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SUMMARY

A novel flow cytometric procedure was established for use in evaluating the *in vitro* antimalarial activity of tetramethylpiperidine (TMP)- substituted phenazines. The flow cytometric procedure was compared with microscopy and radiometry for efficiency in quantitating the level of parasitemia in malaria cultures. The flow cytometric method compared well, as determined by the Bland and Altman measure of agreement, with both microscopy and radiometry and was chosen for use in this study due to its speed, precision and convenience (includes a fixing step that allows samples to be evaluated at any one time). The TMP-substituted phenazines B4119 and B4158, synthetic derivatives of clofazimine, were evaluated extensively against a drug-sensitive and various drug-resistant lines of *Plasmodium falciparum in vitro* and against *P. berghei* in mice. Parasite growth was measured using microscopic and flow cytometric methods, while heme polymerization was investigated using an infrared spectroscopic procedure. The therapeutic potential of B4119 alone (30mg/kg/day), and in combination with a sub-therapeutic dose of chloroquine (1.25μg/kg/day) was measured in a murine model of experimental infection with *P. berghei*.

B4119 and B4158, but not clofazimine, inhibited the growth of the drug-sensitive strain of *P. falciparum* with respective IC_{50} values of 0.22μM and 0.4μM, while the drug-resistant strains of the parasite were equally sensitive to the TMP-substituted phenazines, indicating a lack of cross-resistance. Augmentation of anti-plasmodial activity was observed when B4119 and B4158 were used in combination with chloroquine or mefloquine. The compounds were capable of inhibiting all blood stages of *P. falciparum*. Pretreatment of erythrocytes with B4119 and B4158 did not prevent merozoite invasion. B4119- and B4158-mediated inhibition of the growth of *P. falciparum* was associated with interference with heme polymerisation to β-haematin *in vitro*. Administration of B4119 to *P. berghei*-infected mice was accompanied by a significant reduction in parasitemia, while additive therapeutic activity was observed when this agent was combined with chloroquine.

The TMP-substituted phenazines B4119 and B4158 are promising, novel anti-plasmodial agents.

OPSOMMING

'n Nuwe vloeisitometriese prosedure is ontwikkel om te gebruik in die evaluering van die *in vitro* antimalaria aktiwiteit van tetrametielpiperidien (TMP)-gesubstitueerde fenasiene. Die effektiwiteit van die vloeisitometriese prosedure om die vlakke van parasitemie in malaria kulture te bepaal is met die mikroskopiese en radiometriese metodes vergelyk. Die vloeisitometriese metode het, soos bepaal deur die Bland en Altman se mate van ooreenstemming, goed met beide die mikroskopiese en radiometriese metodes vergelyk en is vir hierdie studie gekies aangesien dit vinnig, akkuraat en gerieflik is. Hierdie metode het 'n fikseringsstap ingesluit wat dit moontlik gemaak het om die monsters op 'n latere geleentheid te evalueer. Die TMP-gesubstitueerede fenasiene B4119 en B4158, sintetiese derivate van klofasimien, is breedvoerig teen 'n geneesmiddel-sensitiewe en verskeie geneesmiddel-bestande lyne van *Plasmodium falciparum in vitro* en teen *P. berghei* in muise ondersoek. Parasietgroei is deur middel van mikroskopiese en vloeisitometriese metodes bepaal terwyl heem-polimerisasie ondersoek is deur die gebruik van spektroskopiese prosedures. Die terapeutiese potential van B4119 alleen (30mg/kg'dag) en in kombinasie met 'n sub-terapeutiese dosis van chlorokien (1.25µg/kg/dag) is in 'n muis model van eksperimetele infeksie met *P. berghei* bepaal.

B4119 en B4158, maar nie klofasimien, het die groei van die geneesmiddel-sensitiewe stam van *P. falciparum* by IK_{50} waardes van 0.22µM en 0.4µM respektiewelik geïnhibeer, terwyl die geneesmiddel-bestande stamme van die parasiet ewe sensitief was vir die TMP-gesubstitueerde fenasiene, wat op die afwesigheid van kruis-bestandheid dui. Verhoging van anti-plasmodiale aktiwiteit is waargeneem wanneer B4119 en B4158 in kombinasie met chlorokien en meflokien gebruik is. Die verbindings was in staat om alle bloed-stadiums van *P. falciparum* te inhibeer vooraf behandeling van eritrosiete met B4119 en B4158 het nie die indring van merozïete verhoed nie. B4119- en B4158-bemiddelde inhibisie van die groei van *P. falciparum* is met veranderinge in heem polimerisasie tot β-hematien *in vitro* geassosieer. Die toediening van B4119 aan *P. berghei*-geïnfekteerde muise het tot 'n betekenisvolle vermindering in parasitemie gelei, terwyl 'n vermeerdering in terapeutiese aktiwiteit waargeneem is Indians die verbinding met chlorokien gekombineer is. Die TMP-gesubstitueerde fenasiene B4119 en B4158 is belowende, nuwe anti-plasmodiale middels.

LIST OF ABBREVIATIONS

ADCI	Antibody-dependent cellular immunity
AIDS	Acquired immunodeficiency syndrome
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BBIQ	Bisbenzylisoquinolines
Ca	Calcium
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CO_2	Carbon dioxide
CQ	Chloroquine
CQR	Chloroquine resistant
CQS	Chloroquine sensitive
CSA	Chondroitin sulphate A
DHFR	Dihydrofolatereductase
DDT	Dichloro-diethyl-trichloroethane
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding antigen
EIPA	5-(N-ethyl-N-isopropyl) amiloride
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
G3PDH	Glyceraldehyde 3-phosphate dehydrogenase
H	Hydrogen
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
HPIA	Heme polymerization inhibitory activity
ICAM-1	Intercellular adhesion molecule-1
iRBC	Infected red blood cell
K	Potassium
MDR	Multidrug resistance
Mef	Mefloquine

MRC	**Medical Research Council**
MSP-1	**Merozoite surface protein-1**
Na	**Sodium**
NaCl	**Sodium chloride**
NAD	**Nicotinamide dinucleotide**
NADPH	**Nicotinamide adenine dinucleotide phosphate (reduced)**
NHE	**Na+/H+ exchanger**
NMRP	**National Malaria Research Programme**
NPPB	**5-nitro-2-(3-phenylpropylamino) benzoic acid**
NRA	**Nucleoside releasing agent**
O_2	**Oxygen**
PABA	**Para-aminobenzoic acid**
PBS	**Phosphate buffered saline**
PCT	**Parasite clearance time**
***PF*EMP-1**	***Plasmodium falciparum* erythrocyte membrane protein-1**
***PF*EMP-2**	***Plasmodium falciparum* erythrocyte membrane protein-2**
***PF*HRP-1**	***Plasmodium falciparum* histidine rich protein-1**
PG	**Prostaglandin**
PGE_2	**Prostaglandin E_2**
Pgh1	**P-glycoprotein homologue-1**
PKC	**Protein kinase C**
PLA_2	**Phospholipase A_2**
PRR	**Parasite reduction ratio**
PSD	**Sulfadoxine/pyrimethamine**
PVM	**Parasitophorous vacuolar membrane**
Rb	**Rubidium**
RBC	**Red blood cell**
RESA	**Ring-infected erythrocyte surface antigen**
RNA	**Ribonucleic acid**
TCA	**Tricarboxylic acid**
TMP	**Tetramethylpiperidine**
TSP	**Thrombospondin**

VCAM-1	**Vascular adhesion molecule-1**
WHO	**World Health Organization**

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Figure 42 : Effects of intra peritoneal administration of chloroquine (0.25µg/kg/day) together with oral administration of B4119 (30mg/kg/day) on the survival rate of *P. berghei*-infected Balb/C mice.

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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 ; Kremsner *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 oocyte 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).

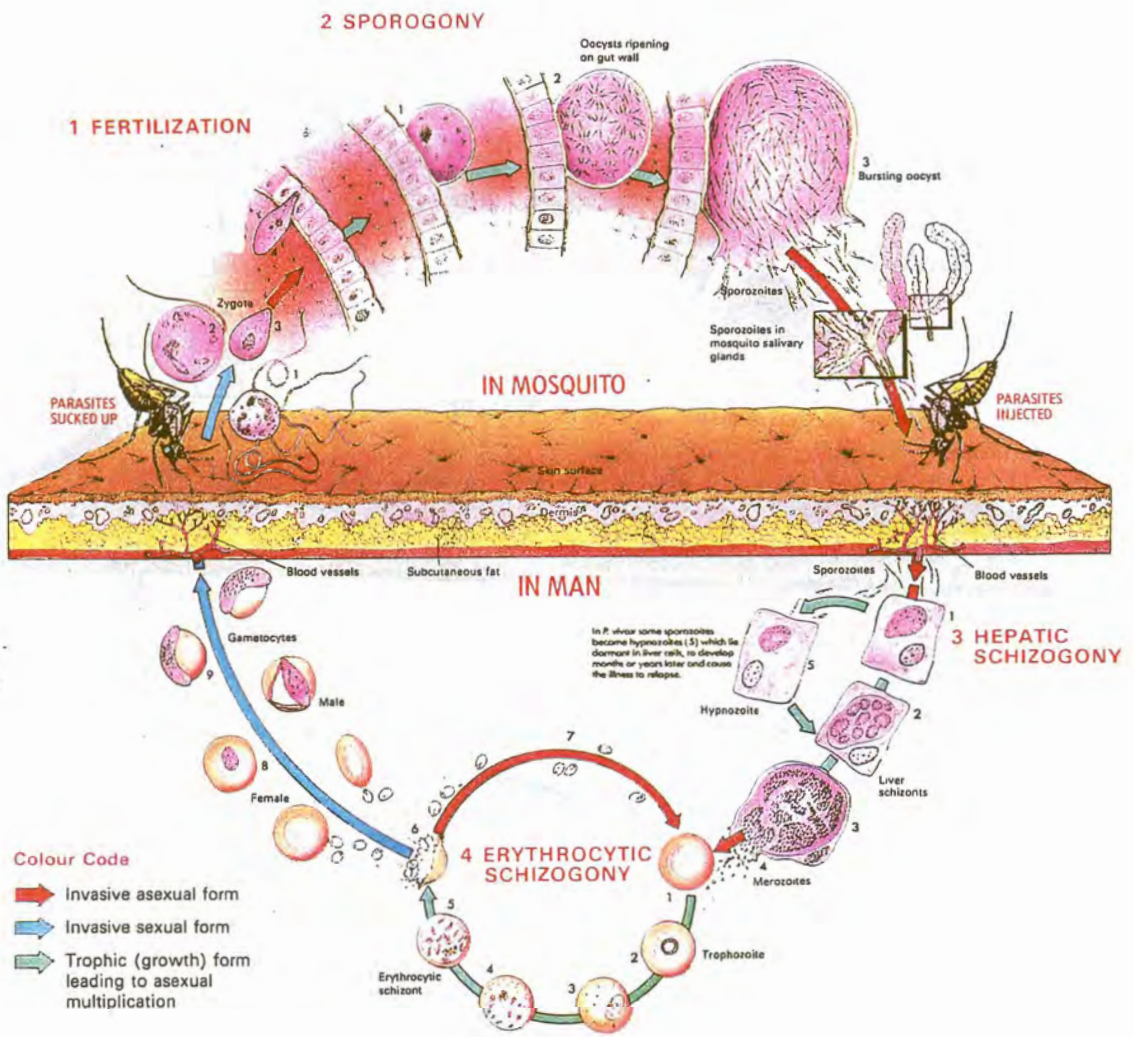


Figure 1 : The life cycle of *Plasmodium falciparum* (Knell, 1991).

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.

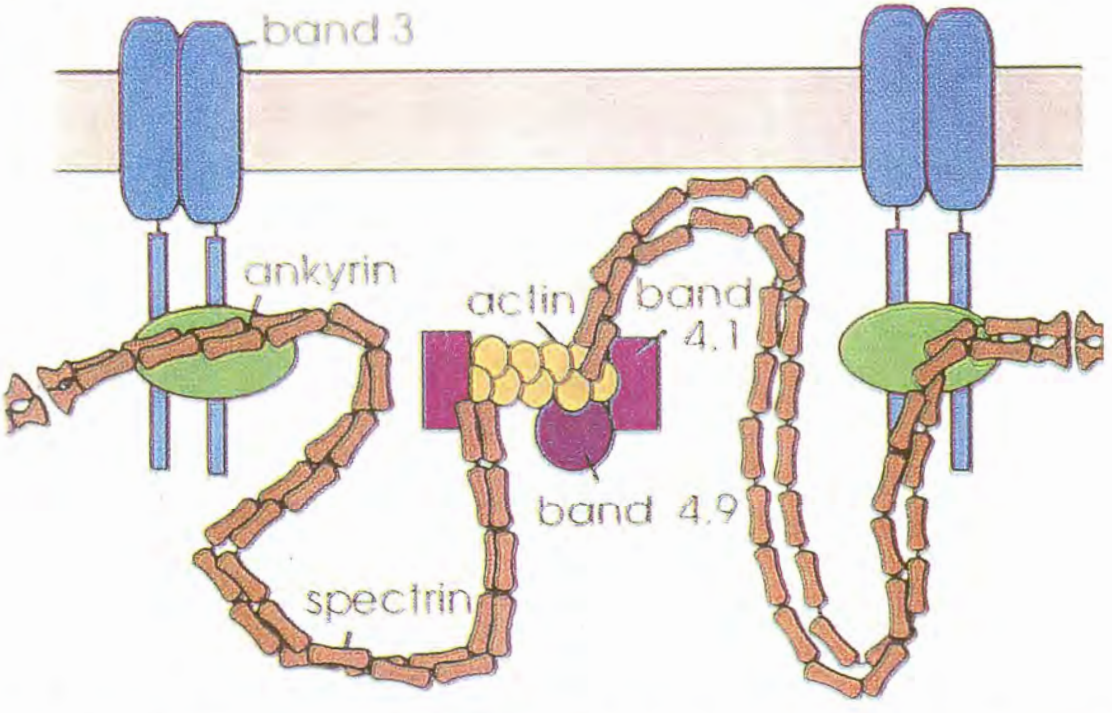


Figure 2 : Schematic presentation of the erythrocyte membrane and organization of its proteins.

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.

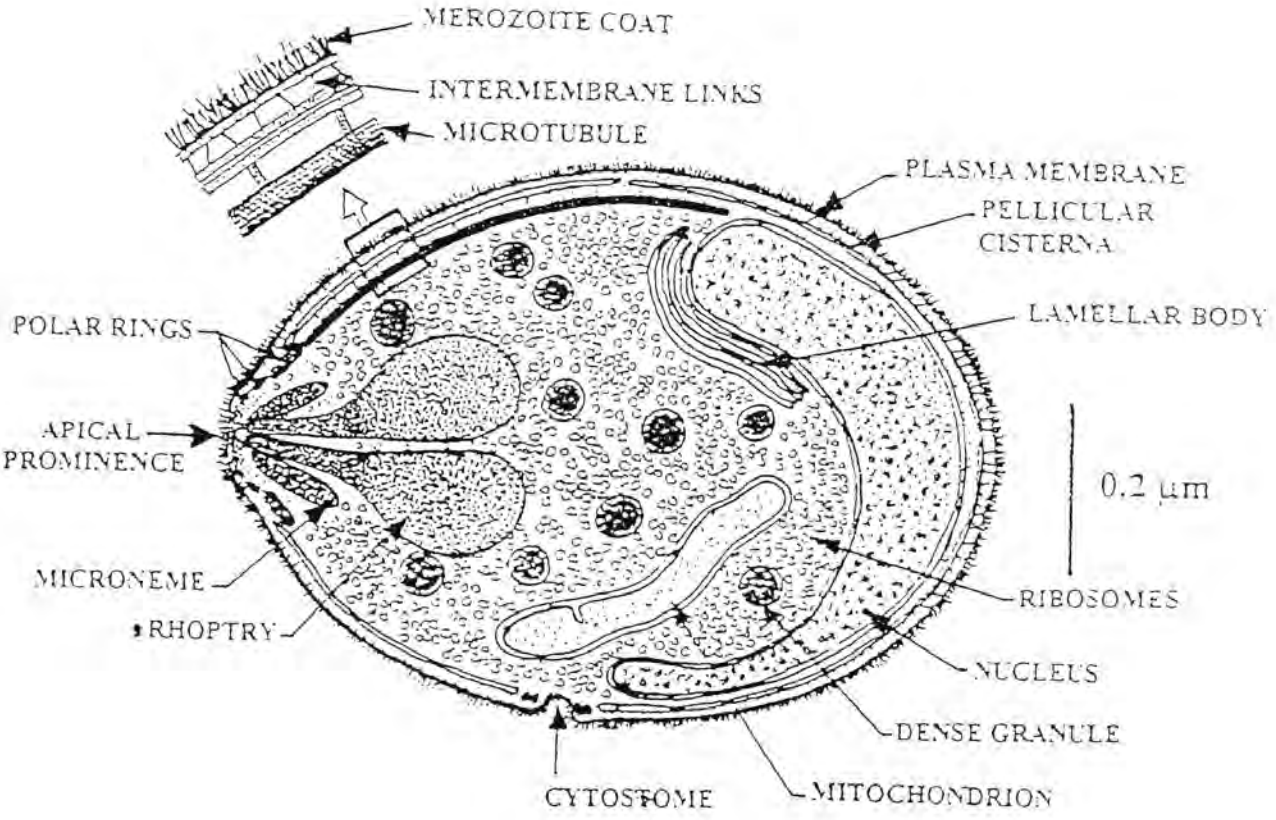


Figure 3: The structure of *Plasmodium falciparum* merozoite.

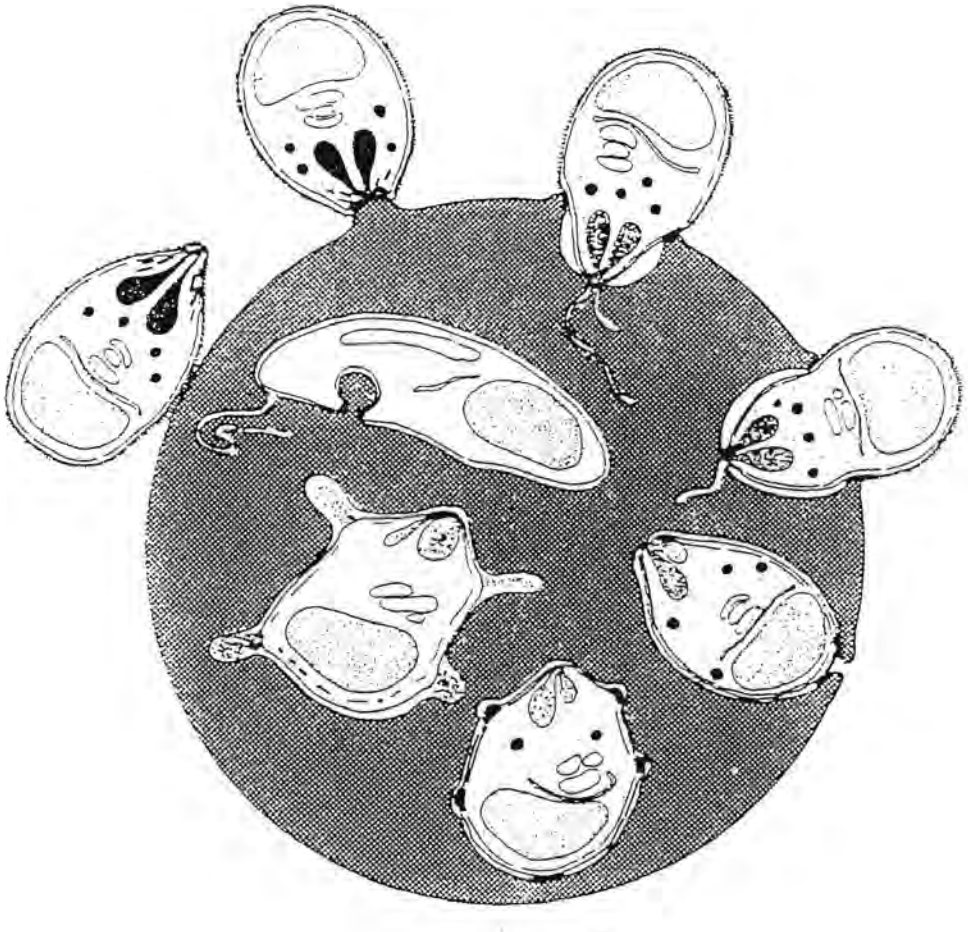


Figure 4 : Schematic presentation of the invasion of erythrocytes by merozoites.

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 glycophorins 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 glycophorins is sialic acid-dependent. Neuraminidase and trypsin cleave part of the glycophorin 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 O_2 and CO_2 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).

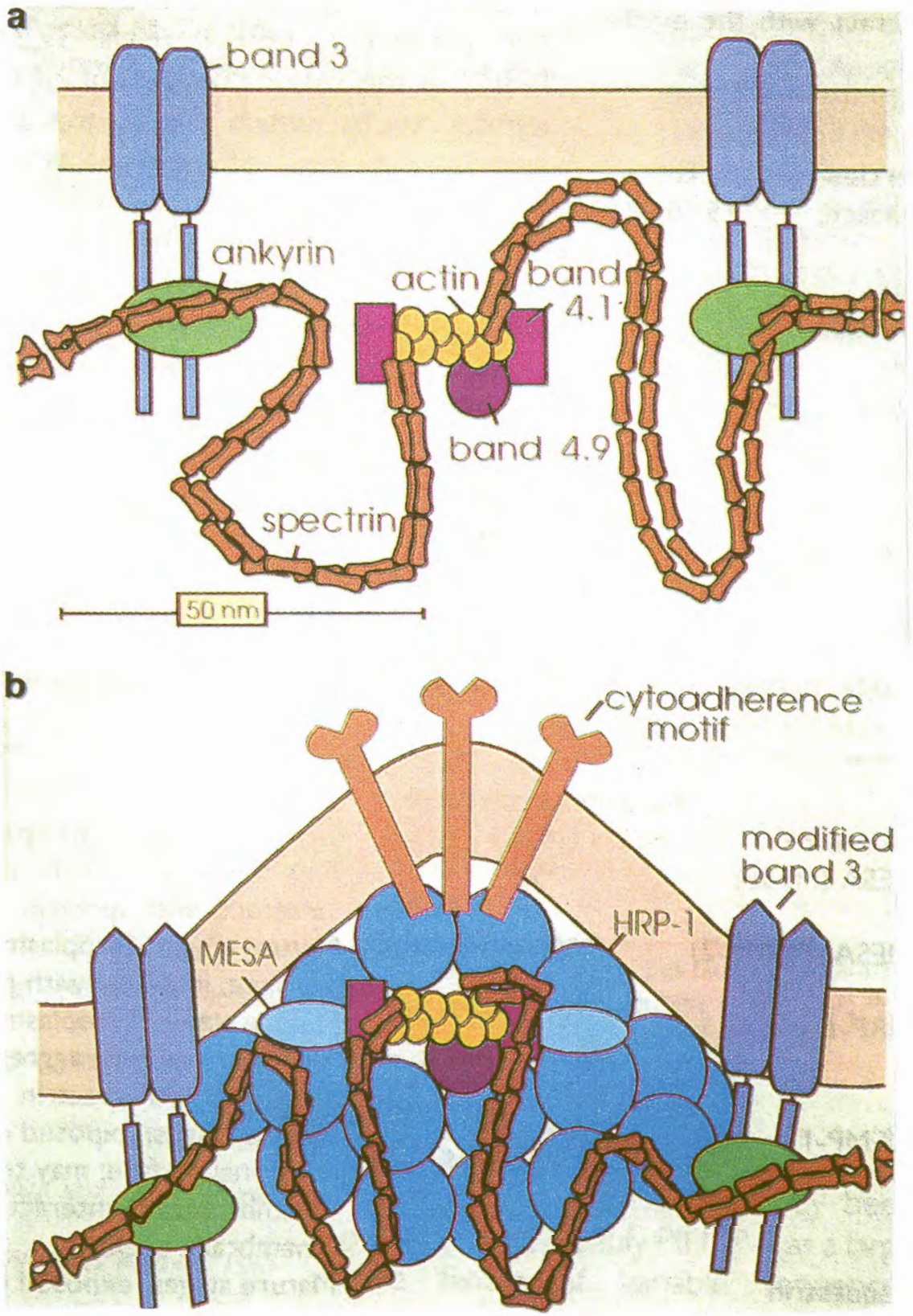


Figure 5 : Diagramatic presentation of the membrane structural differences between normal (a) and parasite-infected (b) red blood cells.

