



## CHAPTER 1

### Introduction

*“It hides in the dark, silent, waiting... Then as dusk approaches it strikes – fast! Deadly! Malaria is a killer! In Africa, it is one of the worst serial killers of all time...”*

#### 1.1 The statistics

Worldwide, there are 109 malaria endemic countries as surveyed in 2008, with 45 of these in Africa. In 2006, 3.3 billion people were at risk of contracting malaria of which 1.2 billion people reside in Africa. Two hundred and forty-seven million people were infected with malaria in 2008 resulting in 1 million deaths, with 91% of these in Africa and 85% due to children younger than 5 years of age (Figure 1.1) (World Malaria Report 2008). In Africa, a child dies every 30 seconds due to the devastating impact of malaria (Greenwood *et al.*, 2005). In eastern Uganda, children can expect to be infected with malaria once every 2 months, even with the use of bednets and artemisinin combination therapies (ACT's) (Price, 2000) and in the rest of Africa a child can have an average of 1.6 to 5.4 clinical episodes of malaria fever every year (World Malaria Report 2008). This is clearly in stark contrast to the Millennium Development Goals (MDG) that were adopted by 189 nations and signed by 147 heads-of-state and governments during the United Nations (UN) Millennium Summit in September 2000. The MDG's 8 goals include the eradication of hunger and poverty, provision of primary education, gender equality, improved maternal health and reduction in child mortality, to combat various diseases like Human Immunodeficiency Virus (HIV) and malaria, environmental sustainability and finally the development of global partnerships. Of particular interest is MDG goal 6, which aims to reduce malaria infection and mortality, and especially child mortality by 2015.

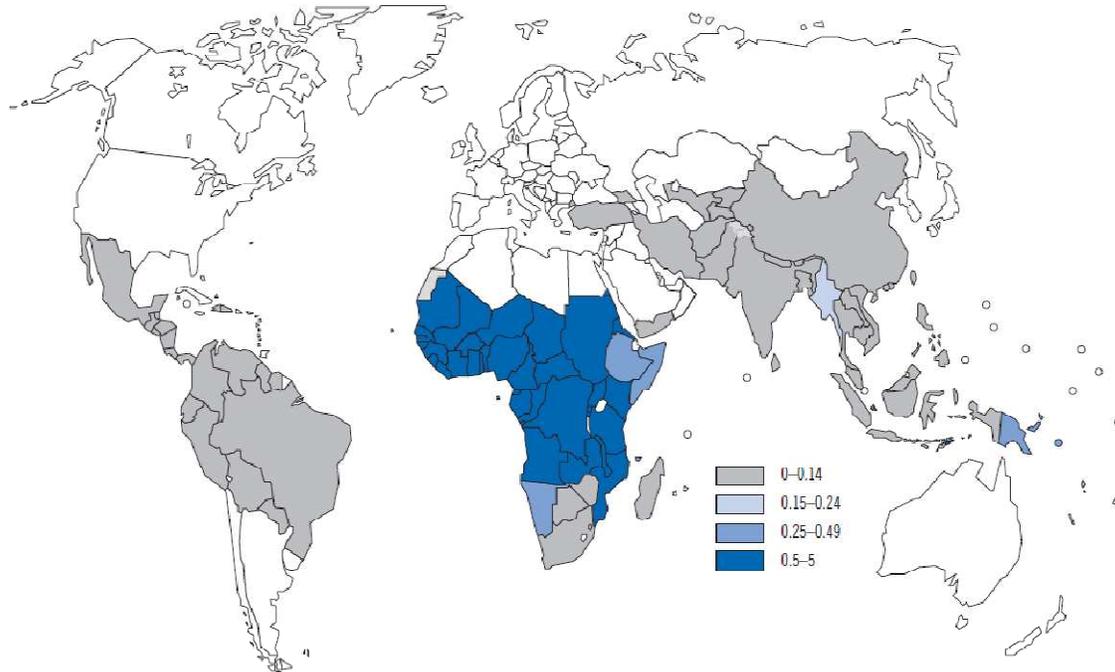


Figure 1.1: Estimated worldwide deaths (in millions) from malaria in 2006 as given by the 2008 WHO report ([www.who.int](http://www.who.int)).

A global map of endemicity of malaria is lacking since WHO maps only provide estimations of malaria incidence (Figure 1.1). The Malaria Atlas Project (MAP) generated a total of 8938 *P. falciparum* parasite rate (*PfPR*) surveys, of which 7953 passed the strict criteria to be included in a global database. This data was captured from 1985 until currently (2010), of which more than 50% of the data is representative from 2000 onward. This database is currently used to predict malaria endemicity and incidence with geographic visualisation (Figure 1.2) (Hay *et al.*, 2009, Guerra *et al.*, 2008, Guerra *et al.*, 2007). In the future, it is aimed to also produce a map on *P. vivax* endemicity, but unfortunately data for this is still lacking (Hay *et al.*, 2009).

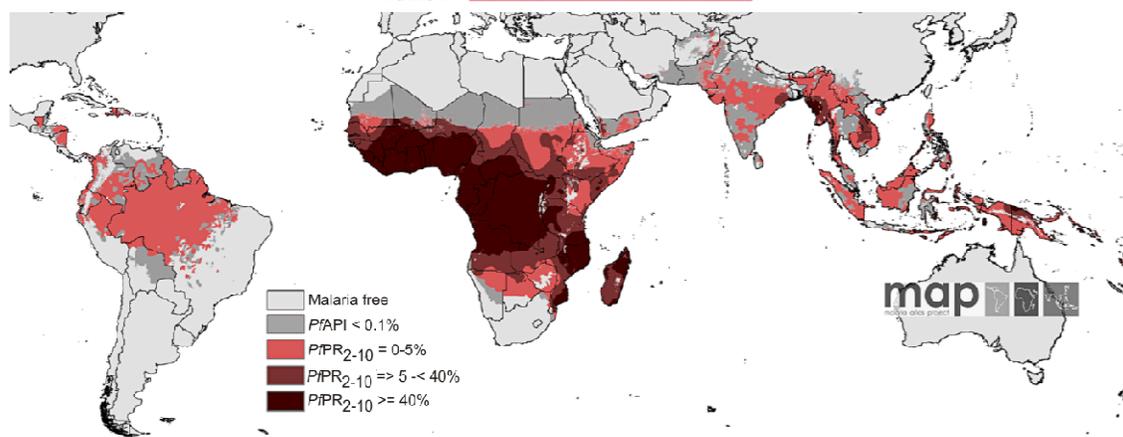


Figure 1.2: Distribution of *P. falciparum* (Hay *et al.*, 2009).

The map is categorized as low risk  $PfPR_{2-10} \leq 5\%$ , light red; intermediate risk  $PfPR_{2-10} > 5$  to 40%, medium red; and high risk  $PfPR_{2-10} \geq 40\%$ , dark red. Unstable risk areas is medium grey where  $PfAPI < 0.1$  per 1000 pa or no risk in light grey. All red areas are representative of  $PfAPI > 0.1$  per 1000 pa.  $PfAPI$  is the *P. falciparum* annual parasite incidence.  $PfPR_{2-10}$  is *P. falciparum* prevalence rate corrected to the 2-10 year age group

## 1.2 The economic burden of malaria

During the rainy season in the province of Garki, Nigeria, a person would be bitten an average 174 times per night by mosquitoes of the genera *Anopheles gambiae* (Gallup J.L. & Sachs J.D., 2001). In Kou Valley in Burkina Faso, a person would be bitten 158 times per night by *A. gambiae*, with the average total mosquito bites reaching an astounding 35 000 per year (Gallup J.L. & Sachs J.D., 2001). Even with these horrible statistics, most people neither have bednets nor do they have proper prophylaxis, and are therefore constantly reinfected with malaria. This has a direct economic impact on mostly already poverty stricken countries, as people are unable to work or go to school, therefore resulting in an overall reduction in productivity (Greenwood *et al.*, 2005). It is not surprising that the 33 richest countries are malaria free (Gallup J.L. & Sachs J.D., 2001). The economic burden of malaria can be seen in the fact that the Gross Domestic Product (GDP) in endemic countries can decrease by as much as 1.3% per year.

Reasons for the deterioration of malaria in some parts of Africa may be attributed to environmental changes like climate instability, global warming, war and civil disturbances, increasing travel around the world, HIV infection, increasing drug resistance, and increasing insecticide resistance (Tatem *et al.*, 2006, Greenwood & Mutabingwa, 2002, Greenwood, 2002). However, in recent years, 7 out of 45 African countries and 22 countries outside of Africa, with small populations with active interventions were able to reduce the total number of malaria cases and malaria related deaths when compared to data from 2000. Four African countries, Eritrea, Rwanda, Sao Tome and Principe, as well as Zanzibar in Tanzania, were able to reduce their malaria burden by 50% between



2000-2007 by means of aggressive malaria control. A huge success story is the United Arab Emirates, which was the first malaria endemic country since the 1980's to be certified malaria free by the WHO, and now forms part of the 92 malaria free countries around the world. Of the 109 countries affected by malaria, 82 are in the control stage of malaria elimination, 11 countries are in pre-elimination, 10 in elimination stages and 6 countries are preventing re-introduction of malaria (World Malaria Report 2008).

### 1.3 History of malaria

Malaria has been known as a killer disease for centuries with Hippocrates already describing fevers, mostly correlating to swamps, hence the Italian name “*mal' aria*” meaning “bad air”. Ancient Romans were affected by malaria due to the marshes around Rome (Gardiner D.L. *et al.*, 2005). The first challenge to the miasma theory (stench from decaying matter) came from Louis Pasteur and Robert Koch who demonstrated that microbes were responsible for certain diseases. Later, this was followed by Edwin Klebs and Corrado Tommasi-Crudeli who claimed in 1879 the isolation of “*Bacillus malariae*” as causative agent for malaria, although this theory was soon disregarded (Guillemin, 2002). In 1880, Alphonse Laveran (1845-1922) observed the first malaria gametocyte in the blood of a French soldier in Algeria, a discovery that won him the Nobel Prize in 1907. In 1897, Ronald Ross (1857-1932) identified *Plasmodium* parasites within the *Anopheles* mosquito and demonstrated that malaria is transmitted from an infected mosquito to the human host. This achievement won him Knighthood and the Nobel Prize in 1902 (Hagan & Chauhan, 1997). The final piece of the puzzle came from Short and Garnham who in 1948, described schizonts in the livers of monkeys and thereby completed the life cycle of *Plasmodium* (Gardiner D.L. *et al.*, 2005).

