

CHAPTER 2

This chapter consists of the literature review. The focus of this chapter is on malaria, artemisone and the Pheroid® drug delivery system.

CHAPTER 2

LITERATURE REVIEW

1. Malaria

1.1. Introduction

Malaria is potentially a life-threatening disease caused by infection with *Plasmodium* protozoa. (WHO, 2010a). A French army surgeon, Charles Louis Alphonse Laveran, observed parasites in a malaria patient's blood, Dr Ronald Ross discovered that mosquitoes are malaria vectors and an Italian professor, Giovanni Battista Grassi, later showed that *Anopheles* mosquitoes act as the only vectors for transmitting human malaria (Tuteja, 2007).

Malaria remains an immense and persistent burden of disease on humanity. According to estimates, 216 million malaria cases were reported in 2010, of which 91% were due to *P. falciparum* with an estimated 655 thousand deaths worldwide (WHO, 2012). There was a decrease in the estimated malaria cases reported since 2005, which were 244 million cases. Since 2005, the European Region, followed by the Region of the Americas had the major decline in malaria cases with a decrease of 86% and 42% respectively (WHO, 2010a). Since 2000 the globally estimated incidence and deaths of malaria have reduced by 17 and 26% respectively. Although there is a decrease, it is clear that the internationally agreed target of 50% reduction by 2010 has not been attained (WHO, 2010a). In Africa, a child dies of malaria every 30 seconds (Kokwaro, 2009). Approximately 86% of global deaths were reported to be children under the age of 5 years (WHO, 2012). In 2010, 81% of cases were in the African region, with 13% in South-East Asia and 5% in Eastern Mediterranean regions (WHO, 2012). Of these the total estimated number of cases, only 11% were confirmed cases as reported by the national malaria control programme (NMCP) (WHO, 2012).

Malaria is currently endemic in over 100 countries and is commonly associated with some of the poorest countries in the world. The disease is in itself a hindrance to economic development (Ashley *et al.*, 2006) (Figure 1.1). The poor and rural families do not have access to preventative measures that are essential to malaria control; they may live far from the closest healthcare facility and cannot afford treatment (Kokwaro, 2009). These countries are visited by more than 125 million people from non-endemic countries every year and well over 10 thousand travellers are reported to fall ill after returning home; however, underreporting means that the real figure may be as high as 30 thousand (WHO, 2010c).

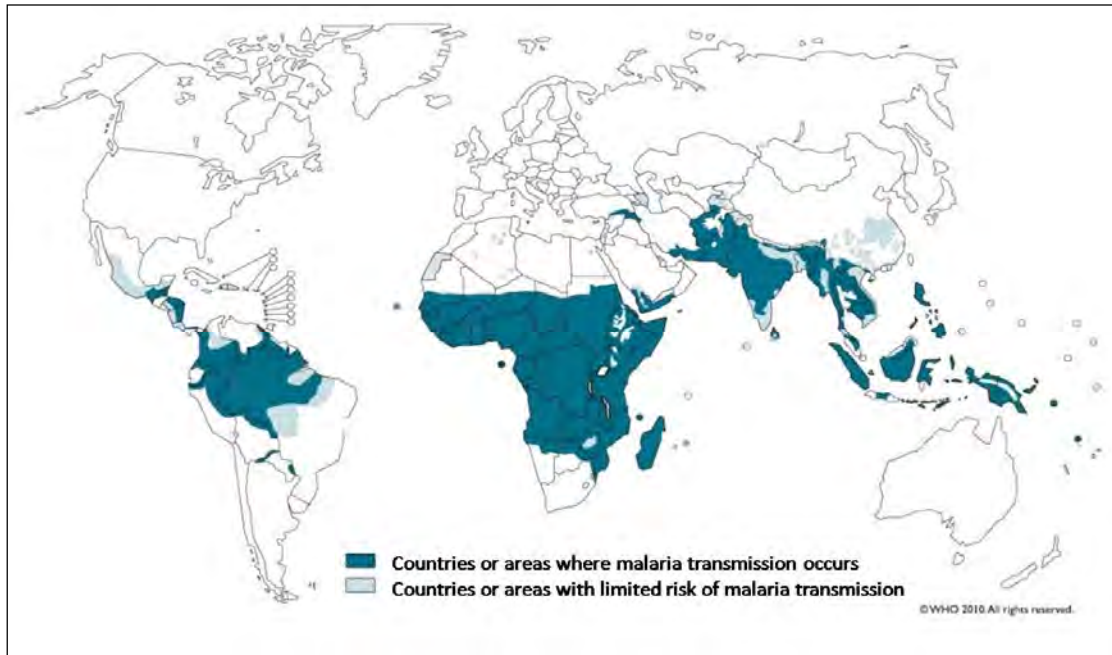


Figure 1.1: Countries and areas at risk of malaria transmission in 2009 (reprinted from WHO, 2010c).

1.2. The malaria parasite

Malaria is caused by a eukaryotic single-celled *Plasmodium* protozoan parasite transmitted by an infected female *Anopheles* mosquito vector (Tuteja, 2007; WHO, 2010b). Approximately 60 of the 400 species of anopheline mosquitos throughout the world are malaria vectors under natural conditions (Tuteja, 2007). The four *Plasmodium* species known to cause malaria in humans include *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, and *Plasmodium malariae* (WHO, 2010b). Of these species, *P. vivax* and *P. falciparum* occur most frequently, while *P. ovale* and *P. malariae* are not lethal and are relatively rare (Daily, 2006). The life cycle of the malaria parasite (Figure 1.2) is extremely complex, consisting of a sexual stage (sporogony), which occurs within the mosquito, and an asexual stage (schizogony), which occurs in the host (Aly *et al.*, 2009)..

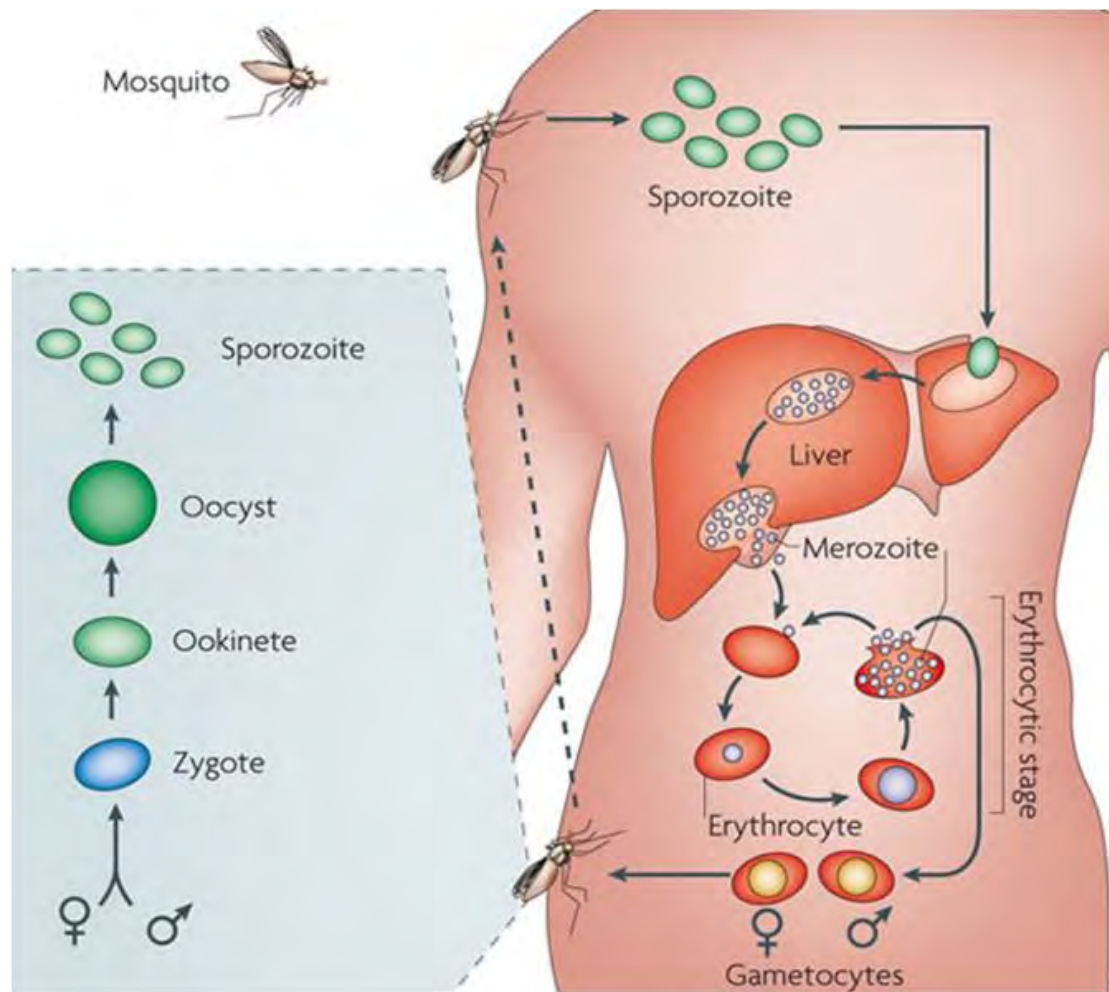


Figure 1.2: Illustration of the life cycle of the malaria parasite (reprinted from Dondorp *et al.*, 2010 with permission from Nature Publishing Group).

Infection of the human host with the parasite is initiated when an infected female *Anopheles* mosquito takes a blood meal. The mosquito injects saliva containing sporozoites into the host. The sporozoites travel rapidly to the liver where they pass through the Kupffer cells and infect hepatocytes. There they undergo asexual multiplication, generating schizonts, which when they rupture, release merozoites into the blood stream. The merozoites invade erythrocytes where they undergo erythrocytic schizogony proceeding through young ring form stages to trophozoites to mature schizonts. The mature schizonts rupture and the released merozoites invade new red blood cells and start a new erythrocytic schizogony cycle (Aly *et al.*, 2009). Because the contents of the infected erythrocytes on rupture stimulate the production of tumor necrosis factor and other cytokines, the clinical symptoms of malaria are associated with the erythrocytic phase of infection (Tuteja, 2007). After several cycles, some of the intra-erythrocytic parasites undergo sexual development into

gametocytes. Ingestion of gametocytes by a mosquito during a blood meal activates gametogenesis, the formation of gametes, in the mosquito midgut lumen. In the mosquito, male and female gametes fuse to form a zygote. The zygote transforms into an oocyst, which forms sporozoites. When these sporozoites are released, they migrate to the salivary glands of the mosquito and infect the next host upon a blood meal (Aly *et al.*, 2009).

1.3. Symptoms

1.3.1 Uncomplicated malaria

Malaria symptoms can develop as soon as 6-8 days after infection or as late as several months (Tuteja, 2007). The early signs and symptoms are non-specific and can commonly lead to misdiagnosis as influenza, hepatitis, gastroenteritis or meningitis (Walker *et al.*, 2010). Paroxysms begin with an initial 'cold' stage (sensation of cold, shivering), a 'hot' stage (fever, headaches, vomiting; seizures in young children) and a 'sweating' stage (sweats whilst their temperature returns to normal, tiredness) in which the patient has rigors and shakes excessively. This paroxysm lasts between 6-10 hours and is followed by an asymptomatic period and subsequent paroxysms if the patient is not treated. The attacks occur every second day with the *P. falciparum*, *P. vivax*, and *P. ovale* and every third day with *P. malariae* infection (CDC, 2010).

1.3.2. Complicated (severe) malaria

Severe malaria is when the infections are complicated by serious organ failures or abnormalities in the patient's blood or metabolism and it is considered a medical emergency in need of immediate and aggressive treatment (CDC, 2010). Severe malaria is usually complex and has several key pathogenic manifestations such as generalized convulsions, severe normocytic anaemia, hypoglycaemia, metabolic acidosis with respiratory distress, fluid and electrolyte disturbances, acute renal failure, acute pulmonary oedema, acute respiratory distress syndrome (ARDS), circulatory collapse, shock, septicaemia jaundice, hemoglobinuria, high fever, coagulation failure, prostration and impaired consciousness. Malaria is especially dangerous to pregnant women and small children because they have a much higher risk of developing severe malaria. Premature delivery, low birth weight and increased mortality in the newborn are common (WHO, 2010b).

1.4. Diagnosis

Prompt and accurate diagnosis is a vital part of malaria management (Kokwaro, 2009). Because symptoms overlap with those of many other diseases and conditions, the use of clinical diagnosis solely for malaria has very low specificity and sensitivity and may result in over-treatment. The World Health Organisation recommends confirmation of clinical suspicion of malaria by a laboratory test: either a blood film for microscopy or a rapid diagnostic test (RDT) (WHO, 2011). Thick blood smears are used for species diagnosis based on morphological characteristics and the thin smears are used for quantification of the parasitized red blood cells (Tuteja, 2007). Figure 1.3 illustrates the differences in morphology between the *Plasmodium* species (Coartney *et al.*, 1971). However, at the community level, where access to malaria diagnosis and treatment is needed, high-quality microscopy services are not widely available. This is due to microscopy requiring trained staff, well-maintained equipment, a regular supply of reliable reagents, clean water and electricity, and a well-executed quality management system (WHO, 2011).

For these reasons, RDTs were developed in the early 1990s (WHO, 2011). By using a finger-prick of blood, RDTs detect malaria antigens in only a few minutes. They employ lateral flow immunochromatography to detect either *P. falciparum* specific histidine-rich protein 2 (HRP2), species-specific or pan-specific *Plasmodium* lactate dehydrogenase (pLDH) or pan-specific aldolase (Walker *et al.*, 2009; WHO, 2010b). Because RDTs do not require water, electricity or laboratory facilities and can easily be performed in remote rural settings, these allow widescale access to diagnostic testing, which could not be done with microscopy alone. Although RDTs also require training and regular supervision, they do not depend on the availability of trained laboratory technicians (WHO, 2011). Other diagnostic tests involve the use of the polymerase chain reaction (PCR) and serology. PCR detects plasmodium specific nucleic acid sequences and serology detects antibodies present in the blood against the *Plasmodium* parasites (WHO, 2010b).