The knobs are composed of parasite derived proteins. These are *Plasmodium falciparum* erythrocyte membrane protein-1 (*PF* EMP-1) and sequestrin, as well as host-derived protein, altered Band 3 protein (*Pf*alhesin) (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 (*PF*HRP-1), *Plasmodium falciparum* erythrocyte membrane protein-2 and -3 (*PF*EMP-2 and -3). *PF*HRP-1 is the major constituent of the conical knobs and together with *PF*EMP-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 *i*RBC'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 *i*RBC, 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).

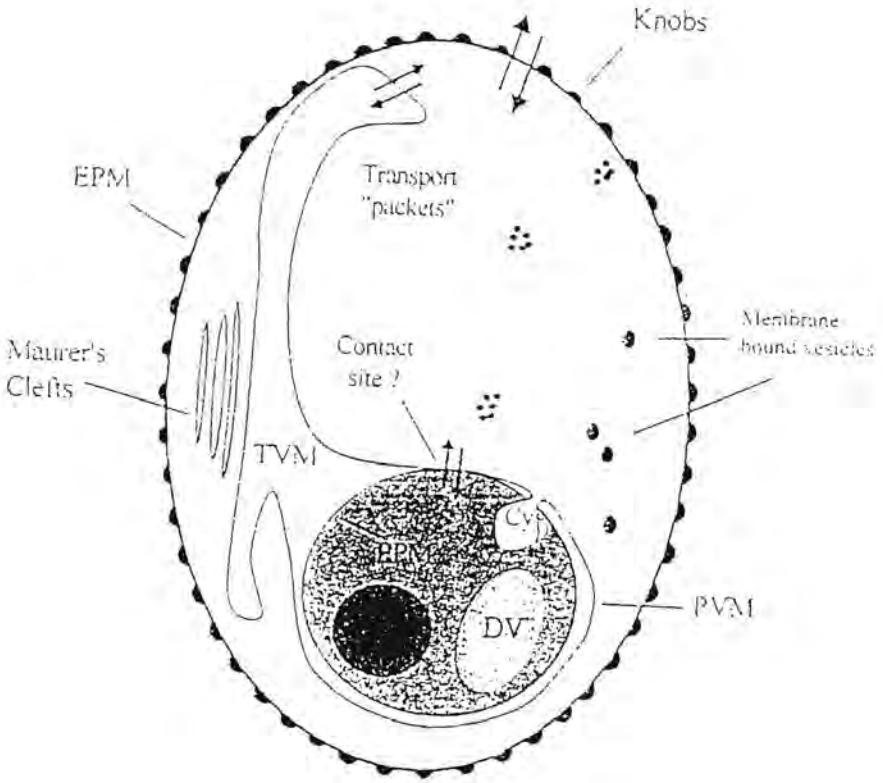


Figure 6 Schematic presentation of the membranous structures and transport pathways of the infected erythrocyte. TVM, tubovesicular membrane network; PPM, parasite plasma membrane; PN, parasite nucleus; DV, digestive vacuole; EPM, erythrocyte plasma membrane; PVM, parasite vacuole membrane; ↓↑, new trafficking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

Properties of the neotransporters include **(a)** anion selectivity with high permeability for chloride ions **(b)** high permeability for cations © 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 pinocytic vesicles where it is degraded into globin and toxic heme (Slater *et.al*, 1991 ; Meshnick, 1996). Globin is converted by aspartic protease (plasmephins) 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.

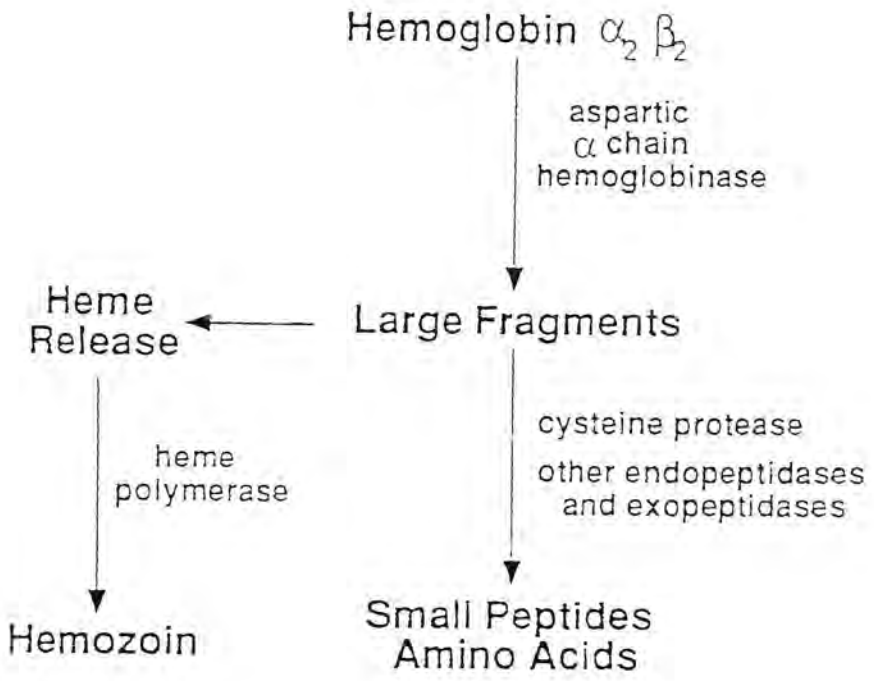


Figure 7 : Proposed haemoglobin degradation pathway in *Plasmodium falciparum*

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 malarias (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, mepracrine 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

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 determine 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 this 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 cloroquine resistance were observed from the southern African states of Botswana, Mozambique, Angola, Namibia, Swaziland and Zimbabwe (Freese *et.al*, 1991 ; Freese *et.al*, 1993). Cloroquine 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.

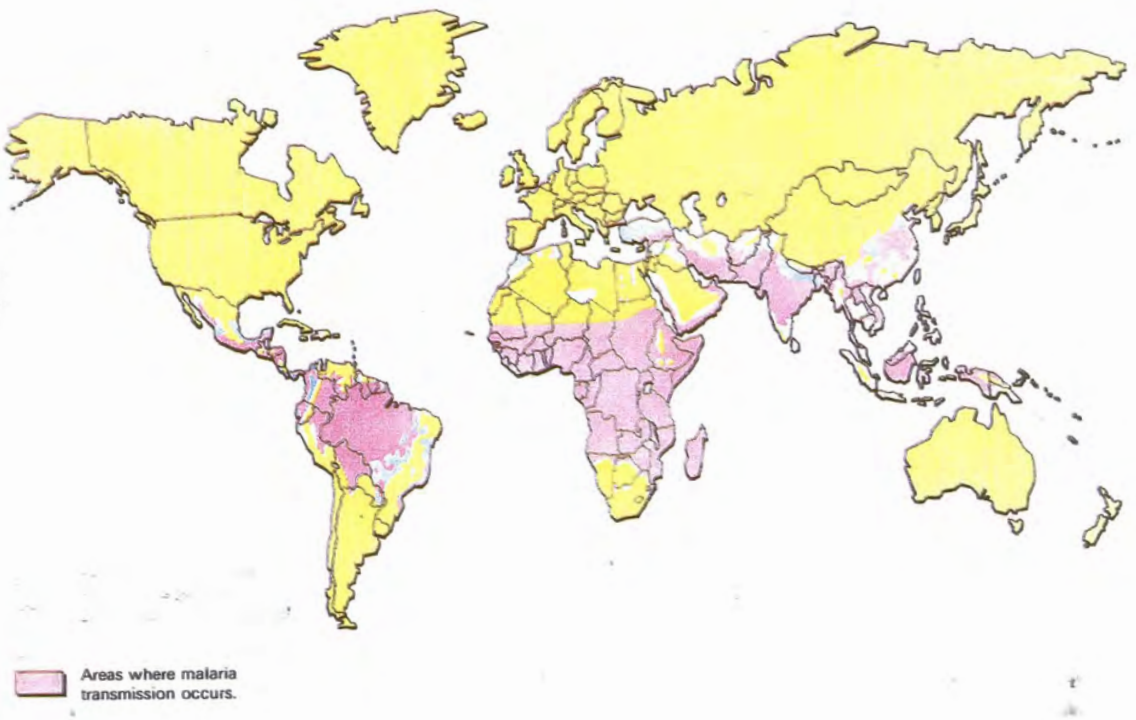


Figure 8 : Geographical distribution of chloroquine resistance.

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-lifes 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 acidotrophic 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 ; Wünsch *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 inessential (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 (Frean, 1996 ; Vial, 1996 ; Butler, 1997) and

(vi) identify potent vaccine candidates with either anti-sexual or -assexual 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 *Autuos* 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 *Plasmodiun 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 (SP*f*66) 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 E_2 (PGE_2) 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 elctron-transporting membrane-bound enzyme of phagocytes that is sensitive to activation by leucoattractantants, 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 resceptor. 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 activitry *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 phospholipaseA_2(PLA_2)-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 PLA_2 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 PLA_2 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).

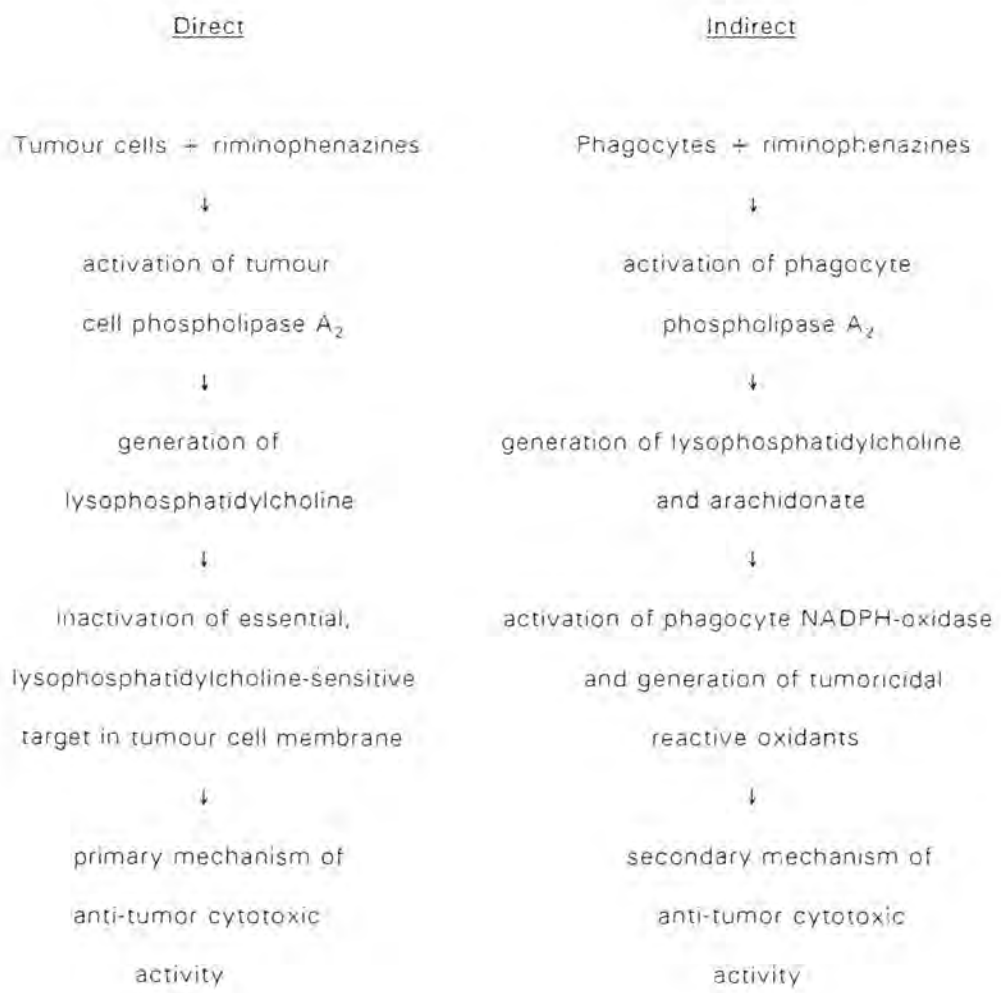


Figure 9 : Pathways of riminophenazine-mediated anti-tumor activity.

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 actvity 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.*

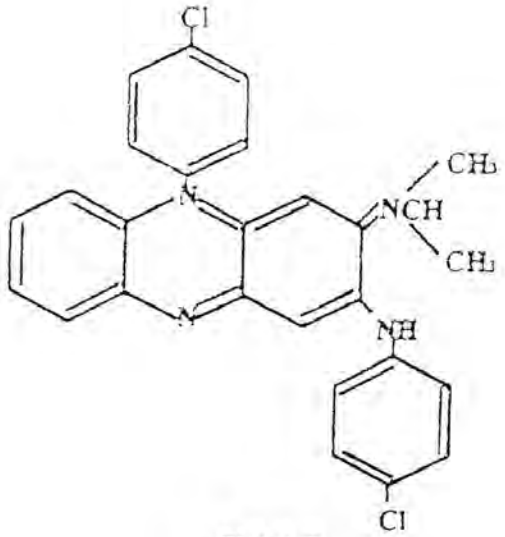
3 effects on the growth of the various stages of the parasite i.e stage-specific activity.

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

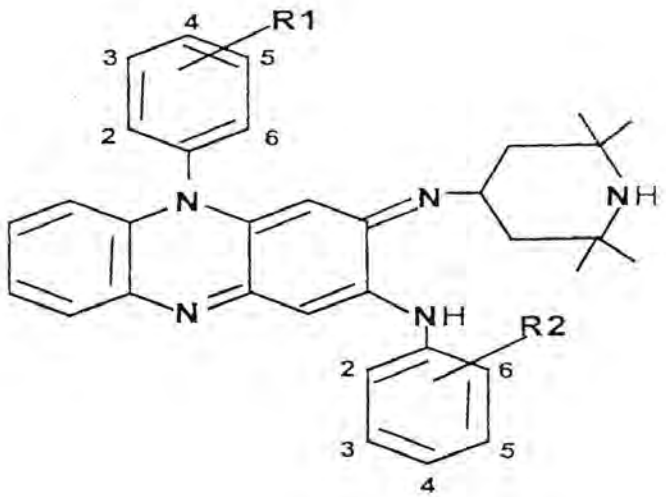
5 haem polymerization inhibitory activity (HPIA).

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-itercalatively 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

200µl 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 4^0C 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 -20^0C. The plasma was heat-inactivated at 56^0C 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 (20µL) at 2% parasitaemia and 5% haematocrit were incubated with serial dilutions of chloroquine (20µL) ranging from 4nM to 250nM (final dilution) in 96-well microculture plates made up to 200µL 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, 150µL of medium was replaced with hypoxanthine-free medium containing tritiated hypoxanthine (1µCi). 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, 100µL of medium was removed from the wells and replaced with 100µL of the fixation solution. The plates were stored overnight at 4°C. The fixed parasite cultures (25µL) were stained with 0.5mL of a thiazole orange solution (0.26µg/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 ± SEM of parasite growth in drug-treated wells and IC_{50} concentrations were obtained using the Graphpad Instat2 progamme. 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 ([^{3}H]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.

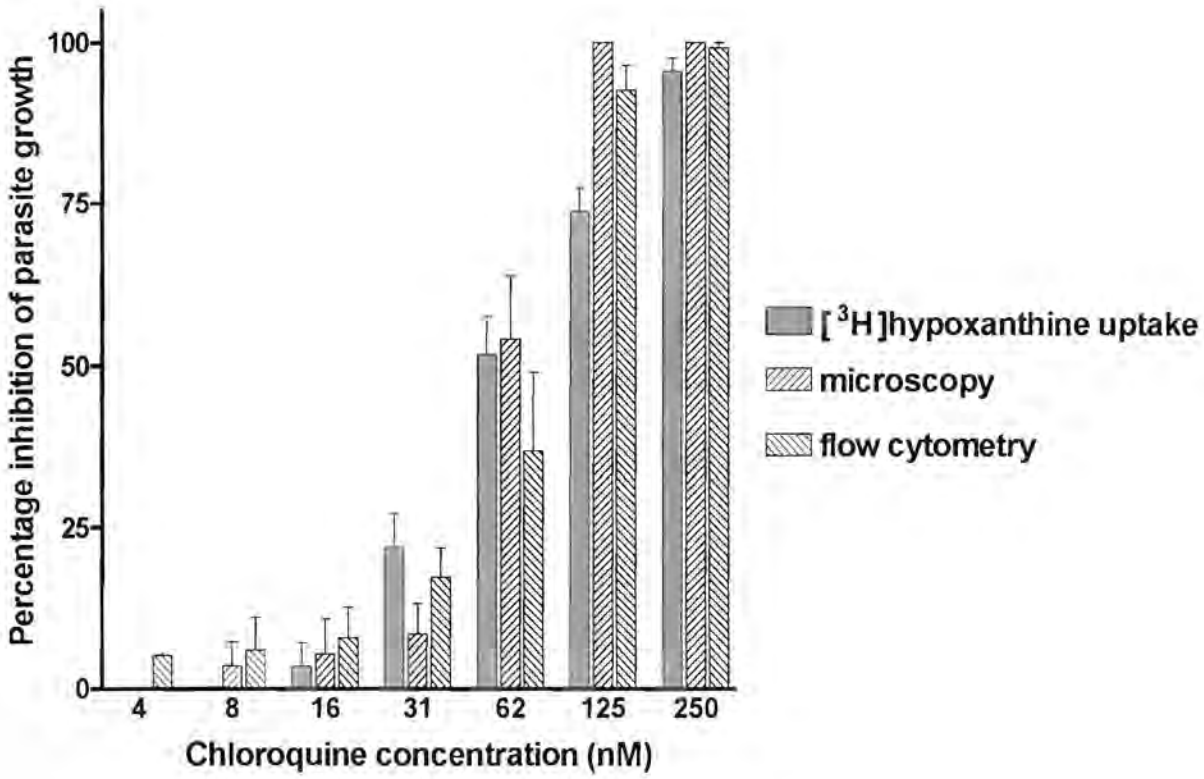


Figure 11: Evaluation of parasitemia in malaria parasite cultures using flow cytometric, radiometric and microscopic methods. Results are expressed as percentage inhibition of parasite growth and show mean values and SEM of five different experiments for each method

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-(2,26,6-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 (NMRP) 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 20µL ring-infected erythrocyte suspension (2% parasitemia and 0.5% final haematocrit), and 180µL of complete medium with B4119 and B4158 [(1-8µM) or (0.2-2µg/mL)] or without the drug (control). The total volume in all wells was 200µL. 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 IC_{50} for chloroquine of this parasite strain increased from 0.075 to 0.38µM in the absence of drug pressure. To each well of a microtiter plate was added 20µL of a ring- infected red cell suspension. For the drug combination assays, 20µL of both chloroquine diphosphate (0.044 to 0.50µM) or mefloquine hydrochloride (0.001 to 0.065µM) were added to the wells, either individually or in combination with 20µL of either B4119 (0.4µM) or B4158 (0.8µM). The suspension was made up to 200µL 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 micrototer plate was added 20µl of synchronized ring and late trophozoites parasite suspensions (5% parasitemia and 0.5% haematocrit), and 180µL of parasite culture medium with B4119 and B4158 (1 to 8µM) or without (control) the drug. The total volume in all wells was 200µL. The plates were placed in a modular chamber, gassed for 15 min and then incubated for 12 hours at 37^0C. 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 [(1-8μM) or (0.2-2μg/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 (20μL) 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 200μL with complete culture medium. Wells containing 20μL of iRBC suspension in 180μL of the culture medium were also included. The plates were gassed and sealed in the modular gas chambers and incubated at 37^{0}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 ± SEMs and statistical analysis performed using the Student's t-test. The IC_{50} 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 [(1-8μM) or (0.2-2μg/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 [(1-8μM) or (0.2-2μg/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 IC_{50} values in Table 1. The IC_{50} 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 IC_{50} value for chloroquine increased from 0.075μM to 0.38μM. Combining the TMP-substituted phenazines, B4119 (0.4μM) and B4158 (0.8μM), with low concentrations of chloroquine (0.044-0.50μM) or mefloquine (0.01-0.065μM) 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 8μM). 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 1μM 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 8μM. 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 2μM) but significantly ($p < 0.05$) interfered with the maturation of the ring forms into trophozoites at 4 and 8μM.

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

	IC_{50} values (µM) of experimental compounds			
Drugs	**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

IC_{50} 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 ± 9 (t)	51.7 ± 9 (t)
1	20 ± 7 (r)	21 ± 6 (r)
2	13 ± 4 (r)	16 ± 5 (r)
4	8 ± 2 (r)	10 ± 3 (r)
8	9 ± 3 (r)	8 ± 3 (r)

B

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

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

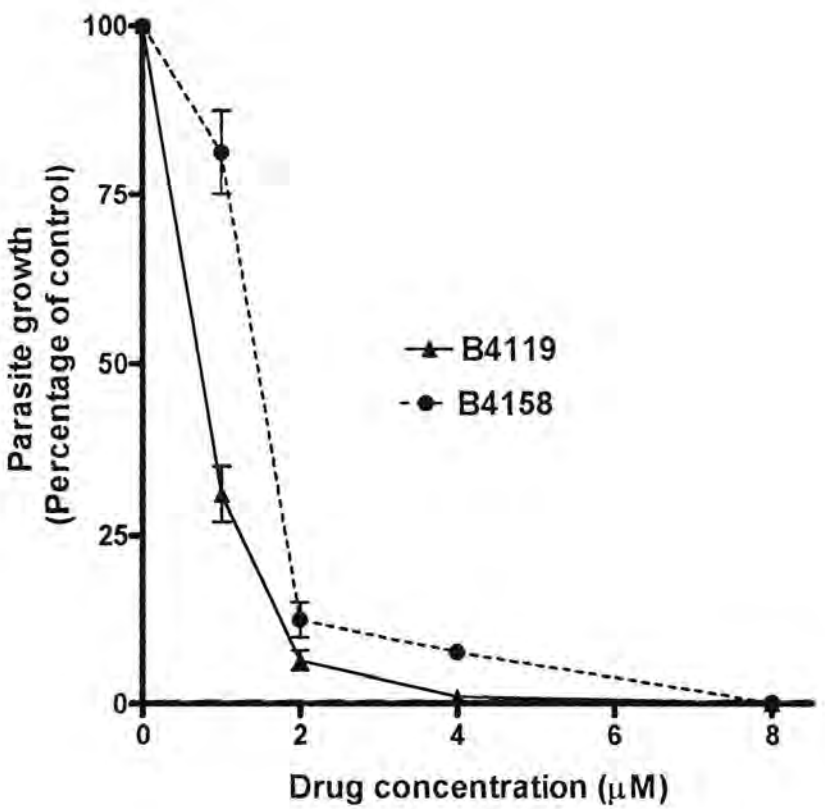


Figure 12 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the PfUP10 strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as the mean percentage parasitemia of the drug-free control systems ± SEM of three different experiments.