### 1.4 Life cycle

Malaria is caused by the protozoan parasite, *Plasmodium*, that occurs in 4 major disease causing species: *P. vivax*, *P. malariae*, *P. falciparum*, and *P. ovale*. *P. falciparum* is the most virulent, and causes the most severe form of malaria (Carter & Mendis, 2002). Recent findings has established the enzoonotic transmission of the simian malaria parasite *P. knowlesi*, that was previously only found in nature in macaques, to humans (Bronner *et al.*, 2009). The Plasmodial parasite has a complex life cycle that consists of both a vertebrate and invertebrate host. Malaria is transmitted by the bite of female *Anopheles* mosquitoes, occurring mainly between sunrise and sunset. Unfortunately, Africa is home to some of the most effective malaria vectors including *A. gambiae* and *A. funestus* (Mons B. *et al.*, 1997). The highest risk of contracting malaria is at the end of the rainy season or soon thereafter as this is also the time when the mosquito vectors are most

abundant. When an infected female mosquito takes a blood meal, malaria sporozoites are released from the saliva into the subcutaneous tissue of the human host (Figure 1.3 B).

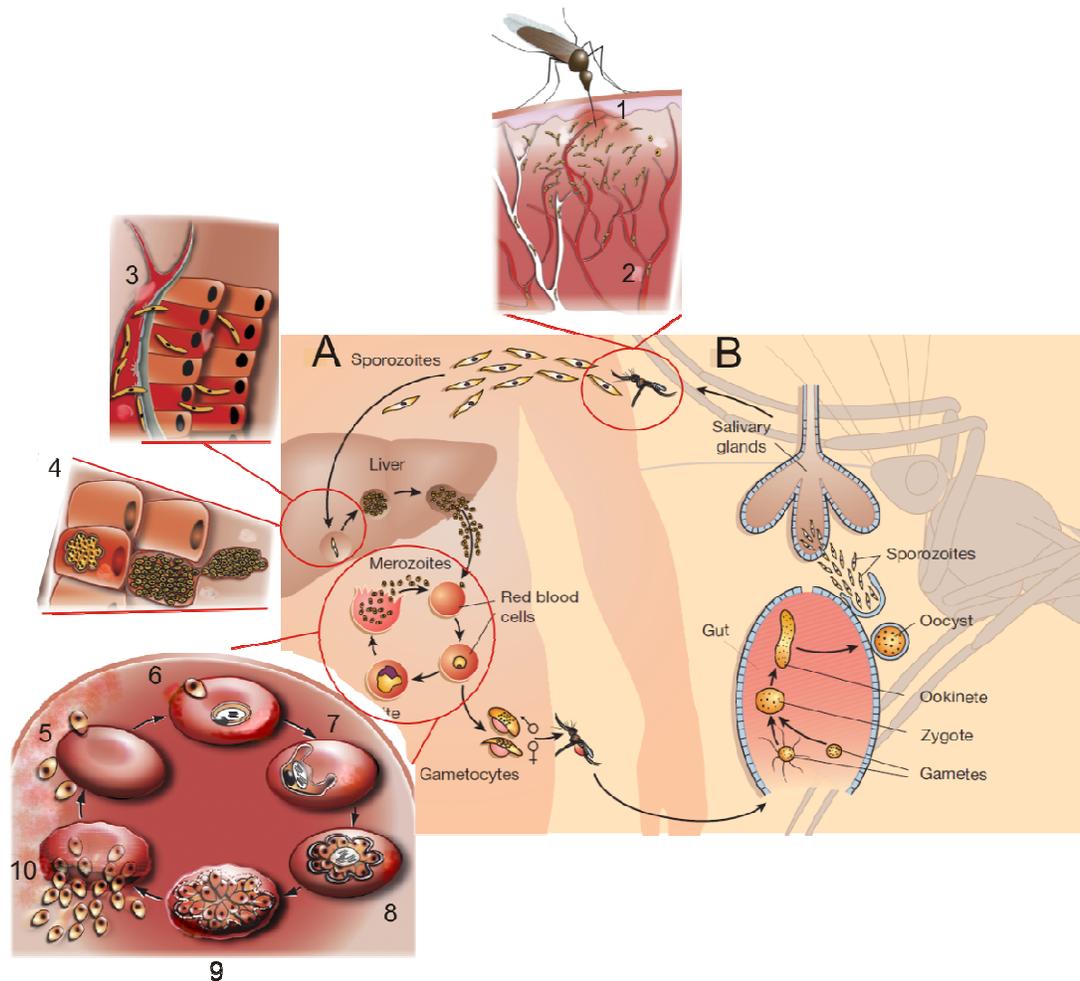


Figure 1.3: The Plasmodial life cycle. Compiled from (Wirth, 2002, Silvie *et al.*, 2008).

A: The asexual life cycle in the human host. B: The sexual life cycle in the mosquito. 1: Intradermal sporozoite injection when the female mosquito takes a blood meal. 2: Sporozoites migrate to the blood vessels to be distributed through the blood circulation. 3: Sporozoites invade the hepatocytes in the liver. 4: The parasites mature and multiply in the liver to ultimately release merozoites in membrane-shielded merosomes. 5: Start of the intraerythrocytic developmental cycle by the invasion of an erythrocyte by a merozoite. 6: Ring stage. 7: Trophozoite stage. 8: Schizont stage. 9: Preparation to release merozoites from the erythrocyte. 10: Merozoite egress. The released merozoites will then re-invade an erythrocyte to start the intraerythrocytic developmental cycle again.

The sporozoites progress to the liver where they will invade hepatocytes and develop into schizonts. This hepatocytic incubation period of malaria is 7 to 15 days but may also take up to 3 months (Silvie *et al.*, 2008). *P. vivax* is able to produce hypnozoites that can reside within the liver for months therefore causing malaria relapses months or sometimes years after infection. Usually, after 6 to 10 days, the schizonts will multiply and discharge 10 000 to 30 000 merozoites from the hepatocytes into the bloodstream (Yu *et al.*, 2008). The merozoites will invade erythrocytes where they will multiply within their 48 hour asexual life cycle. This intraerythrocytic development cycle



(IDC) consists of the development into the ring stage, followed by the trophozoite stage and finally the schizont stage in which the parasite will prepare itself for re-invasion of erythrocytes by the production and release of 8-32 merozoites (Figure 1.3 A) (Bozdech *et al.*, 2003, Bannister *et al.*, 2000). This cycle will continue until the death of the host occurs or death of the parasites due to drug treatment or immune responses of the human host. A proportion of the asexual parasites will develop into sexual gametocytes, which can be taken up by another mosquito when it bites an infected human host (Wirth, 2002). Within the mosquito gut, the gametocytes will differentiate into male and female gametes that can fuse to form a zygote, which is the only diploid stage in an otherwise mainly haploid life cycle. In the mosquito midgut, the zygote differentiates into an ookinete and finally matures into a sporozoite-filled oocyst (Wirth, 2002). The oocyst migrates out of the mosquito gut to release sporozoites that are able to migrate to the mosquito salivary gland therefore enabling the mosquito to infect a human host and completing the life cycle of the Plasmodial parasite (Sinden & Billingsley, 2001).

## 1.5 Pathogenesis

The most common symptoms of malaria include fevers, chills, headaches, muscular aching, weakness, vomiting, coughing, diarrhoea, abdominal pain and may therefore be commonly mistaken for flu (Clark & Cowden, 2003). Early diagnosis and treatment can be life saving and therefore it is important that travellers to malaria endemic areas monitor their health after visits to malaria areas and seek medical advice once they fall ill (World Malaria Report 2008). The rupture of infected erythrocytes and invasion of new erythrocytes is also the main cause of pathogenesis. Uncomplicated malaria has a cyclical occurrence with coldness, followed by rigor and fever as well as sweating every 48 hours corresponding to the lysis of infected erythrocytes and the release of merozoites and subsequent re-invasion of new erythrocytes.

Merozoites that are released into the bloodstream to invade erythrocytes do not pierce the erythrocyte but forms a deep invagination that encloses the parasite within the parasite vacuolar membrane (PVM) (Garcia *et al.*, 2008). Invasion can be divided into several stages that include initial adhesion, re-orientation of the merozoite apical surface, junction formation, generation of the PVM and movement of the merozoite into the parasite vacuole, sealing of the parasite vacuole, discharge of granules onto the parasite vacuole, and finally merozoite transformation into ring stage parasites (Iyer *et al.*, 2007). Initial adhesion is mediated mainly by merozoite surface protein-1 (MSP-1) that is an integral membrane protein on the surface of merozoites (Cowman & Crabb, 2002). Initial attachment to the erythrocyte by MSP-1, is followed by re-orientation of the merozoite apical end towards the erythrocyte surface which is mediated by apical membrane



antigen-1 (AMA-1). Duffy binding proteins and reticulocyte binding-like (*Pf*RBL) proteins are important for junction formation (Cortes, 2008), while entry into the parasite vacuole is mediated by the erythrocyte binding antigens (Silvie *et al.*, 2008).

Knob formation seems essential for erythrocyte adhesion by rosetting as well as sequestering, and is one of the major disease complications associated with clinical episodes of malaria that include impaired microvascular flow, hypoxia, reduced metabolite exchange, and cerebral malaria (Garcia *et al.*, 2008, Starnes *et al.*, 2009). The occurrence of rosetting and sequestration is one of the major differences between *P. falciparum* and *P. vivax*, since erythrocytes infected with *P. vivax* cannot sequester and therefore also does not result in the life-threatening symptoms associated with *P. falciparum*. Rosetting and sequestering is mediated due to the export of erythrocyte membrane protein-1 (*Pf*EMP-1) to the surface of the erythrocyte to protect the parasite against the host immune responses. *Pf*EMP-1 is able to bind various receptors, that include intracellular adhesion molecule 1 (ICAM), E-selectin, CD36, CD31, and hyaluronic acid (HA) ultimately resulting in rosetting and sequestration of infected erythrocytes (Artavanis-Tsakonas *et al.*, 2003).

