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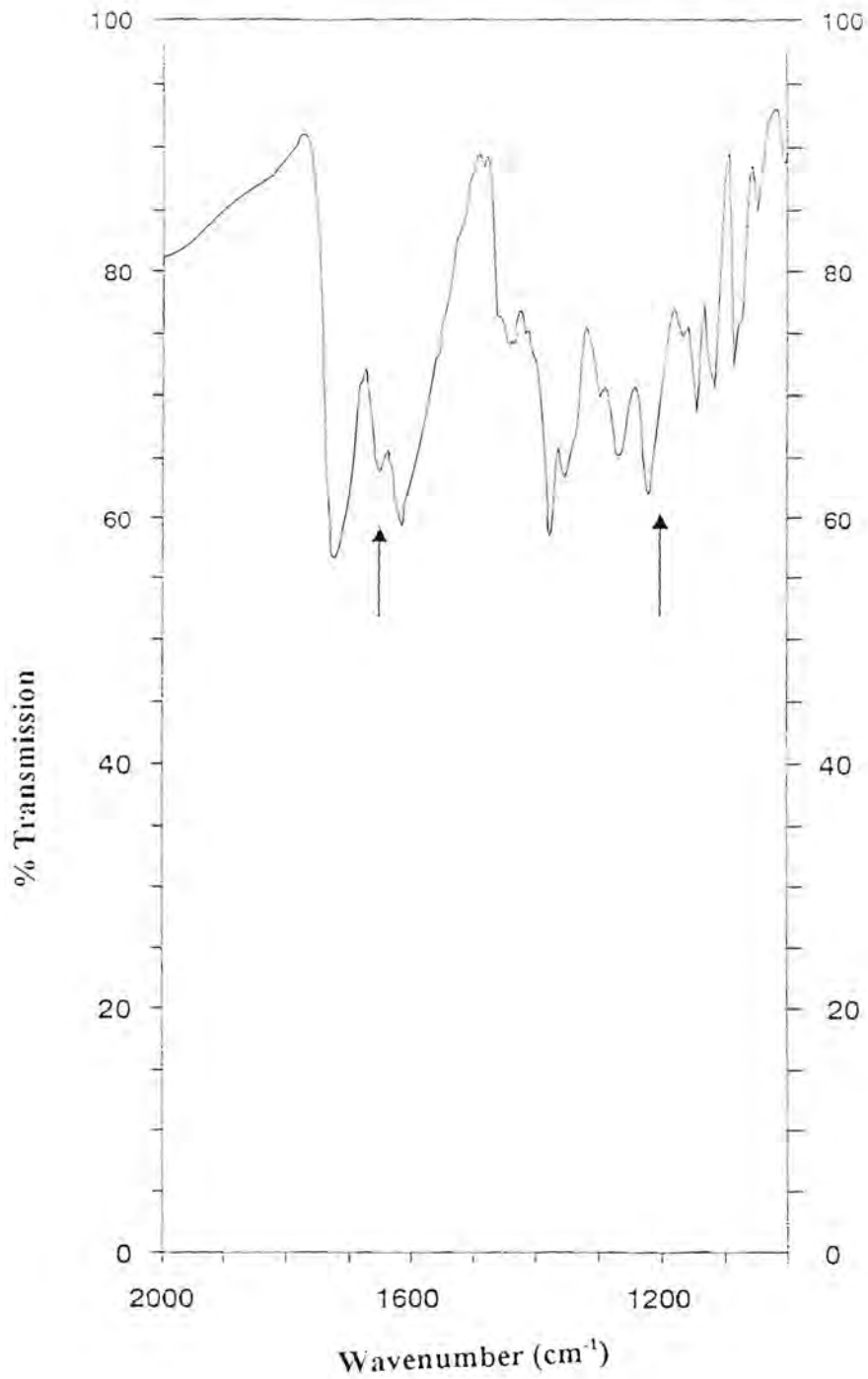
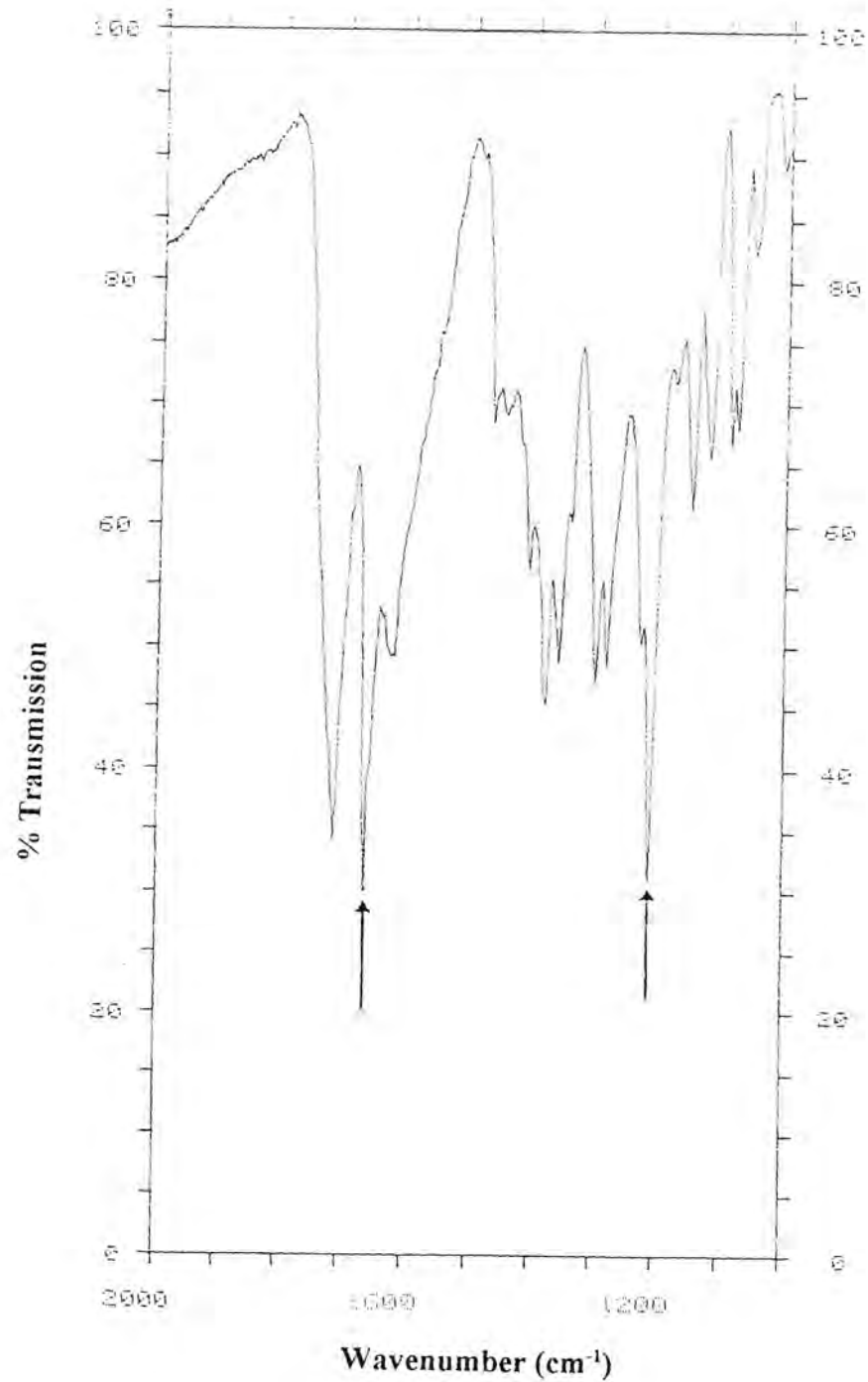**Figure 24** : Infrared spectra of haematin after 0 min incubation in 12.9M acetic acid, pH 5, 60°C

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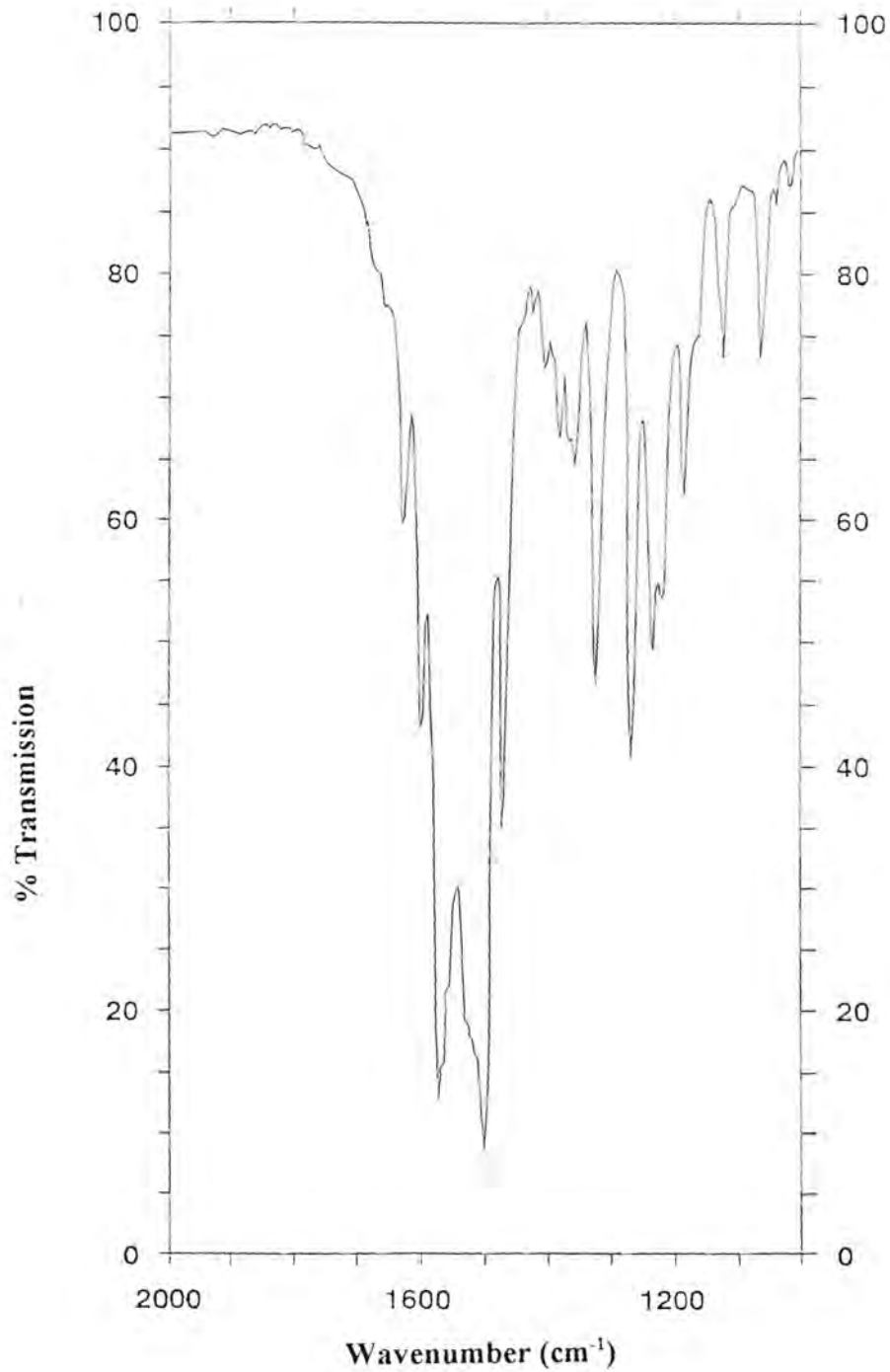
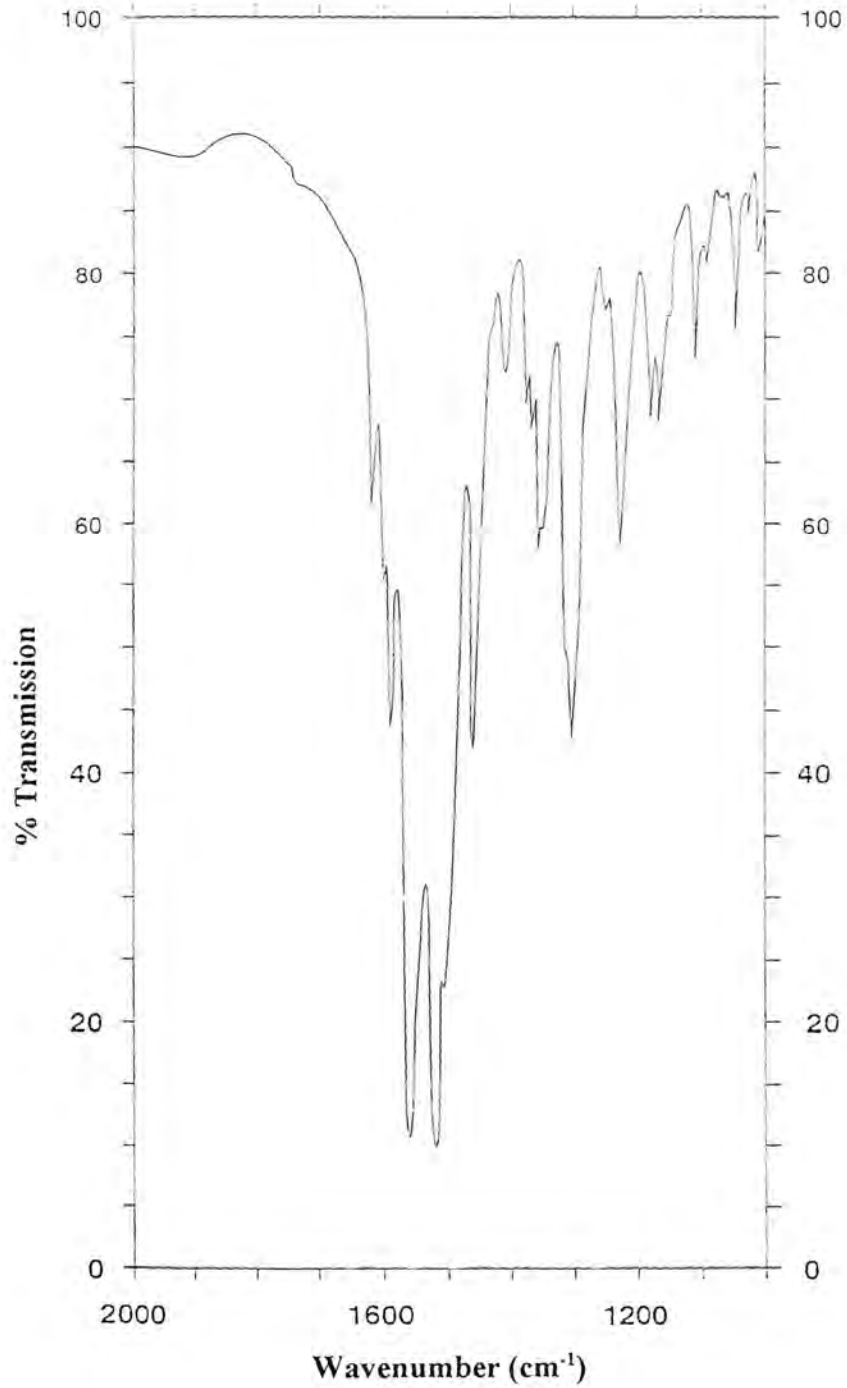**Figure 26** : Infrared spectra of B4119 after 30 min incubation in 12.9M acetic acid, pH 5, 60°C

