP10 strain of *P. falciparum* was synchronized to the ring-stage of development as described in section 2.3.3. To the wells of a 96-well microtiter plate was added 20 μ L of synchronized ring and late trophozoites parasite suspensions (5% parasitemia and 0.5% haematocrit), and 180 μ L of parasite culture medium with B4119 and B4158 (1 to 8 μ M) or without (control) the drug. The total volume in all wells was 200 μ L. The plates were placed in a modular chamber, gassed for 15 min and then incubated for 12 hours at 37°C. At the end of the incubation period, the ability of the ring forms to develop into trophozoites, as well as that of

schizonts to mature and re-infect new red cells as ring-stage parasites was measured using microscopy (single wells) and flow cytometry (duplicate wells) as described in sections 2.3.4.2 and 2.3.4.4.

3.3.4.4 Invasion assay of drug-treated erythrocytes

A stringently synchronized strain of *P. falciparum* (PfUP10) was grown in a 800ml flask (instead of the usual 250mL flask) and a low haematocrit in order to increase parasite levels. Parasite cultures were ready for use after reaching about 65% parasitemia. This parasite suspension was obtained from a tightly synchronised ring stage parasites (15% parasitemia) cultured over two growth cycles in 50mL of culture medium and low haematocrit which will decrease the growth inhibitory effect of lactic acid secreted into medium by metabolically active parasites. Normal erythrocyte suspensions (0.5% haematocrit) in complete culture medium were incubated with B4119 and B4158 [(1-8 μ M) or (0.2-2 μ g/mL)] or without the drug (control) for 24 hours. The red cells were then washed twice with culture medium to remove extracellular phenazines and then resuspended to 0.5% haematocrit. Equal aliquots (20 μ L) of B4119/B4158-treated red cells and late trophozoite infected red cell suspension (60% parasitemia and 0.5% haematocrit) were added to 96-well microtiter plates and made up to 200 μ L with complete culture medium. Wells containing 20 μ L of iRBC suspension in 180 μ L of the culture medium were also included. The plates were gassed and sealed in the modular gas chambers and incubated at 37°C. After 24 hours, the ability of the parasites to invade drug-treated red cells was evaluated and in the following 24 hours the maturation of ring forms into trophozoites was also evaluated microscopically as in section 2.3.4.2.

3.3.5 Expression and statistical analysis of results

Data from experiments are expressed as mean percentages \pm SEMs and statistical analysis performed using the Student's t-test. The IC₅₀ values were calculated using the Graphpad Instat2 programme.

3.4 RESULTS

Direct anti-plasmodial activity of B4119 and B4158 *in vitro*. The effects of the two TMP-phenazines (B4119 and B4158) on the growth of the *Plasmodium falciparum* strains *in vitro* were measured by either microscopy or flow cytometry as it has been documented that results obtained using these two methods are comparable. The *in vitro* anti-plasmodial activities of the two agents against sensitive and resistant strains of *Plasmodium falciparum* are shown in Figures 12, 13, 14 and 15 respectively. These results show that compounds B4119 and B4158 possess significant ($p < 0.05$) anti-plasmodial activity in all parasite strains used at the concentrations tested [(1-8 μ M) or (0.2-2 μ g/mL)]. The results in Table 1 indicate that clofazimine, the parent molecule, did not exhibit any activity against the laboratory strain (PfUP10) at the concentrations tested [(1-8 μ M) or (0.2-2 μ g/mL)] and that B4119 was the most active of the three agents. Both TMP-substituted phenazines inhibited the growth of the chloroquine-sensitive and -resistant parasite strains without any obvious difference in potency as indicated by the IC₅₀ values in Table 1. The IC₅₀ values in Table 1 also indicate that the parasite strains responded to the TMP-substituted phenazines with the following order of sensitivity : RSA16 >PfUP10 >RSA17 >RSA9.

Chloroquine- and mefloquine-sensitizing activities of B4119 and B4158 *in vitro*. The effects of mefloquine and chloroquine on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* in the presence or absence of the TMP-substituted phenazines, B4119 and B4158 are shown in Figures 16, 17, 18 and 19 respectively. For these experiments, the PfUP10 laboratory strain of *P. falciparum* was passaged for two months under proper culture conditions without cryopreservation such that the IC₅₀ value for chloroquine increased from 0.075 μ M to 0.38 μ M. Combining the TMP-substituted phenazines, B4119 (0.4 μ M) and B4158 (0.8 μ M), with low concentrations of chloroquine (0.044-0.50 μ M) or mefloquine (0.01-0.065 μ M) resulted in inhibition of parasite growth ($P < 0.05$) that was greater than that observed with the individual compounds i.e chloroquine and mefloquine.

Stage-specific activity of B4119 and B4158 *in vitro*. The stage-specific effects of B4119 and B4158 on the PfUP10 laboratory strain of *Plasmodium falciparum* are shown in Figures 20 and

21 respectively. Both B4119 and B4158 significantly ($p < 0.05$) inhibited the growth of the first (ring forms to trophozoites) and particularly the last (trophozoites/schizonts to rings) stages of the parasite growth cycle for all the concentrations tested (1 to $8\mu\text{M}$). These results were confirmed by microscopical examination of Giemsa-stained slides in Table 2 (**A** and **B**). Results in Table 2A show that both drugs exhibit pronounced inhibitory effects on the first phase of parasite growth at $1\mu\text{M}$ in a dose-related manner, whereas results in Table 2B show that both agents inhibited the growth of trophozoites at all 4 concentrations tested, especially at higher concentrations of 4 and $8\mu\text{M}$. At these concentrations no rings could be seen. The effects of the agents on the two cycles of parasite maturation result in a pronounced inhibitory effect when a 48 hour parasite growth cycle is evaluated as shown in Figure 12. Microscopic evaluation of the stage-specific effects of both agents (Table 2) was confirmed with flow cytometric procedures.