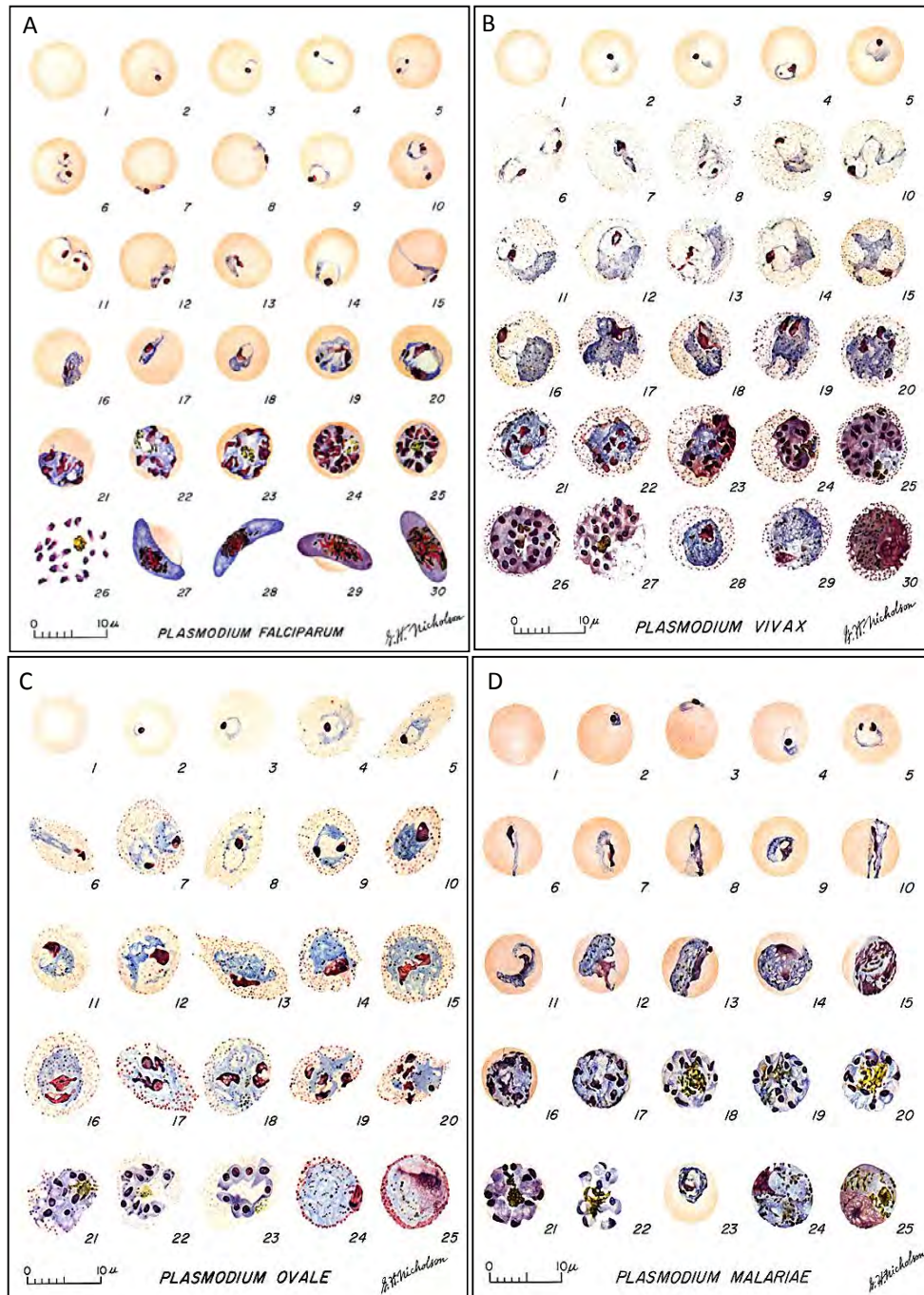


Figure 1.3: Illustrations of the different stages of A) *P. falciparum*, B) *P. vivax*, C) *P. ovale* and D) *P. malariae* in thin blood smears. Normal red blood cell (1); Ring-stage trophozoites (A:2-10, B:2-6, C:2-5, D:2-5); Trophozoites (A:11-18, B:7-18, C:5-15, D:6-13); Schizonts (A:19-26, B:19-27, C:16-23, D:14-22); Developing gametocyte (D: 23); Mature female macrogametocytes (A:27-28, B:28-29, C:24, D:24); Mature male microgametocytes (A:29-

30, B:30, C:25, D:25) (Reprinted from Coartney *et al.*, 1971 with permission from CDC-DPDx)

1.5. Control strategies

1.5.1. Malaria control policies and strategies

The WHO defines malaria control as “reducing the malaria disease burden to a level at which it is no longer a public health problem” (WHO, 2010a). The aim of the WHO antimalarial treatment policy are to a) decrease morbidity and mortality through fast and complete cure of infection; b) decrease the frequency and the period of malaria infection during pregnancy, and c) limit or ideally eradicate the transmission of malaria (WHO, 2010a). Malaria control has two arms consisting of malaria prevention and case management. Malaria prevention consists of mosquito vector control through removal of mosquito breeding sites, use of insecticides and human contact prevention via the use of screens and bed nets (White, 2004).

1.5.2. Malaria prevention through vector control

Malaria vector control as malaria prevention method is only efficient if high coverage is reached and maintained. Two powerful and broadly applied interventions are long-lasting insecticide-treated mosquito nets (LLINs) and indoor residual spraying (IRS). They function in reducing human-vector contact and reducing the lifespan of female mosquitoes and, therefore, transmission (WHO, 2012). From 2008 until the third quarter of 2010, more than 254 million ITNs have been delivered to African countries. The percentage of households owning at least one ITN increased from approximately 27% in 2007 to 42% in 2010. The majority of ITNs distributed are long-lasting ITNs (LLINs). The efficacy of ITNs decreases with time and, therefore, the LLINs needs to be replaced since their lifespan are 3 years, otherwise malaria cases and deaths may be reinforced. IRS also increased in Africa from 10 million in 2005 to 73 million in 2009 (WHO, 2010a).

In order to explain the other control strategies such as malaria preventive chemotherapy (section 1.5.5) and case management with antimalarial drug treatment (section 1.5.6), a brief overview of the existing antimalarial drugs will proceed these sections.

1.5.3. Antimalarial chemotherapy

Existing antimalarial drugs are structurally categorized into quinolines, antifolates, antibiotics and peroxides.

1.5.3.1. Quinolines

Quinine (QN) **1** was isolated from the bark of the *Cinchona* tree in 1820 and until the 1930s it was the sole effective antimalarial agent (Kaur *et al.*, 2009). Chloroquine (CQ) **5** was first synthesized in 1934 and became the most widely used antimalarial drug by the late 1940s. Because of its excellent clinical efficacy, limited host toxicity, ease of use and simple, cost-effective synthesis CQ became a highly successful drug (O'Neil, *et al.*, 2012). Besides QN and CQ, the other known antimalarial drugs from this family include quinidine **2**, mefloquine (MQ) **3**, amodiaquine (AQ) **6**, and primaquine **9**. Other drugs that have been developed include the bis-quinoline piperazine **7** and the fluorenemethanol lumefantrine (LF) **4** (Kaur *et al.*, 2010). QN, quinidine, MQ and LF are classified as arylmethanols since they contain an aminomethyl residue attached to the benzylic hydroxyl group, while CQ, AQ, piperazine and pyronaridine **8** are classified as 4-aminoquinolines, and primaquine as an 8-aminoquinoline.

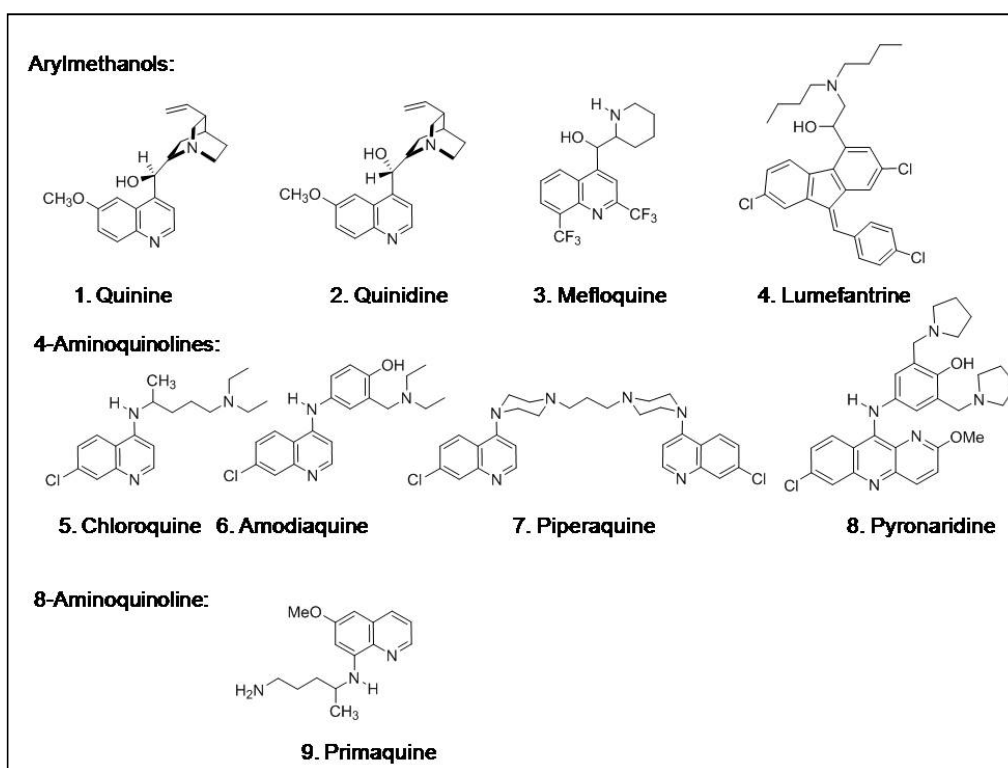


Figure 1.4: Structure of several quinoline antimalarial drugs.

In an effort to overcome resistance, the preparation of new quinolines through modification of the carbon side chain of CQ was the focus of much research. Many compounds were made but very few of these were successful. The most notable is ferroquine **10** (Figure 1.5),

that is also the first organometallic antimalarial drug candidate to enter clinical trials (Dubar *et al.*, 2008; Barends *et al.*, 2007). Another approach is to use agents such as verapamil **11** and imipramine **12** to reverse resistance to chloroquine (Krogstad *et al.*, 1987; Martin *et al.*, 1987; Egan & Kaschula, 2007). These compounds, however, are toxic at the concentrations required for resistance reversal. Also, hybrid compounds that have antimalarial activity and CQ-resistance-reversing ability such as the compound **13** known as 'reversed chloroquine' (RCQ) have been prepared. This compound has lower IC₅₀ values than CQ for both CQ sensitive and resistant *P. falciparum* strains (Burgess *et al.*, 2006). Compounds such as molecule **14**, a 4-amino-7-chloroquinolines with dibenzylaminoethylene side chains, has the ability to both inhibit hemozoin formation and by-pass CQ resistance (Zishiri *et al.*, 2011).

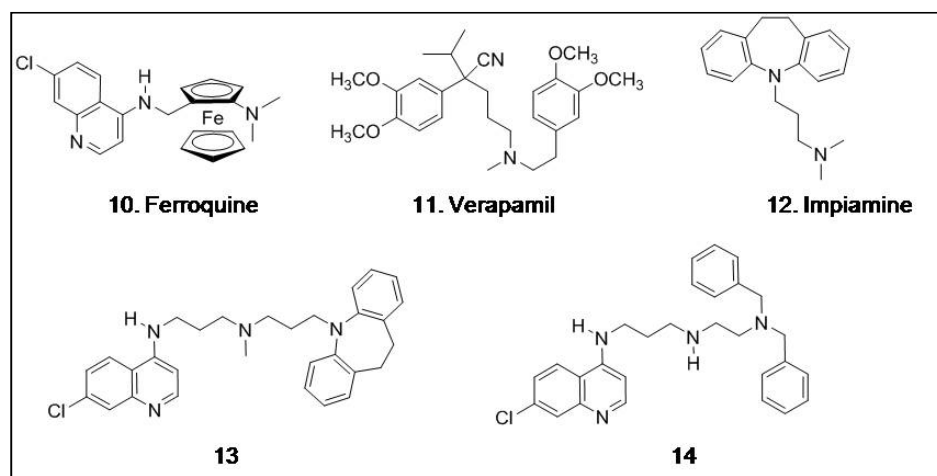


Figure 1.5: Structures of several resistance reversal agents and modified chloroquine molecules.

1.5.3.2. Antifolates

The antifolate class of antimalarial drugs was discovered in the 1940s. They have the ability to inhibit folate metabolism and thereby disrupt the biosynthesis of amino acid and nucleotides. Rapidly dividing cells, such as cancer cells and malaria parasites, are particularly sensitive to antifolates. The parasites are not able to utilize exogenous folates and rely on folate biosynthesis (Hyde, 2007b). Dihydrofolate reductase (DHFR) and dihydropteroate synthase (DHPS) are the two important enzymes for the folate synthesis which are targeted by antifolates (Mital, 2007). Pyrimethamine **15** and proguanil **16** are DHFR inhibitors, while sulfadoxine **17** and dapsone **18** are DHPS inhibitors (Figure 1.6). The DHFR and DHPS inhibitors are often used in combination (Lu *et al.*, 2010, Hyde, 2007b). Malarone, a commercially available antifolate drug combination, consists of

atovaquone and proguanil. Atovaquone **19** is an effective antimalarial drug but is associated with high recrudescence rates and decreased parasite susceptibility following treatment (Chiodine *et al.*, 1995; Looareesuwan *et al.*, 1996). Proguanil on the other hand, has weak antimalarial activity, but demonstrated synergistic activity *in vitro* with atovaquone. However, atovaquone is expensive to prepare, and resistance to atovaquone appears to develop rapidly (Baggish & Hill, 2002).

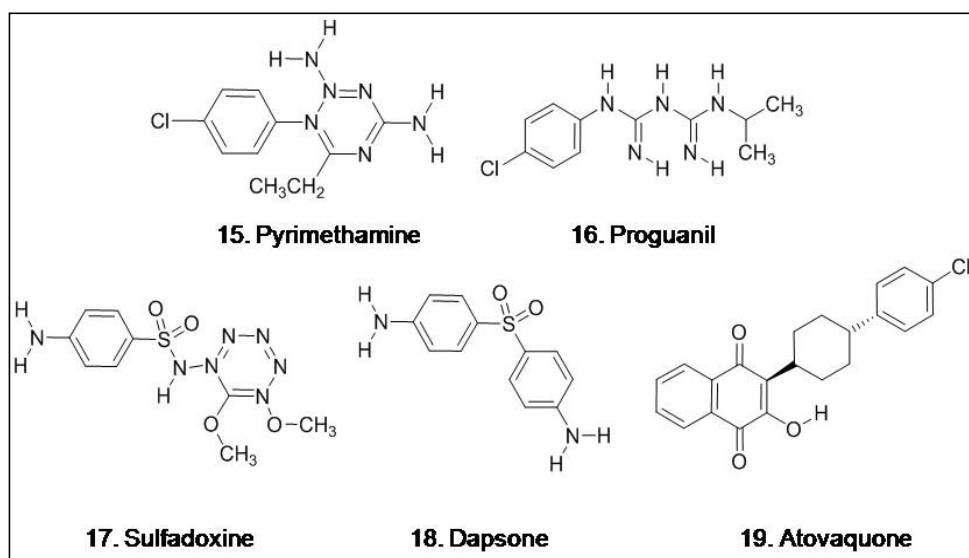


Figure 1.6: Structures of several antifolate antimalarial drugs.

1.5.4.3. Antibiotics

Many antibiotics, such as the fluoroquinolones, tetracyclines, lincosamides and macrolides classes, have antimalarial activity. They are only used in combination therapy with other antimalarial drugs since their parasitological response is slow and are not highly active against the malaria parasite (Dahl & Rosenthal, 2007). Deoxycycline **21** (Figure 1.7), a semi-synthetic derivative of tetracycline **20** was first developed in 1967. This class of antibiotics inhibit protein synthesis by binding to the 30S ribosomal subunit, thereby blocking the binding of aminoacyl-tRNA to mRNA-ribosome complex (Leggat, 2009). Clindamycin **22** is a semi-synthetic lincosamide that inhibits protein synthesis by binding to the 50S ribosomal subunit, thereby interfering with the formation of initiation complexes and aminoacyl translation reactions (Reusser, 1975). Resistance to these antibiotics is known (Leggat, 2009; Spizek & Rezanka, 2004).

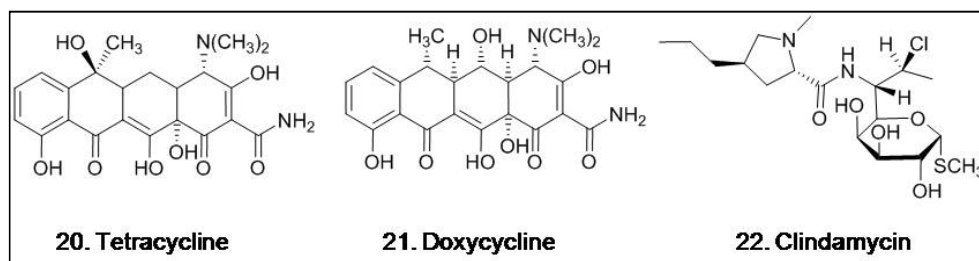


Figure 1.7: Structures of some antibiotic antimalarial drugs.