Figure 27 : Infrared spectra of B4158 after 30 min incubation in 12.9M acetic acid, pH 5, 60°C
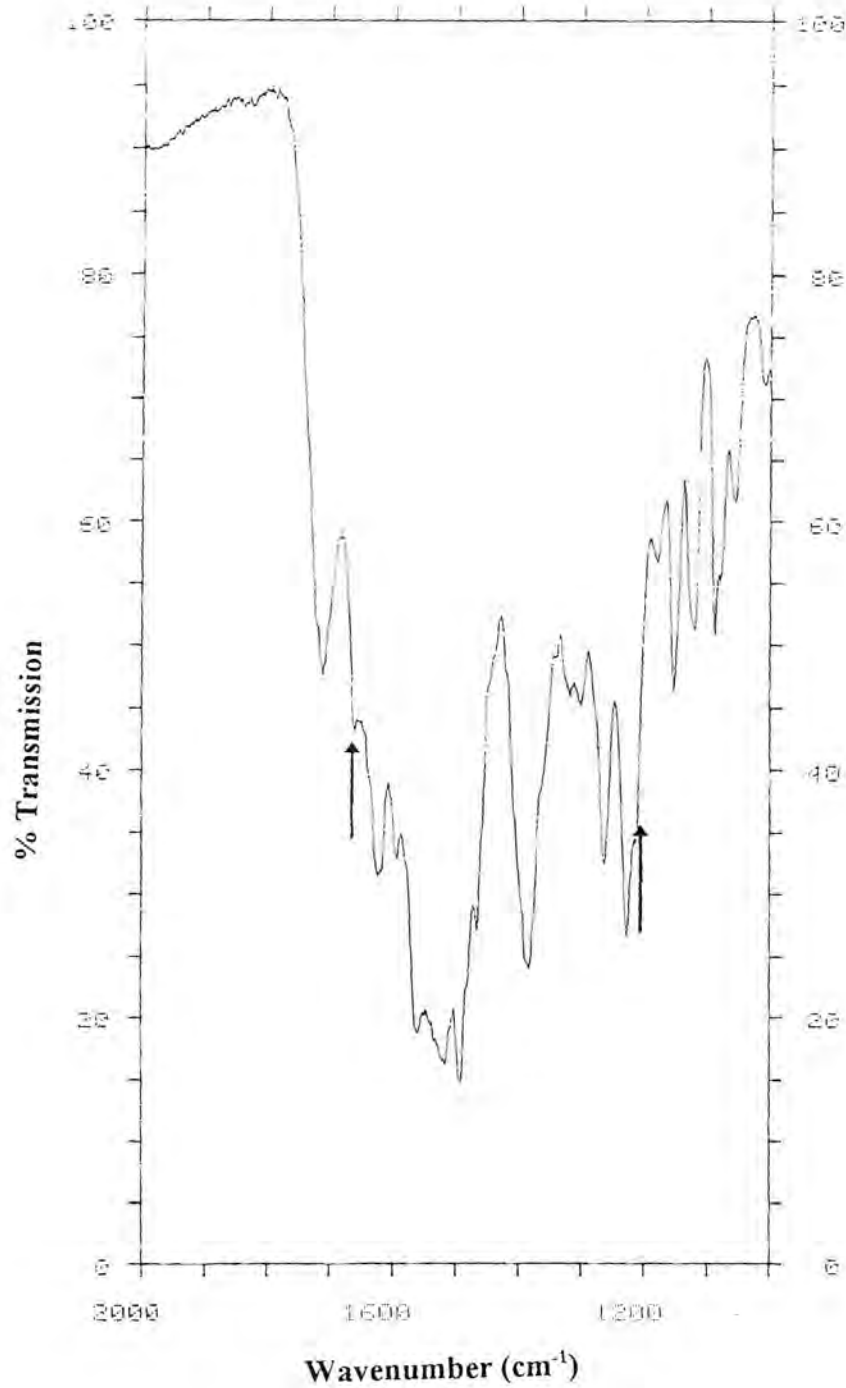
Figure 28 : Infrared spectra of B4119-treated haemin solution after 30 min incubation in 12.9M acetic acid, pH 5, 60°C. The absence of the Soret band is indicated by arrows.
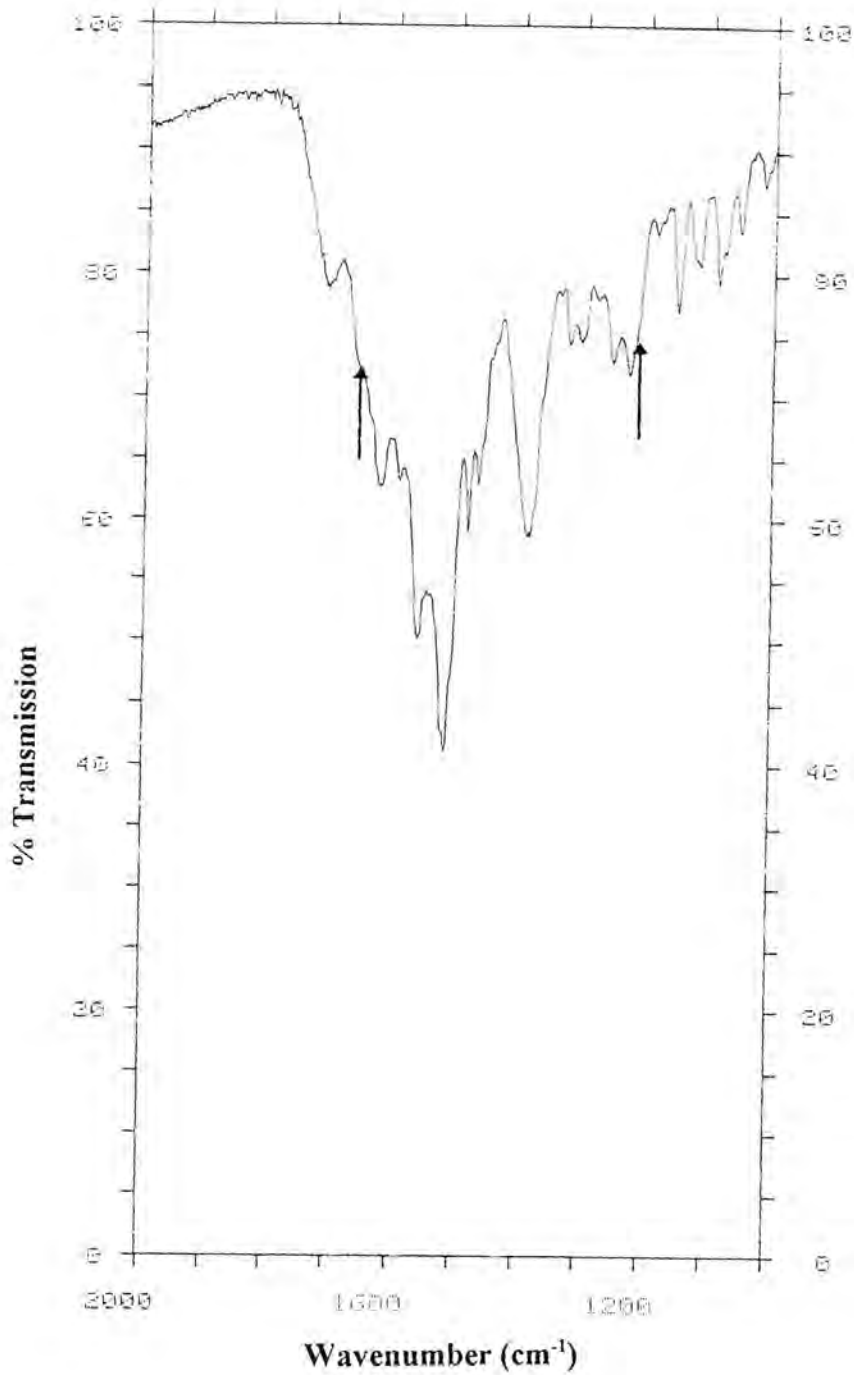
85

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 : To 800μl 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 $35 \pm 10\%$ and $55 \pm 12\%$ 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

Concentration (μM)	Cellular lactate (mg lactate /2×10^9 erythrocytes/ml) after treatment with:	
	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

| Concentration (µM) | Cellular ATP (nanomoles ATP / 2×10^9 erythrocytes/ml) after treatment with: | |
	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.*
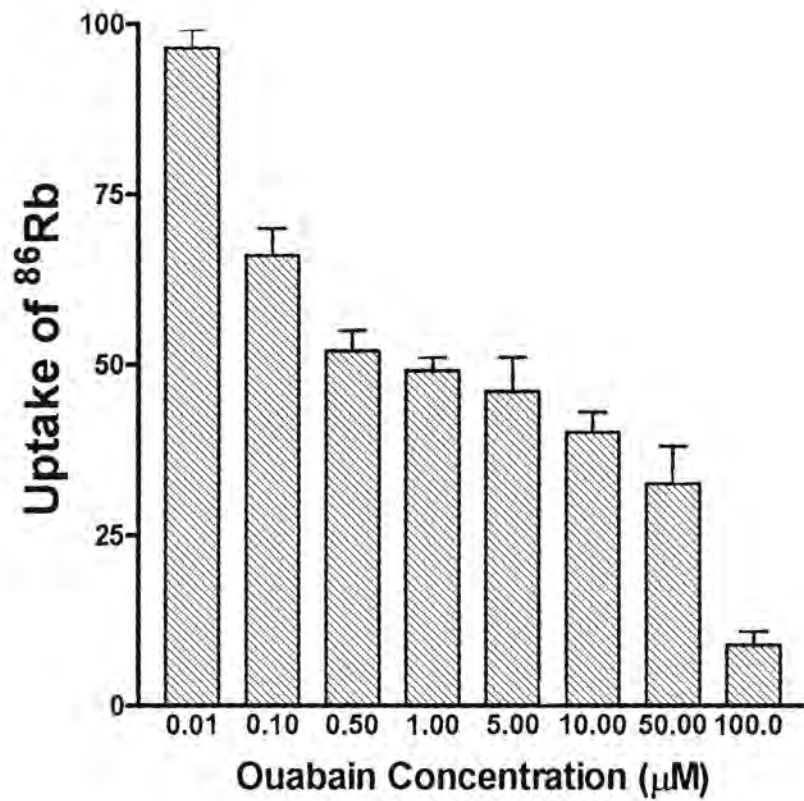
95

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.