Effects of pretreatment of erythrocytes with B4119 and B4158 on parasite invasion and maturation. The ability of B4119 and B4158 to inhibit merozoite invasion and maturation of the resultant rings into trophozoites was also investigated microscopically and the results are shown in Figures 22 and 23 respectively. Both compounds had no noticeable effect on the invasion process at any of the concentrations tested (1 to $2\mu\text{M}$) but significantly ($p < 0.05$) interfered with the maturation of the ring forms into trophozoites at 4 and $8\mu\text{M}$.



Table 1: Sensitivity of *Plasmodium falciparum* to standard antimalarial drugs, as well as to clofazimine, B4119 and B4158 *in vitro*.

Drugs	IC ₅₀ values (µM) of experimental compounds			
	PfUP10	RSA17	RSA16	RSA9
Chloroquine	0.074	0.08*	0.06*	0.16*
Quinine	ND	0.59*	0.65*	0.67*
Sulfadoxine/pyrimethanine	ND	2.30*	2.10*	6.20*
B4119	0.22	0.24	0.22	0.40
B4158	0.4	0.60	0.30	0.70
Clofazimine	> 8	ND	ND	ND

IC₅₀ values are the means of three experiments conducted in duplicate. ND, not determined.

* Values obtained from Freese, 1993(a).

Table 2 : Microscopic evaluation of the *in vitro* stage-specific antiplasmodial activity of B4119 and B4158 for the first (rings to trophozoites) (A) and last (trophozoite/schizont to ring forms) (B) stages of parasite development.

A

Drug concentration (μM)	Parasitemia (%)	
	B4119	B4158
0	51.7 \pm 9 (t)	51.7 \pm 9 (t)
1	20 \pm 7 (r)	21 \pm 6 (r)
2	13 \pm 4 (r)	16 \pm 5 (r)
4	8 \pm 2 (r)	10 \pm 3 (r)
8	9 \pm 3 (r)	8 \pm 3 (r)

B

Drug concentration (μM)	Parasitemia (%)	
	B4119	B4158
0	86 \pm 14 (r)	86 \pm 14 (r)
1	29 \pm 11 (r and t)	28 \pm 7 (r and t)
2	19 \pm 7 (r and t)	24 \pm 11 (r and t)
4	10 \pm 2 (t)	17 \pm 3 (t)
8	5 \pm 1 (t)	8 \pm 2 (t)

Results in the table represent the mean percentage parasitemia \pm SEMs of three experiments where r and t represent ring and trophozoite parasite forms respectively.

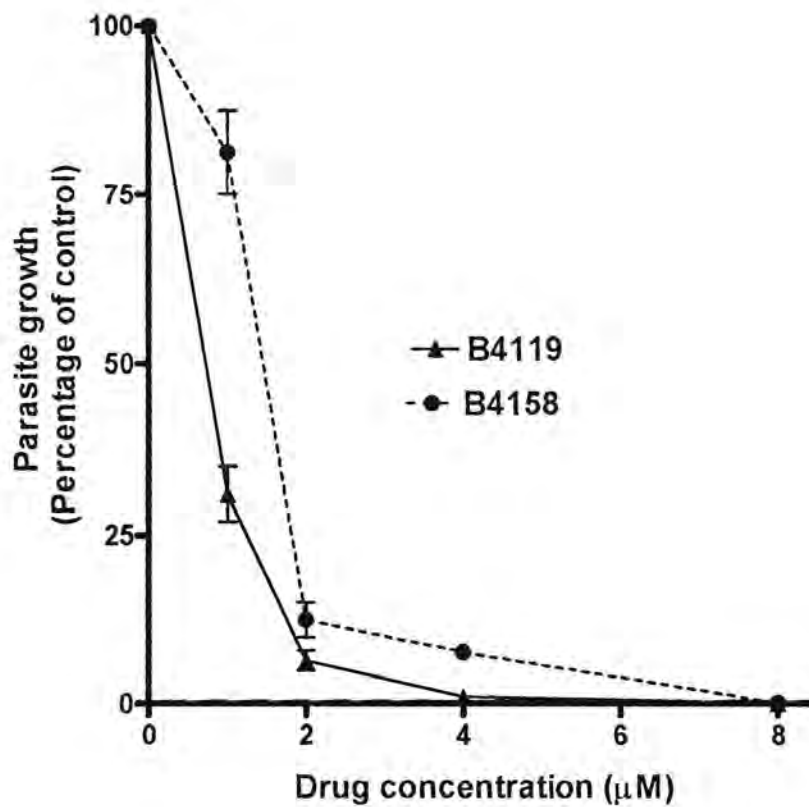


Figure 12 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the PfUP10 strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as the mean percentage parasitemia of the drug-free control systems \pm SEM of three different experiments.