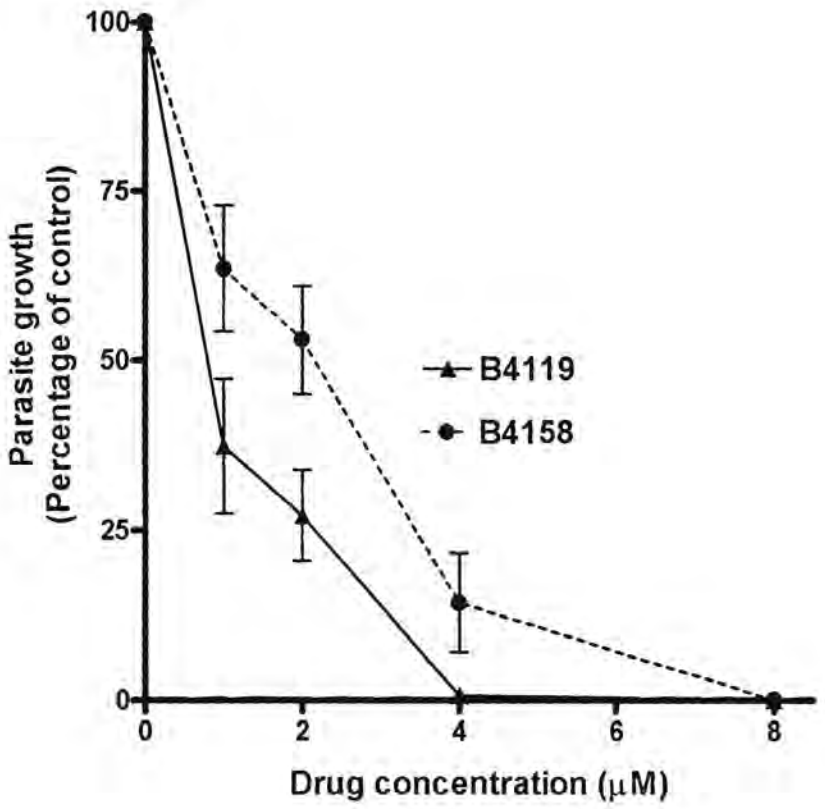


Figure 13 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against RSA17 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems ± SEM of two different experiments.

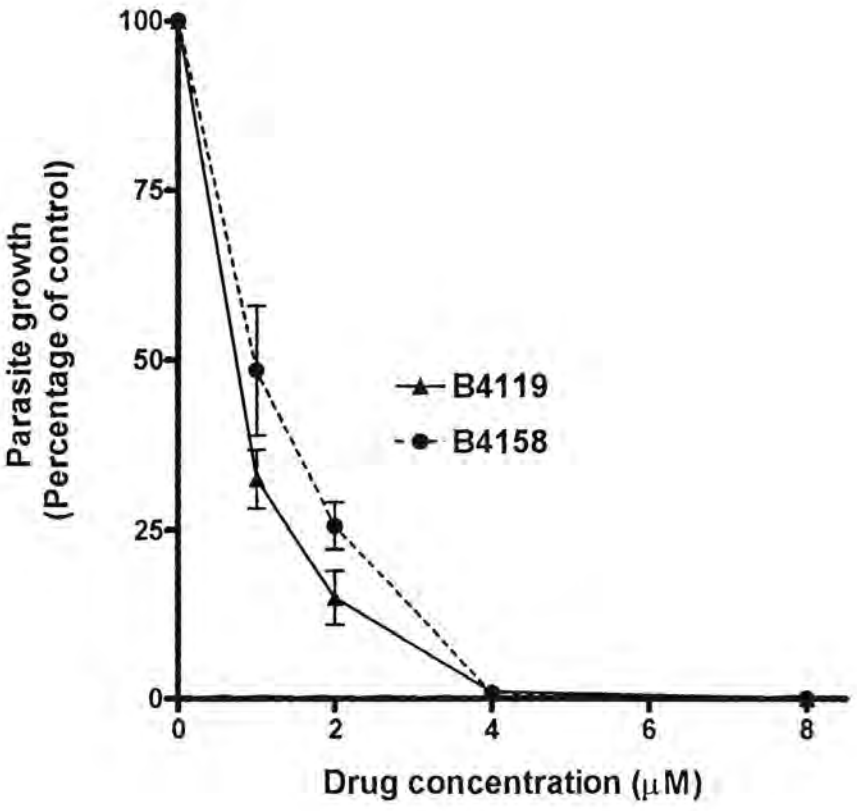


Figure 14 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA16 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems ±SEM of two different experiments.

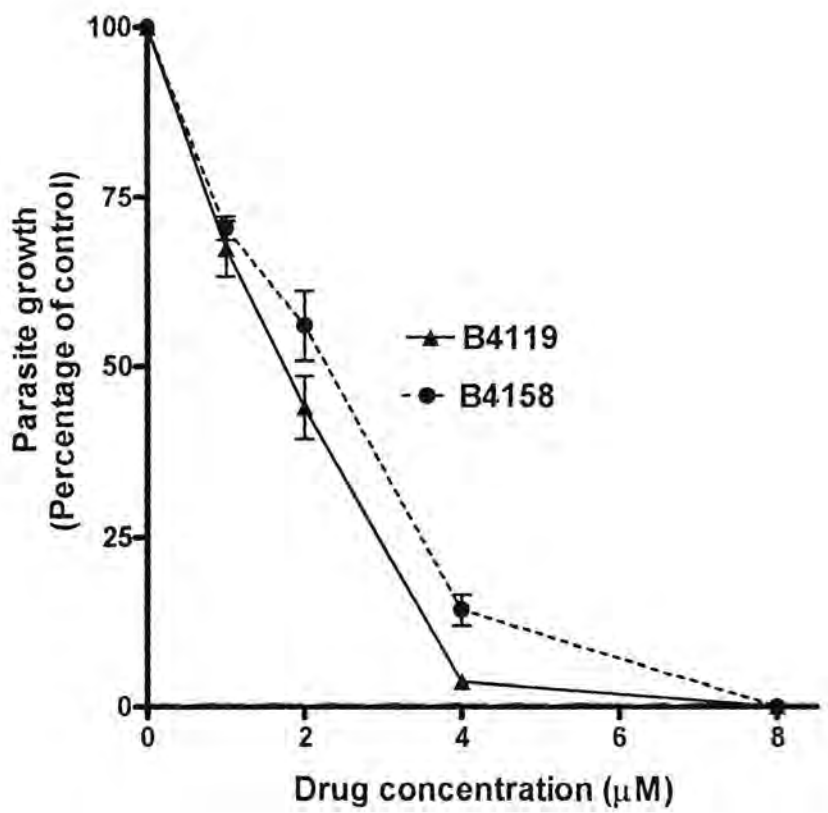


Figure 15 : Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA9 strain of *Plasmodium falciparum* using microscopy. Results are expressed as the mean percentage parasitemia of the drug-free control systems ± SEM of two different experiments.

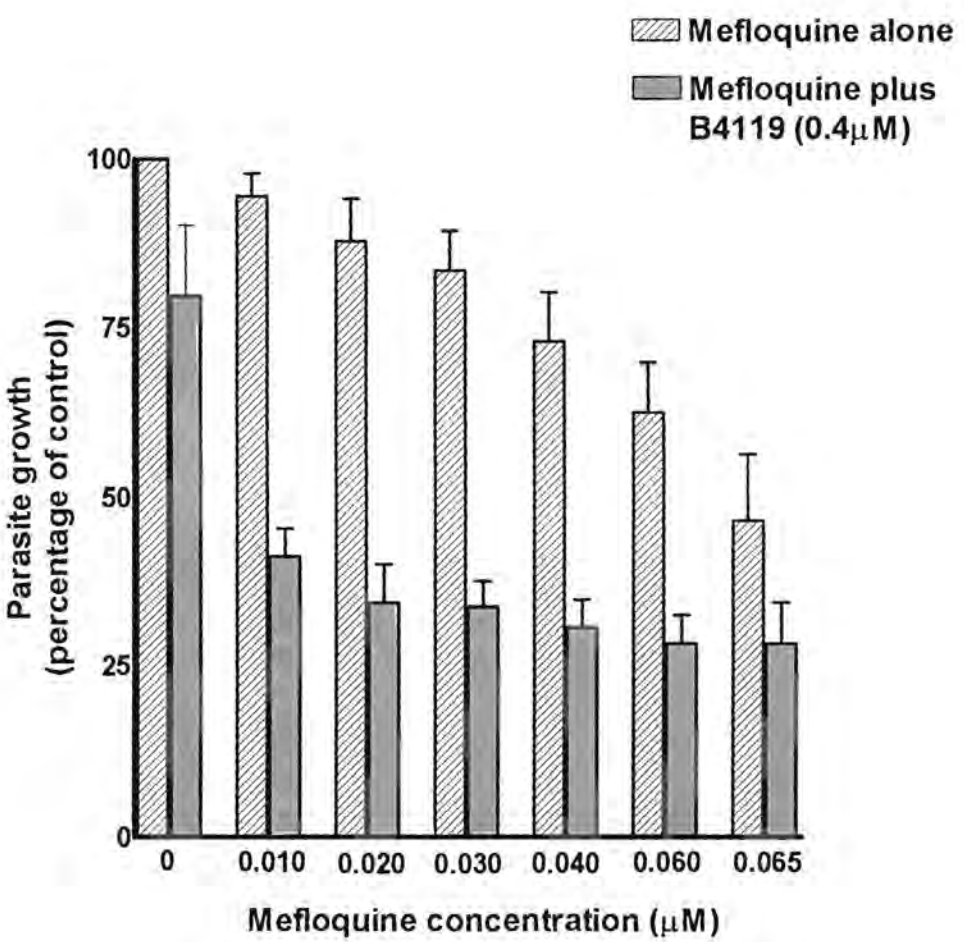


Figure 16 : Effects of B4119 (0.4μM) and mefloquine (Mef) (0.010 - 0.065μM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as mean percentage ± SEM of three different experiments.

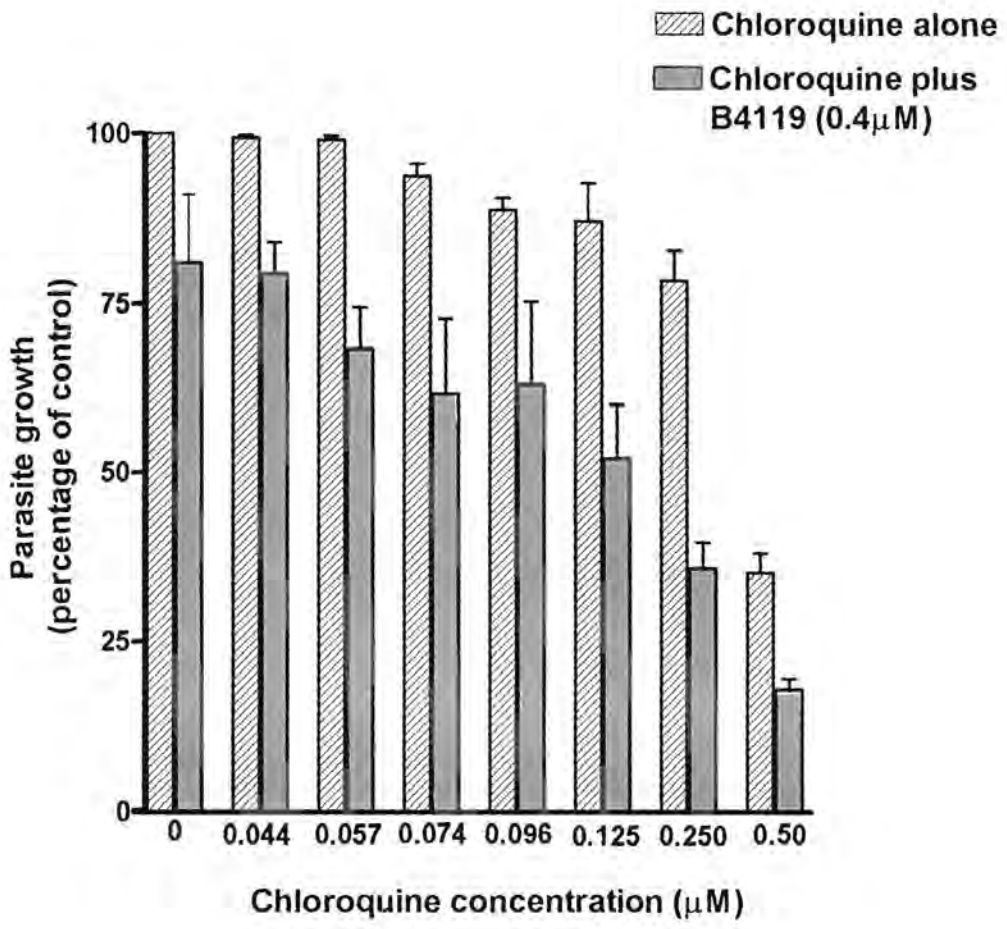


Figure 17 : Effects of B4119 (0.4μM) and chloroquine (CQ) (0.044 -0.50μM) individually and in combination on the growth PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results are expressed as mean percentage ± SEM of three different experiments.

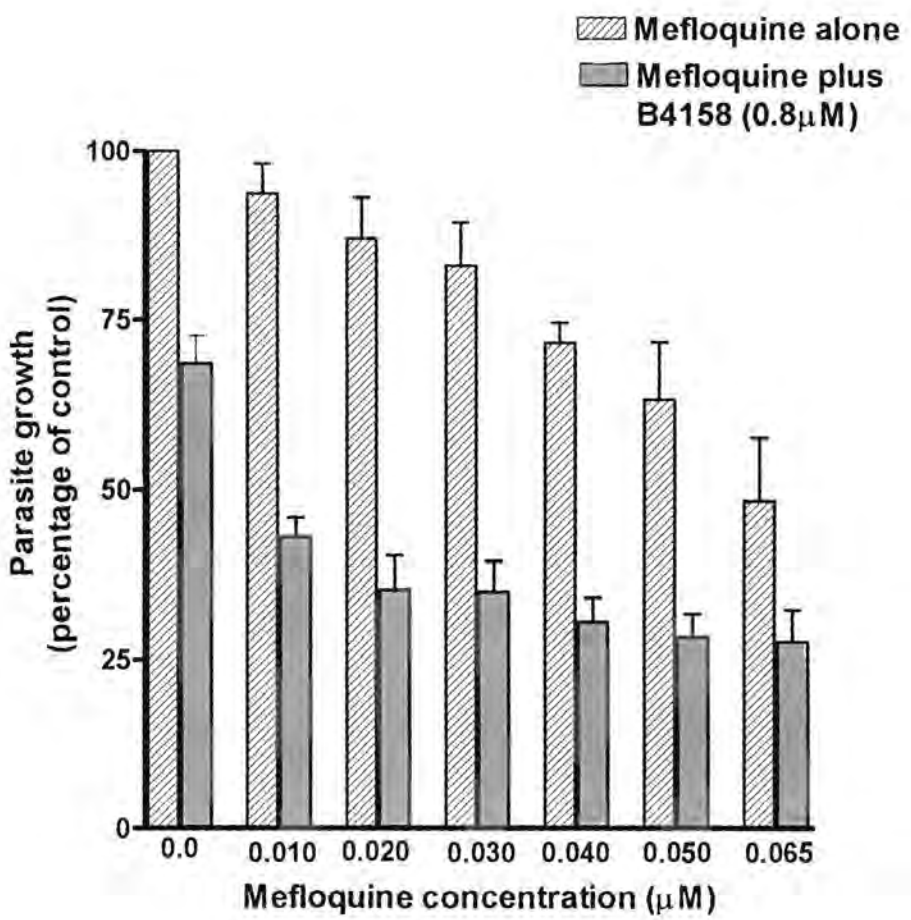


Figure 18 : Effects of B4158 (0.8μM) and mefloquine (Mef) (0.010 - 0.065μM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry . Results represent the mean percentage± SEM of three different experiments.

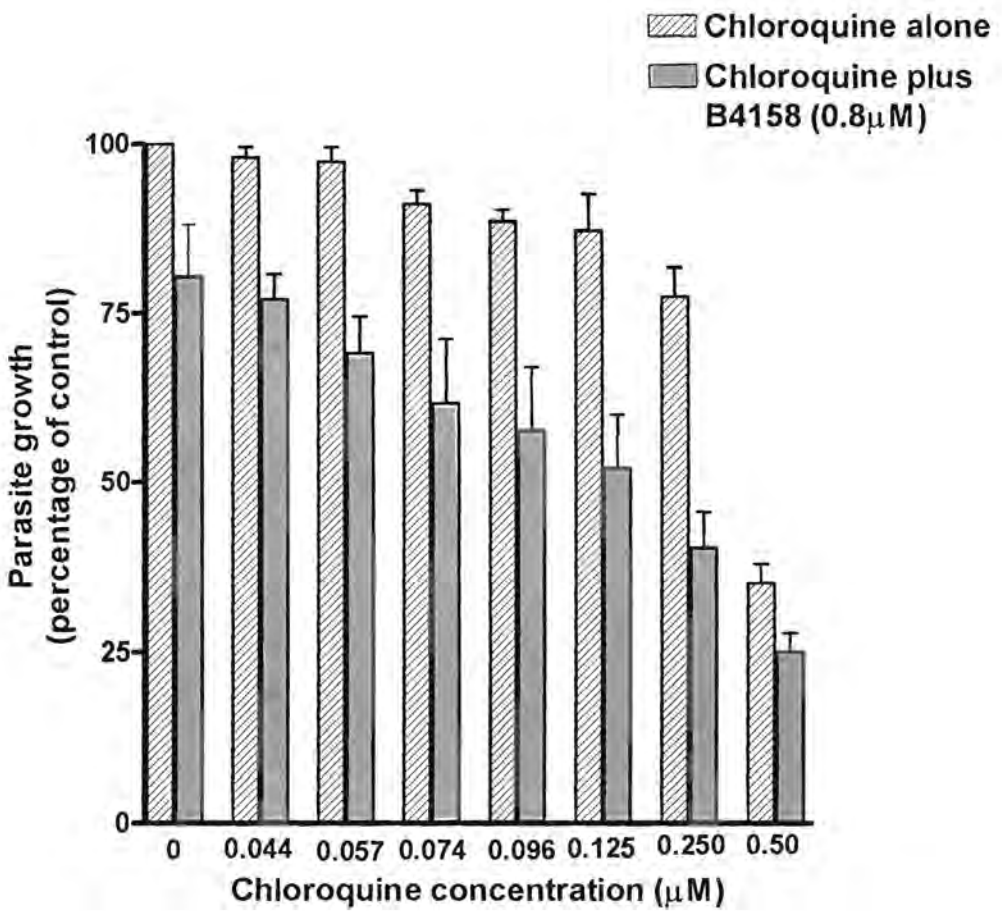


Figure 19 : Effects of B4158 (0.8μM) and chloroquine (CQ) (0.044 -0.50μM) individually and in combination on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using flow cytometry. Results represent mean percentage± SEM of three different experiments.

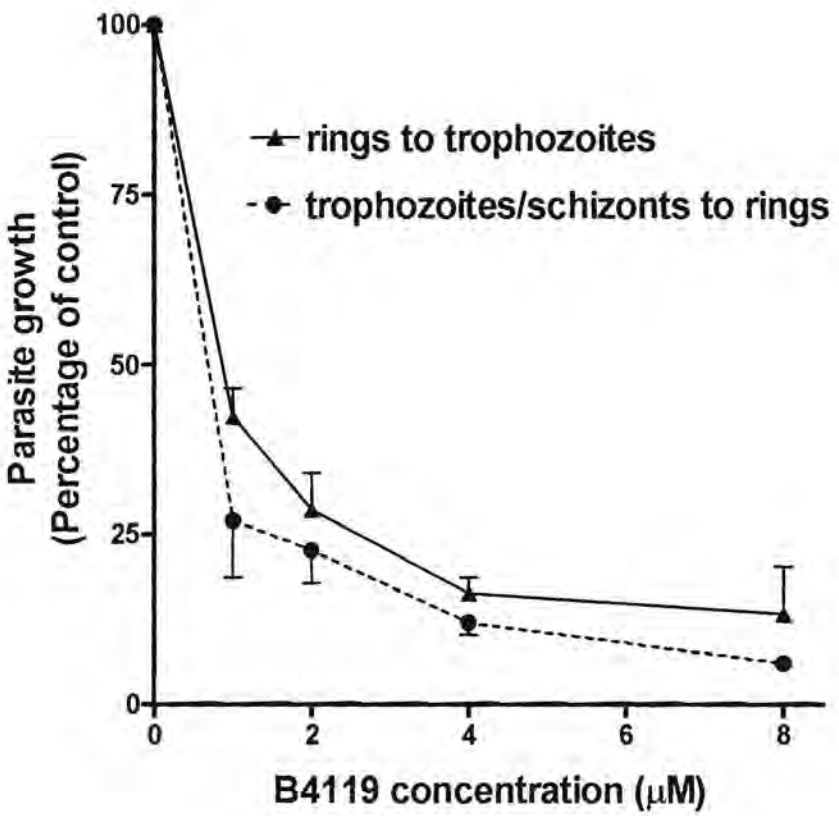


Figure 20 : Stage-specific antimalarial activity of B4119 for the first (ring to trophozoites/schizonts) and last (trophozoites/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry. Data from three experiments conducted in duplicate are expressed as mean percentage parasitemia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

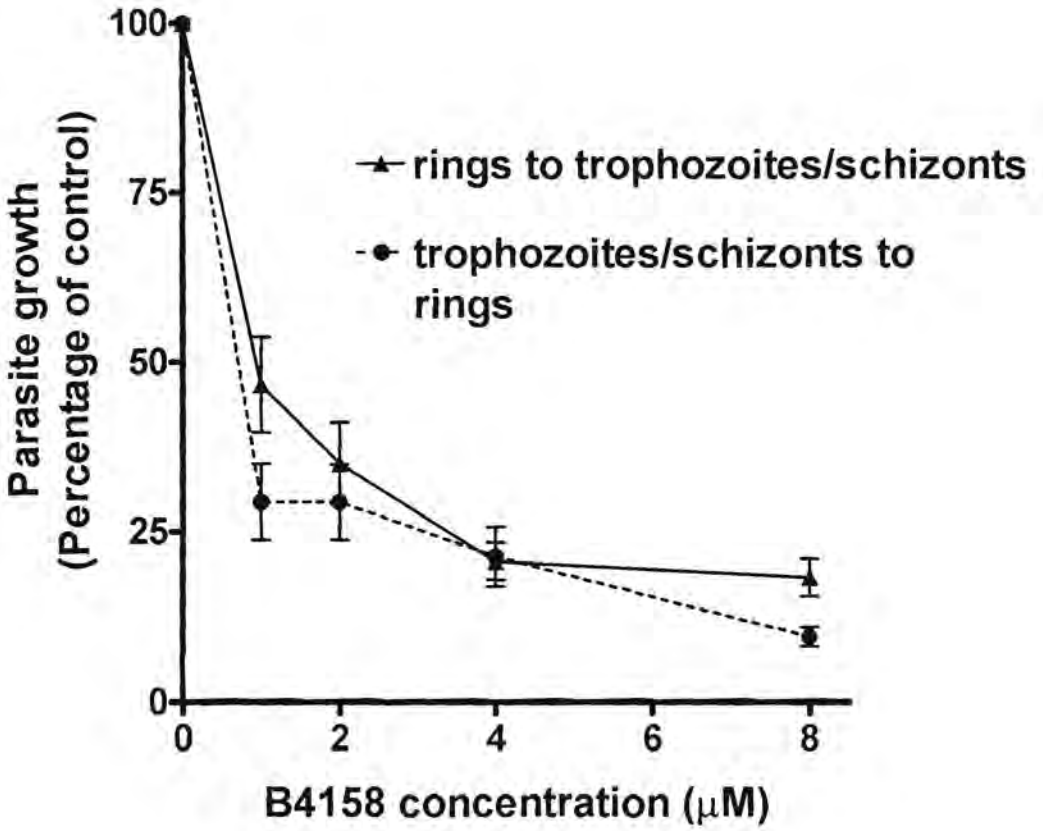


Figure 21 : Stage-specific activity of B4158 for the first (rings to trophozoites/schizonts) and last (trophozoite/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry. Data from three experiments conducted in duplicate are expressed as mean percentage of parasitemia of the drug-free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