1.5.3.3. Peroxides

Terpene peroxides have remarkable antimalarial activities. Yingzhaosu A **28** and Qīnghāosu (artemisinin) **23**, the two sesquiterpene peroxides, are extracted from Chinese medicinal plants (Figure 1.8) (Opsenica & Šolaja, 2009). Artemisinin is obtained from the plant *Artemisia annua*, so-called sweet wormwood or sweet Annie (Woodrow *et al.*, 2005). For over 2000 years, this plant has been used in traditional Chinese medicine for the treatment for chills and fevers (Balint, 2001). During the latter half of the 20th century artemisinin was discovered and recognized as an antimalarial agent by Chinese scientists (Krishna *et al.*, 2008). In the 1970's, the Chinese also prepared the derivatives dihydroartemisinin (DHA) **24**, artemether **25** and artesunate **26** during a collaborative effort (collectively referred to as 'Project 523'). These derivatives of artemisinin are collectively known as the 'artemisinins' (Krishna *et al.*, 2008).

These compounds have become vital antimalarial drugs with potent activity against the multidrug-resistant strains of *P. falciparum* malaria (Vivas *et al.*, 2007). They have a high efficacy and destroy a wider range of parasite blood stages (asexual as well as sexual) compared to the other classes of antimalarial drug (Vivas *et al.*, 2007; White, 2002). Chemically, artemisinin is a sesquiterpene trioxane lactone containing a peroxide bridge, which is essential for its activity. The structures of the common artemisinins are depicted in Figure 1.8.

Artemisone **27** is a promising new artemisinin derivative. Artemisone is superior to both artemisinin and DHA in terms of activity and safety (Haynes, 2006). For the purpose of this thesis, the literature relevant to the artemisinins will be discussed in detail in section 2.

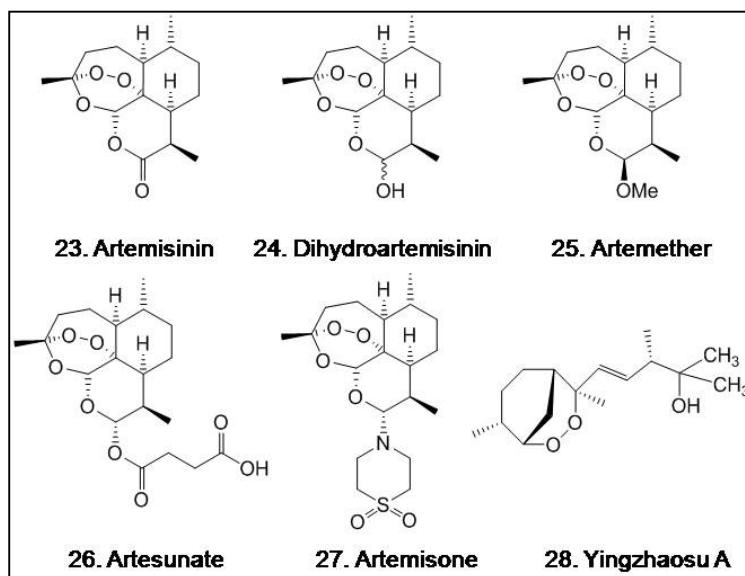


Figure 1.8: Structures of artemisinin, its derivatives and Yingzhaosu A.

Cyclic peroxides such as endoperoxides **29**, 1,2,4-trioxanes **30**, 1,2,4,5-tetraoxanes **31** and 1,2,4-trioxolanes **32** have received a great deal of synthetic attention recently (Figure 1.9).

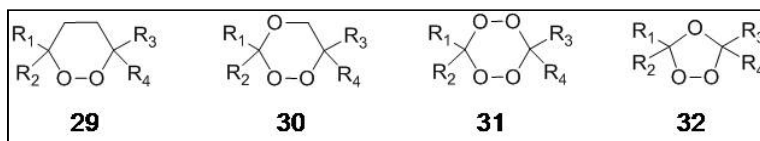


Figure 1.9: Peroxides types that may potentially supplement artemisinin antimalarial drugs.

Synthetic endoperoxides based on another Chinese endoperoxide-containing compound, Yingzhaosu A **28**, such as arteflene **33** (Hofheinz *et al.*, 1994), and the bicyclic endoperoxide **34** (Korshin *et al.*, 2002) have been prepared (Figure 1.10). These endoperoxides showed good *in vitro* and *in vivo* antimalarial activity but these analogues are too expensive to prepare. Another natural source of stable cyclic peroxides is marine sponges (Fattorusso *et al.*, 2002; Fattorusso *et al.*, 2006). Although the antimalarial activity of these compounds were 50-fold less than that of artemisinin, plakortin **35** (Huggs & Faulker, 1978; Cafieri *et al.*, 1999) and dihydroplakortin **36** (Figure 1.10) showed an additive effect when used in combination with chloroquine.

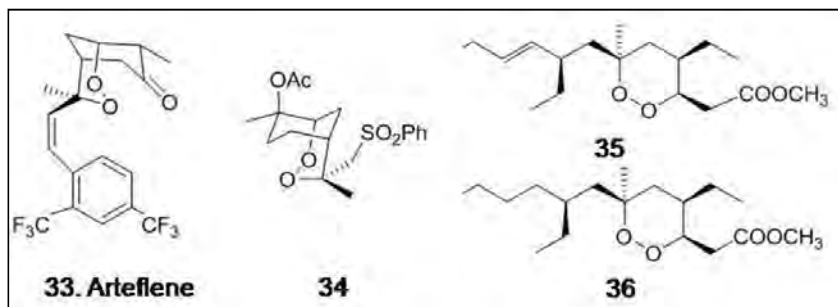


Figure 1.10: Structures of several cyclic endoperoxides.

Synthetic 1,2,4-trioxanes have the same structure as the active pharmacophore of the artemisinins. Tricyclic trioxanes such as **37** and **38** have quite good antimalarial activities (Jefford *et al.*, 1993) and the spirocyclopentyl trioxane **39** has comparable antimalarial activity comparable to that of artemisinin (Figure 1.11) (Robert *et al.*, 2001). Trioxanes **40a-i**, **41a-i** and **42a-i** are amino-functionalized spiro-1,2,4-trioxanes with very good *in vivo* oral activity, similar to that of arteether, against the *P. yoelii nigeriensis* strain in mice (Singh *et al.*, 2010a,b).

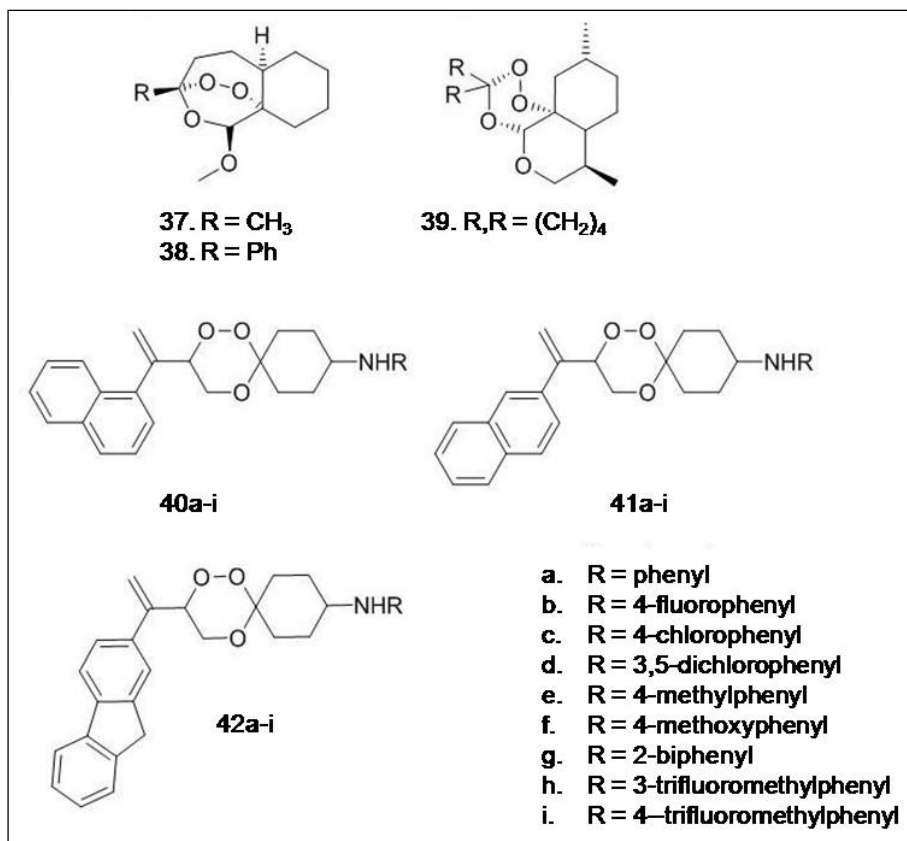


Figure 1.11: Structures of several totally synthetic 1,2,4-trioxanes.

1,2,4-Trioxolanes, or ozonides also have antimalarial activity. The ozonide known as arterolane, OZ277 or RBx-11160 **43** (Vennerstrom, 2004) is the most potent fully synthetic peroxidic antimalarial compound (Figure 1.12). It has an *in vitro* potency higher than those of chloroquine and mefloquine and is almost as active as artemether and artesunate against K1 (chloroquine-resistant) and NF54 (chloroquine susceptible) strains of *P. falciparum*. Moreover, it is highly effective *in vivo* against *P. berghei* in mice (Valecha *et al.*, 2010). Its half-life is only about two- to threefold longer than that of DHA as established from Phase I clinical trials. However, it is unstable in the plasma of malaria patients when it was administrated as monotherapy (Valecha *et al.*, 2010). Phase III clinical trials in the form of an arterolane maleate/piperaquine phosphate combination are in process (Muraleedharan *et al.*, 2009; Olliaro & Wells, 2009).

Ozonide OZ439 **44** (Charman *et al.*, 2011) was designed to achieve a single-dose oral cure, and to increase the *in vivo* half-life and blood exposure profile compared with that of artemisinin derivatives and arterolane. OZ439 is now in Phase II clinical trials, and was part of the Medicines for Malaria Venture (MMV) portfolio at the end of the 2th quarter, 2012. Additionally, three potential combination partner drugs have been selected, and the development plans and the non-clinical work to evaluate which are compatible with OZ439 are underway (MMV, 2013).

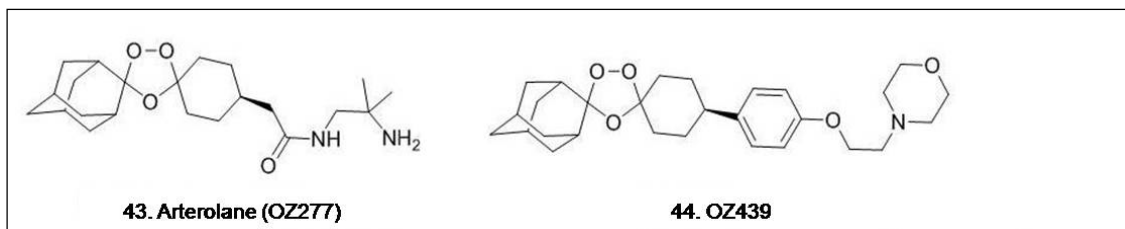


Figure 1.12: Structures of the 1,2,4-trioxolanes OZ277 and OZ439.

Dispiro-1,2,4,5-tetraoxanes, also known as ketone diperoxide, contain two endoperoxide groups with antimalarial properties first reported in 1992 (Vennerstrom, 1992). Compound **45** had antimalarial activity of approximately six-fold less than that of artemisinin and, therefore, its structure was used as a template for the design of other compounds (Figure 1.13) (Vennerstrom, 2000). Tetraoxane RKA216 **46** has an *in vitro* antimalarial activity comparable with those of artemisinin and artemether. A dosage of 30 mg/kg cured mice infected with the *P. berghei* ANKA strain (Amewu *et al.*, 2006). RKA 182 **47** shows superior *in vitro* and *in vivo* activity compared to artemether and artesunate (O'Neill, 2010). Second

generation analogues **48a-j** of RKA182 have improved metabolic activity compared to RKA 182. These analogues show very good *in vitro* activity in the low nanomolar range and several demonstrate promising oral activity in the *P. berghei* ANKA mice model (Marti *et al.*, 2011).

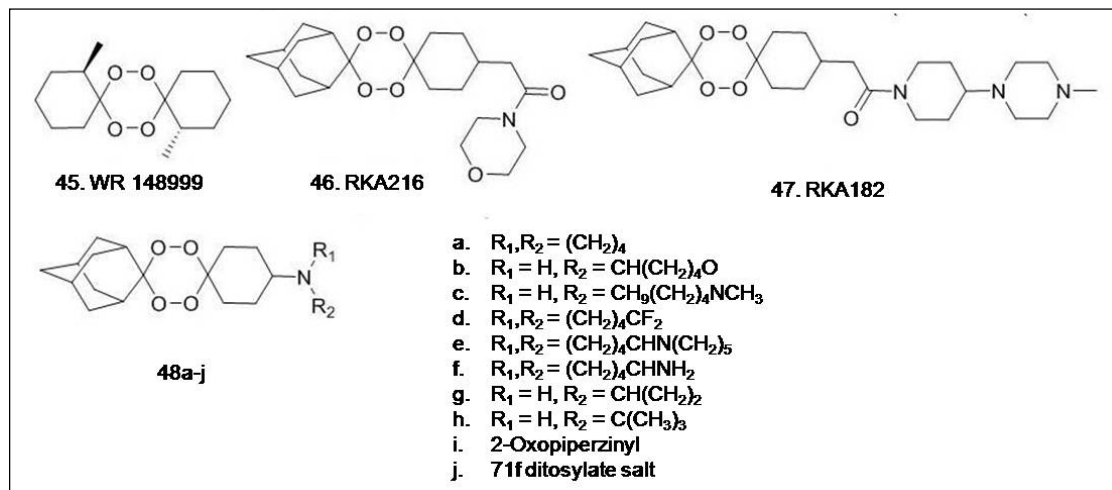


Figure 1.13: Structures of several of the 1,2,4,5-tetraoxanes.

A recent approach of antimalarial drug design involves linking two molecules, each with intrinsic activity, into a single hybrid molecule (Muregi & Ishih, 2010). When a trioxane, trioxolane or tetraoxane is covalently linked to a quinoline, the new hybrid molecules are called trioxaquinines, trioxolaquinines and tetraoxaquinines respectively (Araújo *et al.*, 2009). Compounds DU 1102, 1106 and 1108 **49a-c** and DU 1302 **50** are trioxaquinines that possess higher antimalarial activity against sensitive or resistant strains than each of the individual fragments (Figure 1.14) (Dechy-Cabaret *et al.*, 2000, Dechy-Cabaret *et al.*, 2004; Araújo *et al.*, 2009).

Coslédan *et al.* (2008) developed 120 trioxaquinines and trioxalaquinines from 2003–2006 with antimalarial activity (IC₅₀ values) *in vitro* against both CQ-sensitive and –resistant *P. falciparum* isolates ranging from 5 to 74 nM. Among these compounds, the antimalarial activities of 72 were evaluated in mice and 25 were then evaluated with preclinical tests. The trioxaquine PA1103 **51** possesses antimalarial activity against both chloroquine-resistant and –sensitive strains of *P. falciparum* (IC₅₀ values ranging from 7 to 24 nM), that is within the same range of activity as artemisinin, it showed good bioavailability and low toxicity. It was selected for drug development, but due to restructuration of Sanofi, is no longer under development. (Coslédan *et al.*, 2008).

Synthetic peroxide hybrids **52 a-b** are the most potent examples of 1,2,4-trioxalaquines. They seem to be generally more potent than their semi-synthetic trioxaquine counterparts but the trioxolane is found to be less stable than trioxanes due to metabolism (Araújo *et al.*, 2009). Compound **53** and **54** are two examples of tetraoxaquinines with **53** being several times more active than artemisinin in *in vitro* studies against CQ resistant and sensitive strain of *P. falciparum* (Loup *et al.*, 2007).