Severe malaria will cause a 100% mortality rate if left untreated and even when treated still results in 15% mortality (World Malaria Report 2008). Symptoms of severe malaria include amongst others, splenomegaly, severe headaches, cerebral ischemia, cerebral malaria, hepatomegaly, hypoglycemia, and hemoglobinuria with renal failure, and finally coma and death (de Ridder *et al.*, 2008). High risk individuals include pregnant woman and children as well as travellers. Severe malaria has many similarities to sepsis, and for this reason sepsis has been used as a model in elucidating the pathogenesis of malaria as disease (Mackintosh *et al.*, 2004). “Malaria toxin” is released upon lysis of the erythrocytes due to merozoite release. This “malaria toxin” is identified as glycosylphosphatidylinositol (GPI) which subsequently induces the release of tumor necrosis factor (TNF) to activate a network of cytokines to mediate cellular defence, resulting in illness of the host (Grau *et al.*, 1989). Production of pro-inflammatory cytokines is central to malaria as disease with many of these mediators also active in various infectious diseases. Disease pathology as a result of cytokine induction include fever, hypoglycaemia, bone marrow depression, coagulopathy, hypotension, and the possible destruction of infected erythrocytes (Clark & Cowden, 2003). Both lymphotoxin (LT $\alpha$ ) and TNF will induce high levels of IL-6 and induce arginine dependent nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) which is able to kill parasites (Anstey *et al.*, 1996). Cytokine-mediated protection against malaria is mediated by the action of macrophages that are able to generate nitric oxide as a reactive oxygen species resulting in the



stimulation of T-cells. Cerebral malaria is typically associated with increased mRNA and protein levels of TNF, IL-2 and LT $\alpha$  (Brown *et al.*, 1999, Engwerda *et al.*, 2002).

Malaria in pregnant women poses a severe health risk to both mother and the unborn child. One of the main reasons is the fact that infected erythrocytes from the placenta bind specifically to chondroitin sulphate A (Fried *et al.*, 2006, Ricke *et al.*, 2000), compared to ICAM and CD36 in adults (Maubert *et al.*, 1998, Ricke *et al.*, 2000, Rogerson *et al.*, 2007). During normal pregnancy the cytokine balance is shifted towards a Th2-type response to ensure a safe pregnancy, while in malaria infected pregnancies the balance is shifted towards Th1 as a result of the malarial infection. Malarial infection increases the levels of TNF $\alpha$ , IFN $\gamma$ , IL1 $\beta$  and IL-2 which severely affects the risk of stillbirths, abortions and congenital malaria (Rogerson *et al.*, 2007).

Children under the age of 5 and immuno compromised individuals are also at risk of severe malaria. Severe malaria in children may often result in anaemia, learning impairments and brain damage (World Malaria Report 2008). In Africa, the severity of malarial infections is worsened even further by the extremely high incidence of HIV infections that affect both children and adults. A susceptible immunity and impaired cytokine response poses a risk of severe complications and death due to malarial infection (Rogerson *et al.*, 2007, de Ridder *et al.*, 2008). This is worsened even more by the fact that there seems to exist an antagonistic interaction between certain antimalarials and the various antiretroviral protease inhibitors commonly used for HIV infection (He *et al.*, 2009).

## 1.6 Eradication efforts against malaria

World War II was followed with huge malaria eradication efforts across all continents. These programmes made extensive use of insecticides like dichloro-diphenyl-trichloroethane (DDT) and antimalarials like chloroquine as prophylaxis (Hemingway J., 2004). By the 1950's, malaria was eliminated from Australia, Europe, and the USA (Figure 1.4). Unfortunately, these early eradication efforts failed in Africa and Asia. Today, various malaria eradication efforts have been renewed. One such an effort is the "Roll Back Malaria" partnership, a global partnership initiated by WHO, United Nations Development Programme (UNDP), The United Nations Children's Fund (UNICEF), and the World Bank in 1998. The aim of the "Roll Back Malaria" partnership is to work with national governmental organisations and private companies to enable the reduction of the human and socio-economic burden of malaria. This is done mainly by the provision of bednets and the necessary malarial drugs in rural areas affected by the harsh impact of malaria. To combat malaria,

the WHO recommend the use of long lasting insecticidal nets (LLIN), ACT's, indoor residual spraying of insecticides (IRS) and intermittent preventive treatment (IPT) during pregnancy.

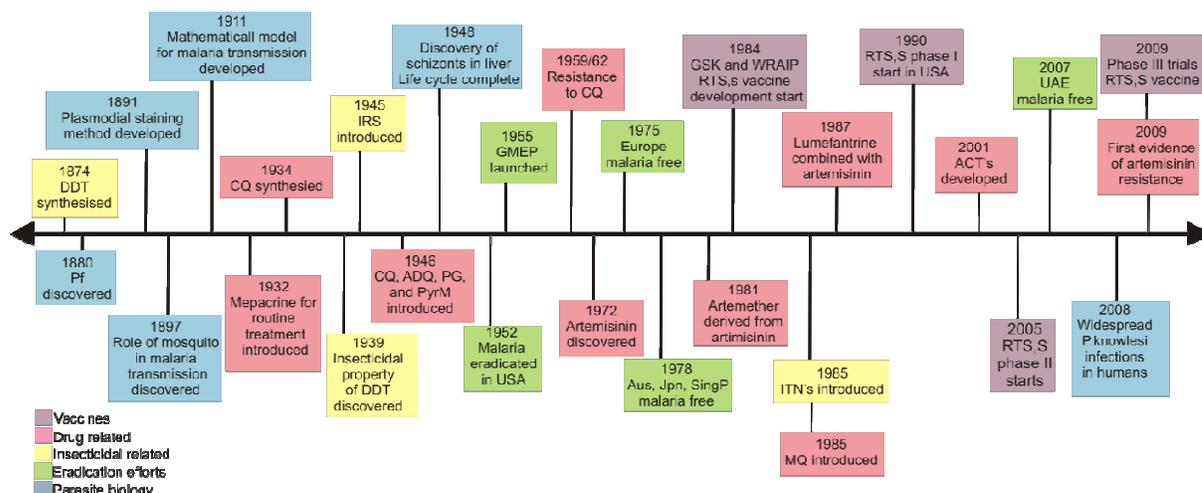


Figure 1.4: Timeline of some of the most important milestones in the fight against malaria. Created from (Vangapandu *et al.*, 2007, Hyde, 2005, WHO)

The South African Department of Health currently recommends mefloquine (MQ), doxycyclin or atovaquone/proguanil as appropriate chemoprophylaxis for use in South Africa . The Centre for Disease Control recommends the use of atovaquone/proguanil, doxycycline, chloroquine (CQ) or MQ (only in areas without CQ and MQ resistance) or primaquine, as chemoprophylaxis depending on which malaria endemic country is to be visited . Compliance is essential when taking chemoprophylaxis. MQ has to be taken weekly, at least 1 week before entering a malaria area and 4 weeks after leaving the malaria area. Doxycyclin has to be taken daily 2 days before entering a malaria area and continue for 4 weeks after leaving the malaria area. Atovaquone-proguanil is preferred for shorter stays since it needs to be taken daily 2 days before entering a malaria area and continued for 7 days after leaving the malaria area. The choice of prophylaxis depends on various factors that include the age and weight of a patient, medical conditions, and activities that the patient will embark on like scuba diving. For female patients it is also necessary to consider if the patient is pregnant or breastfeeding . No antimalarial prophylactic regimen gives complete protection, but it may be useful to alleviate the severity of the illness. Chemoprophylaxis and treatment of *P. falciparum* malaria is becoming difficult due to increasing resistance of the parasite to all known drugs. This is also the reasoning for the WHO to establish the “ABCD of Malaria Protection” (World Malaria Report 2008).

- Awareness of the risk of malaria
- Avoid being Bitten
- Chemoprophylaxis
- Diagnosis and treatment as soon as possible

### 1.6.1 Insecticide resistance – the use of spraying and bed nets

The main purpose of IRS is to reduce transmission of malaria from the mosquito to its human host by elimination of the vector found within houses. Unfortunately, a huge challenge is the increase in resistant mosquitoes to existing insecticides, especially DDT, and against the pyrethroids. Insecticide treated nets (ITN) may assist in the prevention of malaria infection, since it is able to reduce transmission of malaria from the mosquito to the human host. In 2000, only 1.7 million children living in malaria endemic countries had access to ITN's, but this number has now increased to 20.3 million children in 2007. Unfortunately, this still leaves 89.6 million (81.5%) children without nets and extremely vulnerable to infection (Noor *et al.*, 2009). In 18 African countries surveyed by the WHO, it was determined that 34% of households owned an ITN of which 23% children and 27% pregnant woman slept under (World Malaria Report 2008). Unfortunately, this still leaves 66% of African households without an ITN and therefore at an increased risk of contracting malaria.