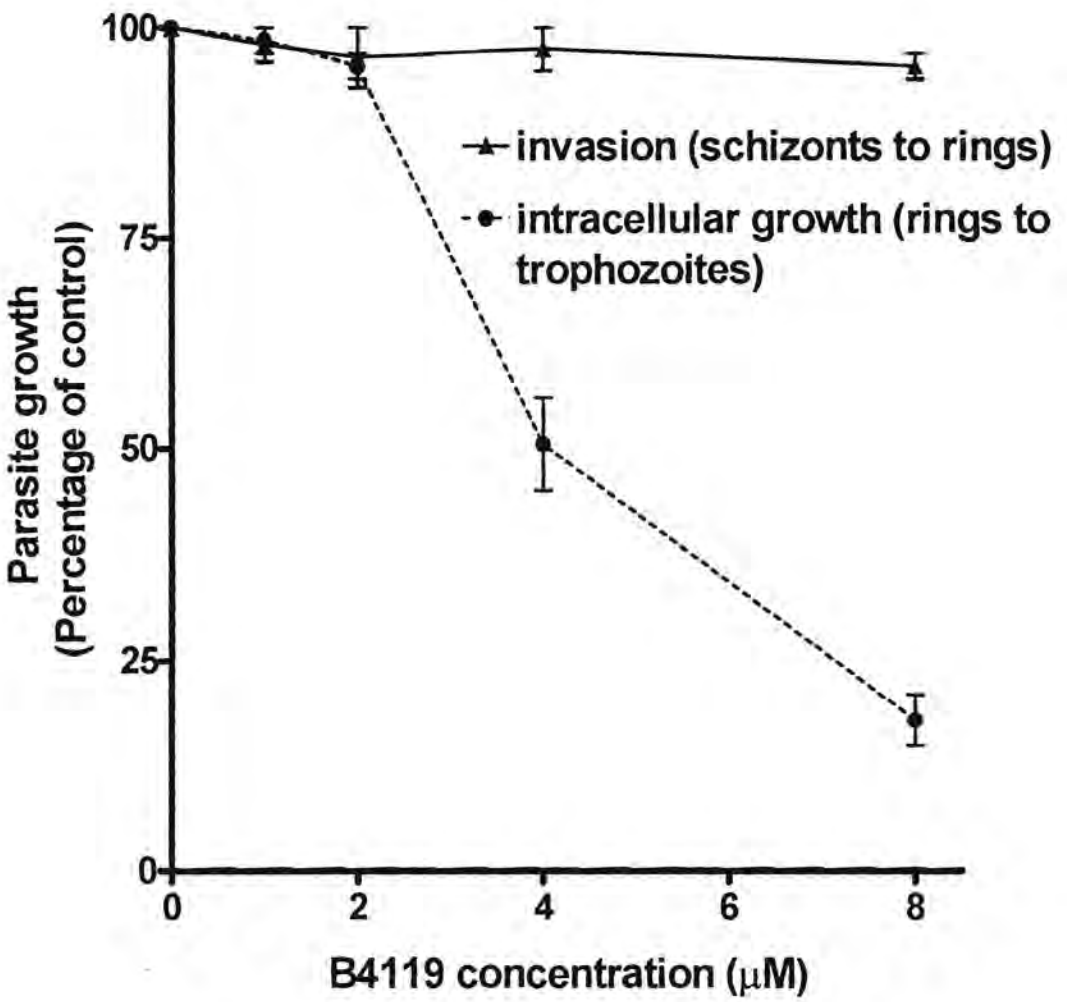


Figure 22 : Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4119 pre-treated red cells using microscopy. Data from three experiments conducted in duplicate are presented as mean percentage parasitemia of the drug-free control system ± SEMs.

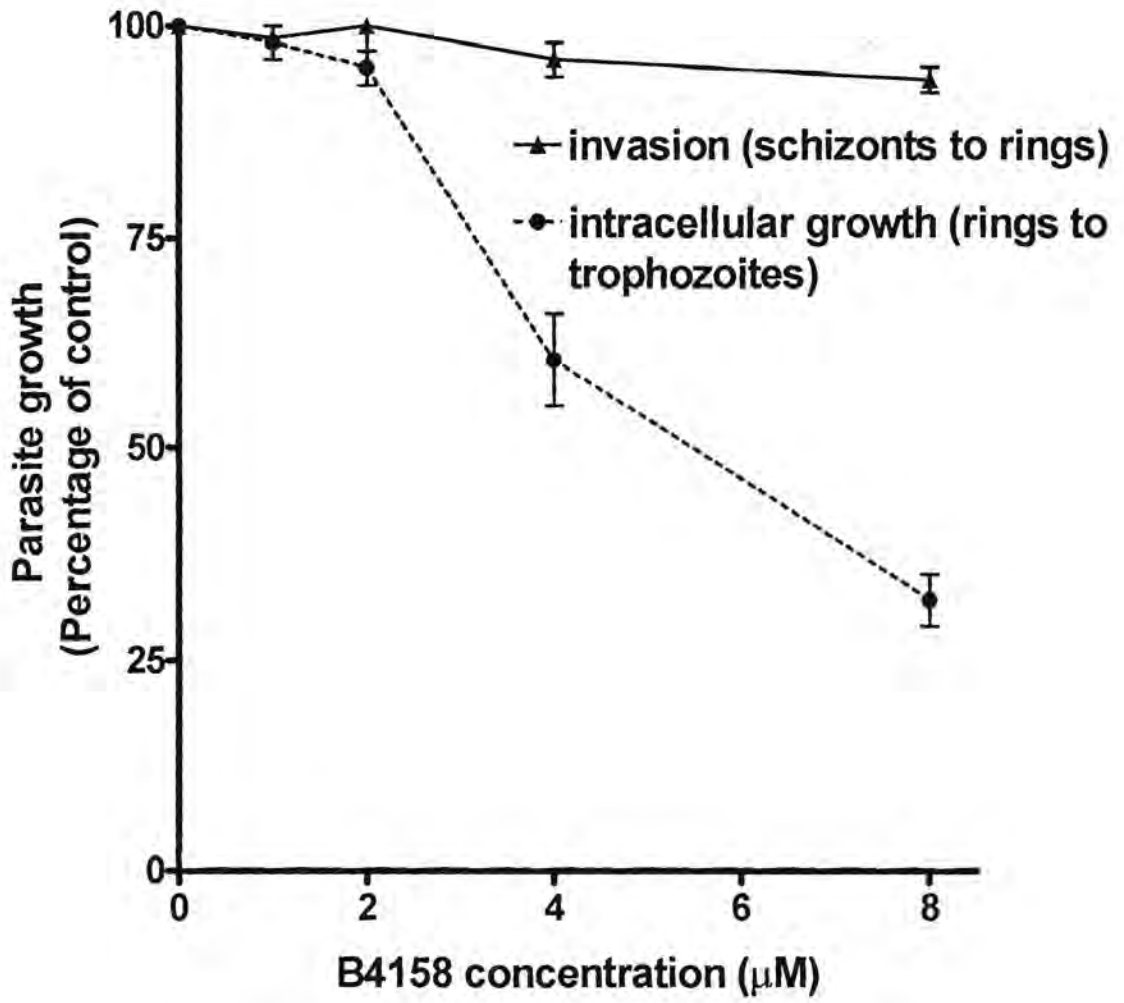


Figure 23 : Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4158 pre-treated red cells using microscopy. Data from three experiments conducted in duplicate are presented as mean percentage parasitemia of the drug-free control systems ± SEMs.

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 0.38μM) 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 8μM 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.

CHAPTER 4

HEME POLYMERIZATION INHIBITORY ACTIVITY (HPIA) OF B4119 AND B4158: AN INFRARED SPECTROMETRIC STUDY

4.1 INTRODUCTION

Intraerythrocytic parasites digest their host cell cytosol (Rosenthal and Meshnick, 1996) producing amino acids required for protein anabolism and parasite maturation (Sherman, 1979). During this catabolic activity, haemoglobin releases free heme that is toxic for biological membranes and parasite enzymes (Fitch *et.al*, 1982; Gluzman *et.al*, 1994). Plasmodium parasites lack heme oxygenase that can cleave the toxic heme into an open-chain tetrapyrrole. Consequently, heme is not excreted from the parasite. Instead, heme is detoxified by conversion to hemozoin or ferriprotoporphyrin IX (malaria pigment). Hemozoin is made of a polymer of hemes linked between the central ferric ion of one heme and a carboxylate side-group oxygen of another (Goldberg, 1994). Hemozoin is released along with the merozoites when infected erythrocytes burst and is taken up by macrophages. It has been found to decrease the phagocytic activity of these cells as well as altering cytokine production profiles of macrophages, thus contributing to the immunopathogenesis of malaria (Sullivan *et.al*, 1996).

The uniqueness of the heme detoxifying property of malaria parasites has been identified as a possible drug target [Meshnick, 1996(a)]. The main target for quinoline antimalarials e.g chloroquine and quinine is the parasite's heme polymerization process (Basilico *et.al*, 1998). Heme can be polymerized *in vitro*, non-biologically i.e in the absence of proteins or peptides from commercial haemin at 60^0C and acidic pH to form a polymer called β-haematin. β-haematin has similar chemical, spectroscopic and biological properties as the native malaria pigment, hemozoin. Quinoline antimalarials have been shown to inhibit both forms of polymerization (Egan *et.al* 1994; Dorn *et.al*, 1998) and this is directly related to their antimalarial (parasite growth inhibition) potency (Raynes *et.al*, 1996). The activity of chloroquine and amodiaquine have been shown to be directly dependent on the saturable binding of the drugs to haematin and that the inhibition of heme polymerization may be secondary to this binding. The chloroquine-resistance mechanism regulates the access of chloroquine to haematin (Bray, *et.al*, 1998).

A radiolabelled heme incorporation assay is presently used for evaluating compounds with potential anti-plasmodial potential, but it is expensive and uses trophozoite lysates and radioactive heme and most laboratories are not equipped to use this method routinely (Dorn *et.al*, 1995). Alternatively, drugs can be evaluated for their ability to inhibit non-biological spontaneous formation of β-Haematin from heme using infrared spectrometry (Egan *et.al*, 1994). Basilico and colleagues (Basilico *et.al*, 1998) have recently reported on a novel micro-assay procedure that is an adaptation of the infrared spectroscopic assay. Spontaneous formation of β-Haematin within microtitre plate wells is read at an absorbance of 405nm after solubilization in dimethyl sulfoxide (DMSO). The procedure is inexpensive, rapid and reproducible and can be routinely used for two purposes (I) studying the mechanisms of action of compounds with known antimalarial activity (ii) primary screening of new compounds which will subsequently be evaluated in growth assays because they exhibit heme polymerization inhibitory activity (HPIA).

4.2 AIMS AND OBJECTIVES

The study was designed to evaluate the HPIA of B4119 and B4158.

4.3 MATERIALS AND METHODS

4.3.1 Media and reagents

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

1M HYDROCHLORIC ACID (HCl) [SARCHEM, Krugersdorp. South Africa]

9.8ml HCl

100ml H_2O

0.1M SODIUM HYDROXIDE (NaOH) [HOLPRO ANALYTICS (PTY)LTD, Johannesburg South Africa]

0.4g NaOH

100ml H_2O

BLUE SILICA GEL PELLETS [MERCK, Darmstadt, Germany]

12.9M ACETIC ACID [HOLPRO ANALYTICS (PTY)LTD, Johannesburg, South Africa]

To prepare 500ml of an acetate buffer:

Glacial acetic acid (371ml) was poured into a 500ml measuring cylinder. Sodium Hydroxide (93g) was weighed out into a 200ml beaker and dissolved in 150ml distilled water. The mixture was stirred until a slurry formed. Sodium hydroxide (slurry) was slowly added to glacial acetic acid and pH adjusted to 5.

DRUGS

Pure compounds of B4119 (MW 606) and B4158 (MW 585) were used in powdered form as 3 mole or 42mg.

4.3.2 **Experimental procedures**

Heme polymerization was measured as described previously (Egan *et.al*, 1994). Haemin (15mg) was added to 3ml of 0.1M NaOH and 0.3ml of 1M HCl solutions with or without the powdered forms of the test agents (3 mole). The solutions were heated at 60°C until equilibrated and 1.74ml of 12.9M acetic acid was added. The control solutions were immediately placed on ice to stop β-haematin formation, while solutions with or without (β-hemtin control) were incubated for a further 30 min period. At the end of the incubation period, the reaction mixture was cooled on ice for 5min and then filtered using an 8µm cellulose acetate/nitrate Millipore® filter Type C [Separation Scientific cc, Honeydew, South Africa] and extensively washed with water. The solid precipitate was dried over silica gel and phosphorous pentoxide at room temperature for 48hours. The precipitate (2mg) and potassium bromide (200mg) were ground into a fine powder which was then compressed under 10 tons to prepare discs which were analysed by infrared spectroscopy using a Perkin-Elmer 983 infrared spectrometer [Perkin-Elmer South African LTD, Fairland, South Africa].

4.4 RESULTS

The effects of B4119 and B4158 on heme polymerization are shown in Figures 24 to 29. Addition of acetic acid (to give final concentration of 4.5M, pH 4.5) to haematin (Figure 25) at 60°C, followed by 30 min incubation leads to the formation of β-haematin with a characteristic infrared spectrum which includes peaks at 1210 and 1661 cm^{-1} indicated by arrows in Figure 25. The

infrared spectra of B4119 and B4158 shown in Figures 26 and 27 respectively indicate that both test agents do not have peaks similar to those distinguishing β-haematin. When 3 mole equivalents of B4119 or B4158 were mixed with the haematin (before addition of acetic acid), the formation of β-haematin was inhibited as indicated by the absence of strong peaks at the positions indicated by arrows in Figures 28 and 29 respectively. Although the peak at 1661cm^{-1} may be obscured by drug peaks in both cases, the peak at 1210cm^{-1} clearly appears to be absent in both cases i.e Figure 28 and 29.

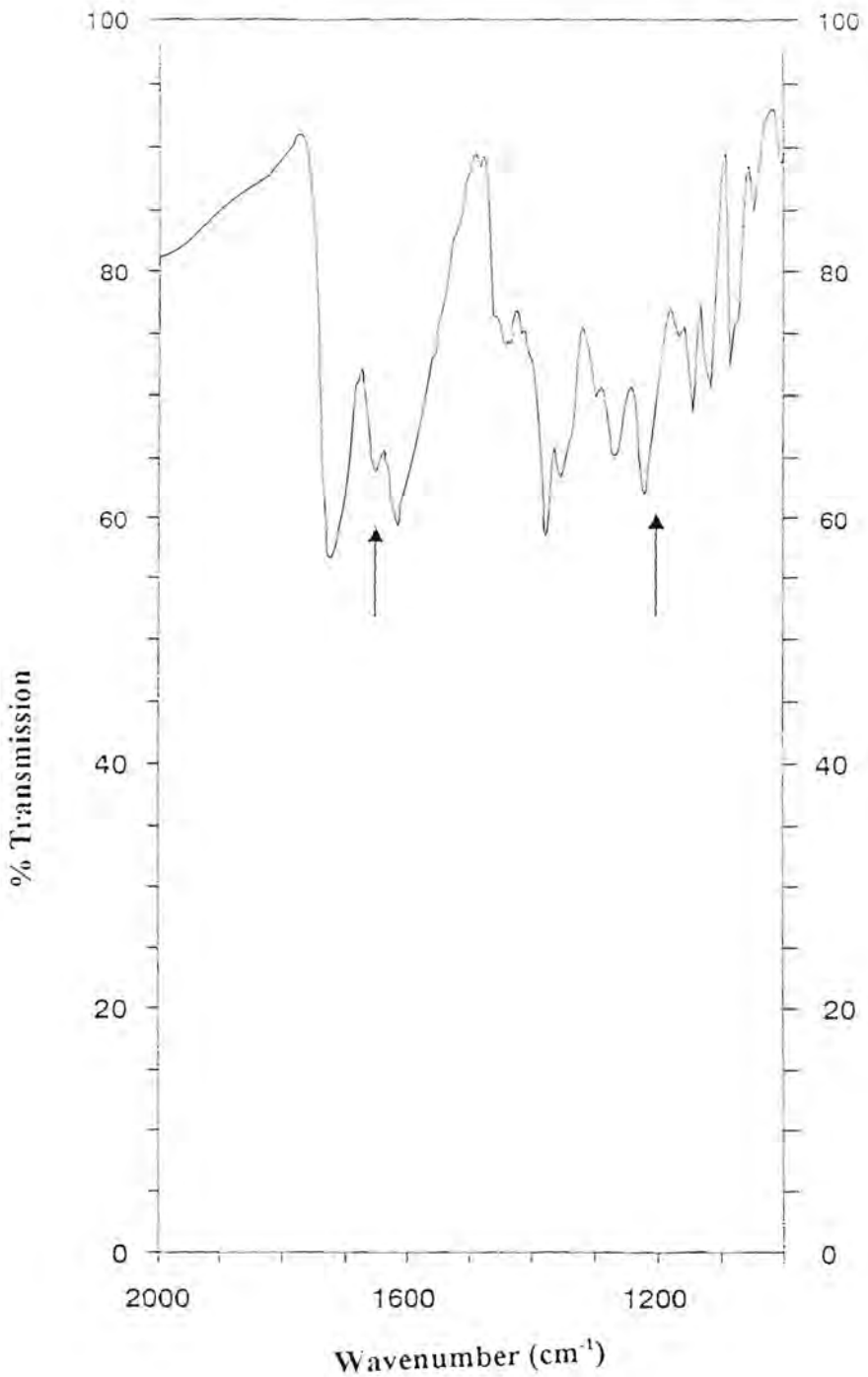


Figure 24 : Infrared spectra of haematin after 0 min incubation in 12.9M acetic acid, pH 5, 60°C

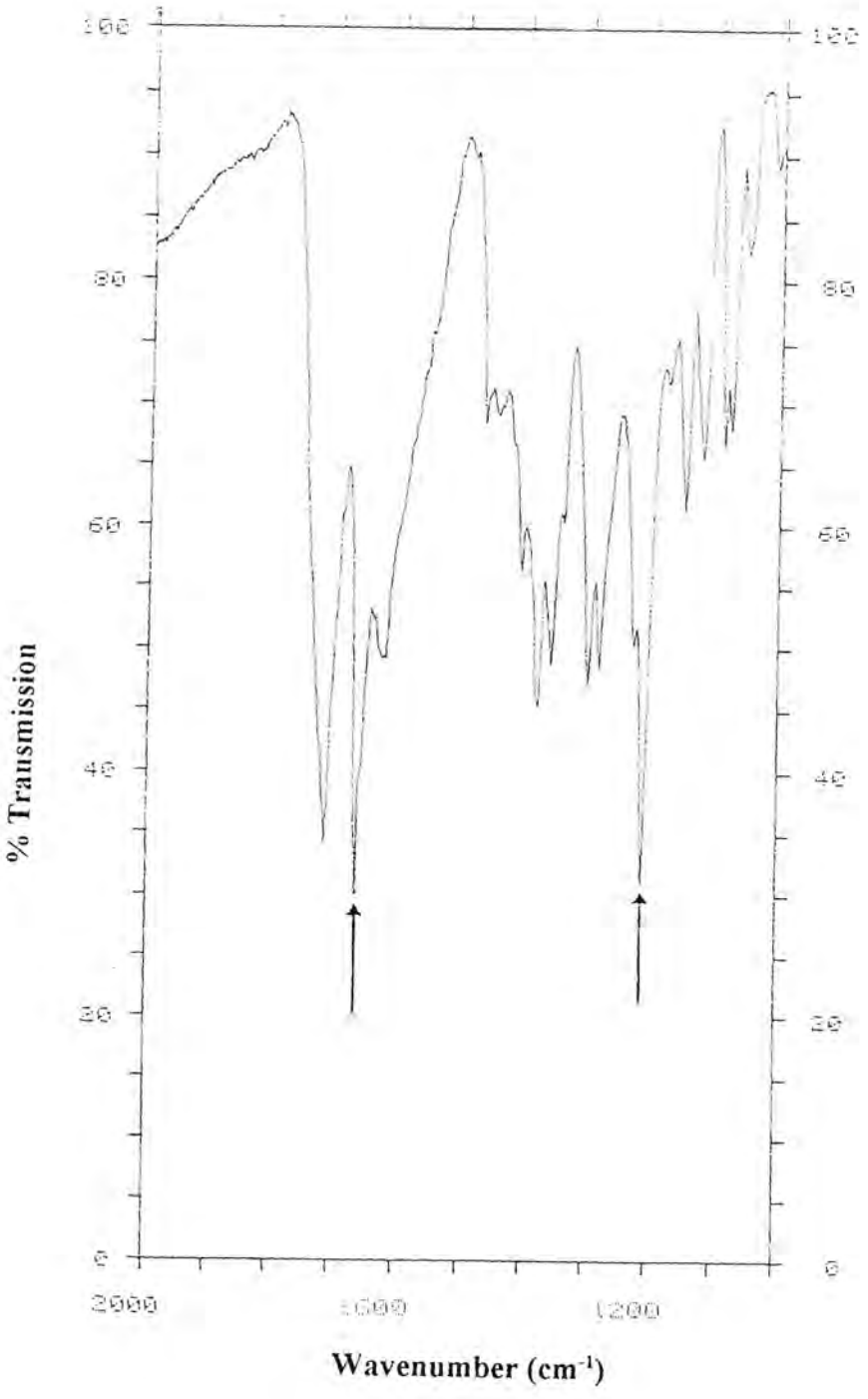


Figure 25 : Infrared spectra of β-haematin after 30 min incubation in 12.9M acetic acid, pH 5, 60°C. The definitive peaks for β-haematin at 1660 cm^{-1} and 1210 cm^{-1} (Soret band) are marked with arrows.

