

**FLOW CYTOMETRIC EVALUATION OF
RIMINOPHENAZINES
AS ANTIMALARIAL AGENTS**

BY

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Dedicated to the memory of

RACHEL MMAPITSO MADUPO

.....with love.....

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INDEX

| | Page |
|---|--------|
| SUMMARY | i |
| SAMEVATTING | ii |
| LIST OF ABBREVIATIONS | iii |
| LIST OF FIGURES | iv |
| LIST OF TABLES | vi |
| CHAPTER 1 | |
| LITERATURE REVIEW | 1 |
| 1. THE MALARIA PARASITE | 2 |
| 1.1 CAUSATIVE AGENTS AND DIAGNOSIS | 2 |
| 1.2 DISTRIBUTION OF MALARIA AREAS | 4 |
| 1.3 CLINICAL FEATURES | 6 |
| 1.4 IMMUNOLOGY OF MALARIA | 6 |
| 1.5 TREATMENT AND PREVENTION OF MALARIA | 7 |
| 1.5.1 <i>Antimalarial agents</i> | 7 |
| 1.5.2 <i>Mechanisms of drug action</i> | 8 |
| 1.5.3 <i>Treatment criteria of malaria infections</i> | 10 |
| 1.6 DRUG RESISTANCE | 10 |
| 1.6.1 <i>Development and spread of drug resistance</i> | 11 |
| 1.6.2 <i>Mechanisms of drug resistance</i> | 14 |
| 1.6.3 <i>Overcoming drug resistance</i> | 16 |
| 2. RIMINOPHENAZINES | 18 |
| 2.1 CLASSIFICATION AND PHARMACOLOGY | 18 |
| 2.2 BIOLOGICAL ACTIVITIES AND THERAPEUTIC APPLICATIONS OF RIMINOPHENAZINES | 19 |
| 3. AIMS AND OBJECTIVES OF THE STUDY | 22 |

CHAPTER 2

| | | |
|-----------|--|-----------|
| 2. | EVALUATION OF PARASITEMIA IN MALARIA CULTURES | 24 |
| 2.1 | INTRODUCTION | 25 |
| 2.2 | OBJECTIVES | 26 |
| 2.3 | MATERIALS AND METHODS | 26 |
| 2.3.1 | <i>Media and reagents</i> | 26 |
| 2.3.2 | <i>Parasite culture maintenance</i> | 28 |
| 2.3.3 | <i>Synchronization of parasite cultures</i> | 29 |
| 2.3.4 | <i>Comparison of methods used to determine parasitemia of malaria cultures</i> | 30 |
| 2.3.4.1 | <u>Exposure of parasite cultures to chloroquine</u> | 30 |
| 2.3.4.2 | <u>Microscopy</u> | 30 |
| 2.3.4.3 | <u>Radiometry</u> | 30 |
| 2.3.4.4 | <u>Flow cytometry</u> | 30 |
| 2.3.5 | <i>Expression and statistical analysis of results</i> | 31 |
| 2.4 | RESULTS | 31 |
| 2.5 | DISCUSSION | 34 |

CHAPTER 3

| | | |
|-----------|--|-----------|
| 3. | IN VITRO ANTIMALARIAL ACTIVITY OF RIMINOPHENAZINES | 35 |
| 3.1 | INTRODUCTION | 36 |
| 3.2 | OBJECTIVES | 36 |
| 3.3 | MATERIALS AND METHODS | 36 |
| 3.3.1 | <i>Media and reagents</i> | 36 |
| 3.3.2 | <i>Parasite cultures</i> | 36 |
| 3.3.3 | <i>Preparation of riminophenazines</i> | 36 |
| 3.3.4 | <i>Flow cytometry</i> | 36 |
| 3.3.5 | <i>Expression and statistical analysis of results</i> | 37 |
| 3.4 | RESULTS | 37 |
| 3.4.a | Direct antimalarial activity of riminophenazine compounds | 37 |
| 3.4.b | Chloroquine-sensitizing activities of riminophenazine agents | 38 |
| 3.5 | DISCUSSION | 45 |

CHAPTER 4

| | | |
|-----------|--|-----------|
| 4. | RIMINOPHENAZINE-MEDIATED MODULATION OF GENERATION OF SUPEROXIDE ANION BY HUMAN NEUTROPHILS | 47 |
| 4.1 | INTRODUCTION | 48 |
| 4.2 | OBJECTIVES | 49 |
| 4.3 | MATERIALS AND METHODS | 49 |
| 4.3.1 | <i>Media and reagents</i> | 49 |
| 4.3.2 | <i>Preparation of neutrophils</i> | 50 |
| 4.3.3 | <i>Measurement of generation of superoxide by neutrophils using the cytochrome C reduction assay</i> | 51 |
| 4.3.4 | <i>Expression and statistical analysis of results</i> | 51 |
| 4.4 | RESULTS | 51 |
| 4.5 | DISCUSSION | 54 |

CHAPTER 5

| | | |
|-----|------------------------------|-----------|
| | CONCLUDING DISCUSSION | 55 |
| 5.1 | CONCLUDING DISCUSSION | 56 |
| | REFERENCES | 59 |

SUMMARY

The *in vitro* antimalarial activity of clofazimine and seven of its analogues, all TMP(tetramethyl-piperidyl group)-derivatives except B669, against the RB-1 and *pfUP-1* laboratory strains of *Plasmodium falciparum* was investigated using a flow cytometric procedure. The flow cytometric method 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 riminophenazine agents were found to exhibit antimalarial action of varying degrees: B669, B4100, B4103, B4112 and B4158 showed the best activity followed by B4121 and B4169. Clofazimine did not exhibit any activity at concentrations up to 2 μ g/ml in this system. Their effective concentrations *in vitro* were comparable to that of standard antimalarial agents such as chloroquine. The agents B4103 and B4112 exhibited additive antimalarial activities when combined with chloroquine. The inclusion of the TMP group and extent of halogenation of six of the riminophenazines tested indicate that it is these structural properties which are the major determinants of the antiplasmodial activity. This is the first study to establish an antiplasmodial activity of riminophenazines and further tests are necessary to establish their antiparasitic mode of action and therapeutic potential in animal models of experimental chemotherapy.

SAMEVATTING

Die *in vitro* antimalaria aktiwiteit van klofasimien en sewe van sy derivate is bepaal met behulp van 'n vloesitometriese prosedure en die gebruik van die laboratoriumstamme van *Plasmodium falciparum* RB-1 en pfUP-1. Die derivate, behalwe klofasimien en B669, bevat almal 'n tetrametielpiperidiel groep (TMP). Die vloesitometriese metode was eerstens met mikroskopie en radiometrie vir effektiwiteit in die meting van die graad van parasitemie in malaria kulture vergelyk. Die vloesitometriese metode het baie goed vergelyk, soos aangedui deur die Bland en Altman bepaling van ooreenstemming, met beide die mikroskopiese en radiometriese metodes en was vir hierdie studie gekies op grond van die spoed waarmee monsters gedoen kan word, akkuraatheid en gerieflikheid daarvan (dit sluit 'n fikseringstap in wat dit moontlik maak om enige hoeveelheid op 'n bepaalde tyd te doen). Die riminofenasiene besit verskillende grade van anti-malaria aktiwiteit. B669, B4100, B4103, B4112 en B4158 het die beste aktiwiteit getoon, gevolg deur B4121 en B4169. Klofasimien het geen aktiwiteit by konsentrasies so hoog as 2 $\mu\text{g/ml}$ in hierdie sisteem getoon nie. Die effektiewe konsentrasies van die aktiewe derivate *in vitro* is vergelykbaar met dié van standaard antimalaria middels soos chloroquine. Die middels B4103 en B4112 het verder additief bygedra tot die aktiwiteit van lae chloroquine konsentrasie. Die insluit van die TMP groep en die graad van halogenering van ses van die riminofenasiene wat getoets is, dui daarop dat hierdie molekulêre strukture 'n belangrike bydrae tot die antiplasmodium aktiwiteit kan lewer. Hierdie is die eerste studie wat die antiplasmodium aktiwiteit van riminofenasiene beskryf. Toekomstige toetse is nodig om hulle antiparasitiese meganisme van werking te ondersoek. Die terapeutiese potensiaal van die middels sal ook verder in diermodelle van eksperimentele chemoterapie bepaal moet word.

LIST OF ABBREVIATIONS

| | | |
|-----------------------------------|-------|---|
| A | | Adenine |
| ADCI | | Antibody-dependent cellular cytotoxicity |
| AIDS | | Acquired Immunodeficiency Syndrome |
| ATP | | Adenosine triphosphate |
| CsA | | Cyclosporin A |
| Cyt C | | Cytochrome C |
| DNA | | Deoxyribonucleic acid |
| EDTA | | Ethylenediamine tetra-acetate |
| ELISA | | Enzyme-linked immunosorbent assay |
| FCS | | Fetal calf serum |
| FMLP | | N-formyl-methionyl-leucyl-phenylalanine |
| HBSS | | Hanks balanced salt solution |
| HIV | | Human immunodeficiency virus |
| H₂O₂ | | Hydrogen peroxide |
| Ig | | Immunoglobulin |
| IL-1 | | Interleukin-1 |
| INF | | Interferon |
| iRBC | | Infected red blood cell |
| MDR | | Multidrug resistance |
| MEM | | Minimum Essential Medium |
| NADPH | | Nicotinamide dinucleotide phosphohydrolase |
| O₂ | | Oxygen |
| PBS | | Phosphate buffered saline |
| <i>pfEMP-1</i> | | <i>Plasmodium falciparum</i> erythrocyte membrane protein-1 |
| <i>pfmdr-1</i> | | <i>Plasmodium falciparum</i> multidrug resistance gene-1 |
| PG | | Prostaglandins |
| Pgh-1 | | P-glycoprotein homologue-1 |
| P-gp | | P-glycoprotein |
| PLA₂ | | Phospholipase A ₂ |
| PMA | | Phorbol myristate acetate |

| | | |
|------------|-------|---------------------------------|
| PKC | | Protein kinase C |
| RNA | | Ribonucleic acid |
| RNI | | Reactive nitrogen intermediates |
| ROI | | Reactive oxygen intermediates |
| SDS | | Sodium dodecyl sulphate |
| SOD | | Superoxide dismutase |
| T | | Thymine |
| TMP | | Tetramethyl piperidyl |
| TNF | | Tumour necrosis factor |
| USA | | United States of America |
| WHO | | World Health Organization |

LIST OF FIGURES

| | Page |
|--------------------------|---|
| <u>FIGURE 1</u> : | The life cycle of <i>Plasmodium falciparum</i> . ⁶ 3 |
| <u>FIGURE 2</u> : | Geographical distribution of malarious areas. ⁶ 5 |
| <u>FIGURE 3</u> : | Geographical distribution of chloroquine resistance. ⁴⁶ . . . 13 |
| <u>FIGURE 4</u> : | Molecular structure of riminophenazine compounds used in this study. 23 |
| <u>FIGURE 5</u> : | Evaluation of parasitemia in malaria parasite cultures using flow cytometric, radiometric and microscopic methods . 32 |
| <u>FIGURE 6</u> : | The Bland and Altman measure of agreement between methods used in evaluating parasitemia in malaria parasite cultures. 33 |
| <u>FIGURE 7</u> : | Direct antimalarial activity of riminophenazine compounds against the RB-1 laboratory strain of <i>Plasmodium falciparum</i> 39 |
| <u>FIGURE 8</u> : | Direct antimalarial activity of riminophenazine compounds against the pfUP-1 laboratory strain of <i>Plasmodium falciparum</i> 40 |
| <u>FIGURE 9</u> : | Priming effects of riminophenazines (0.5µg/ml) on the generation of superoxide by human neutrophils (10 ⁶ /ml) in the presence of FMLP (0.1µM). 53 |

LIST OF TABLES

| | Page |
|--|-------------|
| <u>TABLE 1</u> : The IC ₅₀ (50% inhibitory concentration) values of the various riminophenazine molecules for the RB-1 laboratory strain of <i>Plasmodium falciparum</i> | 41 |
| <u>TABLE 2</u> : The antimalarial activity of chloroquine alone and in combination with the riminophenazine agents against the RB-1 laboratory strain of <i>Plasmodium falciparum</i> | 42 |



CHAPTER 1

LITERATURE REVIEW

1. THE MALARIA PARASITE

Malaria, like HIV infection, tuberculosis and malnutrition, still poses a threat to public health in most tropical countries worldwide. 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.^{1,2} More than 80% of the world's malaria casualties occur in Africa, with around a million children under the age of five dying annually.^{3,4}

South Africa does not escape infections by malaria parasites. The South African health authorities noted an upsurge in malaria notifications and clinical cases during the first three months of 1993 and this has prompted public interest and research.⁵

1.1 CAUSATIVE AGENTS AND DIAGNOSIS

The microorganisms causing malaria are of the family Plasmodia. Only four Plasmodium species are known to cause disease in man and they are *Plasmodium falciparum*, *P.vivax*, *P.ovale* and *P.malariae*. The disease is caused by a parasite-infected Anopheles mosquito which introduces the asexual blood forms of the parasite into the bloodstream of the human host.⁶

