PRECLINICAL EVALUATION OF THE POSSIBLE ENHANCEMENT OF THE EFFICACY OF ANTI-MALARIAL DRUGS BY PHEROID TECHNOLOGY™

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Natasha Langley
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"If we take as our standard of importance
The greatest harm to the greatest number
Then there is no question that malaria is the most important
of all infectious diseases."

~Sir Macfarlane Burnet

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ABSTRACT

Malaria is currently one of the most imperative parasitic diseases of the developing world. Current effective treatment options are limited because of increasing drug resistance, treatment cost effectiveness and treatment availability. Novel drug delivery systems are a new approach for increased efficacy in the treatment of the disease. Pheroid™ technology, a proven drug delivery system, in combination with anti-malarial drugs was evaluated in this study. The aim of this study was to evaluate the possible enhancement of the efficacy of the existing anti-malarial drugs in combination with Pheroid™ technology.

The efficacy of existing anti-malarial drugs in combination with Pheroids was investigated *in vitro* with a chloroquine RB-1-resistant strain of *P. falciparum.* Two different Pheroid formulations, vesicles and microsponges, were used and the control medium consisted of sterile water for injection. Parasitaemia levels were determined microscopically and expressed as a percentage. An *in vivo* pilot study was also conducted using the *P. berghei* mouse model. The mice were grouped into seven batches of three mice each. The control group was treated with a Pheroid vesicle formulation only. Three of the groups were treated with three different concentrations of chloroquine dissolved in water namely 2 mg/kg; 5 mg/kg and 10 mg/kg bodyweight (bw) respectively, while the other three groups received the same three concentrations of chloroquine entrapped in Pheroid vesicle formulations. The measure of parasite growth inhibition (percentage parasitaemia), the survival rates and the percentage chemosuppresion was determined. In the *in vivo* study, all concentrations of chloroquine entrapped in Pheroid vesicles showed suppressed parasitaemia levels up to 11 days post infection. From day 11, the parasitaemia increases rapidly and becomes higher than that in groups treated with chloroquine in water. Chloroquine entrapped in Pheroid vesicles showed improved activity against a chloroquine resistant strain (RB-1) *in vitro.* The efficacy was enhanced by 1544.62%. The efficacy of mefloquine, artemether and artesunate in Pheroid microsponges were enhanced by 314.32%, 254.86% and 238.78% respectively. It can be concluded that Pheroid™ technology has potential to enhance the efficacy of anti malaria drugs.

Key words: Malaria, Chloroquine, Mefloquine, Artemether, Artesunate, Pheroid™ technology, *P. falciparum, P. berghei.*

UITTREKSEL

Malaria is een van die belangrikste infektiewe siektes en die effektiwiteit van geneesmiddels wat huidiglik vir die behandeling daarvan gebruik word, begin problematies raak. Faktore soos toenemende geneesmiddel weerstandbiedendheid, geneesmiddelbeskikbaarheid en die koste-effektiwiteit van behandeling beperk die mate waartoe effektiewe behandeling toegepas kan word. Innoverende geneesmiddel draersisteme, soos die Pheroid™ geneesmiddel draersisteem, en die moontlike bydrae wat dit tot die verhoging in effektiwiteit van bestaande geneesmiddels kan lewer, is in hierdie studie ondersoek.

Die effek van chloroquine, mefloquine, artemether en artesunaat, in kombinasie met Pheroid™ tegnologie, is op malaria geïnfekteerde eritrosiete ondersoek in 'n *in vitro* weefselkultuurstudie. Eritrosiete is geïnfekteer met 'n chloroquine weerstandbiedende vorm (RB-1) van die *P. falciparum* malariaparasiet. Twee verskillende formules van die Pheroid™ sisteem, naamlik Pheroid-mikrodruppeltjies en Pheroid-mikrosponsies is gebruik om die geneesmiddels af te lewer en steriele water vir inspuiting was die kontroleformule. Parasietvlakke is bepaal met ligmikroskopie. *In vivo* studies is ook gedoen op muise wat vooraf geïnfekteer was met *P. berghei*. Die muise is in sewe groepe ingedeel. Drie groepe het verskillende konsentrasies chloroquine in Pheroid-mikrodruppeltjies ontvang. Drie groepe het verskillende konsentrasies chloroquine opgelos in water, ontvang en die kontrole groep het Pheroid-mikrodruppeltjies sonder enige geneesmiddel ontvang. Die chloroquine geneesmiddelkonsentrasies was 2 mg/kg, 5 mg/kg en 10 mg/kg. Die parasietvlakke op verskillende tydintervalle, die oorlewingkurwes van die muise sowel as die persentasie chemiese onderdrukking van die verskillende doserings is bepaal.

Die *in vitro* studie het aangetoon dat die effektiwiteit van chloroquine in Pheroid-mikrodruppeltjies verhoog is met 1544.62% en dat die effektiwiteit van mefloquine, artemether en artesunaat in Pheroid-mikrosponsies verhoog is met 314.32%, 254.86% en 238.78% onderskeidelik. Die *in vivo* studie het aangetoon dat parasietvlakke in die muise vir al drie die geneesmiddelkonsentrasies in Pheroid-mikrodruppeltjies onderdruk is tot dag 11 van die studie. Die resultate toon duidelik aan dat Pheroid™ tegnologie groot moontlikhede inhou vir malariabehandeling. Addisionele eksperimente moet uitgevoer word om die volle potensiaal van hierdie afleweringsisteem te ondersoek.

Sleutelwoorde: Malaria, Chloroquine, Mefloquine, Artemether, Artesunaat, Pheroid™ tegnologie, *P. falciparum, P.berghei.*

INTRODUCTION AND AIM OF STUDY

Malaria is a very problematic parasitic disease and treatment policies have to be continuously revised and assessed by the World Health Organization because of the failing therapeutic efficacy of anti-malarial drugs currently in use (Bosman & Olumese, 2004). This problem can be directly attributed to the emergence of mono- and multi-drug resistant parasites which render treatment options as ineffective and limited (Bloland, 2001; Renslo & McKerrow, 2006). Indirectly, this leads to increased treatment dosages and the escalating prevalence of dose related adverse effects which is very detrimental to patient compliance (White, 1998).

Various factors are involved when the deterioration of malaria control strategies are scrutinized. Climate stability, global warming, civil disturbances, escalating travel within endemic areas, drug and insecticide resistance all contribute to increasing transmission rates (Greenwood *et al.*, 2005). The development of a safe and effective malaria vaccine has therefore also become a dominant focus area and in aid of hastening the funding, developing and licensing of such a product the *Malaria Vaccine Technology Roadmap* was launched in December 2006 by the World Health Organisation.

The emergence and spread of chloroquine and multi-drug resistant parasites is the most important reason for treatment failures in malaria. The prevalence of this phenomenon markedly reduces our options of drugs to implement in treatment regimes. The implementation of artemisinin based combination therapy has received a lot of attention and is recommended as a preventative measure for the emergence of drug resistance by the World Health Organization (WHO, 2006).

A great need for alternative treatment options of the disease has therefore become eminent and as a part of the drug discovery processes it is of paramount importance to explore new strategies concerning the treatment and the chemoprophylaxis of malaria. These strategies could include the possible enhancement of compound efficacy by the incorporation of a novel drug delivery system like the Pheroid™ drug delivery system which can be classified as a novel, patented, colloidal system based on Pheroid™ technology.

In light of all the given facts regarding this novel drug delivery system the main objectives of this study was to examine the possibility of (i) increasing the efficacy of

mono therapy drugs and (ii) to possibly decrease the dosage requirements for malaria treatments by Pheroid™ technology. When evaluating this project in its entirety it can be considered to be a stepping stone with the aim of formulating a new dosage form for the treatment of malaria in the near future with mono- and combination therapy strategies.

The aims of the study were:

1. To evaluate the *in vitro* efficacy of chloroquine, mefloquine and the artemisinin derivates, artemether and artesunate, against a chloroquine resistant strain.

2. To evaluate the *in vitro* efficacy of the above mentioned drugs in combination with Pheroid™ vesicles against a chloroquine resistant strain.

3. To evaluate the *in vitro* efficacy of the above mentioned drugs in combination with Pheroid™ micro sponges against a chloroquine resistant strain.

4. To evaluate the efficacy of chloroquine alone and in combination with Pheroid™ vesicles in an *in vivo* mouse model.

As hypothesis it is stated that the efficacy of chloroquine, mefloquine, artemether and artesunate will be increased in combination with Pheroid™ formulations in an *in vitro* model. The efficacy of chloroquine in combination with Pheroid™ vesicles will be increased in an *in vivo* mouse model.

CHAPTER 1

Malaria

1.1 Introduction

Malaria is currently one of the most imperative parasitic diseases of the developing world. Globally 350-500 million cases of malaria occur each year of which more than 1 million people die. The highest mortality rate is found amongst children under the age of 5 years in the Sub-Saharan region of Africa (CDC-(A), 2004). Due to the emergence and spread of drug resistant parasites, mortality figures have risen in recent years. This poses eminent health and economic problems for populations situated in malaria endemic areas and undisputedly contributes to the worldwide burden of the disease (WHO, 2006). Various factors are involved when the deterioration of malaria control strategies are scrutinized. Climate stability, global warming, civil disturbances, escalating travel within endemic areas as well as drug- and insecticide resistance all contribute to the increasing transmission rates (Greenwood *et al.*, 2005). A great need has arisen for the development of a safe and effective malaria vaccine and in aid of hastening the funding, developing and licensing of such a product the *Malaria Vaccine Technology Roadmap* was launched in December 2006 by the World Health Organisation. Their aims and objectives are to develop a vaccine by the year 2025 that will be 80% effective against the clinical disease and that will provide protection against the disease for at least four years. Young children and pregnant woman will be the focus groups of vaccine development, seeing that they are the most vulnerable and susceptible to the disease, especially *P. falciparum* malaria that is by far the most destructive parasitic strain (Basco, 2007).

1.2 Malaria in South Africa

South Africa has an estimated population of 40 million people and approximately 10%, or roughly 4 million, of these people live in the malaria risk areas. The areas in South Africa that are considered to be malaria endemic are the low-altitude parts of the Limpopo province, Mpumalanga province and KwaZulu-Natal as can be seen in figure 1.1. The effects of malaria presented much worse in the past, in comparison with statistics today. In 1932, for example, 22 132 deaths from malaria in KwaZulu-Natal

where reported in a population of 1 819 000 million people rendering a mortality rate of 1.2%. Disease incidence rates were equally devastating for Mpumalanga and the Limpopo province during this period. Various control strategies were considered but the employment of DDT, a popular insecticide, in indoor residual spraying proved to be very effective and caused reported malaria cases to decline rapidly (Tren & Bate, 2004).

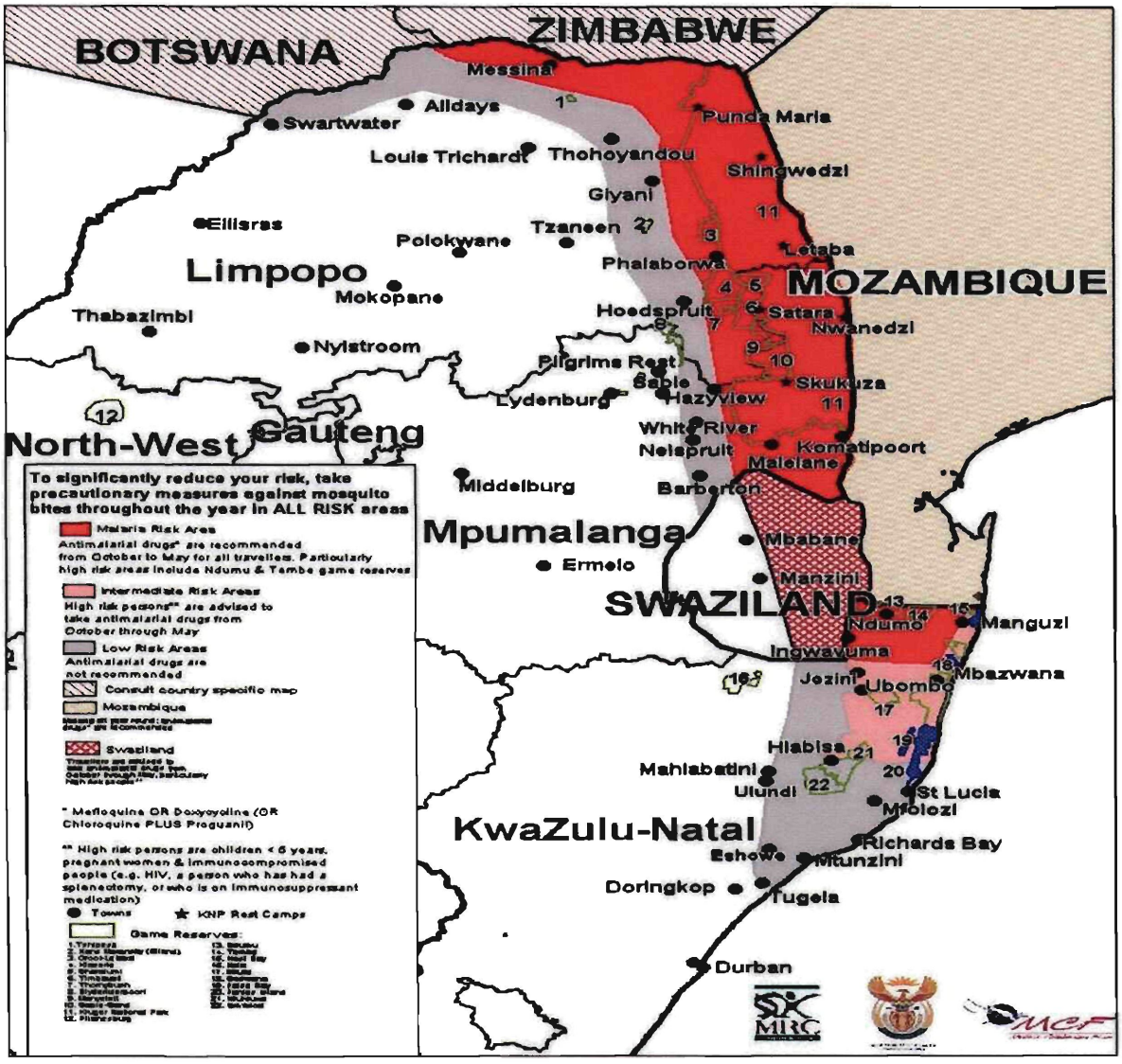


Figure 1.1 Depiction of malaria endemic areas in South Africa (Malaria in Southern Africa, Update 2005).

Due to environmentalist pressure and political debates, the use of DDT was discontinued in the late nineties with devastating effects. The malaria infection rate increased dramatically and this can evidently be seen in figure 2 representing data on annual reported malaria cases and deaths per year from 1971 to 2002. Resistance emerged towards synthetic peroxide insecticides (anti-mosquito insecticides) and sulfadoxine/pyrimethamine (malaria treatment drugs) which also contributed to the increasing infection rate (Tren & Bate, 2004).

The South African government was left with no other choice but to reintroduce DDT. This took place in KwaZulu-Natal, which was worst affected at the time, in 2000 with remarkable results.

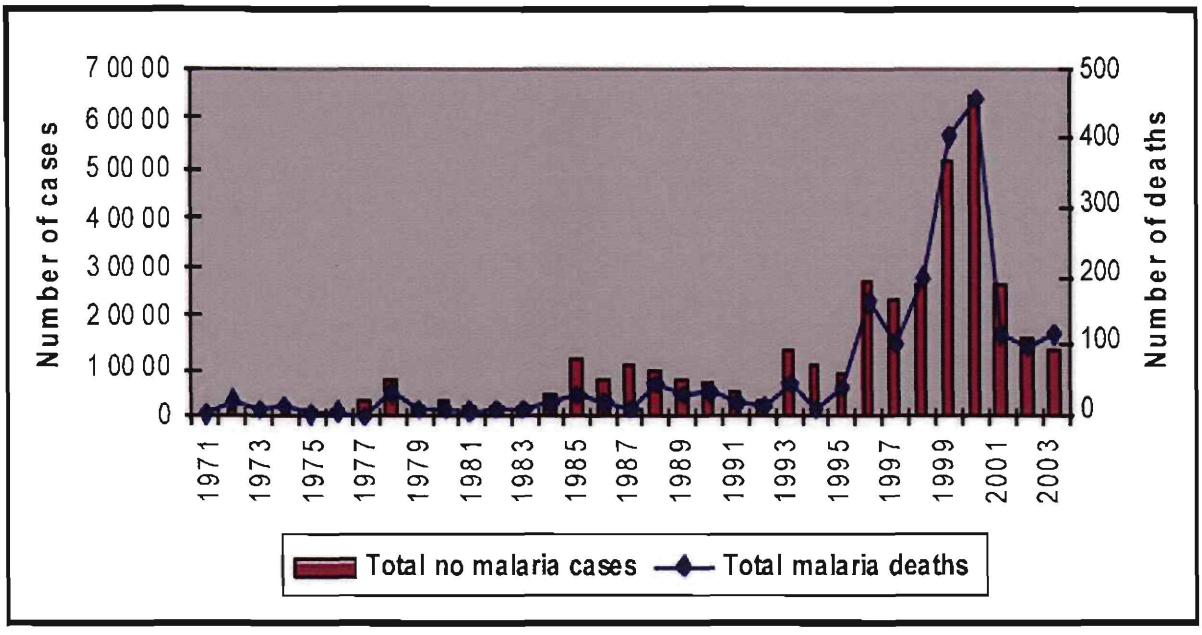


Figure 1.2. Documented annual number of malaria cases and deaths in South-Africa (1971-2003) (The South African Dept of Health, National Malaria Update, Dec 2003).

Currently, South Africa's malaria status is well within containable boundaries. Control measures, including chemoprophylaxis and treatment regimes, are firmly in place and with the scientific resources and adequate funding available, the South African Department of Health can ensure that this deadly disease will be managed according to international standards (Tren & Bate, 2004).

1.3 Epidemiology

The term epidemiology is defined by three very important factors:

- what causes the disease;
- malaria incidence, distribution; and
- disease control (CDC-(A), 2004).

Implementing control strategies to optimize preventative measures will be discussed in section 1.7.

1.3.1 Cause of the disease

Malaria is an infectious disease caused by parasites of the *Plasmodium* genus. The parasites are primarily hosted by female *Anopheles* mosquitoes, which act as vectors transmitting the protozoan organisms to humans when feeding (Quekett, 2005).

There are four known species that infect humans: *P. falciparum, P. vivax, P. ovale* and *P. malariae. P. falciparum*, however, can be held liable for the majority of severe cases and deaths that occur (Kakkilaya, 2006).

1.3.2 Incidence and distribution

The incidence of malaria is subjective to numerous variables. Climate changes and the presence of humans, female *Anopheles* mosquitoes and malaria parasites are of the most important key elements. Not only do they influence the incidence of the disease but also the global disease distribution (CDC-(B), 2004). Malaria can be found worldwide especially in the tropical areas of sub-Saharan Africa as seen in figure 1.3. It can clearly be seen that Africa is the continent worst effected by the disease. Other areas effected to a slighter degree are South-East Asia, Central America, South America, India and the Pacific Islands.

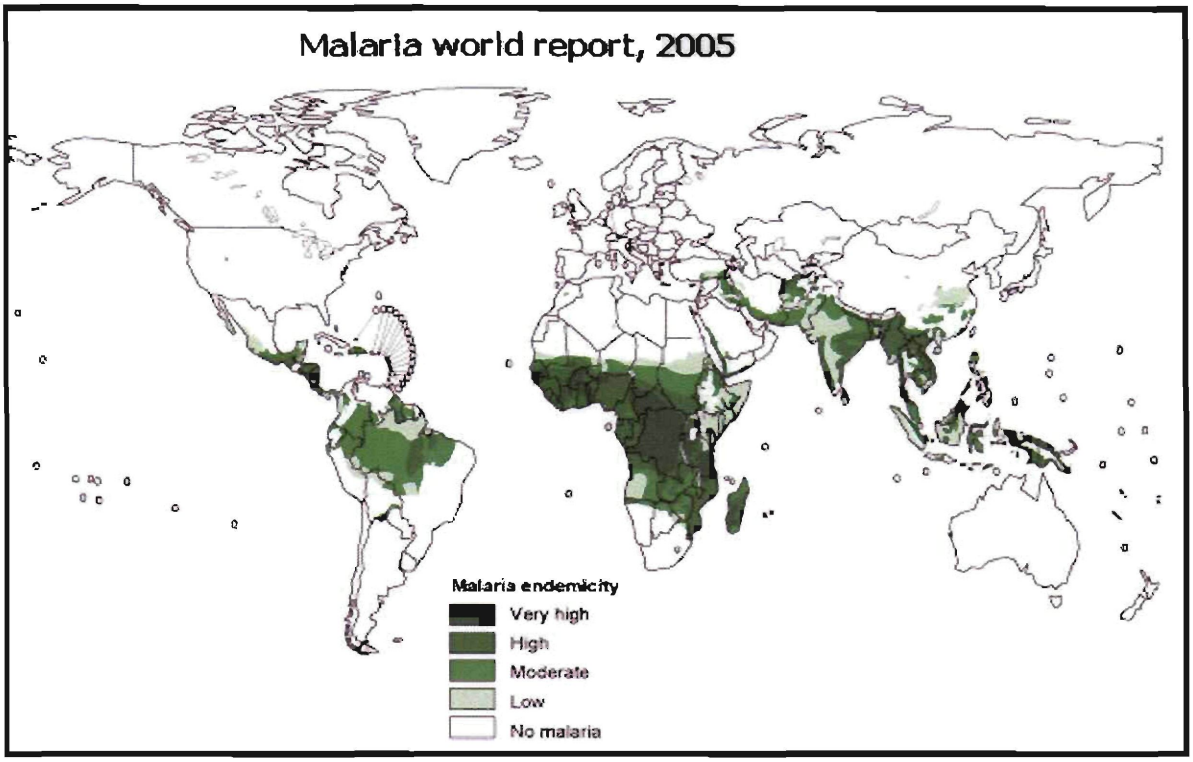


Figure 1.3 Areas of global malaria endemicity (World Malaria Report, 2005). The map is a depiction of malaria endemic areas indicating where incidence is at its highest.

According to statistics malaria now occurs in 90 countries. Three and a half billion people are fortunate enough to live in malaria free areas and 1.62 billion people live in areas where malaria is increasing. Of the previously mentioned 1.62 billion, 400 million people live in malaria endemic areas unchanged by control measures (Malaria in Southern Africa, Update 2005).

1.4 The parasitic lifecycle

The parasitic lifecycle of the malaria parasite is complicated and multifaceted. It can be separated into three dominant stages:

- pre-erythrocytic schizogony;
- erythrocytic schizogony; and
- sporogony (Wiser, 2003).

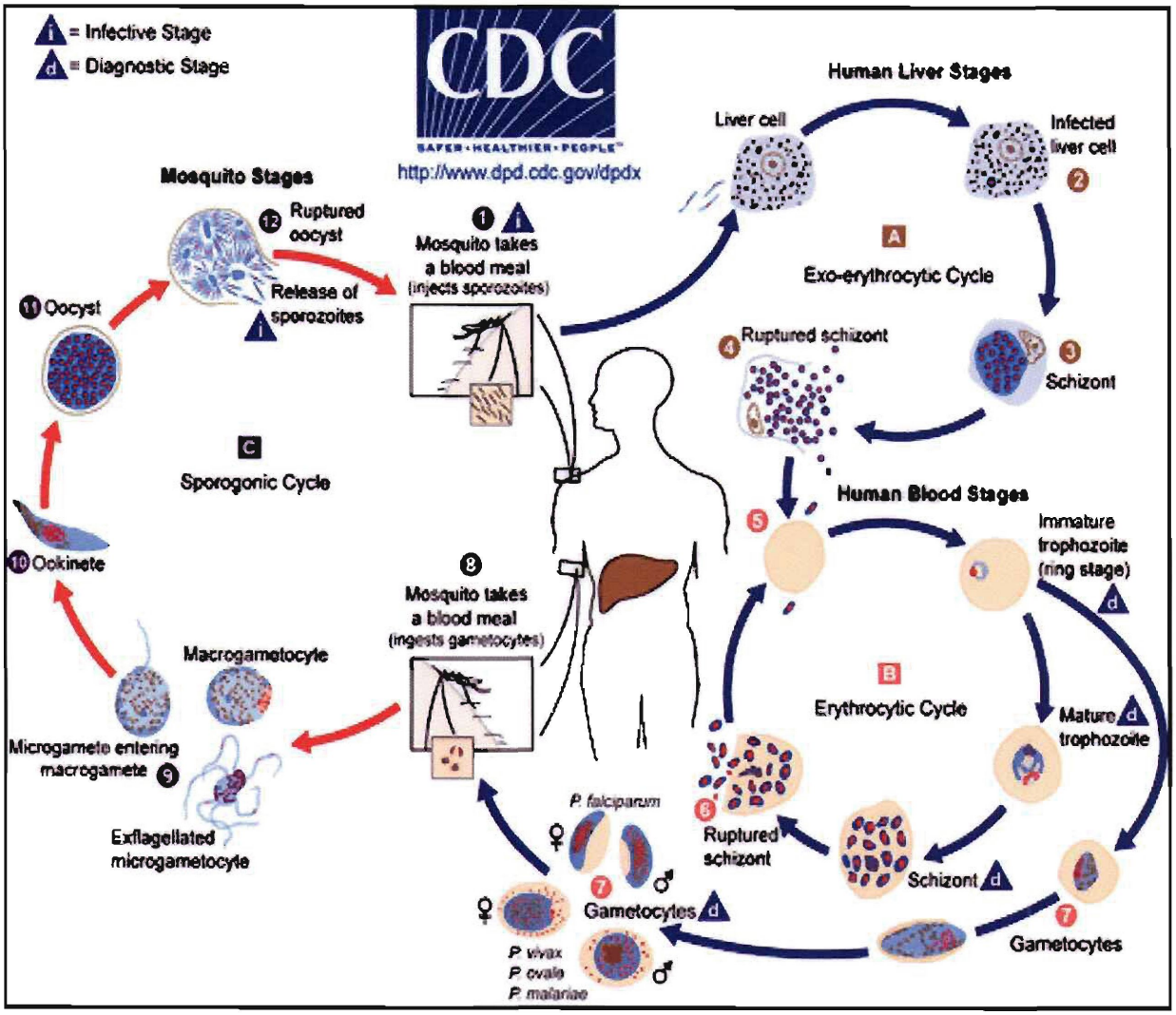


Figure 1.4 A schematic representation of the malaria parasite's life cycle (Adapted from CDC, 2004 – Schema of the presentation of life cycle of malaria, CDC-(B), 2004).