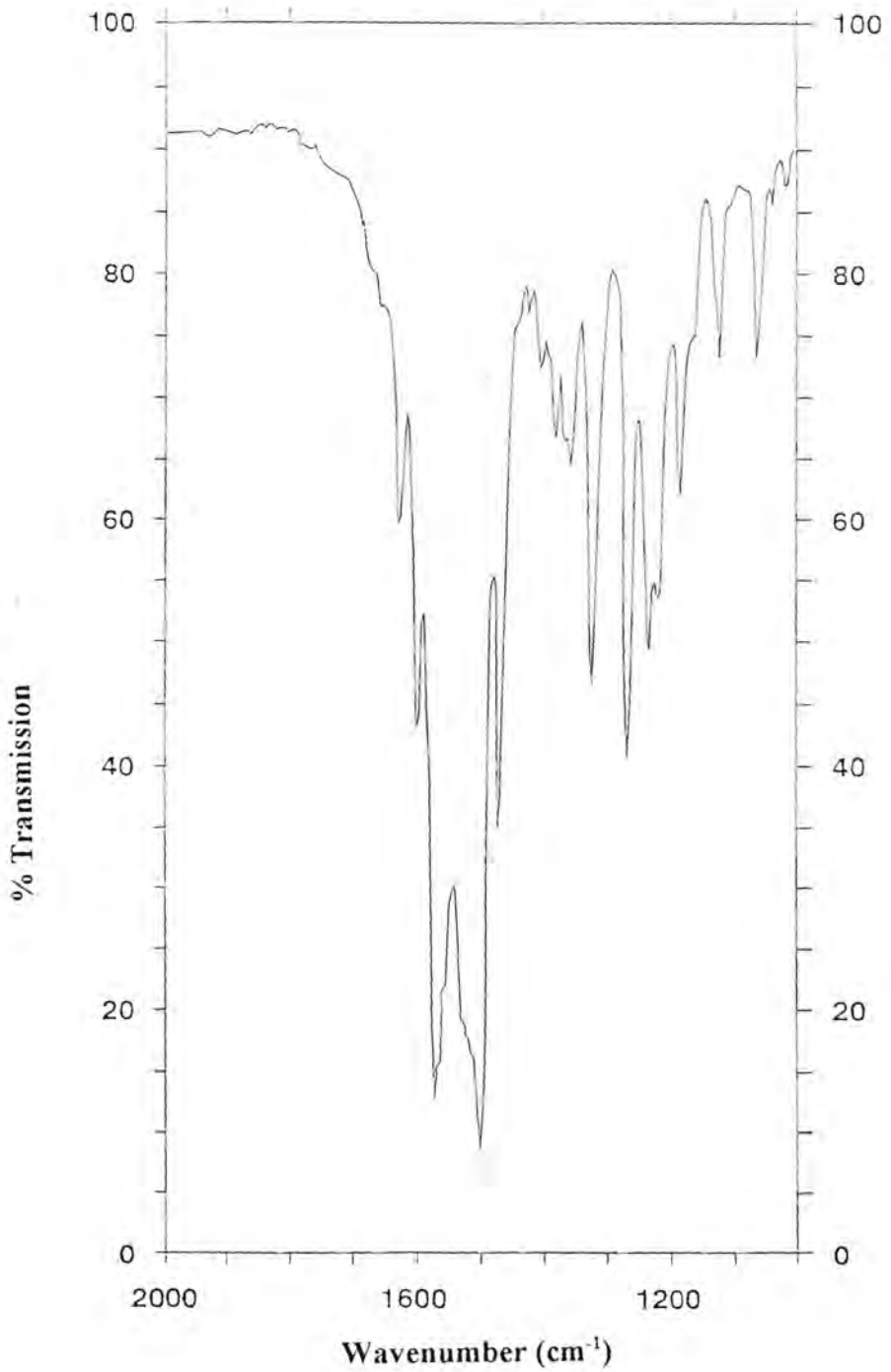


Figure 26 : Infrared spectra of B4119 after 30 min incubation in 12.9M acetic acid, pH 5, 60°C

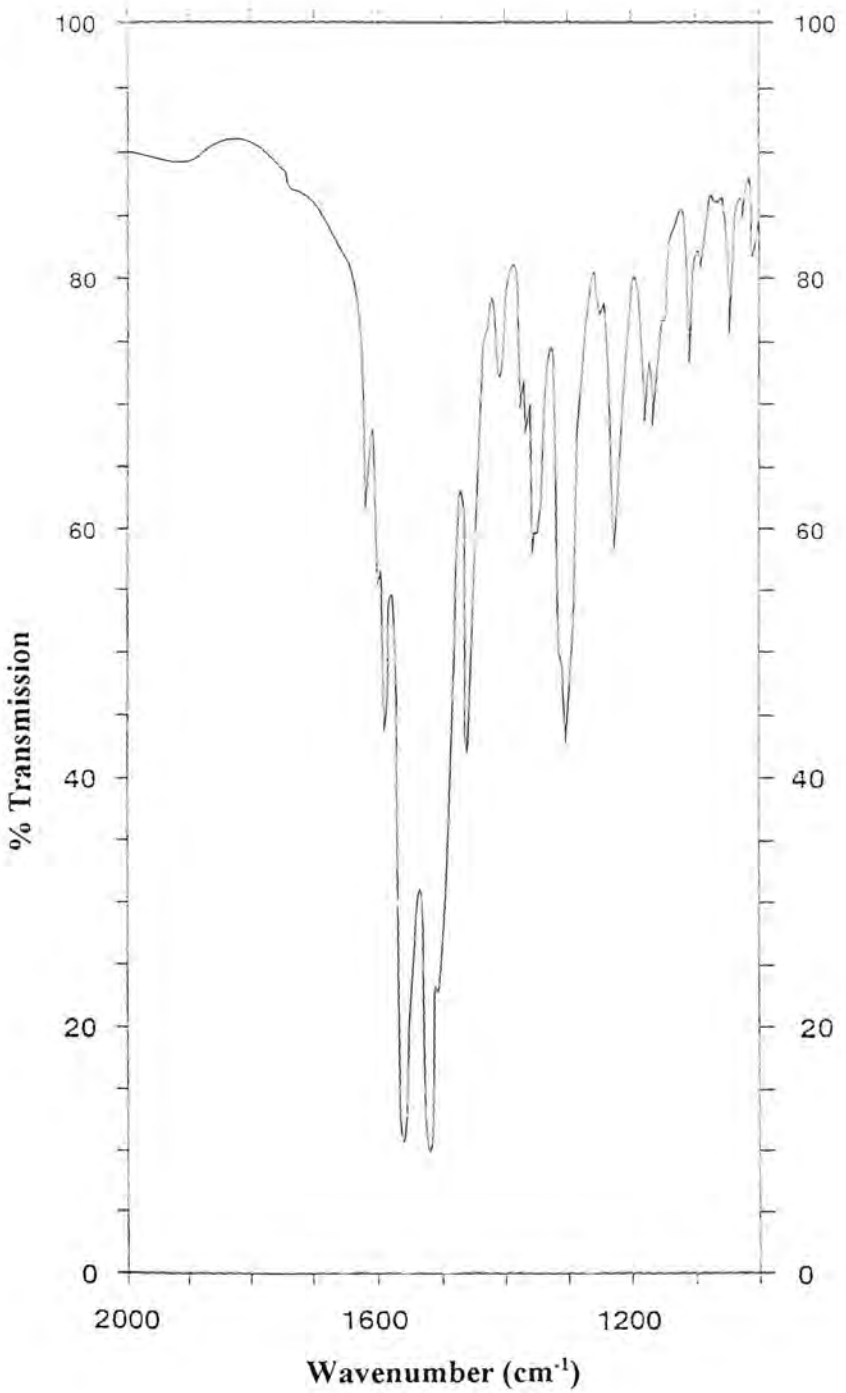


Figure 27 : Infrared spectra of B4158 after 30 min incubation in 12.9M acetic acid, pH 5, 60°C

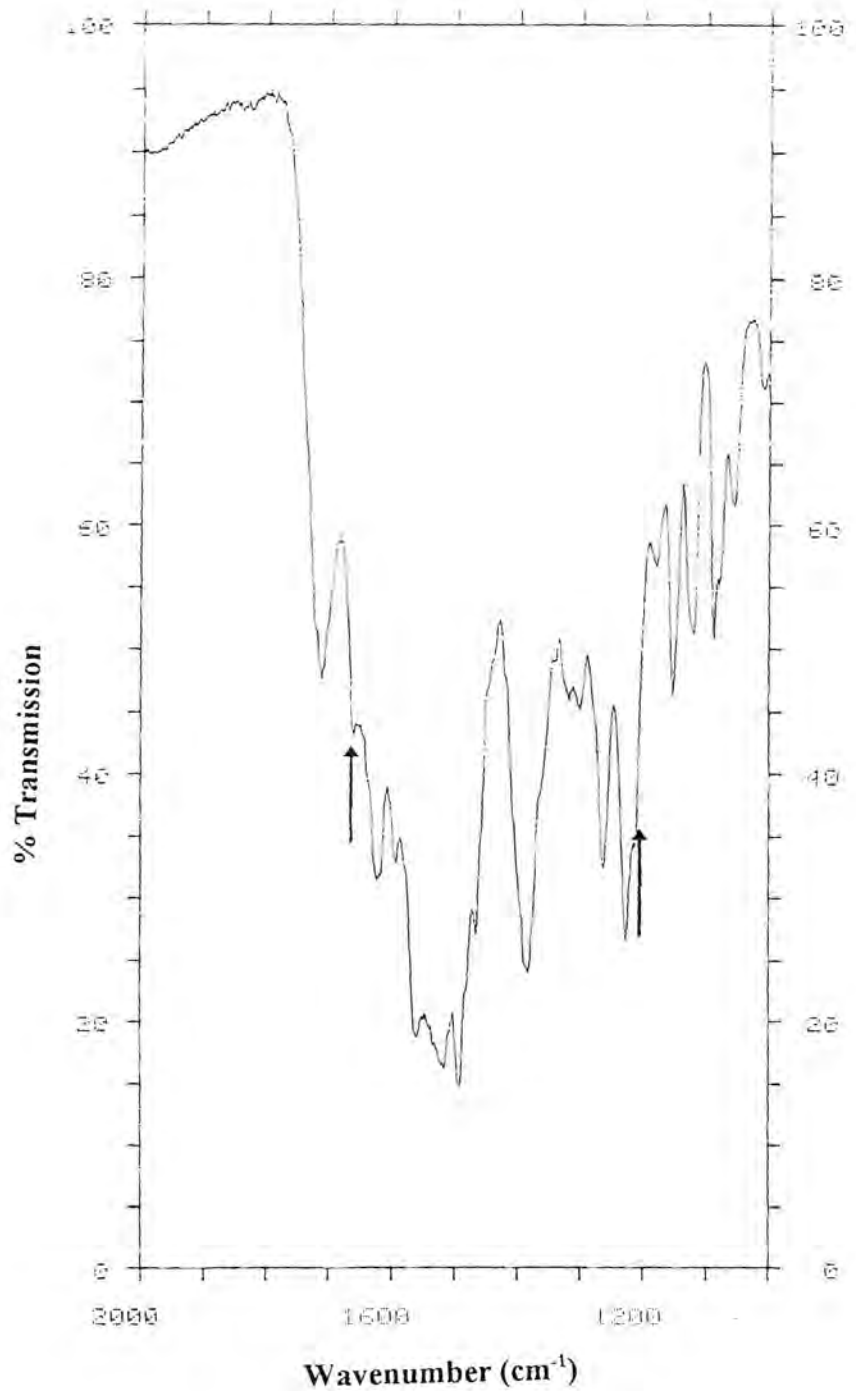


Figure 28 : Infrared spectra of B4119-treated haemin solution after 30 min incubation in 12.9M acetic acid, pH 5, 60°C. The absence of the Soret band is indicated by arrows.

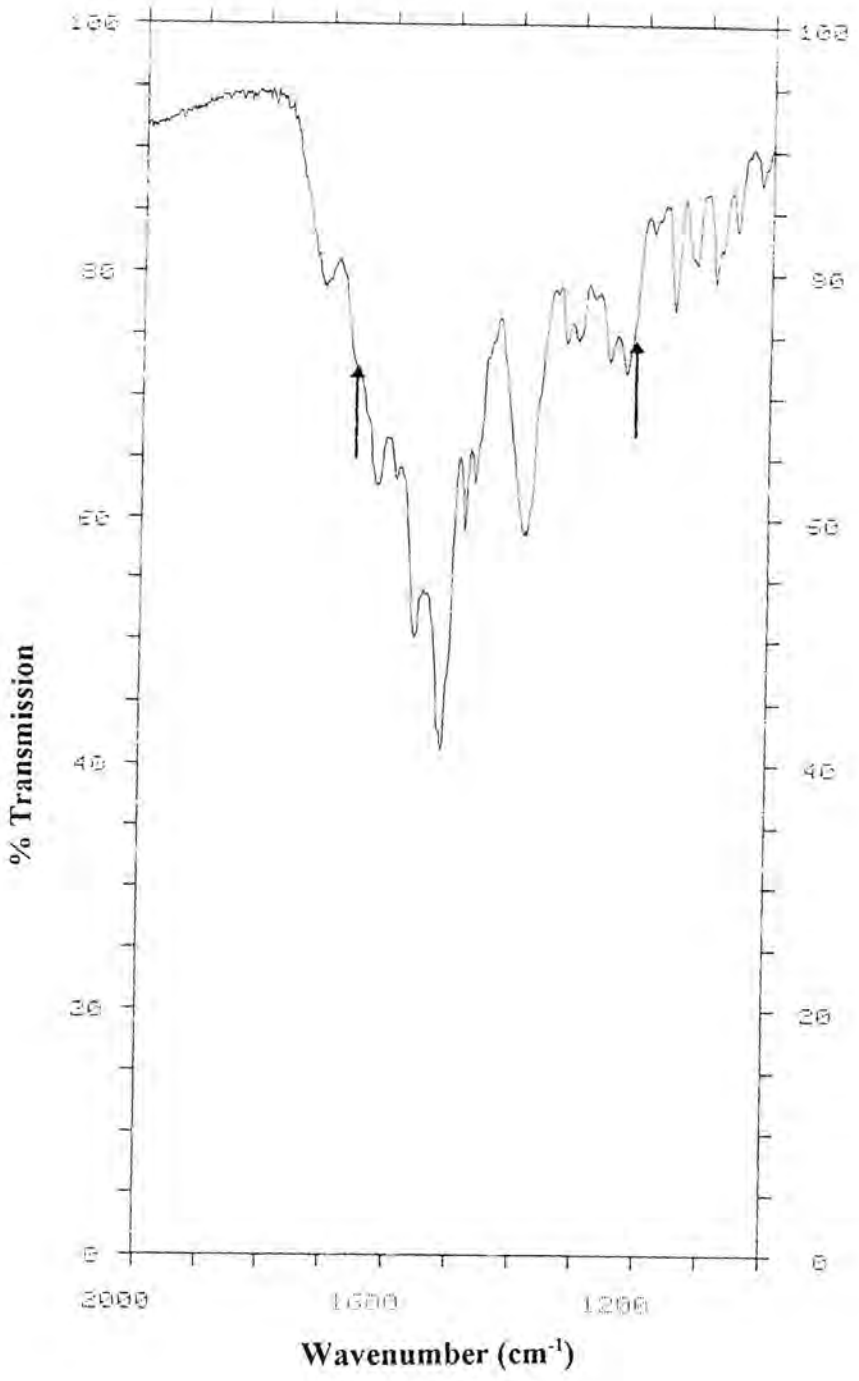


Figure 29 : Infrared spectra of B4158-treated haemin solution after 30 min incubation in 12.9M acetic acid solution, pH 5, 60°C. The absence of the Soret band is indicated by arrows.

4.5 DISCUSSION

It has been demonstrated in this study that β-haematin forms spontaneously in the absence of proteins. The two characteristic peaks of β-haematin at a wavelength of 1661 cm^{-1} and 1210 cm^{-1} of the infrared spectra represent the iron-carboxylate bond that attaches one heme moiety to the other. This peak is also called the Soret band (Slater *et.al*, 1991; Basilico *et.al*, 1998).

Several mechanisms, most notably interference with heme polymerization, have been proposed to explain the anti-plasmodial activity of chloroquine (Slater, 1993; Olliaro and Yuthavong, 1998). Chloroquine-mediated inhibition of heme polymerization is achieved by formation of heme-chloroquine complexes that are toxic for the parasite (McChesney and Fitch, 1984). In the current study I have observed that B4119 and B4158 are also potent inhibitors of heme polymerization, suggesting that this activity may be a property of lipophilic cationic amphipathic agents, which accumulate in acidic organelles.

If this mechanism of anti-plasmodial activity is common to chloroquine and the phenazine compounds, it clearly raises the issue of the sensitivity of the chloroquine-resistant strains of *Plasmodium falciparum* to B4119 and B4158. This may indicate that chloroquine and the TMP-substituted phenazines inhibit heme polymerization by different mechanisms. Alternatively, the TMP-substituted phenazines may be unaffected by the biochemical mechanisms which promote resistance to chloroquine. In this respect, it is noteworthy that resistance to chloroquine is associated with induction of the P-glycoprotein homologue, Pgh1, in malaria parasites (Bray and Ward, 1993). Interestingly, the TMP-substituted phenazines not only inhibit heme polymerization, but are also potent inhibitors of P-glycoprotein (Van Rensburg *et.al*, 1997).

Alternatively, it may not be valid to suggest that the antimalarial activity of these novel agents against the resistant strains is related to P-glycoprotein inhibitory effects. Krogstad's group (De *et.al*, 1996) has shown that an analogue of chloroquine in which the aminoalkyl side chain is either made longer or shorter than it is in chloroquine is fully active against resistant strains. Apparently the resistance mechanism recognises the side chain and not the quinoline ring. B4119 and B4158 are sufficiently different to evade this mechanism. Clofazimine might inhibit β-haematin formation *in vitro* and the reason it is not antimalarial is that it lacks the basic side chain which is responsible for accumulation in the food vacuole. The TMP-group of the two novel compounds probably has

this role and they will very likely accumulate in the food vacuole, as does chloroquine. It is probably this property which differentiates the antimalarial activity of the new derivatives from the inactivity of the parent compound, clofazimine, towards malaria.

Finally, the procedures presented in this work provides a useful and simple tool for screening compounds for potential anti-plasmodial activity as well as in identifying drug targets for active compounds in malaria parasites.

CHAPTER 5

CYTOTOXIC ACTIVITY OF B4119 AND B4158 AGAINST NORMAL HUMAN ERYTHROCYTES

5.1 AIMS AND OBJECTIVES

The prototype riminophenazine, clofazimine, has been reported to be a membrane-active agent which interferes with Na+,K+-ATPase activity in eukaryotic cells (Van Rensburg *et.al*, 1993) and K+-transport systems in Gram-positive bacteria (De Bruyn *et.al*, 1996). To investigate the possibility that the anti-plasmodial effects of B4119 and B4158 were achieved indirectly by cytotoxic effects on erythrocytes as opposed to direct effects on the malaria parasites, the effects of these test agents on erythrocyte viability, Na+,K+-ATPase activity and energy metabolism were investigated.

5.2 MATERIALS AND METHODS

5.2.1 **Media and reagents**

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

DRUGS

Pure substances of B4119 and B4158 were prepared as in section 3.3.3

RPMI-1640 medium (indicator-free) [Highveld Biological Products, Kelvin, SA]

Rubidium-86 [NEN, USA]

Lactate dehydrogenase [Boehringer Mannheim, Germany]
220mU/assay

27mM Nicotinamide dinucleutide [Boehringer Mannheim, Germany]

Triton®X-100 [Boehringer Mannheim, Germany]

ATP kit

- ATP assay mix dilution buffer

reconstitute in 50ml sterile water per vial

- ATP assay mix (firefly luciferase enzyme)

2mg enzyme

1ml ATP dilution buffer

Scintillation fluid[Packard, USA]

3% Gelatin solution

3g powdered gelatin

100ml distilled water

Autoclave

Tris buffer (low K+)

122mM NaCl

4mM KCl

1mM $MgSO_4$

1mM KH_2PO_4

20mM Tris

5mM glucose

pH 7.4

5.2.2 Experimental procedures

5.2.2.1 Preparation of leukocyte-depleted human erythrocytes

Heparinized blood (30ml) was layered onto 20ml Histopague®-1077 in a centrifuge tube and spun at room temperature for 25 min at 300g in a Beckman TJ-6 centrifuge. The lymphocyte layer was removed and the erythrocyte-rich pellet sedimented in 3% gelatin for 15min at 37°C to remove neutrophils. The erythrocytes were washed three times in RPM1-1640 medium by centrifugation at 300g for 5 min. The resultant pellet was resuspended in RPMI-1640 medium to 5% haematocrit before use.

5.2.2.2 ***Drug-mediated haemolysis***

The haemolytic potential of B4119 and B4158 was measured by the release of haemoglobin. To 800µl of serum-supplemented, indicator-free RPMI-1640 was added 100µl red cell suspension (final haematocrit 0.5%) with or without 100µl of test agents (1 - 8µM) and the erythrocytes incubated for 45min, 24 and 48 hours at 37°C. At the end of the incubation period, the tubes were centrifuged at 300g for 5min and the supernatant assayed spectrophotometrically at 405nm for haemoglobin.

5.2.2.3 ***Rubidium-86 uptake by erythrocytes***

The Na+,K+-ATPase activity of uninfected erythrocytes was measured using ^{86}Rb as a tracer (Prasad *et.al*, 1987).

Kinetic studies : Erythrocyte suspensions (2ml, 2 X10^9 cells/ml) were incubated in isotonic Tris buffer with or without 10µl ouabain (100µM), a selective inhibitor of Na+,K+-ATPase, for 15min at 37°C. Rubidium-86 (34mBq specific activity, at a final concentration of 2µCi/ml) was added to all the tubes and the reaction terminated by the addition of ice-cold PBS at intervals of 0, 30, 60, 90 and 120min. The cells were washed three times with PBS at 300g for 5min. TritonX-100 (0.5ml of a 0.1% solution) and 5% TCA (0.5ml) were added to the pellet to solubilise the cells and precipitate haemoglobin respectively. The tubes were then centrifuged at 400g for 10min and 200µl of the supernatant added to 3ml scintillation cocktail in 5ml scintillation vials. Radioactivity was measured in a Tricarb 2100-TR scintillation counter [Packard Instrument Company, Illinois, USA]. Na+,K+-ATPase-mediated uptake of ^{86}Rb is taken as the difference in uptake of the cation in systems with or without ouabain.

Effects of B4119 and B4158 on ^{86}Rb uptake by erythrocytes : Packed erythrocytes (2ml) were added to 18ml of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100µl of erythrocyte suspension (0.5% final haematocrit) with or without 100µl of test agents (1 - 8µM) was added and incubated for 45 min and 48 hours at 37°C. At the end of the incubation periods the cells were washed three times with, and resuspended in 2ml isotonic Tris buffer at a concentration of 2 X10^9 cells/ml with or without ouabain (10µl of a 100µM concentration). Rubidium-86 (2µCi) was then added to the tubes which were incubated for a further 2 hours. Reactions were terminated by addition of cold

PBS and the cells washed three times with PBS. Triton X-100 (0.5ml of a 0.1% solution) and 5% TCA (0.5ml) were then added to the pellet to disrupt the cells and precipitate haemoglobin respectively. The tubes were spun at 400g for 10min and 200µl of the supernatant was added to 3ml scintillation fluid in scintillation vials. Na+,K+-ATPase activity was determined as above.

5.2.2.4 ***Erythrocyte metabolic activity***

The metabolic activity of red cells was evaluated from measurements of intracellular ATP and lactate levels (as indices of glycolytic activity) at concentrations of 1 and 2µM of the test agents after a 48 hour incubation period. A similar experimental set-up was used as in section 5.2.2.3. However, in this case the cells were washed twice with indicator- and serum-free RPMI following incubation and then lysed, and proteins precipitated by addition of 1ml 0.6N perchloric acid to the pellets. The tubes were then centrifuged at 400g for 10 min and the protein-free supernatants removed and restored to neutral pH using 10N NaOH.