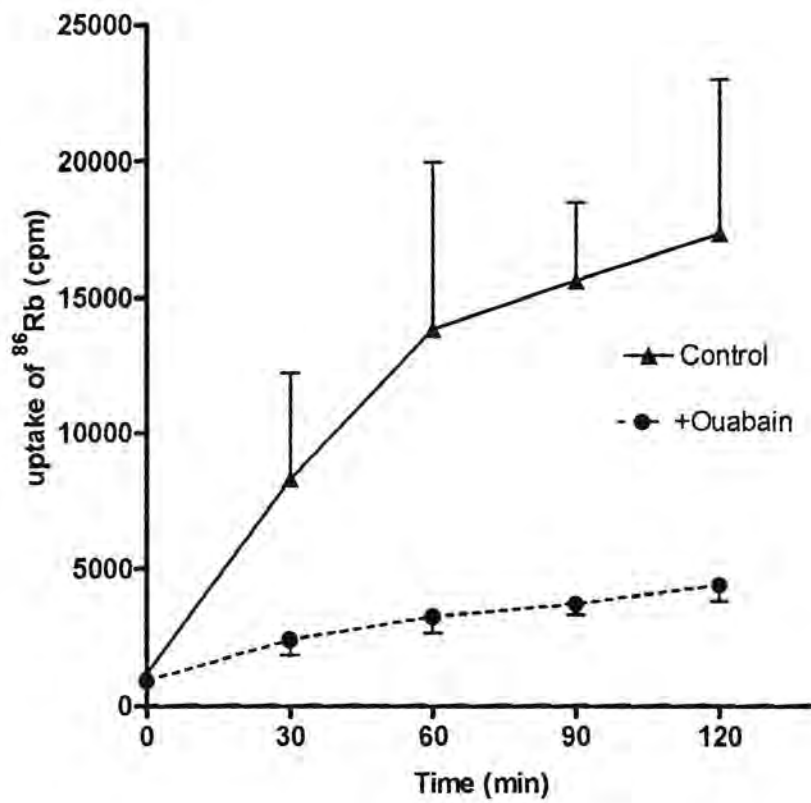
96

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).
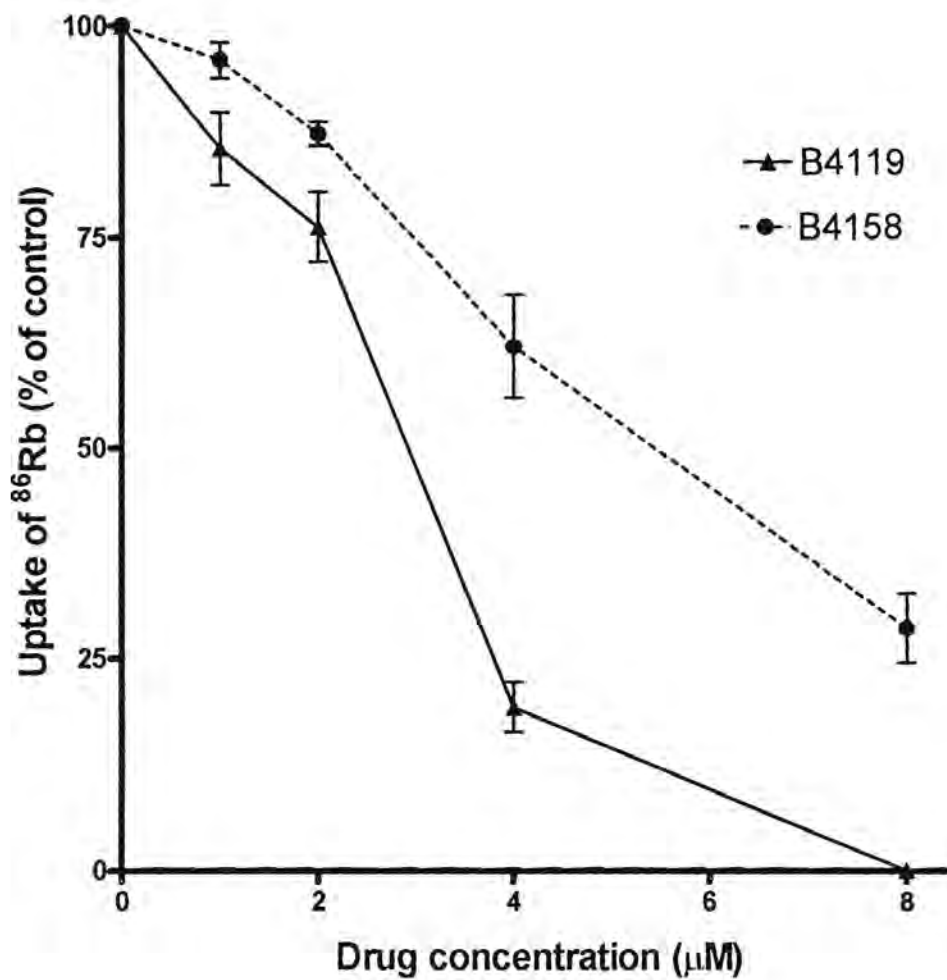
Figure 32 : Effects of B4119 and B4158 on ^{86}Rb uptake by erythrocytes after 45min incubation period. Results from 3 experiments are presented as mean percentage ^{86}Rb uptake of the drug-free control system ± SEMs. The absolute value for uptake of ^{86}Rb by the drug-free control systems was 19627 ± 2145 cpm.