Plasmodium falciparum is the most dangerous of the four parasites because it can invade erythrocytes of any age producing overwhelming parasitemias that can lead to death. The other human malaria parasites have no potential to cause mortality because they produce minimal parasitemias and are limited to infect young [*P.vivax*] and [*P.ovale*] or old [*P.malariae*] red blood cells.²

The life cycle of all human malaria species is generally the same. It comprises of an exogenous sexual stage (sporogony) of multiplication in the Anopheles mosquito and an endogenous asexual phase (schizogony) of multiplication in the human host. The life cycle of *Plasmodium falciparum* is depicted overleaf (Figure 1).⁶

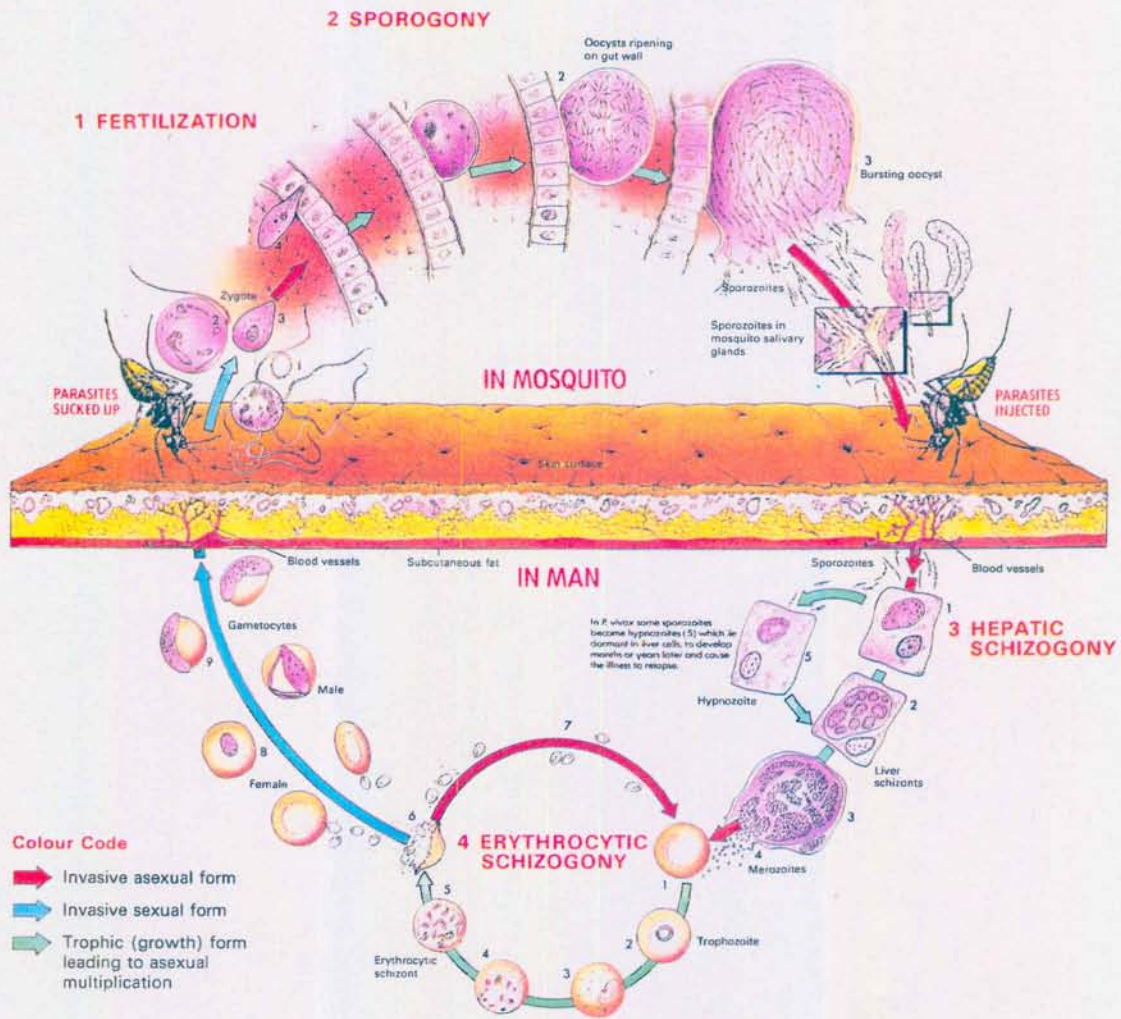


Figure 1 : The life cycle of *Plasmodium falciparum*.⁶

A definite diagnosis of malaria is established by the presence of parasites in the host's red blood cells. There are three main diagnostic procedures for malaria infections⁷ :

1. Microscopic evaluation of Giemsa-stained blood smears.
2. Serological tests including immunofluorescence, immunoprecipitation, haemagglutination, radio immunoassays and enzyme immunoassays such as ELISA (enzyme-linked immunosorbent assay).
3. Molecular biology detection tests that use DNA and RNA probes targeted against parasite-specific nucleotide sequences.

1.2 DISTRIBUTION OF MALARIA AREAS⁶

Cases of infection with malaria are widely distributed throughout the world. Malaria is endemic in tropical areas with rainfall throughout the year and the indigenous people are exposed to continuous and constant transmission of the infection. These endemic areas include tropical Africa e.g Papua New Guinea, central America e.g Venezuela and South-east Asia e.g Thailand.

Drier tropical areas with sporadic rainfalls encounter malaria transmissions during wet seasons. Epidemics vary yearly, being either high or rare. Such epidemics can be found in South Africa e.g Mpumalanga. Although the transmission of malaria in Europe has been combatted, there is an increase of clinical cases of "imported malaria" occurring as a consequence of extensive travelling by the population. Various socio-economic and environmental factors predispose the world's populace to malaria infection. The geographical distribution of malarious areas is shown in Figure 2 (overleaf).⁶

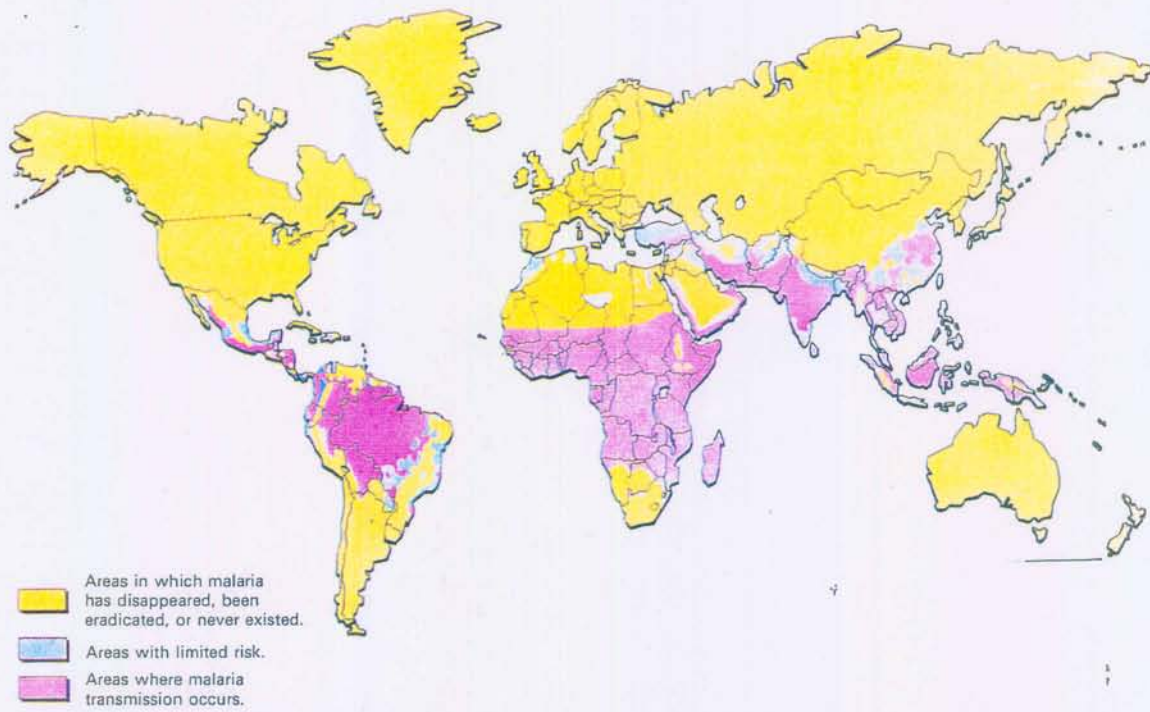


Figure 2 : Geographical distribution of malarious areas.⁶

1.3 CLINICAL FEATURES

The uncomplicated form of the disease is characterised by a febrile illness with headache, muscular discomfort, weakness and malaise as presenting symptoms. As the disease progresses, periodic fever develops and the other organ systems are involved.⁸ The severe form of the malaria, as it occurs in the case of *Plasmodium falciparum* infections, causes a clinical picture associated with severe anaemia, respiratory distress, renal failure, acidosis, hypoglycaemia, disseminated intra vascular coagulation, pulmonary edema and cerebral malaria leading to coma and death.^{9,10}

1.4 IMMUNOLOGY OF MALARIA

Protective immunity to malaria develops after several years of exposure to the parasite-infested Anopheles mosquito. The immunological mechanisms probably require cell-mediated responses and protective antibodies produced against asexual blood forms and soluble antigens that are released transiently during the invasion process.^{11,7}

Humoral immunity plays an important role in the control and elimination of malarial parasites and its effectiveness has been extensively studied in *in vitro* assays, animal models and human malaria infections.¹² The IgG antibody class has been found to protect rodents against *Plasmodium berghei* infections and the antisera blocked the invasion of merozoites into red cells in this model.¹² These protective antibodies were found to be ineffective *in vitro* unless acting in combination with monocyte-macrophages¹³, but the passive transfer of IgG antibodies from immune individuals to severely infected recipients did offer some protection.¹⁴

The research work of Bouharoun-Tayoun and Druhile¹³ described the malarial humoral immunity comprising of (i) cytophilic antibodies; IgG1 and IgG3, binding to monocytes via Fc receptors to promote antibody-dependent cellular immunity (ADCI)¹² and (ii) noncytophilic antibody classes; IgG2, IgM and IgG4, that block the entrance of merozoites into non-infected erythrocytes. There is ample evidence from *in vivo* depletion studies and adoptive transfer of T-cell clones¹⁵ that the CD8⁺-

(cytotoxic/suppressor) T-cells as well $\gamma\delta$ T-cells offer T-cell mediated protection in malaria infections.^{16,17,18} The T-cells elaborate cytokines including, interleukin-1, gamma-interferon and tumour necrosis factor that activate macrophages and neutrophils.^{19,20} This leads secondarily to phagocytosis and destruction of malarial parasites by the activated phagocytes²¹ which secrete a battery of antiplasmodial reactive oxygen intermediates²² and reactive nitrogen intermediates.²³

The *Plasmodium falciparum* parasite has evolved tactics to evade the host's immune attack.²⁴ The most acceptable and plausible mechanism of immune attack evasion documented so far is antigenic variation of the *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1).²⁵ This large protein is synthesised by the parasite and expressed in knob-like structures on the infected erythrocyte membrane and it serves to attach the infected red cell to the vascular endothelium. This property is advantageous because (i) the defective infected red cell can evade splenic destruction and (ii) since surface epitopes are regularly varied, the immune system is rendered ineffective.

1.5 TREATMENT AND PREVENTION OF MALARIA

The success in proper control and prevention of malaria depends on measures taken against both the parasite and the mosquito vector. The rising incidence of the disease is primarily due to the prevalence of insecticide-resistant Anopheles mosquitoes and multidrug-resistant *Plasmodium falciparum*.²⁶⁻²⁹ An array of antimalarial agents has been developed and vaccines directed against the *Plasmodium falciparum* sporozoites are presently undergoing clinical trials for safety, immunogenicity and efficacy in human volunteers.^{27,30,31}

1.5.1 Antimalarial agents

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.^{32,33} Chloroquine remains the chemotherapeutic agent for the treatment of all malaria species worldwide. Chloroquine was developed in the 1940's and its efficiency, safety, stability, low cost and ease of production made

it the most widely used synthetic antimalarial.³⁴

Drug research led to the discovery of other antimalarials which are also now in general use.