### 1.6.2 Vaccines

Another step toward the eradication of malaria is through the development of an effective malaria vaccine. The ideal vaccine must be able to provide complete immunity against the disease or prevent severe disease and death. Unfortunately, genetic variability of the parasite is hampering vaccine development. Four stages of the parasite life cycle has been targeted as possible vaccine candidates including the pre-erythrocytic (when infected with sporozoites), the human hepatic stage, the erythrocytic and the gametocyte stages (Graves & Gelband, 2006). Vaccines directed towards the pre-erythrocytic stages aim to completely prevent infection while blood stage vaccines aim to reduce and hopefully eliminate parasites upon infection. Gametocyte vaccines on the other hand aim to prevent transmission of the parasite to the vector. The most advanced pre-erythrocytic vaccine to date is the RTS,S/AS01 vaccine developed by GlaxoSmithKline in a process that has already started in 1984 at the Walter Reed Army Institute of Research (Ballou, 2009) (Figure 1.4). It consists of the antigenic C-terminus of the parasite's circumsporozoite protein (CSP) fused to the hepatitis B surface antigen and is expressed in the form of virus-like particles in *Saccharomyces cerevisiae*. Phase I and Phase IIa clinical trials on Gambian adults (Bojang *et al.*, 2009), 2002 Mozambiquean children aged 1-4 years (Sacarlal *et al.*, 2008) as well as infants (Aponte *et al.*, 2007) used the AS02 oil-in-water adjuvant system and showed promising protection by RTS,S against malaria infection. The AS02 adjuvant was replaced with the RTS,S/AS01 which contains liposomes as adjuvant, and applied in Kenya and Tanzania with over 800 infants between 5-17 months and showed 55% efficacy over a follow-up period of 8 months (Bejon *et al.*, 2008b, Bejon

*et al.*, 2008a). The previously used AS02-adjuvant was well tolerated, but the new AS01-adjuvant had similar safety with higher humoral immunogenicity, a favourable Th1 cell immune response and a trend towards higher vaccine immunogenicity (Kester *et al.*, 2009, Lell *et al.*, 2009). RTS,S/AS01 given in three doses, rather than a single dose, provided better results in Ghanaian and Gabonese children (Owusu-Agyei *et al.*, 2009, Lell *et al.*, 2009). Phase III clinical trials for RTS,S/AS01 started in May 2009 and include sites in Kenya, Tanzania, Malawi, Mozambique, Gabon, Ghana, and Burkina Faso. Should results be promising, the product could only be ready for recommendation and registration at the earliest by 2014 (World Malaria Report 2008).

Other vaccine candidates have also been pursued over time but with less success to date than that obtained with RTS,S/AS01. Asexual blood stage vaccines aim to protect against malaria as disease rather than the infection, but has been less successful to date. Various MSP's have been investigated as vaccine candidates with little success in clinical trials conducted to date in Kenya and Mali. Another vaccine, the Combination B vaccine (MSP/RESA), consisting of two merozoite surface proteins together with a ring infected erythrocyte surface antigen (RESA) showed good immunogenicity and is being investigated further (Graves & Gelband, 2006). Another joint venture by Walter Reed Army Institute of Research and GlaxoSmithKline resulted in the FMP2.1 (AMA-1/AS02) vaccine candidate which showed host immunity and safety in phase I trials (Polhemus *et al.*, 2007, Spring *et al.*, 2009) and is presently in phase II trials in Mali. The FMP2.1/AS02 (A) vaccine candidate consists of FMP2.1 which is a recombinant protein based on AMA-1 from *P. falciparum* strain 3D7. Another approach to vaccine development is transmission blocking vaccines that are based on the prevention of sporozoite development in the mosquito salivary glands. Various surface protein antigens are in development but is hampered by the problematic protein expression of these proteins. The use of irradiated attenuated *P. falciparum* sporozoites is also underway in phase I trials, but may pose safety, technical and logistical problems (Ballou, 2009).

## **1.7 Currently used drugs and drug resistance**

Antimalarial drugs are probably the cornerstone of the malaria elimination effort with the use of ITN's and IRS strengthening the efforts against combating malaria. Unfortunately, the harsh reality is that even with these efforts, people living in endemic malaria areas will still contract malaria and without cheap and affective drugs, many more people will succumb to the devastating effect of malaria. The problem is compounded by the lack of new antimalarials. The tragedy is that all existing antimalarial drugs are actually only derivatives of certain core structures and can be grouped into three main classes; the quinolines (quinine, chloroquine, mefloquine, primaquine), the anti-folates (sulfadoxine, pyrimethamine) and the most recent drugs, the artemisinin derivatives



(artemisinin, artemether, dihydroartemisinin) (Na-Bangchang & Karbwang, 2009). Certain antibiotics (doxycyclin, clindamycin) also display antimalarial properties. There is an increasing spread of drug and insecticide resistance due to the evolutionary pressure put on both the mosquito and the parasite. The Thai-Cambodian border is historically the first site of emerging resistance to antimalarials, and has now also seen the first signs of resistance to treatment with artemisinin (Noedl *et al.*, 2008). This could result in a tragedy for all malaria endemic countries and as rightfully noted by Prof Ogobaro Doumbo during the 5<sup>th</sup> MIM Conference, Nairobi, Kenya, 2009: “*Artemisinin resistance is a Tsunami coming into Africa*”. The development of resistance to currently used drugs may be due to several factors that include the overuse of antimalarial drugs, inadequate therapeutic treatments of infections, parasite adaptability at genomic and metabolic levels and fast proliferation rates of the parasite that allows new generations to be formed in a very short time (Hyde, 2007, Olliaro & Taylor, 2003). The mechanisms of resistance to these drugs involve the modification of drug transport systems, increased synthesis of inhibited enzymes (Nirmalan *et al.*, 2004b), an increase in enzymes that can inactivate the drug and finally the use of alternative pathways (Vangapandu *et al.*, 2007). Unfortunately, except for the folate drugs, both the mode-of-action as well as the mechanism of resistance is poorly understood (Na-Bangchang & Karbwang, 2009).

### 1.7.1 Chloroquine

Chloroquine (CQ) is part of the quinoline family of drugs and was synthesized in 1934. Also part of the quinoline family is quinine (QN) which is extracted from cinchona bark and was one of the first antimalarial drugs. CQ provided antimalarial treatment for 8 decades and was the cornerstone of malaria eradication in the 1950's and 1960's (Figure 1.4). The main advantage of CQ was its fast action against the blood stages, low toxicity, good bio-availability and pharmacokinetics as well as its low production cost, therefore making it the ideal drug for Africa (Santos-Magalhaes & Mosqueira, 2010). Unfortunately, to its disadvantage, CQ has a very long half life (1-2 months) which may be one of the reasons for the emergence of resistance to CQ, which was first observed in 1962 in Thailand, and later in Africa (Gregson A. & Plowe C.V., 2005, Na-Bangchang & Karbwang, 2009) (Table 1.1). The mode-of-action of CQ is based on the accumulation of the drug within the food vacuole, which will eventually interfere with the polymerisation of toxic heme monomers into hemozoin, which is part of the parasite's detoxification process. CQ enters the food vacuole (pH of ~4.5-5.0) possibly by diffusion and then accumulates within the food vacuole due to pH trapping of the protonated drug at the low pH within the food vacuole (Figure 1.5). CQ will then form a complex with heme ferriprotoporphyrin IX which ultimately leads to the toxic effect of the

drug on the parasite (Vangapandu et al., 2007). CQ resistance occurs due to mutations in the *Pfcr* gene (located on chromosome 7) that expresses the chloroquine resistance transporter (*PfCRT*), a transmembrane protein located on the digestive vacuole. Mutations of this gene were also found in CQ resistant field isolates (Djimde *et al.*, 2001). Modification in the P-glycoprotein homologue (*Pgh1*) gene is also implicated in CQ and mefloquine (MQ) resistance (Santos-Magalhaes & Mosqueira, 2010). It is an analogue of glycoproteins found in cancer cells that function as pumps to expel cytotoxic drugs (Le Bras & Durand, 2003). Therefore, CQ resistant strains are proposed to accumulate less CQ within the parasite.

MQ and halofantrine (HF) were developed by the US Army and are both aryl amino alcohol derivatives of quinine (Figure 1.5). They are all blood stage specific and acts on hemoglobin digestion probably similarly to the mode-of-action of CQ (Vangapandu *et al.*, 2007). QN accumulates in the food vacuole and therefore inhibits the formation of hemozoin biocrystals, hence leading to the formation of toxic heme within the parasite (Figure 1.5). QN has traditionally been used to treat cerebral malaria despite its toxicity when given intravenously and may also lead to serious cardiovascular or central nervous system toxicity. Complacency is also associated with QN since it must be taken orally three times daily for seven days therefore resulting in rapid resistance development to QN by the parasite (Na-Bangchang & Karbwang, 2009). MQ, which also induces the formation of toxic heme complexes within the parasite food vacuole, was developed during the Vietnam War to treat US soldiers (Figure 1.5). Side effects associated with MQ include nausea, vomiting, diarrhoea, and several severe neurological effects that include hallucinations, sleep disturbances, psychosis and delirium (Table 1.1). Primaquine (PQ) is a schizontocide used for prophylaxis against all types of malaria. It is active against schizonts and gametocytes and in *P. vivax* is able to prevent malaria relapse due to the presence of hypnozoites. Unfortunately, it is a very toxic drug with adverse side effects that includes anorexia, cramps, chest weakness, and anaemia (Santos-Magalhaes & Mosqueira, 2010) (Table 1.1). The mechanism of resistance to QN has not been elucidated. MQ resistance seems to be associated with mutations in the *Pfmdr1* gene resulting in increased drug efflux (Vangapandu *et al.*, 2007).