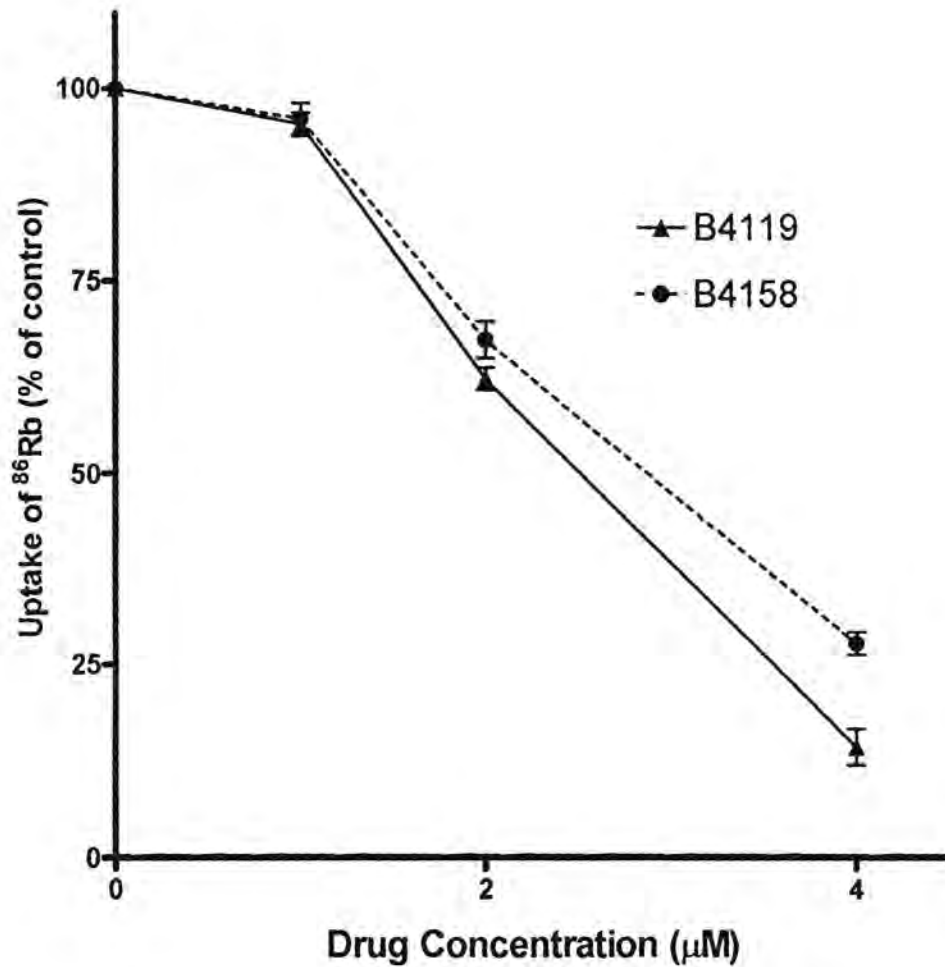
Figure 33 : Effects of B4119 and B4158 on ^{86}Rb uptake by erythrocytes after 48 hours incubation period. Results from 3 experiments are presented as mean percentage ^{86}Rb uptake of the drug-free control system ± SEMs. The absolute value for uptake of 86Rb by drug-free control systems was 21789 ± 1356 cpm.
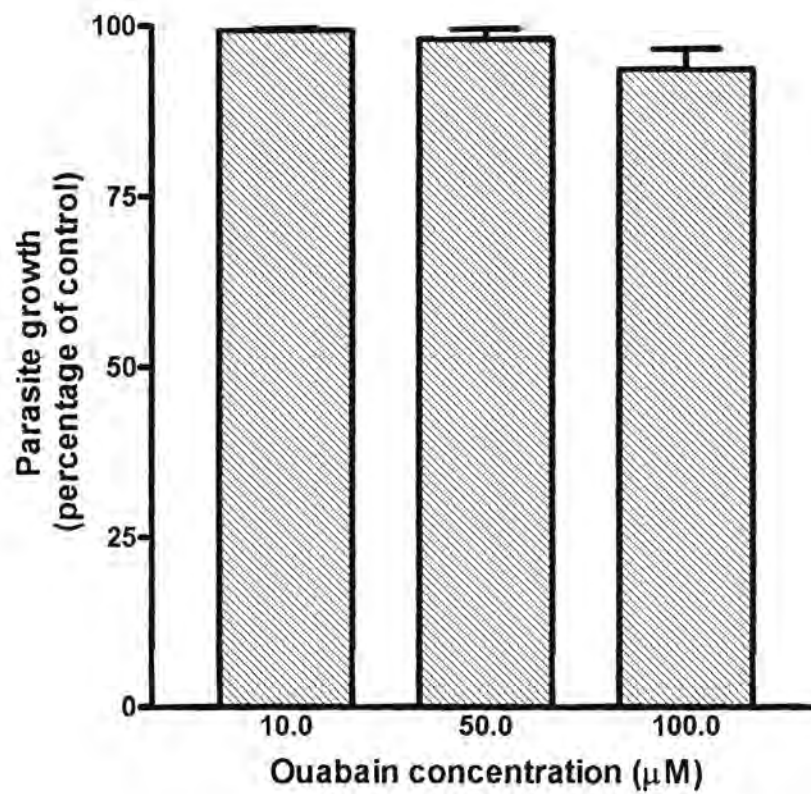
Figure 34 : Effects of ouabain on the growth of the PfUP10 laboratory strain of *Plasmodium falciparum* using microscopy. Results from 2 experiments are presented as the mean percentage of parasite growth of the ouabain-free control system ± SEMs.

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 ($8\mu M$), 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 $2\mu M$ 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 $2\mu M$ 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 2×10^6 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

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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).
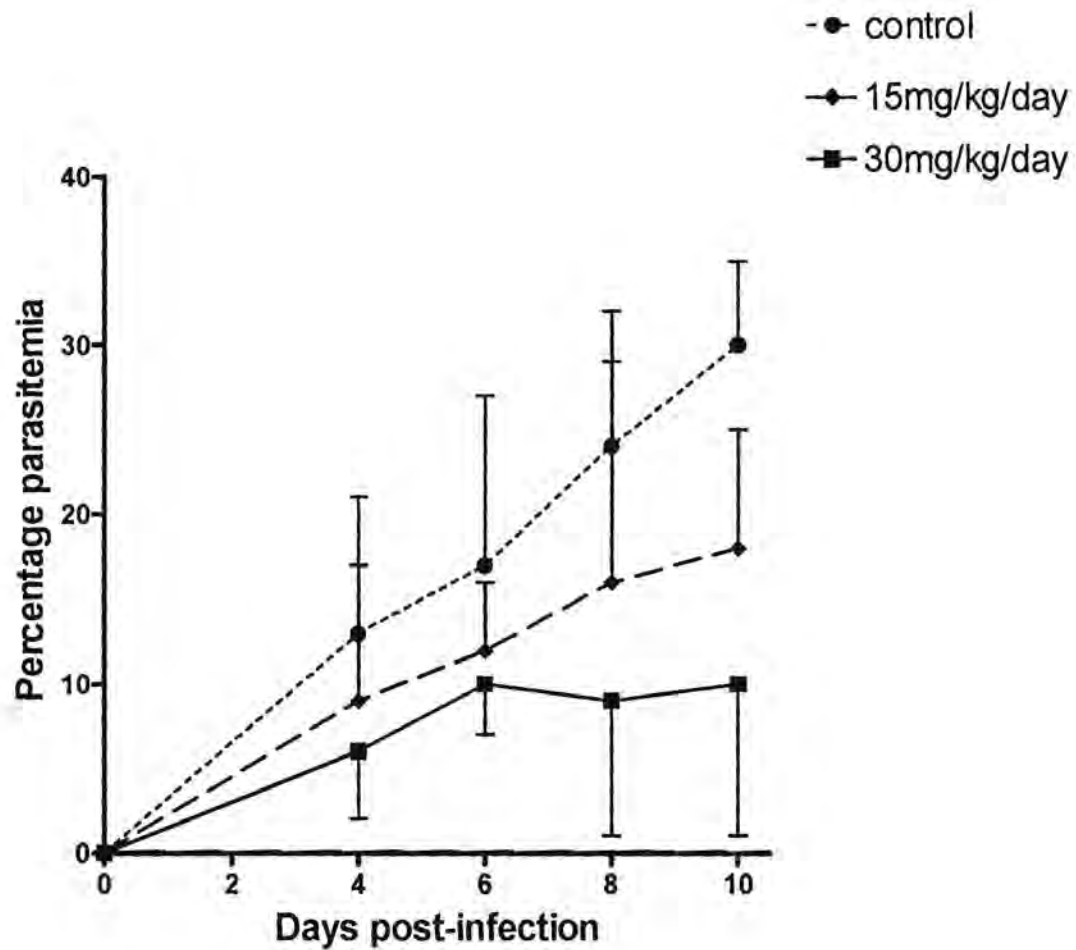

Figure 35 : Effects of oral administration of B4119 (15 and 30mg/kg/day) on parasite growth in *P. berghei*-infected Balb/C mice.