These antimalarial agents are divided into six classes^{7,8,35,36}:

1. **Arylaminoalcohols** : quinine, mefloquine, quinidine and halofantrine.
2. **4-aminoquinolines** : chloroquine, amodiaquine, mepacrine and pyronaridine.
3. **Sulfones** such as diaminodiphenyl sulfone and sulfadoxine and **sulphonamides** such as sulfalene and cotrimoxazole.
4. **Biquanides** such as proquanil, **triazine derivatives** such as cycloquanil and chlorcycloquanil and **diamine derivatives** such as pyrimethamine.
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*

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.³⁷ This lack of motivation 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.⁷

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*.^{7,8}

Chloroquine inhibits parasite growth through the following mechanisms :

- * It binds to the parasite's DNA thus inhibiting protein synthesis and causing parasite death.^{34,38}
- * Chloroquine binds to ferriprotoporphyrin IX, a product of infected red blood cells' (iRBC) haemoglobin degradation , forming a complex toxic for the parasite.^{7,32,37}
- * Chloroquine is lysosomotropic i.e it accumulates within the parasite to attain steady state levels that are toxic for the parasite.^{34,39,7}

Pyrimethamine / Sulfadoxine (Fanzidar)

Both drugs are blood schizontides and pyrimethamine is active against all human plasmodium species while sulfadoxine is used in the treatment of falciparum malaria only. Fanzidar acts by interrupting the parasite's folic acid synthesis thus inhibiting parasite nucleoprotein synthesis.⁷

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.⁸

Artemisinin derivatives

These drugs are primarily blood schizonticides that are valuable for treatment of chloroquine- and quinine-resistant malaria in severe, complicated malaria. The agents exert toxic effects on the parasites by concentrating in the parasites food vacuole and inhibiting haem polymerization.⁷

Antibiotics (doxycycline)

These molecules are active in the endo-exoerythrocytic cycle and inhibit parasite growth by interfering with normal ribosomal protein synthesis.⁷

Atovaquone

This experimental agent is active against all growing stages of the malaria parasite and potentiates the activities of tetracycline and proquanil. It is structurally similar to coenzyme Q and disrupts the mitochondrial electron transport chain of the parasite.⁷

1.5.3 Treatment criteria for malaria infections

Plasmodium falciparum has the ability to exhibit increasing resistance to virtually all drugs developed 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 the availability of the drug.

A mild, uncomplicated form of malaria is treated with first-line agents like chloroquine or primaquine. Cases of uncomplicated chloroquine-resistant and severe malaria are curable with alternative antimalarials like mefloquine, quinine, halofantrine and fanzidar. Whenever possible, the artemisinin derivatives should be used in combination with other drugs especially mefloquine.^{38,39,7,40,41} Recommendations for malaria treatment in South Africa have been published.^{42,43,44} Chloroquine is the chemotherapeutic agent 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.

1.6 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 parasite destroyed by increased drug doses tolerated by the host, or complete i.e parasite withstand maximum drug doses tolerated by the host.^{45,46} The global resurgence of malaria is due mainly to the advent of drug-resistant parasites and insecticide-resistant mosquito vectors. Chloroquine resistance has already been reported in other human plasmodial strains. The multi-

drug resistance phenomenon of this parasite aggravates complications encountered when trying to unravel the drug resistance puzzle.^{37,39,47}

Responses of *Plasmodium falciparum* to drugs are graded according to the following categories^{7,8,45}:

1. Sensitivity - clearance of blood parasitemia within seven days of the first day of treatment with no recrudescence.
2. RI resistance - clearance of parasitemia as in sensitivity but with delayed recrudescence.
3. RII resistance - marked reduction of parasitemia without clearance.
4. RIII resistance - persistently high parasitemia.

1.6.1 ***Development and spread of drug resistance***

Drug resistance was first described in Brazil in 1910 after treatment failure in malaria clinical cases with a quinine regimen.⁴⁶ Chloroquine-resistance in *Plasmodium falciparum* emerged in the 1950's in both South America and South-east Asia⁴⁶ and it was first reported in Africa in 1979 from both Kenya and Tanzania.^{48,49} Infections with resistant falciparum malaria are now common in areas where these strains are endemic. In 1980, cases of resistance to chloroquine were observed from the southern African states of Botswana, Mozambique, Angola, Namibia, Swaziland and Zimbabwe. Chloroquine resistance in South Africa was first reported in 1985 from Mpumalanga, Kwazulu/Natal and Northern Province in Venda.⁵⁰⁻⁵⁴

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.^{55,56,57} This resistance pattern has since spread to South America (Brazil) and some African states (Burkina Faso) at alarming rates.⁵⁸ Mefloquine resistance is associated with halofantrine and quinine resistance but not chloroquine.⁵⁹

Multi-drug resistance patterns involving quinine/mefloquine/halofantrine or fanzidar

-12-

have also been detected and this is a serious public health threat in Thailand, Indonesia, Papua New Guinea and Pakistan.^{58,60-63} The geographical distribution of chloroquine resistance is shown in figure 3 overleaf.⁴⁶

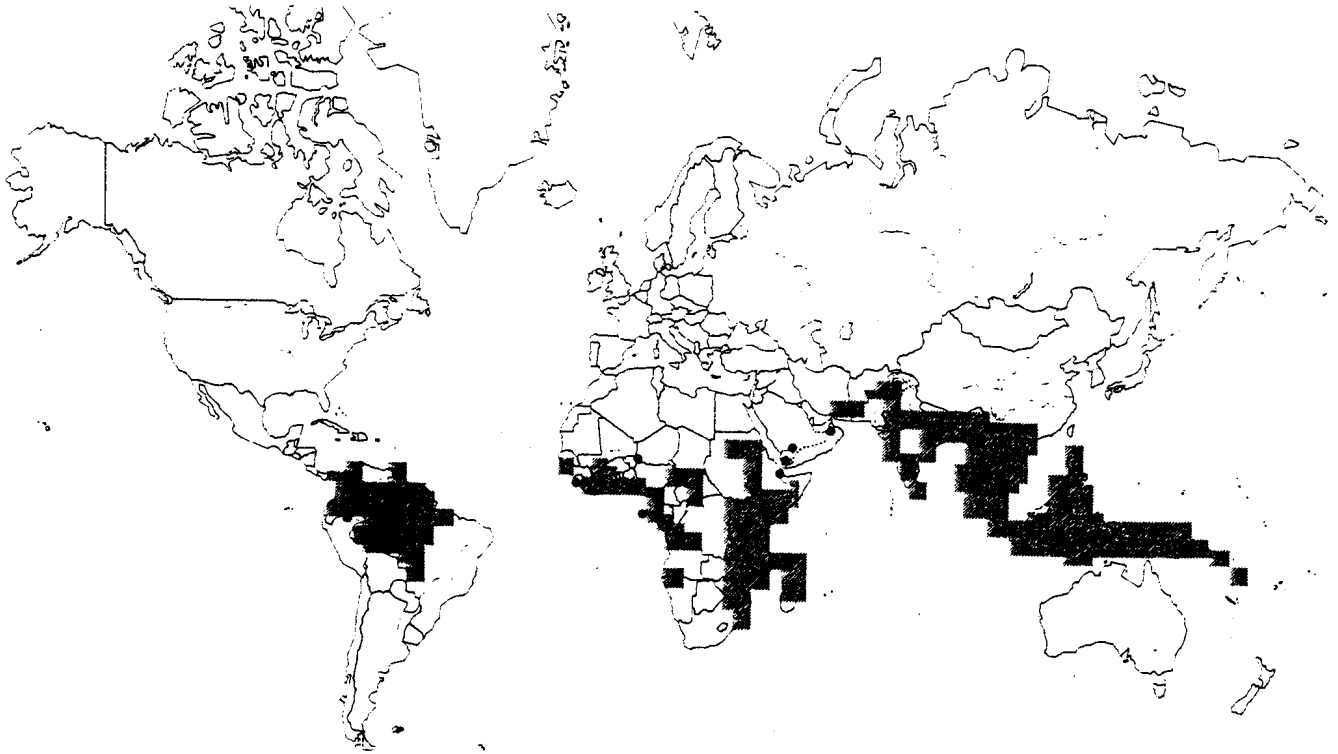


Figure 3 : Geographical distribution of chloroquine resistance.⁴⁶

1.6.2 *Mechanisms of drug resistance*

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.^{64,65}

There are striking similarities between drug resistance in *Plasmodium falciparum* and the MDR phenomenon occurring in mammalian tumour cells.⁶⁶ 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 overexpression of a 170kDa protein molecule, called 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.^{67,68}

Chloroquine, a weak base, acts on the iRBC by moving down a concentration gradient and accumulating in the parasite's food vacuole to lethal concentrations as shown by autoradiographic studies with ³H-chloroquine.³⁷ The accumulated chloroquine reaches steady state levels that are toxic to the parasite and lead to disruption of the parasite's architecture and death. This lysosomotropic property has been proposed as the basic mechanism by which chloroquine inhibits plasmodial growth and development.^{34,69,70}

Chloroquine-resistant *Plasmodium falciparum* strains have enhanced drug effluxing capacity as compared to their chloroquine-sensitive counterparts.⁷¹ Amplification of *Plasmodium falciparum* MDR genes (pfmdr1) and expression of the P-glycoprotein homologue 1 (Pgh1) has been documented.⁷² The localization of Pgh1 molecule on the membrane of the food vacuoles of chloroquine- and mefloquine-resistant isolates of *P. falciparum* supports the proposal that reduced sensitivity to these two antimalarials is due to increased drug effluxing mediated by an ATP-driven P-

glycoprotein pump.⁷³⁻⁷⁵ According to the above hypothesis, if Pgh1 acts as an efflux pump in chloroquine resistance, an increase in the expression of this protein will be expected to show a concomitant increase in levels of chloroquine resistance. This mechanism of chloroquine resistance has however been invalidated through research studies conducted by a group led by Alan Cowman^{73,66} in Australia and another led by Donald Krogstad⁷⁴ in the United States of America (USA).

These two groups discovered that :

(i) The Pgh1 is expressed at the same levels in both chloroquine-resistant and susceptible strains, (ii) the level of chloroquine resistance in strains expressing high amounts of Pgh1 is not greater than in those resistant strains expressing lower levels of Pgh1 and (iii) some chloroquine-resistant-linked nucleotide changes in the *pfmdr* homologues are predictive of the chloroquine resistance status of the parasite. It was suggested, not concluded, from their studies that overexpression of Pgh1 is not related to levels of chloroquine resistance and that the nucleotide sequence alterations of the *pfmdr* gene may be directly or indirectly involved in the mechanisms of drug resistance. The above mechanisms still remain disputed.^{76,77,78}

An alternative mechanism studied to account for reduced chloroquine sensitivity and accumulation in target parasites is that of the reduced force of drug uptake.⁶⁵ The food vacuole of *Plasmodium falciparum* also has on its membrane, an ATPase proton pump that maintains low pH in this vesicles. The acidic pH of the food vacuole is essential for the proper functioning of the parasite's biological machinery and most notably, it creates a steep concentration gradient necessary for uptake of chloroquine.⁷⁹⁻⁸¹ Studies have shown that the pH of food vacuoles from drug-resistant parasites is less acidic than that of drug-sensitive strains. A weakened ATPase proton pump leads to high vacuolar or vesicular pH and decreased chloroquine uptake and accumulation resulting in parasite insensitivity to drugs upon exposure.^{70,71}

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^{70,67}:

- (i) Antitumour agents permeate into target cells more slowly than antimalarial agents.
- (ii) 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 return to normal while this is not the case with malaria parasites.
- (iii) The MDR reversal agents in cancer cells will totally restore drug sensitivity, while drug resistance modulators in malaria parasites lead only to partial restoration.
- (iv) 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 agents.

Overall, a number of mechanisms can account for drug resistance in *Plasmodium falciparum* :

- (i) enhanced efflux of chloroquine from resistant parasites by an ATP-driven P-glycoprotein pump, (ii) weakened ATPase proton pump and (iii) variation or alteration of the drug target site (v) weakened permeability barrier that decreases chloroquine accumulation in food vacuoles and (vi) combination of mechanisms (i) and (ii).

1.6.3 **Overcoming drug resistance**

Scientists have employed a number of strategies to combat chloroquine resistance in falciparum malaria. The options currently under investigation include (i) use of drug-resistant reversing agents, (ii) development of novel antimalarial agents with direct antimalarial activity and that can bypass the parasite's drug resistance mechanisms and (iii) development of vaccines that can enhance the patient's

immunity.

A number of agents that potentiate the activities of existing antimalarials have already been tested.⁸² Calcium channel blockers (e.g verapamil and calcium channel antagonists of various chemical classes),^{83,84} tricyclic antidepressants (e.g desipramine)^{85,86}, tricyclic antihistamines (e.g cyproheptadine)⁸⁷, phenothiazines and calmodulin inhibitors⁸⁸ have been shown to reverse chloroquine resistance in *Plasmodium falciparum in vitro* and *in vivo* in *Aotus* monkeys⁸⁹ 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 protein-binding activities of the agent.^{90,91} On the other hand, clinical studies with verapamil are complicated by the deleterious toxic side effects of this drug in humans.⁸⁸ Rasoanaivo et. al⁹² have also shown the importance of plant products as drug resistance modulators *in vitro*. Most of the first antimalarials were derived from plant products. They have isolated and characterised three new isoquinoline alkaloid dimers from Jumelle plants and named them hervelines A, B and C. These plant derivatives have proved to have direct antimalarial and chloroquine-potentiating activities in chloroquine-resistant *Plasmodium falciparum*. Some exploratory studies have documented that prostaglandin derivatives (PGE1),⁹³ cytochrome-p450 mixed-function oxidase inhibitors (metyrapone)⁹⁴ and iron-chelating agents (desferrioxamine)⁹⁵⁻⁹⁸ also sensitize resistant strains of the falciparum parasites to chloroquine.

