THE ANTIPLASMODIAL ACTIVITIES OF THE TETRAMETHYLPIPERIDYL-SUBSTITUTED PHENAZINES, B4119 AND B4158

by

Marema Ephraim Makgatho
B.Sc (Medical Science) (University of the North)
M.Sc (Medical Immunology) (University of Pretoria)

Submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy (Medical Immunology)

In

The Faculty of Medicine
Department of Immunology
University of Pretoria
Pretoria

September 1999
To the memory of my beloved mother

NGWANABOTLOU MONICA CHUENE
ACKNOWLEDGEMENTS

Thanks to the following people for contributing to the success of my studies:

My supervisor and promoter, Prof. Connie Medlen, for constant support, encouragement and humour.

Prof. Ronald Anderson for his contribution and advise during the course of my study.

South African Medical Research Council (MRC) for financial support.

Dr. J.F O'Sullivan, Department of Chemistry, University College Dublin, Republic of Ireland, for supplying pure substances of phenazine compounds.

Mrs Martie Madgwick for her time spent over the final typing of this manuscript.

My colleagues for constant motivation and support.

Finally, to my family; Mapitso, Monica, Lucy, Maphela and Makgati for their emotional and spiritual support.
# TABLE OF CONTENTS

## CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1. **THE MALARIA PARASITE**
   1.1 Life cycle
   1.2 Morphology and growth of blood stages
   1.3 Host response to infection

2. **ERYTHROCYTE MEMBRANE STRUCTURE AND FUNCTION**
   2.1 Lipid bilayer
   2.2 Transmembrane molecules
   2.3 Membrane skeleton

3. **THE ERYTHROCYTE AND MALARIA PARASITE INVASION**

4. **THE MALARIA-INFECTED ERYTHROCYTE**
   4.1 Membrane modifications
   4.2 Transport and metabolic properties

5. **TREATMENT AND PREVENTION OF MALARIA INFECTIONS**
   5.1 Antimalarial drugs
   5.2 Mechanisms of drug action
   5.3 Treatment criteria of malaria infections
   5.4 Pharmacokinetic and pharmacodynamic properties of antimalarial drugs
   5.5 Drug resistance
      5.5.1 Development and spread of drug resistance
      5.5.2 Mechanisms of drug resistance
      5.5.3 Overcoming drug resistance

6. **RIMINOPHENAZINES**
   6.1 Classification and pharmacology
   6.2 Clinical applications
      6.2.1 As anti-inflammatory and pro-oxidative agents
      6.2.2 As anti-tumour agents
      6.2.3 As antimicrobial agents
1.7 AIMS AND OBJECTIVES OF THE STUDY

CHAPTER 2
EVALUATION OF PARASITEMIA IN MALARIA CULTURES

2.1 INTRODUCTION
2.2 AIMS AND OBJECTIVES
2.3 MATERIALS AND METHODS
2.3.1 Media and reagents
2.3.2 Parasite culture maintenance
2.3.3 Synchronization of parasite cultures
2.3.4 Comparison of methods used to determine parasitemia in malaria cultures
2.3.4.1 Exposure of parasite cultures to chloroquine
2.3.4.2 Microscopy
2.3.4.3 Radiometry
2.3.4.4 Flow cytometry
2.3.5 Expression and statistical analysis of results
2.4 RESULTS
2.5 DISCUSSION

CHAPTER 3
IN VITRO ANTIMALARIAL ACTIVITIES OF TMP-SUBSTITUTED PHENAZINES, B4119 AND B4158

3.1 INTRODUCTION
3.2 AIMS AND OBJECTIVES
3.3 MATERIALS AND METHODS
3.3.1 Media and reagents
3.3.2 Parasite laboratory isolates
3.3.3 Preparation of drugs
3.3.4 Experimental procedures
3.3.4.1 Direct anti-plasmodial activity of B4119 and B4158 in vitro
3.3.4.2 Chloroquine- and mefloquine-sensitizing activities of B4119 and B4158
3.3.4.3 Stage-dependent effects of B4119 and B4158 on the growth of P. falciparum in vitro
3.3.4.4 Invasion assay of drug-treated erythrocytes
3.3.5 Expression and statistical analysis of results
3.4 RESULTS
3.5 DISCUSSION