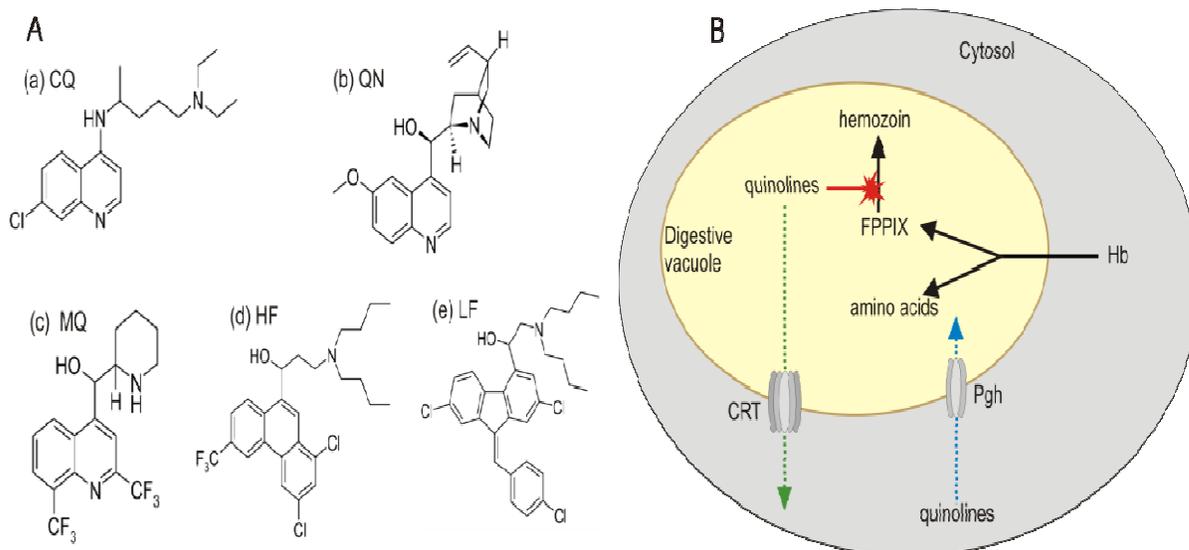


Figure 1.5: Proposed mode-of-action of quinoline-based drugs. Compiled from (Schlitzer, 2008, Hyde, 2007, Djimde *et al.*, 2001)

A: Structures of various quinoline drugs. (a) Chloroquine (CQ), (b) Quinine (QN), (c) Mefloquine (MQ), (d) Halofantrine (HF), (e) Lumefantrine (LF). B: Proposed mode-of-action of quinoline based drug. Quinolines prevent the formation of hemozoin (as indicated in red) during hemoglobin digestion within the food vacuole of the parasite. The proposed mechanism of resistance is also indicated in blue and green. Mutations in Pgh transporter protein will result in reduced import of CQ, while mutations in the *PfCRT* gene will result in the *PfCRT* transporter protein having increased ability to expel CQ from the food vacuole, therefore resulting in decreased CQ levels within the parasite food vacuole and therefore decreased efficiency.

## 1.7.2 Antifolates

Sulfadoxine/Pyrimethamine combination therapy (SP) has been used to replace CQ in many African countries. Unfortunately, due to the slow elimination of the drug, resistance soon prevailed (Na-Bangchang & Karbwang, 2009) (Table 1.1). Sulfadoxine (SDX) inhibits the dihydroopterate synthase (DHPS) domain of the hydroxymethylpterin pyrophosphokinase/dihydroopterate synthase (HPPK/DHPS) bifunctional enzyme complex (Figure 1.6). DHPS is only found in the parasite and not in the human host, therefore making it a good target. Pyrimethamine (PYR), proguanil and cycloguanil (CG) are able to inhibit dihydrofolate reductase (DHFR) activity of the dihydrofolate reductase/thymidylate synthetase (DHFR/TS) bifunctional enzyme complex, and are able to bind more strongly to the DHFR enzyme from the parasite than that of its human orthologue. Antifolates attack all stages of the parasite in the erythrocytic cycle and can inhibit the early development stages in the liver and mosquito (Vangapandu *et al.*, 2007). These drugs are able to block DNA replication in the parasite by blocking the synthesis of folates that are necessary for DNA metabolism. Resistance to PYR, CG and chlorocycloguanil are as a result of point mutations in the DHFR enzyme, while mutations in the DHPS gene are responsible for resistance to the sulfa-drugs (Na-Bangchang & Karbwang, 2009, Bacon *et al.*, 2009).

The atovaquone/proguanil combination was only introduced in 1997 as a prophylaxis and has a mechanism of synergy that is not yet fully understood (Table 1.1). Atovaquone (AVQ) is a structural analogue of coenzyme Q that plays a role in the electron transport chain (Figure 1.6). It works on the principle that blockage is obtained from the iron-sulfur protein that is required for electron transfer to cytochrome c1 from ubihydroquinone that is bound to the cytochrome b within complex III. Inhibition with this drug will result in the membrane potential changing and ultimately leading to arrest of parasite respiration and a lack of pyrimidine biosynthesis, with the added advantage that this drug does not affect the human mitochondria. Resistance occurs due to specific point mutations in cytochrome b (Hyde, 2007).

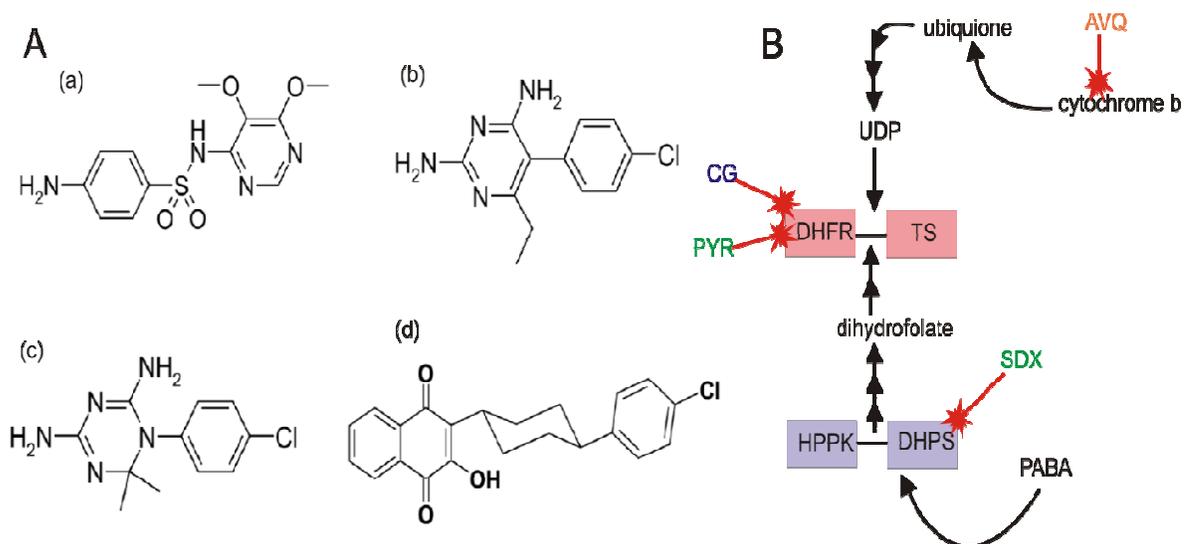


Figure 1.6: Proposed mode-of-action of anti-folate drugs. Compiled from (Hyde, 2007, Schlitzer, 2008, Le Bras & Durand, 2003).

A: Structures of various anti-folate drugs. (a) Sulfadoxine (SDX), (b) Pyrimethamine (PYR), (c) Cycloguanil (CG), (d) Atovaquone (AVQ). B: Proposed mode-of-action of anti-folate drugs. Drug target indicated in red. PYR and CG inhibits the activity of DHFR resulting in tetrahydrofolate depletion within the parasite. SDX inhibits the activity of DHPS resulting in dihydrofolate depletion within the parasite. AVQ is a prophylactic drug only and disrupts the membrane potential of the parasite. Resistance to PYR, CG and SDX are obtained by point mutations within their respective drug targets.

### 1.7.3 Artemisinin

The only currently effective drug is artemisinin and derivatives thereof. Artemisinin derivatives are used in clinical applications and are predominantly used in combination with other drugs (Hyde, 2005). The advantage of artemisinin is its short half live, and therefore unlikely resistance development, although the first signs of resistance to artemisinin has been reported in Western Cambodia (Noedl *et al.*, 2008) (Table 1.1). This calls for urgent containment measures, since

recrudescence is already seen in 30% of patients receiving artemisinin as a mono-therapy in Cambodia compared to 10 % in North-Western Thailand (Dondorp *et al.*, 2009).

Artemisinin was first extracted from the Chinese plant *Artemisia annua*, more commonly known as sweet wormwood or “qinghao”, and was used by Chinese herbal medicine practitioners for at least 2000 years. The naturally occurring compound has poor bio-availability and therefore derivatives have been made. The most important artemisinin derivatives are artesunate, artemether, arteether and dihydroartemisinin (Meshnick S.R., 2002) of which sodium artesunate is the most effective derivative being able to reduce parasite numbers  $\sim 10^4$ -fold in 48 hours (Hyde, 2007). Artemisinin is a fast acting drug that acts on all forms of the blood stages as well as gametocytes, but not on the liver stage or transmission to the mosquito. Due to the rapid increase in antimalarial drug resistance by the parasite, the WHO recommends the use of ACT's rather than mono-therapy for the treatment of malaria (World Malaria Report 2008). An ACT will include an artemisinin-based drug in combination with another antimalarial drug in order to prevent development of resistance to the artemisinin drugs that are currently the last line of defence against malaria. The principle entails that the parasites that may escape the fast acting artemisinin are then killed by the slower acting partner. The WHO recommends the following therapeutic options for ACT-based treatment of uncomplicated and severe *falciparum* malaria: artemether/lumefantrine; artesunate/amodiaquine; artesunate/sulfadoxine/pyrimethamine (only in areas with sulfadoxine/pyrimethamine efficacy); artesunate/mefloquine; and, dihydroartemisinin/piperaquine. These ACT's should be administered for at least 3 days for an optimum effect. Absorption of the ACT's are also enhanced when administered in combination with a fatty meal (WHO Guidelines for the treatment of malaria, 2010). The use of ACT's have impacted positively on the malaria situation, since at least 40 countries in Africa now prefer the use of ACT's for first line treatment of malaria. Two-hundred and fifty million treatments of CoArtem<sup>®</sup> (artesunate/lumefantrine) were delivered to Africa at the end of July 2009 in the fight against malaria. The combined use of CoArtem (artemether/lumefantrine combination therapy) and increased efforts of IRS together with the provision of ITN's have resulted in a 66% decrease in malaria-related deaths in Zambia. Similarly in Kwa-Zulu Natal, South Africa, the use of CoArtem as first line treatment in combination with renewed vector control efforts resulted in a 97% decrease in malaria-related deaths in 2003 (Barnes *et al.*, 2009). CoArtem has few adverse side effects and also claims safety during pregnancy although this may be somewhat controversial (Falade & Manyando, 2009) (Table 1.1).

Two possible modes-of-action for artemisinin have been proposed, although it seems that both mechanisms depend on the activation of the peroxide group that will form free radicals (Figure 1.7).