Cyclosporin A (CsA), a cyclic undecapeptide, attained clinical use in organ transplantation due to its immunosuppressive properties. It has been shown to exhibit antimalarial activities both in laboratory strains and animal models of malaria but cannot be used to treat clinical malarial infections because of its cost, toxicity and immunosuppressive properties.⁹⁹ Although CsA reverses P-glycoprotein-mediated MDR in tumour cells, this was not the case in chloroquine-resistant *Plasmodium falciparum*.¹⁰⁰

Researchers have also opted for the development of new antimalarials. The WHO previously contributed about 30% of its budget to malaria eradication and treatment programmes, but the funding has declined to around 4% in 1995. In the USA, an average of 231 million dollars and 12 years is necessary to take a chemical from the laboratory to the pharmacy shelf and of every 3000 compounds tested, only one reaches the final test phase. These pharmacoeconomic data demonstrate that developing a new antimalarial agent is a long, expensive and high risk venture for pharmaceutical companies which expect high returns for their investments.¹⁰¹⁻¹⁰³

Wilbur Milhous and colleagues at the Walter Reed Army Institute of Research (home to the largest antimalarial drug-screening programme in the world) in Washington (USA) have so far discovered mefloquine and halofantrine as alternative antimalarials to chloroquine and are currently running clinical trials for the primaquine replacement agent, WR238605, and other agents including WR243251 and WR268668.^{101,104,105} Outside the USA, extensive clinical trials are being conducted by the Burroughs-Wellcome group on atovaquone and several analogues of the artemisinin series, first discovered by the Chinese.^{101,106,107,41}

2. RIMINOPHENAZINES

2.1 CLASSIFICATION AND PHARMACOLOGY

Riminophenazines are derivatives of phenazine compounds that were first discovered by Barry et al.¹⁰⁸ The prototype drug structural formula is a three-ringed phenazine core called anilinoaposafranine. The compounds are further classified according to the various substituent groups occurring in the side chains surrounding the phenazine ring. These substituent groups in the side chain give rise to two more classes of phenazines called aposafranones and imidazophenazines.^{109,110}

Clofazimine or Lamprene (B663) is the prototype riminophenazine agent first synthesized in 1957 as an antituberculosis agent in the laboratories of the Irish Medical Research Council in Dublin¹⁰⁸ and a variety of its analogues have been synthesized to date^{110, 111} all of which are highly lipophilic, generally insoluble in

water, slightly soluble in ethanol and soluble in dioxan, dimethyl sulfoxide and methyl cellosolve.¹¹² Clofazimine concentrates highly in tissues of high fat content, with reticuloendothelial components and high vascularity such as the liver but its levels are hardly detectable in the brain. Clofazimine has a half-life of approximately 70 days and is primarily excreted through bile.¹¹³

The most common side effects associated with the use of clofazimine in the treatment of various disease states are reddish-brown pigmentation of the skin, conjunctivae and urine. These side effects are not serious and usually disappear after cessation of treatment. Serious side effects associated with clofazimine usage have been reported, but they are time- and dose-related, and include drug crystal deposition in the gut and spleen causing abdominal discomfort and splenic infarction respectively.^{110,112,113}

2.2 BIOLOGICAL ACTIVITIES AND THERAPEUTIC APPLICATION OF RIMINO-PHENAZINES

Clofazimine is primarily an antileprosy agent. However a broad spectrum of biological activities have been attributed to it and its analogues. Investigations by Savage et. al¹¹⁴ into the structural properties of clofazimine and its analogues indicate that the potent prooxidative activities of the riminophenazine agents are dependent on the substituent chemical moiety at position 2 of the phenazine nucleus and the chlorination patterns in the para-position of the phenyl- and analino-rings of these agents.

Van Rensburg and colleagues¹¹⁵ have shown that clofazimine and B669, a clofazimine analogue more potent than the parent molecule, exhibit antimicrobial activities against Gram-positive bacteria while Gram-negative bacterial species remain insensitive. Treatment of Gram-positive and Gram-negative bacteria with clofazimine and B669 results in enhanced activity of microbial phospholipase-A₂ (PLA₂), causing generation and accumulation of lysophospholipids that are selectively antimicrobial for Gram-positive organisms and mycobacteria.

Originally it was thought that clofazimine could exert its direct antimicrobial activities by binding to the guanine bases of DNA, that are more abundant in bacteria than in mammalian cells, thus retarding proliferation of bacterial cells.¹¹⁶ However, the relevance of this mechanism has been questioned.¹¹⁵ A recent study by De Bruyn and colleagues (in press)¹¹⁷ has served to establish the possible target for lysophospholipid-mediated antimicrobial activities of riminophenazines to be the K⁺-transport system. Their results also demonstrate that the K⁺-transport systems of Gram-negative bacteria are either resistant or inaccessible to riminophenazines and lysophospholipids.

Clofazimine and B669 also potentiate the antimicrobial activity of human and murine phagocytes by enhancing the activities of lysosomal enzymes and the respiratory burst enzyme, NADPH-oxidase.¹¹⁸ NADPH-oxidase is the oxidant-generating and electron-transporting membrane-bound enzyme of phagocytes that is activated by leucoattractants, cytokines and opsonized particles.¹¹⁹ The production of reactive oxidants is mediated by a variety of signal transduction components of the phagocyte membrane including phospholipase-A₂, -C and -D that act individually or in combination depending on the membrane binding signal. The secondary messengers generated by these transduction mechanisms serve to activate cytosolic protein kinase C (PKC) that, through phosphorylation-dependent mechanisms, initiates the prooxidative activities of NADPH-oxidase.^{118,120,121} These prooxidative interactions of clofazimine and B669 with phagocytes may contribute to the antimicrobial mechanisms of the riminophenazines.

Various riminophenazine agents have also been shown to have anti-inflammatory and immunosuppressive effects on leukocytes. These properties result from the ability of the agents to contribute to the production of immunosuppressive prostaglandins (PG), especially prostaglandin E₂ by polymorphonuclear and mononuclear leucocytes.¹²² Anderson and Smit¹²³ have also shown that clofazimine and B669 inhibit the proliferation and the Na⁺-, K⁺-ATP-ase activity of human lymphocytes by a lysophospholipid-dependent mechanism.

Van Rensburg et. al ^{124,125} have documented that clofazimine and its analogue, B669 inhibit the growth and multiplication of cancer cell lines, including cell lines possessing intrinsic resistance such as the human hepatocellular carcinomas (HepG2, PLC and Mahlavu), human colorectal carcinoma (CaCo2) and human cervix epithelioid carcinoma (HeLa). The antitumour activity of these agents is caused by a unique dual mechanism,^{126, 127} one which involves PLA₂-dependent oxidative and the second, a PLA₂-dependent non-oxidative mechanism. The oxidative pathway involves the production of tumoricidal oxidants by activated phagocytes upon exposure to riminophenazines whereas the non-oxidative pathway is detected in the absence of phagocytes. In the last setting, riminophenazine treated tumour cells attain increased activity of phospholipase-A₂ with subsequent inactivation of lysophospholipid-sensitive Na⁺, K⁺-ATPase, an enzyme which is essential for cellular metabolism and proliferation. It has also been documented that oral administration of clofazimine and B669 delays the development of carcinogen-induced tumours in rats and mice.¹²⁸

In another study it was found that clofazimine and B669 reverse P-glycoprotein-mediated MDR in a small cell lung cancer cell line and are even superior to cyclosporin A (CsA) in sensitizing tumour cells to standard anticancer drugs such as vinblastine and doxorubicin. The MDR neutralizing activity of the riminophenazines is possibly achieved by inhibition of the ATPase activity of P-glycoprotein.¹²⁴

Clofazimine is also useful in the treatment of other non-mycobacterial chronic inflammatory conditions such as vitiligo, generalised pustular psoriasis, pyoderma gangrenosum and discoid lupus erythematosus.^{108,110,111,117} It is also one of the standard drugs used for the treatment of leprosy and since the development of clofazimine-, dapsone- and rifampicin-resistant *M.leprae* several active clofazimine analogues were discovered under the auspices of the WHO.¹¹¹ Clofazimine is also useful in the antimicrobial combination chemotherapy of *Mycobacterium avium* complex infections in AIDS patients. Although it also inhibits the growth of tubercle bacilli *in vitro* as well as in animal models of experimental infection, it has never

been used successfully in the treatment of tuberculosis.¹²⁹

3. AIMS AND OBJECTIVES

Although riminophenazines exhibit antimicrobial and antitumour activities as well as MDR reversal properties, they have never been tested in parasite / malarial systems. As a result of an intensive screening programme on the molecular, structural and functional activities of riminophenazine compounds at the Department of Immunology, University of Pretoria, it has been found that a tetramethylpiperidyl (TMP) group on position 2 of the phenazine nucleus increased the antitumour and antimicrobial activities of these agents (unpublished results).

For this study, the antimalarial activities of six new riminophenazine compounds, all of them with a TMP group (B4100, B4103, B4112, B4121, B4158 and B4169), as well as clofazimine and B669, were evaluated for their antiplasmodial properties as well as for prooxidative interactions with human neutrophils. The structures of compounds used in this study are shown in Figure 4 overleaf.

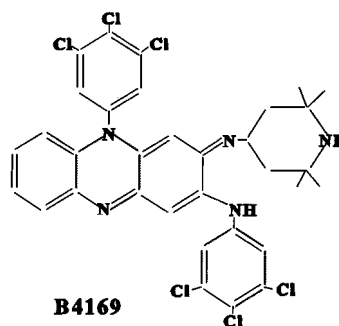
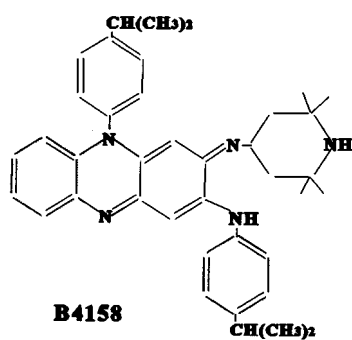
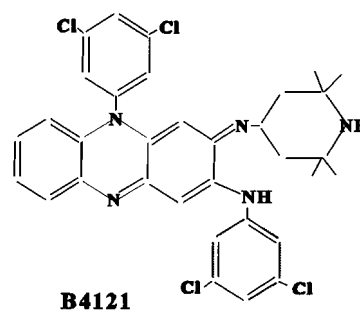
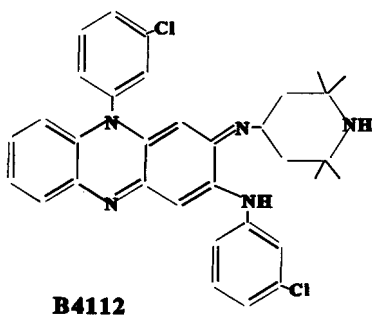
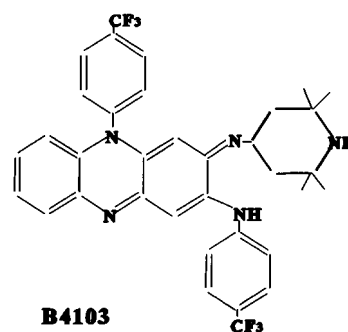
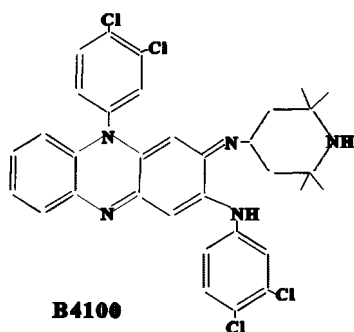
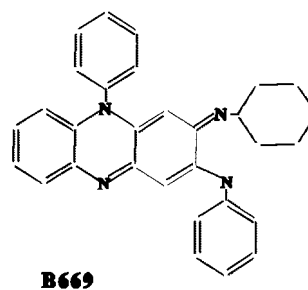
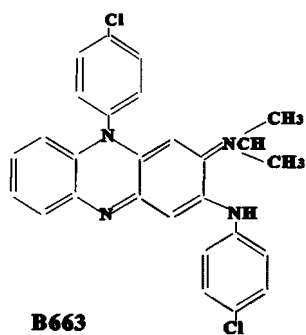


Figure 4 : Molecular structures of rimonophenazine compounds used in this study.

CHAPTER 2

**EVALUATION OF PARASITEMIA IN MALARIA
CULTURES**

2.1 INTRODUCTION

Scientists in pharmaceutical companies, 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 parasite viability of malaria-parasitized erythrocyte cultures include the microscopic evaluation of Giemsa-stained slides and measurement of the amount of radiolabelled nucleotide (hypoxanthine) incorporated into viable parasites i.e radiometry. These methods, although still in use, are time-consuming, insensitive and subject to human error.¹³⁰

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 characteristics of their protein or DNA content, light scattering properties or antigenic makeup.¹³¹ The viability of intraerythrocytic hemoparasites 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 non-viable parasites. Numerous dyes or fluorochromes have been used for assessing viability of parasites such as *Plasmodium falciparum*, *Plasmodium yoelii*, *Plasmodium berghei*, *Babesia bovis* and *Trypanosoma 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 and providing quantitative data.^{130,131}

Initial studies have employed the use of fluorescent dyes like propidium iodide, mithramycin, Feulgen-acriflavine, hydroethidine, acridine orange and 33258 Hoechst 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 effect on cell viability and growth

and (iii) require prompt sample analysis.^{130, 132}

Hoechst 33258, a *bis*-benzimidazole dye, binds non-intercalatively to A-T-rich DNA sequences that are abundant in the plasmodium genome and can be used to quantitate parasitemia in fixed malaria cultures, but conflicting results are still obtained due to the brief incubation or storage period of fixed cultures and concentration of the dye used. The above drawback is also observed when using acridine orange.¹³²⁻¹³⁴

In 1987 Makler and Lee¹³⁵ 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, (iii) the results obtained from the flow cytometric procedure correlate well with both microscopy and radiometry and (iv) flow cytometry allows parasite counting to be performed with speed, precision and sensitivity, and consequently can serve as a valuable tool for mass screening of potential antimalarial agents.