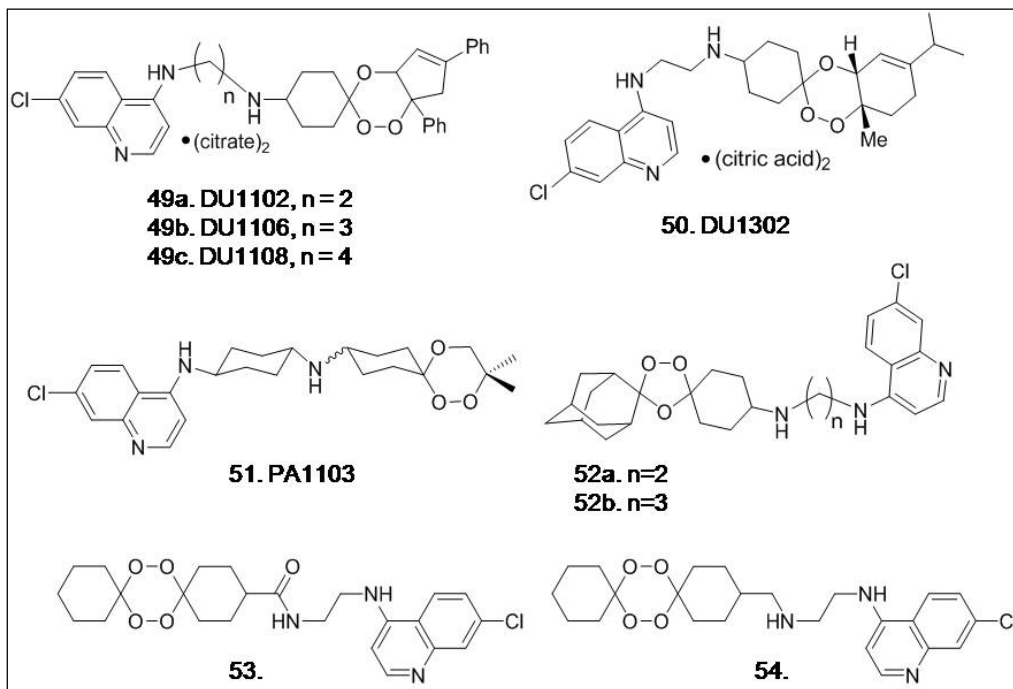


Figure 1.14: Structures of examples of trioxaquinines, trioxolaquines and tetraoxaquinines.

1.5.4. Antimalarial chemotherapy resistance

Affordable, commonly available antimalarials that are eliminated slowly from the body such as chloroquine, and sulfadoxine-pyrimethamine (SP) were used mostly for case management (White, 2004). Resistance to these antimalarials has become widespread throughout Africa and Asia and poses great challenge to medicine and development of effective antimalarial drugs (Daily, 2006; White, 2004; WHO, 2010a). The development of malaria drug resistance is a crucially important health concern and places great pressure on the control of malaria in affected countries (Wongsrichanalai *et al.*, 2002). Two of the four malaria parasite species known to naturally infect humans, have shown resistance to antimalarial drugs, namely *P. falciparum* and *P. vivax* (Boland, 2001). Drug resistance in *P. falciparum* accounts for most of the malaria disease burden (Wongsrichanalai *et al.*, 2002).

Resistance of *P. falciparum* to chloroquine was first reported from the Thai-Cambodian border regions in 1957, and on the Colombian-Venezuelan border shortly afterwards. Resistance spread to Sub-Sahara Africa during the late 1970s and 1980s, with profound consequences (Paynes, 1987; Wellems & Plowe, 2001). The use of CQ is now confined to limited areas of North Africa, Central America and the Caribbean region (Müller & Hyde, 2010). Several strains of *P. falciparum* developed resistance to the majority of the frequently used antimalarials (Wongsrichanalai *et al.*, 2002) (Table 1.1).

Table 1.1: Dates when purified antimalarial drugs were introduced and of the first reports of antimalarial drug resistance (Wongsrichanalai *et al.*, 2002, Kaur *et al.*, 2009)

Antimalarial drug	Introduced	1st reported resistance
Quinine	1820	1910
Chloroquine	1945	1957
Proguanil	1948	1949
Sulfadoxine-pyrimethamine	1967	1967
Mefloquine	1977	1982
Atovaquone	1996	1996

There are serious concerns about the emergence of artemisinin resistance. Reports indicate that prolonged *P. falciparum* parasite clearance times in four countries in the Greater Mekong subregion are now occurring in patients treated with ACTs: Cambodia, Myanmar, Thailand and Vietnam (Noedl, *et al.* 2008.; Dondorp, *et al.* 2009; Phyo *et al.* 2012; WHO, 2012) (Figure 1.15). Initially, parasite isolates taken from patients displaying increased tolerance to ACTs did not show reduced susceptibility to either dihydroartemisinin (DHA) or artesunate *in vitro* (Dondorp, *et al.*, 2009), but more recently, such isolates are now displaying enhanced tolerance. Isolates from Pailin and Ratanakiri, areas of artemisinin resistance and susceptibility in western and eastern Cambodia, respectively, also did not show reduced susceptibility with the classical isotopic test, but after a 6-h pulse exposure to 700 nM dihydroartemisinin, the survival rate of exposed ring-stage parasites was 17-fold higher in isolates from Pailin (median, 13.5%) than in those from Ratanakiri (median, 0.8%). Therefore, the ring stages survived drug exposure by cell cycle arrest and resumed growth upon drug removal (Witkowski *et al.*, 2013).

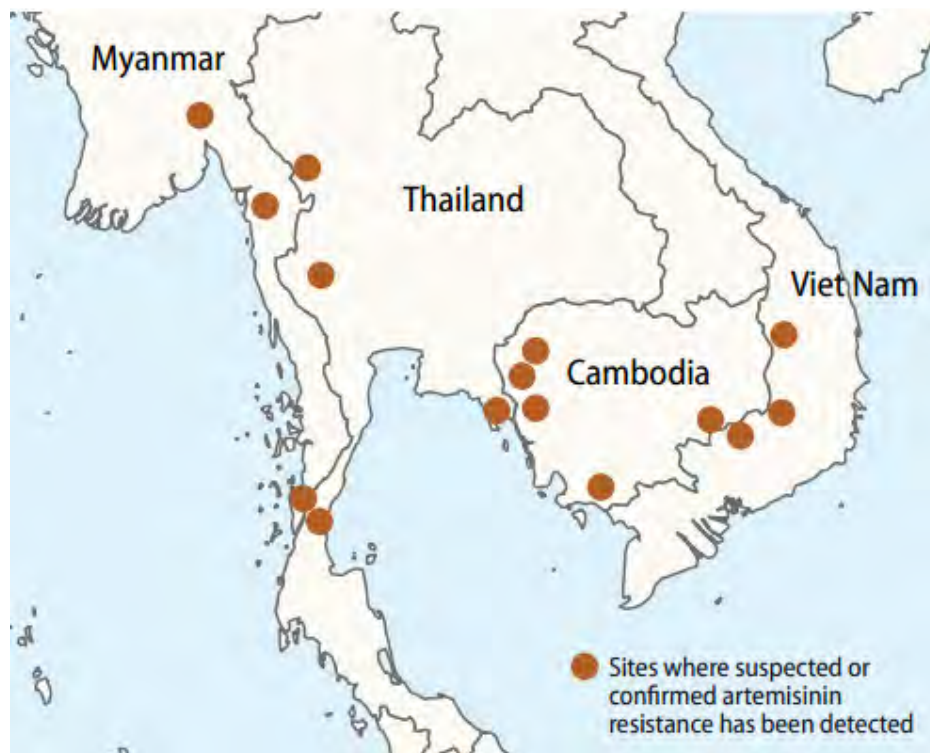


Figure 1.15: Sites where suspected or confirmed artemisinin resistance has been detected in therapeutic efficacy studies conducted from 2007 to 2012 (reprinted from WHO, 2012).

Resistance of malaria parasites arises from several factors, including the misuse of antimalarial chemoprophylaxis, poor compliance or inappropriate dosing regimens, a high level of parasite and an enormous parasite proliferation rate (Hyde, 2007a). Poor drug quality may also contribute to drug resistance because of poor manufacturing practices, counterfeiting or drug deterioration through poor handling and storage. Concurrent illness may also influence antimalarial drug resistance. Poorer parasitological responses to both chloroquine and SP were found in malnourished children. Similarly, individuals infected with the human immunodeficiency virus (HIV) may also have a low parasitological response. Malnutrition among African children under the age of 5 has been estimated to be 30% and HIV infection among children is estimated to be between 4 and 5 million (Boland, 2001).

A phenomenon known as cross-resistance occurs whereby parasite resistance to one drug may be acquired for by another similarly acting drug without being exposed to both drugs (White, 2004). Therefore, because many antimalarial drugs frequently used are chemically similar, development of resistance to one can facilitate development of resistance to others (Boland, 2001). Antimalarial resistance spreads because the resistant parasites have a higher survival rate in the presence of the antimalarial than sensitive parasites and

consequently leads to the transmission of resistant rather than sensitive parasites. Also, resistant infections are more likely to have increased rates of recrudescence and respond slower to treatment than sensitive infections. This will probably increase gametocyte densities, which in turn increases transmission, compared with drug-sensitive infections (White, 2004).

Rapid parasitological confirmation by microscopy or by RDTs is recommended in all patients with suspected malaria before treatment is started. This should be done in order to prevent unnecessary treatment with anti-malarial drugs. Treatment solely on the basis of clinical suspicion should only be considered when a parasitological diagnosis is not possible (WHO, 2010d).

1.5.5. Preventive chemotherapy

Chemoprophylaxis is the administration of an antimalarial drug to ensure that the drug's concentration level in the blood is above the concentration that inhibits parasite growth and maintained for the duration of the malaria risk period (Greenwood, 2006). It reduces the risk of fatal disease if the subject adheres to the recommended drug regimen, but none of the present prophylactic regimens offers total protection against malaria (WHO, 2010c).

Chemoprophylaxis should be started before departure to the risk area. Antimalarials to be taken on a daily basis should be started the day before arrival in the risk area, whereas chloroquine (where it is still used or prescribed) should be started 1 week before and mefloquine should be started 2–3 weeks (if side effects occur the patient should switch to another prophylactic regimen) before arrival in the risk area. It should be taken frequently during the stay in the malaria risk area, and since parasites may still emerge from the liver after departure, the drugs should be taken continually for 4 weeks after departure. However, atovaquone–proguanil, which is the exception, can be stopped 1 week after departure. Travellers should also be instructed on the possible late-onset of *P. ovale* and *P. vivax area*, if they visited a risk area for these species (WHO, 2010c). Travellers to endemic countries are often misinformed and lack compliance with preventive measures (Wyler, 1993).

Administration of antimalarial prophylactic regimens must be cognisant of known contraindications and all may elicit adverse reactions (Wyler, 1993). Atovaquone-proguanil should be avoided by pregnant woman, infants weighing less than 11 kg, women breast-feeding these infants or patients with severe renal impairment. Atovaquone-proguanil is well tolerated and the most common side-effects include abdominal pain, nausea, vomiting and

headache (Gkrania-Klotsas, 2007; CDC & Brunette, 2013). Chloroquine phosphate or hydroxychloroquine sulfate may cause gastrointestinal problems, headache, blurry vision, insomnia and may exacerbate psoriasis. Retinopathy only occurs with high dosages of chloroquine but is unlikely when chloroquine is used for routine weekly malaria prophylaxis (Gkrania-Klotsas, 2007; CDC & Brunette, 2013). Doxycycline may cause photosensitivity, esophagitis and increased frequency of vaginal yeast infections, as well as long-term staining of teeth and nails (Gkrania-Klotsas, 2007).

Pregnant woman, infants, children younger than 8 years and persons allergic to tetracyclines should avoid doxycycline. Mefloquine may cause gastrointestinal problems, headache, insomnia, abnormal dreams, visions, depression, anxiety disorder, and dizziness and sporadically paresthesia, tremor, ataxia, impatience, mood changes, panic attacks, poor memory, confusion, hallucinations, aggression, paranoia, and encephalopathy (CDC & Brunette, 2013). Therefore, persons with a history of seizures, depression, anxiety and psychiatric disorders should not use mefloquine as prophylaxis. Persons with cardiac conduction abnormalities should also avoid mefloquine treatment (CDC & Brunette, 2013). The FDA has added a boxed warning, the most serious kind of warning about these potential problems, to the labeling of mefloquine because they had received rare reports of neurologic side effects (dizziness, loss of balance, and ringing in the ears) that were permanent (FDA, 2013).

Intermittent preventive treatment (IPT) is the administration of a full therapeutic course of an effective antimalarial treatment at specified time points to a defined population at risk of malaria, without screening for parasitaemia, with the aim of preventing mortality and morbidity (Greenwood, 2006). To reduce the maternal anaemia and low birth weight commonly associated with malaria in pregnancy, IPTs are recommended (Briand, 2007). Pregnant women at risk of *P. falciparum* infection in countries with moderate to high malaria transmission should receive IPTs sulfadoxine-pyrimethamine (SP) at each scheduled antenatal care visit. The first IPT-SP dose should be administered as early as possible during the 2nd trimester of pregnancy and at least 1 month apart up to the time of delivery. Intermittent preventive treatment in infants (IPTi) for all infants at risk of *P. falciparum* infection in Sub-Saharan African countries with moderate to high malaria transmission, all infants should be administered during the routine immunization programme. IPTis are administered during immunization with the DTP2, DTP3 and measles vaccines in three separate doses of sulfadoxine-pyrimethamine (SP), as recommended by the WHO (WHO, 2012).

Seasonal malaria chemoprevention (SMC) is the administration of full treatment courses to children aged 3 to 59 months during the malaria season. Children in highly seasonal malaria transmission across the Sahel subregion of Africa should receive SMC of amodiaquine plus sulfadoxine-pyrimethamine at monthly intervals commencing at the start of the transmission (WHO, 2012).

1.5.6. Case management: Treatment

1.5.6.1. Uncomplicated malaria

Antimalarial combination therapy (ACT) is the concurrent application of two or more antimalarial drugs with differing modes of action, in so far as this is established. The combinations are often more efficient than monotherapy and in the case of *de novo* parasite mutations leading to resistance to one of the combination drugs during the infection, it is generally expected that the infection will be eradicated by the partner drug. Non-artemisinin based combination treatments include sulfadoxine-pyrimethamine plus chloroquine (SP+CQ) or amodiaquine (SP+AQ), but because of resistance to these drugs as monotherapy, their efficacy in combinations have also been compromised.

The World Health Organisation (WHO) guidelines now recommend therapeutic combinations of artemisinin with other antimalarial agents known as the artemisinin-based combination treatments (ACTs) for uncomplicated *P. falciparum* malaria (WHO, 2012). This is done to forestall the possibility of the spread of resistance and, therefore, the choice of ACT will be based on the level of resistance of the partner drug in the combination in a specific region (WHO, 2010a, 2010b). The artemisinins cause a 100 – 1000 fold reduction in parasite numbers per asexual cycle of the parasite, which is superior to any other currently available antimalarial. This radical reduction of parasite numbers causes a rapid resolution of the symptoms. The artemisinins also clear gametocyte stages, thereby reducing gametocyte carriage and also the spread of malaria while *in vitro* evidence suggests that chloroquine may actually induce gametocytogenesis. Because the artemisinins are eliminated rapidly, they are combined with slowly eliminated antimalarials. The partner drug protects the artemisinin component and the artemisinin component protects the partner drug to some extent. Current combinations recommended by the WHO include artemether-lumefantrine (AL), artesunate-amodiaquine (AS + AQ), artesunate-mefloquine (AS + MQ), and artesunate- sulfadoxine-pyrimethamine (AS + SP) as well as the recently added dihydroartemisinin plus piperazine (DHA + PPQ) (WHO, 2012).