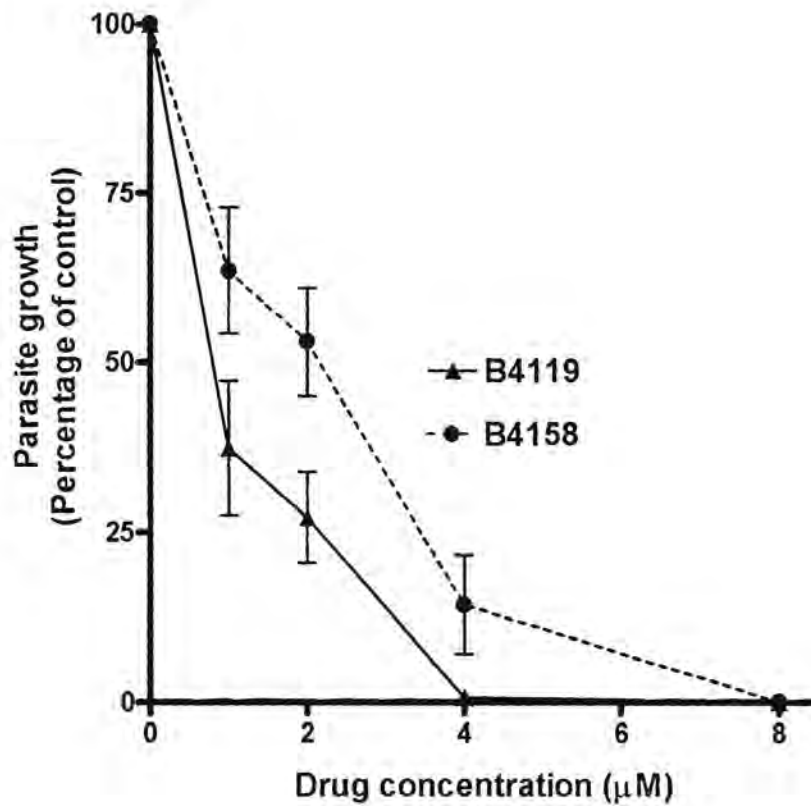


Figure 13 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against RSA17 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems \pm SEM of two different experiments.

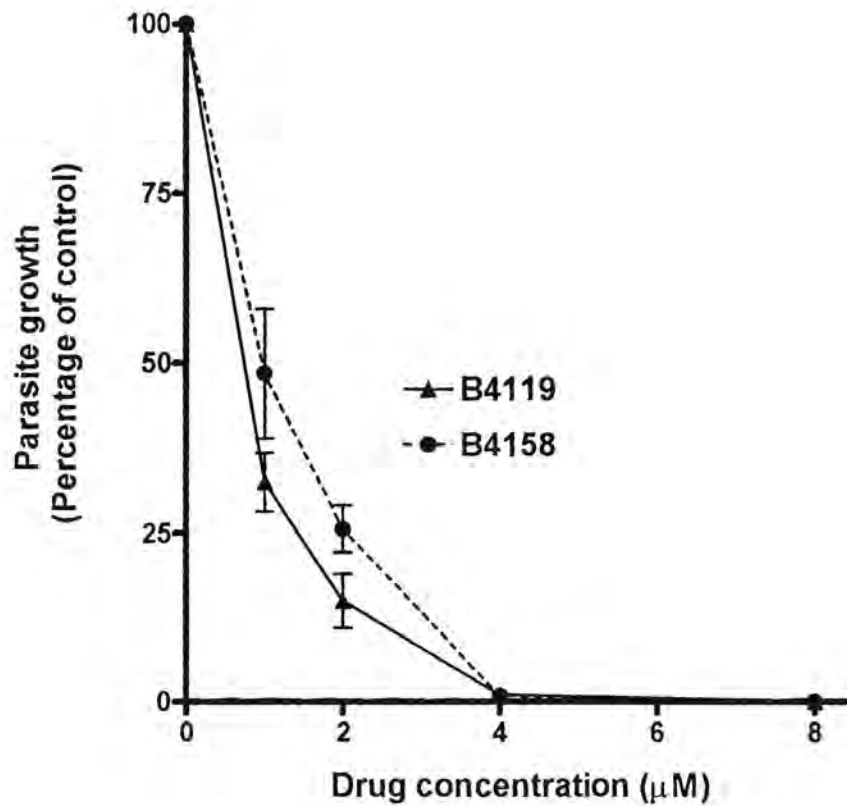


Figure 14 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA16 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems \pm SEM of two different experiments.

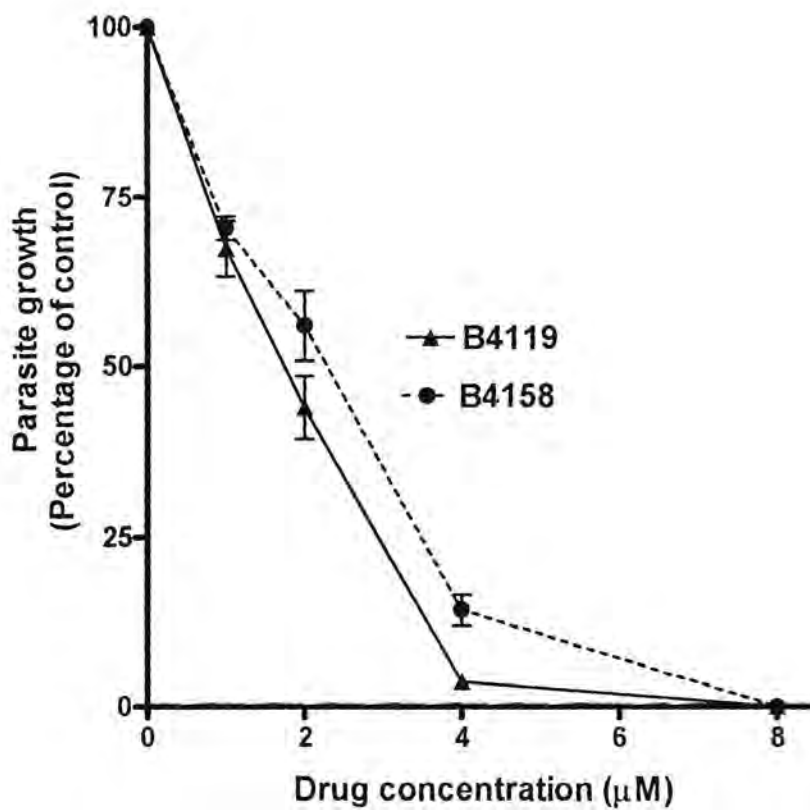


Figure 15 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA9 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems \pm SEM of two different experiments.