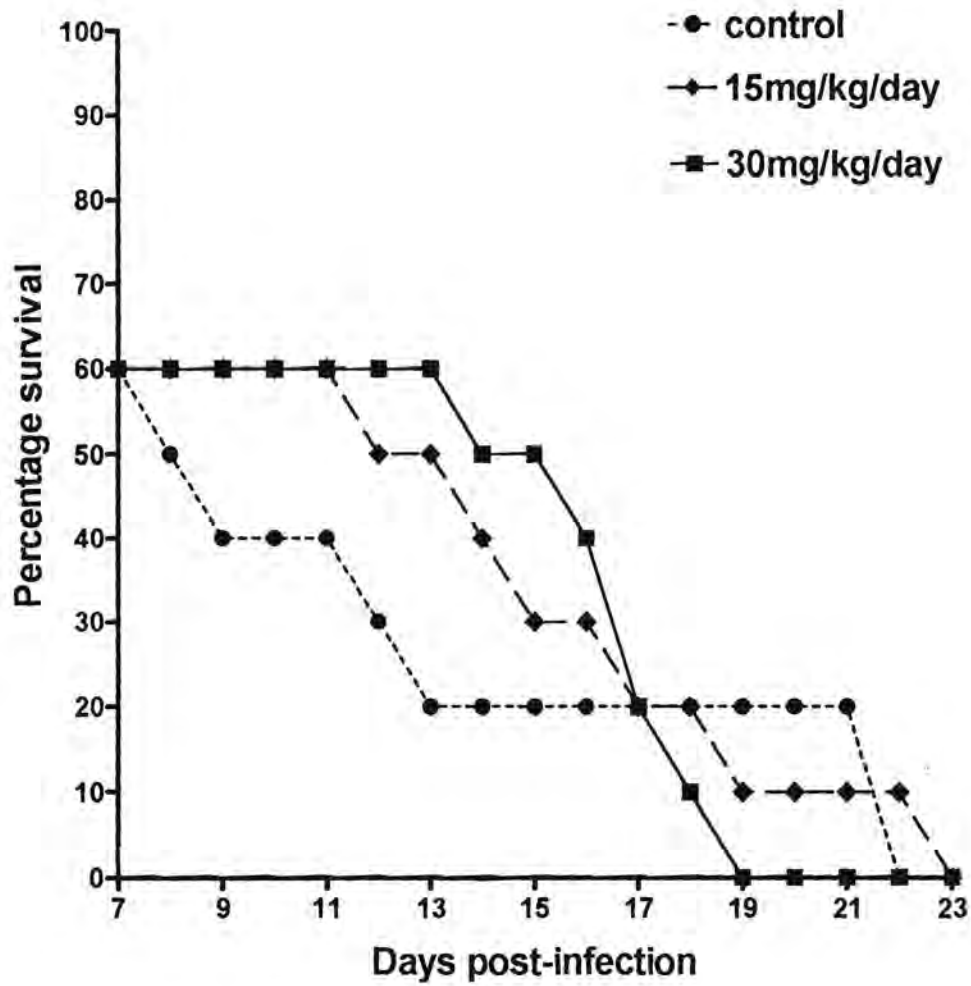
Figure 36: Effects of oral administration of B4119 (15 and 30mg/kg/day) on the survival rate of mice infected with *P. berghei*.
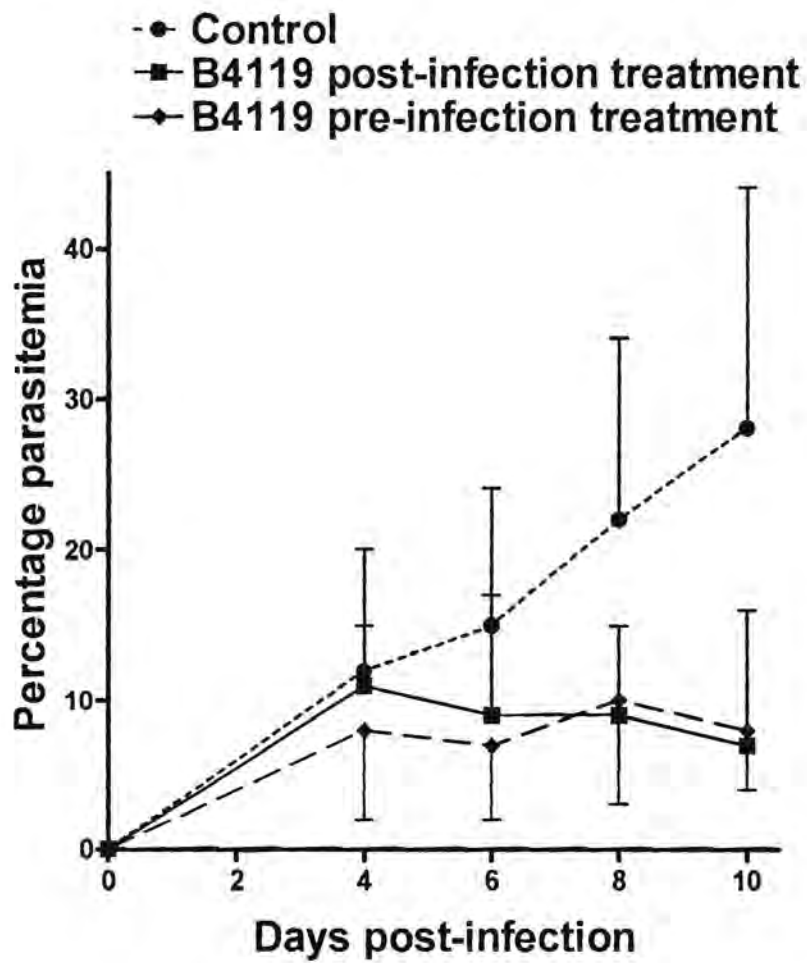
Figure 37 : Effects of the pre- and post-infection treatment compared to postinfection treatment only with 30mg/kg/day B4119 on growth of *P. berghei* parasites.