A 3-day course of treatment with an artemisinin is required to ensure at least three post-treatment asexual cycles of the parasite and subsequently result in a parasitemia reduction of at least 90%. This reduces the potential for resistance, because the slowly eliminated partner drug only needs to eliminate the remaining 10% of the parasitemia (WHO, 2010a, 2010b).

For the treatment of uncomplicated *P. falciparum* malaria during the first trimester of pregnancy, 7-day quinine plus clindamycin are recommended. In the case where this treatment fails, artesunate plus clindamycin should be given for 7 days. If treatment with the recommended quinine plus clindamycin fails or if the only treatment available is ACTs, then ACT's may be given. For the treatment of uncomplicated *P. falciparum* malaria during the second and third trimesters of pregnancy, 7-day artesunate plus clindamycin or 7-day quinine plus clindamycin or ACTs that is active in the region are recommended. Standard antimalarial treatment including ACTs, with the exception of dapsone, primaquine and tetracyclines such as doxycycline, should be given to lactating women. Infants and young children should receive ACTs. Atovaquone-proguanil, artemether-lumefantrine or quinine plus doxycycline or clindamycin should be given to travellers returning to non-endemic countries (WHO, 2010a, 2010b).

Chloroquine is recommended for the treatment of uncomplicated *P. vivax* malaria in chloroquine-sensitive infection, or an appropriate ACT where *P. vivax* resistance to chloroquine has been documented. The chloroquine and ACT treatments should be combined with a 14-day course of primaquine to avoid relapses, because primaquine eliminates hypnozoites (WHO, 2012; Daily, 2006). The ACT, artesunate plus sulfadoxine-pyrimethamine is not effective against *P. vivax* in many places. In mild-to-moderate glucose-6-phosphate dehydrogenase deficiency (G6PD) deficiency, primaquine may be given at reduced dose levels, but is contraindicated in severe G6PD deficiency (WHO, 2010a, 2010b).

1.5.6.2. Severe/complicated malaria

With severe malaria, treatment should be started immediately with injectable artesunate and followed by a complete course of an effective ACT as soon as oral medications can be taken. Parenteral antimalarials recommended for adults are rectal and intramuscular (IM) artesunate, quinine (IM) or artemether (IM) (WHO, 2012). Because of the unpredictable absorption of IM artemether, it should only be used if the alternatives are not available (WHO, 2010a, 2010b).

2. Artemisone, a novel artemisinin derivative

2.1. Introduction

Since drug-resistant malaria caused previous antimalarial drugs to be ineffective and posed great challenge to medicine and development of effective antimalarial drugs (White, 2004; WHO, 2010a), the choice of treatment for malaria has consequently changed from the failing chloroquine and sulfadoxine-pyrimethamine to artemisinin-based combination treatment (White, 1997; WHO, 2010b).

Apart from being expensive to manufacture, there are other limitations to the use of artemisinins. They are soluble in many aprotic solvents, but show poor solubility in both water and oil, making intravenous injection (IV) difficult (Li & Zhou, 2010). Their fast metabolism imposes a short half-life (Ilett *et al.*, 2002:1015; Kerb *et al.*, 2009). The high recrudescence rates of these compounds – that is re-emergence of parasitaemia after initial subsidence in the absence of an external infection – is indirectly due to these short half-lives, and limits their effectiveness (Woodrow *et al.*, 2005). According to animal studies, embryotoxicity is also a concern. In addition, neurotoxicity of the artemisinins is observed in *in vitro* (Fishwick *et al.*, 1995; Mclean & Ward, 1998; Wesche *et al.*, 1994; Schmuck *et al.*, 2002) and *in vivo* (Brewer, *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997; Nontprasert *et al.*, 1998) assays. Nevertheless, the artemisinins still show considerably less toxicity than the other antimalarials (Woodrow *et al.*, 2005).

Artemisone is a new derivative of artemisinin. It is a semi-synthetic 10-alkylamino-artemisinin synthesized from dihydroartemisinin (Haynes *et al.*, 2004). Artemisinin and DHA are used as antimalarial drugs, but artemisone is superior to both in terms of activity and safety (Haynes, 2006). Compared to the most widely used artemisinin derivative, artesunate, it shows enhanced *in vitro* antimalarial activity against chloroquine-sensitive and -resistant *P. falciparum* cell lines (Haynes *et al.*, 2006). It also displays enhanced *in vivo* activity against chloroquine sensitive *P. berghei* and chloroquine resistant *P. yoelii* rodent models. (Vivas *et al.*, 2007, Haynes *et al.*, 2006). In *Aotus* monkey-*P. falciparum* models artemisone also showed enhanced activity (Haynes *et al.*, 2006; Obaldia *et al.*, 2009). In Phase IIa trials no neurotoxicity was observed, it was well tolerated in humans and had a curative effect at dose levels of approximately one third of artesunate (Krudsood *et al.*, 2005; Nagelschmitz *et al.*, 2008). The lack of neurotoxicity of artemisone is fundamental, given that artemisinins, as discussed earlier are neurotoxic in neuronal cell cultures *in vitro* (Fishwick *et al.*, 1995; Mclean & Ward, 1998; Wesche *et al.*, 1994; Schmuck *et al.*, 2002)

and in animal models (Brewer, *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997; Nontprasert *et al.*, 1998).

Artemisone was developed by a consortium involving Hong Kong University of Science and Technology (HKUST), Bayer AG, then Bayer Health Care until 2007 when all intellectual property was transferred to HKUST (personal communication, RK Haynes; 2013). Thereafter, Medicines for Malaria Venture (MMV) funded a pharmaceutical company Penn Pharmaceuticals in Wales to look at decomposition under thermal stress testing. Although the drug has been withdrawn from the active development portfolio of MMV, they still continue to test artemisone against resistant isolates. The world's supply of artemisone – about 60 kg – is now in the hands of Cipla Mumbai Pty. Ltd. (personal communication, RK Haynes, 2013).

Figure 2.1 highlights the global portfolio of anti-malarial drugs in the pipeline organized by development stage as of March 2012 (Anthony *et al.*, 2012). Artemisone is in a grey box with a dashed border (figure 2.2). This indicates that as of March 2012, artemisone development did not have active MMV involvement (dashed border) and that it was defined as “on hold” (grey box) because no significant developmental progress was made publicly in the last year.

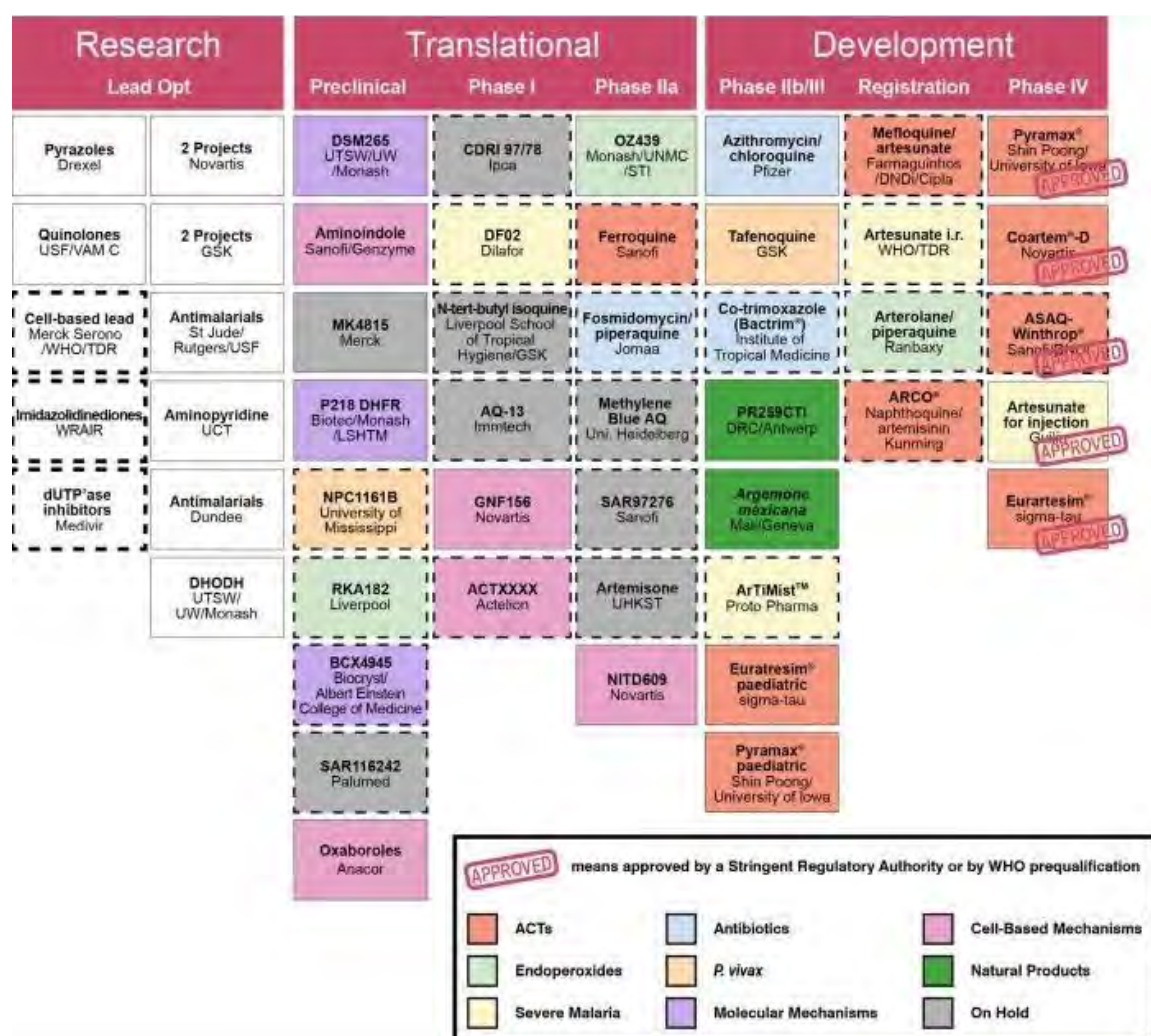


Figure 2.1: The global portfolio of anti malarial medicines under development (as of March 2012; reprinted from Anthony *et al.*, 2012 with permission from Biomed Central).

2.2. Synthesis and physico-chemical properties

Although the total synthesis of artemisinin has been carried out on several occasions, the syntheses are far too expensive to warrant commercial use. However, a biotechnological approach that leads to a late biochemical precursor of artemisinin in *A. annua* has been developed. This late precursor, known as artemisinic acid, can then be converted into artemisinin in a relatively few number of steps (Paddon *et al.*, 2013).

Artemisinin is easily extracted from *Artemisia annua*, and this extraction method constitutes the major source of artemisinin at the moment (Vonwiller *et al.*, 1993; Haynes & Vonwiller, 1994; Woodrow *et al.*, 2005; Haynes, 2006). Artemisinin is converted into dihydroartemisinin (DHA) by reduction (Haynes *et al.*, 2006). Artemisone is synthesized

from DHA in two ways. The one route (Figure 2.2 route a) involves the treatment of DHA (1) with trimethylsilyl chloride (TMSCl) and then with trimethylsilyl bromide (TMSBr) resulting in a bromide intermediate (3). The other, simpler route (Figure 2.2, route b) to follow involves the treatment of DHA (1) with a mixture of sodium bromide (NaBr) and then with TMSCl in toluene followed by the amine. The treatment of the intermediate bromide (3) with the amine nucleophile results in the formation of an intermediate (4) which can be converted to artemiside (5). Oxidation of artemiside (5) produces artemisone (7) and sulfoxide (6) (Haynes *et al.*, 2006).

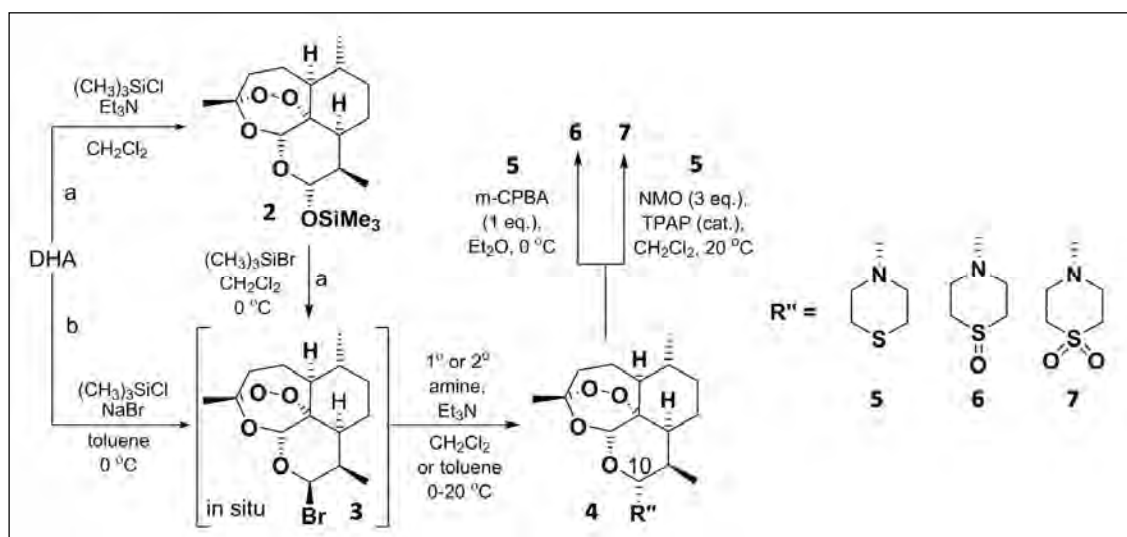


Figure 2.2: The synthesis of artemisone from dihydroartemisinin (modified from Haynes *et al.*, 2006 with permission from Wiley online interscience).

Artemisone is a highly crystalline compound that is stable under various temperatures. In Table 1 the aqueous solubility and Log P (octanol–water partition coefficient) values of artemisinin and artemisinin derivatives are given. Artemisone is partly water-soluble with an aqueous solubility of 89 mg/L at pH 7.2, which lies above the minimum aqueous solubility requirements for a drug (Haynes *et al.*, 2006). It has a low log P of 2.49 at pH 7.4. Because of this low Log P value, artemisone is classified as a non-lipophilic drug; this may in part be responsible for the relatively low neuro- and cytotoxicity in *in vitro* and *in vivo* assays (Nagelschmitz *et al.*, 2008).

Table 2.1: Aqueous solubility and octanol–water partition coefficients (Haynes *et al.*, 2006).

	Artemisinin	DHA	Artesunate	Artemether	Artemisone
Solubility (mg/L) ^a	63	nd	565	117	89
Log P ^b	2.94	2.35, 2.73	2.77	3.98	2.49

^a Aqueous solubility at pH 7.2. ^b Octanol–water partition coefficient for the neutral compound determined at pH 7.4, except that for artesunate which was determined at pH 2 by HPLC. Because DHA exists as two epimers in solution, log P appears as two values. nd: Due to decomposition, the solubility of DHA could not be measured

2.3. The mechanism of action of artemisinins

Since artemisinin was discovered in the 1970s and after considerable research efforts on artemisinin, there remains substantial controversy over the mechanism of action of artemisinins on malaria parasites (O’Neil *et al.*, 2010; Haynes *et al.*, 2013). The endoperoxide bridge within the 1,2,4-trioxane is essential for activity, as confirmed by years of structural-functional research (Wang, 2010). 1-Deoxyartemisinin, which lacks an endoperoxide bridge, is ineffective in killing parasites (Haynes & Krishna, 2004).