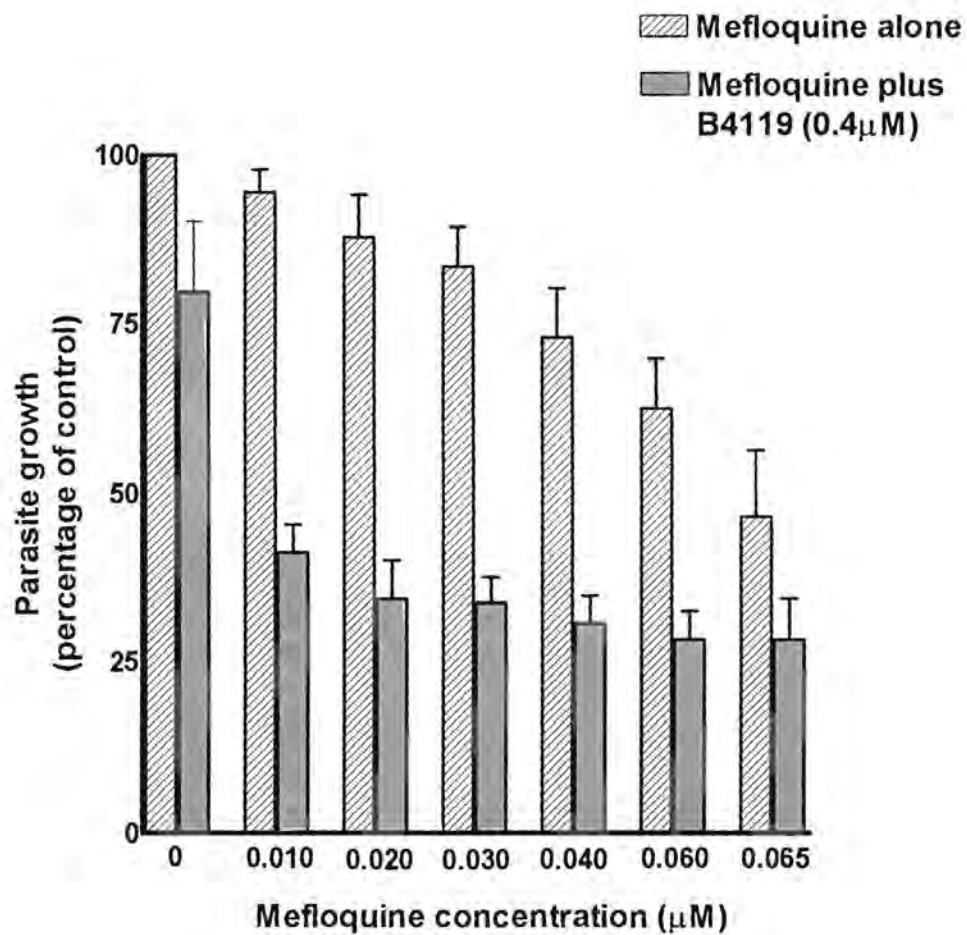


Figure 16 : Effects of B4119 (0.4µM) and mefloquine (Mef) (0.010 - 0.065µM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as mean percentage \pm SEM of three different experiments.

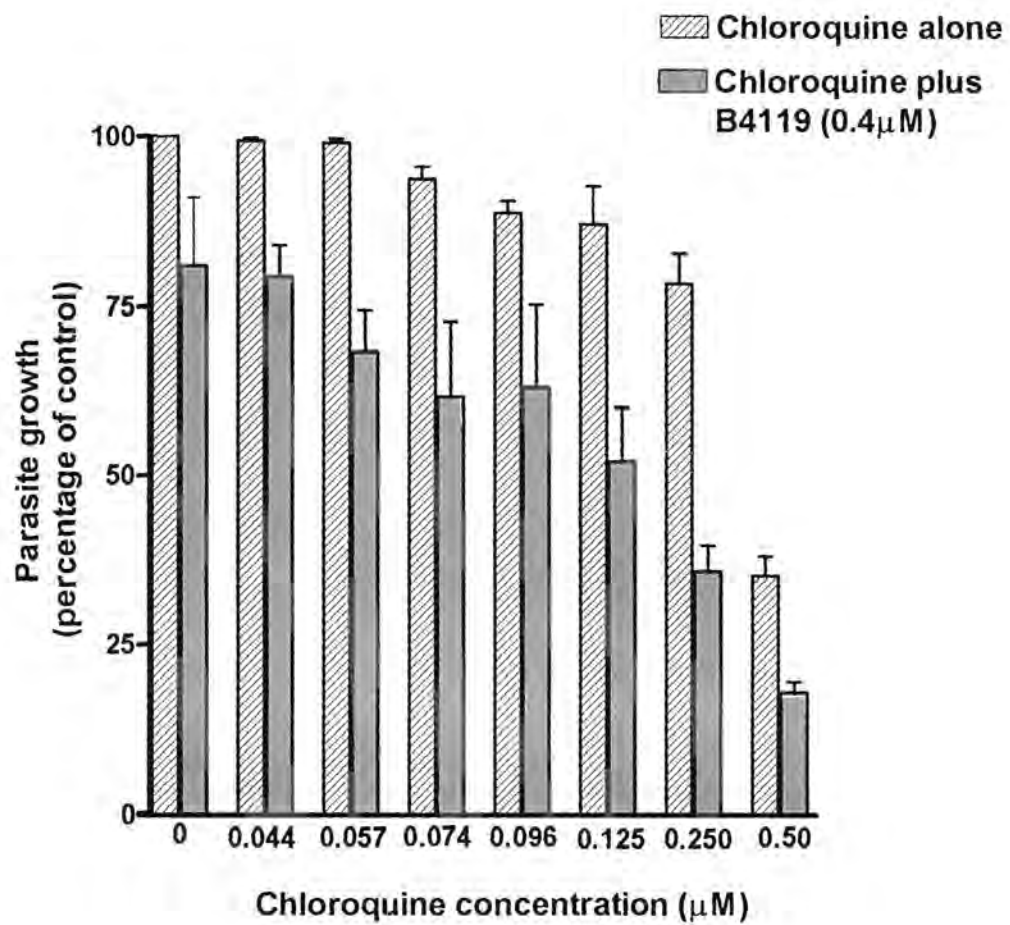


Figure 17 : Effects of B4119 (0.4µM) and chloroquine (CQ) (0.044 -0.50µM) individually and in combination on the growth PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as mean percentage ± SEM of three different experiments.