CHAPTER 4
HEME POLYMERIZATION INHIBITORY ACTIVITY (HPIA) OF B4119 AND B4158: AN INFRARED SPECTROMETRIC STUDY
4.1 INTRODUCTION
4.2 AIMS AND OBJECTIVES
4.3 MATERIALS AND METHODS
4.3.1 Media and reagents
4.3.2 Experimental procedures
4.4 RESULTS
4.5 DISCUSSION

CHAPTER 5
CYTOTOXIC ACTIVITY OF B4119 AND B4158 AGAINST NORMAL HUMAN ERYTHROCYTES
5.1 AIMS AND OBJECTIVES
5.2 MATERIALS AND METHODS
5.2.1 Media and reagents
5.2.2 Experimental procedures
5.2.2.1 Preparation of leukocyte-depleted human erythrocytes
5.2.2.2 Drug-mediated haemolysis
5.2.2.3 Rubidium-86 uptake by erythrocytes
5.2.2.4 Erythrocyte metabolic activity

5.2.2.5 Direct anti-plasmodial activity of ouabain in vitro

5.3 RESULTS

5.4 DISCUSSION

CHAPTER 6

PLASMODIUM BERGHEI MOUSE MODEL: ANTIPLASMODIAL ACTIVITY OF B4119

6.1 INTRODUCTION

6.2 AIMS AND OBJECTIVES

6.3 MATERIALS AND METHODS

6.3.1 Media and reagents

6.3.2 Mouse parasite culture maintenance

6.3.3 Drug studies

6.4 RESULTS

6.5 DISCUSSION

CHAPTER 7

CONCLUDING DISCUSSION

REFERENCES
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 IC₅₀ 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 ingestuít wat dit moontlik gemaak het om die monsters op 'n latere geleentheid te evalueer. Die TMP-gesubstitueerde 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 om 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 eksperimentele infeksie met P. berghei bepaal.

B4119 en B4158, maar nie klofasimien, het die groei van die geneesmiddel-sensitiewe stam van P. falciparum by IK₅₀ 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 meflokiëen gebruik is. Die verbinding was in staat om alle bloed-stadiums van P. falciparum te inhibeer vooraf behandeling van eritrosiete met B4119 en B4158 het nie die inring van merozietel 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-geïnfecteerde muise het tot 'n betekenisvolle verminderind in parasitemie geleit, 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
CO₂: 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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRC</td>
<td>Medical Research Council</td>
</tr>
<tr>
<td>MSP-1</td>
<td>Merozoite surface protein-1</td>
</tr>
<tr>
<td>Na</td>
<td>Sodium</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NAD</td>
<td>Nicotinamide dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate (reduced)</td>
</tr>
<tr>
<td>NHE</td>
<td>Na+/H+ exchanger</td>
</tr>
<tr>
<td>NMRP</td>
<td>National Malaria Research Programme</td>
</tr>
<tr>
<td>NPPB</td>
<td>5-nitro-2-(3-phenylpropylamino) benzoic acid</td>
</tr>
<tr>
<td>NRA</td>
<td>Nucleoside releasing agent</td>
</tr>
<tr>
<td>O₂</td>
<td>Oxygen</td>
</tr>
<tr>
<td>PABA</td>
<td>Para-aminobenzoic acid</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCT</td>
<td>Parasite clearance time</td>
</tr>
<tr>
<td>PFEMP-1</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein-1</td>
</tr>
<tr>
<td>PFEMP-2</td>
<td><em>Plasmodium falciparum</em> erythrocyte membrane protein-2</td>
</tr>
<tr>
<td>PFHRP-1</td>
<td><em>Plasmodium falciparum</em> histidine rich protein-1</td>
</tr>
<tr>
<td>PG</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGE₂</td>
<td>Prostaglandin E₂</td>
</tr>
<tr>
<td>Pgh₁</td>
<td>P-glycoprotein homologue-1</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLA₂</td>
<td>Phospholipase A₂</td>
</tr>
<tr>
<td>PRR</td>
<td>Parasite reduction ratio</td>
</tr>
<tr>
<td>PSD</td>
<td>Sulfadoxine/pyrimethamine</td>
</tr>
<tr>
<td>PVM</td>
<td>Parasitophorous vacuolar membrane</td>
</tr>
<tr>
<td>Rb</td>
<td>Rubidium</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>RESA</td>
<td>Ring-infected erythrocyte surface antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic acid</td>
</tr>
<tr>
<td>TMP</td>
<td>Tetramethylpiperidine</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
</tbody>
</table>
VCAM-1
WHO

Vascular adhesion molecule-1
World Health Organization
LIST OF FIGURES

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

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

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

Figure 4: Schematic presentation of the invasion of erythrocytes by a merozoite.