1.4.1 Pre-erythrocytic schizogony

Human infection is initiated when sporozoites, situated in the salivary glands of the mosquito, are injected into the host during a mosquito feeding. The sporozoites enter hepatic circulation and migrate to the liver where they invade hepatocytes (liver cells) and then undergo asexual replication. The replicative process of the sporozoites is called pre-erythrocitic schizogony and it produces progeny called merozoites in great numbers. The hepatocyte host cells rupture and release these merozoites into the circulation where they invade red blood cells (Wiser, 2003).

1.4.2 Erythrocytic schizogony

Once inside the erythrocyte the merozoites develop into (i) early trophozoite/ring form, (ii) trophozoites, and (iii) schizonts. Mature schizonts each contain approximately 20 merozoites. When lysis of the erythrocyte occurs these merozoites are then released into the blood stream to invade uninfected erythrocytes. When cell rupture occurs not only are the merozoites released but antigens and waste products as well, resulting in the intermittent fever paroxysms associated with the clinical symptoms of the disease. This cycle continues repetitively and in synchronisation every 48h for most *Plasmodium* species (Tuteja, 2007). A small amount of merozoites differentiate to generate micro- and macrogametocytes (female and male gametocytes, respectively). The gametocytes are inactive in human hosts but are vital for transmitting the disease back to the vector (Wiser, 2003).

1.4.3 Sporogony

When feeding on an infected human a mosquito may ingest gametocytes. In the midgut of the mosquito the gametocytes undergo gametogenesis to produce micro- and macrogametes. The gametes fuse and become fertilized to form a zygote. The zygote converts to an ookinete that is able to penetrate the cell wall of the midgut. An oocyst is then produced and undergoes sporogony. As end result a number of sporozoites are created which migrate to the salivary glands of the mosquito, ready to be transmitted to the next host (Wiser, 2003).

1.4.4 Symptoms and manifestations of malaria

The symptoms and manifestations of malaria characteristically present as periodic fever paroxysms that occur in 48 or 72 hour intervals. The severity of these paroxysms depend upon numerous factors such as the type of *Plasmodium* species causing infection and the immunity level and general health of the individual. It can be classified as uncomplicated or severe malaria. The paroxysms consist of three notable stages and are described in table 1.1. These symptoms are generally associated with uncomplicated malaria (Wiser, 2003).

Table 1.1 Classification of malaria paroxysm symptoms (Wiser, 2003).

Cold stage	• Experiencing an intense cold sensation • Extreme shivering • Elevated body temperature • Lasts between 15-60 minutes
Hot stage	• Experiencing an intense hot sensation • Elevated body temperature • Severe headache, nausea, fatigue, dizziness, anorexia, myalgia • Lasts between 2-6 hours
Sweating stage	• Profuse sweating • Abating body temperature • Exhaustion and fatigue • Lasts between 4-6 hours

Severe malaria generates more complicated manifestations and it occurs in 90% of all *P. falciparum* infections. In most cases it is fatal. Two distinctive features of severe malaria are cerebral malaria and severe anaemia. Other important presentations include the following:

- respiratory distress;
- renal failure;
- hypoglycaemia;
- circulatory collapse;
- coagulation failure; and
- impaired consciousness, prostration, jaundice, intractable vomiting (Parasitaemia ≥2%) (Pasvol, 2005).

1.5 Malaria diagnosis

Promptly diagnosing malaria is considered to be an integral part of efficiently treating the disease. Symptoms associated with uncomplicated malaria are not specific and can easily be confused with other general viral infections. Therefore a sound diagnostic

opinion should not only be based on physical findings but on laboratory testing as well. Various methods have been developed to aid the process (CDC- (C), 2004).

1.5.1 Microscopy

Microscopy is still considered to be the 'gold standard' for laboratory confirmation of the disease. A combination of thick and thin Giemsa stained blood smears are made and examined under a light microscope. Thick smears allow for the confirmation of parasites present and thin smears for specie identification and parasitaemia quantification. Where malaria is suspected, smears should be made every 6-12 hours for 2-3 successive days before a diagnosis is made. This diagnostic method is laborious, very time consuming and requires a relatively large quantity of culture reagents and parasites to conduct an accurate evaluation. It can be a useful method to implement in moderately equipped laboratories to obtain an initial evaluation of the possible anti-parasitic activity of a limited number of drugs (Gkrania-Klotsas & Lever, 2007; Basco, 2007).

1.5.2 Antigen detection methods

Antigen detection methods were first and foremost designed to be used in the field and to render fast results where microscopic methods are not available. They are more commonly known as 'Rapid Diagnostic Tests' (RDTs) or 'Malaria Rapid Diagnostic Devices' (MRDDs). These tests detect antigens such as histidine rich protein-2 (HRP-2) present only in *P. falciparum* infections or parasite lactate-dehydrogenase (pLDH) found in infections caused by all four *Plasmodium* species. RDTs have a very specific mechanism of action. Dye labelled antibodies bind to malaria parasites in a blood sample and are captured and made visible on a strip of nitrocellulose. When the microscopic dye particles accumulate on the nitrocellulose strip a visible control line will appear indicating whether parasites are present in the sample or not. Blood samples are obtained by a simple finger prick of the patient. RDTs are relatively expensive and their accuracy levels need to be improved but they provide an accurate and rapid diagnosis when required (Gkrania-Klotsas & Lever, 2007; WHO, 2004).

The use of rapid diagnostic test are strongly encouraged by the World Health Organisation especially in remote, poorly resourced malaria endemic areas. They have longer shelve-lives which reduces the possibility of wastage and lightens the pressure on supply lines (WHO, 2004).

1.5.3 Molecular diagnosis

A molecular diagnosis is based on polymerase chain reaction (PCR) techniques. PCR techniques identify *Plasmodium* DNA, mRNA and small subunit rRNA and can be used for diagnostic purposes or treatment follow-up evaluations. They are exceptionally sensitive and have the ability to distinguish between the different *Plasmodium* species and identify mixed infections. PCR techniques are costly to implement and require a great deal of proficiency in order to conduct the tests and to evaluate results (Gkrania-Klotsas & Lever, 2007).

1.6 Control strategies

Control strategies consist of various approaches to contain the disease and is considered to be a multi-faceted process. All approaches should be implemented concurrently to achieve optimum results.

1.6.1 Vector control

This approach to control the disease can be achieved by either (i) reducing vector density, (ii) interrupting the lifecycle of the mosquito or (iii) creating a barricade between the human host and the mosquito thus preventing the mosquito from feeding. In order to reduce vector density, biosystem modifications can be implemented to control problematic populations. Completely eradicating mosquito populations can be achieved by interrupting their life cycle specifically with organisms feeding on mosquito larvae, destroying breeding sites. A synthetic barricade refers to the usage of insecticide treated bed-nets, indoor residual spraying of insecticides, repellents and wearing protective clothing (Tripathi *et al.*, 2005).

1.6.2 Chemoprophylaxis

Chemorophylactic agents and can be categorized according to two mechanisms of action:

- inhibiting asexual blood stage development (chloroquine, mefloquine, doxycycline and primaquine); and
- inhibiting development of parasites in the pre-erythrocytic stage in the liver (atovquone-proguanil) (Ashley *et al.*, 2006).

There are a number of factors that need to be taken into consideration before prescribing malaria chemoprophylactic agents. The patient's medical history, drug safety and tolerability, drug efficacy due to patterns of parasite drug resistance and the level of malaria endemicity of the travel destination should all be taken into account. The patient should also be informed that even if the medication is administered correctly, chemoprophylaxis only provides 75%-95% protection (Checkley & Hill, 2007).

1.7 Malaria treatment

1.7.1 Drug resistance

"Drug-resistant malaria' can be defined as an infection that survives a deliberate attempt to eradicate it using a standard drug protocol." (Hastings & Watkins, 2006).

It materialises with evolutionary single or multiple point mutations in the *Plasmodium* genome rendering parasites that are drug insensitive (Shanks, 2006). The emergence and spread of this phenomenon has greatly affected the control and treatment of malaria in endemic countries especially concerning *P. falciparum* infections which account for most of the disease burden. It has been documented that strains of *P. falciparum* has reached stages of multiple drug resistance towards chloroquine, sulphadoxine-pyrimethamine and mefloquine which places major limitations on treatment options (Wongsrichanalai *et al.*, 2002).

Reasons for the development of drug resistance include drug-use patterns, compound characteristics, human host, parasite, vector and environmental factors (Wongsrichanalai *et al.*, 2002). Artemisinin based combination therapy is currently the treatment of choice for drug resistant malaria and it is of great importance that the

efficacy of this therapeutic regimen is maintained because no other effective alternatives exist to surmount this hurdle (Wongsrichanalai *et al.*, 2002).

1.7.2 World Health Organisation guidelines

The 'World Health Organisation (WHO) guidelines for the treatment of malaria' is an encompassing document that has been released by the WHO in 2006. It provides logical, international, evidence-based information in aid of developing policies and protocols for the effective treatment of malaria. Obtainable treatment regimens from the guidelines are focussed in particular on uncomplicated and severe malaria taking global drug resistance patterns and economic health service capacities into account.

Due to the fact that extensive drug resistance has developed towards mono-therapies, the WHO advises the use of combination therapy to counter this effect. Active compounds used as combination therapy should have different modes of action assuring that parasites present in the host will be eliminated by either one of the compounds or both. This increases treatment efficacy, shortens the duration of treatment and decreases the risk of drug resistant parasite formations. Artemisinin based combination therapies are at present considered unsurpassed in effectively treating all types of malaria infections (WHO, 2006).

1.7.3 Treatment objectives

The main treatment objectives include the following:

- alleviating symptoms;
- preventing disease relapse; and
- preventing disease distribution (Kakkilaya, 2006; CDC- (C), 2004).

In order to achieve these objectives the severity of the disease, type of infection, location of infection and the patient's medical history should be assessed in all presenting cases of malaria. The information gathered from such investigations are vital for prescribing the correct treatment especially if the patient should have a cardiac disease, suffer from epilepsy, be pregnant or present with any other health conditions (Kakkilaya, 2006).

1.7.4 Anti-malarial treatment regimes

1.7.4.1 South African treatment regimes

The following tables are adapted from the Standard Treatment Guidelines and Essential Drug List, 2003 Edition published by the South African Department of Health. It includes the treatment regimens for both uncomplicated (Table 1.2) and complicated (Table 1.3) *falciparum* malaria. It also summarizes the treatment regimens for non-*falciparum* malaria, including malaria caused by *P. malariae* (Table 1.4), *P. vivax* and *P. ovale* (Table 1.5).

Table 1.2 Treatment regimen for uncomplicated *P. falciparum* malaria.

<table>
<tr><th></th><th>First-line treatment</th><th colspan="3">Alternative treatment</th></tr>
<tr><td>Adults</td><td>Sulphadoxime/pyrimethamine (500/25mg):

Single dose of 3 tablets orally.</td><td colspan="3">Quinine:

600 mg tablets every 8 hours for 7 days.</td></tr>
<tr><td rowspan="2">Paediatric</td><td>Quinine:

10 mg/kg every 8 hours for 7-10 days.</td><td colspan="3">Initiated 2-3 days after Quinine treatment.

<u>< 8 years:</u> Clindamycin

10 mg/kg, orally, every 12 hours

<u>> 8 years:</u> Doxycycline

4 mg/kg orally and immediately followed by 2mg /kg for 7 days or until thin smears are negative. Taken with meals or a full glass of fluid.</td></tr>
<tr><td>Artemether-Lumefantrine:

Given orally with a fatty-based meal or liquid to ensure adequate absorption.</td><td colspan="3">First dose given immediately. Second dose after 8 hours and subsequent doses 2 times daily for 2 days.</td></tr>
<tr><td></td><td>Weight</td><td>Artemether</td><td>Lumefantrine</td></tr>
<tr><td></td><td>10-15 kg</td><td>20 mg</td><td>120 mg</td></tr>
<tr><td></td><td>15-25 kg</td><td>40 mg</td><td>240 mg</td></tr>
<tr><td></td><td>25-35 kg</td><td>60 mg</td><td>360 mg</td></tr>
<tr><td></td><td>> 35 kg</td><td>80 mg</td><td>480 mg</td></tr>
</table>

Table 1.3 Treatment regimen for severe and complicated *P. falciparum* malaria.

Adults	Quinine: 600 mg, orally, every 8 hours for 7 days.
	Quinine IV (1ml = 300 mg quinine salt): Loading dose: 20 mg/kg in dextrose 5% in sodium chloride solution 0.9%. 5 -10 ml/kg, depending on the patients fluid balance, over a 4 hour period. Maintenance dose: 10 mg/kg in dextrose 5% in sodium chloride solution 0.9%, 8 hours after the loading dose. Repeat the maintenance dose every 6 hours until oral medication can be given. Continue with treatment for 7 days or until thin smears are negative. **Plus/Either:** Doxycycline: 200 mg dose immediately starting on day 3 of quinine treatment. Thereafter 100 mg daily for 7 days or until thin smears are negative. **OR** Sulphadoxime-Pyrimethamine (500/25 mg): 3 Tablets given orally as a single dose on day 3 of quinine treatment.
Paediatric	Quinine IV Infusion: Diluted in 5 ml/kg dextrose 5% or NaCl 0.9%. Administer 20 mg/kg over 4 hours, then 10 mg/kg over 4-6 hours with 8 hour intervals until the patient can take oral treatment. **Then** 2-3 days after IV treatment was initiated and the patient can swallow Quinine: 10 mg/kg every 8 hours to complete a 7-10 day course. **Plus:** <u>< 8 years:</u> Clindamycin: 10 mg/kg orally every 12 hours for 7 days. <u>> 8 years:</u> Doxycycline: 4 mg/kg immediately, then 2 mg/kg daily for 7 days or until thin smears are negative. Treatment should be taken with meals or a full glass of fluid.

Table 1.4 Treatment regime for *P. malariae* malaria.

Adult	Chloroquine: 600 mg orally immediately, 300 mg after 6-8 hours, 300 mg 24 hours after the initial dose and 300 mg 48 hours after the initial dose.
Paediatric	Chloroquine: 10 mg (base)/kg administered as a single dose, then 5 mg (base)/kg 6, 24 and 48 hours respectively after the initial dose.

Table 1.5 Treatment regime for *P. vivax* and *P. ovale* malaria.

Adult	Chloroquine: 600 mg (base) orally immediately, 300 mg after 6-8 hours, 300 mg 24 hours after the initial dose and 300 mg 48 hours after the initial dose. **Followed by** Primaquine phosphate: 15 mg orally on a daily basis for 14 days.
Paediatric	Chloroquine: 10 mg (base)/kg orally as a single dose, then 5 mg (base)/kg given 6, 24 and 48 hours respectively after the initial dose.

From the data it can therefore be concluded that the following drugs are essential in the adult and paediatric treatment regimes of malaria in South Africa.

- **Uncomplicated *P. falciparum* malaria:**

  Adults - sulphadoxime/pyrimethamine.

  Paediatric – quinine and artemether/lumifantrine.

- **Severe and complicated *P. falciparum* malaria:**

  Adults – quinine, doxycycline and sulphadoxime/pyrimethamine.

  Paediatric – quinine, clindamycin and doxycycline.

- ***P. malariae* malaria:**

  Adult – chloroquine.

  Paediatric – chloroquine.

- ***P. vivax* and *P. ovale* malaria:**

  Adult – chloroquine and primaquine.

  Paediatric – chloroquine.

1.7.4.2 Internationally accepted treatment regimens

Table 1.6 – 1.11 are adapted from *Dr. B.S. Kakkilaya's Malaria Web Site*, Updated April 14, 2006 and provides a summary on the international treatment guidelines.

Table 1.6 Treatment regimen for uncomplicated malaria.

Age (Years):	Chloroquine: 1 Tablet = 150 mg base 5ml Suspension = 50 mg Base				Primaquine:		*SP:
	1st Dose	2nd Dose	3rd Dose	4th Dose	*P. vivax* / Mixed	*P. falciparum* Single Dose (14 days)	
0 – 1	75 mg	37.5 mg	37.5 mg	37.5 mg	Nil	Nil	¼ Tablet
1 – 5	150 mg	75 mg	75 mg	75 mg	2.5 mg	7.5 mg	½ Tablet
5 – 9	300 mg	150 mg	150 mg	150 mg	5 mg	15 mg	1 Tablet
9 – 14	450 mg	225 mg	225 mg	225 mg	10 mg	30 mg	2 Tablets
> 14	600 mg	300 mg	300 mg	300 mg	15 mg	45 mg	3 Tablets

***Sulphadoxime/Pyrimethamine**

Table 1.7 The recommended dose spacing for treatment with chloroquine.

	1st Dose	2nd Dose	3rd Dose	4th Dose
Starting treatment at mid-day	Stat	After 6 hours	After 24 hours	After 48 hours
Starting treatment by evening	Stat	After 12 hours	After 24 hours	After 36 hours
Starting treatment on the following day	Stat	2nd and 3rd doses given together after 24 hours		After 48 hours

Table 1.8 Parenteral chloroquine treatment for complicated, drug sensitive *P. falciparum* malaria.

Intravenous infusion	10 mg (base)/kg in isotonic fluid over a period of 8 hours (max ≤ 600 mg) followed by 15 mg/kg (max ≤ 900 mg) over a period of 24 hours.
Intramuscular/ Subcutaneous injections	3.5 mg (base)/kg every 6 hours (max ≤ 200 mg); or 2.5 mg (base)/kg every 4 hours (max ≤ 150 mg).

Table 1.9 Treatment of complicated or chloroquine resistant *P. falciparum* malaria with quinine.

Quinine	
Intravenous administration	Adults: 7 mg salt/kg over 30 minutes immediately followed by 10 mg/kg diluted in 10 ml/kg isotonic fluid over 4 hours. After a 4 hour interval 10 mg/kg over 4 hours repeated every 8-12 hours until the patient is able to swallow. **OR** 20 mg salt/kg diluted in a 10 ml/kg isotonic fluid, infused over 4 hours followed by 10 mg/kg over 4 hours repeated every 8-12 hours until the patient is able to swallow.
	Paediatric: 24 mg salt/kg diluted in a 10 ml/kg isotonic fluid infused over 4 hours, then 12 mg salt/kg over 4 hours every 8-12 hours until the patient is able to swallow.
Intramuscular administration	20 mg salt/kg diluted to 60 mg/ml by deep intramuscular injection (divide the dosage to two sites of administration). Then 10 mg salt/kg every 8 hours.
Oral administration	Adults: 600 mg (salt) 3 times daily for 7 days.
	Paediatric: +/- 10 mg/kg 3 times daily for 7 days.
A single dose of sulphadoxine-pyrimethamine or tetracycline **OR** doxycycline for 7 days (non-pregnant adults).	

Table 1.10 Treatment of complicated or chloroquine resistant *P. falciparum* malaria with artemisinin derivates.

Artemisinin Derivates	
Artemether 80 mg/ml Injection	Intramuscular administration: 3.2 mg/kg as loading dose, followed by 1.6 mg/kg daily for 5 days or until the patient is able to swallow. Maximum dose: ≤480 mg for adults and ≤ 9.6 mg/kg for children.
(Availible in 40 mg capsules)	Oral administration: 160 mg in two divided doses on the first day, then 80 mg/day for 5 days.
Artesunate 60 mg Powder with a 1 ml 5% sodium bicarbonate ampoule for injection	Parenteral administration: Reconstitute the powder in 1 ml of 5% sodium bicarbonate solution and dilute it with isotonic saline or 5% dextrose to a total of 3 ml for intramuscular and 6 ml or intravenous use. 2.4 mg/kg on the first day (additional 1.2 mg/kg after 4 hours in severe *P. falciparum* malaria), followed by 1.2 mg/kg daily for 7 days or until the patient can swallow.
(Availible in 50 mg tablets)	Oral administration: 100 mg on the first day of treatment, followed by 50 mg daily for 7 days.
Arteether 150 mg/2 ml Injection	Adults: 150 mg intramuscularly once a day for three days.
	Paediatric: 3 mg/kg once a day for three days.

Table 1.11 Treatment of complicated or chloroquine resistant *P. falciparum* malaria with other anti-malaria compounds.

Drug	Treatment
Mefloquine	15–25 mg/kg given as two separate doses 6-8 hours appart (max ≤1500 mg).
Tetracycline	250 mg four times daily for 7 days (patients > 8 years and non-pregnant).
Doxycycline	100 mg twice daily for 7 days (patients > 8 years and non-pregnant).

From the data, it can therefore be concluded that the following drugs are vital components of international treatment regimens.

- **Uncomplicated malaria:**

  Chloroquine, primaquine and sulphadoxime/pyrimethamine.

- **Complicated drug sensitive *P. falciparum* malaria:**

  Chloroquine.

- **Complicated or chloroquine resistant *P. falciparum* malaria:**

  Artemether, artesunate, arteether, doxycycline, mefloquine, tetracycline and quinine.

1.8 Conclusion

Statistics on malaria paint a very vivid picture of the burden of the disease in South Africa and other world countries. This disease is not only responsible for an enormous amount of deaths each year but also undermines the economic development of some of the poorest countries in the world. It has been determined by the Roll Back Malaria (RBM) initiative of the World Health Organisation that the annual economic growth of countries with a high malaria transmission rate has historically been lower than in countries without malaria and that malaria is responsible for a 'growth penalty' of up to

1.3% per year in some African countries (RBM, 2007). As a response to increasing levels of resistance to anti-malarial medicines, the World Health Organisation recommends that conventional mono-therapies, such as chloroquine, amodiaquine or sulfadoxine–pyrimethamine, should be replaced by combination therapies, preferably containing artemisinin derivatives (ACTs – artemisinin-based combination therapies) for *P. falciparum* malaria. The artemisinin derivates play a very important role in the treatment of the disease because of the drug's rapid therapeutic response and activity against multi-drug resistant *P. falciparum* malaria (Geyer, 2001).

Drug efficacy, economic viability, accessibility and patient compliance are all important factors that need to be taken into consideration when implementing treatment regimens, therefore each compound utilized in treating malaria should be carefully considered accordingly. If all preventative measures and treatment options are implemented as prescribed, we could be well on our way to avert epidemics and manage morbidity and mortality because of this disease.

CHAPTER 2

Anti-malarial Compounds and Efficacy Analysis

2.1 Introduction

The emergence and spread of chloroquine and multi-drug resistant parasites is the most important reason for treatment failures in malaria. The prevalence of this phenomenon markedly reduces our options of drugs to implement in treatment regimes. The dramatic increase in morbidity and mortality figures in recent years provides hard evidence as to the severity of the disease and the extent to which it affects the world population (WHO, 2006).

A great amount of emphases is therefore placed on the discovery, development and implementation of effective anti-malarial compounds (Fidock *et al.,* 2004). In this chapter four of the most important anti-malarial drugs namely chloroquine, mefloquine, artemether and artesunate will be discussed. Applicable drug pharmacokinetics, toxicities and mechanisms of action will be highlighted. In addition, the different drug assays used for the *in vitro* and *in vivo* measurement of the efficacy of these drugs will briefly be discussed.