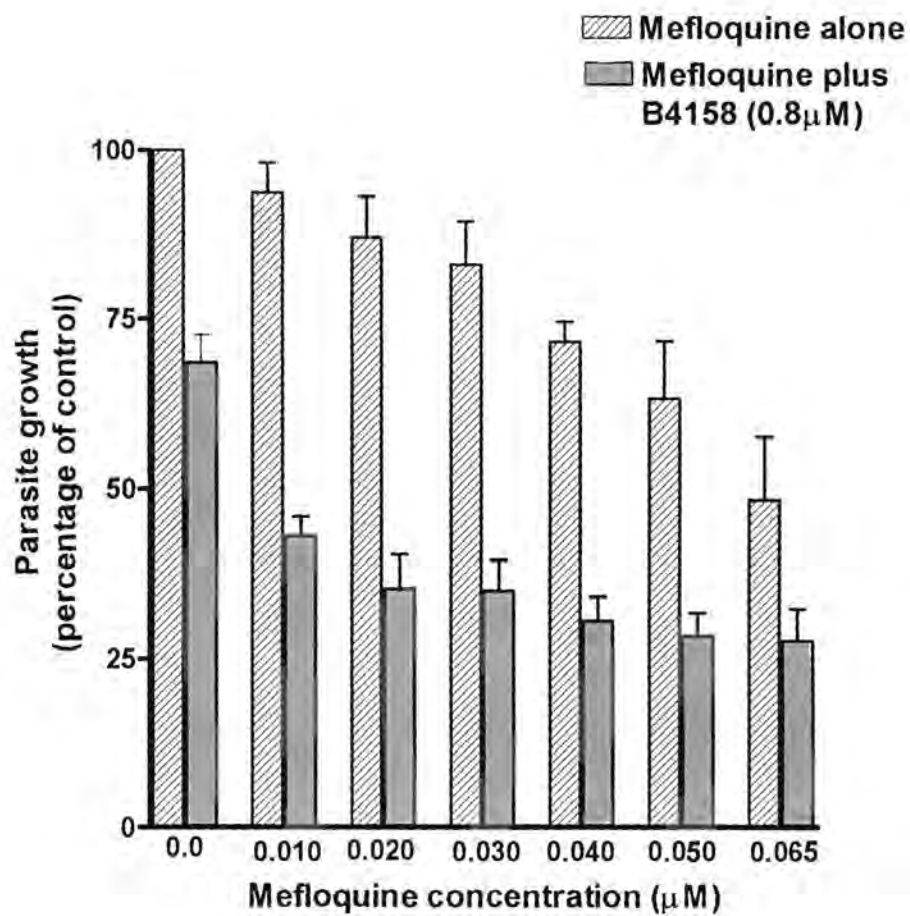


Figure 18 : Effects of B4158 (0.8 μM) and mefloquine (Mef) (0.010 - 0.065 μM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry . Results represent the mean percentage ± SEM of three different experiments.

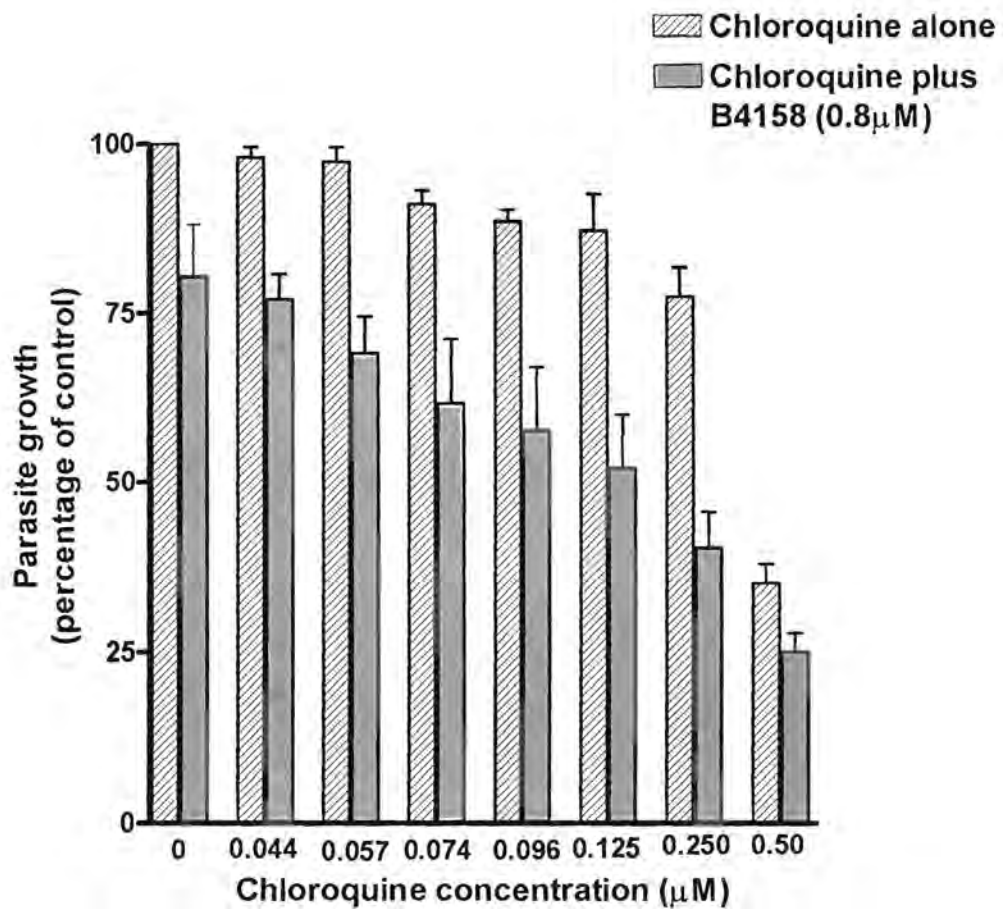


Figure 19 : Effects of B4158 (0.8 µM) and chloroquine (CQ) (0.044 -0.50 µM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results represent mean percentage ± SEM of three different experiments.