Effects of B4119 and B4158 on erythrocyte intracellular ATP levels. ATP levels were measured using the luciferin/luciferase method (Holmsen *et.al*, 1972). A mixture of 20µl erythrocyte supernatant, 100µl nucleoside releasing agent (NRA) and 30µl assay buffer (made up with 50ml distilled water) was prepared in round-bottomed vials and mixed by vortexing. The vial was placed in an LKB Wallace Luminometer and 20µl of enzyme was added into the reaction mixture and the resultant chemiluminescence recorded. The principle of this reaction is as follows: ATP extracted from red cells reacts with oxygen and luciferin and addition of the enzyme (firefly luciferase) leads to formation of oxyluciferin (excited state of luciferin). When oxyluciferin moves to the ground state, it emits light energy that is proportional to the red cell ATP levels. Results are expressed as nanomoles ATP/ 2×10^9 erythrocytes.

Effects of B4119 and B4158 on erythrocyte intracellular lactate levels.

Lactate was assayed spectrophotometrically at 340nm using an enzymatic procedure based on the lactate dehydrogenase (from hog muscle, 220 milliunits/assay; Boehringer-Mannheim, Marburg Germany)-mediated conversion of lactate to pyruvate in the presence of 7mM NAD (Whalfield, 1974). Each assay tube contained 2ml of erythrocyte supernatant and 0.2ml NAD (7mM final concentration). The reaction was initiated by the addition of 20µl lactate

dehydrogenase and the conversion of NAD to NADH monitored spectrophotometrically at 340nm using a Helios gamma spectrophotometer [UNICAM, England]. Lactate concentrations were determined from a standard curve and results expressed as mg lactate /2×erythrocytes.

5.2.2.5 ***Direct anti-plasmodial activity of ouabain in vitro.***

The PfUP10 laboratory strain of *Plasmodium falciparum* was cultured as described in section 2.3.2. Drug testing was carried out in 96-well microtiter plates. To each well was added 20µl ring-infected erythrocyte suspension (2% parasitemia and 0.5% haematocrit), and 180µl of complete medium with or without ouabain (10 - 100µM). The plates were placed in a modular gas chamber, gassed for 15 min. with gas cocktail, sealed and incubated for 48 hours at 37°C. At the end of the incubation period, levels of parasitemia were determined using microscopy as described in section 2.3.4.2.

5.3 RESULTS

Cytotoxic effects of B4119 and B4158 on erythrocytes. Exposure of the cells to 4 and 8µM, B4119 for 24 and 48 hours resulted in 30±7% and 85±11% release of haemoglobin respectively while treatment with B4158 (4 and 8µM) for 24 and 48 hours resulted in 35 ± 10% and 55 ± 12% haemolysis respectively as compared to the control systems. Red cells were unaffected by a 45min exposure to the test agents at all four concentrations tested.

Incubation of erythrocytes with 1 and 2µM of the test agents did not affect ($p > 0.05$) erythrocyte lactate levels (Table 3) after a 48 hour incubation period, while there was a dose-related increase ($p < 0.05$) in ATP levels (Table 4) when the compounds (1 and 2µM) were incubated with the red cells for 48 hours.

Table 3: Effects of exposure of human erythrocytes to B4119 and B4158 on intracellular lactate levels

	Cellular lactate (mg lactate /2×10^9 erythrocytes/ml) after treatment with:	
Concentration (μM)	**B4119**	**B4158**
0	8.3±0.4	8.3±0.4
1	8.2±0.3	7.2±1
2	9±0.7	8.4±0.5

Results of 6 experiments are presented as mean ± SEM.

Table 4 : Effects of exposure of human erythrocytes to B4119 and B4158 on intracellular ATP levels

	Cellular ATP (nanomoles ATP / 2×10^9 erythrocytes/ml) after treatment with:	
Concentration (μM)	**B4119**	**B4158**
0	12±6	12±6
1	24±10	17±5
2	27±5	23± 8

Results of 6 experiments are presented as mean±SEM.

Effects of B4119 and B4158 on erythrocyte Na+,K+-ATPase activity.

The results are shown in Figures 30 to 34. Ouabain (0.01 - 100μM) caused a dose-related inhibition ($p < 0.05$) of rubidium-86 uptake (Figure 30). Kinetic studies (Figure 31) showed that uptake of rubidium-86 increases with time (0 - 120min) and is inhibitable by addition of 100μM ouabain over the same time period. Exposure of the cells to the test agents (1- 8μM) resulted in significant ($p < 0.05$) inhibition of rubidium-86 uptake at concentrations of 4μM and higher, and 2μM and higher for the 45min (Figure 32) and 48 hour (Figure 33) incubation periods respectively.

Effects of ouabain on the growth of *Plasmodium falciparum* PfUP10 laboratory strain.

The results in Figure 34 show that ouabain at concentrations of 10, 50 and 100μM failed ($p > 0.05$) to inhibit the growth of *Plasmodium falciparum*.

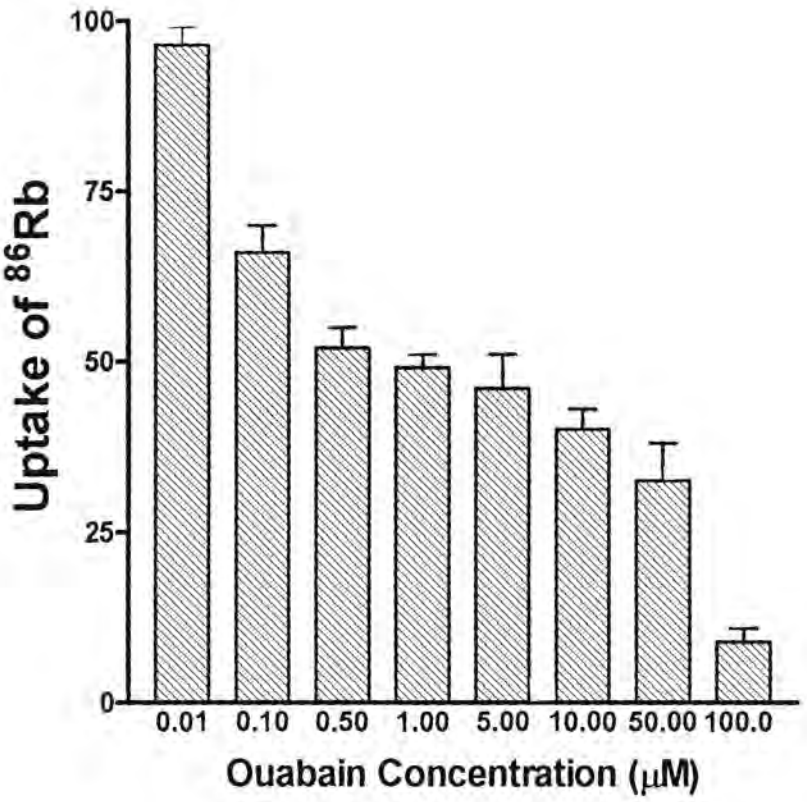


Figure 30 : Effects of ouabain on ^{86}Rb uptake by human leukocyte-depleted erythrocytes. Results of 4-6 experiments are presented as mean percentage ^{86}Rb uptake of the ouabain-free control systems ± SEMs. The mean absolute value for uptake of ^{86}Rb by the drug-free control system was 18371 ± 1125 cpm.

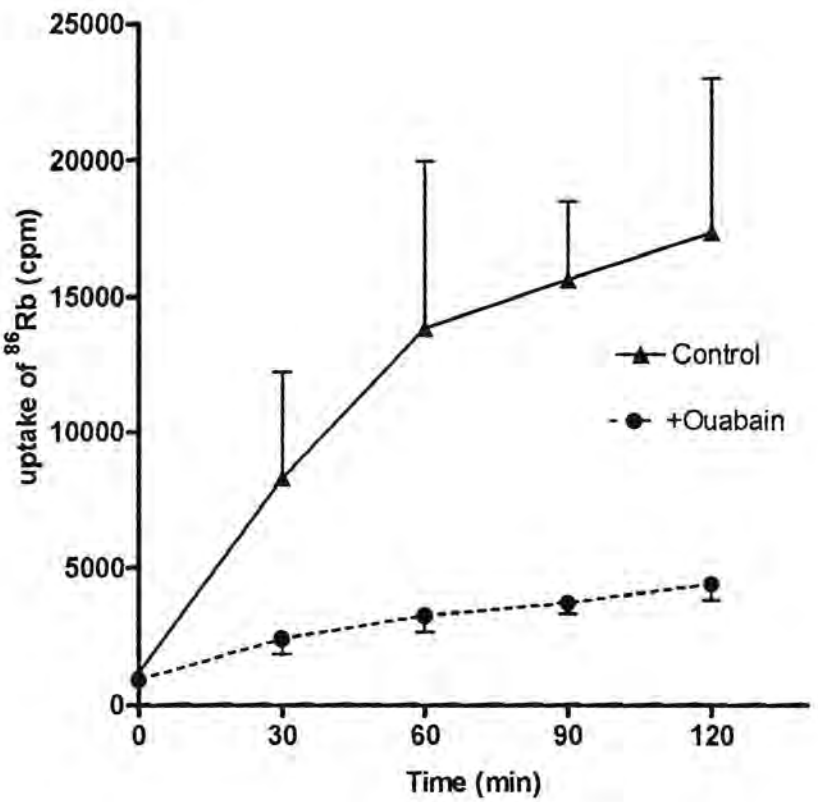


Figure 31 : ^{86}Rb uptake kinetics by erythrocytes with or without ouabain (100μM). Results from 3 experiments are expressed as absolute counts per minute (cpm).

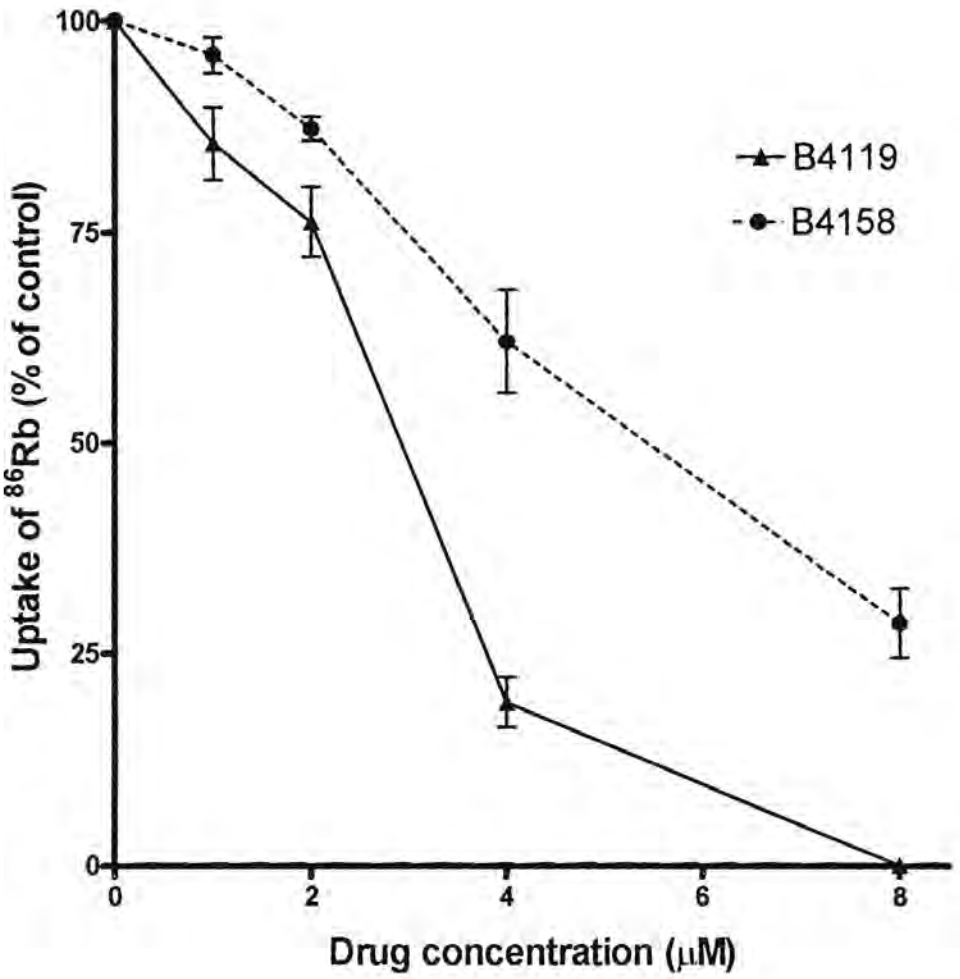


Figure 32 : Effects of B4119 and B4158 on ^{86}Rb uptake by erythrocytes after 45min incubation period. Results from 3 experiments are presented as mean percentage ^{86}Rb uptake of the drug-free control system ± SEMs. The absolute value for uptake of ^{86}Rb by the drug-free control systems was 19627 ± 2145 cpm.

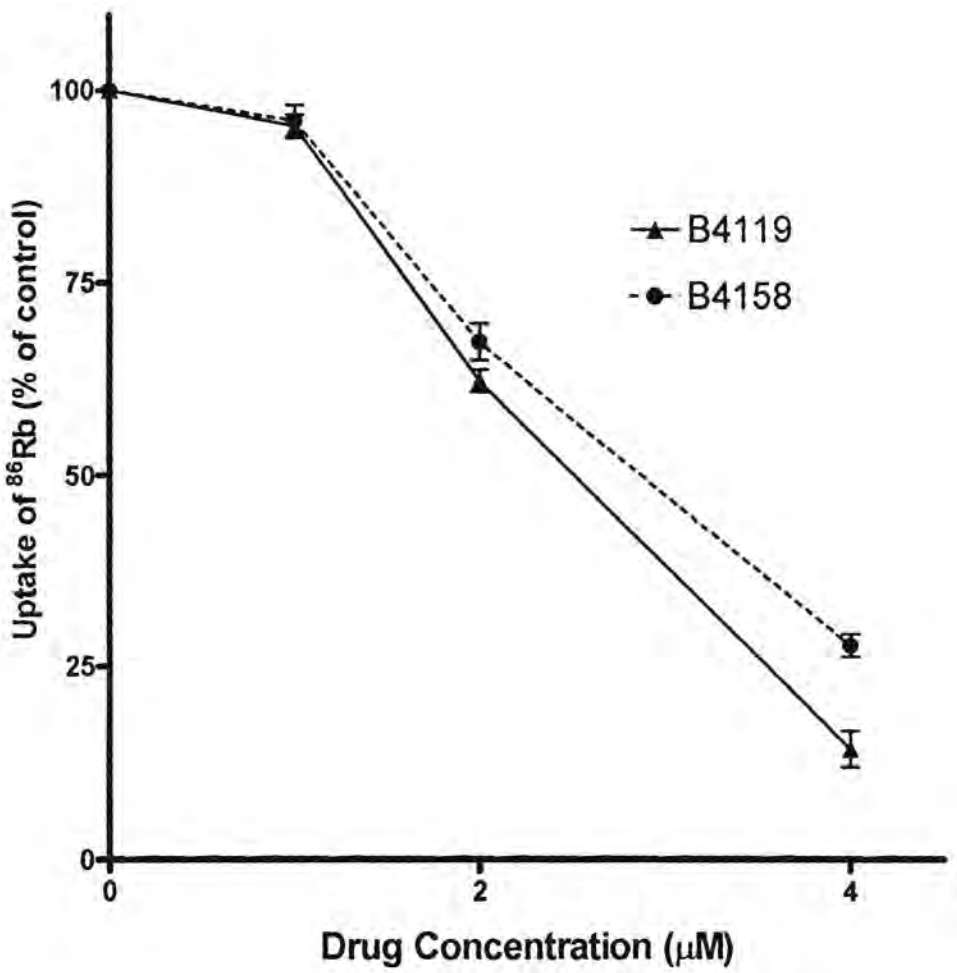


Figure 33 : Effects of B4119 and B4158 on ^{86}Rb uptake by erythrocytes after 48 hours incubation period. Results from 3 experiments are presented as mean percentage ^{86}Rb uptake of the drug-free control system ± SEMs. The absolute value for uptake of 86Rb by drug-free control systems was 21789 ± 1356 cpm.

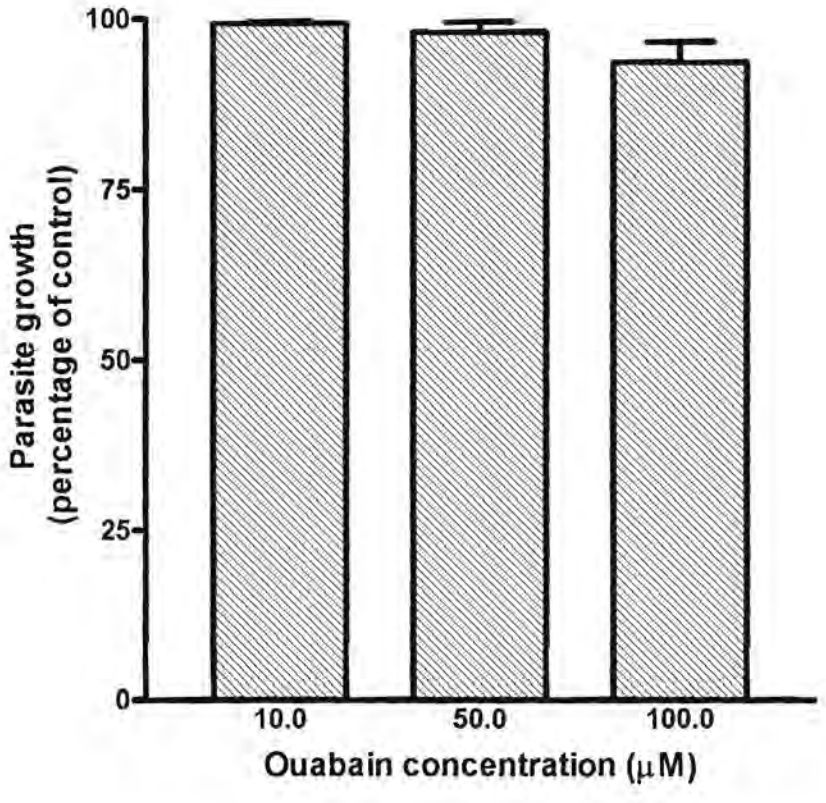


Figure 34 : Effects of ouabain on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using microscopy. Results from 2 experiments are presented as the mean percentage of parasite growth of the ouabain-free control system ± SEMs.

5.4 DISCUSSION

In order to establish whether the observed anti-plasmodial activity of B4119 and B4158 was primary, or secondary to membrane-disruptive or cytotoxic effects on erythrocytes, the viability and metabolic activity of these cells was measured following exposure to the TMP-substituted phenazines for periods varying from 45min to 48 hours. Drug-mediated cytotoxic effects on erythrocytes were investigated using a haemolytic procedure, as well as by measurement of glycolytic activity according to the production of intracellular lactate and ATP in control and drug-treated erythrocytes. At the highest concentration used (8μM), both compounds were cytolytic for erythrocytes after 24 and 48 hours of incubation. However, at lower concentrations at which effective anti-plasmodial activity was evident, neither B4119 nor B4158 affected erythrocyte viability or metabolic activity. These observations demonstrate that at concentrations of 2μM and lower, B4119 and B4158 are selectively active against *Plasmodium falciparum*.

Ouabain, a classical inhibitor of the erythrocyte Na+, K+-ATPase activity, did not inhibit parasite growth at concentrations that effectively inhibited rubidium-86 uptake by erythrocytes. Although B4119 and B4158 inhibited rubidium-86 uptake by erythrocytes, inhibition of Na+,K+-ATPase cannot be implicated as the primary mechanism by which the test agents inhibit parasite growth since ouabain fails to inhibit parasite growth. However, inhibition of erythrocyte Na+,K+-ATPase activity may explain the increased levels of ATP in B4119- and B4158-treated cells due to decreased consumption of this adenine nucleotide.

Taken together, these observations demonstrate that the anti-plasmodial activity of B4119 and B4158, at concentrations of 2μM and lower, is due to direct effects of these agents on *Plasmodium falciparum*. At higher concentrations of the test agents indirect effects on erythrocytes may contribute to the anti-plasmodial action of these compounds *in vitro*.

CHAPTER 6

ANTIPLASMODIAL ACTIVITY OF B4119 IN THE *PLASMODIUM BERGHEI* MOUSE MODEL OF EXPERIMENTAL CHEMOTHERAPY

6.1 INTRODUCTION

The main species of *Plasmodium* that occur naturally in rodents, and which are used to infect laboratory rats and mice, are *P. berghei*, *P. yoelii*, *P. chabaudi* and *P. vinckei* (Garnham, 1980). *Plasmodium berghei* was the first murine malaria parasite to be described by Vincke and Lips in 1948. It was isolated from thicket rats (*Grammomys sudaster*) at Katanga, in the former Zaire (Killick-Kendrick, 1974). This parasite easily infects laboratory mice, rats and hamsters and the strain is still being used in numerous laboratories today. *P. berghei* is large and prefers to infect reticulocytes (Janse and Waters, 1995). A second murine parasite, *Plasmodium vinckei*, was discovered four years later in Katanga and is smaller as compared to *P. berghei* (Landau and Boulard, 1978). *P. berghei* and *P. vinckei* were found to include closely related species, *Plasmodium yoelii* and *P. chabaudi* respectively (Carter and Walliker, 1975 ; Cox and Turner, 1970).

The life cycles of all murine malaria parasites are similar (Cox, 1988). The infection begins with injection of sporozoites from the salivary glands of an infected mosquito *(Anopheles millecampsi)* and exoerythrocytic schizogony occurs in the hepatocytes. The most suitable mosquito for laboratory transmission of all the known species is *Anopheles stephensi*. There is only one phase of exoerythrocytic schizogony which takes 40 - 60 hours for completion. Merozoites from the liver invade red blood cells where they become rings, trophozoites and schizonts yielding about 6 - 18 merozoites each after 24 hours. After re-invasion, the infection continues over a 24 hour cycle (Aikawa and Seed, 1980 ; Homeweed and Neame, 1980).

The murine malaria parasites can be differentiated on the basis of morphology in blood, enzyme characteristics and the rate of development and size of the stages in the liver and blood (Landau

and Boulard, 1978). The distinguishing biological characteristics of *Plasmodium berghei* will be afforded the main focus since the well-studied strain has been used for the next phase of my laboratory research. The early infection occurs in normoblasts, but later there is a preference for immature erythrocytes. The typical parasites are heavily stained and asynchronous.
Multiple infections are common and cause hypertrophy of the host cell (Peters, 1987). Ring forms have two nuclei and the trophozoites and schizonts are compact (non-motile). Free trophozoites and schizonts are common in heavy infections. Schizonts produce varying numbers of merozoites depending on the host; namely 6 - 10 in mice, 16 in rats and hamsters and 16 - 18 in thicket rats (Cox, 1988). The asexual cycle takes 22 - 25 hours. The rate of multiplication results in logarithmic increases in parasite numbers within the blood cells and the host dies, or the immune system intervenes (Mons et.al, 1985). The gametocytes are large and fill, or nearly fill the host cell. The sporogonic cycle is rapid as compared to the other parasites (Killick-Kendrick, 1974). This strain of murine malaria possesses lactate dehydrogenase as a distinguishing enzyme characteristic.