2.2 Classification of anti-malarial compounds

Four compounds were chosen for the purposes of this study namely chloroquine, mefloquine, artemether and artesunate. These compounds are classified according to the stage of the parasitic lifecycle they affect (Summarized in Table 2.1) . There are four basic categories:

- **Blood schizontocides:** Eliminates parasites in the human red blood cells thus affecting the erythrocytic stage.
- **Tissue schizontocides:** Prevents invasion of malaria parasites into red blood cells in the pre-erythrocytic stage.
- **Gametocytocides:** Eliminates sexual forms of the parasites in hepatic circulation preventing re-uptake and thus infection of the mosquitoes.

- **Sporontocides:** Prevents sporogony from taking place in the mosquito (Sweetman, 2002).

Table 2.1. Principle anti-malarial compounds chosen for the purposes of this study (Sweetman, 2002).

Compound	Anti-malarial group	Activity
Chloroquine	4-Aminoquinolines	Blood schizontocide
Mefloquine	4-Methanolquinolines	Blood schizontocide
Artemether and Artesunate	Sesquiterpene lactones	Blood schizontocides

2.3 Chloroquine

2.3.1 Introduction

Chloroquine has been the drug of preference for malaria treatment and chemoprophylaxis since as early as the 1940's. The popularity of this compound is based on its efficacy, low risk of side-effects and cost effectiveness. It was extensively and negligently utilized in population-based dosing regimens in the 1960's. Thousands of tons were distributed to Brazil to be included in table salt formulations in a massive attempt to eradicate the disease. Before long drug resistance against chloroquine was reported and has since then, spread at a remarkable speed (Foley & Tilley, 1998). The chemical structure of chloroquine is depicted in figure 2.1.

Severe *P. falciparum* infections are unresponsive towards the drug but it can still be used to treat uncomplicated chloroquine sensitive *P. falciparum* infections as well as *P. ovale* and *P. malariae* infections (Wolff, 1997).

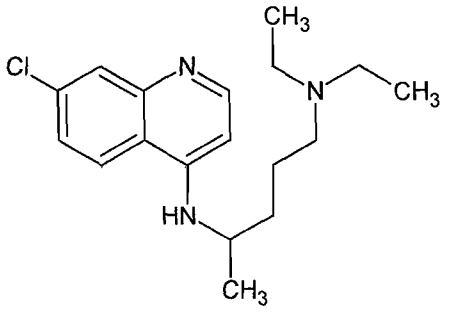


Figure 2.1 The chemical structure of chloroquine.

2.3.2 Pharmacokinetics

Chloroquine is a synthetic 4-aminquinoline, available in tablet form as a phosphate salt and as a hydrochloride injection. Absorption of the compound takes place speedily and almost entirely (90%) in the gastrointestinal tract (Kakkilaya, 2006) and even more swiftly following intramuscular and subcutaneous administration (WHO, 2006). Peak plasma concentration levels are reached within three hours after drug administration and it is distributed rapidly throughout the body including the placenta and breast milk. An initial loading dose is necessary to initially achieve the desired plasma concentrations. This is due to the fact that widespread sequestration of the compound takes place in the liver, spleen, kidneys and lungs rendering a 100-1000 L/kg volume of distribution. It is primarily metabolized in the liver to mono-desethylchloroquine and is excreted in the urine after a slow release from bodily tissues. Chloroquine has a half-life of three to five days and an elimination half-life of one to two months (Katzung, 2001).

2.3.3 Toxicity

Chloroquine has a narrow safety margin making it quite dangerous. An acute overdose can be lethal, but generally the drug is well tolerated by all users. There are two principle adverse effects that are limiting in practice and this is the compounds' unpleasant taste and prevalence of pruritus, which can be very severe in dark-skinned patients. Other less frequent adverse effects include headache, gastro-intestinal disturbances, nausea, vomiting, diarrhoea and various skin eruptions (WHO, 2006).

2.3.4 Mechanism of action

The mechanism of action of chloroquine as an anti-malarial compound remains uncertain and several hypothesis thereof exist. The hypothesis include: DNA intercalation, chloroquine inhibiting parasite haemoglobin degradation, the effect of chloroquine in the acidic parasitic food vacuole (weak-base theory) and heme polymerisation of which the last three theories are most likely to be possible (Foley & *Tilley, 1998*).

2.3.4.1 DNA intercalation

The 4-aminoquinolines, chloroquine in particular, demonstrate strong interactions with DNA. These compounds have the ability to inhibit DNA replication and RNA synthesis. It has been suggested that this ability might be part of the mechanism of action of these compounds. However, the plasmodial enzymes involved in DNA replication have been identified and do not appear to be direct targets of the mechanism of action of chloroquine (Folley & Tilly, 1998).

2.3.4.2 The inhibition of haemoglobin degradation by chloroquine

Chloroquine is classified as a rapid acting blood schizonticide which means that it is only active against mature schizonts in human red blood cells. At this stage of the life cycle, the parasites actively digest haemoglobin. It is therefore assumed that chloroquine in some way or other interferes with the feeding process of the parasites. By endocytosis the parasites ingest small amounts of haemoglobin from the host cytoplasm. Haemoglobin containing vesicles are formed and then transported to a secondary lysosome namely the food vacuole where the haemoglobin is digested. It is believed that the vacuole is the target site of action for chloroquine. Ultra-structural studies support the notion by proving that the food vacuole swells and accumulates undigested haemoglobin vesicles after parasite infected red blood cells are treated with relevant concentrations of chloroquine (Foley & Tilley, 1998; Hoppe *et al.*, 2004).

2.3.4.3 Weak-base theory

Chloroquine is a diprotic weak base and the entrapment of the compound in the acidic food vacuole of the parasite might be explained by an ion-trapping mechanism. When chloroquine is in its unprotonated form it crosses the membranes of the parasite infected red blood cells and via a pH gradient created by the food vacuole being acidic (pH 2.5), accumulates in the vacuole. When the compound molecules reach the vacuole they are protonated in the acidic environment and become membrane impermeable and therefore trapped in the acidic food vacuole (Foley & Tilley, 1998). The vascular pH is elevated by the presence of the now dibasic chloroquine molecules which may inhibit the activity of the parasitic digestive enzymes essential for the degradation process of haemoglobin (Wolff, 1997).

2.3.4.4 Heme polymerisation

The degradation of haemoglobin produces a secondary product known as free heme or ferriprotoporphyrin. It is toxic for the parasites and the induction of hemolysis of host red blood cells and lysis of malaria parasites can be accredited towards the presence of ferriprotoporphyrin. The parasites detoxify this product by converting it to non-toxic crystals of hemezoin with polimerase enzymes that catalyzes the conversion. Chloroquine binds with the ferriprotoporphyrin before this conversion takes place and forms a complex that has toxic effects on the parasites. Ferriprotoporphyrin can therefore be seen as a putative chloroquine receptor. Chloroquine also reduces the activity of the polimerase enzymes without inhibiting the formation of ferriprotoporphyrin from haemoglobin digestion leaving it to accumulate in the food vacuole and kill the parasites (Wolff, 1997; Sullivan *et al.*, 1998).

2.3.5 Chloroquine resistance

An understanding of the mechanism involved in chloroquine resistance is important as levels of resistance to chloroquine and other anti-malarial drugs are increasing. The mechanism involved in chloroquine resistance involve three possibilities. Firstly, it has been reported that drug resistant parasites release pre-accumulated chloroquine up to fifty times faster than any chloroquine sensitive strain, resulting in elevated levels of drug efflux (Bloland, 2001). Secondly an alteration of the parasitic food vacuole pH could compromise the pH gradient that stimulates chloroquine uptake into the vacuole

and thirdly the possibility that intracellular chloroquine transporters namely the chloroquine resistance transporter (CRT) and the P-glycoprotein homologue (Pgh1) might be missing or altered in the parasites molecular constituency (Becker & Kirk, 2004). *Plasmodium falciparum* species have shown the greatest measure of resistance towards chloroquine and the specific transporters involved have been identified as the *P. falciparum* chloroquine resistance transporter (PfCRT) and the multi-drug resistance-1 transporter (PfMDR1) (Valderramos & Fidock, 2006).

All these theories consequentially lead to the diminishing of chloroquine anti-malarial activity eliminating this valuable compound as an effective and affordable treatment option (Foley & Tilley, 1998).

2.4 Mefloquine

2.4.1 Introduction

Mefloquine hydrochloride is a synthetic 4-quinoline methanol that is chemically related to quinine. It is proven to have remarkable anti-malarial activity against all four *falciparum* species especially against chloroquine-resistant isolates but there is a growing concern about toxicity and emerging resistance developing towards the drug (Foley & Tilley, 1998). Figure 2.2 depicts the chemical structure of the compound.

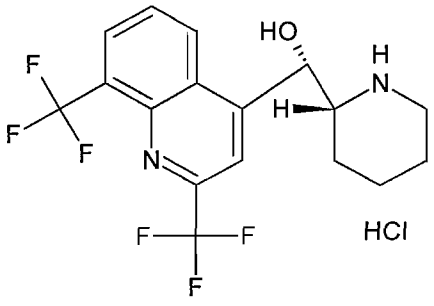


Figure 2.2 The chemical structure of mefloquine.

2.4.2 Pharmacokinetics

Mefloquine is well tolerated in oral dosage forms but can cause severe local irritations when administered parenteraly. It is absorbed reasonably well from the gastro-intestinal tract and within 18 hours of administration peak plasma concentrations are reached (Katzung, 2001). It is highly plasma bound (98%), undergoes entero-hepatic recycling, and is extensively distributed throughout the human body. The elimination half-life of mefloquine is approximately 21 days, which in the case of a malaria infection, is reduced to roughly fourteen days possibly because entero-hepatic circulation is disrupted during infection. The compound is metabolized in the liver and excreted primarily in the bile and faeces. Small amounts of mefloquine can also be found in breast milk (WHO, 2006).

2.4.3 Toxicity

Mefloquine is often prescribed for malaria chemoprophylaxis. Adverse effects experienced with weekly dosing include quite a number of conditions, most frequently nausea, vomiting, abdominal pain, anorexia, diarrhoea, headache, dizziness, loss of balance, dysphoria, somnolence and other sleeping disorders like insomnia and abnormal dreams. Neuropsyciatric disorders are of greater importance and can occur as frequently as 1 in every 200 patients treated in Africa and one in every twenty patients receiving mefloquine as treatment after a severe malaria infection, which should be a matter of grave concern (WHO, 2006).

2.4.4 Mechanism of action

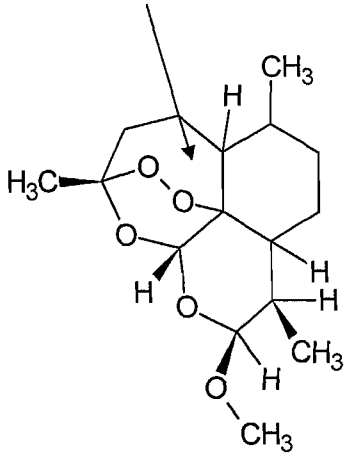
Like chloroquine, mefloquine is also classified as a blood schizontocide which targets the erythrocytic stage of parasite development. The mechanism of action of mefloquine has been compared to that of chloroquine but it is currently not clear whether these two anti-malarial agents share the same target sites. Ultra-structural studies suggest that mefloquine causes morphological changes in the parasitic food vacuole. These morphological changes involve the degranulation of hemozoin rather than the clumping of pigments as noticed with chloroquine. Mefloquine therefore alters a different step in the parasite feeding process than chloroquine. Studies also indicate that mefloquine inhibits heme polymerisation, but to a lesser extent than chloroquine (Foley & Tilley, 1998).

2.5 Artemisinin derivates: Artesunate and Artemether

2.5.1 Introduction

Artemisinins are plant extracts derived from the *qing hao* herb. *Qing hao* is also known as *Artemisia annua* or sweet wormmood and it has been used by Chinese civilisations for many centuries as a treatment for fever. It was discovered in 1971 at the Beijing Pharmaceutical Institute that an ether extract form *qinghao* had anti-malarial activity against *P. bergei,* a rodent malaria parasite isolate, and *P. cynomolgi,* a primate malaria parasite isolate. Since then a crystalline material was purified and the chemical structure of artemisinin determined, opening the way to well developed studies focusing on modifications to the artemisinin structure to improve solubility and stability. This led to the development of two of the most important compounds (chemical structures in figure 2.3) for the treatment of malaria namely artemether and artesunate (Haynes & Krishna, 2004).

Peroxide structure of artemether



Peroxide structure of artesunate

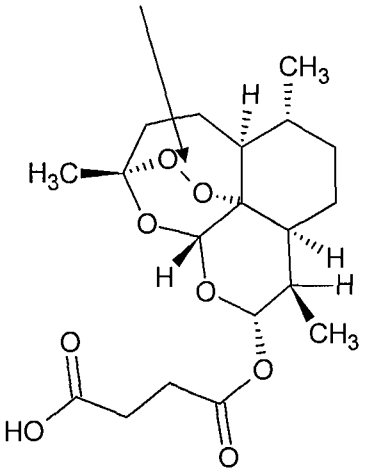


Figure 2.3 The chemical structures of artemether and artesunate.

2.5.2 Pharmacokinetics

Artemether is the more lipid soluble, methyl ether derivate of dihidroartemisinin and has a molecular weight of 298.4 g/mol. It is available in capsules and tablets of different strengths as well as ampoules of injectable solutions for parenteral use. Peak plasma

concentrations are reached within 2-3 hours after oral administration and generally after 6 hours when given as intramuscular injection. It should be noted that absorption can be slow and erratic and peak plasma concentrations in some cases, are only reached after 18 hours. It is 95% bound to plasma proteins and is transformed to its active metabolite, dihidroartemisinin when metabolized. Cytochrome P_{450} enzyme CYP_3A_4 mediates biotransformation of the compound. The elimination half-life of artemether is 1 hour but can be prolonged after intramuscular administration due to continued absorption (WHO, 2006).

Artesunate on the other hand is the water soluble sodium salt of the hemisuccinate ester of artemisinin. Although the compound is water soluble it has little stability in aqueous solutions at a neutral or acidic pH. It can be formulated and administered as tablets, rectal capsules, intramuscular and intravenous injections making it a very versatile compound. It is very rapidly absorbed. Peak plasma concentrations are reached within 1.5 hours, 2 hours and 0.5 hours respectively after oral, rectal and intramuscular administration. The percentage of drug that is plasma bound has not yet been determined. Artesunate is entirely converted to its active metabolite dihydroartemisinin and is very quickly eliminated from the hepatic circulation, with an approximate elimination half-life of 45 minutes (WHO, 2006).

2.5.3 Toxicity

The toxicity profiles of artemether and artesunate are similar to that of artemisinin. All of the above mentioned compounds are very well tolerated and are considered to be safe treatment options. Mild adverse effects like gastrointestinal disturbances, dizziness, tinnitus, reticulocytopenia, neutropenia, elevated liver enzyme values and electrocardiographic abnormalities have been reported. Type 1 hypersensitivity reactions, about 1 in every 3000 patients, are the only adverse reactions of a more serious nature that have been reported. Animal studies that where conducted with artemotil and artemether revealed neurotoxicity as adverse effect after high dosages of the compounds where administered but these results have not yet been substantiated in humans. How well artemisinins are tolerated during the first trimester of pregnancy have not yet been evaluated and treatment in pregnancy should therefore be avoided until more information regarding this topic is available (WHO, 2006).

2.5.4 Mechanism of action

Artemisinin and its derivates are currently the most valuable group of anti-malarial agents. Although these compounds are highly effective, no exact molecular target for anti-malarial activity has been identified and only speculations have been made regarding a specific mechanism of action. A few of the most popular hypotheses, encompassing diverse theories will be discussed.

The chemical structure of the artemisinins contain a peroxide structure within the 1,2,4-trioxane system which is vital for anti-malarial activity (Figure 2.3). Studies in mice have indicated that peroxides like *tert*-butyl hydroperoxide quickly, but selectively eliminate parasites within infected erythrocytes only by inducing haemolysis. Therefore this hypothesis on the anti-malarial activity of the artemisinins is based on the formation of reactive oxygen species (hydroxyl, alkoxyl, protonated superoxide or peroxyle radicals) within the infected erythrocytes. It is suggested that the presence of the reactive oxygen species are greatly enhanced by exogenous peroxide which will ultimately overpower the anti-oxidant defence system of the parasites. No such effects have been observed in unparasitized mice (Krishna *et al.*, 2004).

It has also been proposed that the formation of reactive oxygen species can be enhanced through the iron (Fe^{2+}) - dependent Fenton process. Fe^{2+} is a by-product of haemoglobin digestion by the malaria parasites and is found in the food vacuole. The peroxide bridge in artemisinins is cleaved by Fe^{2+} and this leads to an increased level of oxygen-centred or alkoxyl centred radicals and carbon-centred and neutral products or reactive intermediates. Theoretically these reactive intermediates show anti-parasiticidal activity when reacting with essential, sensitive bio-molecules in the parasites. Artemisinins are then, based on this proposed mechanism, considered to be pro-drugs that depend on the formation of active anti-malarial intermediates after ferrous interactions (Haynes & Krishna, 2004).

Lastly, a scientifically proven theory has been put to the test involving the selective inhibition of PfATP6, a Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase (SERCA), by artemisinins. PfATP6 is a SERCA ortologue of *P. falciparum* which transfers Ca^{2+} from the cytosol of the cell to the lumen of the sarcoplasmic reticulum. It is the only SERCA-type Ca^{2+}-ATPase sequence that can be found in the parasites genome. Thapsigargin is a sesquiterpene lactone like artemisinin and a very specific SERCA Ca^{2+}-ATPase inhibitor. Studies indicate that both thapsigargin and artemisinin inhibit PfATP6 with equal potency levels. The distribution of artemisinin in infected erythrocytes was

illustrated by using a fluorescent artemisinin derivate. The compound, like fluorescent labelled thapsirgargin, was distributed to membranous structures in the cytoplasm of the parasites and contrary to believe, did not localize in the parasitic food vacuole. It was also found that by adding a Fe^{2+} chelator together with artemisin, decreased the inhibition of PfATP6. All these results indicate that artemisinins inhibits the PfATP6 outside the parasitic food vacuole, after activation by iron (Eckstein-Ludwig, 2003).

2.6 Malaria drug *in vitro* sensitivity assays

In vitro drug sensitivity assays measure the intrinsic sensitivity of malaria parasites. All available assays are designed to determine how novel or existing anti-malarial compounds influence parasitic growth and development in an *in vitro* environment (Noedl *et al.*, 2003). It is a vital research tool than can be implemented in:

- drug development;
- investigating drug resistance and its effect on treatment failures;
- validating possible candidates as molecular markers of drug resistance;
- indirectly measuring plasma drug concentrations in bioassays; and
- examining trends in changing parasite drug sensitivity levels (Basco, 2007).

Existing assay systems can be categorised into three groups:

- a direct microscopic analyses involving the visual counting of parasites;
- The integration of parasites and radioisotope precursors; and
- non-radioactive techniques (Basco ,2007).

2.6.1 Direct microscopic and visual assays

Direct microscopic evaluation of parasiteamia is in essence the same as the diagnostic method described in section 1.5.1 (chapter 1). The 48-hour test and its variants, microtechniques and the WHO microtest, other alternative microtechniques and visual agglutination tests are all part of this category. With exception to the visual agglutination test all of these methods involve a physical examination of microscopic slides to determine parasiteamia levels of parasitized samples before exposure to anti-

malarial compounds and controls as well as afterwards. These tests are easy to perform, but controversy exists regarding the objectivity of the person doing the microscopic evaluation (Basco, 2007).

2.6.2 Radio-isotopic precursor assays

A number of radiolabelled precursors, including nucleic acids, proteins and phospholipids have been used to measure parasite mutation. In cultures that contain uninfected human erythrocytes and platelets combined with malaria parasites, the parasites are the only actively dividing cells. The erythrocytes and the platelets do not synthesize DNA, RNA, proteins or membranes and the leukocytes do not multiply, but tend to disintegrate over a few days. Incorporating radiolabelled precursors is therefore an indirect measurement of the parasites metabolic activities (Basco, 2007).

Examples of DNA precursors are [^{3}H]adenosine, [^{3}H]xanthine and [^{3}H]hypoxanthine. [^{3}H]Hypoxanthine is the principal purine base used by *P. falciparum* and also the preferred radioisotope to utilize *in vitro* (Basco, 2007). [^{3}H]hypoxanthine is also considered to be the "gold standard" of *in vitro* assays. The use of this precursor assures that the effect of compounds on parasitic growth can be measured with speed, accuracy and a remarkable sensitivity. This assay is diverse and can be applied for different applications including drug screening, experimental studies, evaluating parasite cross-resistance potential and the response of parasite field-isolates to anti-malarial compounds.

Before starting the assay, the malaria parasite culture has to be maintained under the most favourable conditions and the correct parasitaemia (0.25%-0.5%) must be established. [^{3}H]hypoxanthine can be added to each well containing infected erythrocytes in either different compound concentrations or a control medium, after the first 24-hours of incubation. After a 48-hour incubation period is completed the results can be analysed and quantified with a liquid scintillation counter (Basco, 2007).

2.6.3 Non-radioactive based assays

Non-radioactive techniques include methods such as fluorescence-activated cell sorter assays, fluorometric assays, ELISA-based and non-ELISA-based colorimetric methods (Basco, 2007). *In vitro* assays grant an objective view of inherent drug sensitivity in

comparison with *in vivo* assays because host-related factors such as host immunity and drug failure are excluded when evaluating obtained results. These methods are safe, essentially without any risk for patients and diversely applicable (Noedl *et al.*, 2003).

2.6.3.1 Flow cytometry

Flow cytometry is an automated non-radioactive assay method used to determine a variety of properties of cells suspended in a suitable medium as they flow, one by one, through a column. The assay can be rapidly conducted, it is objective, accurate and extremely DNA specific. A very important application of this method is to measure the sensitivity of *P. falciparum* to anti-malarial compounds. After a 48-hour incubation period of infected erythrocytes with test compounds have been completed, the samples are stained with a fluorescent DNA dye and analysed. Depending on the type of dye used, the stained cells are excited with an ultra-violet light or a laser. Uninfected erythrocytes possess no DNA content and will therefore show no measure of fluorescence. In contrast, infected erythrocytes fluorescence increases proportionally as trophozoites develop into schizonts as more DNA is produced. Results are given in the form of histograms showing the number of cell counts against increasing fluorescence intensity. Flow cytometry assays can differentiate between the various asexual stages of parasite development. It is recommended, however, that other assays must also be done in order to validate results obtained from flow cytometry (Basco, 2007).

2.6.3.2 Fluorometric assays

This assay is non-radioactive, accurate and easy to apply. All these qualities add to the possibility of implementing this assay in anti-malarial drug sensitivity studies in field laboratories as well as in drug screening. Anti-malarial compounds are tested in 24- or 96-well plates and no additional adding of reagent are required. Distilled water or saponin is added to the wells after the incubation period is completed to lyse the erythrocytes. The samples are centrifuged and the packed pellets are dissolved in guanidinium or sodium dodecyl sulphate solution and then stained with DNA-binding fluorochromes. A flow cytometer can be used to detect and measure the DNA content of the infected erythrocytes. Mini-fluorometers, fluorescence spectrophotometers or

fluorescence-activated microplate readers can also be implemented to measure the fluorescence intensity of the samples. All standard operating procedures should be performed by skilled technicians that are experienced in the manipulation of small volumes of reagents to ensure that results obtained are as accurate as possible. The available fluorometric assays are unfortunately not as sensitive as other methods (Basco, 2007).

2.6.3.3 Non-ELISA-based colorimetric assay

Lactate dehydrogenase (LDH) plays an important role in the carbohydrate metabolism of human malaria parasites. LDH metabolizes pyruvate to lactic acid and regenerates NAD, which is a necessity for the production of ATP. An essential biochemical characteristic of *Plasmodium* LDH (pLDH) that distinguishes it from human LDH is its ability to rapidly employ 3-acetylpyridine adenine di-nucleotide, which is a NAD analogue, as the coenzyme to produce pyruvate from lactate. Studies have indicated that by measuring pLDH activity in the presence of 3-acetylpyridine adenine di-nucleotide it can be determined whether *P. falciparum* is present or not. This colorimetric assay method is reliable and quick but is not sensitive enough for routine field application and it does not have the ability to differentiate between the phases of the parasitic lifecycle (Basco, 2007).

The assay is conducted in 24- or 96-well plates. The plates are frozen and thawed at the end of the incubation period and the haemolysed content of each well is transferred to a second well plate. Malstat™ solution consisting of 3-acetylpyridine adenine di-nucleotide coenzyme and L-lactate substrate is added and mixed with the well content. Nitro blue tetrazolium and phenazine ethosulfate are added in a final step in order to measure to what extent the reduced form of 3-acetylpyridine adenine di-nucleotide is produced. Nitro blue tetrazolium turns blue in its reduced form. Results are obtained with a spectrophotometer (Basco, 2007).