2.2 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 the Sigma chemical corporation (St.Louis, USA).

PARASITE CULTURE MEDIUM

RPMI-1640 complete medium [Highveld Biological Products, Kelvin, S.A.]; 500ml



-27-

Supplemented with :

22mg Hypoxanthine / 8ml distilled water

5mg/ml Gentamicin

150mg/ml Glutamine

50ml blood A+ human serum

WASH MEDIUM

Parasite culture medium without A+ human serum

CRYOPRESERVATION OR FREEZING 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 Hemagglutination buffer [BBL Microbiology Systems, Cockeysville, MD]

1000ml distilled water

10mM Chloroquine Diphosphate salt

2.5mg chloroquine diphosphate salt

0.5ml distilled water

2.4 Ci/mMol Tritiated hypoxanthine [Amersham Life Science International, Buckinghamshire, England]

Thiazole orange (1mg/ml ethanol) was kindly provided by Prof. L. Visser, Dept. of Biochemistry, University of Pretoria.

Giemsa stain [MERCK, Darmstadt, Germany]

2.3.2 *Parasite culture and maintenance*

A laboratory strain of low grade chloroquine-resistant (RB-1) *P.falciparum* was kindly provided by Dr. B.L Sharp [National Malaria Research Programme, MRC Durban].⁵¹

(A) Erythrocyte and serum preparation

* Venous blood from blood group O+ donors, without previous exposure to malaria and not under any antimalarial or antiinflammatory medication, was drawn in 5ml EDTA vacutainer tubes. The blood was centrifuged thrice in wash medium at 350g for 5 min with subsequent aspiration of the leucocyte supernatant after every centrifugation step. The final red cell pellet was resuspended in the wash medium and stored at 4°C for not more than ten days. The wash medium was exchanged with fresh medium every second day for proper preservation of the erythrocytes.

* Venous blood samples from five donors with blood group O+ or A+ and no known previous exposure to malaria were drawn into glass vacutainer tubes. The blood was allowed to clot and then centrifuged at 350g for 5 min. The supernatant was aseptically collected into 50ml centrifuge tubes, pooled and stored at -20°C. The serum was heat-inactivated at 56°C for 45 min before adding to the culture medium.

(B) Cryopreservation

Cultures containing more than 2% ring-forms were suitable for storing in liquid

nitrogen. Cultures were collected into 15ml centrifuge tubes and centrifuged at 200g for 5 min. After removing the supernatant, 0.5ml freezing medium and red blood cells were added to the cell pellet and mixed. Aliquots of 1ml were placed into cryopreservation tubes and stored in liquid nitrogen .

(C) Thawing and establishment of parasite cultures

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 5 min. This procedure was repeated thrice. The parasite cultures were maintained as described by Trager and Jensen with modifications.¹³⁶ Briefly, cultures were established from the pellet by transferring 10ml complete culture medium and 100µl O+ human erythrocytes into culture flasks. Cultures were gassed and incubated at 37°C.

(D) Gassing of cultures

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

2.3.3 Synchronization of parasite cultures

A synchronization step was necessary to obtain only the ring-stage forms of the parasite for carrying out the assays. This procedure can be performed if the percentage parasitemia in parasite cultures exceeds 5%. Ring-stage synchronization was achieved by sorbitol lysis as first described by Lambros and Vanderberg with modifications.¹³⁷ 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 10 mins. 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 to determine parasitemia of malaria cultures***

2.3.4.1 Exposure of parasite cultures to chloroquine

The RB-1 laboratory strain of *P. falciparum* was used for all the assays performed in this section. Ring-stage-infected erythrocyte cultures (20ul) at 2% parasitemia and 5% haematocrit were incubated with serial dilutions of chloroquine (20ul) ranging from 4nM to 250nM in 96-well microculture plates and made up to 200 μ l with complete culture medium. Wells without chloroquine and with chloroquine at 1000nM served as positive and negative control parameters respectively. The plates were placed in modular gas chambers, gassed for 15 mins. with the gaseous cocktail, 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 5 min. After staining, the slides were washed with running tap water, air-dried and investigated under a light microscope using the 100X objective. The level of parasitemia was determined by counting 300 red blood cells.

2.3.4.3 Radiometry

Twenty-four hours before the end of the incubation period, 150 μ l of the medium was removed from each well and 150 μ l hypoxanthine-free medium containing tritiated hypoxanthine (1 μ Ci) was added. After 18 hours, the cultures were harvested on filter discs by a cell harvester and the radioactivity counted in a Tri-carb 2100-TR scintillation counter [Packard Industrial Company, Illinois, U.S.A]. All treatments were performed in quadruplicate.

2.3.4.4 Flow cytometry

At the end of the incubation period, 100 μ l of medium was removed from the wells and 100 μ l of the fixation solution added to each well. The plates were stored overnight at 4°C. The fixed parasites (25 μ l) were stained with 0.5ml of a thiazole

orange solution (0.26 μ g/ml), reconstituted in PBS, in test tubes. The tubes were incubated at room temperature for an 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, Hialeah, FLA, USA].

2.3.5 *Expression and statistical analysis of results*

Results of each assay are expressed as percentage inhibition \pm SEM of parasite growth in drug-treated wells. Statistical analysis of the data was performed using the Student's t-test for paired values and Bland and Altman measure of agreement.¹³⁸

2.4 RESULTS

The RB-1 laboratory strain of *Plasmodium falciparum* was incubated with chloroquine at concentrations ranging between 4nM and 250nM for 48 hours. The extent of inhibition of parasite growth was assessed using microscopy, radiometry (³H]hypoxanthine uptake) and flow cytometry. All three methods, as shown in Figure 5, showed a dose-related inhibition of parasite growth by chloroquine which was evident at a concentration of 62nM and statistically significant ($p < 0.05$) at 125nM and higher. The Bland and Altman measure of agreement¹³⁸, depicted in Figure 6, revealed good agreement between the flow cytometric 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 IC₅₀ values (the concentration necessary to inhibit 50% of parasite growth) of chloroquine for the RB-1 strain using flow cytometry, microscopy and hypoxanthine uptake assay (radiometry) were 76nM, 70nM and 76nM respectively.

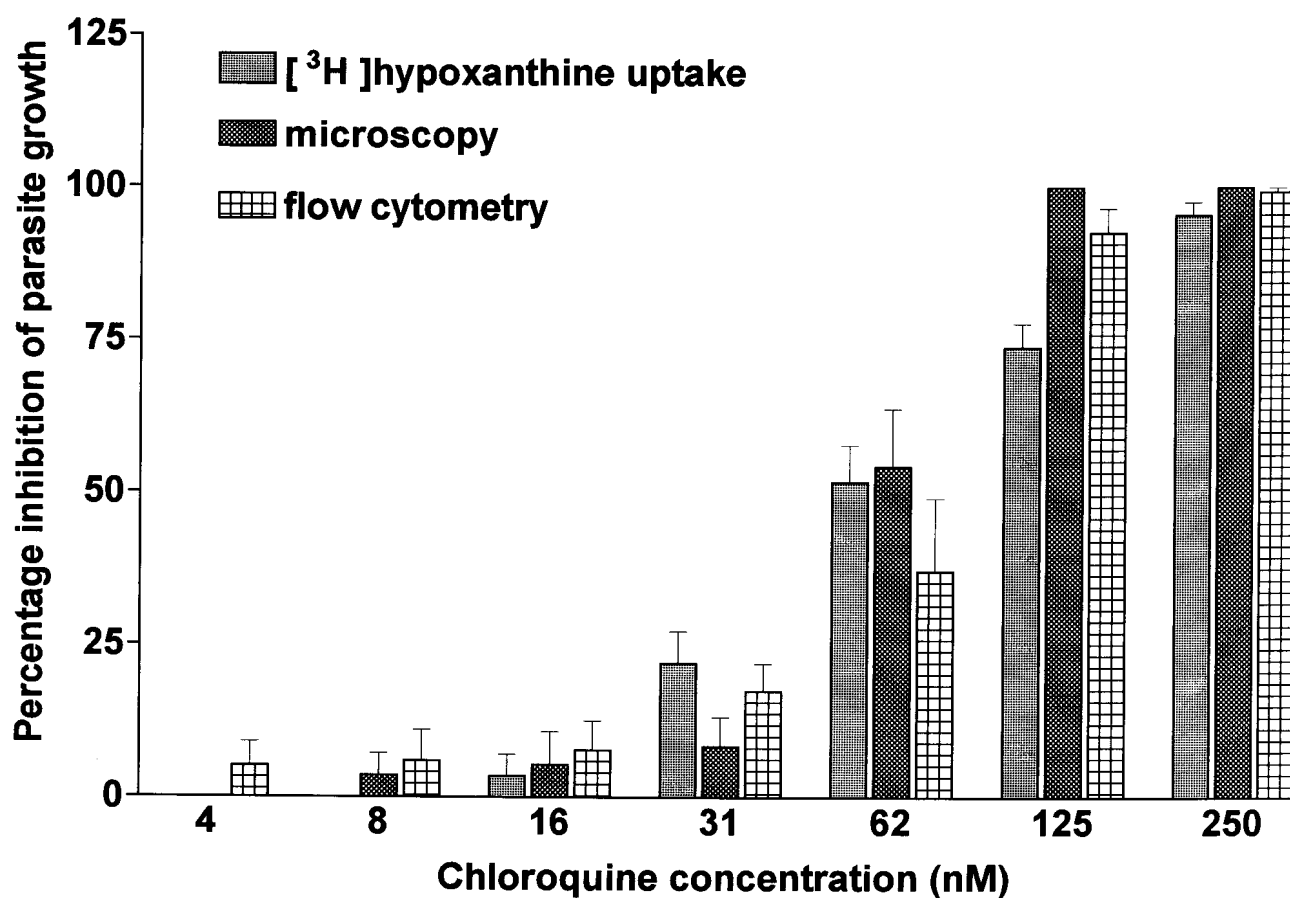


Figure 5 : Evaluation of parasitemia in malaria parasite cultures using flow cytometric, radiometric and microscopic methods. Results are expressed as percentage inhibition of parasite growth and show mean values and SEM of five different experiments for each method.

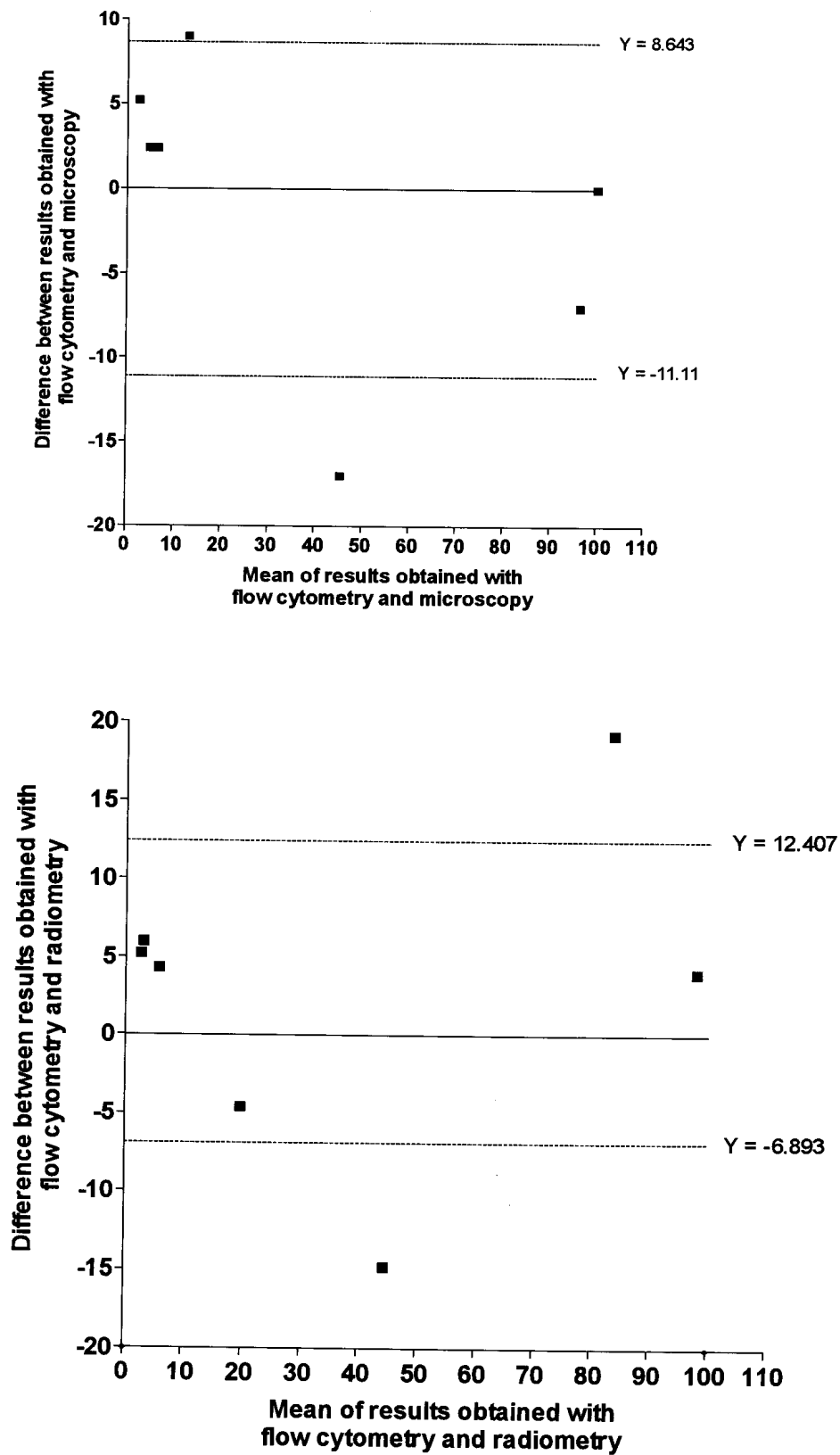


Figure 6 : The Bland and Altman measure of agreement between methods used in evaluating parasitemia in malaria parasite cultures.