The first proposed mechanism is that artemisinin interferes with sarco/endoplasmic reticulum calcium-dependent ATPase (SERCA). Upon treatment with artemisinin,  $Fe^{2+}$  is activated which will enable the inhibition of the SERCA-like *Pf*ATP6 ATPase transporter. *Pf*ATP6 is the only SERCA-type  $Ca^{2+}$  ATPase in the parasite and is completely inhibited by artemisinin. SERCA maintains the  $Ca^{2+}$  ion concentrations that play a role in signalling and post-translational processing of proteins. Artemisinin binds to *Pf*ATP6 by hydrophobic interactions allowing cleavage of the peroxide bridge by iron that will then generate carbon-centred radicals ultimately resulting in enzyme inhibition and parasite death (Eckstein-Ludwig *et al.*, 2003, Krishna *et al.*, 2006). The second proposed mechanism is the production of reactive species. The heme or iron catalyses the peroxide bridge of the drug causing the formation of free radicals that will ultimately lead to protein alkylation (de Ridder *et al.*, 2008, Vangapandu *et al.*, 2007). Resistance may be by mutations in the *Pf*atp6 gene (Jambou *et al.*, 2005).

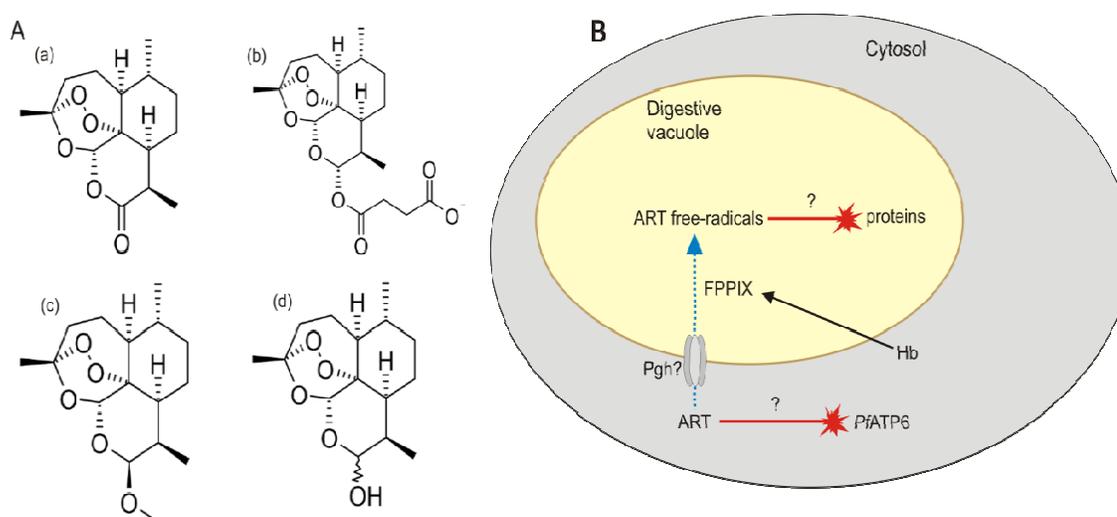


Figure 1.7: Proposed mode-of-action of artemisinin based drugs. Compiled from (Hyde, 2007, Schlitzer, 2008, de Ridder *et al.*, 2008, Jambou *et al.*, 2005)

A: Structures of various artemisinin based drugs. (a) Artemisinin (ART), (b) Artesunate, (c) Artemether, (d) Dihydroartemisinin. B: Proposed mode-of-action of artemisinin based drugs. The first proposed mode-of-action is by interference with *Pf*ATP6, while the second proposed mode-of-action is by the production of radicals that will damage parasite proteins. Resistance occurs due to mutations in the *Pf*atp6 gene.

The reality of the current malaria situation is that parasite resistance to drugs is on the increase. The problem at the moment is that there is no replacement drug available in the near future, and the possibility of a vaccine may be a reality but still far in the future. The availability of the *Plasmodium* genome (Gardner *et al.*, 2002) may impact on the quality of human health but needs to be exploited. It may provide a basic understanding of the *Plasmodium* parasite, and this may be used to develop effective vaccines, new drugs and improved diagnostics (Duraisingh M. *et al.*, 2006).

Table 1.1: Summary of currently used drugs. Compiled from (de Ridder *et al.*, 2008, Vangapandu *et al.*, 2007, Jambou *et al.*, 2005, Hyde, 2007, Schlitzer, 2008, Le Bras & Durand, 2003, Djimde *et al.*, 2001)

| Drug  | Pharma name                        | Discoverer | Half life                                   | Mw (g/mol) | Formulae  | Cellular Target  | Advantages  | Disadvantages   | Prophylaxis or treatment |
|---|------------------------------------|------------|---|------------|---|--|---|---|--------------------------|
| <b>Quinolines</b>                                       |                                    |            |   |            |   |  |   |   |                          |
| Chloroquine   | Resochin<br>Dawaquin<br>Daramal    | 1934       | 1-2 months                                  | 436.0      | C <sub>18</sub> H <sub>26</sub> ClN <sub>3</sub>                      | Heme metabolism  | Fast acting in erythrocytic stage, hydrophilic, good bio-availability, cheap                  | Macular retinopathy, widespread resistance, itching                           | Both                     |
| Quinine   | Quininmax<br>Aflukin               | 1633       | ~18 h                                       | 324.4      | C <sub>20</sub> H <sub>24</sub> N <sub>2</sub> O <sub>2</sub>         | Heme metabolism  | Fast action in erythrocytic stage, hydrophilic, oral route, good bio-availability             | Not well tolerated, adverse side effects, hypoglycemia, neurotoxicity         | Treatment                |
| Mefloquine  | Lariam                             | 1963       | 2 to 4 weeks                                | 378.3      | C <sub>17</sub> H <sub>16</sub> F <sub>6</sub> N <sub>2</sub> O       | Heme metabolism  | Potent in RBC stage   | Severe neuropsychiatric reactions, depression, "suicide", long half-life      | Mostly prophylaxis       |
| Amodiaquine   | Camoquine<br>Flavoquine            |            | 5.2 ± 1.7 min                               | 355.9      | C <sub>20</sub> H <sub>22</sub> ClN <sub>3</sub> O                    | histamine N-methyltransferase inhibitor                            | Effective against CQ resistant strains  | Hepatotoxic<br>No longer marketed in US                                       | Treatment                |
| Halofantrine  | Halfan                             | 1960s      | 6 to 10 days                                | 500.4      | C <sub>26</sub> H <sub>30</sub> Cl <sub>2</sub> F <sub>3</sub> N<br>O | Withdrawn from market  | oral  | cardiac arrhythmias only for treatment due to erratic absorption and toxicity | Treatment                |
| <b>Anti-folates</b>                                     |                                    |            |   |            |   |  |   |   |                          |
| Pyrimethamine   | Daraprim                           | 1951       | 96 h  | 248.71     | C <sub>12</sub> H <sub>13</sub> ClN <sub>4</sub>                      | Folate synthesis inhibition of DHFR                                | Oral use, prophylaxis and treatment   | may deplete folic acid in humans  | Both                     |
| Sulfadoxine   |                                    |            | 150-200 h                                   | 310.33     | C <sub>12</sub> H <sub>14</sub> N <sub>4</sub> O <sub>4</sub> S       | Structural analog of PABA inhibits DHPS                            | Oral use, prophylaxis and treatment   |   | Both                     |
| <b>Antibiotics</b>                                      |                                    |            |   |            |   |  |   |   |                          |
| Doxycycline   | Vibramycin<br>Monodox<br>Doxyhexal | 1960s      | 18-22 h                                     | 444.43     | C <sub>22</sub> H <sub>24</sub> N <sub>2</sub> O <sub>8</sub>         | Impairment of apicoplast genes resulting in abnormal cell division | Used for prostatitis, sinusitis, syphilis, chlamydia, in malaria prophylaxis                  | Delayed antimalarial effect, slow acting                                      | Prophylaxis              |
| <b>Artemisnins</b>                                      |                                    |            |   |            |   |  |   |   |                          |
| Artemesinin, Dihydroartemesinin, Artesunate, artemether |                                    |            | 30 min                                      |            |   | Inhibits PfATP6 outside food vacuole                               | Safe, well tolerated, fast acting, gametocytocidal, schizonticidal, no wide spread resistance | dose dependent, short half life, low bio-availability, poor water solubility  | Treatment only           |
| <b>Combinations</b>                                     |                                    |            |   |            |   |  |   |   |                          |
| Artemether (AM)-lumefantrine (LM)                       | CoArtem®<br>Lumerax                | 1987       | LM 4-6 days, AM 30 min<br>AM 20mg, LM 120mg |            |   | Many targets, heme metabolism, protein metabolism                  | Well-tolerated, meet WHO criteria for safety and quality                                      | Expensive, not for use during pregnancy                                       | Treatment                |
| Pyrimethamine   | Fansidar                           |            | 100 - 231 h SDX, 54 - 148 h PYR,            |            |   | Synergistic action against   | synergistic action,   | Not for use in pregnancy,   | Treatment,               |



|   |                   |       |  |   |   |  |                       |
|---|-------------------|-------|--|---|---|--|-----------------------|
| (PYR)-sulfadoxine (SDX)                               | Laridox           |       | SDX 500mg, PYR 25mg  | folate biosynthesis   | schizonticidal, blood stages, effective against CQ resistance                             | skin reactions   | no longer prophylaxis |
| Chlorproguanil (CPG)-dapsone (DS)                     | LapDap            | 1980s | CPG 12 h, DS 20 h<br>CPG 80 mg, 100 mg DS                                      | Synergistic on folate biosynthesis<br>CP inhibits DHFR and DS inhibits DHPS           | Fast elimination times, lower tendency towards resistance                                 | Toxicity at high concentrations  |                       |
| Dihydroartemisinin(DHA) - Piperazine Phosphate (PPQ)  | Duo-Cotecxin      |       | DHA 2 h, PPQ 9 days<br>DHA 40mg, PPQ 320mg                                     | Synergistic combination   | active against the asexual forms, schizonts, gametocytes oral, good for resistant strains | Only orally, nausea, diarrhoea, loss of appetite, not during pregnancy | Treatment             |
| Artesunate (AS) - Mefloquine (MQ)                     | Artequin          |       | AS 30 min and MQ 21 days<br>AS 200 mg, MQ 250 mg                               | Schizonticidal action   | Oral, good for resistant strains, good tolerability, short treatment duration             | Side effects on nervous and digestive system                           | Treatment             |
| Artesunate (AS) - Amodiaquine                         | ASAQ Larimal      | 2007  | AS 30 min and ADQ 6 min<br>AS 100 mg and ADQ 270 mg                            | Schizonticidal action   | Affordable, effective against erythrocytic stages   | Dizziness, itching, headache, photosensitivity                         | Treatment             |
| Artesunate (AS) Pyrimethamine (PYR)-sulfadoxine (SDX) | Artidox           |       | 100 - 231 h SDX, 54 - 148 h PYR,<br>AS 30 min<br>AS 50 mg, SDX 500mg, PYR 25mg | AS has schizonticidal action<br>SP has synergistic action against folate biosynthesis | Oral use  | Not during pregnancy, abdominal pain, nausea                           | Treatment             |
| Atovaquone (AV) - Proguanil (PG)                      | Malarone, Melanil | 1997  | AV 2-3 days PG weeks<br>250 mg AV, 100 mg PG                                   | Synergistic combination, folate biosynthesis and pyrimidines                          | Short usage period  | Prophylaxis only   | Prophylaxis only      |