2.6.3.4 ELISA-based assays

Various assay methods have been developed in this category that involve non-radioactive procedures, detection of low parasitaemia levels and the cost-effective interpretation of obtained data. The assays can be performed in 24- or 96-well plates following methods used for radioisotopic assays, except that no radiolabelled precursor

has to be added. After incubation, aliquots from the content of each well is transferred to a 96-well microtitre plate in order to be washed and prepared for ELISA reading (Basco, 2007). ELISA (enzyme-linked immunosorbent assay) can be described as an immunochemical method that is able to detect small amounts of specific proteins. In an ELISA assay the antibody against the protein of interest is immobilized on a solid support. A solution that contains the protein is applied to the antibody-coated surface. The protein then binds to the antibody and any unbound proteins are washed away. A second antibody, which is covalently linked to an easily assayed enzyme, is added. Any remaining unbound antibody-linked enzymes are washed away. A product is formed when the enzyme catalyses a substrate and is detected by a spectrophotometer (Voet & Voet, 1995).

The same principles as those for the rapid diagnostic tests (section 1.5.2) to diagnose malaria were used to develop the double-site enzyme-linked lactate dehydrogenase immunodetection assay (DELI). To ensure complete haemolysis and release of pLDH, test plates are frozen and thawed three times after incubation is completed. A phosphate-buffered salt solution is used to dilute the lysate in each well between 1/20-1/200 times, a very important step to follow if the absorbance levels in drug-free control wells has to be obtained. The diluted lysate is transferred to a monoclonal antibody pre-coated 96-well plate. Standard ELISA procedures are performed with a second biotinalyted monoclonal antibody, streptavidin-peroxidase conjugate and peroxidase substrate (Basco, 2007).

Histidine-rich proteins (HRPs) is the focus point of another ELISA-based assay available. The method revolves around the detection and quantification of HRP II proteins produced specifically by *P. falciparum* parasites. HRP II is secreted into human erythrocytes and extracellular compartments actively during late ring and trophozoite stages of the parasitic lifecycle. Parasite metabolism and HRP II production is stopped when incubated with an active compound. HRP II is therefore used as an indicator of *in vitro* parasite multiplication. Standard ELISA procedures are followed as with the DELI assay, but the HRP II assay has fewer steps. A simplified protocol for field use was also implemented and a commercial kit is available for field studies (Basco, 2007).

2.7 Malaria drug *in vivo* assays

In vivo assays are the most traditional approach to assess the therapeutic response of test compounds. Assays are initiated with rodent models, of which the *P. bergei, P. yoelii, P. chabaudi* and *P. vinckei* are extensively used. There are quite a number of variables to take into consideration when implementing rodent or mouse models during experimental design and interpretation. The choice of rodent malaria and mouse strain can significantly influence the degree and course of infection while lethality and synchronicity directly affects test results. Table 2.2 lists the four mentioned rodent models and their key characteristics.

Table 2.2 The characteristics of malaria rodent mouse models (adapted from Fidock *et al.*, 2004).

	Rodent *plasmodium* species			
	P. berghei	*P. yoelii*	*P. chabuadi*	*P. vinckei*
First isolated	1948	1965	1965	1952
Cycle	Asynchronous	Asynchronous	Synchronous	Synchronous
Periodicity	22-25 hours	22-25 hours	24 hours	24 hours
Host cell	Reticulocytes	Reticulocytes	Mature red blood cells	Mature red blood cells
Merozoites per schizont	Twelve to eighteen	Twelve to eighteen	Six to eight	Six to twelve
Primary use	Drug screening	Liver stage biology and vaccine studies	Mechanism of drug resistance and antigenic variation	Not specified

Primate models also play an important role in preclinical drug development. Certain strains of *P. falciparum* are well characterized in *Aotus* and *Siamiri* monkeys. Primate models give accurate information and a clear prediction of human efficacy, pharmacokinetics and confirmation of the test drug candidate providing a coherent transition to clinical studies (Fidock *et al.*, 2004). For the purposes of this study only the

P. berghei mouse model was used. This model was ideal for drug screening with Pheroid™ technology and will be discussed in chapter 5.

2.8 Conclusion

The early diagnosis and prompt treatment of malaria is one of the most important procedural components of the global strategy to control this devestating disease. The effectiveness of this intervention is highly reliant on anti-malarial drugs like chloroquine, mefloquine, artemether and artesunate which should not only be safe and effective, but also available, affordable and acceptable to the population at risk. The rational use of effective anti-malarial drugs also is a key component which not only reduces the risk of severe disease, death and shortens the duration of the illness, but also contributes to slowing down the development of the parasite's resistance to anti-malarial drugs (Geyer, 2001).

In vitro and *in vivo* drug assays play an important role in the screening of drugs as part of the novel drug development process. Thereby possible new anti-malarial drugs can be identified. These assays are also vital for the continuous evaluation of existing drugs in order to determine whether they are still effective as anti-malarials against uncomplicated, complicated and drug resistant malaria (Fidock *et al.*, 2004; Geyer, 2001).

CHAPTER 3

The Pheroid™ Drug Delivery System

3.1 Introduction

The Pheroid™ (further referred to as Pheroid/s) drug delivery system is a novel, patented, colloidal system based on Pheroid technology. It is inimitable and very unique and can be described as a submicron emulsion type formulation that consists primarily of modified essential fatty acids (Grobler, 2004). In this study it was evaluated in terms of the possible enhancement of the therapeutic efficacy of anti-malarial compounds.

3.2 Structural characteristics

Pheroids are stable micron- and submicron-sized structures that are evenly distributed in a dispersion medium (continuous phase). Their size, structure and morphology can be modified in order to entrap, transport and deliver pharmacologically active compounds to selected target sites (Grobler, 2004). Each structure, or Pheroid, generally consists of three phases: an aqueous phase consisting of sterile water, an oil phase and a dispersed gas phase which is associated with the oil phase. The structure does not contain any phospholipids or cholesterol but has a characteristic lipid bi-layer. They are formed by a self-assembly process that is similar to that of low energy emulsions and micro-emulsions. No lyophillization or hydration of the lipid components are required (Grobler *et al.*, 2007).

The oil phase contains a combination of essential fatty acids, mostly ethylated and pegylated polyunsaturated fatty acids in cis-formation (Grobler *et al.*, 2007). Some of the functions of the fatty acids include cell membrane integrity maintenance, energy homeostasis, immune system modulation with for example prostaglandins and leukotriens and they play an important role in some of the regulatory aspects involved in programmed cell death. The Pheroid drug delivery system therefore has therapeutic qualities of its own (Grobler, 2004).

A unique component of the drug delivery system is the gas phase consisting of nitrous oxide (N_2O), which is distributed throughout the dispersion medium. Nitrous oxide is described as a volatile anesthetic compound that is soluble in water and in fat. It contributes to the miscibility of the fatty acids in the dispersion medium, the self-assembly process and the stability of formed Pheroids (Grobler *et al.*, 2007).

Molecular modeling studies indicate that there is a measure of interaction between the fatty acids and the nitrous oxide with stable Pheroid structures as result. The combination of these two components provide an efficient transportation model for hydrophobic and hydrophilic drugs. It was noted in controlled experiments that the stability and efficacy of various formulations had decreased dramatically if the essential fatty acids or the nitrous oxide was absent from the formulation (Grobler *et al.*, 2007).

3.2.1 Classification of the Pheroid drug delivery system

The chosen method of manufacturing as well as the specific compounds used in formulation determines the type of Pheroid produced. The three main types are:

1. lipid-bilayer vesicles in nano- and micrometer sizes;
2. microsponges; and
3. pro-Pheroid containing depots or reservoirs (Uys, 2006).

The lipid-bilayer vesicle sizes range from 80-300 nanometers and 0.5-1.5 micrometers. The microsponges range between 0.5-5.0 micrometers. The size of the Pheroid depots or reservoirs are determined by the amount of pro-Pheroid that is contained within the structure. Sizes and shapes obtained for the various Pheroid types are reproducible within a controlled environment (Steyn, 2006; Uys, 2006).

The micrographs in figure 3.1 which were obtained by confocal scanning laser microscopy clearly show active compounds entrapped in various types of Pheroids. Optical sectioning with the laser permits scanning through the structures without causing any physical interference. The Pheroids are made visible by labeling them with the fluorescent marker Nile Red. The fluorescence emitted by the Nile Red is detained in the red spectrum. The Pheroid structures are visualized at different depths because they are not all situated in the same focal plane (Grobler *et al.*, 2007)

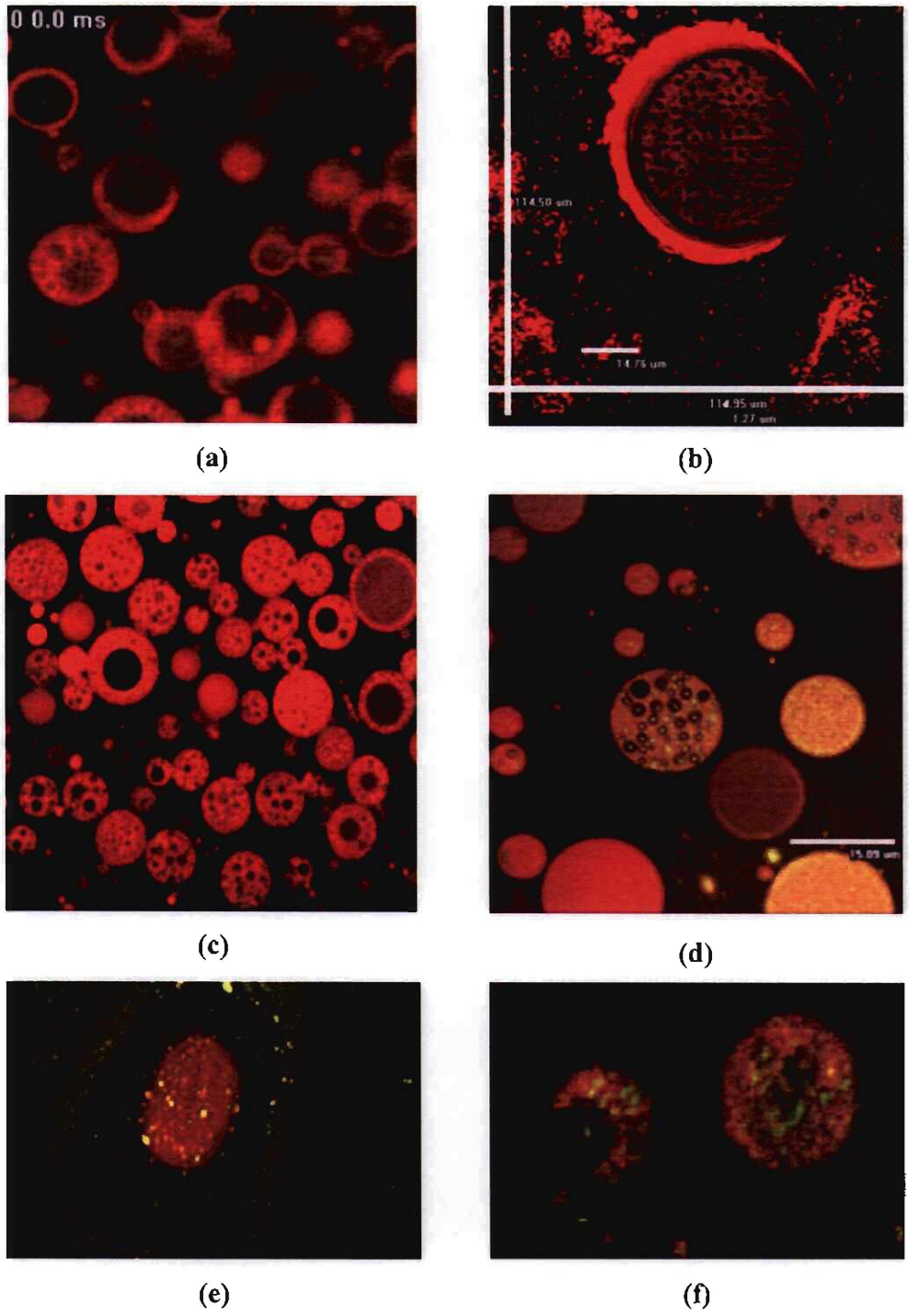


Figure 3.1 (a): A mixture of liposome-like bilayer vesicles and nanosponges with a mean diameter of 200 nm. Hydrophilic compounds can be entrapped in the central hydrophilic aqueous space of the nanosponges and hydrophobic compounds can be entrapped in the membrane area. **(b)**: A Pheroid microsphere, diameter of 35-45 microns, releasing its actives. **(c)** and **(d)**: Three phased multiple colloids, (w/o/w

emulsions), structures in (c) have sub-micron diameters and those in (d) up to 15 micron. **(e)**: A hydrophobic core depot formulation that is pH dependent. It contains the lipid phase of the Pheroid formulation, a surrounding hydrophilic zone and an outer vesicle-formation and release zone. They range in size from 5-10 microns. **(f)**: Red fluorescent Pheroid structures containing on average 13 auto fluorescent active compound molecules (Grobler *et al.*, 2007).

3.3 The Pheroid drug delivery system in comparison with other lipid-based delivery systems

Substantial differences can be seen when the Pheroid drug delivery system is compared to other conventional macromolecular carriers like lipid-based delivery systems, therefore it is very important that the Pheroid drug delivery system should not be confused with other existing delivery systems. Table 3.1 gives a summary of similarities, differences and main advantages of the Pheroid drug delivery system.

Table 3.1 Fundamental characteristics and main advantages of the Pheroid drug delivery system in comparison with other lipid-based delivery systems (Grobler, 2004).

The Pheroid delivery system	Other delivery systems
Pheroids, as seen with cytokine studies, don't provoke any immune responses in the human body.	Generally, some of the components of a delivery system are foreign substances to the human body, for example artificial polymers.
Pheroids do not cause cytotoxicity in cells and they contribute to the maintenance of cell membrane integrity.	Liposomal systems will either increase or be the cause of cytotoxicity in human cells and influence cell integrity.
All *in vitro* studies conducted showed that drug resistance was reduced or eliminated. A possible mechanism of action could be that Pheroids allow release of active compounds beyond the membrane zone and drug efflux pumps found in drug resistant organisms. Theories are still under investigation.	Other delivery systems tend to cause adverse immune responses and may contribute to drug resistance because their composition prevents release of active compounds beyond the drug efflux pumps found in resistant organisms.

Pheroids have polyphilic properties enabling active compounds with different solubility's, including insoluble drugs, to be entrapped by the system.	Most other drug delivery systems are either lipophilic or hydrophobic.
Pheroids reduce the volume of distribution of small molecule drug compounds resulting in increased concentrations of the active at the target site. A specific toxicity is decreased while the enhancement of a narrow therapeutic index can be achieved.	Liposomes that encapsulate small molecule chemotherapeutic agents have shown similar results.
Due to the nature of the Pheroid composition bioavailability of active compounds are enhanced by Pheroid inhibition of the drug efflux mechanism in the intestinal lumen.	There are no liposomal delivery systems that present with this feature.
Microsponge type Pheroids are well suited for combination therapies. One compound can be entrapped in the interior volume and the other in the sponge-like spaces of the structure. The geographical separation of active compounds reduce the risk of drug interactions and related adverse effects.	Combination therapies are of problematic nature in most drug delivery systems available.

3.4 Pharmaceutical applicability of the Pheroid drug delivery system

A variety of in depth studies were conducted with the aim of improving our understanding of this very unique delivery system. The efficacy enhancement of oral and parenteral therapies were tested with therapeutic compounds and preventative vaccines. The results obtained confirmed the following abilities of the delivery system:

- decreased time to onset of drug action;
- the increased delivery of active compounds;
- the reduction of the minimum inhibitory concentration of active compounds;
- the increase in therapeutic efficacy;
- reducing cytotoxicity;
- gene entrapment and transferal to cell nuclei ; and

- the reduction and suggested elimination of drug resistance (Grobler, 2004).

Some examples of the applications of Pheroid technology is described briefly below.

3.4.1. Therapy of tuberculosis

In vitro and *in vivo* studies conducted with drugs used in current tuberculosis treatment regimens have been tested in combination with the Pheroid drug delivery system with promising results. A bioequivalence study was performed to determine whether the Pheroid delivery system would remain as effective in oral administration as was proven to be in topical applications. Results revealed that there was an increase in the plasma levels of these anti-microbial drugs after oral administration which would suggest that the drugs entrapped in the Pheroid drug delivery system were absorbed to a greater extent from the gastrointestinal tract. The increased rate of absorption led to an increased cellular response. Therapeutic concentrations of the drugs entrapped in the Pheroids were maintained for longer and the circulatory time of the drugs were extended which would indicate that the exposure of the tuberculosis bacillus to the anti-microbials were increased (Grobler, 2004).

The minimum inhibitory concentration (MIC) of the drugs was decreased due to the fact that the delivery of the drugs to the target cells where increased by the Pheroid delivery system. A lower dosage of the drugs was used to obtain similar therapeutic effects and a decrease in side-effects were observed together with an increase in patient compliance. This could aid the prevention of multi-dug resistance (Gobler, 2004).

3.4.2 Vaccines

Vaccines are a vital part of disease control and prevention strategies. An indirect relationship has been observed for vaccine immunogenicity and safety. Human immune responses to synthetic and recombinant peptide vaccines administered with standard adjuvants tend to be poor, which makes it quite obvious that there is an urgent need for effective adjuvants to enhance the immunogenicity and immunostimulatory properties of vaccines (Grobler, 2004).

The efficacy of a commercially available *hepatitis B* vaccine and a *hepatitis* vaccine incorporated in a Pheroid formulation was investigated and compared. Non-

recombinant *hepatitis B* vaccines are generally based on the use of the surface molecules of the virus as antigen. For the comparative animal studies, different formulations of this peptide-based vaccine were used namely the peptide, the peptide with alum as an adjuvant and the peptide incorporated in a Pheroid formulation. The use of the Pheroid as a drug delivery system led to more than a 10-fold increase in the efficacy of the peptide-based *hepatitis B* vaccine as measured by an antibody response. The Pheroid has an obvious dual role in vaccinology, firstly as a delivery system for disease specific antigens and secondly as an immuno-stimulatory adjuvant (Grobler, 2004).

The efficacy of a commercially available virus-based *rabies* vaccine and a *rabies* vaccine incorporated in a Pheroid formulation was also investigated and results obtained were compared. An inactivated virus was used in the formulation of the *rabies* vaccines. For the comparative animal studies, different formulations of the virus were used, namely the inactivated virus incorporated in a Pheroid formulation and the inactivated virus with alum (aluminium hydroxide) as adjuvant. The inactivated virus incorporated in a Pheroid formulation showed a 9-fold increase in antibody response when compared to the other formulation (Grobler, 2004).

3.4.3 Peptide drugs

Pheroid technology proved to be an important factor in the delivery of peptide and protein drugs. Key advantages of the drug delivery system for such studies would include the increased delivery of active compounds, penetration of most known barriers in the body and cells and an increased therapeutic efficiency. Recent studies were done involving the delivery of the peptide drug calcitonin by using the Pheroid drug delivery system. Results obtained in one of the studies indicated that the quaternised chitosan derivative, TMC, showed the highest absorption enhancement of salmon calcitonin orally. Pheroid microsponges were also able to increased the absorption of the salmon calcitonin but to a slightly lesser extent (Uys, 2006; Lubbe, 2007).

3.5 The relevance of essential fatty acids in Pheroid technology formulations for the treatment of malaria

In certain malaria endemic areas, evidence has shown that some people are highly susceptible and others do not suffer or are resistant to *P. falciparum* malaria infection. The precise reason for this has not yet been determined. Neither cell-mediated or humoral immunity sufficiently explains this occurrence of events which raises the possibility that other factors like long-chain polyunsaturated fatty acids (LCPUFAs) and free radicals are involved. It has been reported in previous studies that the direct exposure of *P. falciparum* parasites to LCPUFAs like arachidonic acid ($C_{20\ 4,n-6}$), eicosapentaenoic ($C_{20:5,n-3}$), docosahexaenoic acid ($C_{22:6,n-3}$), docosahexaenoic acid methyl ester ($C_{22:6,n-3}$), docosanoic acid ($C_{22:0}$), linoleic acid ($C_{18:2,n-6}$) and oleic acid ($C_{18:1,n-9}$) has induced intra-erythrocytic degeneration and parasite death. Growth inhibition assays were conducted on all of the fatty acids mentioned and data collected showed that the ability of the fatty acids to inhibit *P. falciparum* varied and is partially dependent on the degree of unsaturation of the fatty acid structure. Arachidonic acid, eicosapentaenoic acid, linoleic acid and docosahexaenoic acid methyl ester all showed marked inhibition but the best results were achieved with docosahexaenoic acid which caused >90% parasite death repeatedly. Docosanoic acid and oleic acid showed little effect (Kumar & Das, 1999; Kumaratilake *et al.*, 1992; Kumaratilake *et al.*, 1997). It is important to note that essential fatty acids are a vital component of the Pheroid drug delivery system and therefore might exert the same therapeutic effects as discussed.

The effects of the essential fatty acid docosahexaenoic acid ($C_{22:6,n-3}$) was also evaluated on *P. berghei*. The study conclusively stated that the mice treated with this specific fatty acid showed significant decreases in parasitaemia levels throughout the course of the treatment. The parasitaemia was not only decreased but maintained at a lower level than the initial parasitaemia in comparison to two other groups, one treated with DPPS (DL-α-dipalmitoyl phosphatidylcholine) and the other with saline. Results from the fatty acid treated groups were much more favourable (Kumaratilake *et al.*, 1992). It is important to note that Pheroids also contain essential fatty acids and that this may be applicable in malaria treatment.

Not only has it been noted that LCPUFAs have a cytotoxic effect on *P. falciparum* but also that they are able to stimulate free radical generation (Kumaratilake *et al.*, 1992). Mammalian cells such as macrophages, neutrophils and mast cells have been shown to produce nitric oxide, a potent free radical. Nitric oxide (NO) and its oxidized forms

are classified as reactive nitrogen intermediates which have known antiviral and antibacterial activities *in vitro*. Studies were conducted to determine their anti-parasitic activity and clearly indicated that when nitric oxide interacts with oxygen, nitrite and/or nitrate are produced and that these reactive nitrogen intermediates are toxic to *P. falciparum* parasites (Rockett *et al.*, 1991). These facts are very important considering that the mechanism of action of compounds like the artemisinin derivates (section 2.4), artemether and artesunate, are also based on the generation of reactive oxygen species. This could hypothetically potentiate the measure of activity of the compound on intra-erythrocytic parasites targeting the parasites anti-oxidant defence systems. However, the Pheroid drug delivery system contains nitrous oxide and not nitric oxide and it should have no relevance in the treatment of malaria.

3.6 Conclusion

The Pheroid drug delivery system has unique characteristics which allows for a variety of pharmaceutical applications to be explored. It can be diversely applied to different areas of research. The Pheroid drug delivery system might play a prominent role in the possible enhancement of the efficacy of drugs used to treat infectious parasitic and bacterial diseases like malaria and tuberculosis and could play a vital role in the development of preventative treatments like vaccines.

CHAPTER 4

The Effect of Pheroid™ Technology on the Efficacy of Anti-malarial Compounds: *In Vitro* Evaluation

4.1 Introduction

In vitro studies are a vital component in the anti-malarial drug discovery process. It is primarily used to determine parasite drug susceptibility or the measure to which parasite growth is inhibited and is based on the cultivation of *P. falciparum, in vitro* in human erythrocytes (Fidock *et al.*, 2004; Noedl *et al.*, 2003). This research tool provides valuable information for the development and evaluation of current drug policies (Bosman *et al.*, 2001). This chapter describes how *in vitro* methods were implemented in this study to determine if the efficacy of existing anti-malarial compounds could be enhanced by entrapment in two Pheroid formulations namely Pheroid vesicles and Pheroid microsponges.

4.2 Methods and materials

4.2.1 Materials

RPMI-1640, D-(+)-glucose, HEPES, hypoxanthine, sodium chloride, sorbitol and gentamycin were purchased from Sigma (South Africa). Sodium bicarbonate and Giemsa stain were obtained from MERCK (South Africa) and Albumax II serum replacement from GIBCO (New Zealand). The EDTA vaccutainers used for blood collection are available from BD Biosciences (South Africa). Chloroquine phosphate, artemether and artesunate were all purchased from Sinoway Industrial CO. LTD. (Xiamen, China) and mefloquine hydrochloride from Sifaviton S.p.A (Mairano, Italy). A special gas mixture containing 5% O_2, 5% CO_2 and 90% N_2 was purchased from Afrox (Germiston). The two Pheroid formulations were supplied by the North-West University, Unit for Drug Research and Development (Potchefstroom).