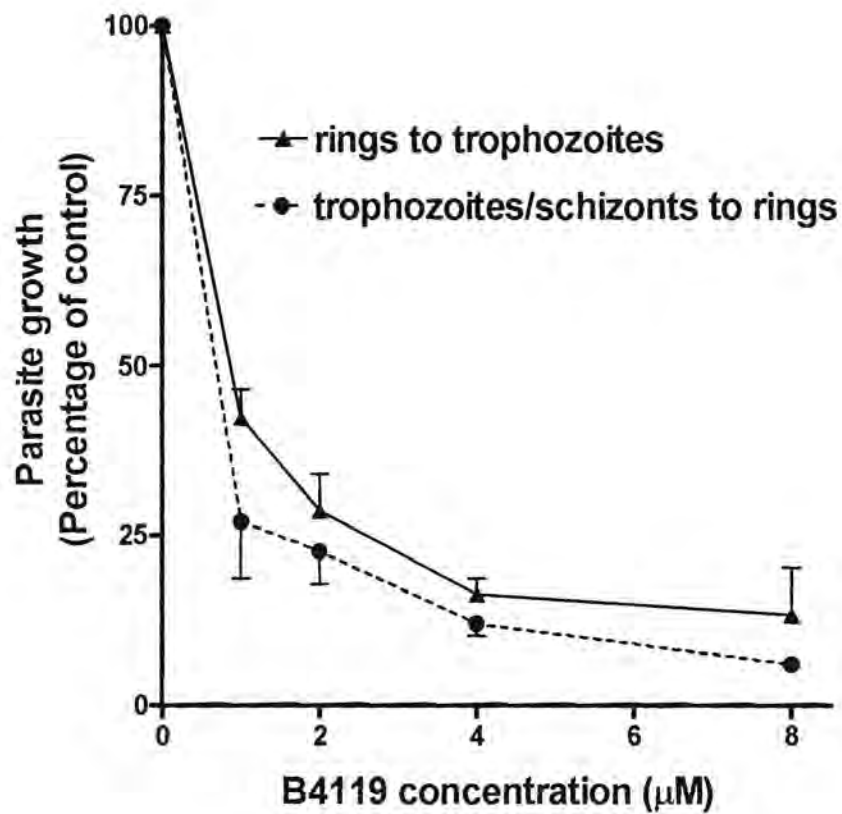


Figure 20 : Stage-specific antimalarial activity of B4119 for the first (ring to trophozoites/schizonts) and last (trophozoites/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry. Data from three experiments conducted in duplicate are expressed as mean percentage parasitemia of the drug-free control systems \pm SEMs.

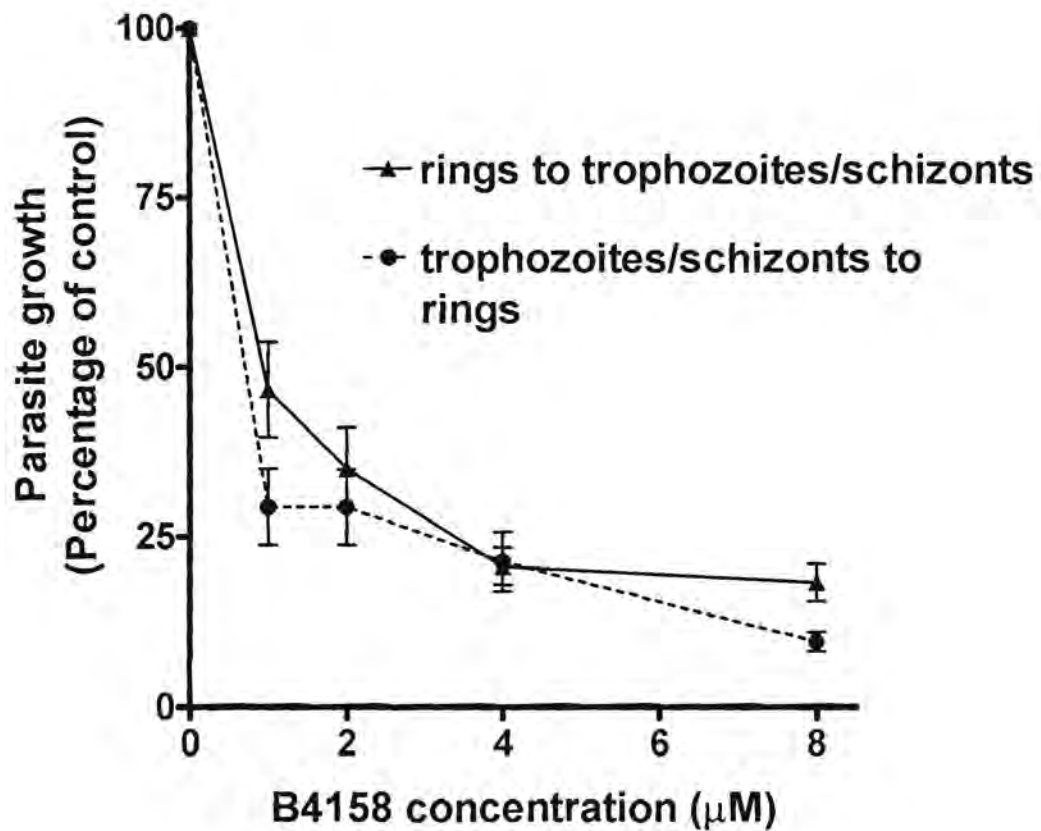


Figure 21 : Stage-specific activity of B4158 for the first (rings to trophozoites/schizonts) and last (trophozoite/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry. Data from three experiments conducted in duplicate are expressed as mean percentage of parasitemia of the drug-free control systems \pm SEMs.