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 occur.

The 95% confidence intervals (Figure 6) 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.¹³³ However, the use of thiazole orange allows for a fixing procedure which permits analysis of samples at a later more convenient time. The results of all three methods suggest that the laboratory strain of *Plasmodium falciparum* (RB-1) used in this study is of low grade resistance with an average chloroquine IC₅₀ of 74nM.

The flow cytometric procedure yielded results comparable to those obtained by the microscopic and radiometric methods but the following advantages should however make flow cytometry the preferred method of analysis : (i) it is sensitive (we were able to detect antimalarial activity at chloroquine concentrations as low as 4nM using this method) and lends itself to rapid evaluation of the effects of pharmacological agents on the growth of malaria parasites in erythrocyte cultures, (ii) the procedure offers the means to determine *P.falciparum* parasitemia 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 amount of parasite nucleoproteins present in the red cell.¹³⁵



CHAPTER 3

IN VITRO ANTIMALARIAL ACTIVITY OF RIMINOPHENAZINES

3.1 INTRODUCTION

Riminophenazines exhibit antimicrobial activities but have never been tested in parasitic/malarial systems. The flow cytometric method proved superior, as shown in Chapter 2, for screening new antimalarial agents. This procedure operates on the principle that solely infected erythrocytes stain with thiazole orange dye and as such the percentage parasitemia in the infected cells will therefore be directly proportional to the intensity of emitted fluorescence light.

3.2 OBJECTIVES

The objectives of this part of the study were to determine possible direct and chloroquine-sensitizing activities of riminophenazines on the laboratory strains of *Plasmodium falciparum* using the flow cytometric procedure.

3.3 MATERIALS AND METHODS

3.3.1 *Media and reagents*

See section 2.3.1

3.3.2 *Parasite cultures*

The RB-1 (used in Chapter 2) and a chloroquine-sensitive *pfUP-1* (kindly provided by Prof. B. Louw, Department of Biochemistry, University of Pretoria) laboratory strains of *Plasmodium falciparum* were used in this study. The parasite cultures were maintained as described in Sections 2.3.2 and 2.3.3.

3.3.3 *Preparation of riminophenazines*

Pure substances of eight riminophenazine agents [B663, B669, B4100, B4103, B4112, B4121, B4158 and B4169] were kindly provided by Dr. J.F. O'Sullivan. All drugs were dissolved as stock solutions in ethanol, at concentrations of 2mg/ml. The drugs were diluted in culture medium and used at concentrations ranging from 0.125µg/ml - 2µg/ml.

3.3.4 *Flow cytometry*

Both laboratory strains of *Plasmodium falciparum* were cultured as described in

section 2.3.3. Ring-stage infected erythrocyte cultures (20 μ l) at 2% parasitemia and 5% haematocrit were added to the wells of 96-well microculture plates containing 140 μ l and 160 μ l culture medium. Chloroquine (20 μ l and final concentrations of 31 and 62nM) and the riminophenazine agents (20 μ l at final concentrations of 0.125 - 2 μ g/ml) were added to the experimental wells either individually or in combination (in the case of the RB-1 strain only). Drug-free control wells as well as wells containing an excess of chloroquine (1000nM) serving as background systems were included with each experiment. The plates were incubated for 48 hours as described in section 2.3.3.1 and the level of parasitemia assessed using a flow cytometric procedure as described in section 2.3.3.4.

3.3.5 **Expression and statistical analysis of results**

Results are expressed as percentage inhibition \pm SEM of parasite growth in drug-treated wells and statistical analysis performed using the Student's t-test for paired values.

3.4 RESULTS

3.4.a **Direct antimalarial activity of riminophenazine compounds**

The extent of inhibition of parasite growth was measured using the flow cytometric method. Results obtained with the RB-1 strain (Figure 7) show significant ($p < 0.05$) direct antimalarial activity by the agents B669, B4100, B4103, B4112 and B4158 (concentrations of 0.5 μ g/ml and higher) whereas B4121 and B4169 were only active at the highest concentration (2 μ g/ml) tested. Clofazimine did not exhibit any significant antimalarial activity at any of the concentrations tested. Similar results were obtained with the *pfUP-1* strain of *Plasmodium falciparum* (Figure 8). The 50% inhibitory concentrations (IC₅₀) for the agents used in this study are as shown in Table 1. The results in this table indicate that agents B4158, B4112 and B669 kill 50% of the malaria parasites at concentrations lower than 0.5 μ g/ml while the other compounds, except B663 which showed no antimalarial activity at concentrations tested, achieved the same activity at concentrations of between 0.5 and 1 μ g/ml.

3.4.b Chloroquine-sensitizing activities of riminophenazine agents

The antimalarial effects of a combination of chloroquine (31 and 62nM) with the experimental agents (0.125 to 2 μ g/ml) are shown in Table 2. Although the experimental agents (0.125 - 2 μ g/ml) did not increase the sensitivity of the malaria parasite to chloroquine (31 and 62nM), additive effects were observed in combinations using B4103 (0.25 μ g/ml and 62nM chloroquine) and B4112 (0.25 μ g/ml and 62nM chloroquine).

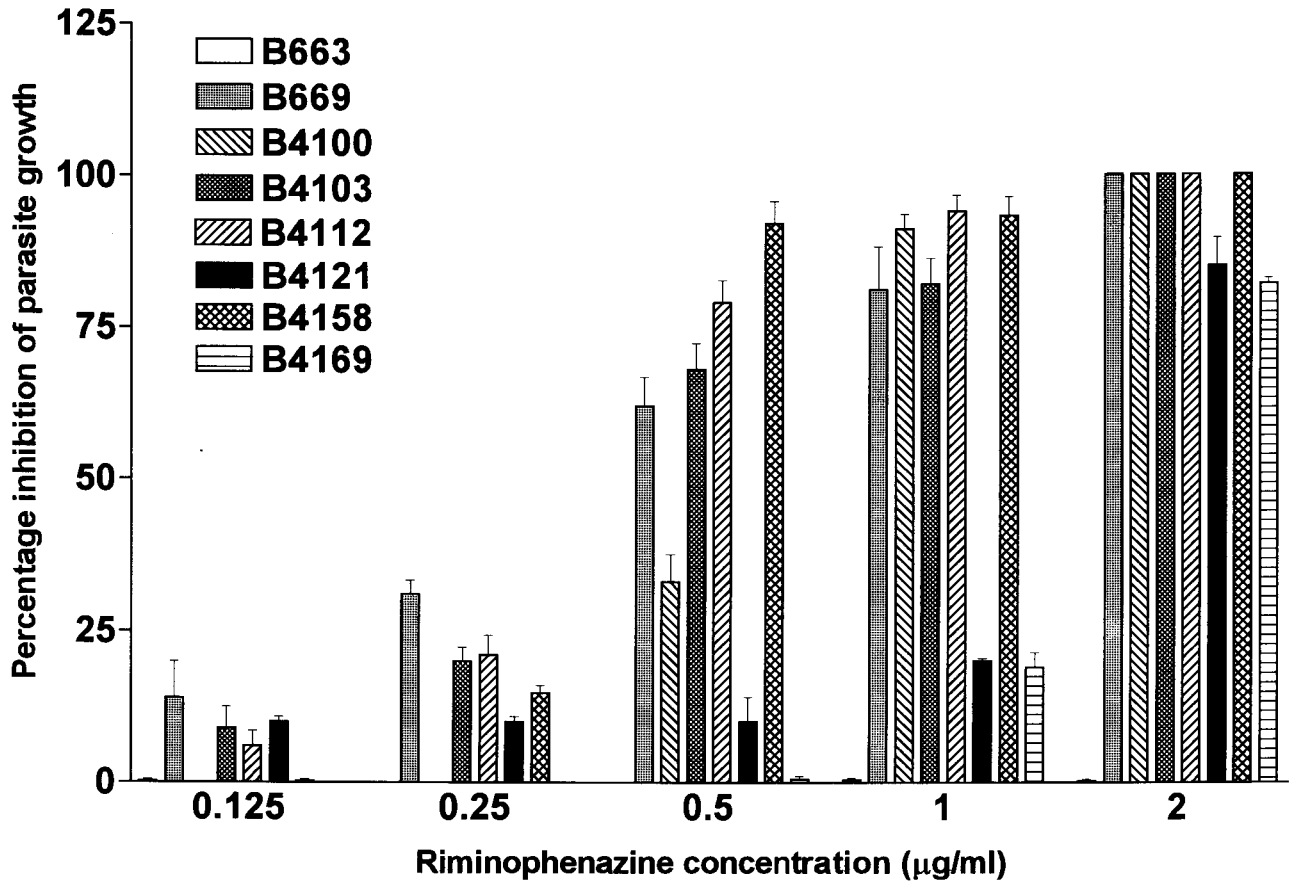


Figure 7 : Direct antimalarial activity of rimonophenazine compounds against the RB-1 laboratory strain of *Plasmodium falciparum*. Results are expressed as the mean percentage inhibition of parasite growth by the rimonophenazine compounds (0.125 - 2µg/ml) plus SEM of four different experiments.

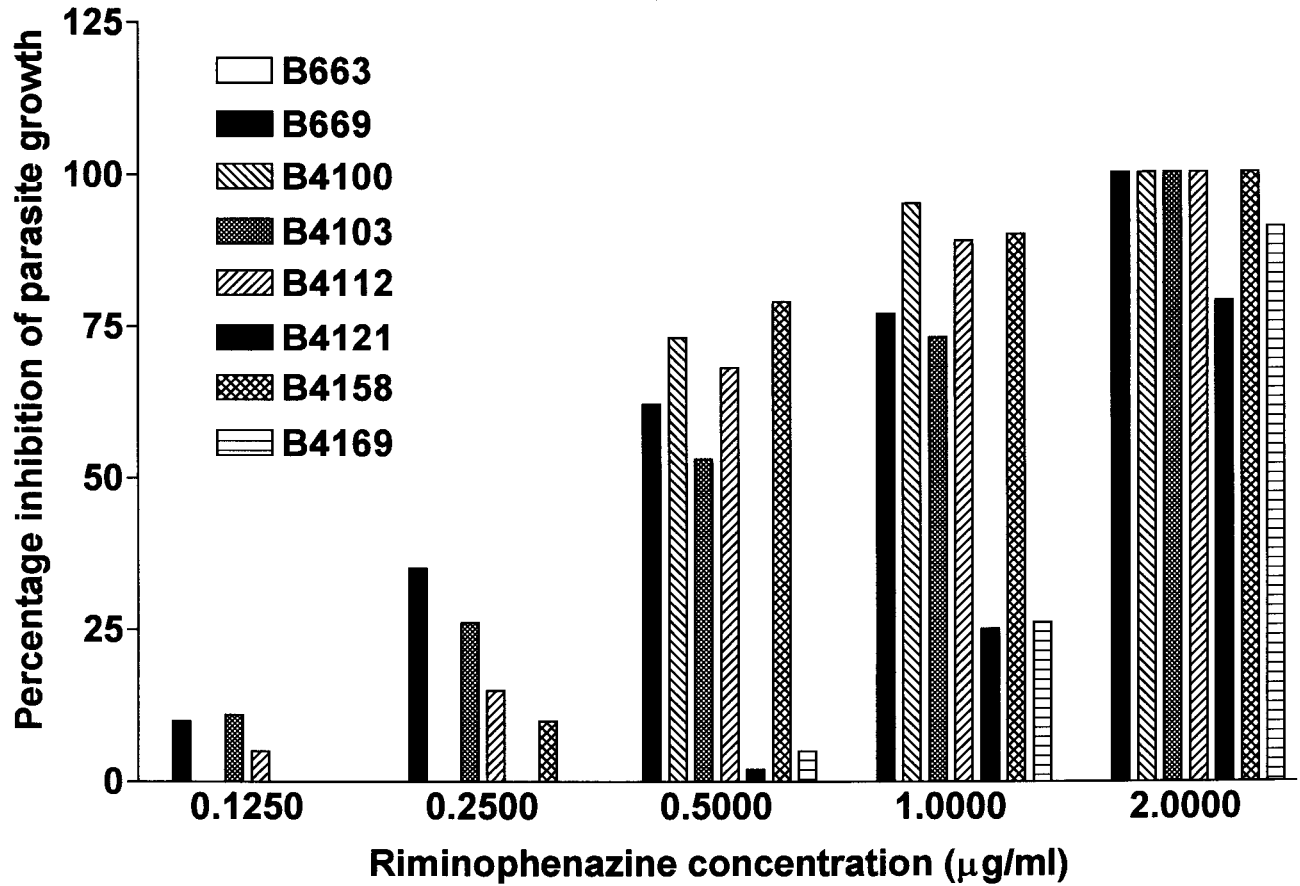


Figure 8 : Direct antimalarial activity of riminophenazine compounds against the pfUP-1 laboratory strain of *Plasmodium falciparum*. Results are expressed as the mean percentage inhibition of parasite growth by the riminophenazine compounds (0.125 - 2µg/ml) of a single experiment run in duplicate.