## 1.8 New drug targets

The first step in drug discovery is the identification of novel drug targets that are absolutely essential for parasite survival (Na-Bangchang & Karbwang, 2009). An important point for consideration during drug discovery is the fact that the parasite resides inside the erythrocyte and that a successful drug should be able to cross multiple membranes (Santos-Magalhaes & Mosqueira, 2010). Ideally, an antimalarial should be selective, curative and have no toxicity for the human host, have good oral bio-availability, and allow short treatment duration in order to avoid complacency. Drug development can be broken down into 4 main steps that include target identification, target validation, identification of lead inhibitors, and optimisation of those inhibitors regarding their pharmacological and toxicological properties. A drug target is validated when it is proven to be essential for growth and survival (Cowman & Crabb, 2003). Target selectivity is indicated by sequence differences between parasite and host or absence in the host. The *PfDHFR* gene shares only 27% homology with its human counterpart with the majority of divergence occurring in the active site (Yuvaniyama *et al.*, 2003). This results in the extremely tight binding of PYR to *PfDHFR*. The apicoplast is a plastid-like organelle related to the chloroplast found in plants and is the major centre for fatty acid metabolism, isoprenoid and heme synthesis which are not found in the human host (McLeod *et al.*, 2001). Specific pathways that can be targeted include the shikimate pathway which is not present in mammals and is therefore a target worth exploiting. Seven enzymes are involved within this pathway that converts erythrose-4-phosphate and phosphoenolpyruvate to chorismate, which is then utilized by various other pathways including the production of p-aminobenzoic acid (pABA) utilized in the folate pathway (Table 1.2). Secondly, the Plasmodial protein farnesyltransferase (*PfPFT*) is active in the isoprenoid biosynthesis and plays a role in post-translational modifications. PFT inhibitors have been used in the treatment of human cancers and are therefore worth exploiting. The parasite also has its own antioxidant enzymes to protect it from oxidative stress and include 3 enzymes (superoxide dismutase, glutathione peroxidase and catalase), which together with the redox enzymes can be viable drug targets (Muller, 2004) (Table 1.2).

Polyamine metabolism is a target in cancer therapy as well as in some other parasitic diseases and is therefore worth exploiting in the Plasmodial parasite (Muller *et al.*, 2008, Clark *et al.*, 2010), and is discussed in more detail in the following section.

**Table 1.2: Summary of potential new drug targets.** Compiled from (Jana & Paliwal, 2007, Vangapandu *et al.*, 2007, Olliaro & Yuthavong, 1999, Fatumo *et al.*, 2009)

| Target/pathway                           | Enzymes                                     | Inhibitor                       | DIndex <sup>a</sup> | Reference   |
|--|---|---------------------------------|---------------------|---|
| Polyamine biosynthesis                   | S-adenosyl-L-homocysteine hydrolase         | Neplanocin A                    | 0.8                 | (Kitade <i>et al.</i> , 1999, Shuto <i>et al.</i> , 2002) |
|  | Adenosine deaminase                         | Coformycin                      | 1                   | (Tyler <i>et al.</i> , 2007)                              |
|  | Spermidine synthase                         | Cyclohexamine                   | 0.6                 | (Haider <i>et al.</i> , 2005)                             |
|  | AdoMetDC                                    | MDL73811                        | 0.8                 | (Wright <i>et al.</i> , 1991)                             |
|  | ODC   | DFMO                            | 0.8                 | (Berger, 2000)  |
| Vitamine B synthesis                     | Pyridoxal kinase                            | Aminophylline                   | n/d                 | (Delport <i>et al.</i> , 1990)                            |
| Apicoplast                               | Fab H                                       | Thiolactomycin                  | n/d                 | (He <i>et al.</i> , 2004)                                 |
|  | Fab I                                       | Triclosan                       | n/d                 | (McLeod <i>et al.</i> , 2001)                             |
| Shikimate pathway                        | 5-enolpyruvyl shikimat 3-phosphate synthase | Glyphosphate                    | 0                   | (McConkey, 1999)  |
|  | Chorismate synthase                         | 6-S-fluoroshikimate             | 0.6                 | (McRobert <i>et al.</i> , 2005)                           |
| Hemoglobin metabolism/proteases          | Plasmeprin I, II,                           | Leupeptin, pepstatin            | 0.8                 | (Coombs <i>et al.</i> , 2001)                             |
|  | Falcipains                                  | Vinyl sulfones, chalcones       | 0.6                 | (Rosenthal <i>et al.</i> , 1996)                          |
| Pyrimidine synthesis, electron transport | DHODase                                     | Pyrazofurin                     | 1                   | (Biagini <i>et al.</i> , 2003)                            |
|  | Thymidylate synthase                        | 5-fluoroorotate                 | 1                   | (Jiang <i>et al.</i> , 2000)                              |
| Purine salvage, DNA/RNA                  | HGPRT                                       | Allopurinol                     | n/d                 | (Sarma <i>et al.</i> , 1998)                              |
|  | Topoisomerase I                             | Irinotecan                      | 1                   | (Azarova <i>et al.</i> , 2007)                            |
|  | DNA topoisomerase II                        | Levofloxacin                    | 0.8                 | (Kicska <i>et al.</i> , 2002)                             |
| Glycolysis                               | Hexokinase                                  | Brefeldin A                     | 0.6                 | (Wanidworanun <i>et al.</i> , 1999, Kumar & Banyal, 1997) |
| Transporters                             | Hexose transporter                          | O-3-hexose derivatives          | 0.4                 | (Joet <i>et al.</i> , 2003)                               |
| Isoprenoid biosynthesis                  | DOXP reductoisomerase                       | Fosmidomycin                    | 0                   | (Nallan <i>et al.</i> , 2005)                             |
|  | Protein farnesyltransferase                 | FTI-2153                        | n/d                 | (Ohkanda <i>et al.</i> , 2001)                            |
| Redox system/ oxidant defense            | Thioredoxin reductase                       | 5,8-dihydroxy-1,4-naphtoquinone | 0.9                 | (Luersen <i>et al.</i> , 2000)                            |
|  | Gamma-GCS                                   | Buthionine sulfoximine          | 0.6                 | (Meierjohann <i>et al.</i> , 2002)                        |
|  | GST   | Hemin                           | 0.6                 | (Fritz-Wolf <i>et al.</i> , 2003)                         |
|  | Glutathione reductase                       | Selenocysteine                  | 0.9                 | (Muller, 2004)  |
| Mitochondrial system                     | Cytochrome c oxidoreductase                 | Atovaquone                      | n/d                 | (Krungkrai <i>et al.</i> , 1997)                          |
| Membrane biosynthesis                    | Phospholipid biosynthesis                   | G25                             | n/d                 | (Roggero <i>et al.</i> , 2004)                            |
| Protein kinases                          | Various protein kinases                     | Xestoquinone                    | n/d                 | (Doerig & Meijer, 2007)                                   |

<sup>a</sup>DIndex is the druggability index given by the TDR database ([www.tdrtargets.org](http://www.tdrtargets.org)). The DIndex is a composite score consisting of a weighted normalised sum in order to predict the likelihood of a protein being druggable. The DIndex values range from 0 to 1. A larger score is an indication that the protein is more likely to be a druggable target. n/d: not determined. HGPRT: hypoxanthine-guanine-xanthine phosphoribosyltransferase, DOXP: 1-deoxy-D-xylose-5-phosphate, GCS: glutamylcysteine synthetase, Fab H:  $\beta$ -ketoacyl-ACP synthase III, Fab I: enoyl-ACP reductase, DOHDase: dihydroorotate dehydrogenase. GST: glutathione S-transferase. CDK: Cyclin dependent protein kinases. AdoMetDC: S-adenosylmethionine decarboxylase. ODC: Ornithine decarboxylase.

## 1.9 Polyamines

Polyamines are small flexible polycations that are represented by 3 basic polyamines which include the diamine putrescine (1,4-diaminopropane), the tri-amine spermidine [N-(3-aminopropyl)-1,4-diaminobutane] and the tetra-amine spermine [N,N'-bis(3-aminopropyl)-1,4-butanediamine] (Figure 1.8). At physiological pH, these polyamines are positively charged and are therefore capable of electrostatic interaction with nucleic acids, DNA, RNA and proteins (Heby *et al.*, 2007) (Figure 1.8). The interaction of polyamines with various macromolecules may lead to stabilisation of DNA, and the regulation of transcription and replication. Polyamines also have a very important role in cellular differentiation, proliferation, growth and division (Pignatti C. *et al.*, 2004, Geall A.J. *et al.*, 2004, Assaraf Y.G. *et al.*, 1987).