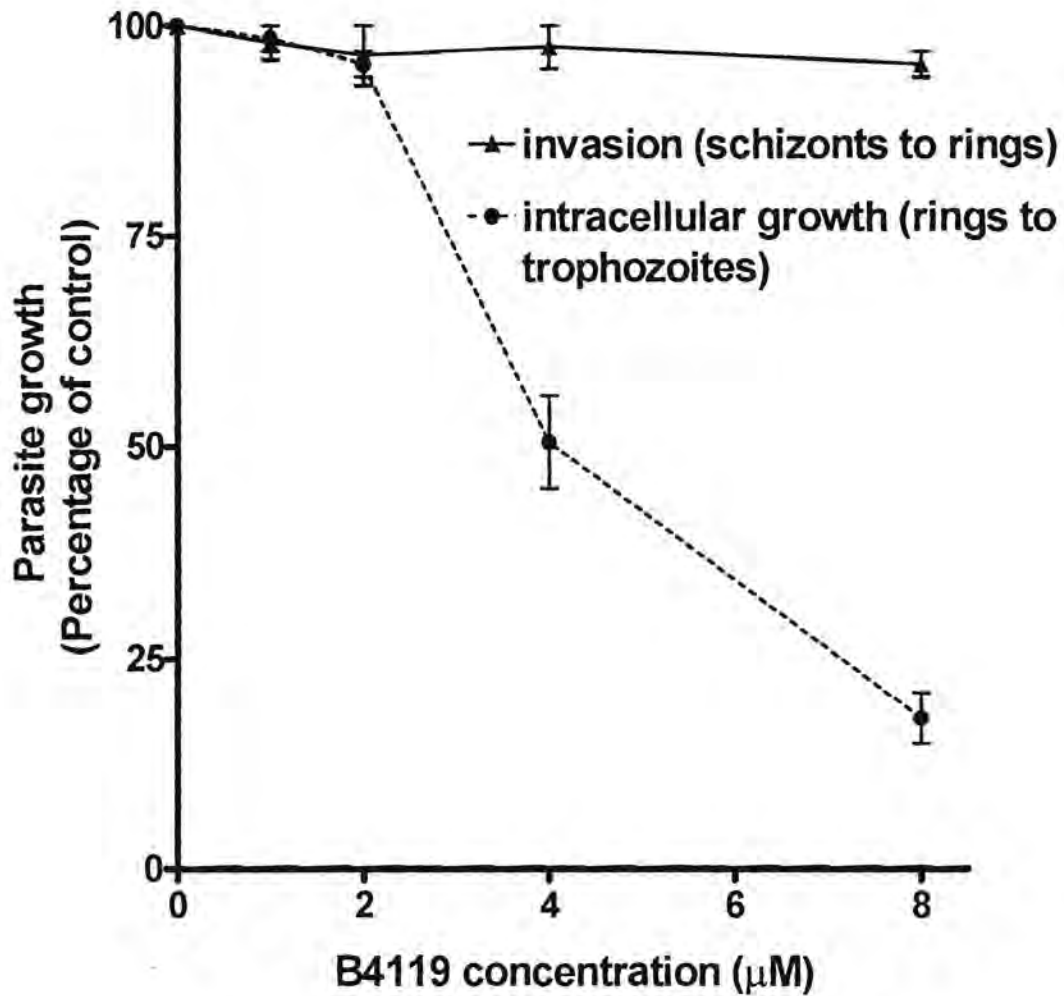


Figure 22 : Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4119 pre-treated red cells using microscopy. Data from three experiments conducted in duplicate are presented as mean percentage parasitemia of the drug-free control system \pm SEMs.