Artemisinins are regarded by some as prodrugs, requiring generation of active intermediates after encountering ferrous iron. These active intermediates are considered to be carbon-centered free radicals or reactive oxygen species (ROS) (Cui & Su, 2009). As peroxides are known sources of reactive oxygen species (ROS), it is proposed that the mechanism of action of artemisinins are connected to the generation of ROS like hydroxyl, alkoxyl, (protonated) superoxide or peroxy radicals within the parasitized erythrocyte (Haynes & Krishna, 2004). Studies suggest that artemisinins alter parasite oxidative stress and diminish the antioxidant and glutathione (GSH) levels in the parasite (Meshnick, 2002; Krungkrai *et al.*, 1987; Ittarat *et al.*, 2003).

Concerning the ring opening of artemisinins during bioactivation, two models have been proposed, *i.e.* the reductive scission model and the open peroxide model. The reductive scission model proposes that ferrous heme or non heme Fe²⁺ binds to artemisinin. Consequent electron transfer causes reductive scission of the peroxide bridge to generate oxygen centred radicals, which in turn rearrange to give carbon centered radical (Olliaro *et al.*, 2001; Wu *et al.*, 1998). Iron was found to interact with the peroxide to produce a primary carbon centred radical or a secondary carbon centred radical. These carbon-centered radicals may target heme-binding proteins and proteinases involved in the degradation of haemoglobin (Haynes & Krishna, 2004).

The open peroxide model proposes that the ring opening of artemisinins is accomplished by protonation of the peroxide or by complexation with Fe^{2+} (Olliaro *et al.*, 2001). In this model it was also proposed that iron may act as a Lewis acid to facilitate ionic bioactivation of artemisinin, leading to cleavage of the endoperoxide bridge and subsequent formation of an unsaturated hydroperoxide by the incorporation of water. The Fenton degradation of the hydroperoxide also produces a hydroxyl radical. These products may act as oxidants or produce ROS that may be responsible for the antimalarial activity of artemisinins (Haynes *et al.*, 2007).

Heme is generated from digestion of hemoglobin in the food vacuole of the malaria parasite. The involvement of intraparasitic heme in the activation of artemisinins is, therefore, also hypothesized. This may also explain the selective toxicity of artemisinins toward malaria parasites (Stocks *et al.*, 2007). Evidence against this heme activation hypothesis has also emerged. For instance, the efficiency of the conversion of synthetic artemisinin derivatives to C-centered radicals does not correlate with antimalarial activity and the C-centered radicals are too short-lived to have any intermolecular interaction (Haynes *et al.*, 2004; Haynes *et al.*, 2007; Haynes *et al.* 2013).

Furthermore, artemisinins are active against early ring stage malaria parasites that contain very little hemozoin, as well as other parasite species such as *Toxoplasma* and *Babesia* that do not contain hemozoin (Skinner *et al.*, 1996; Jones-Brando *et al.*, 2006; Kumar *et al.*, 2003; Haynes *et al.* 2013). However, more recent studies revealed that, by using fluorescence microscopy and image analysis, hemoglobin is released from heme in ring stage parasites and may be an iron based activator which is responsible for artemisinin activity against the ring stage parasites (Abu-Bakar *et al.*, 2010). Fluorescent artemisinin derivatives examined in living parasites using confocal microscopy showed that the localisation of artemisinin in living parasites is not associated with the food vacuole, where digestion of hemoglobin occurs (Eckstein-Ludwig *et al.*, 2003).

2.3.1. *The biological target(s) of artemisinins*

2.3.1.1. *Haem and haem model alkylation*

It has been suggested that hemoglobin is degraded by a series of protease enzymes to release peptides and amino acids required for development within the malaria parasite and that the concurrent build-up of hemozoin is potentially toxic to the parasite by means of pro-oxidant activity and/or interaction with food vacuole proteins (Egan, 2008). It has also been proposed that heme–artemisinin adducts are formed when artemisinin-derived radicals react

with free hemin, heme present in the hemozoin and hemoglobin *in vitro* (Hong *et al.*, 1994; Kannan, *et al.*, 2002; Kannan, *et al.*, 2005) and that these adducts interact with the *P. falciparum* histidine-rich protein II (PfHRP II), to displace the heme from PfHRP II (Kannan *et al.*, 2002; Loup *et al.*, 2007). This reaction is then proposed to inhibit heme polymerization and hemozoin formation, resulting in heme build up, which is toxic to the parasite. Contradicting this, *in vivo* studies showed that hemozoin formation was not inhibited by artemisinin treatment (Meshnick, 1996). The various theories have been summarized, and it is concluded that all have problems in adequately accounting for the nanomolar activities of the artemisinins (Haynes *et al.* 2013).

2.3.1.2. Protein targets

Incubation of malaria parasites with radiolabeled artemisinins revealed binding of artemisinin with several proteins, of which the translationally controlled tumor protein (TCTP) orthologue of mammalian and other cells have been identified. So far the function of TCTP in malaria parasites and the significance of its binding to artemisinin has not been identified (Bhisutthibhan *et al.*, 1998; Bhisutthibhan *et al.*, 1999). Also, there is only low-affinity interaction ($K_d > 10 \mu\text{M}$) between TCTP and dihydroartemisinin (Bhisutthibhan *et al.*, 1998; Eichhorn *et al.*, 2013; Pandey *et al.*, 1999).

Also, sarcoplasmic reticulum Ca^{2+} -transporting ATPases (SERCAs) have been proposed as specific targets of artemisinins based on the structural similarities between the sesquiterpene moieties of artemisinins and thapsigargin, a potent inhibitor of SERCAs [88]. It was suggested that artemisinins may act in a similar way, specifically inhibiting the SERCA of malarial parasites (PfATP6, Eckstein-Ludwig *et al.*, 2003). Convincing evidence supporting this hypothesis includes demonstrated specificity for inhibition of the PfATP6, exceptional correlation ($R^2 > 0.9$) between assays for inhibiting PfATP6 in the oocyte model and eradication of malaria parasites, antagonism between thapsigargin and artemisinins, and the inhibition of PfATP6 is also Fe^{2+} -dependent (Haynes & Krishna, 2004). In an artificial system where PfATP6 is expressed in *Xenopus laevis* oocytes, the inhibition of PfATP6 by artemisinin is specific and artemisinin and thapsigargin antagonise each other when they are assayed in parasite cultures (Eckstein-Ludwig *et al.*, 2003).

On the contrary, in this system, the inhibitory concentrations of artemisinins are 30 fold higher than the *in vitro* concentrations of artemisinins used in *P. falciparum* culture. 1-Deoxyartemisinin, which lacks an endoperoxide bridge, did not inhibit PfATP6 in this model, or kill parasites in culture unless it was used at very high concentrations ($>10 \mu\text{M}$, Eckstein-

Ludwig *et al.*, 2003). In the same *Xenopus* oocyte system, when a single amino acid (L263E) in PfATP6, which is potentially involved in hydrophobic interactions with artemisinins, is changed, no inhibition of the enzyme by artemisinin occurs (Uhlemann *et al.*, 2005.). Furthermore, the SERCA in *Toxoplasma gondii* (TgSERCA) is also inhibited by artemisinins or thapsigargin (Nagamune *et al.*, 2007). Supporting the PfATP6 hypothesis, a polymorphism in the gene encoding PfATP6 has been associated with the *in vitro* resistance to artemether in *P. falciparum* field isolates (Krishna, 2006).

2.3.1.3. Mitochondria

Inhibition of the ETC and inner membrane potential of the mitochondria of both the sexual and asexual stages of parasites by artemisinins has also been described in parasites. By using classic ETC inhibitors of complex I–IV, they also showed that the ETC of *Plasmodium* parasites is similar to that of mammals. They proposed that the iron group in the cytochrome centre induces the formation of radicals and may be responsible for ETC inhibition (Krungkrai *et al.*, 1999). This proposed mechanism does not explain the specificity of artemisinin activity towards malaria parasites. However, Wang *et al.* (2010) suggest that species differences in mitochondria explain the specificity of the artemisinins (Wang *et al.*, 2010).

Results from a yeast (*Saccharomyces cerevisiae*) model suggest that NADH dehydrogenase in the mitochondrial electron transport chain might be a target for artemisinins as deletion of the gene encoding this enzyme led to artemisinin resistance and overexpression of this gene increased sensitivity to artemisinin. Consequently, it was proposed that the mitochondrion has a dual role in the mechanism of artemisinins, where the ETC is responsible for artemisinin activation, which generates local ROS, which consecutively hinder mitochondrial function by the depolarization of the mitochondrial membrane. Depolarization of the mitochondrial membrane affect pyrimidine biosynthesis, which is involved in nucleic acid production and, therefore, may cause parasite death (Li *et al.*, 2005). In more recent studies, it was demonstrated that artemisinin acts in a similar manner in malaria as in the yeast model. By labelling the malarial mitochondria with Mitotracker Red and using a monoclonal antibody against artesunate, it was shown that most of the artesunate is colocalized to malarial mitochondria. Reactive oxygen species (ROS) formation was induced by artemisinin in isolated mitochondria from yeast and malaria but not in mammalian mitochondria. Deoxyartemisinin, which lacks an endoperoxide bridge, had no effect on membrane potential or ROS production in malarial mitochondria. By adding desferrioxamine,

an iron chelator, ETC activity and artemisinin-induced ROS formation was considerably reduced (Wang *et al.*, 2010).

Furthermore, neurotoxicity of the artemisinins is observed in *in vitro* (Fishwick *et al.*, 1995; Mclean & Ward, 1998; Wesche *et al.*, 1994; Schmuck *et al.*, 2002) and *in vivo* (Brewer *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997; Nontprasert *et al.*, 1998) assays. Schmuck *et al.* (2002) proposed that artemisinins affect the mitochondrial inner membrane potential in neuronal-cell cultures. Artemisone, however, unlike the first generation artemisinins, is not neurotoxic, supporting the view that artemisone does not affect mitochondrial inner membrane potential (Haynes *et al.*, 2006).

2.4. Pharmacokinetics

All artemisinins have relatively short half-lives of 1–3 h after oral administration, which is the most frequent route of administration (Gordi, 2012). The majority of artemisinin derivatives are converted primarily to dihydroartemisinin (DHA), also a potent antimalarial. Artemisinin itself is not metabolised to DHA. The degree of conversion to DHA differs between the derivatives. Artesunate is very quickly hydrolysed to DHA. Following oral artesunate administration, artesunate and its main metabolite, DHA, were measured in the plasma. Peak artesunate concentrations (C_{max}) were reached in less than one hour (T_{max}) and an elimination half-life ($t_{1/2}$) of 20 - 45 minutes was observed. Peak DHA concentrations (C_{max}) were reached in less than two hours post-dose; with DHA $t_{1/2}$ values of 30-90 minutes (Morris *et al.*, 2011). Artemether and arteether are also converted to DHA (Woodrow *et al.*, 2005). For artemisinin, the peak artemisinin concentrations were reached in 100 minutes (T_{max}) and an elimination half-life ($t_{1/2}$) of 37 minutes was observed (Benakis *et al.*, 1997).

The pharmacokinetics of artemisinins are consistent with rapid absorption and rapid elimination. It is characterized by intersubject variability in plasma drug concentrations, high oral clearance of 150-300 litres/h, a low to moderate apparent volume of distribution of 6-20 liters/kg, and elimination half-lives up to 5 h (Ashton *et al.*, 1998; Duc *et al.*, 1994; Mordi *et al.*, 1997; Na-Bangchang *et al.*, 2004; Teja-Isavadharm *et al.*, 1996; Teja-Isavadharm *et al.*, 2001; Zhang *et al.*, 2001). The pharmacokinetics of artemisone correlates well to those of the artemisinins. After the last dose of the 3-day course of 80 mg artemisone, intersubject variability occurred and artemisone was rapidly absorbed with T_{max} values of about 1.5 h after dosing, oral clearance of 237 litres/h, apparent volume of distribution of 13.7 litres/kg and elimination half-life values of 3.1 h. In the single-dose artemisone study, artemisone was rapidly absorbed, with median T_{max} values increasing from 0.25 to 0.87 h for the five

dose groups (10, 20, 30, 40 and 80 mg). Moderate to high intersubject variability of the $C_{\max}$ was observed in the groups with a $C_{\max}$ increasing less than dose proportionally. After the single 80-mg dose, artemisone had a geometric mean maximum concentration of 140.2 ng/mL, a short elimination half-life ($t_{1/2}$) of 2.79 h, a high oral clearance of 284.1 liters/h and a large volume of distribution of 14.50 litres/kg (Nagelschmitz *et al.*, 2008).

The three important metabolites of artemisone and the degradation product, DHA, were measured simultaneously with the parent drug. The highest plasma concentrations were found for M1, followed by M2 and M3, after administration of the solution and tablets, with lower exposures and slightly shorter half-lives than for the parent compound. After the 80-mg dose $C_{\max}$ values were 121.1 ng/mL for M1, 108.9 ng/mL for M2 and 74.3 ng/mL for M3, compared to the $C_{\max}$ of 140.2 ng/mL for the parent compound. The AUC was 315.4 ng h/mL for M1, 379.9 ng h/mL, for M2 and 294.8 ng h/mL for M3 compared to the AUC of 281.6 ng/mL for the parent compound. The $t_{1/2}$ was 2.78 h for M1, 1.36 h for M2 and 1.59 h for M3 compared to a $t_{1/2}$ of 2.79 h for the parent compound. Although the plasma concentrations of dihydroartemisinin were low with $C_{\max}$ values of 10 ng/mL after the 80 mg dose, it is important to note that no DHA was observed in *in vitro* studies (Nagelschmitz *et al.*, 2008; Haynes *et al.*, 2006).

2.5. Metabolism

The fast metabolism of artesunate (by CYP2A6), artemether (by CYP3A4 /A5) and arteether (by CYP3A4 (secondary contribution of CYP2B6 and CYP3A5)) to dihydroartemisinin and of dihydroartemisinin to α -difydroartemisinin- β -glucuronide by UGT1A9 and UGT2B7 causes artemisinins to have a short half life (Ilett *et al.*, 2002; Kerb *et al.*, 2009). These short half lives attributes to the high recrudescence rates of these compounds and, therefore, limits their effectiveness (Woodrow *et al.*, 2005).

Artemisone is also metabolised by CYP 3A4 and metabolites M1, M2 and M3 are the primary metabolites formed. There are species-differences in the formation of the metabolites. In human liver microsomes, labelled artemisone produced metabolites M1-M5 after 30 minutes. Of the total radioactivity (TRA), M1 was responsible for approx. 34%, M2 for approx. 19%, M3 for approx. 16% and M4 and M5 for smaller amounts of less than 6% each. When artemisone was incubated with dog microsomes a large amount of M1 and a small amount of M3 were formed. With rat microsomes artemisone formed smaller amounts of M1 and large amounts of M2 and M3. In these systems, no DHA was detected. Plasma from rats after oral artemisone treatment contains large amounts of M2 and M3 and small

amounts of M1, M4, and M5. Because of the good correlation between the *in vitro* profiles from rat liver microsomes and hepatocytes and the *in vivo* plasma profile of metabolites of rats, human artemisone metabolism will likely compare well with the *in vitro* profiles from human microsomes and hepatocytes (Haynes *et al.*, 2006).

CYP3A4 is regarded as the vital enzyme for phase I biotransformation of artemisone in humans. These phase I reactions include the dehydrogenation in the thiomorpholine-dioxide moiety leading to metabolite M1, monohydroxylation in the methyl-oxepane and methylcyclohexyl moieties leading to the metabolites M2 and M3 respectively (Figure 2.4). Metabolites M4 and M5 are formed by combination of the primary biotransformation reactions (Haynes *et al.*, 2006). Inhibition of CYP3A4 mediated metabolism of artemisone was achieved with the CYP3A4 inhibitors trolleandomycin, azamulin, and ketoconazole (Haynes *et al.*, 2006).

During the treatment of human hepatocytes with 4 – 10000 ng/L of artemisone induction of CYP1A2, CYP3A4, CYP2B6 and CYP2C19 not clinically significant although a borderline inductive effect on CYP2B6 and CYP3A4 was observed at the highest concentration. Artemisone had no inhibitory effects on CYP isoforms when incubated with standard substrates and recombinant enzymes responsible for the biotransformation of these substrates (isoforms CYP1A2, -2A6, -2C9, -2C19, -2D6, -2E1, and -3A4).