4.2.2 Cultivation of *P. falciparum*

The complete culture medium consisted of 5.2 g RPMI 1640 basal medium, 2.0 g D-(+)-glucose, 3.0 g HEPES, 0.044 g hypoxanthine, 0.6 ml gentamycin (40 mg/ml), 18 ml 5% sodium bicarbonate solution and 2.5 g Albumax II as serum replacement made up to 500 ml with double distilled water. All components were filtered under a vacuum through a 0.22 µm filter. Wash medium, used to wash fresh erythrocytes and remove white blood cells, contains the same components except the Albumax II.

A RB-1 chloroquine resistant *P. falciparum* strain was used in this study. The strain was stored in liquid nitrogen for long-term purposes. The isolates were thawed in a waterbath at 37 °C and the content then transferred under sterile conditions to a centrifuge tube. After 12% NaCl and 1.6% NaCl solutions were respectively added and each solution mixed with the content of the centrifuge tube for ten to twenty seconds, fresh erythrocytes were added. The cells were then transferred to a 75 cm^3 culture flask (MERCK, South Africa), 10ml of preheated culture medium was added and the culture was gassed for a minimum of 30 seconds with a gas mixture containing 5% O_2, 5% CO_2 and 90% N_2. The flasks were sealed airtight and incubated at 37 °C.

Erythrocytes (group B^+) were obtained from human whole blood and collected in 10ml EDTA-vaccutainers with anticoagulant. The erythrocytes were washed with the wash medium and centrifuged to separate the serum and leucocytes from the erythrocytes. The top layer, supernatant and leukocyte buffy coat were removed and the process was repeated two to three times in an attempt to remove all the leukocytes. Leukocytes are detrimental to parasite growth. The erythrocytes were then re-suspended in wash medium and stored at 4 °C. These erythrocytes were uninfected and important for the routine cultivation of the culture as they are a source of nutrients as well as the site for parasite asexual replication.

The culture was synchronised in the ring stage by transferring the culture flask content into a 50 ml centrifuge tube and adding 4.0 ml of a 5% sterile sorbitol (preheated to 37 °C) solution to the parasite pellet. At least 20 ml of culture is required. It was mixed well and left for five minutes. As a last step 8 ml of a 8% glucose solution is added and mixed well with the culture and then centrifuged for five minutes at 2000 rpm. The supernatant was then removed; the cells re-suspended in culture medium in a culture flask, fresh erythrocytes were added and gassed with the special gas mixture before incubating once again.

The parasite cultures were maintained every second day by the replacement of culture medium and removal and replacement of erythrocytes until the parasitaemia reached 10.6% at 100% heamatocrit. It was then adjusted to a preferred 0.1% parasitaemia and 5.5% haematocrit for incubation purposes.

4.2.3 Pheroid formulations

Two Pheroid formulations were used in the experiment namely Pheroid vesicles in a 1:250 strength formulation and a full strength Pheroid microsponge formulation. The specific composition and characteristics of the formulations are kept confidential for commercial purposes. The negative control medium consisted of sterile water for injection.

Compound serial dilutions were made with the four test compounds in 24-well plates. The stock solution of each compound was prepared and continuously diluted with (i) Pheroid vesicles (1:250) dilution, (ii) Pheroid microsponges and (iii) control medium. Therefore three sets of the same concentration range of each compound was constituted for incubation with the *P. falciparum* parasites with only the diluent being varied. The test compounds were entrapped in the Pheroids by adding the desired amount of stock solution of each drug to the respective Pheroid formulations to obtain the desired concentration before the serial dilutions were made. The concentration ranges each consisted of 12 data points. The four test compounds were:

- Chloroquine phosphate (Sinoway) with concentrations between 0 nM-1000 nM.
- Mefloquine hydrochloride (Sifaviton) with concentrations between 0 nM-500 nM.
- Artemether (Sinoway) with concentrations between 0 nM-100 nM.
- Artesunate (Sinoway) with concentrations between 0 nM-50 nM.

The specific concentration ranges were chosen based on concentration ranges and IC50 values of the respective drugs from results of previous studies. *In vitro* anti-malarial studies done between 1998-2003 on the drug susceptibility on *P. falciparum* in Thai border areas with chloroquine and mefloquine used concentration ranges of 5-500nM and 1-200nM respectively. It also indicated the drugs' geometric mean inhibitory concentrations (GIC_{50}) to be 74.33 (45.2-122.2) for chloroquine and 22.2 (10.8-45.6) for mefloquine (Chaijaroenkul *et al.*, 2005). Studies conducted on *P.*

falciparum in Cambodia used chloroquine, mefloquine and artesunate in concentration ranges as follow; 5-5120 nM, 1-1024 nM and 0.05-512 nM for chloroquine, mefloquine and artesunate respectively. Drug GIC_{50} values were indicated to be 124.7 (11.4-972), 20.70 (4.5-110.1) and 1.06 (0.18-11.90) for the respective drugs (Lim *et al.*, 2004). In a study conducted with African isolytes the IC_{50} values for atemether were in a very narrow range 0.8-15.2 nM, the mean being 3.43 nM. The concentration range for the drug was established to be 0.8-100 nM (Pradines *et al.*, 1998)

4.2.4 *In vitro* growth inhibition assay

The procedure was carried out in 96-well plates. Each compound was incubated in a separate well plate and a row was left open on the plate between concentration ranges in order to minimize the possibility of cross contamination. A total volume of 200 µl per well was used; 180 µl was allocated for infected erythrocytes and 20 µl for the test compound in dilution. The compound dilutions were transferred form the 24-well plates to the 96-well plates under sterile conditions, the infected erythrocytes were added and then mixed well. The well plates were placed in incubation containers and gassed for approximately five minutes with the special gas mixture containing 5% O_2, 5% CO_2 and 90% N_2. Incubation took place in a CO_2 free incubator for 48 hours. After 48 hours the parasites have completed a life cycle and were in the ring stage again. The parasitaemia was determined using ring infected red blood cells.

4.2.5 Method of analysis

Microscopy was used as analytical method in assessing the efficacy of the test and reference formulations in this study. Thin film smears were made of each incubated sample by placing a drop of parasite culture obtained from a well in the 96-wellplate where incubation took place. The sample was smeared across the length of the slide by using a second slide and allowed to air dry. The dried smears were fixed with methanol and stained with Giemsa solution for 3-5 minutes. The Giemsa solution consisted of approximately 0.5 ml of phosphate buffer and ten to twenty drops of concentrated Giemsa stock solution. The smears were rinsed with water to remove unbound stain and the slide allowed to dry. The microscopic analysis was performed on a confocal microscope, using a 100x oil immersion objective for magnification (Nikon PCM 2000 with digital camera DMX 1200, The Netherlands). A drop of

microscope immersion oil was placed on the slide and then viewed. On average, 10000 cells were examined per slide. The % parasiteamia was determined for each slide, using the following formula:

$$\% \text{ Parasiteamia} = \frac{\text{Number of parasite infected erythrocytes}}{\text{Number of erythrocytes}} \times 100$$

The data was analyzed using Microsoft Excel™ and GraphPad Prism™. Results were presented as column graphs. Complete tables of the results are presented in Annexure A where exact parasitaemia percentages along with the corresponding drug concentrations and Pheroid formulations are stipulated. The area under the curve was determined with GraphPad Prism™ for each comparative data set, plotted and also presented as column graphs.

The area under the curve can be described as an integrated measure of a measurable effect or phenomenon. It is used as a cumulative measurement of drug effect in pharmacokinetics. GraphPad Prism™ computes the area under the curve by using the trapezoid rule which is a method for approximating a definite integral by evaluating the integrand at two points. The following formula is used to calculate the area under the curve:

$$\frac{\Delta X\,(Y1 + Y2)}{2}$$

It is used for each adjacent pair of points defining the curve. ΔX is the difference between the values on the x-axis of the adjacent points of the curve and Y1 anY2 would be their corresponding values on the Y-axis.

4.5 Results and discussion

4.5.1 Chloroquine

Chloroquine phosphate (CQ) was entrapped in Pheroid vesicles, Pheroid microsponges and dissolved in sterile water for injection. The concentration range of 0 nM to 1000 nM was used consistently throughout the experiment for all three of the formulations. Data obtained from the incubation of parasite infected erythrocytes with

both chloroquine in Pheroid vesicles and Pheroid microsponge formulations and chloroquine in water were compared. It is important to note that all the experiments with the four test drugs were conducted on a chloroquine resistant (RB-1) *P. falciparum* strain of human malaria with a starting parasitaemia of 0.1% (section 4.2.2).

Figure 4.1 illustrates the effects of (i) chloroquine in water and (ii) chloroquine entrapped in Pheroid vesicles on parasitaemia levels after a 48 hour incubation period. When evaluating the results obtained with the drug in water it is noticeable that the parasitaemia levels are high and that there is a great degree of fluctuation regardless of the dose concentration. The chloroquine in water formulation has little to none inhibitory effects on the growth of the parasites. Parasitaemia levels are more or less the same for the highest concentration (1000 nM) and for the 0 nM well containing no drug. The parasitaemia levels were 0.49% and 0.56% respectively. A sudden but slight increase in parasitaemia appears in die 25 nM concentration area, reaching 0.92% that might be attributed to experimental error.

The data obtained for the drug in Pheroid vesicles look much more promising. Parasitaemia levels of 0% were obtained from 200 nM to 1000 nM, therefore effective parasite growth inhibition is achieved at those concentrations. An elevated parasitaemia level can be seen at the 5 nM concentration (in agreement to the sudden but slight increase noticed in the control formulation at 25 nM) and this could also be attributed to experimental error. Overall the parasitaemia levels are much lower after incubation with the Pheroid vesicle formulation compared to the chloroquine in water formulation with levels ranging between 0.48% to 0% from the lowest to the highest drug concentrations for the Pheroid vesicle formulation.

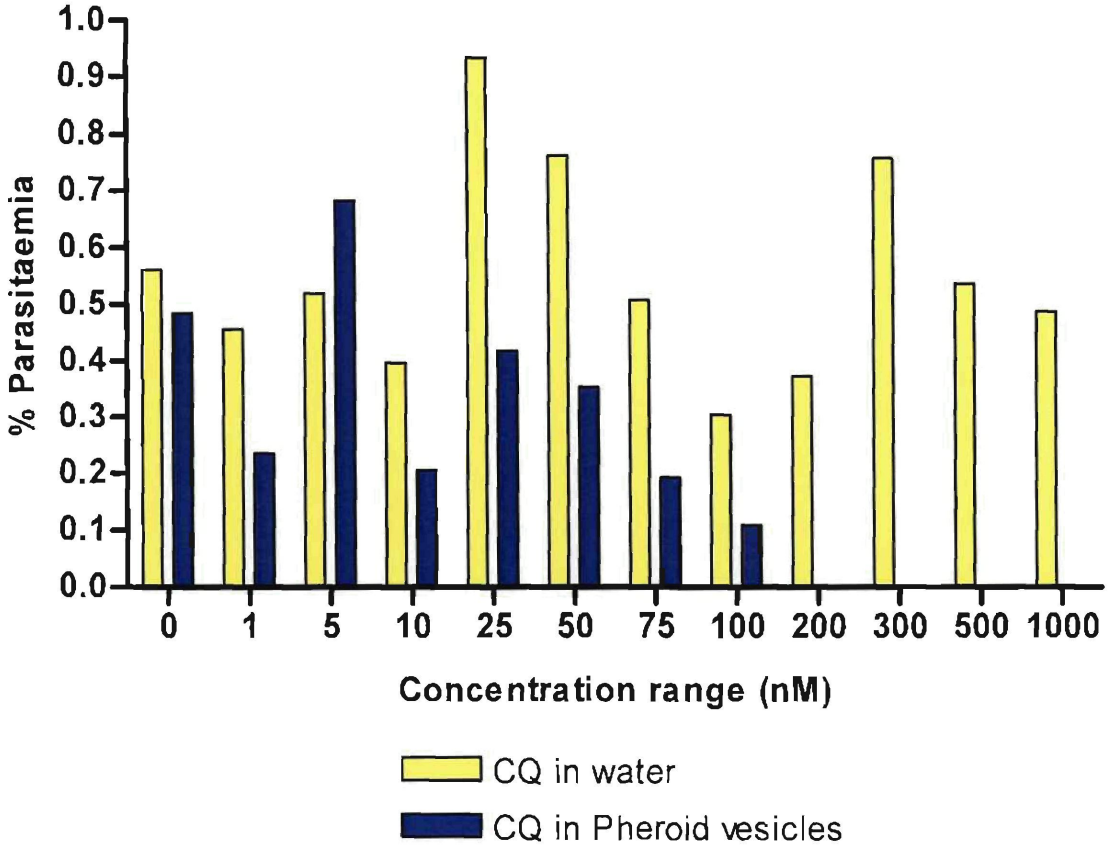


Figure 4.1 Parasitaemia determined *in vitro* for chloroquine (CQ) entrapped in a Pheroid vesicle formulation as well as in water with drug concentrations ranging from 0 nM to 1000 nM.

Figure 4.2 shows the effects of (i) chloroquine in water and (ii) chloroquine entrapped in Pheroid microsponges on parasitaemia levels after the 48 hour incubation period. The drug entrapped in the Pheroid microsponge formulation shows a marked increase in efficacy, as was the case with the Pheroid vesicle formulation but to a slightly lesser extent. Parasitaemia levels of 0% were also obtained with 300 nM to 1000 nM concentrations, showing evidence of parasite growth inhibition. The parasitaemia levels vary between 0.57% and 0% from the lowest to the highest drug concentrations for the Pheroid microsponge formulation.

From these results it can clearly be seen that chloroquine in water is not effective against the chloroquine resistant strain (RB-1), which was expected. Chloroquine entrapped in Pheroid vesicles and Pheroid microsponges increased the efficacy of

chloroquine dramatically. This is probably due to the action of Pheroids. Pheroids act by transporting the entrapped drug molecules across cell membranes and in this case probably across the parasite membrane. This probably bypasses the membrane transport mechanisms usually involved in transporting chloroquine for instance the *P. falciparum* chloroquine resistance transporter (PfCRT). This could probably lead to the reversal of chloroquine resistance but needs to be further investigated. It can also be seen from the results that chloroquine entrapped in Pheroid vesicles are more effective than Pheroid microsponges. This is probably due to the way in which chloroquine is entrapped in the Pheroid vesicles. The chemical structure of chloroquine may also play an important role in the entrapment mechanism.

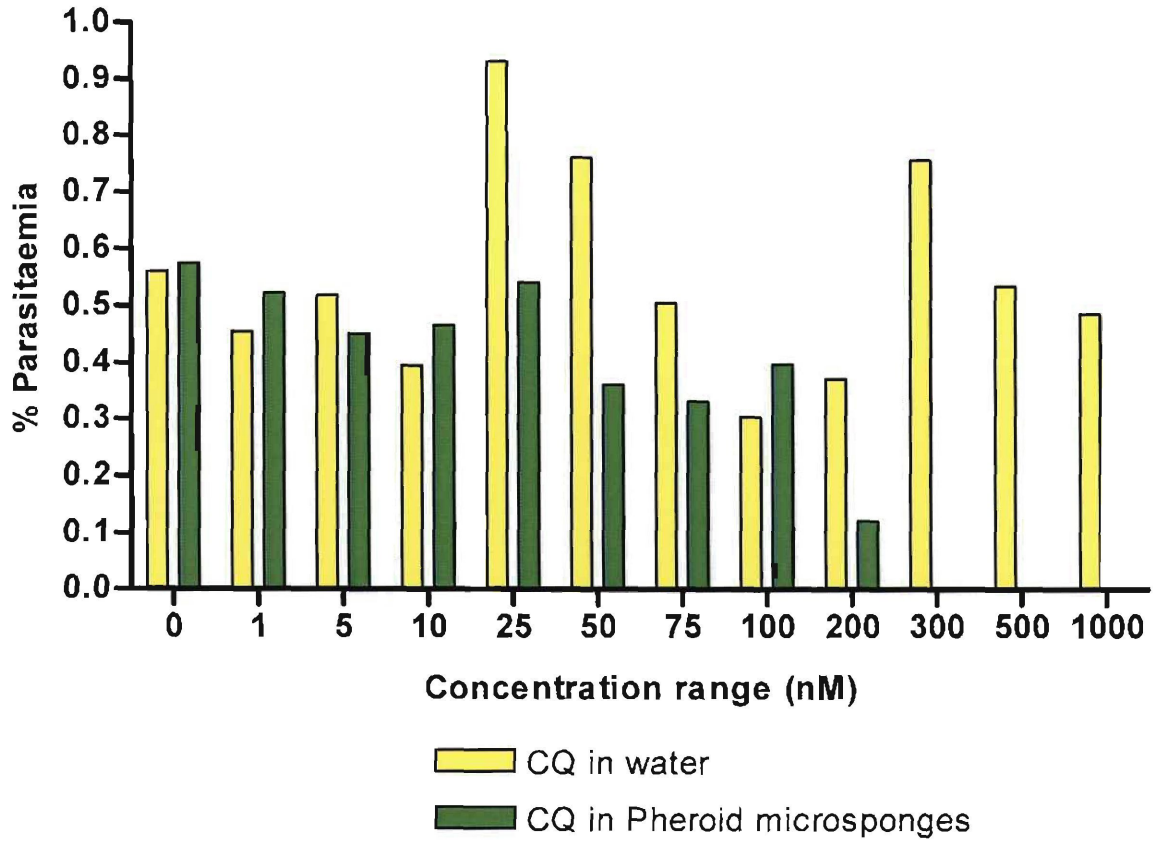


Figure 4.2 Parasitaemia determined *in vitro* for chloroquine (CQ) entrapped in a Pheroid microsponge formulation as well as in water with drug concentrations ranging from 0 nM to 1000 nM.

4.5.2 Mefloquine

Mefloquine hydrochloride (MQ) was entrapped in Pheroid vesicles, Pheroid microsponges and dissolved in sterile water for injection. The concentration range of 0 nM to 500 nM was used consistently throughout the experiment. Data obtained from the incubation of parasite infected erythrocytes with mefloquine in both Pheroid vesicle and Pheroid microsponge formulations and mefloquine in water were compared.

Figure 4.3 depicts the effects of (i) mefloquine in water and (ii) mefloquine entrapped in Pheroid vesicles on parasitaemia levels after a 48 hour incubation period. Parasitaemia levels for mefloquine in water are highest in the well containing no drug and in the well with the lowest concentration (1 nM) reaching 0.56% and 0.58% respectively. As the concentration of the drug is increased the parasitaemia levels decreased, showing that the drug in sterile water for injection (control medium) has an inhibitory effect on parasitic growth. There was no absolute suppression of parasitic growth observed in the control, the lowest parasitaemia levels are seen with the highest drug concentration (500 nM), and was determined as 0.82%.

Complete suppression of parasitaemia levels was observed with mefloquine entrapped in the Pheroid vesicles at 300 nM and 500 nM. The parasitaemia levels show a very slight but not significant increase between 0 nM and 5 nM and from there it decreases until 0% is reached at 300 nM with the exception of the sudden increase at 15 nM. This was also seen in graphs 4.1 and 4.2 with chloroquine and could be attributed to experimental errors made during execution of the study. Overall parasitic growth is inhibited largely with the Pheroid vesicle formulation compared to the control formulation, reaching 0.44% (0 nM) and 0% (500 nM) parasitaemia for the Pheroid vesicle formulation.

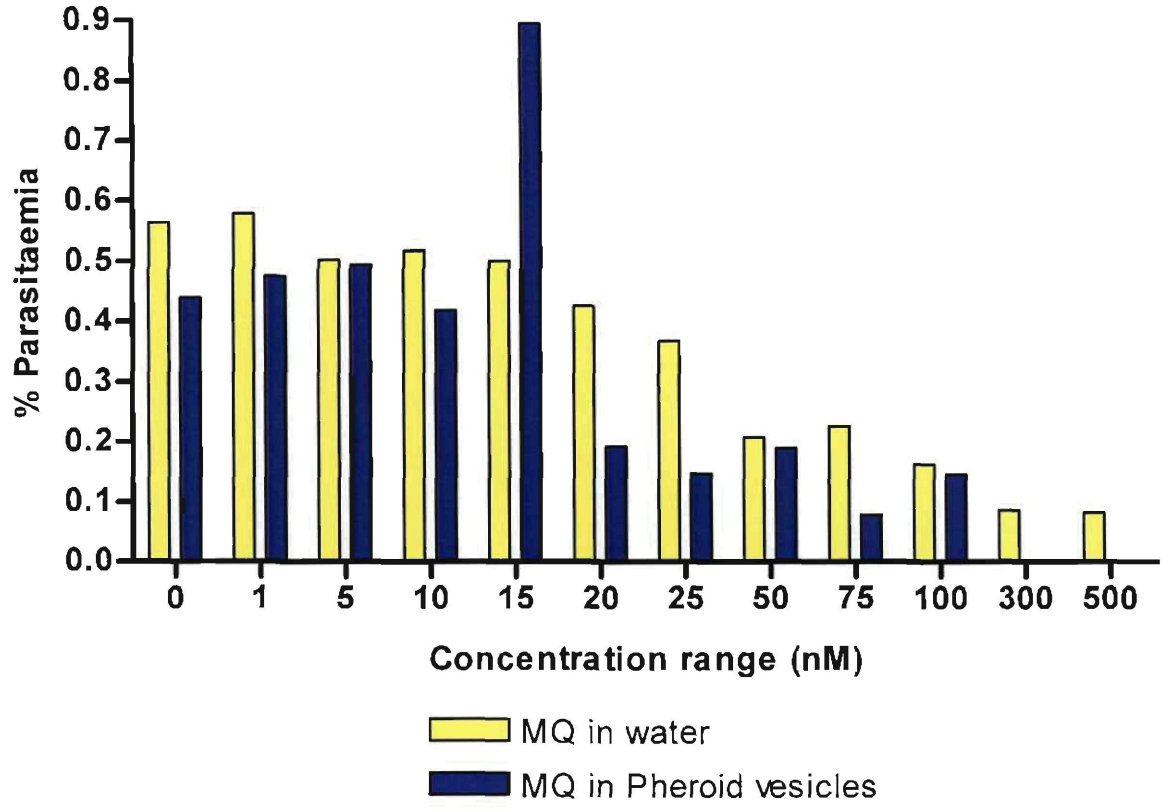


Figure 4.3 Parasitaemia determined *in vitro* for mefloquine (MQ) entrapped in Pheroid vesicles and in water with drug concentrations ranging from 0 nM to 500 nM.

Figure 4.4 gives the effects of (i) mefloquine in water and (ii) mefloquine entrapped in Pheroid microsponges on parasitaemia levels after the 48 hour incubation period. The effects of the drug in Pheroid microsponges were more pronounced compared to Pheroid vesicles. Complete parasite growth inhibition can be seen at concentrations of 100 nM to 500 nM. The parasitaemia levels range between 0.525% and 0.00% at 0 nM and 500 nM respectively. It is evident from these results that mefloquine in water is not completely effective against the chloroquine resistant strain. It does, however, seem to be more effective than chloroquine, resulting in a lower percentage parasitaemia. Mefloquine is a more lipophilic drug which appears to act on different targets than chloroquine (Foley & Tilley, 1998). This could possibly explain the results found with mefloquine in water.

Mefloquine entrapped in Pheroid formulations appears to be effective at high concentrations. Mefloquine entrapped in Pheroid microsponges appear to be more effective than mefloquine entrapped in Pheroid vesicles. This can possibly be attributed to the lipophilic nature and structure of mefloquine, whereby it is better entrapped in Pheroid microsponges.