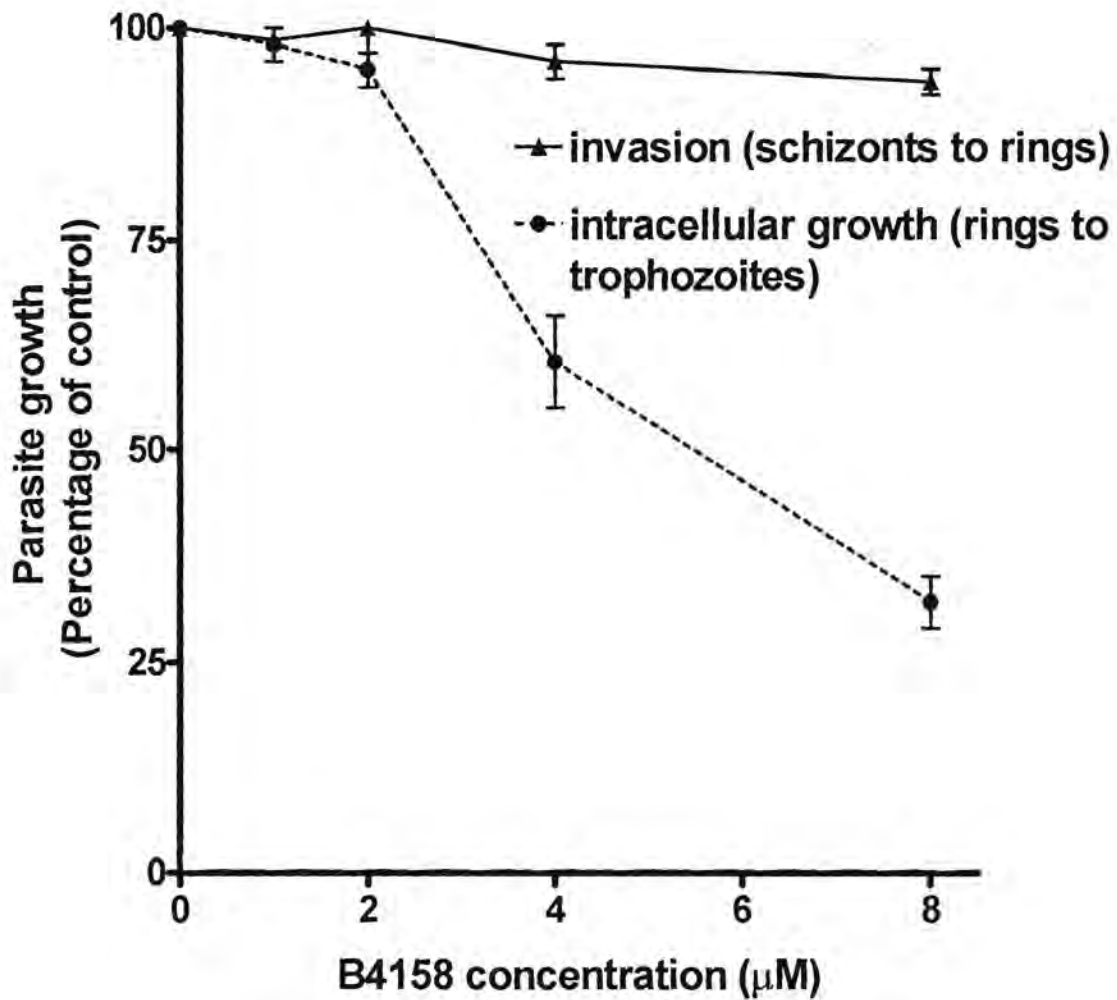


Figure 23 : Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4158 pre-treated red cells using microscopy. Data from three experiments conducted in duplicate are presented as mean percentage parasitemia of the drug-free control systems \pm SEMs.

3.5 DISCUSSION

In the current study I have observed that clofazimine, primarily an anti-mycobacterial agent, does not possess anti-plasmodial activity at therapeutically relevant concentrations *in vitro*. This observation is in agreement with a previous report (Sheagren, 1968). Interestingly, however, substitution of the isopropyl substituent on the imino functional group at position 2 of the phenazine nucleus of clofazimine with a TMP group resulted in acquisition of activity against *P. falciparum*. Of the seven TMP-substituted phenazines, all of which varied according to the number and type of substituents on the aniline and phenyl rings attached at positions 3 and 10 of the phenazine nucleus respectively, two, B4119 and B4158, exhibited anti-plasmodial activity which was comparable with that of chloroquine. B4119, the most potent of the two agents, is halogenated whereas B4158 is isopropylated at their respective aniline and phenyl rings.

These agents were investigated for activity against drug-resistant strains of *P. falciparum*. Both agents, at sub-microgram concentrations, were found to be active against all the drug-resistant strains of the malaria parasite, the level of susceptibility in the case of chloroquine-resistant strains being similar to that of chloroquine-sensitive strains. These observations suggest that the sensitivity of *P. falciparum* to the TMP-substituted phenazines is unaffected by the mechanisms which confer resistance to conventional antimalarial agents. Chloroquine and quinine are lysosomotropic weak bases which accumulate within food vacuoles, killing the parasite by mechanisms related to interference with haem polymerization (Ridley, 1997). Sulfadoxine/pyrimethamine is an antimetabolite which inhibits plasmodial folate metabolism, resulting in parasite death (Peterson *et.al*, 1990).

Considerable interest remains in the identification of compounds which potentiate the activity of classical antimalarials to decrease toxicity and counter development of drug resistance without compromising clinical efficacy (Winter *et.al*, 1997; Yeo *et.al*, 1997). Both B4119 and B4158 potentiated the activity of mefloquine as well as that of chloroquine, suggesting that combinations of the conventional anti-plasmodial agents with the TMP-substituted phenazines may have therapeutic potential. The change in chloroquine sensitivity (from 0.074 to 0.38 μ M) that occurred in the absence of drug pressure during continued culturing of the PfUP10 strain of *P. falciparum* indicates that changes in culturing conditions may affected the sensitivity patterns that emerge due

to selection of chloroquine-resistant clones (Freese *et.al*, 1991).

Stringently synchronised cultures were used to investigate the stages of growth at which the parasite is most vulnerable to the TMP-substituted phenazines. In these experiments B4119 and B4158 were added to ring and late trophozoite cultures of parasites. The test agents were found to interfere with parasite development at both stages of the life cycle, with the late phase of parasite development (the last 24 hours) being most sensitive. These observation suggest that B4119 and B4158 affect metabolic events which are essential for parasite survival throughout the life cycle.

Because the prototype riminophenazine, clofazimine, has been reported to be a membrane active agent (Van Rensburg *et.al*, 1993; De Bruyn *et.al*, 1996), the ability of B4119 and B4158 to alter erythrocyte membranes and inhibit parasite invasion and maturation was also investigated. Schizont-infected red cell suspensions were allowed to rupture and release merozoites *in vitro*. Neither agent inhibited erythrocyte invasion by merozoites. However, the resulting ring forms failed to mature into trophozoites at 4 and 8 μ M of both compounds. This observation suggests that the TMP-substituted phenazines do not alter the structure of the erythrocyte receptor for parasite invasion (glycophorin-A) (Gratzer and Dluzewski, 1993) and that the parasite adherence structures on the red cell surface are insensitive to B4119 and B4158. The compounds therefore act subsequently on the development and maturation of rings into trophozoites. These results imply that the target sites for the TMP-substituted phenazines are located intracellularly and may be indispensable for parasite maturation. Laboratory work conducted in Chapter 4 will serve to identify the antimalarial mode of action of these two compounds.