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

Figure 6: Schematic presentation of permeation pathways in the host membrane of infected and normal red blood cells.

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

Figure 8: Geographical distribution of chloroquine resistance.

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

Figure 10: Molecular structures of clofazimine and the TMP-substituted phenazines.

Figure 11: Evaluation of parasitemia in malaria parasite cultures using flow cytometric, radiometric and microscopic methods.

Figure 12: Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the PfUP10 (chloroquine-sensitive) strain of *Plasmodium falciparum* using flow cytometry.
Figure 13: Direct antimalarial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA17 (Chloroquine-sensitive and quinine-resistant) strain of *Plasmodium falciparum* using microscopy.

Figure 14: Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA16 (quinine-resistant) strain of *Plasmodium falciparum* using microscopy.

Figure 15: Direct anti-plasmodial activity of the TMP-substituted phenazines, B4119 and B4158, against the RSA9 (sulfadoxine/pyrimethamine-resistant) strain of *Plasmodium falciparum* using microscopy.

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.

Figure 17: Effects of B4119 (0.4μM) 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.

Figure 18: Effects of B4158 (0.8μM) and mefloquine (Mef) (0.010 - 0.065μM) individually and in combination on the growth of the PfUP10 strain of *Plasmodium falciparum* using flow cytometry.

Figure 19: Effects of B4158 (0.8μM) and chloroquine (CQ) (0.044 - 0.50μM) individually and in combination on the growth of the PfUP10 strain of *Plasmodium falciparum* using flow cytometry.

Figure 20: Stage-specific antimalarial activity of B4119 for the first (rings to trophozoites) and last (trophozoites/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry.
Figure 21: Stage-specific antimalarial activity of B4119 for the first (rings to trophozoites) and last (trophozoites/schizonts to rings) 24 hours of the parasite life cycle using flow cytometry.

Figure 22: Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4119 pre-treated red cells using microscopy.

Figure 23: Invasion (schizonts to rings) and growth (rings to trophozoites) of *Plasmodium falciparum* in B4158 pre-treated red cells using microscopy.

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

Figure 25: Infrared spectra of β-haematin after 30min incubation in 12.9M acetic acid, pH 5, 60°C.

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

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

Figure 28: Infrared spectra of B4119-treated haemin solution after 30min incubation in 12.9M acetic acid, pH 5, 60°C.

Figure 29: Infrared spectra of B4158-treated haemin solution after 30min incubation in 12.9M acetic acid solution, pH 5, 60°C.

Figure 30: Effects of ouabain on $^{86}$Rb uptake by human leukocyte-depleted erythrocytes.

Figure 31: $^{86}$Rb uptake kinetics by erythrocytes with and without ouabain (100μM).
Figure 32: Effects of B4119 and B4158 on $^86$Rb uptake by erythrocytes after 45min incubation period.

Figure 33: Effects of B4119 and B4158 on $^86$Rb uptake by erythrocytes after 48 hours incubation period.

Figure 34: Effects of ouabain on the growth of the PfUP10 laboratory strain of Plasmodium falciparum using microscopy.

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 P. berghei-infected Balb/C mice.

Figure 37: Effects of pre- and post-infection treatment with B4119 (15 and 30mg/kg/day) on parasite growth of P. berghei-infected Balb/C mice.

Figure 38: Effects of pre- and post-infection treatment with B4119 (15 and 30mg/kg/day) on the 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 intra peritoneal administration of chloroquine (1.25 - 25μg/kg/day) on the survival rate of P. berghei-infected Balb/C mice.

Figure 41: Effects of intra peritoneal administration of chloroquine (0.25μg/kg/day) and oral administration of B4119 (30mg/kg/day) singly and in combination with chloroquine on parasite growth in P. berghei-infected Balb/C mice.
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.
LIST OF TABLES

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

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

Table 3: Effects of exposure of human erythrocytes to B4119 and B4158 for 48 hours on intracellular lactate levels.

Table 4: Effects of exposure of human erythrocytes to B4119 and B4158 for 48 hours on intracellular ATP levels.