Table 1 : The IC_{50} (50% inhibitory concentration) values of the various riminophenazine molecules for the RB-1 laboratory strain of *Plasmodium falciparum*.

| RIMINOPHENAZINE MOLECULE | IC_{50} ($\mu\text{g/ml}$) |
|---------------------------------|---|
| B663 | > 2 |
| B669 | 0.4 |
| B4100 | 0.6 |
| B4103 | 0.5 |
| B4112 | 0.4 |
| B4121 | 1 |
| B4158 | 0.3 |
| B4169 | 1 |

Table 2 : The antimalarial activity of chloroquine alone and in combination with the riminophenazine agents against the RB-1 laboratory strain of *Plasmodium falciparum*. Data of four experiments are presented as the mean percentage inhibition \pm SEMs of the corresponding drug-free control systems.

* Additive inhibitory effects.

| | CHLOROQUINE CONCENTRATION (nM) | | |
|--|--------------------------------|-------------|-------------|
| | 0 | 31 | 62 |
| Clofazimine ($\mu\text{g/ml}$) | | | |
| 0 | - | 14 \pm 2 | 27 \pm 3 |
| 0.125 | 0 | 10 \pm 3 | 29 \pm 2 |
| 0.25 | 0 | 13 \pm 2 | 22 \pm 4 |
| 0.5 | 0 | 12 \pm 1 | 19 \pm 3 |
| 1 | 0 | 12 \pm 3 | 25 \pm 2 |
| 2 | 0 | 15 \pm 3 | 28 \pm 1 |
| B669 ($\mu\text{g/ml}$) | | | |
| 0 | - | 28 \pm 1 | 42 \pm 4 |
| 0.125 | 14 \pm 6 | 20 \pm 3 | 37 \pm 4 |
| 0.25 | 31 \pm 2 | 14 \pm 2 | 43 \pm 1 |
| 0.5 | 62 \pm 5 | 57 \pm 2 | 59 \pm 5 |
| 1 | 80 \pm 8 | 76 \pm 8 | 90 \pm 6 |
| 2 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 |
| B4100 ($\mu\text{g/ml}$) | | | |
| 0 | - | 17 \pm 1 | 48 \pm 2 |
| 0.125 | 0 \pm 0 | 14 \pm 2 | 41 \pm 8 |
| 0.25 | 0 \pm 0 | 17 \pm 2 | 54 \pm 8 |
| 0.5 | 33 \pm 4 | 47 \pm 8 | 78 \pm 7 |
| 1 | 91 \pm 2 | 97 \pm 2 | 98 \pm 3 |
| 2 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 |



TABLE 2 continued

| | CHLOROQUINE CONCENTRATION (nM) | | |
|--|--------------------------------|-------------|--------------|
| | 0 | 31 | 62 |
| B4103 ($\mu\text{g/ml}$) | | | |
| 0 | - | 13 ± 2 | 45 ± 5 |
| 0.125 | 6 ± 2 | 21 ± 4 | 39 ± 11 |
| 0.25 | 21 ± 3 | 24 ± 4 | $70 \pm 2^*$ |
| 0.5 | 79 ± 3 | 88 ± 6 | 87 ± 10 |
| 1 | 94 ± 2 | 98 ± 1 | 100 ± 0 |
| 2 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| B4112 ($\mu\text{g/ml}$) | | | |
| 0 | - | 20 ± 5 | 45 ± 5 |
| 0.125 | 6 ± 2 | 21 ± 4 | 39 ± 12 |
| 0.25 | 21 ± 3 | 24 ± 1 | $70 \pm 2^*$ |
| 0.5 | 79 ± 3 | 88 ± 6 | 87 ± 10 |
| 1 | 94 ± 2 | 98 ± 1 | 100 ± 0 |
| 2 | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| B4121 ($\mu\text{g/ml}$) | | | |
| 0 | - | 14 ± 9 | 38 ± 2 |
| 0.125 | 10 ± 1 | 10 ± 5 | 28 ± 3 |
| 0.25 | 10 ± 1 | 10 ± 2 | 32 ± 4 |
| 0.5 | 10 ± 4 | 11 ± 4 | 24 ± 3 |
| 1 | 20 ± 1 | 29 ± 5 | 36 ± 3 |
| 2 | 85 ± 4 | 86 ± 13 | 93 ± 7 |



TABLE 2 continued

| | CHLOROQUINE CONCENTRATION (nM) | | |
|--|--------------------------------|-------------|-------------|
| | 0 | 31 | 62 |
| B4158 ($\mu\text{g/ml}$) | | | |
| 0 | - | 20 \pm 5 | 48 \pm 3 |
| 0.125 | 1 \pm 0 | 15 \pm 2 | 38 \pm 2 |
| 0.25 | 14 \pm 1 | 23 \pm 7 | 47 \pm 3 |
| 0.5 | 92 \pm 3 | 89 \pm 3 | 100 \pm 0 |
| 1 | 93 \pm 3 | 99 \pm 1 | 100 \pm 0 |
| 2 | 100 \pm 0 | 100 \pm 0 | 100 \pm 0 |
| B4169 ($\mu\text{g/ml}$) | | | |
| 0 | - | 13 \pm 2 | 42 \pm 6 |
| 0.125 | 1 \pm 0 | 10 \pm 2 | 26 \pm 2 |
| 0.25 | 1 \pm 0 | 10 \pm 1 | 26 \pm 1 |
| 0.5 | 1 \pm 0 | 13 \pm 3 | 24 \pm 4 |
| 1 | 19 \pm 2 | 16 \pm 4 | 40 \pm 4 |
| 2 | 82 \pm 1 | 92 \pm 3 | 94 \pm 2 |

3.5 DISCUSSION

There are several classes of drugs used to treat malaria caused by *Plasmodium falciparum*. These agents encompass the oldest recognised antimalarial agents (quinine), the agents used in World War II (chloroquine and quinacrine) and newer agents used for chloroquine-resistant *Plasmodium falciparum* (mefloquine and halofantrine).⁶⁷

The search for new antimalarial agents is paramount due to continual development of clinical drug resistance by the parasite, as well as deleterious side effects associated with most antimalarial agents developed to date. The present study describes the *in vitro* antimalarial activities of riminophenazine agents of which clofazimine, the parent molecule, was primarily used for the treatment of leprosy. The flow cytometric procedure was preferred over microscopy and radiometry for determining the parasitemia in malaria cultures.

The eight riminophenazine agents used in this study can be divided into three groups based on the extent to which they inhibit parasite growth of the RB-1 strain directly :-

Group I : this group has the highest antimalarial activity and includes the following agents : B669, B4100, B4103, B4112 and B4158. These agents achieve statistically significant inhibition of parasite growth at concentrations between 0.5 μ g/ml and higher ($p < 0.05$).

Group II : this group shows moderate antimalarial activity and includes only B4121 and B4169. These two agents are only active at a concentration of 2 μ g/ml ($p < 0.05$).

Group III : clofazimine is the only agent in this group and does not exhibit any statistically significant antimalarial activity ($p > 0.05$) at the highest concentration tested (2 μ g/ml).

These results were confirmed by a single experiment using another strain of *Plasmodium falciparum* (pfUP-1) as shown in Figure 8.

Some of the riminophenazine agents exhibited antimalarial activity against the RB-1

strain at a concentration as low as $0.5\mu\text{g/ml}$, a concentration easily attainable in serum (serum concentrations for clofazimine of 1 - $2\mu\text{g/ml}$ have been documented).¹³⁹ All the agents, except clofazimine exhibited antimalarial activity of varying degrees depending on the extent and type of halogenation or methylation.

Two of the three most active agents (B669 and B4158) contained no halogens whereas the third (B4112) contained only two chlorine atoms. None of the agents used in this study, showed any chloroquine potentiating activities against this low grade chloroquine-resistant *Plasmodium falciparum* (RB-1) as shown in Table 2, although additive interactions of the riminophenazines with chloroquine were evident in the case of B4112 and B4103.



CHAPTER 4

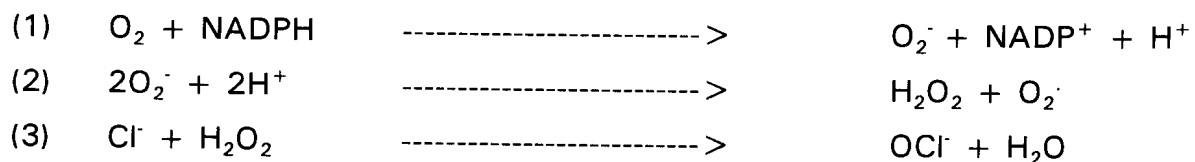
RIMINOPHENAZINE-MEDIATED MODULATION OF GENERATION OF SUPEROXIDE ANION BY HUMAN NEUTROPHILS

4.1 INTRODUCTION

Phagocytes, especially neutrophils, undergo marked changes upon exposure to various stimuli, leading to an increase in oxygen consumption as well as glucose metabolism and an increase in the production of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These metabolic events are collectively termed the respiratory burst and result in the production of microbicidal oxidants known as reactive oxygen intermediates (ROI).¹⁴⁰

The respiratory burst derives from the activation of a dormant enzyme system in neutrophils called NADPH-oxidase, with subsequent reduction of oxygen to superoxide at the expense of NADPH. The metabolism of glucose through the hexose monophosphate shunt serves to replenish the NADPH that has been consumed by the superoxide forming systems. Dismutation of superoxide by superoxide dismutase (SOD) leads to generation of hydrogen peroxide that can also in turn react with chloride ions, a reaction catalysed by myeloperoxidase, to produce hypochlorite, an extremely potent oxidant that is considered the most effective microbicidal agent.¹⁴¹

The chemical reactions responsible for production of these antimicrobial reactive species are shown below :



Reactions 1,2 and 3 are catalysed by NADPH oxidase, superoxide dismutase and myeloperoxidase respectively.

The production of reactive oxygen species, notably superoxide, can be measured by procedures including chemiluminescence, nitroblue tetrazolium reduction, oxygen consumption assays, spin trapping of superoxide radicals and reduction of cytochrome c. The method used in the present study to measure superoxide production by human neutrophils is the superoxide dismutase (SOD) -inhibitable reduction of cytochrome c. The principle of this procedure is based on the finding that superoxide from activated neutrophils reduces cytochrome c and this reaction

is terminated in the presence of superoxide dismutase.¹⁴¹

4.2 OBJECTIVES

The aim of the investigations described in this part of the study was to investigate possible prooxidative activities of novel riminophenazine agents (B663, B669, B4100, B4103, B4158 and B4169) with human neutrophils and to correlate this parameter with their antimalarial activity, because enhanced production of oxidants by phagocytes is an alternative mechanism of antimalarial activity.^{22,23}

4.3 MATERIALS AND METHODS

4.3.1 *Media and reagents*

Unless otherwise indicated, all reagents and chemicals used were obtained from Sigma Chemical Co., St. Louis, U.S.A.

Ficoll hypaque

Dissolve 90g Ficoll [Pharmacia Fine Chemicals, Uppsala, Sweden] in 1000ml distilled water

Add 160ml 75% Metrizoate [Nyegaard and Co., Oslo, Norway]

Adjust density to 1.074 at room temperature and autoclave

Hanks balanced salt solution (HBSS)[Highveld Biological Products, Kelvin, S.A]

Indicator-free and without antibiotics

Adjust pH to 7.4 with 1M NaOH

Phosphate buffered saline (PBS)

Prepared as in section 2.3.1, but pH adjusted to 7.4

3% Gelatin solution

3g powdered Gelatin [MERCK, Darmstadt, Germany]

0.932g FTA buffer

100ml distilled water

0.83% Ammonium chloride solution

8.3g NH₄Cl

1g NaHCO₃

1000ml distilled water

Riminophenazine compounds

The following riminophenazine compounds were used in this part of the study : B663, B669, B4100, B4103, B4158 and B4169 and prepared as in Section 3.3.2.