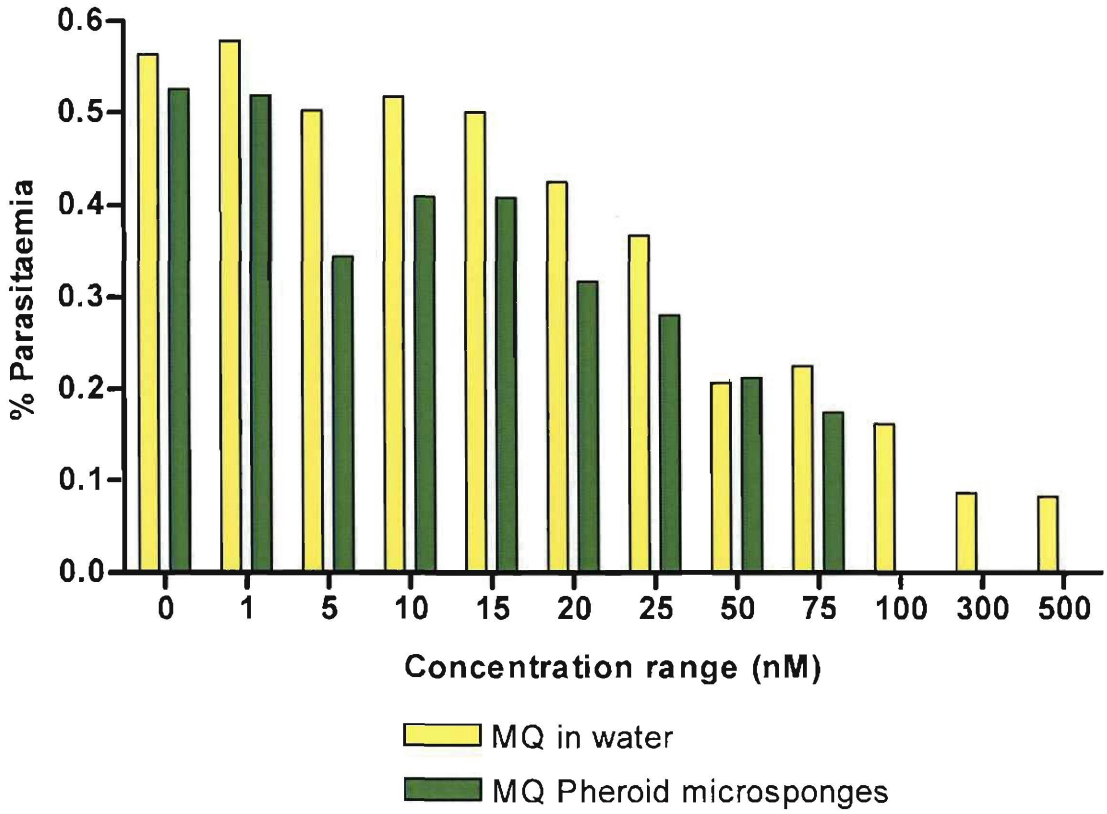


Figure 4.4 Parasitaemia determined *in vitro* for mefloquine (MQ) entrapped in Pheroid microsponges and in water with drug concentrations ranging from 0 nM to 500 nM.

4.5.3 Artemether

Artemether (AM) was entrapped in Pheroid vesicles, Pheroid microsponges and in sterile water for injection. The concentration range of 0 nM to 100 nM was used consistently throughout the experiment for all three formulations. Of the two artemisinin derivates under investigation, artemether is the more lipid soluble compound.

When evaluating the effects of this very active drug on parasitaemia levels, formulated with sterile water for injection, it was noted that the parasitaemia levels fluctuated but steadily decreased as the drug concentration was increased (figure 4.5). The parasitaemia percentage varied between 0.50% to 0% for 0 nM to 100 nM. Complete parasite growth inhibition could be seen only for the 100nM concentration of the drug, and this was not achieved by the incubation of chloroquine and mefloquine in sterile water for injection at the highest concentrations. Incubation with artemether entrapped in Pheroid vesicles showed lower parasitaemia percentages throughout the experiment when compared to the control formulation. Parasitaemia levels ranged from 0.34% to 0% for 0 nM to 100 nM concentrations of artemether. Complete parasite growth inhibition was achieved at 80 nM, 90 nM and 100 nM concentrations of artemether.

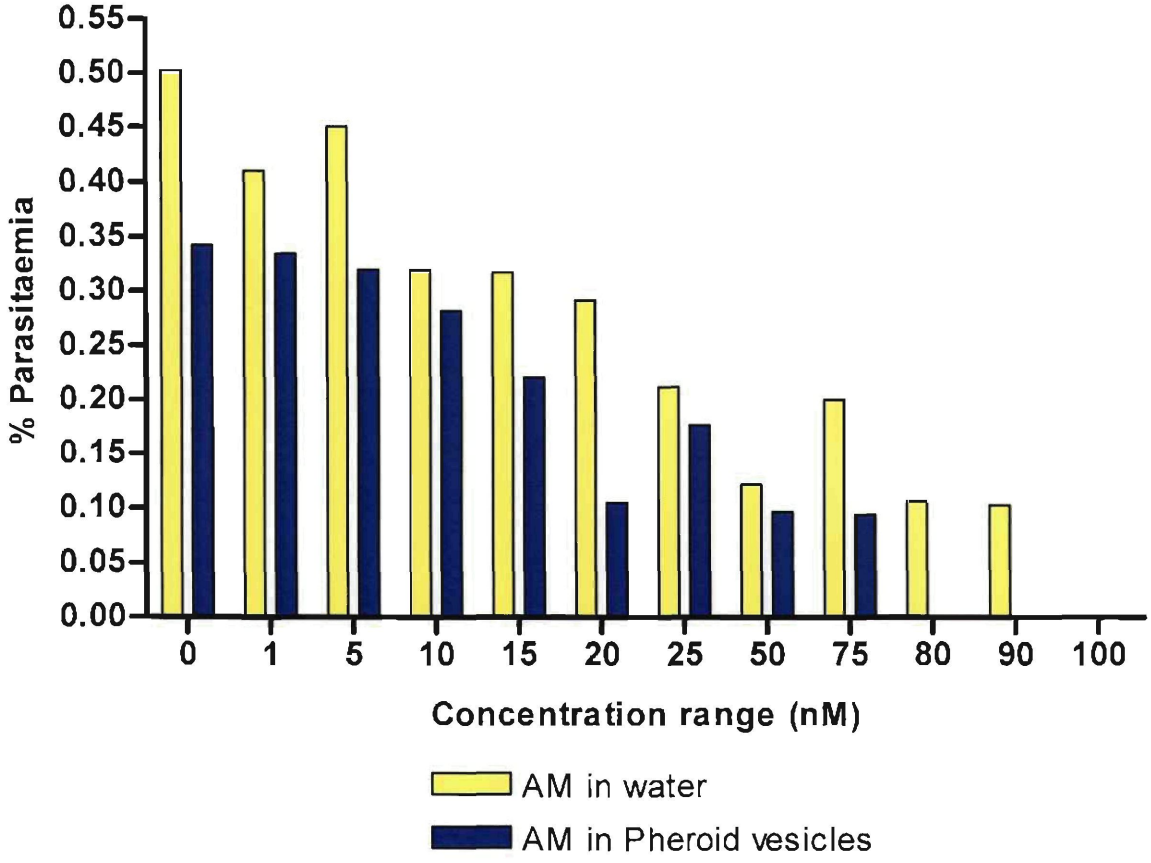


Figure 4.5 Parasitaemia determined *in vitro* for artemether (AM) entrapped in Pheroid vesicles and in water with drug concentrations ranging from 0 nM to 100 nM.

Figure 4.6 compares the results of artemether entrapped in Pheroid microsponges and the control formulation. Better results were obtained with artemether entrapped in Pheroid microsponges compared to artemether entrapped in Pheroid vesicles. Parasitaemia percentages varied between 0.48% and 0.1% for concentrations of 0 nM to 25 nM and from 50 nM to 100 nM 0.00% parasite growth was achieved.

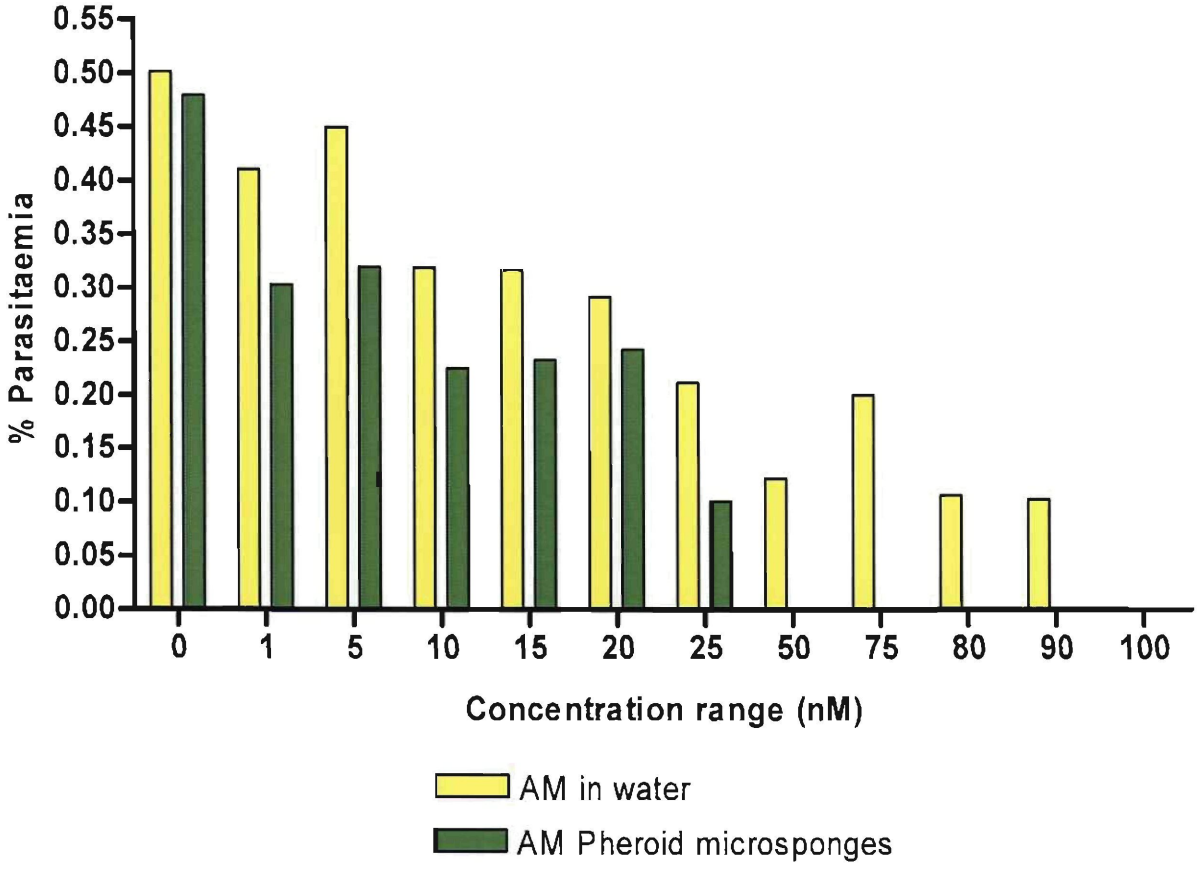


Figure 4.6 Parasitaemia determined *in vitro* for artemether (AM) entrapped in Pheroid microsponges and in water with drug concentrations ranging from 0 nM to 100 nM.

The results indicate that artemether in water is effective against the chloroquine resistant strain. It is also active at much lower concentrations than chloroquine and mefloquine. More important, however, is the fact that the minimum inhibitory concentration of artemether was significantly reduced by the Pheroid formulations. Artemether entrapped in Pheroid microsponges appears to be more effective than

artemether entrapped in Pheroid vesicles. Complete inhibition of parasite growth was seen at a 50 nM concentration for Pheroid microsponges and only at 80 nM for Pheroid vesicles.

4.5.4 Artesunate

Artesunate (AS), which is more water soluble compared to artemether, was entrapped in Pheroid vesicles, Pheroid microsponges and in sterile water for injection. The concentration range of 0 nM to 50 nM was used consistently throughout the experiment for all three of the formulations.

The initial parasitaemia for the 0 nM concentration in the control formulation was higher compared to the other drugs and was measured as 0.67% (figure 4.7). The parasitaemia decreased from 0.67% to 0.10% at 40 nM. No parasitic growth could be seen for the 50 nM concentration. The biggest percentage difference in parasitaemia could be seen between the 0nM concentration and the 1 nM concentration. The 0 nM well only contained sterile water for injection without any artesunate. The parasitaemia decreased from 0.67% to 0.46% when artesunate was added to the formulation. The parasitaemia levels gradually decreased from 1 nM to 40 nM as the drug concentration was increased, giving 0% growth at the highest concentration tested (50 nM).

The results also show that the initial parasitaemia percentage is much lower for drug entrapped in Pheroid vesicles compared to that of the control formulation. The 0 nM well for the Pheroid vesicle formulation contained only Pheroid vesicles with no drug. The parasitaemia percentage was determined to be 0.46%. The parasitaemia levels also gradually decreased until it reached 0.07% at 15 nM, thereafter 0% parasitaemia for concentrations of 20 nM to 50 nM was observed.

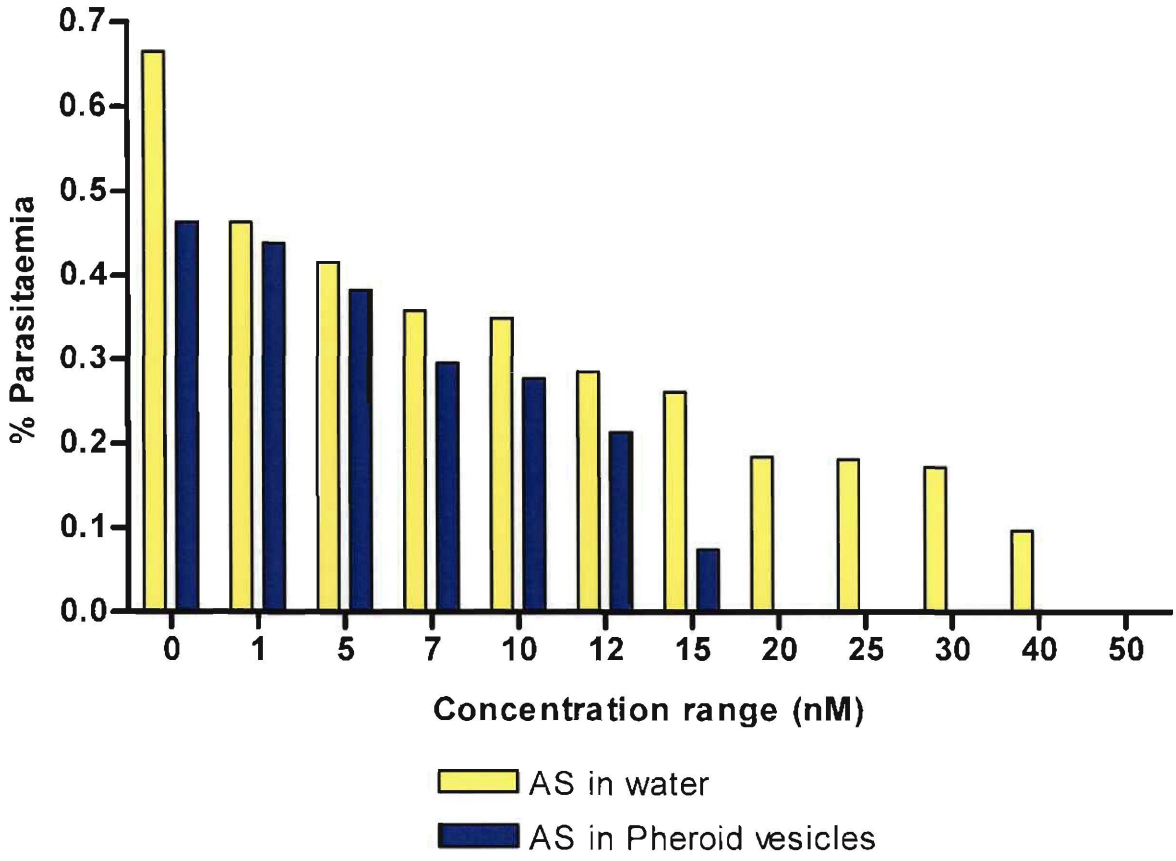


Figure 4.7 Parasitaemia determined *in vitro* for artesunate (AS) entrapped in Pheroid vesicles and in water with drug concentrations ranging from 0 nM to 50 nM.

The results depicted in figure 4.8 show that the initial parasitaemia with the drug entrapped in Pheroid microsponges are very low, reaching only 0.32%. The drug entrapped in the Pheroid microsponge formulation shows a marked increase in efficacy as was seen with the Pheroid vesicle formulation, but slightly less compared to the Pheroid vesicle formulation. Parasitaemia levels of 0% were also obtained between 25 nM to 50 nM concentrations of artesunate, showing evidence of parasite growth inhibition. The parasitaemia levels vary between 0.32% and 0.10% for the 0 nM to 20 nM concentration range. There is a steady decrease in the parasitaemia percentages as the dose of the drug is increased. Parasitaemia levels are therefore directly proportional to the dose of the drug.

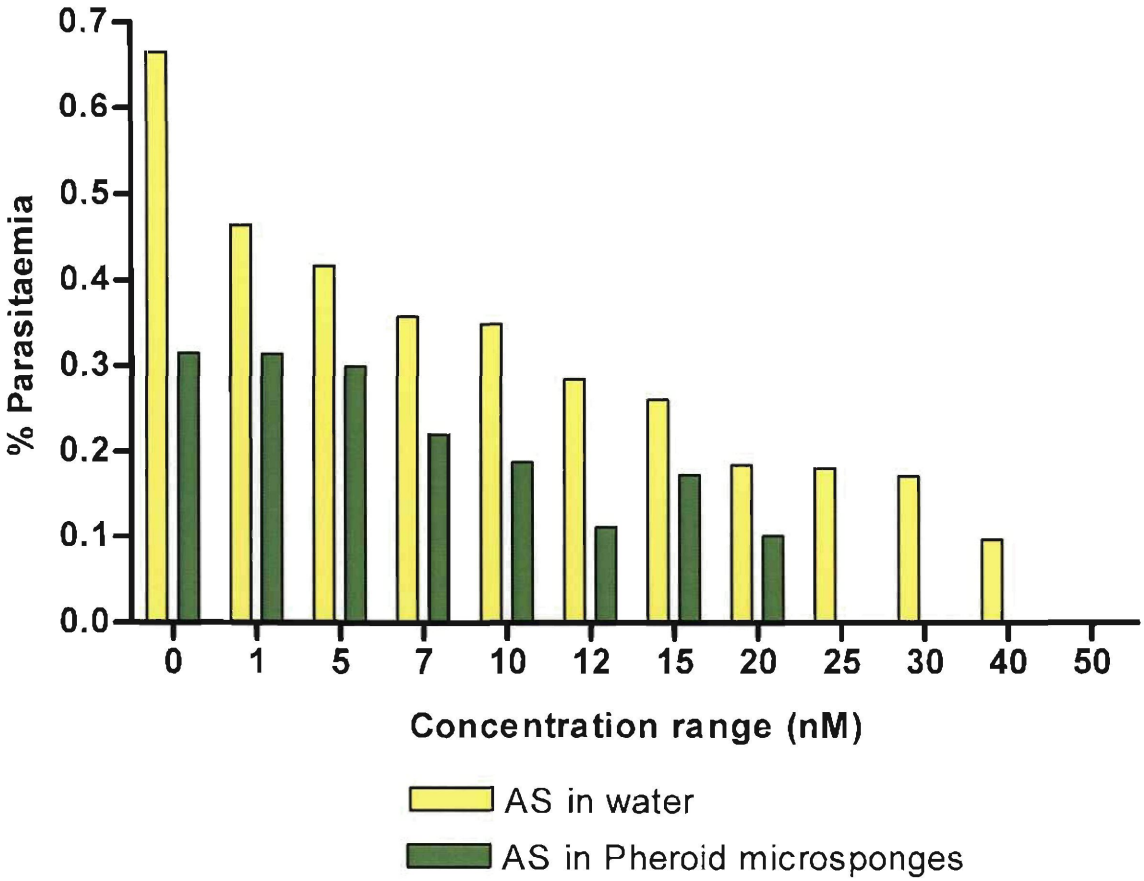


Figure 4.8 Parasitaemia determined *in vitro* for artesunate (AS) entrapped in Pheroid microsponges and in water with drug concentrations ranging from 0 nM to 50 nM.

Artesunate appears to be more effective compared to artemether by inhibiting parasite growth at lower concentrations. Artesunate also appears to be more effective than chloroquine and mefloquine. As with artemether, artesunate entrapped in Pheroid microsponges seemed to be slightly more effective compared to Pheroid vesicles.

4.5.5 Comparison of obtained results

To determine which of the Pheroid formulations were the most effective, results from each Pheroid formulation was compared with the results of each standard reference formulation (drug in water). The comparative area under the curve for each concentration range of each formulation was calculated and represent the number of parasites not killed by the formulation. The data (table 4.1) was calculated with GraphPad Prism (section 4.2.5). The comparative levels for the area under the curve are illustrated in figure 4.9. In each case the level of parasiteamia is higher in the reference treatment (water formulations) than in the tests treatments (Pheroid formulations). Not only the drug used, but also the entrapment into either Pheroid microsponges or Pheroid vesicles also influenced the level of parasiteamia.

Table 4.1 Percentage enhancement of anti-malarial drugs by Pheroid technology.

	AUC Vesicles	AUC Sponges	AUC Control	Enhancement factor (a)	Percentage enhancement (b)
Chloroquine	*34.74*	73.58	*536.60*	15.44	**1544.00%**
Mefloquine	36.34	*22.63*	*71.13*	3.14	**314.00%**
Artemether	11.96	*7.45*	*19.00*	2.54	**254.00%**
Artesunate	4.732	*4.33*	*10.34*	2.38	**238.00%**

(a) Enhancement factor: CQ (536.60/34.74); MQ (71.13/22.63); AM (19.00/7.45); AS (10.34/4.33).
(b) Enhancement percentage: Enhancement factor x 100%.

By using the data obtained, the enhancement factor and percentage enhancement of each treatment was calculated, as indicated in table 4.1. An increase in the efficacy levels of each compound can clearly be seen, in particular with chloroquine. The increase in efficacy observed for chloroquine in the chloroquine resistant parasites was especially pronounced. The efficacy was enhanced by 1545.00% in the Pheroid vesicle formulation. The efficacy of mefloquine, artemether and artesunate in Pheroid microsponge formulations were enhanced by 314.32%, 254.86% and 238.7% respectively.

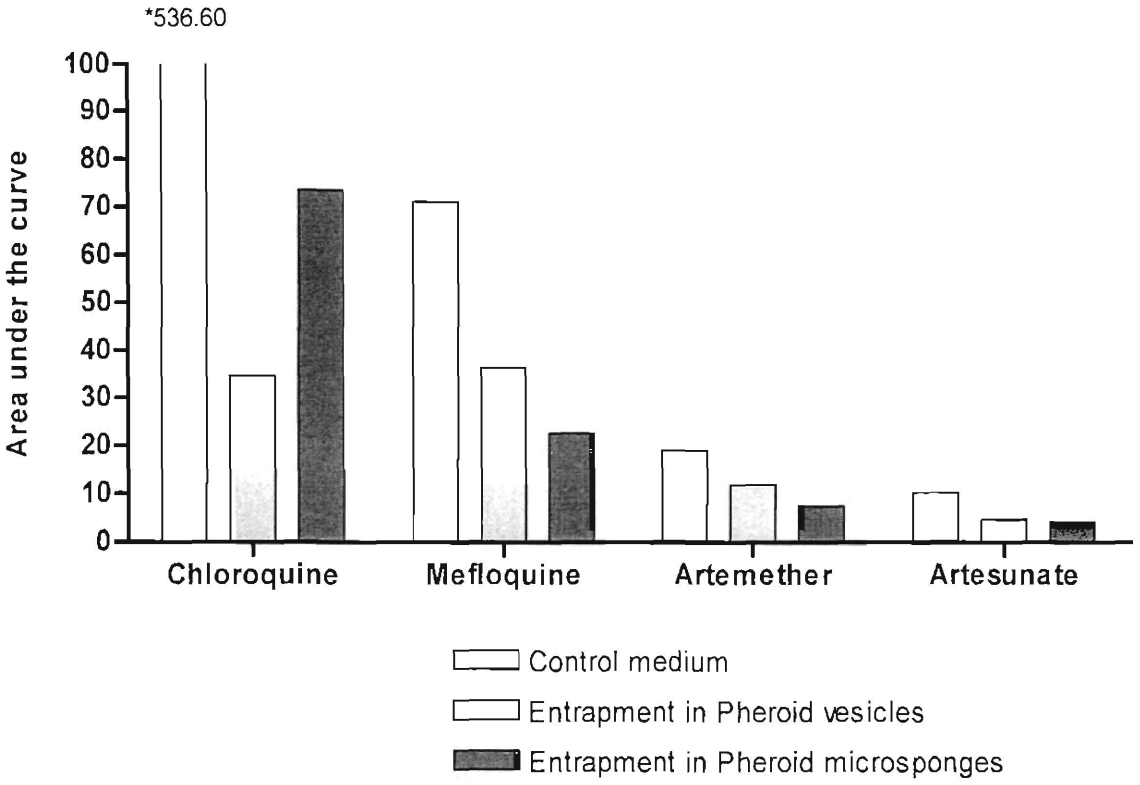


Figure 4.9 Comparative areas under the curve for the test compounds in Pheroid formulations and in the control medium. The graph was adjusted to give a better indication of the results. The chloroquine control continues to reach a value of *536.60, as is indicated in the graph.

4.6 Conclusion

The obtained results indicate that chloroquine in water is not effective against the chloroquine resistant parasite strain, as was expected, but chloroquine entrapped in the Pheroid formulations showed very promising results. Results obtained showed that chloroquine entrapped in Pheroid vesicles were more effective compared to chloroquine entrapped in Pheroid microsponges. The formulation of chloroquine in Pheroid formulations were conclusively effective against the chloroquine resistant parasite strain, thereby possibly being able to reverse choroquine resistance. Mefloquine in water was also not completely effective against the chloroquine resistant parasite strain but the drug entrapped in the Pheroid formulations showed similar

results as obtained with chloroquine. Mefloquine entrapped in Pheroid microsponges showed better results compared to mefloquine entrapped in Pheroid vesicles. Artemether and artesunate both were very effective against the chloroquine resistant parasite strain and showed exceptional increases in drug efficacy in comparison with the chloroquine and mefloquine results. Both compounds even showed better results when entrapped in Pheroid microsponges and to some extent also when entrapped in Pheroid vesicles.