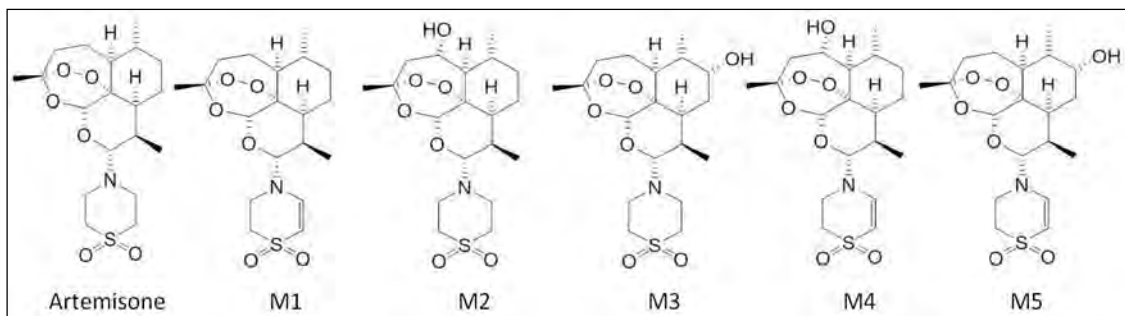


Figure 2.3: Artemisone and metabolites M1–M5

2.6. Antimalarial Activities

Artemisinins are the most potent and rapid antimalarial drugs available. They kill parasites resistant to other drugs. Artemisinin therapy causes a reduction in parasite burden of larger than 90% within 24 hours (Norsten & White, 2007). A limitation of the artemisinins is the high recrudescence rates of the infection 4–6 weeks after the initial artemisinin therapy (Gordi, 2012). The World Health Organisation (WHO) guidelines now recommend therapeutic combinations of artemisinin with other antimalarials with longer half-lives, known as the artemisinin-based combination treatments (ACTs) for uncomplicated *P. falciparum* malaria (WHO, 2010b)

The antimalarial activities of artemisone, as well as other artemisinins, synthetic peroxides and standard malaria drugs *in vitro* against *P. falciparum* are summarized in Table 2.2 and 2.3. The values are given as IC₅₀ values, that is the amount of drug required to inhibit half of parasites from developing (Desjardins *et al.*, 1979). Artemisone has shown excellent *in vitro* activity against both chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Vivas *et al.*, 2007; Delves *et al.*, 2012; Marfurt *et al.*, 2012). When compared to the current artemisinin 'gold standard' artesunate, artemisone displays superior activities in the *in vitro* screens.

Table 2.2: *In vitro* and *ex vivo* activities (IC₅₀ nM) of artemisone and other artemisinins, synthetic peroxides and standard antimalarial drugs against *P. falciparum* as well as *P. falciparum* (*Pf*) and *P. vivax* (*Pv*) field isolates (Vivas *et al.*, 2007; Delves *et al.*, 2012; Marfurt *et al.*, 2012)

Compound	3D7 ^a	K1 ^a	D6 ^b	W2 ^b	TM90C2A ^b	FC27 ^c	K1 ^c	<i>Pf</i> field isolates ^c	<i>Pv</i> field isolates ^c
Artemisone	0.88	1.23	0.8	0.5	0.6	1.9	2.0	1.9	0.7
Artesunate	9.44	5.18	5.0	3.3	4.1	15.4	10.3	4.5	4.0
Artemiside	-	-	-	-	-	4.0	4.3	2.5	3.0
Artemisinin	-	-	11.1	7.1	15.1	-	-	-	-
Artemether	-	-	3.5	3.5	5.1	-	-	-	-
DHA	-	-	-	-	-	10.0	9.6	6.4	4.9
OZ277	-	-	1.1	1.6	1.5	10.9	20.0	3.6	4.6
OZ439	-	-	2.8	3.3	3.2	11.1	18.6	2.1	4.5
Chloroquine	9.73	255.86	8.8	268.3	105.8	39.9	168.7	91.4	48.3
Piperaquine	-	-	8.6	9.6	7.9	-	-	-	-
Amodiaquine	20.3	40.89	4.6	10.8	5.8	-	-	-	-
Pyronaridine	-	-	4.1	9.5	4.6	-	-	-	-
Lumefantrine	7.46	2.56	4.1	1.6	5.9	-	-	-	-
Mefloquine	6.11	1.41	8.8	4.4	14.5	-	-	-	-

3D7 - chloroquine-sensitive; K1 – chloroquine, pyrimethamine and cycloguanil resistant; FC27 - chloroquine-sensitive; K1 - chloroquine, pyrimethamine and cycloguanil resistant; D6 - chloroquine-sensitive; W2 - chloroquine-resistant; TM90C2A – chloroquine and mefloquine resistant; Field isolates taken from malaria patients in Timika, Papua Province, Indonesia. ^aVivas *et al.*, 2007; ^bDelves *et al.*, 2012; ^cMarfurt *et al.*, 2012)

Table 2.3: *In vitro* activities (IC₅₀ ng/mL) of artemisone and other artemisinins against *P. falciparum* W2 and *P. falciparum* field isolates from patients who did not respond to ACTs (unpublished data, personal communication with Ding & Haynes, 2013)

Compound	Isolates (IC ₅₀ ng/mL)	W2 (IC ₅₀ ng/mL)	Isolate/W2 ratio
Artemisone	0.95	0.51	1.86
Artemiside	5.44	3.95	1.38
Artesunate	3.26	2.17	1.50
DHA	3.19	1.37	2.33
DHA internal control	2.22	1.24	1.79
DHA internal controll	2.2	1.3	1.69

Artemisone had an efficacy of 4 to 10 times greater than that of artesunate in *in vivo* screens in the four-day Peters model (Peters & Robinson, 1999) against various rodent malaria lines (Tables 2.4-2.5). It is important to note that since artemisone is more potent than artesunate against the *P. yoelii* artemisinin-resistant line, the possibility exists that artemisone may oppose artemisinin-resistant *P. falciparum* in the future. Tendencies towards slight antagonism between artemisone and chloroquine, amodiaquine, tafenoquine, atovaquone or pyrimethamine and synergisms with artemisone and mefloquine, lumefantrine or quinine in *in vitro* studies were observed. In *in vivo* studies, artemisone showed various degrees of synergism and mefloquine, chloroquine or clindamycin *in vivo* (Vivas *et al.*, 2007).

Table 2.4: *In vivo* activity of artemisone and artesunate against *P. Berghei* N and *P. Yoelii* NS rodent malaria lines in a Peters' four-day screen (Haynes *et al.*, 2006).

Compound	<i>P. berghei</i> N ED ₉₀ mg/kg		<i>P. berghei</i> N Artesunate index		<i>P. yoelii</i> P ED ₉₀ mg/kg		<i>P. yoelii</i> P Artesunate index
	sc	po	sc	po	sc	po	sc
Artesunate	7.2	7.1	1.0	1.0	22.0	-	1.0
Artemisone	1.5	3.1	4.8	2.3	3.9	5.0	5.6

sc - subcutaneous route; po - oral route.; *P. Berghei* N - chloroquine-sensitive; *P. Yoelii* NS - CQ-resistant; ED₉₀ calculated from parasite counts in peripheral blood on Day 4; Artesunate index: ED₉₀ artesunate/ ED₉₀ of compound.

Table 2.5: *In vivo* activity of artemisone and artesunate against rodent malaria lines in a Peters' four-day screen (Vivas *et al.*, 2007).

Line	Route	Artemisone		Artesunate	
		ED ₉₀ (mg/kg)	I ₉₀	ED ₉₀ (mg/kg)	I ₉₀
<i>P. berghei</i> NY	sc	9.62	1.0	41.21	1.0
	po	11.67	1.0	111.94	1.0
<i>P. berghei</i> P	sc	1.92	0.2	18.20	0.4
<i>P. berghei</i> KFY	sc	0.83	0.1	5.38	0.1
<i>P. yoelii</i> NS	sc	11.30	1.0	49.54	1.0
	po	27.99	1.0	179.47	1.0
<i>P. yoelii</i> ART	sc	12.13	1.1	87.50	1.8
<i>P. chabaudi</i> AS	sc	1.38	1.0	19.68	1.0

sc - subcutaneous route; po - oral route.; *P. berghei* NY - drug-susceptible; *P. berghei* P - primaquine-resistant; *P. berghei* KFY - sulfadoxine/pyrimethamine-resistant; *P. yoelii* NS - chloroquine-resistant; *P. yoelii* ART - artemisinin-resistant; *P. chabaudi* AS – drug susceptible; I₉₀ = ED₉₀ resistant line/ED₉₀ parent line

In an *ex vivo* efficacy assay, healthy saimiri monkeys received oral artemisone or artesunate treatments of 30 mg/kg. The plasma samples drawn after dosage (time intervals of 1,2,3,4 and 6h) were assayed against *P. falciparum* K1 strains. Artemisone showed superior and more sustained activity when compared to artesunate. In another study, *P. falciparum* (FVO strain) infected Aotus monkeys received artesunate and artemisone treatments of 10 mg/kg for 3 days. Within 24h following treatment, parasites were cleared in the artemisone treated group but in the group treated with artesunate, parasites were still present after 49h. Except for one artemisone treated monkey that was cured, recrudescence occurred in the other monkeys, but with recrudescence occurring later in the artemisone than in the artesunate groups.

Recrudescence is common to all artemisinins. For this reason, as well as to reduce the possibility of resistance, artemisone will best be used in conjunction with a drug with a longer half life for example mefloquine or amodiaquine. In the same study, infected monkeys receiving a single oral dose of artemisone (10 mg/kg) in combination with mefloquine (MFQ, 5 mg/kg) were completely cured, while recrudescence occurred with a lower MFQ dose of 2.5 mg/kg. Administration of a 3 day oral dose of artemisone (10 mg/kg per day) in combination with amodiaquine (AQ, 20 mg/kg per day) also cured the infected monkeys (Table 2.6) (Haynes *et al.*, 2006; Obaldia *et al.*, 2009). When both drugs were given alone for 3 days, monkeys were not cured Treatment with artemisone (30 mg/kg/day) and clindamycin (CM; 100 mg/kg/day)

showed that 3 days of treatment with this drug combination also cured infected monkeys (Obaldia *et al.*, 2009). Artemisone displayed greater efficacy than artesunate with a curative dose one third that of artesunate in phase II trials with non-severe malaria patients (Krudsood *et al.*, 2005).

Table 2.6: Responses of malaria-infected monkeys to oral treatment with artemisone alone or with artemisone combined with mefloquine, amodiaquine or clindamycin (Obaldia *et al.*, 2009).

No of days treated	Drug(s)	Dosage (mg/kg) daily	No. of monkeys		Parasite recrudescence (days)
			Treated	Cured	
1	Artemisone	20	1	0	No clearance
	Artemisone	30	1	0	No clearance
	Artemisone + Mefloquine	10; 2.5	1	0	24
	Artemisone + Mefloquine	10; 5	2	2	None
	Artemisone + Mefloquine	10; 12.5	1	1	None
	Artemisone + Amodiaquine	30; 20	3	0	11, 11, 12
	Amodiaquine	20	3	0	No clearance
2	Artemisone + Amodiaquine	30; 30	3	2	19
3	Artemisone + Amodiaquine	10; 20	3	3	None
	Amodiaquine	20	3	0	9, 11, 16
	Artemisone	10	3	0	17, 17, 19
	Artemisone + Clindamycin	30; 100	3	3	None
	Artemisone	30	2	1	22

2.7. Toxicity

Although the artemisinins still have considerable less significant toxicity than the other antimalarials (Woodrow *et al.*, 2005), the administration of artemisinins seems to be linked with neurotoxicity and embryotoxicity in *in vitro* and *in vivo* models (D'Alessandro *et al.*, 2007). Neurotoxicity of the artemisinins is observed in *in vitro* (Fishwick *et al.*, 1995; Mclean & Ward, 1998; Wesche *et al.*, 1994; Schmuck *et al.*, 2002) and *in vivo* (Brewer *et al.*, 1994; Kamchonwongpaisan *et al.*, 1997; Nontprasert *et al.*, 1998) assays.

No neurotoxicity was observed for artemisone in *in vitro* studies using primary neuronal brain stem cell and cortical neuron cultures from fetal rats. Cytotoxicity, neurofilament integrity and intracellular ATP levels, also a marker of cytotoxicity, were measured. Of the artemisinins, DHA displayed the greatest neurotoxicity with a “no observable effect concentration” (NOEC) of less than 0.001 mg/mL on neurofilaments. Artemisone supplied NOECs and IC₅₀ values of greater than 10mg/mL for the cytotoxicity endpoint, ATP level measurements and neurofilament integrity.

According to animal studies, the embryotoxicity of artemisinin derivatives are of concern. Preclinical studies in rodents showed that high oral dose and low injectable dose levels of artemisinins can induce fetal death and congenital malformation. This however can only be induced for a brief period during early embryogenesis (Dellicour *et al.*, 2007; Clark *et al.*, 2004; Longo *et al.*, 2006). Since 1989 to 2009 no embryotoxicity has been observed in clinical trials in pregnant women (including first trimester pregnancies) exposed to artemisinins or ACTs (McGready *et al.*, 2001; Adam *et al.*, 2006). However, for safety reasons, artemisinins should be avoided during the first trimester of pregnancy (Li & Weina, 2010). There seems to be a connection between embryotoxicity and defective angiogenesis and vasculogenesis in certain embryo development phases (D'Alessandro *et al.*, 2007).

Artemisone is not metabolised to DHA. When the anti-angiogenic properties of both artemisone and DHA were compared in different model systems, artemisone was significantly less anti-angiogenic than DHA in all the experimental models. From a clinical point of view, in these studies doses of 10–20 times higher than those observed in the plasma of treated patients from the phase IIa clinical trial (Krudsood *et al.*, 2005) were used (D'Alessandro *et al.*, 2007). Together with the superior anti-malarial activity and negligible neurotoxicity, artemisone has an advantage in clinical use over the current used artemisinin derivatives. Therefore, artemisone can potentially be used against malaria during pregnancy, but *in vivo* animal studies should first be carried out (D'Alessandro *et al.*, 2007).

It has also to be considered that ART is potentially genotoxic and mutagenic. Artesunate has been shown to be cytotoxic for mammalian cells, including DNA repair-defective Chinese hamster cell lines (Li *et al.*, 2008), and a large panel of cell lines of different tumor origin (Efferth *et al.*, 2003; Kelter *et al.*, 2007). The genotoxicity were only observed upon chronic treatment of cells with the drug, whereas 1-h pulse treatment was without any effect. It is therefore important to note that the artemisinins have short half-lives in the body and therefore, such short exposures should remain without cytotoxic effect in mammalian cells. The genotoxic/mutagenic potential of artemisone was assessed in two *in vitro* (the Salmonella Microsome test, also known as Ames test, for point mutations and the cytogenetics *in vitro* assay for clastogenicity) and one *in vivo* (Micronucleus test) assays. All tests were negative and therefore artemisone is considered as non-genotoxic (Herbold, 2002a, 2002b, 2002c).

3. Drug delivery of artemisinins

Artemisinins have low aqueous solubility that results in poor and inconsistent absorption and low bioavailability upon oral administration (White, 2008). Another limitation is their short half-lives, which demand frequent drug administration. This, in turn, may lead to non-compliance, recrudescence and possible development of resistance (Dondorp *et al.*, 2010; Gautam *et al.*, 2009). One approach to improve the bioavailability, patient compliance and reduce side effects of an active compound is the use of drug delivery (Gardner, 1987; Speiser, 1998). Lipid-based drug delivery systems, consisting of oils, surfactants, and co-solvents, are often used to enhance absorption and the bioavailability of poorly water-soluble drugs (Nanjwade *et al.*, 2011).