5mM FMLP (N-formyl-L-methionyl-L-leucyl-L-phenylalanine)

5mg / 2ml DMSO

Cytochrome c

3.5mg Cytochrome C / 3.5ml HBSS

Superoxide dismutase, from bovine liver

70ul SOD / 1ml HBSS

4.3.2 Preparation of human neutrophils [ficoll method]

Neutrophils were prepared by a method modified from that described by Ferrante and Thong.¹⁴² About 30ml of heparinized blood was layered onto 20ml of ficoll-hypaque in a centrifuge tube and spun at room temperature for 25 min. at 300g in a Beckman TJ-6 centrifuge. After removal of the plasma and lymphocyte layers, the neutrophil and erythrocyte fractions were sedimented in an abundance of 3% gelatin for 15min. at 37°C to remove the red blood cells. The leucocyte-rich supernatant was centrifuged at 160g for 10 min. at room temperature and the residual red cells were lysed with a 0.83% NH₄Cl solution on ice for 10 min. The cells were washed with PBS at 110g and the resultant pellet resuspended in indicator-free Hanks balanced salt solution (HBSS) at pH7.4 before use.

4.3.3 ***Measurement of generation of superoxide by neutrophils using the cytochrome c reduction assay***

The generation of superoxide anion by human neutrophils was assessed by the superoxide dismutase inhibitable reduction of cytochrome c as described by Babior and colleagues.¹⁴³ Each assay was performed in triplicate. The riminophenazine agents (0.5 μ g/ml, final concentration) were added to pre-warmed neutrophils (1 x 10⁶ cells / ml) in indicator-free HBSS. After a 5 min incubation period, 100 μ l of superoxide dismutase (SOD) (100units/ml final concentration) were added into appropriate tubes followed by 100 μ l cytochrome c (0.1mM, final concentration).

After a further 5min incubation, FMLP (0.1 μ M) was added as stimulant of superoxide production to experimental systems. The reactions were terminated after 5min. by addition of ice-cold PBS and placing the tubes on ice. A set of control tubes for FMLP alone, solvent systems and baseline parameters were included. The tubes were centrifuged for 10 min. at 4°C and 100g. The optical density of the supernatants was measured at 550nm using a Pye Unicam SP 1700 ultraviolet spectrophotometer.

4.3.4 ***Expression and statistical analysis of results***

Results are expressed as nanomoles reduced cytochrome c / 10⁶ neutrophils/ 5min \pm SEM . Statistical analysis of the data was performed using the Student's t-test for paired values.

4.4 RESULTS

Figure 9 shows that only B669 and clofazimine tested at a concentration of 0.5 μ g/ml caused a significant enhancement of neutrophil superoxide production ($p < 0.05$) when compared to the FMLP-control. The other riminophenazine agents, all TMP-derivatives (B4100, B4103, B4158 and B4169), had no significant effect on cellular superoxide release ($p > 0.05$). A comparison between figure 7 and 9 indicates that (i) B669 exhibits both antimalarial and prooxidative activities at 0.5 μ g/ml,(ii) clofazimine exhibits prooxidative but not antimalarial activities at the same concentration (0.5 μ g/ml) whereas (iii) the TMP-riminophenazines ; B4100,

B4103 and B4158 show antimalarial activities with no corresponding prooxidative activities at $0.5\mu\text{g/ml}$ while B4169 does not exhibit either of these two activities at $0.5\mu\text{g/ml}$.

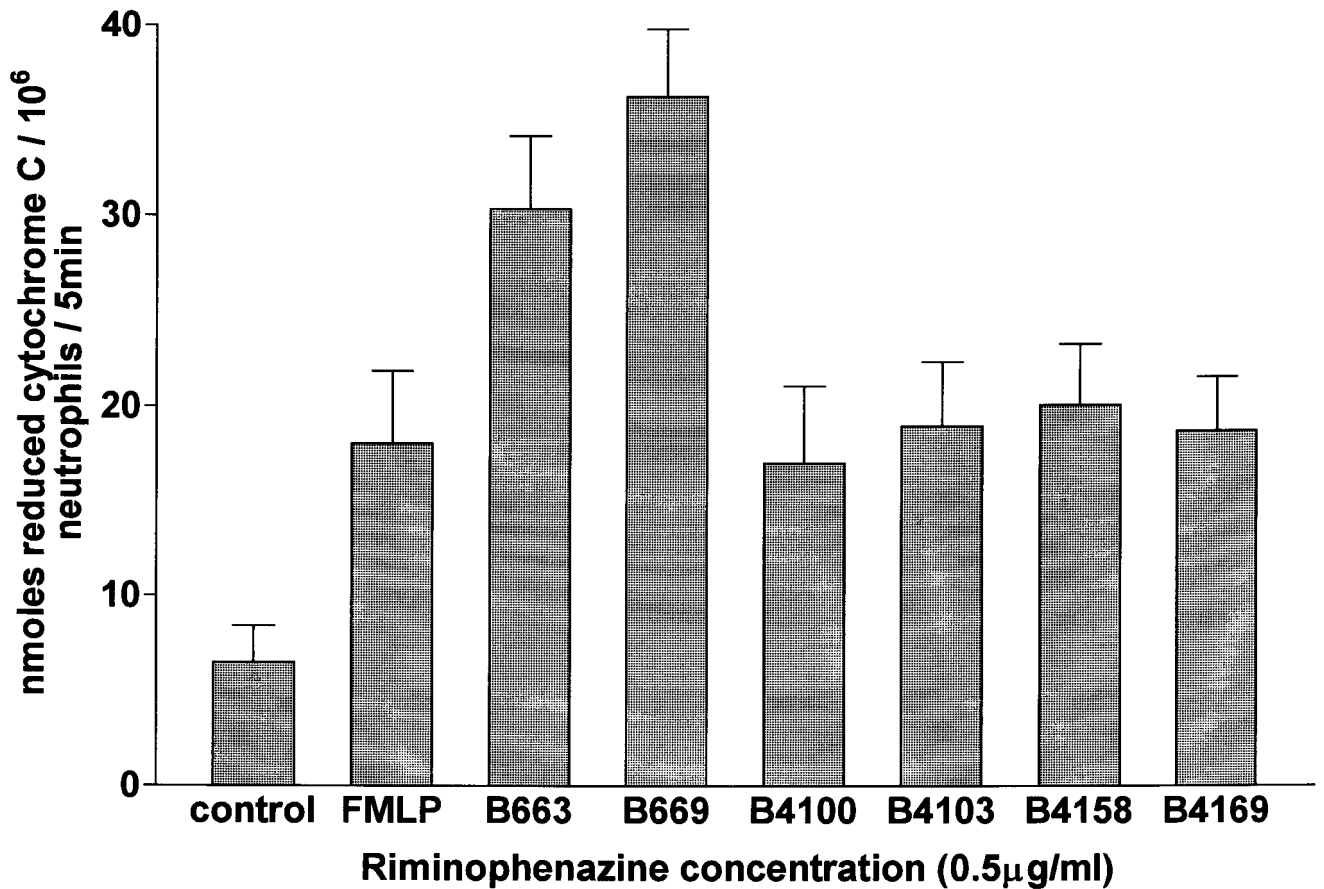


Figure 9 : Priming effects of riminophenazine (0.5 μg/ml) on the generation of superoxide by human neutrophils (10⁶/ml) in the presence of FMLP (0.1 μM). Results are expressed as nanomoles reduced cytochrome c/10⁶ neutrophils / 5 min and show mean values and SEM of five different experiments for each riminophenazine agent.

4.5 DISCUSSION

The respiratory burst of human neutrophils is important for host defence against pathogens and any pharmacological agent that enhances the generation of microbicidal oxidants surely boosts the host defence. Anderson et. al¹²¹ have shown that clofazimine, although unable to promote spontaneous production of reactive oxidants at biologically significant concentrations, can prime neutrophils for enhanced generation of reactive oxidants in response to different stimuli including FMLP, PMA (phorbol myristate acetate), calcium ionophore, opsonized zymosan and arachidonic acid.

Riminophenazines (dihydrophenazine derivatives with an imino group at position 2 of the phenazine nucleus) with the imino group of cycloalkyl nature, such as B669 have been shown¹¹⁴ to enhance superoxide production by FMLP-activated neutrophils. Halogenation of the para-position of the phenazine ring plays no critical role in these prooxidative activities.

The present study confirms previous findings since, as shown in Figure 9, B669 and clofazimine significantly ($p < 0.05$) potentiate superoxide production by neutrophils. The agents with a TMP group at position 2 of their phenazine rings (B4100, B4103, B4158 and B4169) fail to prime FMLP-activated neutrophils to generate superoxide ($p > 0.05$). Comparison of Figures 7 and 9 show that the only agent that was active in both systems was B669. Clofazimine has been shown in Chapter 3, Figure 7 to exhibit no antimalarial activity although an enhanced production of reactive oxidants¹¹⁰ by neutrophils was noted.

Lack of pro-oxidative activity of the TMP-riminophenazines may be attributed to the following : (i) agents were screened at fixed concentrations and might be active at higher or lower concentrations, (ii) agents may require a longer preincubation period, (iii) use of other stimuli of membrane-associated oxidative metabolism such as PMA, calcium ionophore and opsonized zymosan may be necessary and (iv) it might be essential to evaluate the superoxide scavenging property of the TMP-derivatives.



CHAPTER 5

CONCLUDING DISCUSSION

5.1 CONCLUDING DISCUSSION

Despite strong efforts to control it, malaria remains a cause of great concern, particularly in developing countries.¹⁴⁴ 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.⁵⁸ Multidrug resistance has been reported from Africa and Asia, and this includes resistance to chloroquine, sulfadoxine/pyrimethamine, mefloquine, quinine, pyrimethamine and amodiaquine.¹⁴⁵ In some situations, resistance *in vivo* has been reported to all antimalarial drugs except artemisinin and its derivatives and cross-resistance exists among mefloquine, quinine and halofantrine.¹⁴⁶ Indiscriminate use of most of the parasitocidal drugs has led to adverse side-effects such as neurotoxicity in the case of artemisinin derivatives, and early emergence of resistance to the agent which compromises the therapeutic efficacy of these drugs in the treatment of severe and complicated malaria. Recent reports in the media (Pretoria News and The Sunday Times, March 1996) about the high mortality rate due to malaria infections in endemic areas of Northern Kwazulu/Natal, Swaziland and the North-eastern Transvaal also highlight the urgent need for effective antimalarial agents and disease control strategies. Brian Sharp and Janet Freeze from the South African National Malaria Research Programme,¹⁴⁷ have recommended that (a) appropriate drugs in correct dosages be administered and (b) health education for both the community as well as health workers is fundamental to the control of drug-resistant malaria.

Difficulties in establishing superior chemoprophylaxis for plasmodium infections are further compounded by inferior performance of current techniques used for screening *in vitro* antimalarial activity of new agents. The classical procedures used to carry out this task, i.e microscopy and radiometry, are labourious and time-consuming especially when large numbers of samples must be evaluated. Microscopic examination of large numbers of Giemsa-stained blood smears requires qualified laboratory staff. Moreover, radiometry requires long incubation periods (24

- 72 hours) followed by sample processing and scintillation counting.^{130, 135}

The flow cytometric procedures utilized 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 33258 Hoechst and ethidium bromide required modifications of the incubation media, the results obtained not always being comparable with those of microscopic methods.¹³²⁻¹³⁴ 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 numbers of samples are analysed in a short period, 2 - 3 hours), the fixing step incorporated in this method enables one to store samples for staining and analysis at a convenient time (this eliminated the principal drawback when availability of the flow cytometer doesnot coincide with completion of the assay). Moreover the method is simple (all washing and centrifugation steps are eliminated). The agreement, as measured by the Bland and Altman test,¹³⁸ between this method and the two classical assays, such as microscopy and radiometry,¹³¹ clearly demonstrates that future screening of new antimalarial agents can be confidently performed using flow cytometry.

The work presented in this dissertation also shows that riminophenazines, although dissimilar in chemical structure from other antimalarial agents, inhibit growth of the RB-1 strain of *Plasmodium falciparum* at comparable concentrations of 0.125 - 2 μ g/ml. Interestingly, the IC₅₀ for chloroquine (0.4 μ g/ml) is similar to those of most active riminophenazines B4158, B4112 and B669 being 0.3, 0.4 and 0.4 μ g/m respectively. Since the concentrations at which some of these agents inhibit the growth of the malaria parasite are therapeutically attainable *in vivo* (according to serum levels documented for clofazimine)¹³⁹ and because the original compound is relatively non-toxic (side-effects associated with clofazimine chemotherapy are pigmentation of skin, conjunctivae and urine, abdominal discomfort and splenic infarction that normally disappear with cessation of treatment)^{110,113} these agents should be attractive contenders for evaluation in the treatment of malaria infections

caused by *Plasmodium falciparum*. The TMP component and type and extent of halogenation of the riminophenazines used in this study (Figure 4) seems to be very crucial for the observed antiplasmodial activity of these agents.

Interestingly, the TMP-riminophenazines, unlike clofazimine and B669, do not interact pro-oxidatively with human phagocytes, suggesting that the observed direct interactions of these agents with malarial parasites are primarily responsible for the observed antiplasmodial activity. Future investigations will focus on the biochemical mechanisms of the anti-plasmodial activity of these agents, as well as on their chemotherapeutic properties in animal models of experimental chemotherapy.



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