In general, it is clear that the Pheroid drug delivery system has the potential to increase the delivery of anti-malarial compounds, reducing the minimum inhibitory concentration and increasing the therapeutic efficacy of the active compounds, especially for chloroquine. More extensive studies have to be done to confirm these results and to establish valid mechanisms of action.

CHAPTER 5

Preliminary In Vivo Efficacy Study

5.1 Introduction

An increase in the efficacy levels of chloroquine, mefloquine, artemether and artesunate were observed in the *in vitro* study (chapter 4) when entrapped in Pheroid formulations in comparison to the respective drugs dissolved in water. From the results of the *in vitro* study it was clear that the percentage enhancement of the efficacy observed for chloroquine was the highest of the four drugs used and therefore it holds merit for further investigation *in vivo*.

5.1.1 Rodent malaria parasite models

The relevance of rodent malaria parasites is that they are practical models to implement in experimental studies. These models have proved to be analogous to primate and human malaria parasites regarding aspects such as structure, physiology and the parasite life cycle. It provides valuable information on various topics such as the developmental biology of malaria parasites, parasite host-interactions, vaccine development and compound testing (Janse, 2007).

5.1.2 Variables and pharmaceutical applications

A few variables have to be taken into consideration when using rodent malaria models (section 2.2) which includes the type of parasite and mouse strain. These variables allow a variety of assays that can be performed. *P. chabaudi* and *P. vinckei,* for example, generates a high parasitaemia and synchronous infections enables studies on parasite stage specificity. Certain rodent malaria parasites are more susceptible to test compounds than others, for example *P. chabaudi* and *P. vinckei* are more sensitive to iron chelators and lipid biosynthesis inhibitors than *P. berghei*. The type of mouse strain used determines the course of infection. There are existing models that are even to some extent similar to studies on chronic infection (Fidock *et al.*, 2004)

5.2 The *P. berghei* mouse model

This model is most widely used for efficacy studies and is ideal for drug screening. It can be described as a four-day suppressive test that measures the efficacy of four daily doses of a test compound by comparing the blood parasitaemia on the fourth day after infection and the mouse survival time of infected and uninfected mice. Rodent infection is initiated by needle passage from an infected mouse to a uninfected mouse through the intra-peritoneal or the intravenous routes of administration. A small inoculum in the range of 10^6-10^7 infected erythrocytes are administered. Test compounds can be administered to the rodents via different routes including intra-peritoneal, intravenous, subcutaneous or the oral route. The test compounds are evaluated with at least four different doses to determine the drug concentration that produces a 50% and 90% reduction in parasitaemia *in vivo* in the rodent malaria model, thereby providing valuable information regarding the relative potency and oral availability of the compounds. Although these *in vivo* models are valuable it is of great importance to note that the drug sensitivity of a given malaria rodent parasite doesn't always represent that of *P. falciparum*. This is because rodent parasites and their hosts are diverged from the human parasites and human hosts. Results obtained from such studies should therefore be carefully compared and assessed to determine their relevance in human disease (Fidock *et al.*, 2004).

5.3 Experimental: Study design and *in vivo* model

Based on the results obtained in the *in vitro* efficacy study (chapter 4), chloroquine was selected for a pilot *in vivo* efficacy study. The *P. berghei* efficacy assay was conducted at the Department of Pharmacology, Medical School, University of Cape Town under the supervision of Dr Pete J. Smith.

5.3.1 Infection and examinition

The C57 black 6 rodent strain was used for the experiment. The animals were housed in a closed, contained environment and had free access to a standard diet and tap water. The mice were infected with the chloroquine sensitive *P. bergei* ANKA strain at 10^6 cells/ml. Inoculation of the mice with a 10^7 cells/ml usually results in a 2-5% parasitaemia 65-70 hours after inoculation (Gibbons *et al.*, 2007). There was no indication given as to which method was used to conduct the inoculation. Blood

samples were taken 24 hours post infection to determine parasitaemia. The investigator at UCT did not indicate the method of analysis used to determine parasitaemia. Physical examinations were also performed to determine any visible distress or weight changes in the mice. The mice were treated according to ethical guidelines approved by the Animal Ethics Committee of the University of Cape Town.

5.3.2 Experimental design

From the results obtained in the *in vitro* study (section 4.5) the Pheroid vesicle formulation was specifically chosen to conduct the assay using chloroquine. Chloroquine concentrations for the *P. berghei in vivo* model usually range between 2 mg/kgbw and 10 mg/kgbw. The experiment was performed on an established infection (Rane test) (Elufoiye & Agbedahunsi, 2004). The mice were grouped into seven batches of three mice each. One group served as a control. This group was treated with a Pheroid vesicle formulation only. Three of the groups were treated with three different concentrations of chloroquine dissolved in water namely 2 mg/kg; 5 mg/kg and 10 mg/kg bodyweight (bw) respectively, while the other three groups received the same three concentrations of chloroquine entrapped in Pheroid vesicle formulations. At 24 hours post infection the mice were treated once a day for 4 consecutive days with the different formulations. The mice where monitored for 20 days after treatment or 55 days for the 10 mg/kgbw chloroquine groups (Gibbons *et al.*, 2007; Gumede *et al.*, 2002).

5.3.3 Data analyses

Data analyses was performed with GraphPad Prism version 4. The results were represented as graphs which display the measure of parasite growth inhibition (% parasitaemia) plotted against the number of days for which the mice were monitored. The survival rates of all the groups of mice were compared by using the product limit method of Kaplan and Meier (GraphPad Prism User Guide, 2007). Survival fractions were calculated and used to plot percentage survival vs time. The percentage chemosuppresion was also calculated by using the following equation (Elufoiye & Agbedahunsi, 2004) :

$$\frac{\text{Parasitaemia (\%) in control} - \text{Parasitaemia (\%) in treated group}}{\text{Parasitaemia (\%) in control}}$$

The control sample in each case refers to the specific dose of chloroquine in water. Complete tables of all the results are given in Annexure 2 where exact parasitaemia percentages along with the corresponding timeframe of treatment with the drug in water and entrapped in the Pheroid formulations, are stipulated together with the survival rates of the mice used in the experiment.

5.4 Results and discussion

Figure 5.1 shows the effect of Pheroid vesicles on the parasitaemia levels in the control group of mice. No chloroquine was entrapped in this formulation. The starting parasitaemia was between 2-5%. After the 4 day treatment the parasitaemia was 0%. On day 11 the parasitaemia increased to 5.20%, thereafter it escalated to 45.80% on day 20. It is evident that during the first 11 days Pheroid vesicles suppress parasitaemia levels, keeping the parasitaemia within range of the starting parasitaemia. The results indicate that Pheroid vesicles may have an anti-malarial effect. No logical explanation for this anti-malarial effect exists and it might be advisable to investigate this further.

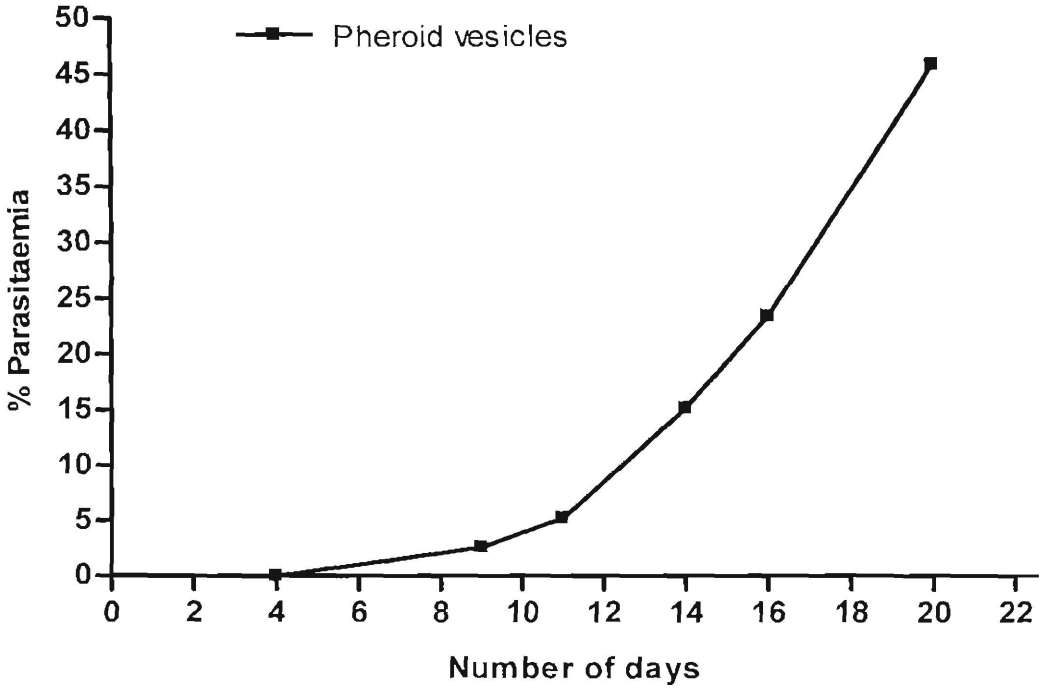


Figure 5.1 The effect of Pheroid vesicles on rodent parasitaemia (%) levels.

Figure 5.2 shows the effect of 2mg/kgbw of chloroquine in water and the comparative Pheroid formulation over a period of 20 days. On day 4 the percentage parasitaemia for the chloroquine in water treatment was higher compared to the chloroquine in Pheroid vesicle formulation. On days 7 and 11 the difference between the two groups became prominent, with a chemosuppression of 96.14% and 69.63% respectively. The parasitaemia percentage was suppressed by the drug in the Pheroid vesicle formulation up to approximately day 11. During this time the parasitaemia varied between 0.34% and 3.04%. From day 11 the parasitaemia levels of the chloroquine in water formulation increase dramatically to 23.6% and 34.3% on days 14 and 16 respectively. However the parasitaemia was also less suppressed for the chloroquine entrapped in Pheriod vesicles and increased to 34.30%, 31.80% and 40,80% on days 14, 16 and 20 respectively.

These results clearly indicate that the Pheroid formulation entrapped with a low dose of chloroquine appear to have increased efficacy during the first 11 days post treatment, compared to the control formulation. Thereafter there appears to be no difference

between the two formulations. No definite explanation could be offered at this stage on why the Pheroid formulation was able to suppress parasitaemia significantly, compared to the chloroquine control formulation, up to 11 days and thereafter was not able to show similar results towards the end of the study period. Further investigations are needed to clarify these results.

The efficacy of chloroquine was also evaluated at a concentration of 5 mg/kgbw in water and in Pheroid vesicles and these results are depicted figure 5.3. As was the case with the 2 mg/kgbw dose, the Pheroid vesicle formulation suppresses the parasitaemia levels up to 14 days. For the 5 m/kgbw dose there appears to be no difference between chloroquine in water and chloroquine in Pheroid vesicles up to 14 days. Although the percentage parasitaemia for the Pheroid vesicles increased to 26.53% after 16 days, it was still lower compared to the 2 mg/kgbw chloroquine treatment parasitaemia levels (31.87%). The percentage chemosuppression was 36.74% on day 11 for the Pheroid formulation (5mg/kgbw) while it was 69.63% on day 11 for the 2 mg/kgbw Pheroid formulation. This indicates a dose dependant effect.

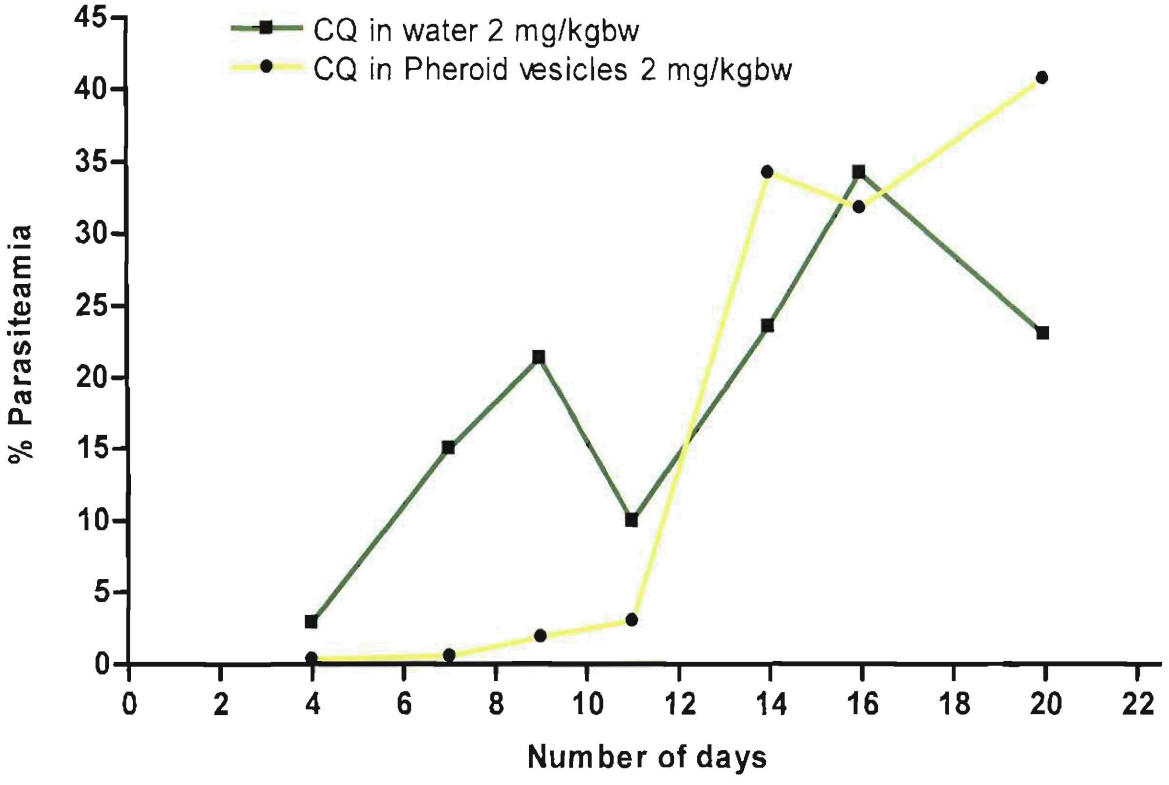


Figure 5.2 The effect of chloroquine (2 mg/kgbw) in water and in Pheroid vesicles on rodent parasitaemia (%).

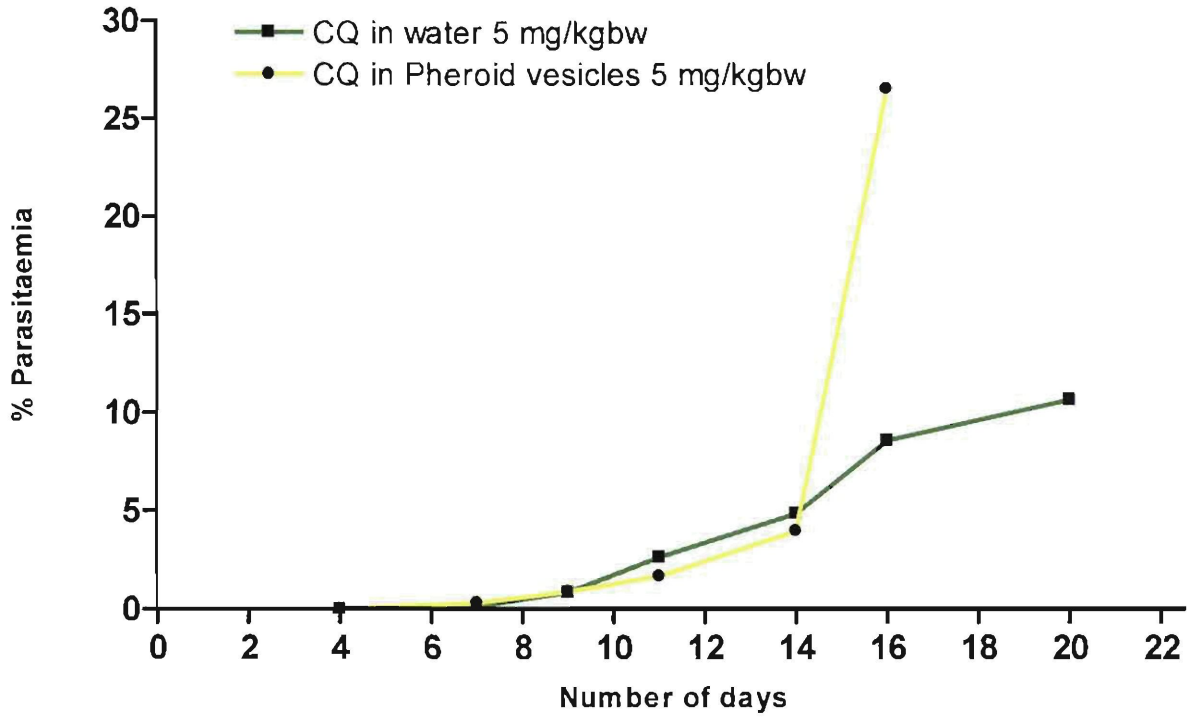


Figure 5.3 The effect of chloroquine (5 mg/kgbw) in water and in Pheroid vesicles on rodent parasitaemia (%).

Figure 5.4 gives the results of the treatment with the 10 mg/kgbw chloroquine formulations. The results shows that there is an initial slight increase in parasitaemia levels on day 7 before it is suppressed by the drug in the Pheroid vesicle formulation from day 11 to day 16, with levels of 0.12%, 0.33% and 0.38% parasitaemia for days 9, 11 and 14 respectively. Compared to the chloroquine in water formulation for the same period, parasitaemia levels range from 0.26% to 0.83%. Chloroquine in Pheroid vesicles at 10mg/kgbw produced 64.14% chemosuppression on day 11 and 54.22% on day 14. On day 16 the % parasitaemia for the Pheroid vesicle formulation increased to 1.43% and to 1.54% on day 20, thereafter it decreased again. Chloroquine in water suppressed the parasitaemia up to day 35. These results are in contrast with previous studies where 10mg/kgbw chloroquine in water lead to 0% parasitaemia after 7 days treatment with the drug in water (Gumede *et al.*, 2002). It is therefore difficult to interpret the results of the chloroquine entrapped in the Pheroid vesicle formulation. Parasitaemia levels were studied up to day 35. The mice were observed further to a total of 55 days, to monitor any further differences between the two treatment groups.

Both groups seemed to be cured after this since parasitaemia levels were less than 1%.

However, in general the obtained results give a good indication that the Pheroid formulations were able to suppress parasitaemia for a period of 11-14 days. The suppression seems to be better when compared to the comparative chloroquine in water formulations. Contrasting results were obtained for the study periods after 14 days and there may be several reasons for this, which is not understood now and needs further investigation.

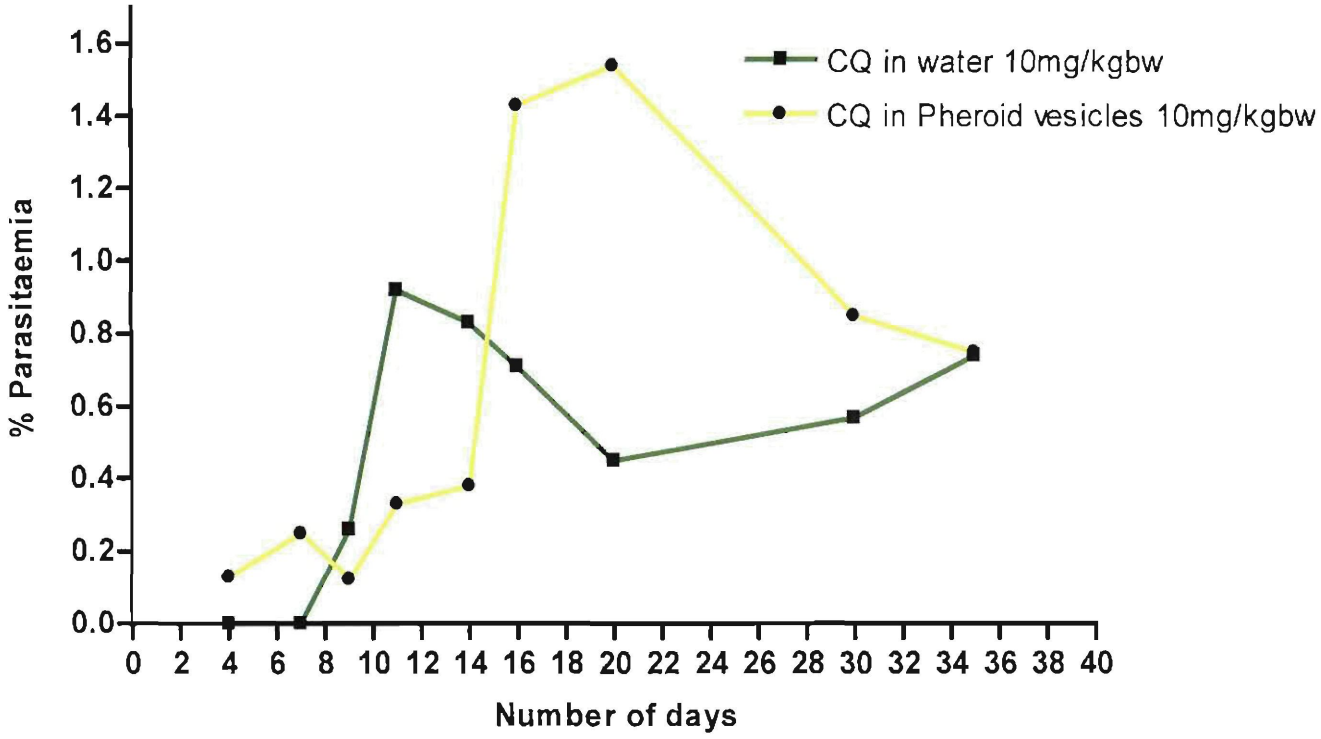


Figure 5.4 The effect of chloroquine (10 mg/kgbw) in water and in Pheroid vesicles on rodent parasitaemia (%).

Figure 5.5 gives a summary of the suppression of parasitaemia by the Pheroid vesicle formulations on day 11. The observed effects of chloroquine in the Pheroid vesicle formulations seemed to reach a maximum on day 11. A definite dose dependent effect can be observed.

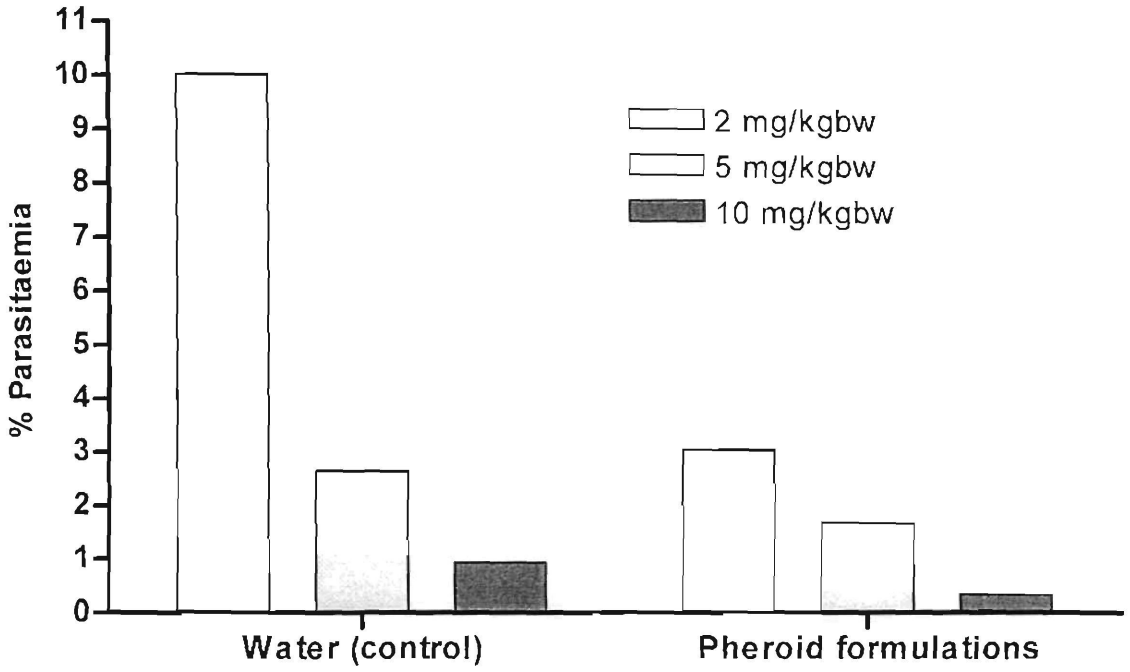


Figure 5.5 The percentage parasitaemia obtained on day 11 for the different chloroquine concentrations in water and in Pheroid vesicle formulations.