Plasmodium berghei causes total mortality in many strains of white mice (Balb/c mice) as compared to the other species, especially *P.yoelii* that causes mild and non-fatal disease in mice and young rats. During the first 6 days of infection, mature erythrocytes are invaded resulting in thrombocytopenia, hemoglonuria and anaemia and the mouse can die in a state of shock (Garnham, 1980). If the mouse survives the first phase of infection then new reticulocytes are invaded and anaemia becomes prevalent causing the animal to die from anoxia in about three weeks (Rudin *et.al*, 1997). This rapid and intense infection course is accompanied by extreme pallor and the animals are weak and cachectic with staring coats. From the experimental point of view a virulent, fulminant infection is necessary for studies on chemotherapy and production of parasite material for immunological or biochemical investigations while a chronic or resolving infection is required for studies on immunity or pathology (Mercado and Coatney, 1951 ; Bordman *et.al*, 1997).

The course of a murine malaria blood infection is markedly affected by intrinsic factors such as strain or species of the host. DBA/2 mice survive for short periods even after infection with low parasitemias of *P. berghei* while the Balb/C mice are relatively resistant. Eugi and Allison (1980)

documented that the A strain mice are susceptible to infection by *P. chabaudi* while the Balb/C and CBA mice are resistant. Cox (1978) noted that the course of a *P. berghei* infection can be depressed or made to be erratic due to concurrent infections with *Eperythrozoon coccoides*. *Plasmodium berghei* infections are ameliorated by keeping the mice on a milk diet free from para-aminobenzoic acid (PABA) which is essential for survival of the erythrocytic stages of the parasite (Ferone, 1977). Recrudescences of parasitemia occur in pregnant mice infected with *P. berghei* (Van Zon and Eling, 1980).

Before the discovery of rodent malaria in 1948 most research work relating to chemotherapy of malaria had been carried out in avian systems using *Plasmodium gallinaceum* in young chicks (Cox, 1988). Since 1948, *P. berghei* has been extensively used with fewer studies performed using *P. yoelii*, *P. chabaudi* and *P. vinckei* (Peters and Howells, 1978). The rodent malaria parasites have been used in four main ways:

a screening of old and new drugs.

b investigation of the mode of drug action.

c induction of resistance to various antimalarials.

d models for maintaining the malaria parasite.

A variety of drug- and host-dependent factors affect the ultimate result obtained for the potential antimalarial activity of a particular compound (Peters, 1980). Host-dependent parameters include the age, sex, diet, strain, environment and diet of the mice while the drug activity will depend on parameters such as the route of drug administration, drug dosage and mode of formulation.

The main tests for evaluating drug activity against *Plasmodium berghei* in mice have been extensively described (Osdene et.al, 1967; Peters, 1980). In the Rane test (Rane and Kinnamon, 1979), mice are infected with an inoculum of 10^6 iRBC intraperitoneally that kills the control mice in 6 days. A single dose of drug is given 3 days after infection and a survival time of more than 12 days in drug-treated mice as compared to controls indicates potential antimalarial activity and 60 days as a cure. This test is thus simple to execute; requiring suitable preparations of the compounds to be tested, intra peritoneal injection of mice with virulent parasite strain, administration of a single drug dose and observation of mortality times. The test has been used by the Walter Reed Army Institute of Research to screen over 200 000 new compounds of which

170 were also tested in monkeys and only mefloquine was developed for human use (Peters, 1975).

An alternative test that is being widely employed for evaluating antimalarial activity of new agents is the Peter's 4-day suppressive test (Peters, 1987). In this procedure, mice are infected with 10^7 iRBC intravenously or intraperitoneally and the test compound administered daily for 4 days beginning from the day of infection. The activity of the test compounds is evaluated as the reduction of parasitemia in drug-treated mice as compared to the controls on day four. The drug sensitive parasite lines K173 and NK65 are most widely used in these two tests. Like the Rane test, the 4-day suppression test is unable to detect activity of antimalarials like proquanil (Peters, 1970). The third group of tests is carried out in *Plasmodium vinckei*-infected mice (Peters, 1987). The mice are inoculated with 10^7 iRBC and the drug is administered a few hours later. Control mice die within 5 - 6 days and an increase in the survival time of 20% indicates potential antimalarial activity. The assessment of antiplasmodial activity against tissue stages has been less frequently studied because of difficulty in maintaining mosquito cultures, or variation in the infectivity rate of the mosquito (Peters, 1975).

The significance of rodent malaria for screening new antimalarial agents and understanding mechanisms of drug action as well as resistance cannot be overlooked. However, rodent malaria models cannot be fully regarded as perfect models of human malaria and they cannot mimic the part played by the host's metabolism and immune responses in the efficacy of drug action (Cox, 1978). The two main problems relate to the exoerythrocytic stages and asexual forms in the blood. The exoerythrocytic forms develop at a rapid rate, even faster than those of *P. knowlesi* (the most rapidly multiplying primate malaria), as compared to the exoerythrocytic forms of human falciparum malaria (Richards, 1984). Therefore drugs that are active for 48 hours will destroy all rodent malaria parasites, but can only prolong multiplication in other forms implying that results obtained with one model cannot be extrapolated to another. The metabolic rate and nuclear division are important determinants of mode of drug action as such their significance in the various models will differ (Sherman, 1984). The asexual stages of *P. berghei* prefer to invade reticulocytes, while in human malaria, *P. falciparum* invades mature erythrocytes. The biochemical and physiological differences in these two cell types will obviously have some effect

on drug activity. The fact that rodent malaria parasites do not sequester in deep tissues as is the case with human forms of the parasites will also affect activity of pharmacological agents directed against the malaria parasites (Boonpucknavig et.al, 1984).

It must therefore be concluded that no rodent malaria model represents a suitable model for human malaria although research using rodent malarias has provided a vast amount of information about malaria parasites as a whole. It is also without doubt that some aspects of the pathology and immunology of rodent malarias resemble the situation in man although the overall makeup is different. Nevertheless, these rodent models are useful in the *in vivo* screening of the anti-plasmodial potential of novel chemotherapeutic agents.

6.2 AIMS AND OBJECTIVES

Laboratory duties performed in this part of the study were designed to investigate the effects of the TMP-substituted phenazine, B4119, alone and in combination with chloroquine on the *in vivo* growth of *Plasmodium berghei* in a mouse model of malaria.

6.3 MATERIALS AND METHODS

6.3.1 Media and reagents

WASH MEDIUM

See section 2.3.1

CRYOPRESERVATION OR FREEZING SOLUTION

See section 2.3.1

NORMAL SALINE

9g NaCl [Sky Chem, Alberton, South Africa]

1L distilled water

PREPARATION OF THE TMP-PHENAZINE B4119

The TMP-phenazine (B4119) was incorporated into rat chows follows:

The drug concentrations were prepared by dissolving 12.5 and 25g B4119 in 50ml ethanol and then adding the solution to 100g of powdered rat chow to obtain 0.0125% and 0.025% B4119 respectively. The drug solutions and rat chow were properly mixed and dried under open air. Control solutions were prepared by adding 50ml ethanol to 100g mouse food.

10mM CHLOROQUINE DIPHOSPHATE SALT

5mg chloroquine diphosphate salt

1ml normal saline

6.3.2 **Mouse parasite culture maintenance, (**Peters, 1987**)**

MICE

Female Balb/C mice, 6 - 8 weeks old, were obtained from colonies maintained at the South African Vaccine Production Company, Johannesburg.

PARASITES

Plasmodium berghei parasites were obtained from Dr. Ian Havlik; Department of Experimental and Clinical Pharmacology, University of the Witswatersrand. The parasites were kept in liquid nitrogen as a 1 : 2 dilution of infected blood in 28% glycerol. The parasite was passaged once in Balb/C mice before use in each experiment. Briefly, a frozen vial was thawed and three mice received a single inoculum of 0.2ml of the parasite suspension intraperitoneally. Parasitemia was allowed to develop to about 20% at which time blood was collected to prepare an infective inoculum for subsequent administration to experimental mice. For preparation of the inoculum, the mice were anaesthetized using halothane (Rhône-Poulenc-Rhorer, Lyon, France) and the thoraxes were opened. Blood was collected in citrate from the dorsal vena carva at the hepatic junction.

The blood was centrifuged at 200g for 5min and the buffy coat of leukocytes as well as excess citrate were removed. The blood from the three mice was then pooled. Part of the suspension was diluted in washing medium to approximately 1 to 2.5 $X10^6$ parasitized red blood cells per 0.2ml of the suspension. The recipient mice were injected with a single inoculum of 0.2ml intraperitoneally. The other part of the parasite suspension was mixed with freezing medium

(28% glycerol) and stored in liquid nitrogen (cryopreservation). The course of clinical infection was monitored by weighing the mice daily and evaluating parasitemia microscopically. Briefly, the tails were pricked with a thin needle and thin smears were prepared on microscope slides and air-dried. After fixing with ethanol, the smears were stained with Giemsa solution (0.2ml Giemsa / 2ml PBS) for 5min. After staining, the slides were washed under running tap water, dried and investigated under a light microscope at a magnification of 787.5. The level of parasitemia was determined by counting 1000 red blood cells per mouse.

6.3.3 **Drug studies**

Effects of B4119 on the growth of *P. berghei*- infected Balb/C mice. Female, 6 - 8 week old, Balb/C mice (randomly bred) were divided into groups of six mice. Mouse food with 0.0125% (15mg/kg/day) and 0.025% (30mg/kg/day) or without B4119 was given concomitantly with intra peritoneal inoculation of parasites (2.5 X 10^6 infected red cells per 0.2 ml suspension). The course of infection and drug effect were evaluated daily starting from day 4 as in section 6.3.2 and treatment was terminated on day 10. Results were expressed as percentage parasitemia, survival rate and weight.

Effects of pretreatment of mice with B4119 on the growth of *P. berghei*. Female Balb/C mice (inbred), 6 - 8 weeks old, were divided into groups of ten mice. Mouse food with 0.025% (30mg/kg/day) B4119 was given three weeks prior to infection (pre and postinfection treatment group). On the day of infection another group of mice was given mouse food with 0.025% (30mg/kg/day) B4119 (postinfection treatment group) and treatment was continued in the latter group. All mice were inoculated intraperitoneally with 2.5 X 10^6 infected red cells per 0.2ml suspension of *P. berghei* parasites. The course of infection and drug effects were evaluated every second day starting from day 4 as detailed in section 6.3.2 and treatment was terminated on day 10. Results are expressed as percentage parasitemia, survival rate and weight.

Effects of chloroquine on the growth *P. berghei*-infected Balb/C mice. Female, 6-8 week old, Balb/C mice (inbred) were divided into groups of five mice. The mice were injected intraperitoneally with 2 X 10^6 infected red cells per 0.2ml suspension of *P. berghei* parasites. On the same day the mice were injected with varying doses of chloroquine (1.25 - 25µg/kg/day in

normal saline). Control mice were injected with 0.2ml normal saline only. The course of infection and drug effects were evaluated every second day from day 4 as detailed in section 6.3.2 and treatment was terminated on day 10. Results are expressed as percentage parasitemia and survival rate.

Effects of combined treatment with chloroquine and B4119 on the growth of *P. berghei*-infected Balb/C mice. Female, 6-8 week old, Balb/C mice (inbred) were divided into groups of ten mice. The mice were injected intraperitoneally with 2 X 10^6 infected red cells per 0.2ml suspension of parasites. On the same day the mice were also injected intraperitoneally with either 0.2ml chloroquine (1.25µg/kg/day) or normal saline and given mouse food containing 0.025% (30mg/kg/day) B4119.The course of infection and drug effects were evaluated every second day from day 4 as detailed in section 6.3.2 and treatment was terminated on day 10. Results are expressed as percentage parasitemia and survival rate.

6.4 RESULTS

***In vivo* anti-plasmodial activity of B4119 against *P. berghei*-infected mice**. To determine the concentration of B4119 that suppresses parasitemia, two doses (15 and 30mg/kg/day) of the drug were evaluated for activity in *P. berghei*-infected mice. A dose-related decrease in parasitemia was observed from day 4 in treated mice as compared to the controls during the first ten days (Figure 35). Significant ($p < 0.05$) inhibition of parasite growth occurred on the eighth ($p = 0.014$) and tenth days ($p = 0.008$) of treatment with 30mg/kg/day B4119, as well as on day 10 ($p = 0.023$) of treatment with 15mg/kg/day B4119 as compared to the control group of mice. Results in Figure 36 show that between day 8 and 13 four of the six control mice died, while one mouse died in the 15mg/kg/day B4119-treated group and all mice were still viable in the 30mg/kg/day B4119-treated group on the same day (day 13). The last mice in the control group, 15mg/kg/day B4119-treated and 30mg/kg/day B4119 treated groups died on days 22, 23 and 19 respectively. There was weight loss in all the mice groups, although there were no significant differences between the groups (results not shown)

Effects of pretreatment of *P. berghei*-infected mice with B4119. B4119 (30mg/kg/day) was identified in the preceding experiments as suppressing murine infection with *P. berghei*. An

experiment was subsequently performed using groups to evaluate the efficacy of administration of B4119 (30mg/kg/day) three weeks prior to infection. Group one was the untreated control group, group two the post infection treatment group and group three the pretreatment group. In group three treatment was continued throughout the course of infection. Results in Figure 37 show that parasitemia was suppressed to the same degree in both the treatment groups compared to the control group with significant ($p < 0.05$) inhibition observed on day 10 of both the postinfection treatment group ($p = 0.005$) and the preinfection treatment group ($p = 0.035$). Of the ten mice used in each group, a single control mouse was still viable on day 16 while 6 and 7 mice were still viable on the same day in the postinfection treatment group and the preinfection treatment groups respectively (Figure 38). There was weight loss in all the mice groups although the was no significant difference between the groups (results not shown).

***In vivo* anti-plasmodial activity of chloroquine against *P. berghei*-infected mice**. In this experiment, infected mice were given different dosages of chloroquine (1.25 - 25µg/kg/day) intraperitoneally to establish a sub-curative chloroquine concentration for use in combination with B4119 (30mg/kg/day) to evaluate the anti-plasmodial activity of a combination these two agents in infected mice. After ten days of treatment with chloroquine, parasites were cleared in mice given between 2.5 and 25µg/kg/day of the drug. An intermediate suppression of parasite growth was observed between days 4 and 8 with an abrupt upsurge from day 9 in mice given 1.25µg/kg/day chloroquine (Figure 39). All control mice died on day 10, while mice treated with 1.25µg/kg/day of chloroquine survived till day 22. Mice given 25µg/kg/day chloroquine did not succumb to infection, whereas of the five mice used, only four survived infection in the groups given 2.5 and 12.5µg/kg/day chloroquine (Figure 40).

***In vivo* anti-plasmodial activity of B4119 in combination with chloroquine**. Chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) in combination caused suppression (incomplete clearance) of parasite growth after ten days of treatment. On day 10, the parasitemia declined to less than 5% in the mice treated with both chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) as compared to those on treatment with either chloroquine or B4119 individually, which were 22% and 18% respectively (Figure 41). The control mice died within nine days after infection, while four and nine mice were still alive respectively out of ten in the group which was treated

with chloroquine and B4119 individually. All the mice in the group given a combination of chloroquine and B4119 were still viable on day 10 (Figure 42).

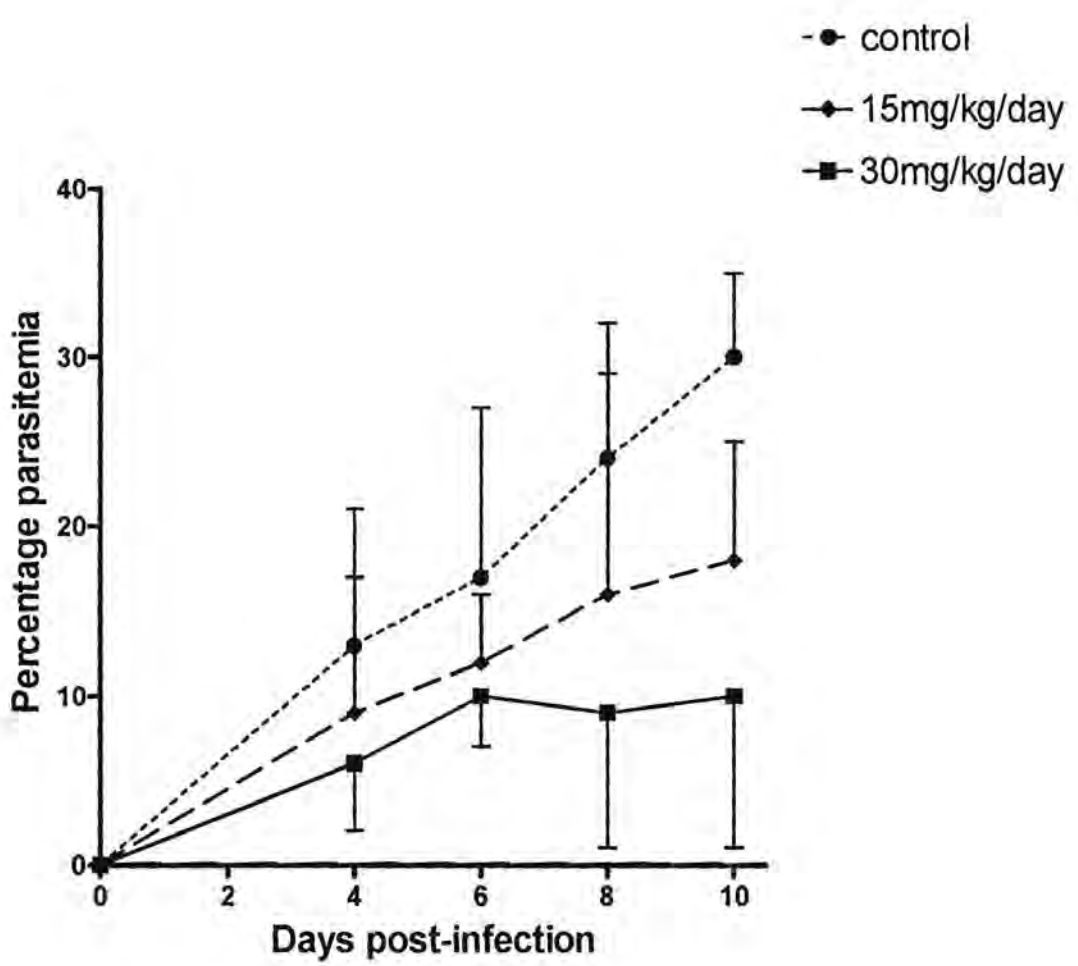


Figure 35 : Effects of oral administration of B4119 (15 and 30mg/kg/day) on parasite growth in *P. berghei*-infected Balb/C mice.

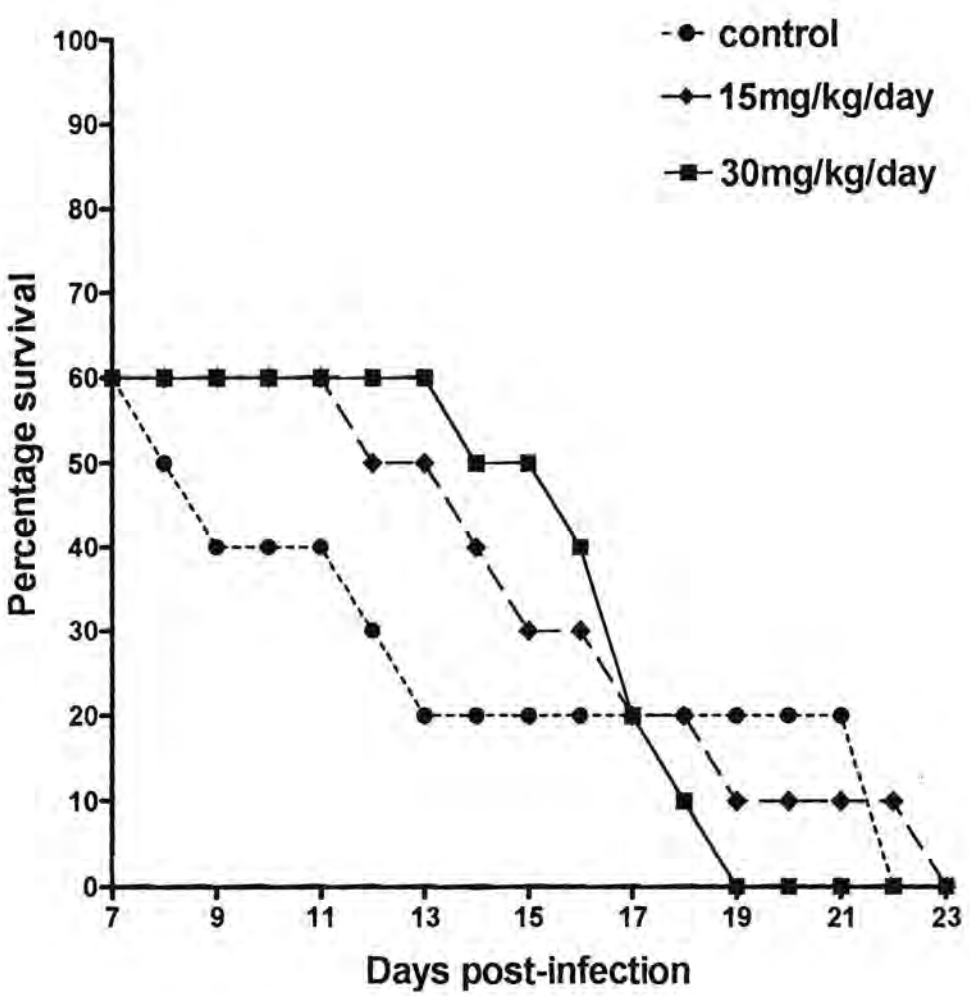


Figure 36: Effects of oral administration of B4119 (15 and 30mg/kg/day) on the survival rate of mice infected with *P. berghei*.

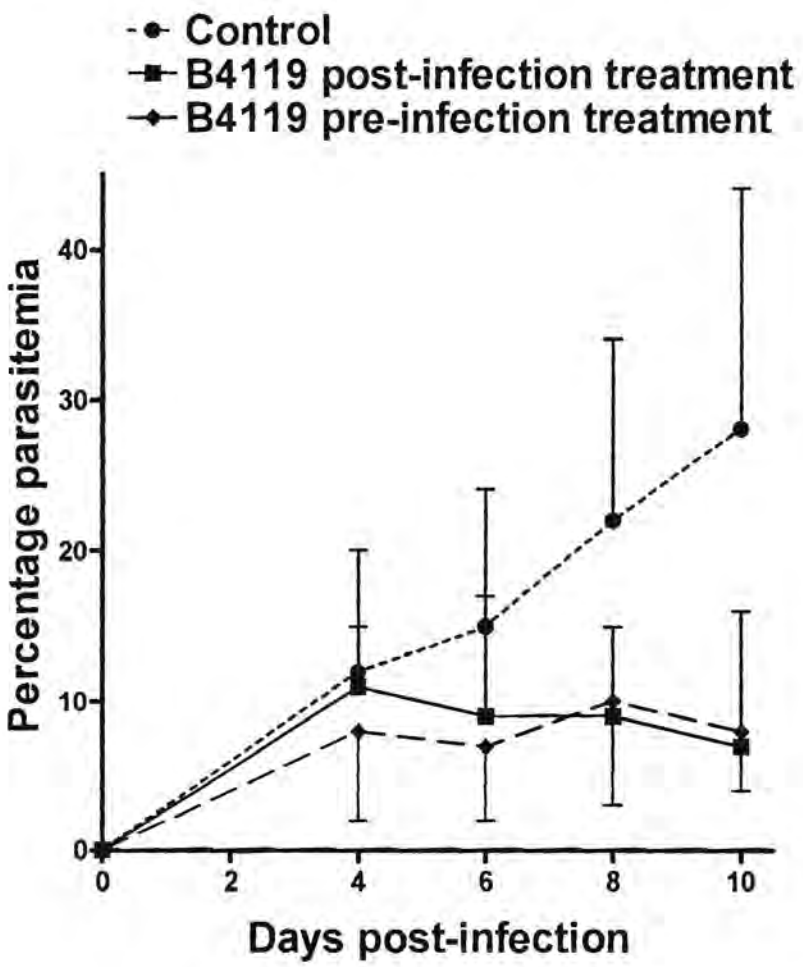


Figure 37 : Effects of the pre- and post-infection treatment compared to postinfection treatment only with 30mg/kg/day B4119 on growth of *P. berghei* parasites.