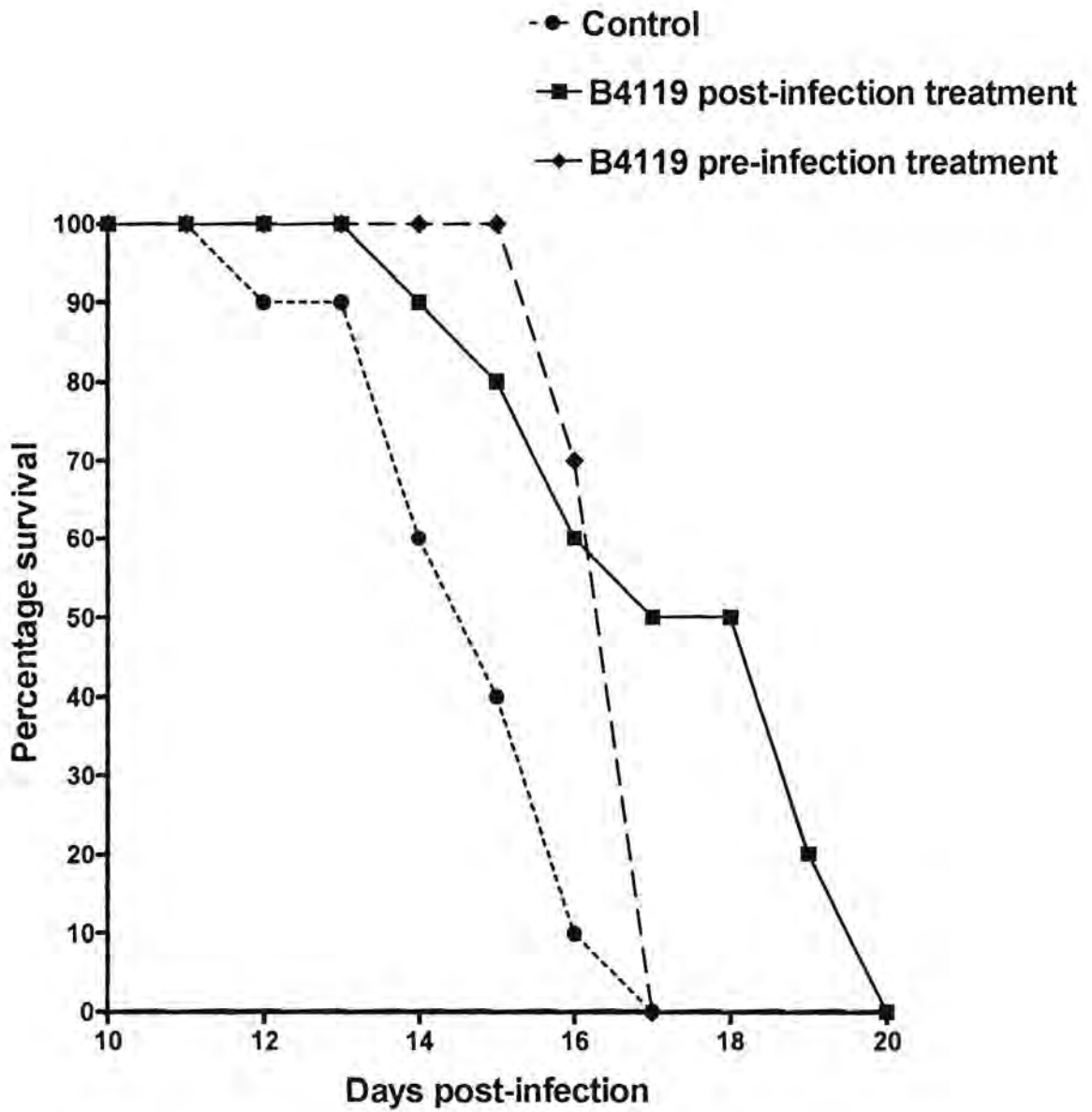
Figure 38 : Effects of pre- and post-infection treatment compared to postinfection treatment only with 30mg/kg/day of B4119 on survival rate of *P.berghei*-infected Balb/C mice.
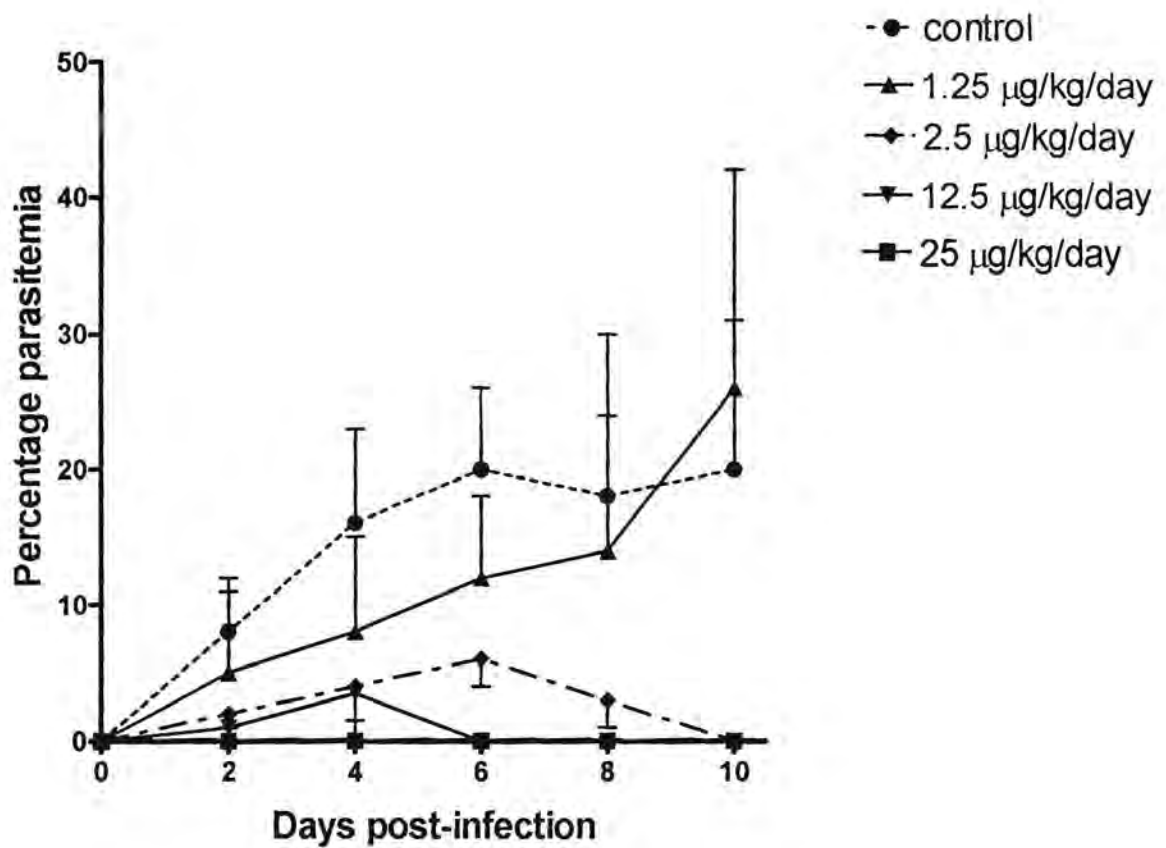
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Figure 39 : Effects of intra peritoneal administration of chloroquine (1.25 - 25µg/kg/day) on parasite growth in *P.berghei*-infected Balb/C mice.