Figure 5.6 depicts the survival rates of the mice used in the seven different groups. Groups D and G, which received doses of 10mg/kg bodyweight chloroquine survived for up to 55 days, and were eventually sacrificed. This means that both groups were cured of *P. berghei* infection. Groups B and E (2 mg/kgbw) all had a 66% survival percentage on day 22 and 0% survival on day 23. Group C (5 mg/kgbw) in water had a 100% survival rate up to day 19 and a 0% survival rate on day 22. The survival rate was the lowest in group F (5mg/kgbw in Pheroid vesicles), with a 33% survival rate on day 10 and a 0% survival rate on day 22. Groups C and F, receiving 5 mg/kgbw chloroquine in water and in Pheroid vesicles appeared to be least cured. This is in

contrast with previous studies were infected mice receiving 5 mg/kgbw chloroquine appeared to be cured (Elufoiye & Agbedahunsi, 2004).

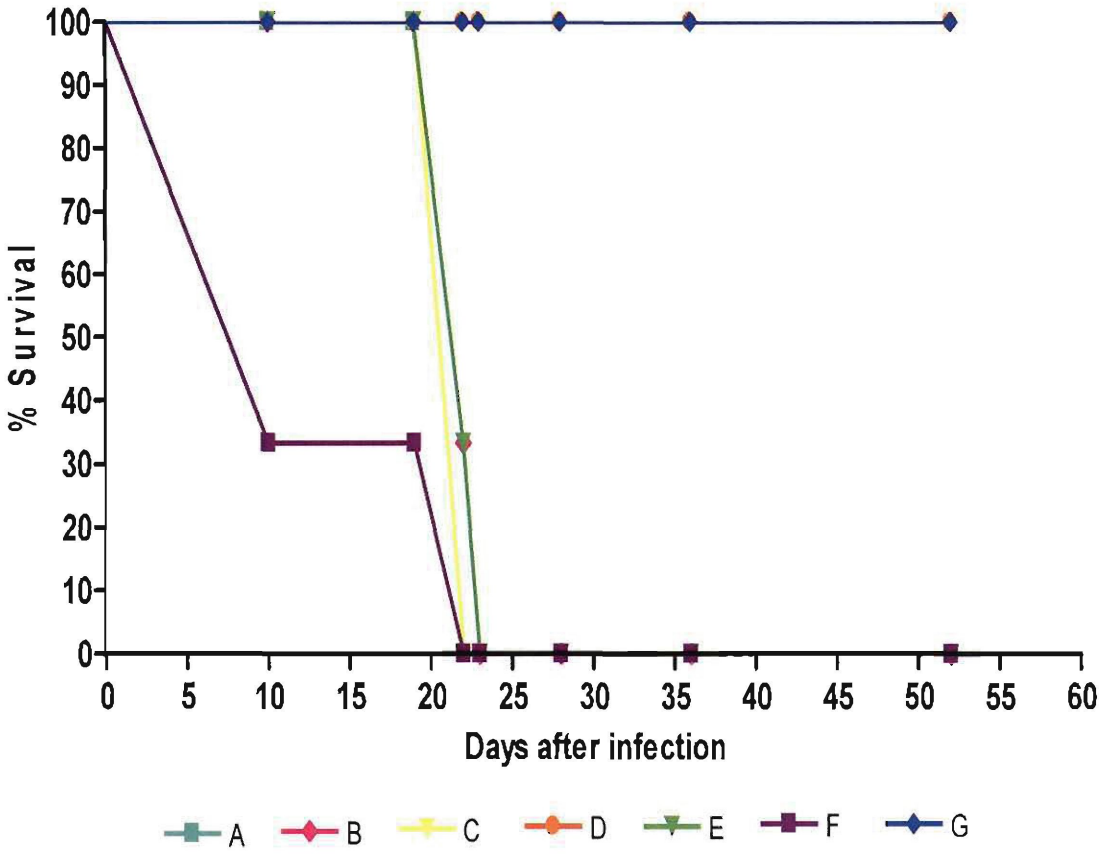


Figure 5.6 Survival rates of the mice receiving treatment of chloroquine in water and chloroquine in Pheroid vesicle formulations. Group A represents the control of only Pheroid vesicles. Chloroquine in water groups; B = 2 mg/kgbw, C = 5 mg/kgbw, D = 10 mg/kgbw. Chloroquine in Pheroid vesicle formulation groups; E = 2 mg/kgbw, F = 5 mg/kgbw, G = 10 mg/kgbw.

5.5 Conclusion

Although the difference in parasitaemia levels in the groups treated with similar chloroquine concentrations is not large and significant throughout the experiment, notable observations could be made. With all three chloroquine concentrations, the groups treated with chloroquine entrapped in Pheroid vesicle formulations show suppressed parasitaemia levels up to 11 days post infection. From day 11, the

parasitaemia increases rapidly and becomes higher compared to parasitaemia levels of the groups treated with chloroquine in water. This was observed in groups dosed at 2 mg/kg bodyweight, 5 mg/kg bodyweight and 10 mg/kg bodyweight. It is unclear at this stage why this happened. A definitive dose dependent effect could be observed for the Pheroid formulations on day 11. When evaluating the survival rates it is difficult at this stage to explain why the rodent groups receiving the highest dose shows a 100% survival rate where the groups receiving lower doses only survived up to day 23 of the study. Various factors influence the interpretation of such survival curves for instance the consistency of criteria entries, constant definition of the end point (time to death), the relation between time of censoring and time to survival, the consistency of average survival rates throughout the duration of the study, taking proportional hazards into account and the definition of the treatment groups before data collection is commenced (GraphPad Prism version 4 User Guide) .

From the obtained results, taking all variables into consideration, it can be concluded that the Pheroid vesicle formulations possibly increase the efficacy of chloroquine up to 11 days post infection with *P. berghei*. However, further studies under more controlled conditions is needed to fully understand possible applications of Pheroid technology in malaria treatment.

SUMMARY AND FUTURE PROSPECTS

Although statistics available on malaria are staggering at this point in time, it is considered to be a very treatable disease if a prompt diagnosis can be made and efficient treatment is given. The two most widely used anti-malarial drugs, chloroquine and sulphadoxine/pyrimethamine are failing as treatment options at an accelerating rate in most malaria endemic regions. Therefore a lot of emphasis is being placed on increased efforts in anti-malarial drug discovery (Fidock *et al.,* 2004). The artemisinin derivates are currently the most active drugs against malaria. These compounds should be carefully prescribed in combination therapies in order to safeguard them against drug resistance and to preserve their use for the future. There are a variety of benefits and drawbacks to available regimens ranging from economic viability to accessibility and patient compliance, therefore each compound utilized in treating malaria should be carefully considered.

In vitro and *in vivo* drug assays play an important role in the screening of drugs as part of the novel drug development process. Thereby possible new anti-malarial drugs can be identified. These assays are also vital for the continuous evaluation of existing drugs in order to determine whether they are still effective as anti-malarials against uncomplicated, complicated and drug resistant malaria (Fidock *et al.,* 2004; Geyer, 2001).

In vitro and *in vivo* assays were implemented in this study to determine if the efficacy of four anti-malarial drugs, namely chloroquine, mefloquine, artemether and artesunate, could be enhanced by incorporating them into the Pheroid™ drug delivery system. Two specific formulations of the drug delivery system was used namely Pheroid vesicles and Pheroid microsponges. The obtained results indicated that this drug delivery system has the potential to enhance the efficacy of the above mentioned compounds *in vitro* and *in vivo.* The minimum inhibitory concentration of the compounds were reduced and the therapeutic efficacy of the active compounds increased, especially for chloroquine. More extensive studies, using *in vitro and in vivo* methods, have to be done to confirm the results and to establish mechanisms of action.

The Pheroid™ drug delivery system could be considered as a new approach to drug delivery and it is recommended that it should be evaluated in future studies according the following:

- compatibility with the nature of malaria as parasitic disease;
- routes of administration;
- intracellular targeting and the determination of drug payloads, levels of toxicity, immunogenicity and protection of the drug against extracellular degradation;
- the shortening of treatment duration by improving the pharmacokinetic or pharmacodynamic profile of test compounds;
- versatility regarding the delivery of combination therapies;
- target selectivity; and
- cost effectiveness (Date *et al.*, 2007).

If all preventative measures and treatment options are implemented for malaria as prescribed the world could be well on its way to avert epidemics and manage morbidity and mortality as a result of this disease. When evaluating this project in its entirety it can be considered to be a stepping stone with the aim of formulating a new dosage form for the treatment of malaria in the near future with mono- and combination therapy strategies.

ANNEXURE A

Analytical Data – *In Vitro* Study

Table A.1 Parasitaemia determined *in vitro* for chloroquine (CQ) entrapped in a Pheroid vesicle and Pheroid microsponge formulations as well as in water with drug concentrations ranging from 0 nM to 1000 nM.

Concentration range (nM)	CQ in Pheroid vesicles (%)	CQ in Pheroid microsponges (%)	CQ in water (%)
0	0.4835	0.5747	0.5608
1	0.2352	0.5228	0.4557
5	0.6818	0.4514	0.519
10	0.2042	0.4662	0.396
25	0.4175	0.5412	0.932
50	0.3533	0.3618	0.761
75	0.1919	0.3318	0.5059
100	0.1091	0.3984	0.304
200	0	0.1223	0.3724
300	0	0	0.7561
500	0	0	0.5352

Table A.2 Parasitaemia determined *in vitro* for mefloquine (MQ) entrapped in Pheroid vesicle and Pheroid microsponge formulations and in water with drug concentrations ranging from 0 nM to 500 nM.

Concentration range (nM)	MQ in Pheroid vesicles (%)	MQ in Pheroid microsponges (%)	MQ in water (%)
0	0.439	0.525	0.563
1	0.475	0.518	0.578
5	0.494	0.344	0.502
10	0.418	0.409	0.517
15	0.894	0.408	0.5
20	0.192	0.317	0.425
25	0.147	0.28	0.367
50	0.189	0.212	0.207
75	0.078	0.174	0.225
100	0.145	0	0.162
300	0	0	0.086

Table A.3 Parasitaemia determined *in vitro* for artemether (AM) entrapped in Pheroid vesicle and Pheroid microsponge formulations and in water with drug concentrations ranging from 0 nM to 100 nM.

Concentration range (nM)	AM in Pheroid vesicles (%)	AM in Pheroid microsponges (%)	AM in water (%)
0	0.4796	0.3424	0.5019
1	0.3033	0.3348	0.4107
5	0.3198	0.3198	0.4504
10	0.2254	0.2814	0.3194
15	0.2333	0.2207	0.3174
20	0.243	0.1048	0.2915
25	0.1006	0.1769	0.212
50	0	0.0967	0.1218
75	0	0.0938	0.2006
80	0	0	0.1062
90	0	0	0.1026

Table A.4 Parasitaemia determined *in vitro* for artesunate (AS) entrapped in Pheroid vesicle and Pheroid microsponge formulations and in water with drug concentrations ranging from 0 nM to 50 nM.

Concentration range (nM)	AS in Pheroid vesicles (%)	AS in Pheroid microsponges (%)	AS in water (%)
0	0.463	0.3151	0.6652
1	0.4382	0.3135	0.4634
5	0.3824	0.2997	0.4162
7	0.2952	0.22	0.3578
10	0.2762	0.1876	0.349
12	0.2126	0.1112	0.2847
15	0.0745	0.1727	0.2606
20	0	0.1007	0.184
25	0	0	0.1807
30	0	0	0.1714
40	0	0	0.0967

ANNEXURE B

Analytical Data – *In Vivo* Study

Table B.1. Parasitaemia and chemosuppresion for chloroquine (2 mg/kgbw) in water and in Pheroid vesicles.

Number of days	CQ in water (%)	CQ in Pheroid vesicles(%)	Chemosuppresion (%)
4	2.90	0.34	0.882759
7	15.01	0.58	0.961359
9	21.40	1.93	0.909813
11	10.01	3.04	0.696304
14	23.60	34.30	-0.45339
16	34.30	31.87	0.070845
20	23.10	40.80	-0.76623
30	-	-	
35	-	-	

Table B.2 Parasitaemia and chemosuppresion for chloroquine (5 mg/kgbw) in water and in Pheroid vesicles.

Number of days	CQ in water (%)	CQ in Pheroid vesicles(%)	Chemosuppresion (%)
4	0.00	0.00	0
7	0.15	0.32	-1.13333
9	0.86	0.90	-0.04651
11	2.64	1.67	0.367424
14	4.85	3.98	0.179381
16	8.59	26.53	-2.08847
20	10.68	-	
30	-	-	
35	-	-	

Table B.3 Parasitaemia and chemosuppresion for chloroquine (10 mg/kgbw) in water and in Pheroid vesicles.

Number of days	CQ in water (%)	CQ in Pheroid vesicles(%)	Chemosuppresion (%)
4	0.00	0.130	0
7	0.00	0.250	0
9	0.26	0.124	0.523077
11	0.92	0.330	0.641304
14	0.83	0.380	0.542169
16	0.71	1.430	-1.01408
20	0.45	1.540	-2.42222
30	0.57	0.850	-0.49123
35	0.74	0.750	

Table B.4 Survival rates of the mice receiving treatment of chloroquine in water and chloroquine in a Pheroid vesicle formulations. Group A represents the control of only Pheroid vesicles. Chloroquine in water groups; B = 2 mg/kgbw, C = 5 mg/kgbw, D = 10 mg/kgbw. Chloroquine in Pheroid vesicle formulation groups; E = 2 mg/kgbw, F = 5 mg/kgbw, G = 10 mg/kgbw.

Number of days	A	B	C	D	E	F	G
4	0.00	2.90	0.00	0.00	0.34	0.00	0.130
7	-	16.10	0.15	0.00	0.58	0.32	0.250
9	2.60	21.50	0.86	0.26	1.93	0.90	0.124
11	5.20	10.01	2.64	0.92	3.04	1.67	0.330
14	15.13	23.60	4.85	0.83	34.30	3.98	0.380
16	23.40	34.30	8.59	0.71	31.87	26.53	1.430
20	45.80	23.10	10.68	0.45	40.80	-	1.540
30	-	-	-	0.57	-	-	0.850
35	-	-	-	0.74	-	-	0.750

ANNEXURE C

Certificates of Analyses

SINOWAY INDUSTRIAL CO.,LTD.

16/F.,HUICHENG COMM.COMPLEX, 839 XIAHE RD.,XIAMEN,CHINA
TEL: 0086-592-5854962 POSTCODE: 361004
FAX: 0086-592-5854960 E-MAIL: sale3@china-sinoway.com

Certificate of analysis

NAME OF PRODUCT: CHLOROQUINE PHOSPHATE
BATCH NO: 0602026 QUANTITY: 0.5KG
MFG DATE: MAR.2006 REP.DATE: MAR.3, 2006
EXP.DATE: FEB.2010

ITEMS	SPECIFICATIONS	RESULTS
CHARACTERS	A WHITE OR ALMOST WHITE CRYSTALLINE POWDER ONE MELTING AT ABOUT 195℃	CONFORMS 195.5℃
IDENTIFICATION	POSITIVE	CONFORMS
SOLUTION S	AS STIPULATED	CONFORMS
APPEARANCE OF SOLUTION	≤BY_5 OR GY_5	<GY_5
PH(10% W/V)	3.8-4.3	4.06
RELATED SUBSTANCE	ONE SPOT ≤1.0% ANY OTHER SPOT ≤0.5%	CONFORMS CONFORMS
HEAVY METALS	≤20PPM	<10PPM
LOSS ON DRYING	≤2.0%	0.42%
ASSAY	98.5-101.0%	100.14%
CONCLUSION	CONFORMS TO BP2003/USP28	

SINOWAY INDUSTRIAL CO.,LTD

REPRESENTED BY:
D B Fine Chemicals (Pty) Limited
P.O Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS 1995 2005

REPRESENTED BY:
D B Fine Chemicals (Pty) Limited
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RIVONIA 2128
Johannesburg
South Africa

10 YEARS 1995 2005

Mod.05/01-8 REV.2 del 22/04/2004

Sifavitor S.p.A.
Via Livelli 1 - 26852 Cassaletto Lodigiano Fraz. Mairano (ITALY)
Tel. 0371-73991 - Fax 0371-73108 - e-mail: sifavitor@mediolast.com

CERTIFICATE OF ANALYSIS

MEFLOQUINE HYDROCHLORIDE

Revision: 24/02/2005 Page 1 of 1

Analysis Number 60604
Analysis Date 03/04/2006

Batch: *8700/03/06* Kg: *232.00*

Formula: [illegible] M.W.: 414.80

Manufacturing Date *17/03/2006* Retest Date *03/2009*

Complies with: *Ph.Eur.-BP - USP*

TEST	RESULTS	SPECIFICATIONS
DESCRIPTION Aspetto	*complies*	white or slightly yellow crystalline powder
IDENTIFICATION Identificazione	*complies* *complies*	(1): IR spectrum (2): chlorides test
APPEARANCE OF SOLUTION Aspetto della soluzione	*complies*	a 5% solution in methanol is clear and not more intensely coloured than reference solution BY7
OPTICAL ROTATION Rotazione ottica	*- 0.02°*	-0.20° to +0.20°
WATER Acqua	*2.04%*	not more than 3.0%
SULPHATED ASH Ceneri solforiche	*0.00%*	not more than 0.1%
HEAVY METALS Metalli pesanti	*complies*	not more than 20 ppm
RELATED SUBSTANCES (HPLC) Impurezze organiche (HPLC)	*(1): < LOD* *(2): < LOD* *(3): 0.03%* *(4): RRT 0.91: 0.01%* *RRT 2.03: 0.01%* *(5): 0.05%*	(1) impurity A nmt 0.10% (2) impurity B nmt 0.10% (3) impurity C nmt 0.20% (4) each unknown impurity nmt 0.10% (5) sum of all impurities nmt 0.50%
ASSAY Titolo	*100.43%*	btw.99.0% and 101.0% with reference to the anhydrous, solvent-free substance (pot.)
RESIDUAL SOLVENTS Solventi residui	*29 ppm*	acetone nmt 200 ppm

REPRESENTED BY:
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P O Box 786
RIVONIA 2128
Johannesburg
South Africa

10 YEARS 1995 2005

03/04/06	03/04/06	03.04.2006
Issue: QUALITY CONTROL	Control: QUALITY ASSURANCE	Approval: PLANT MANAGER

SINOWAY INDUSTRIAL CO.,LTD.

16/F.,HUICHENG COMM.COMPLEX, 839 XIAHE RD.,XIAMEN,CHINA
TEL: 0086-592-5854962 POSTCODE: 361004
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Certificate of analysis

NAME OF PRODUCT: ARTEMETHER
BATCH NO: 060510 QUANTITY:0.5KG
MFG.DATE: MAY.10,2006 EXPIRY DATE:MAY.9,2009
REPORT DATE: MAY.10,2006

ITEMS	STANDARDS	RESULTS
APPEARANCE	WHITE CRYSTALLINE POWDER	CONFORM
ODOR	ODORLESS	CONFORM
MELTING POINT	86℃ TO 90℃	87℃
IDENTIFICATION		
COLOR REACTION	AS STIPULATED	CONFORM
TLC	AS STIPULATED	CONFORM
IR	AS STIPULATED	CONFORM
RELATED SUBSTANCES	AS STIPULATED	CONFORM
CHLORIDE	AS STIPULATED	CONFORM
LOSS ON DRYING	≤0.5%	0.15%
RESIDUE ON IGNITION	≤0.1%	0.05%
HEAVY METAL	≤10PPM	<10PPM
ASSAY	98.0%-102.0%	99.85%
CONCLUSION	CONFORMS TO THE CP2000	

SINOWAY INDUSTRIAL CO.,LTD.

REPRESENTED BY:
D R Fine Chemicals (Pty) Limited
PO Box 708
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Johannesburg
South Africa

10 YEARS
1996 2006

SINOWAY INDUSTRIAL CO.,LTD.

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TEL: 0086-592-5854962 POSTCODE: 361004
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Certificate of analysis

NAME OF PRODUCT: ARTESUNATE
BATCH NO: A051101
MFG.DATE: NOV.1，2005 EXPIRY DATE: NOV.1，2008
REPORT DATE:NOV.2，2005 QUANTITY：0.5KG

ITEMS	STANDARDS	RESULTS
APPEARANCE	WHITE CRYSTALLINE POWDER	CONFORMS
WATER	≤0.5%	0.07%
MELTING RANGE	130-137℃	132.1-134.0℃
IDENTIFICATION	POSITIVE	CONFORMS
PH	3.5-5.0	3.97
CHLORIDE	0.02%	<0.02%
SPECIFIC ROTATION	+10° TO +14°	+11.82°
RESIDUE ON IGNITION	≤0.1%	0.004%
RELATED SUBSTANCES(DHA)	≤1%	<1%
ASSAY	98.0-102.0%	98.84%
CONCLUSION	CONFORMS TO THE CP2005	

SINOWAY INDUSTRIAL CO.,LTD

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10 YEARS 1995 2005

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South Africa
10 YEARS 1995 2005

ANNEXURE D

Conference Participation

28th Annual Conference of the Academy of Pharmaceutical Sciences of South Africa – September 2007

Preclinical evaluation of the possible enhancement of the efficacy of anti-malaria drugs by Pheroid technology™

Natasha Langley, Anne F. Grobler, Lissinda Du Plessis, Awie F. Kotze.

Unit for Drug Research and Development, Faculty of Health Sciences, North-West University, Potchefstroom, 2531.

Introduction:

Malaria is currently one the most imperative parasitic diseases of the developing world. Current effective treatment options are limited because of increasing drug resistance, treatment cost effectiveness and treatment availability. Drug delivery systems could be taken into consideration as a new approach for the increased efficacy in the treatment of the disease. Pheroid™ Technology, a proven drug delivery system, in combination with anti-malaria drugs was evaluated in this study.

Objectives:

The aim of this study was to evaluate the possible enhancement of efficacy of the existing anti-malaria drugs in combination with Pheroid™ technology.

Method:

The efficacy of existing anti-malaria drugs in combination with Pheroids™ were investigated *in vitro* with a chloroquine RB1-resistant strain of *P. falciparum.* Infected red blood cells (parasitemia 0.1%, heamatocrit 5.5%) were incubated for 48 hours with selected concentration ranges of chloroquine, mefloquine, artemether and artesunate in Pheroid formulations and in a control medium. Two distinctively different Pheroid™ formulas, Pheroid vesicles and Pheroid microsponges, were used and the control medium consisted of sterile water for injection. Giemsa stained, thin smear slides were made after a 48 hour incubation period and the parasitaemia levels were determined microscopically by counting the number of parasite infected erythrocytes per 10 000 erythrocytes and expressing it as a percentage.

Results:

The efficacy of chloroquine in Pheroid vesicles was enhanced by 1544.62%. The efficacy of mefloquine, artemether and artesunate in Pheroid microsponges were enhanced by 314.32%, 254.86% and 238.7% respectively. A significant increase in efficacy of chloroquine, mefloquine, artemether and artesunate in Pheroid microsponge and Pheroid vesicles were noted, especially for chloroquine. Pheroid™ technology holds merit for further investigation of its effects in combination with anti-malaria drugs using *in vivo* studies.

The First International Conference on Drug Design, Development and Delivery – Dubai February 2008

An *in-vitro* evaluation of the efficacy of anti-malaria drugs by Pheroid™ technology

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Approximately 300 million people are affected by malaria. Malaria is now mainly confined to Africa, Asia and Latin America. Inadequate health structures, poor socio-economic conditions and the increase in resistance to anti-malarial drugs aggravate the situation. The need to develop drug delivery systems that minimize the likelihood of drug resistant microorganisms requires a multidisciplinary approach. Novel Pheroid™ Technology may be applicable and its effectiveness in combination with anti-malarial drugs was investigated.

The efficacy of existing anti-malarial drugs in combination with Pheroid™ formulations was investigated using a human erythrocyte *in vitro* infection model and a chloroquine resistant strain (RBy1) of *P. falciparum* with selected concentration ranges of chloroquine phosphate, mefloquine, artemether and artesunate. Two distinctively different Pheroid™ formulas, vesicles and microsponges were used. The parasiteamia levels were determined microscopically and the comparative anti-parasitic effect was determined by calculating the total comparative growth inhibitory effect.

The comparative efficacy of chloroquine phosphate in Pheroid™ vesicles was significantly enhanced by 1544.62%. The efficacy of mefloquine, artemether and artesunate in Pheroid™ sponges were enhanced by 314.32%, 254.86% and 238.7 % respectively. This study holds merit for further investigation of the effects of Pheroid™ technology on anti-malarial treatment with *in vivo* methods.

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