In recent studies, lipid-based drug delivery systems were used to improve the anticancer and antimalarial efficacy of the artemisinins. DHA-loaded copolymeric micelles (DHA-CM) and the IC₅₀ values for the human oral carcinoma KB cells significantly decreased from 21.55 for DHA to 18.70 μ M for DHA-CM (Lu *et al.*, 2013). Also, tumor- and tumor lymphatics-homing peptide (LyP-1) conjugated PEG-PCL micelles (LyP-1-PM) were used to deliver artemisinin specifically to both highly metastatic tumor and its lymphatics. This resulted in enhanced anticancer activity against MDA-MB-435S and lymphatic endothelial cells (Wang *et al.*, 2012).

By using liposomes as the drug delivery system, both the pharmacokinetics and antimalarial efficacy of artemisinin were enhanced (Isacchi *et al.*, 2011, Isacchi *et al.*, 2012). Artemisinin was loaded in conventional and polyethylene glycol polyethylene glycol (PEGylated) liposomes and administered via the intraperitoneal route to healthy mice. Free artemisinin was rapidly cleared from plasma and was barely detectable 1 hour after administration while both of the

liposomal formulations had much longer blood-circulation time and artemisinin was still detectable after 3 with the conventional and 24 hours with the PEGylated liposomes. When compared with the free artemisinin, the $AUC_{0-24\text{ h}}$ values were increased by approximately 6 times in both of the liposomal formulations and the half-life of artemisinin was increased by more than 5-fold with the PEGylated liposomes (Isacchi *et al.*, 2011).

When artemisinin and artemisinin-curcumin was loaded into conventional and PEGylated liposomes, the antimalarial activity was enhanced in *Plasmodium berghei* NK-65 infected mice (Isacchi *et al.*, 2012). When artemisinin was administered alone, the parasitaemia levels only decreased after 7 days after the commencing of treatment and there was high variability in artemisinin plasma concentration. However, treatments with artemisinin-loaded conventional liposomes (A-CL), artemisinin-curcumin-loaded conventional liposomes (AC-CL), artemisinin-loaded PEGylated liposomes (A-PL), artemisinin-curcumin-loaded PEGylated liposomes (AC-PL) showed an immediate antimalarial effect, cured all malaria-infected mice within the same time period after inoculation and showed less variability in artemisinin plasma concentrations (Isacchi *et al.*, 2012).

When β -arteether was loaded into self-emulsifying drug delivery systems and administered at a daily dose of 24 mg/kg for 4 days to mice infected with *Plasmodium berghei*, 100% of the treated mice were completely cured for more than 45 days. The antimalarial efficacy was comparable to that of the intramuscular oily solution of arteether and significantly higher than that of an oily solution of β -arteether given orally at the same dose (Memvanga & Pr  at, 2012).

3.1. Pheroid[®] vesicles as a drug delivery system

Although various drug delivery systems are available, they have several limitations i.e. stability, cost and safety issues (Cimato *et al.*, 2004; Hagar *et al.*, 1993; Muller & Keck, 2004). The Pheroid delivery system is a fatty acid-based delivery system that is stable for periods longer than two years at room temperature (Steyn *et al.*, 2011), relatively low manufacturing cost and safe (Grobler, 2009). In December 2003, the Pheroid[®] technology was purchased by the North-West University, South Africa from MeyerZall (Pty.) Ltd. The founders of MeyerZall (Pty.) Ltd., Piet Meyer and Steven Zall, initially developed the technology for the treatment of psoriasis in 1999. The resulting product showed superior efficacy and fewer side effects than comparable products (Grobler, 2009). In 2000, MeyerZall Laboratories established a research team with the aim to explore the potential of the delivery system for wider application than the single topical product. The system was called Emzaloid (derived from the ‘m’ of Meyer, the ‘za’ of Zall and the word “colloid”) and trade-marked (Saunders *et al.*, 1999). Clinical trials carried out in the

UK, Austria, the Netherlands and Boston USA showed that the Emzaloid™-based topical coal tar psoriasis product was superior (n=327) and equivalent (n=60) to the reference products (Goodfield *et al.*, 2003; Tzaneva *et al.*, 2003). Various topical products based on the Emzaloid™ technology were formulated, registered and marketed. Also, faster and deeper absorption of various active compounds with the Emzaloid™ was shown in several transdermal *in vitro* studies (Saunders *et al.*, 1999).

Pheroid® technology is based on Emzaloid™ technology but is not identical. Several differences exist between the manufacturing protocols of Emzaloid™ and Pheroid® systems. According to a patent filed in July 2001, for generation of the Emzaloid™ system, nitrous oxide is used at 200 kPa for 48 hours (Meyer, 2002). For generation of the Pheroid formulation nitrous oxide is used at pressures higher than 150 kPa for 3-4 days. All Pheroid formulations contain d,l- α -tocopherol, while all Emzaloid™ formulations do not contain it and the two formulations also vary in the component ratios. The word Pheroid® is derived from the Greek words “apo” and “phero”, which directly translated mean “to move”, “to ferry” or “to deliver”, and the word colloid (Grobler, 2009).

The Pheroid® is easy to prepare. It is not prepared according to liposomal preparatory principles, but is in a similar manner to that of an emulsion. It consists of an oil phase and a nitrous oxide saturated water phase. The oil phase mainly contains 'Vitamin F' ethyl esters, Kolliphor® EL, DL- α -tocopherol, nitrous oxide and water (Grobler, 2009). The essential fatty acids corresponding to Vitamin F ethyl esters are necessary for various cell functions but cannot be synthesized in the human body (Das, 2006; Dobryniewski *et al.*, 2007). Kolliphor® EL, formerly known as Cremophor® EL, is used to solubilize lipophilic drugs and stabilize emulsions in aqueous systems (Rossi *et al.*, 2007; Gelderblom *et al.*, 2001; Jeong *et al.*, 2007).

The aqueous phase consists of mainly sterile water, but buffers can also be used (Grobler, 2009). The nitrous oxide (N₂O) contributes to the miscibility of the fatty acids, the self-assembly process and the stability of the Pheroid® (Grobler *et al.*, 2008). The stability and efficiency of Pheroid® decreases considerably if the formulation lacks either the essential fatty acids or the nitrous oxide. Also, the combination of these two components supplies an efficient transportation model for hydrophobic and hydrophilic drugs (Grobler *et al.*, 2008).

The pro-Pheroid® formulations only consist of an oil phase saturated with nitrous oxide gas and, therefore, have no water phase or particles. Upon addition of a water phase, spontaneous formation of Pheroid® micro- and nano-particles occurs, entrapping the APIs present in the

formulation into these particles (Grobler, 2009). The Pro-Pheroid® formulation is particularly important when the APIs, for example rifampicin, are unstable in the presence of moisture. Pro-Pheroid® generally contains polyethylene glycol 400 (PEG-400) but any polyethylene glycol or polymeric units may also be used during manufacturing (Grobler, 2009). PEG is a substance that is generally recognized as safe (GRAS list, Food and Drug Administration). The PEG outer coat provides “stealth” characteristics to Pheroid®, permitting increased time in circulation and promotes the avoidance of the mononuclear phagocyte system and liver and spleen accumulation. This increases the therapeutic window and decreases non-specific toxicities (Remsberg *et al.*, 2013).

Pheroids can be manipulated in terms of morphology, size and function (Schlebusch, 2002). The size and structure of the Pheroid® formulations can be manipulated to form lipid-bilayer vesicles, microsponges or pro-Pheroid®, by altering the ratios of components and/or the manufacturing procedure (Grobler, 2009). The typical size of the lipid-bilayer vesicles is between 80-300 nanometer and the size of the micro sponges range between 0.5-5 micrometres (Schlebusch, 2002). The size distribution of the most used vesicle formulation with a fatty acid content of 1.89% was determined with the confocal laser scanning microscopy (CLSM) and membrane filtration (Figure 3.1) (Grobler, 2009).

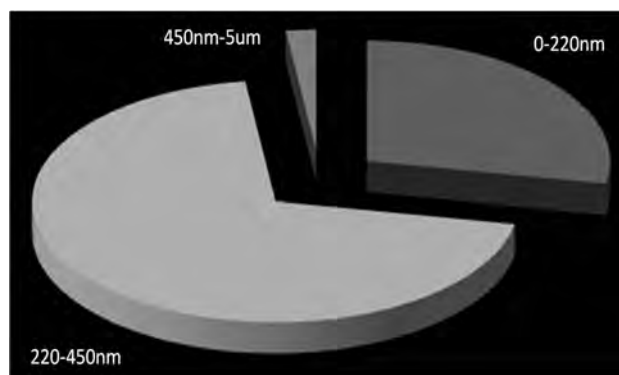


Figure 3.1: Size distribution of a typical Pheroid formulation measured by CLSM (modified from Grobler, 2009 with permission from A. Grobler).

Pheroid® is able to enhance the absorption and/or efficacy of several active ingredients and compounds (Grobler *et al.*, 2006). The entrapment of the pharmacologically active compounds within the Pheroid® creates a safer and more effective formulation than a formulation containing only the active compound (Figure 3.2) (Schlebusch, 2002). In the body, the Pheroids are either metabolised in the mitochondria or the peroxisomes of cells, consequently releasing the API (Grobler *et al.*, 2008).

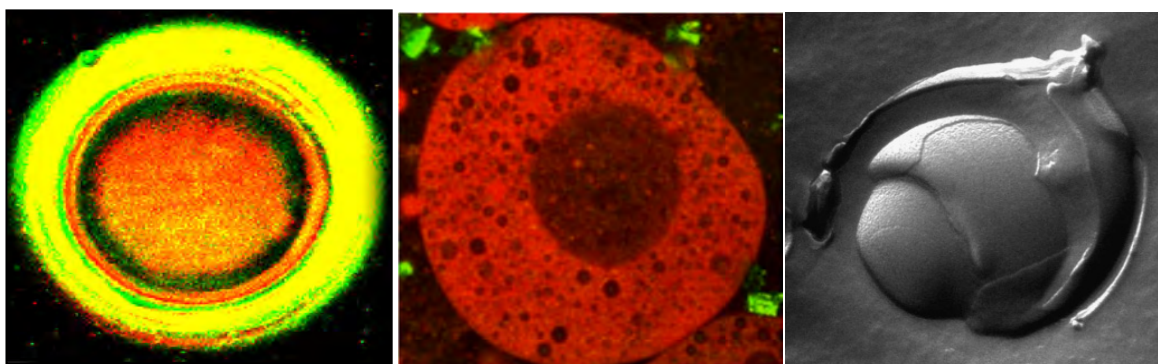


Figure 3.2: Confocal micrographs of (a) Pheroid® vesicle containing rifampicin with a mean diameter of 100 nm, b) Pheroid® microsphere containing Vitamin A with a mean diameter of 1.5 µm and cryo transmission electron microscopy micrograph of c) the multiple layers of the Pheroid® vesicle (personal communication from A. Grobler and A. Botha; 2011)

Pheroid® can be administered by the oral, transdermal and nasal routes. High stability is observed (Steyn *et al.*, 2011; Slabbert *et al.*, 2011). Pheroid® has been successful in the transdermal delivery of anti-infective agents as well as cosmetic products (Grobler *et al.*, 2008; Grobler, 2009). The Pheroid® increased the nasal delivery of calcitonin and recombinant human growth hormone *in vivo* (Du Plessis *et al.*, 2010; Steyn *et al.*, 2010). It resulted in increased drug plasma levels and bioavailability and a decrease in side effects after oral delivery of entrapped tuberculosis drugs (Meyer, 2012). The Pheroid® has also been applied in the delivery of pesticides, micro nutrients and growth regulator (Grobler, 2007; Grobler, 2009). Antimalarial drugs showed superior efficacy when entrapped into the Pheroid® technology (Langley, 2007; Odendaal, 2009; Van Huyssteen, 2010; Van Niekerk, 2010, Steyn 2009).

3.2. Application of Pheroid technology as an anti-malarial (specifically artemisone) drug delivery system

This study focuses on artemisone entrapped in the Pheroid®. *In vitro* and *in vivo* efficacy and *in vivo* bioavailability studies have been carried out for artemisone entrapped in Pheroid®. My study was structured on the basis of the *in vitro* efficacy and *in vivo* bioavailability results obtained by the North-West University (NWU; Steyn, 2010; Steyn *et al.*, 2011). These results show a 4.5 fold increase in the *in vitro* antimalarial efficacy of artemisone when entrapped into the Pheroid® and also a 4.6 fold increased exposure of artemisone *in vivo* when entrapped into the Pheroid®. In a study carried out by the Swiss Tropical and Public Health Institute (STPHI), the *in vitro* efficacy results of Steyn (2010) could not be confirmed (Jourdan, 2011). The STPHI study indicated either a decrease or no change in the IC₅₀ values when artemisone was

entrapped in the Pheroid®. The *in vitro* efficacies are summarized in Table 3.1. The normal assay involved serial dilutions where the Pheroid® concentration was not kept constant throughout the dilution range; therefore, the lower the artemisone concentration, the lower the Pheroid concentration. For the 'constant Pheroid® concentrations assay', the Pheroid® concentrations were kept constant throughout the dilution range. The number of Pheroid® vesicles was kept constant for three different Pheroid® concentrations (1:1000, 1:2000 and 1:4000 (v/v)). When the Pheroid® concentrations were kept constant, similar IC₅₀ values were obtained when compared to the reference. The studies at the NWU were conducted with a constant Pheroid® concentration of 1:2500 (v/v).

In vivo efficacy and *in vivo* bioavailability studies were performed in a murine model (male C57 BL6 mice) at the NWU and at the University of Cape Town (UCT). The *in vivo* efficacy was tested in *P. berghei* infected mice using the Peters' 4-day suppressive test model. In contrast to the *in vitro* efficacy results, the Peters' 4-day suppressive test indicated no significant improvement in the *in vivo* antimalarial activity of artemisone when entrapped into the Pheroid® (Steyn, 2009). With the *in vivo* bioavailability study, the pharmacokinetic parameters of artemisone improved statistically ($p < 0.05$) when entrapped into the Pheroid®. The *in vivo* bioavailability results are summarized in Table 3.2.

Table 3.1: The *in vitro* efficacy results of artemisone reference and artemisone entrapped into the Pheroid® drug delivery system as obtained by the STPHI and NWU.

Results obtained by the NWU ^a – measured in nM						
	Artemisone ref	Artemisone Phe (1:2500)				
RSA11	0.94 ± 0.04	0.21 ± 0.04				
Results obtained by the STPHI ^b – measured in ng/mL						
		Normal assay		Constant Pheroid concentrations assay		
Strain	Artemisone ref	Artemisone Phe	Artemisone Phe (pre-entrapped)	Artemisone Phe (1:1000)	Artemisone Phe (1:2000)	Artemisone Phe (1:4000)
K1	0.18 ± 0.08	0.40 ± 0.26	1.10 ± 0.15	n.d	n.d	n.d
NF54	0.34 ± 0.10	0.60 ± 0.26	1.80 ± 0.18	0.42 ± 0.40	0.35 ± 0.21	0.33 ± 0.23

^a Steyn *et al.*, 2011; ref – reference; Phe - Pheroid®

Table 3.2: The *in vivo* bioavailability results of artemisone reference and artemisone entrapped into the Pheroid® drug delivery system as obtained by the NWU^a.

	T _{max} (h)	C _{max} (ng/mL)	T _{1/2} (h)	AUC _{0-last} (ng h/ml)	AUC _{0-inf} (ng h/mL)
Artemisone ref	0.12 ± 0.01	809.50 ± 98	0.36 ± 0.06	485.70 ± 106.30	604.60
Artemisone Phe	0.55 ± 0.05	1550.00 ± 105.40	1.10 ± 0.26	2219.00 ± 122.70	3094.00 ± 392.10

^a Steyn *et al.*, 2011; ref – reference; Phe - Pheroid®

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