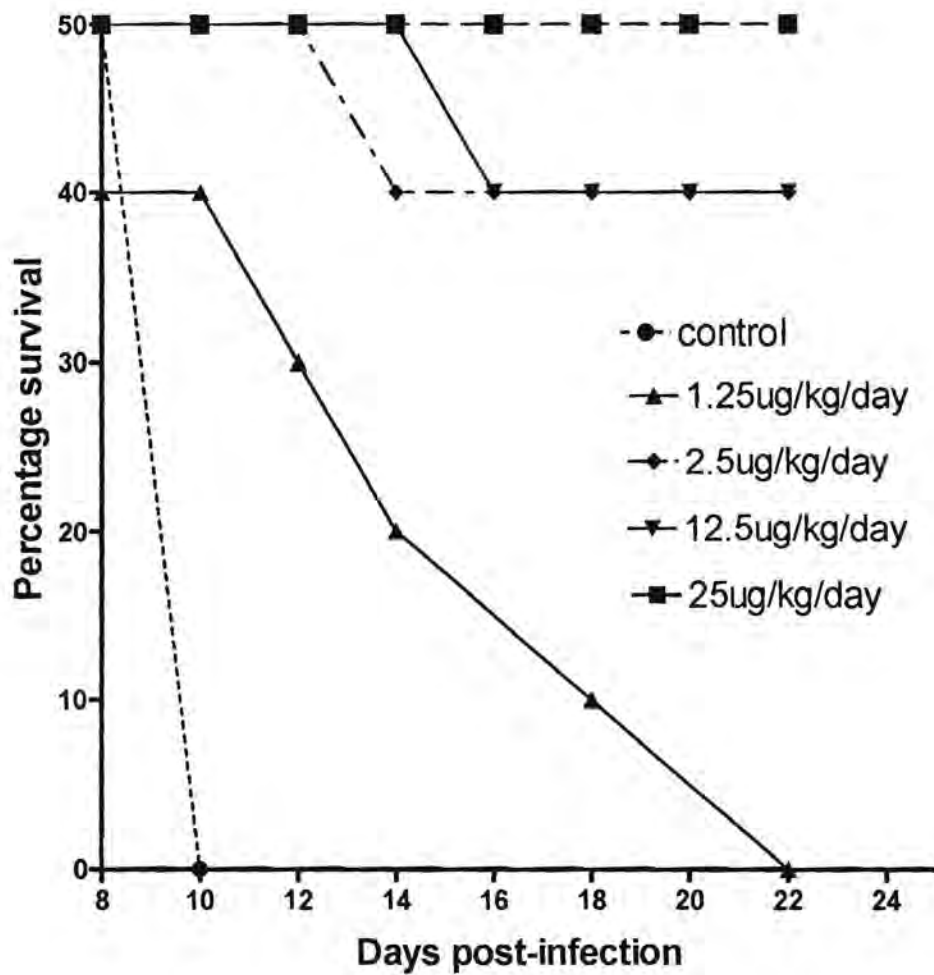
Figure 40 : Effects of chloroquine (1.25 - 25µg/kg/day) on the survival rate of Balb/C mice after infection with *P. berghei* parasites.
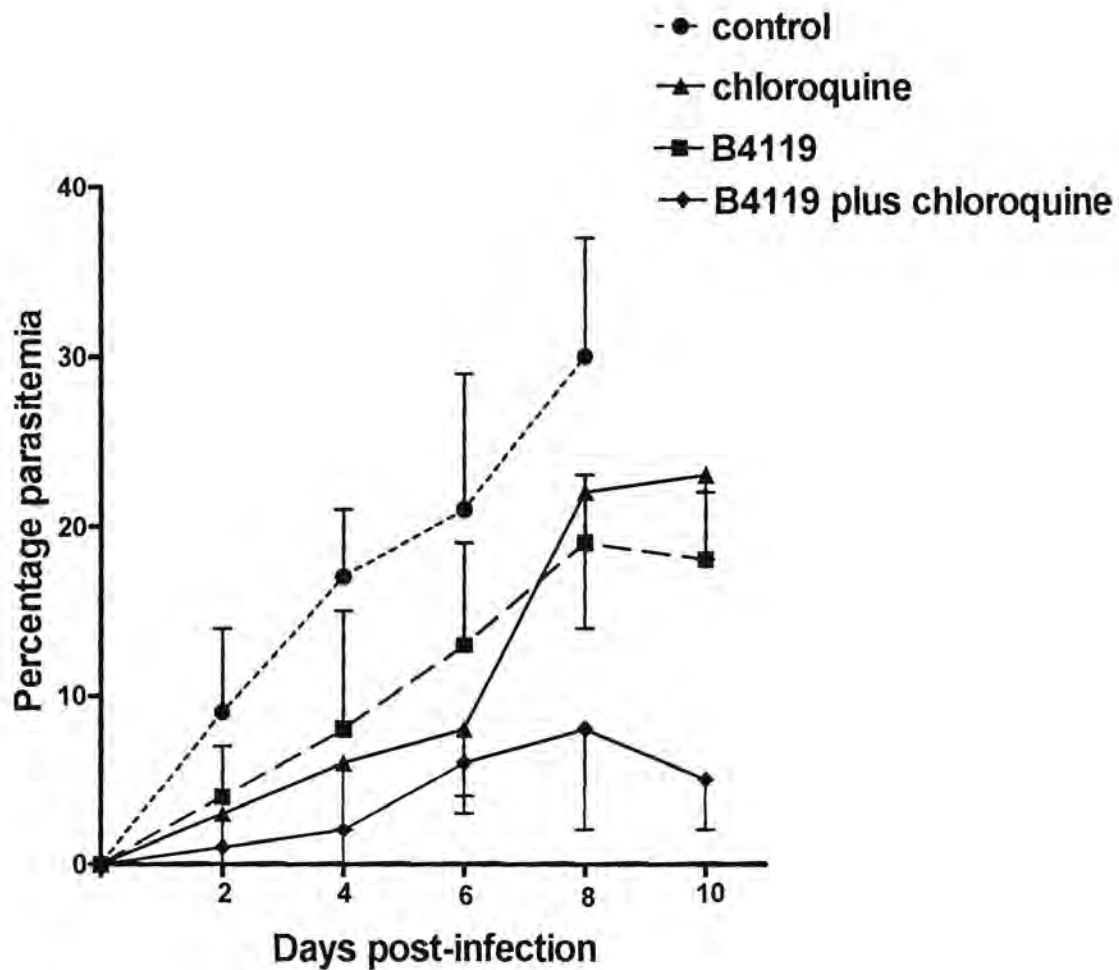
Figure 41 : Effects of chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) individually and in combination on parasite growth of *P.berghei*-infected Balb/C mice.
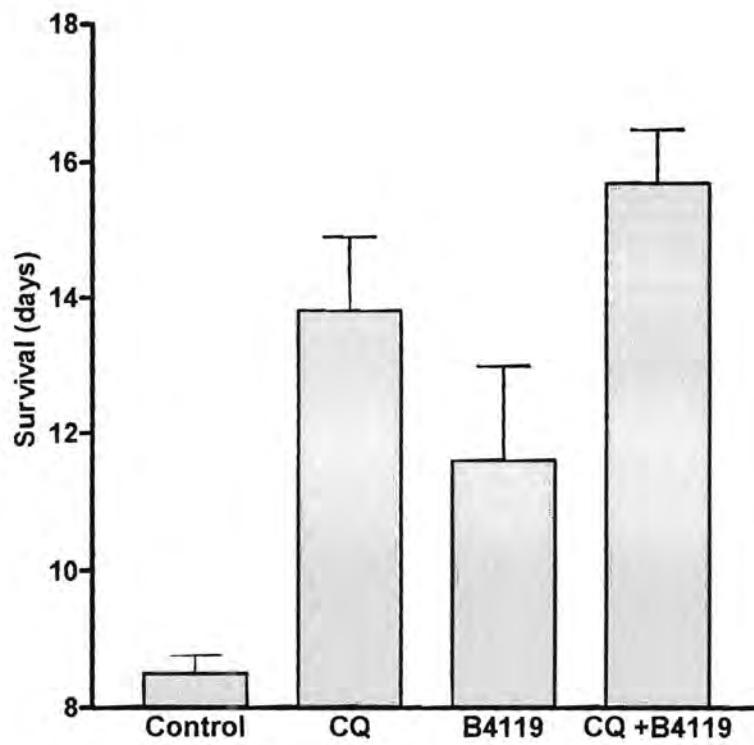
Figure 42: Survival rate of *P.berghei*-infected mice treated with chloroquine (1.25µg/kg/day) and B4119 (30mg/kg/day) singly and in combination.

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.