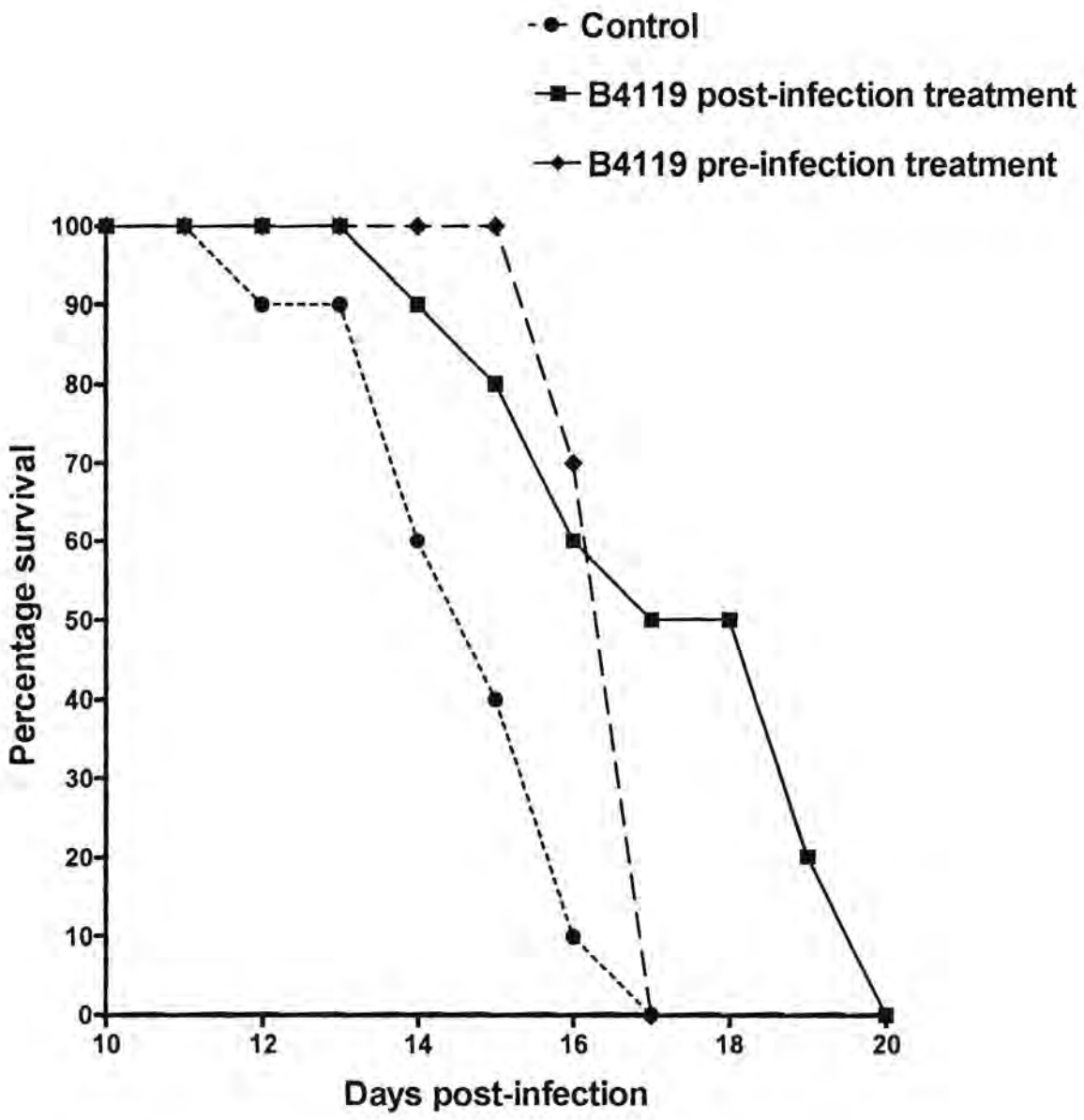


Figure 38 : Effects of pre- and post-infection treatment compared to postinfection treatment only with 30mg/kg/day of B4119 on survival rate of *P.berghei*-infected Balb/C mice.

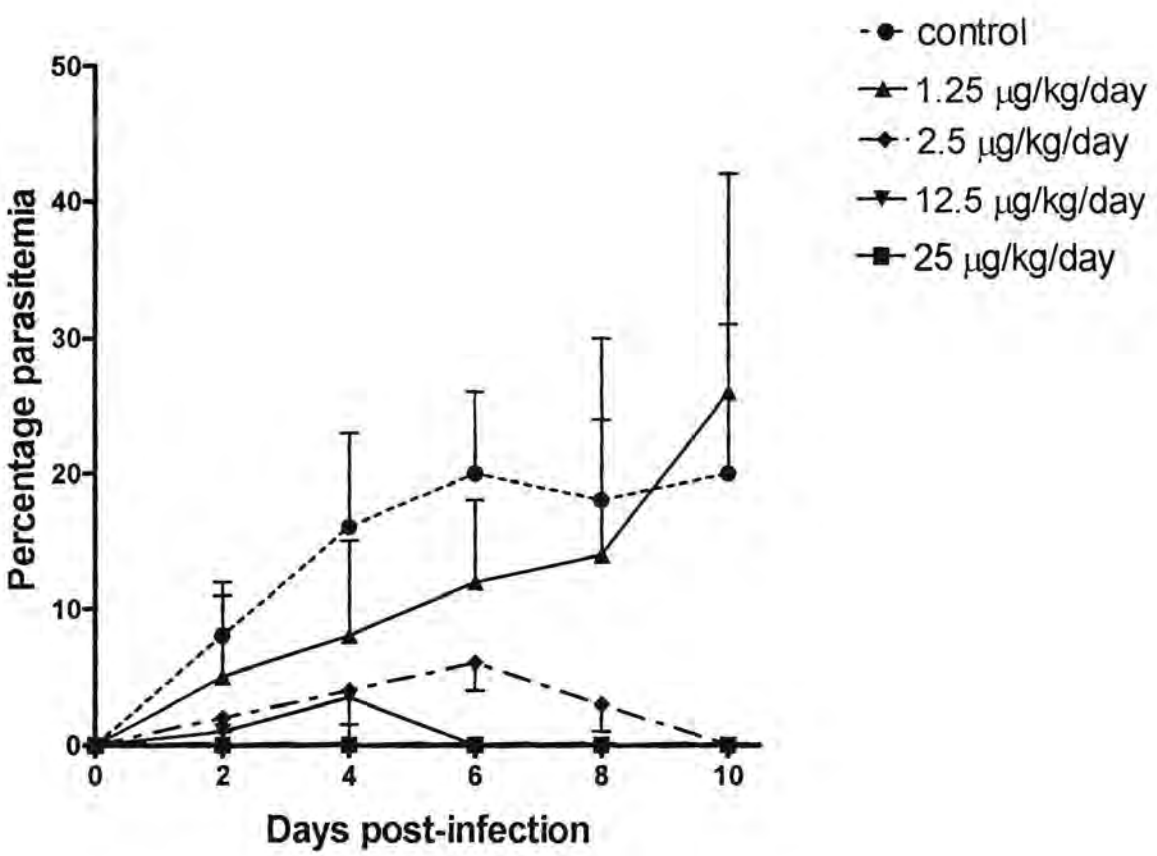


Figure 39 : Effects of intra peritoneal administration of chloroquine (1.25 - 25µg/kg/day) on parasite growth in *P.berghei*-infected Balb/C mice.

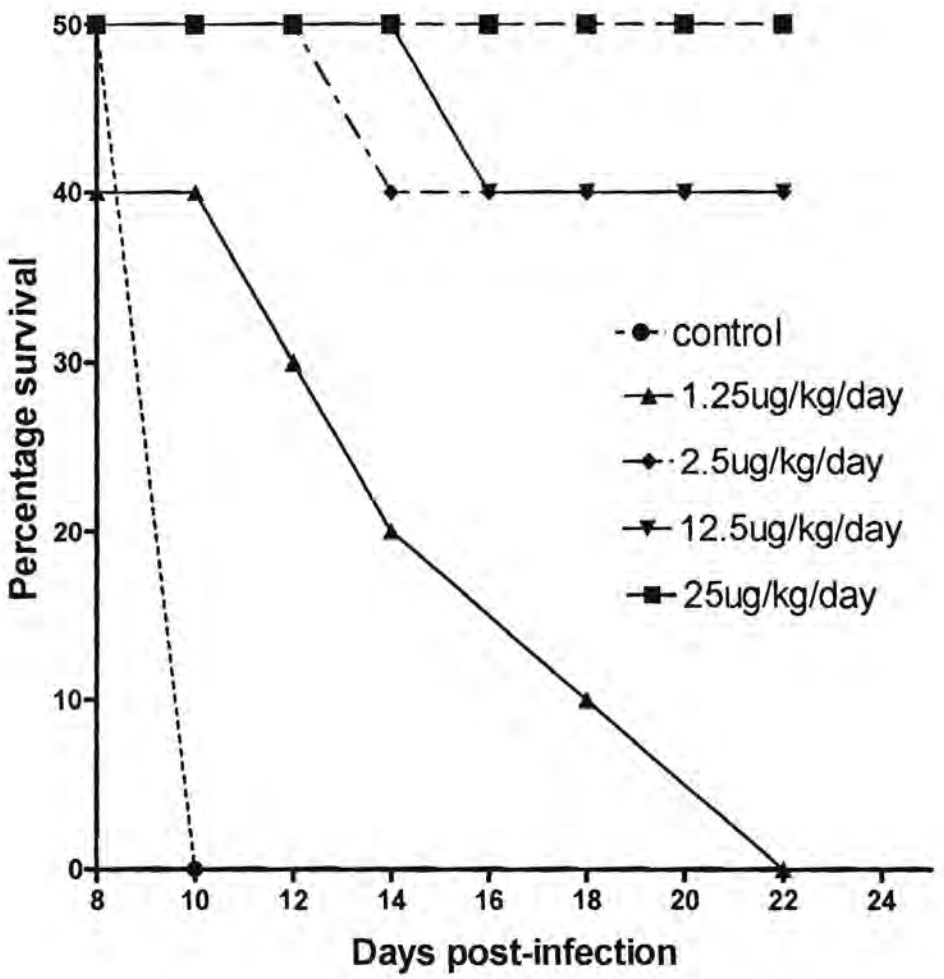


Figure 40 : Effects of chloroquine (1.25 - 25µg/kg/day) on the survival rate of Balb/C mice after infection with *P. berghei* parasites.

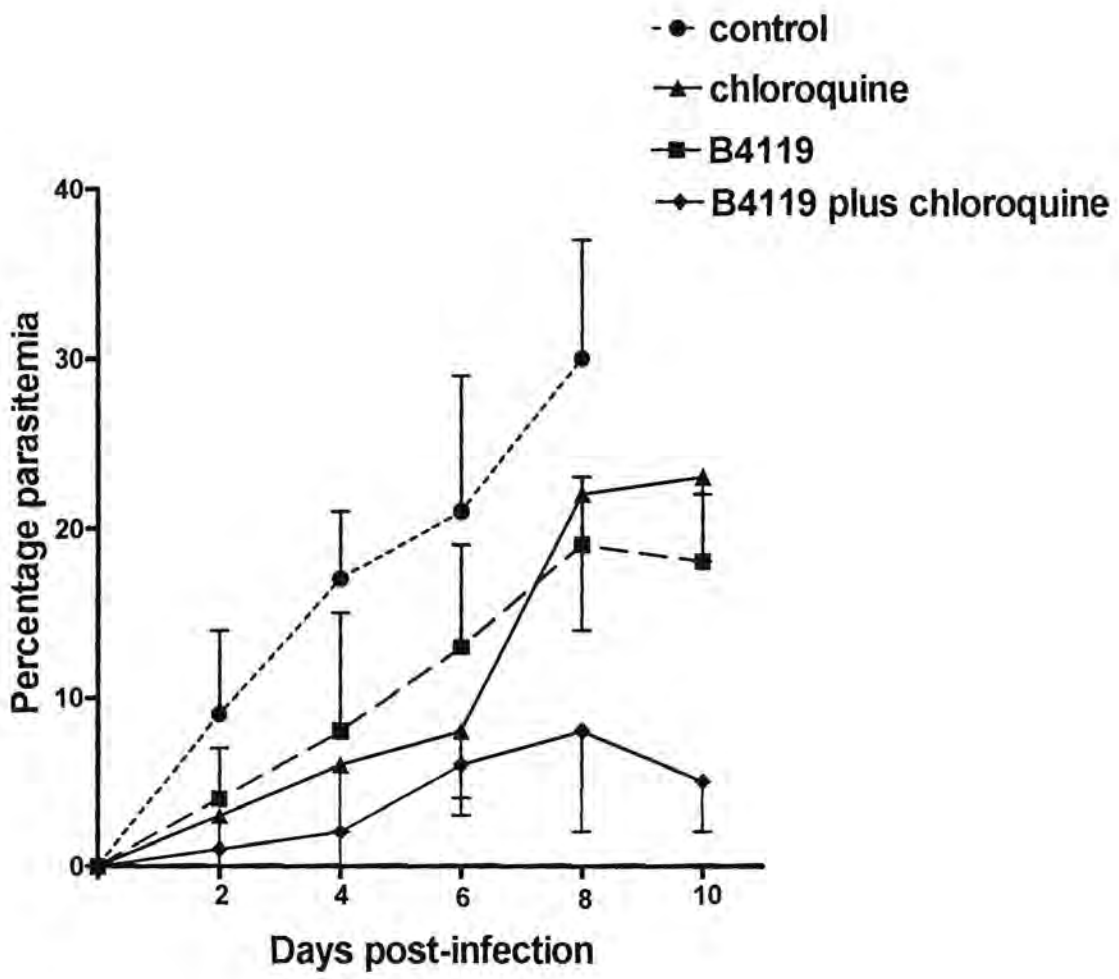


Figure 41 : Effects of chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) individually and in combination on parasite growth of *P.berghei*-infected Balb/C mice.

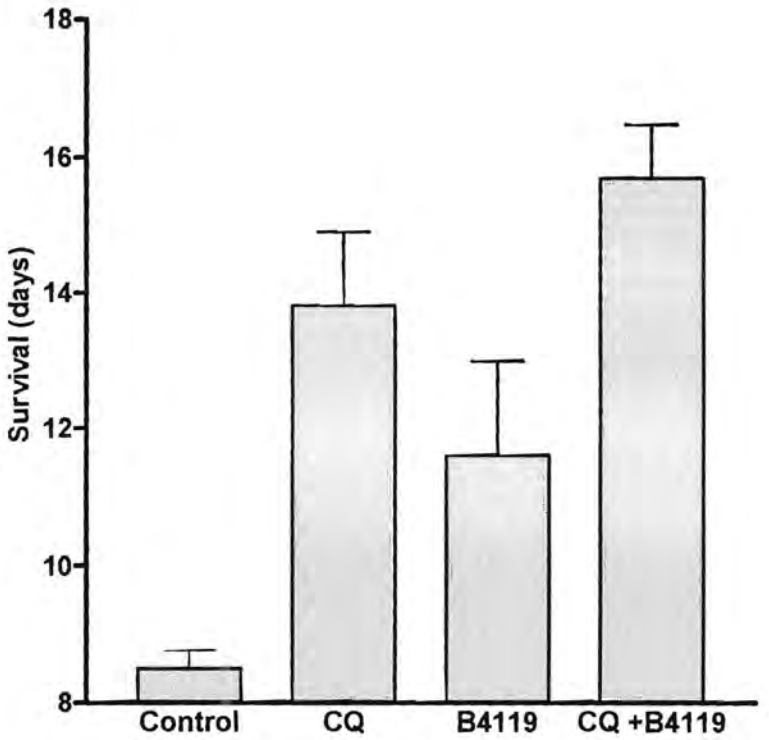


Figure 42: Survival rate of *P.berghei*-infected mice treated with chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) singly and in combination.

6.5 DISCUSSION

The search for new antimalarial compounds ultimately requires an *in vivo* evaluation using an animal model of experimental chemotherapy. The murine model of *P. berghei* used in the current studies is reproducible and can provide useful information on *in vivo* efficacy of anti-plasmodial agents individually and in combination.

In the present study, B4119, rather than B4158, was preferred for evaluation in the murine model of experimental chemotherapy since it exhibited superior *in vitro* anti-plasmodial activities compared to B4158. There is a good correlation between suppression of parasitemia in *P. berghei*-infected mice and the dose of B4119 administered over 10 days, while the drug failed to demonstrate any impressive prophylactic potential. This observation is in agreement with *in vitro* results that indicated failure of drug-treated red blood cells to inhibit merozoite invasion. It is encouraging that treatment of infected mice with B4119 and chloroquine, a classical antimalarial agent, reduced the levels of parasitemia by 95% after 10 days of treatment. However. optimal doses, modes (e.g requirement for carriers such as liposomes or cyclodextrins) and routes of administration remain to be established for the TMP-substituted phenazines.

CHAPTER 7

7.1 CONCLUDING DISCUSSION

Despite major efforts to control it, malaria remains a cause of great concern, particularly in developing countries (Meshnick *et.al*, 1993). To make matters worse, only a few antimalarial drugs are available through regular commercial channels for unlimited clinical use and the development of new agents is costly and time-consuming. Probably the most ominous problem associated with malaria control is the resistance *Plasmodium falciparum* develops to antimalarial drugs (Bjorkman and Phillips-Howard, 1990). Indiscriminate use of most of the parasiticidal drugs has led to adverse side-effects such as neurotoxicity in the case of artemisinin derivatives, and early emergence of resistance to these agents, which compromises their therapeutic efficacy in the treatment of severe and complicated malaria. Doctors Brian Sharp and Janet Freese from the South African National Malaria Research Programme in Durban, have recommended that (a) appropriate drugs in correct dosages be administered and (b) health education for both the community as well as health care workers is fundamental to the control of drug-resistant malaria (Sharp and Freese, 1995).

Difficulties in establishing superior chemoprophylaxis for plasmodium infections are further compounded by the limitations of currently available techniques used for screening the *in vitro* antimalarial activity of new agents. Classical procedures such as microscopy and radiometry, are labourious, especially when large numbers of samples must be processed and evaluated. Microscopic evaluation of large numbers of Giemsa-stained thin blood smears requires qualified laboratory personnel. Moreover, radiometry requires long incubation periods (24 - 72 hours) followed by sample processing and scintillation counting (Makler *et.al*, 1987 ; Wyatt *et.al*, 1991).

Flow cytometric procedures utilize previously incorporated dye and buffer systems that required prompt sample analysis and strict incubation conditions. Use of acridine orange as a tracer for parasite's DNA in infected erythrocytes is associated with nonspecific binding, whilst Hoechst 33258 and ethidium bromide required modifications of incubation media, the results obtained not always comparable with that of microscopic methods (Howard *et.al*, 1979 ; Whaun *et.al*, 1983; Bianco *et.al*, 1986). The present studies describe an improved flow cytometric procedure using

thiazole orange, a membrane permeable dye, as a tracer for parasite-infected red blood cells. This procedure is rapid (large quantities of samples are analysed in a short time period, 2 - 3 hours), the fixing step incorporated in this method enables the storing samples for staining and analysis at a convenient time (this eliminates the principal drawback encountered when availability of floe cytometer does not coincide with completion of the assay). Moreover the method is simple (all washing and centrifugation procedures are eliminated). The agreement, as measured by the Bland and Altman test (Bland and Altman, 1986), between this flow cytometric method and the two classical assays, clearly demonstrate that future screening of new antimalarial agents can be confidently performed using flow cytometry. Further studies are necessary to standardise this method such that its applications can be extended for determining parasite levels from patients samples as well as fresh field isolates.

It has previously been reported that the prototype riminophenazine, clofazimine , which is primarily an antimycobacterial agent (Barry et.al, 1957; Schaad-Lanyi et.al, 1987), does not possess anti-plasmodial activity (Sheagren, 1968). In the current study, I have observed that novel derivatives off clofazimine, TMP-substituted phenazines, do, however, inhibit the growth of *P. falciparum in vitro*. The major structural difference between clofazimine and the TMP-substituted phenazines relates to the nature of the substituent bound to the imino nitrogen functional group at position 2 on the phenazine nucleus. In the case of clofazimine, this position is occupied by an isopropyl group, and by a TMP group in the case of the novel compounds. 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. Importantly, serum concentrations of up to 8μM have been reported in patients with leprosy following oral administration of clofazimine (Schaad-Lanyi et.al, 1987).

Stringently synchronized cultures were used to investigate the stages of growth at which P. falciparum is most vulnerable to the TMP-substituted phenazines. In theses experiments B4119 and B4158 were added to ring and late trophozoite cultures of the parasite. Both test agents were found to interfere with parasite development at both stages of the life cycle, with the late phase of parasite development being most sensitive. Theses observations suggest that B4119 and B4158 affect metabolic events which are essential for parasite survival throughout the life cycle.

B4119 and B4158 were also found to be active against chloroquine-, quinine- and sulfadoxine/pyrimethamine-resistant strains of *P. falciparum*. These observation demonstrates that the sensitivity of the *P. falciparum* to the two phenazine derivatives is unaffected by the mechanisms which confer resistance to the three conventional anti-malarial agents. Chloroquine and quinine accumulate within the parasite's food vacuole killing the parasite by interfering with heme polymerization (Ridley, 1997) while sulfadoxine/pyrimethamine is an antimetabolite combination that starves the parasite of essential folic acid (Peterson et.al, 1990).

Interestingly, B4119 and B4158 were also found to inhibit haem polymerization *in vitro*, suggesting that they share a common mechanism of action with chloroquine and quinine. Assuming that the phenazine agents and chloroquine do indeed share a common primary mechanism of anti-plasmodial action, then the observed sensitivity of the chloroquine resistant strain to B4119 and B4158 may seem somewhat surprising. However, resistance to chloroquine does not appear to be related to altered binding to haem, but rather to decreased accumulation in the parasite food vacuole, possibly as a consequence of the induction of the plasmodial homologue of the P-glycoprotein drug-efflux pump, or to increased vacuolar pH (Bray and Ward, 1993; Slater, 1993). Interestingly, TMP-substituted phenazines not only inhibit heme polymerization, but are also potent inhibitors of P-glycoprotein (Van Rensburg et.al, 1997), a property which may account for their activity against chloroquine resistant strains of the Plasmodium parasite.

Alternatively, it may not be valid to suggest that the anti-plasmodial activity of the phenazine agents is directed against the P-glycoprotein entity of the parasite. It has been shown by Krogstad's group (De *et.al*, 1996) that analogues of chloroquine with either a shortened or elongated aminoalky side chain do inhibit growth of chloroquine-resistant strains because apparently the resistance mechanisms are directed against the side chain and not the quinoline ring. Since the phenazines differ structurally from the quinoline compounds that may be reason enough for their activity against resistant strains. Clofazimine itself is incapable of inhibiting parasite growth since it lacks the basic side chain which is responsible for accumulation n the parasite's food vacuole while its TMP derivatives have.

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