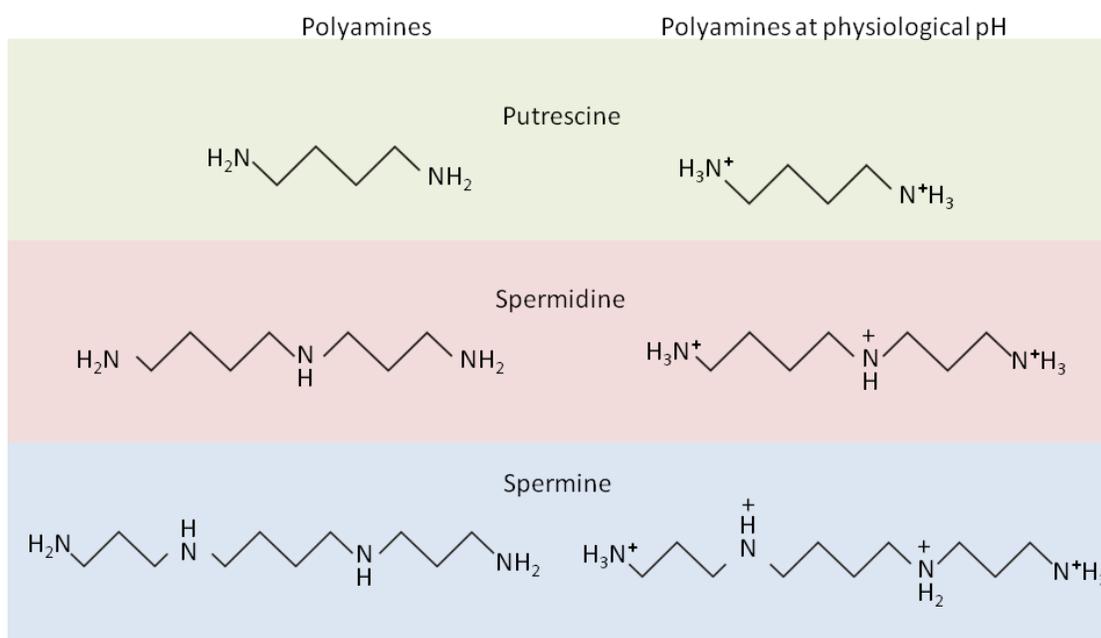


Figure 1.8: Chemical structures of the polyamines, putrescine, spermidine and spermine. The 3 major polyamines at their uncharged states as well as at physiological pH when they are cationic.

In mammalian cells, the cell cycle is regulated by polyamines which are able to affect cell cycle check points and cyclin degradation (Pignatti C. *et al.*, 2004). The depletion of polyamines results in cell cycle arrest at the G1 phase of the cell cycle due to the accumulation of p21 and p27. Polyamines are also said to play a role in cell death and apoptosis. There is increasing evidence that polyamines, cell cycle regulation and apoptosis are closely connected. This is also one of the major issues in cancer research. When polyamine biosynthesis is inhibited by DL- $\alpha$ -difluoromethylornithine (DFMO), apoptosis will be induced by the release of cytochrome c from the mitochondria (Pignatti C. *et al.*, 2004). Complete polyamine depletion will result in an induction of caspase activation and subsequent induction of apoptosis (Pignatti C. *et al.*, 2004).

### 1.9.1 Polyamine synthesis

Polyamine metabolism in mammalian cells uses methionine and arginine as precursors which will then undergo a series of reactions for the formation of the 3 polyamines (Figure 1.9). The polyamine synthetic enzymes in mammalian cells are regulated at the transcriptional, translational and post-translational levels (Muller *et al.*, 2001). ODC activity is regulated by antizyme, which is also able to promote degradation of ODC. The polyamine biosynthetic enzymes are also prone to feedback inhibition of their products. Polyamine metabolism in mammalian cells are more complex than polyamine metabolism within the Plasmodial parasite since various enzymes are present within the mammalian cells that are absent from the parasite. Polyamines can be converted back by interconversion pathways that involve cytosolic N1-acetyltransferase and polyamine oxidase that are specific to spermidine and spermine.

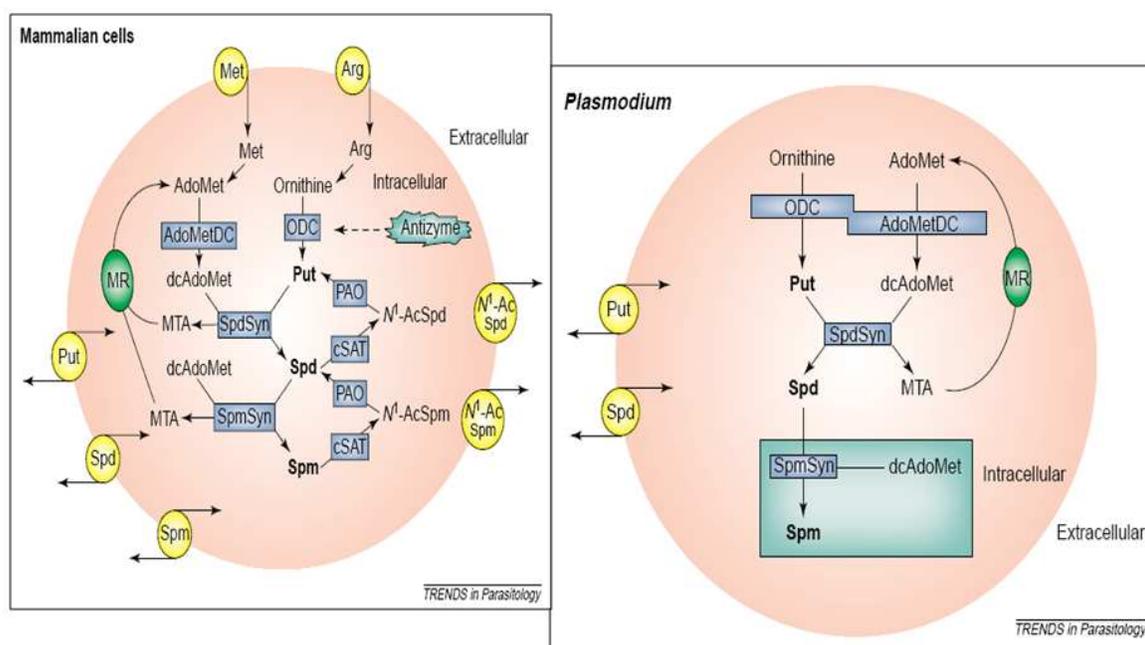


Figure 1.9: Polyamine metabolism in mammalian cells and in *Plasmodium* (Muller S. *et al.*, 2001).

The difference between mammalian cells and that of *Plasmodium* is the bifunctional AdoMetDC/ODC in *Plasmodium* and the simpler polyamine pathway. Ornithine acts as the substrate for ornithine decarboxylase (ODC) which produces putrescine (put). S-adenosylmethionine (AdoMet) is the substrate for S-adenosylmethionine decarboxylase (AdoMetDC) to form decarboxylated AdoMet (dcAdoMet). SpdSyn: Spermidine synthase, Spd: spermidine, spm: spermine, MR: methionine recycling.

In *P. falciparum*, arginase produces ornithine from arginine. Ornithine acts as the substrate for ornithine decarboxylase (ODC) which produces putrescine by the decarboxylation of ornithine. Methionine is utilised by S-adenosylmethionine synthase (AdoMet synthase) in the production of S-adenosylmethionine (AdoMet) which in turn is the substrate for S-adenosylmethionine decarboxylase (AdoMetDC). AdoMetDC decarboxylates AdoMet to form decarboxylated AdoMet (dcAdoMet) of which the aminopropyl group is then donated to spermidine synthase that will add

this to putrescine to form spermidine and ultimately spermine. No spermine synthase activity has been demonstrated in *Plasmodium* but it is assumed that spermidine synthase is able to produce low levels of spermine within the parasite (Haider *et al.*, 2005).

Polyamine metabolism in Plasmodial parasites are controlled by the rate-limiting decarboxylase activities of both AdoMetDC and ODC. An interesting property of Plasmodial polyamine metabolism is the fact that AdoMetDC and ODC form a unique bifunctional Plasmodial AdoMetDC/ODC complex (*PfAdoMetDC/ODC*) with a molecular mass of 330 kDa (Muller *et al.*, 2000). *PfAdoMetDC/ODC* is linked by a hinge and contains parasite specific inserts. Both *PfAdoMetDC* and *PfODC* are able to function independently (Wrenger *et al.*, 2001), although specific inserts have been identified that is important in the modulation of enzyme activity and domain interactions within the parasite (Birkholtz *et al.*, 2004). Feedback regulatory mechanisms have been identified for *PfODC* which is regulated by putrescine (Wrenger *et al.*, 2001), but putrescine has no regulatory effect on the activity of *PfAdoMetDC* (Wells *et al.*, 2006). This is in contrast to *Trypanosoma cruzi* in which putrescine activates AdoMetDC (Clyne *et al.*, 2002). This suggests that polyamine metabolism within Plasmodial parasites are probably regulated by the activities and interactions within the bifunctional *PfAdoMetDC/ODC* complex (Clark *et al.*, 2010).

Another unique difference between mammalian and *P. falciparum* AdoMetDC/ODC is the fact that the Plasmodial bifunctional enzyme has a very long half-life of about 2 hours compared to 15 min of the mammalian counterpart (Muller *et al.*, 2001). This long half-life of *PfAdoMetDC/ODC* has also been determined in Trypanosomes and is therefore worth exploiting (Wrenger *et al.*, 2001). The AdoMetDC activity within Trypanosomes is tightly regulated by prozyme, a property unique to Trypanosomes (Willert & Phillips, 2008). Both prozyme identified in Trypanosomes and antizyme identified in mammals are absent in *Plasmodia*.

High levels of polyamines are often associated with highly proliferating cells like Plasmodial parasites and constitutes 14% of the Plasmodial metabolome, and is therefore the major metabolite present within the Plasmodial parasite (Teng *et al.*, 2009, Olszewski *et al.*, 2009). The host erythrocytes have no polyamine machinery and have therefore trace amounts of polyamines when they are uninfected. Upon invasion of the erythrocytes the polyamine content within the infected erythrocyte is altered due to the activities of the *PfAdoMetDC/ODC* enzyme. Similar to the increase in *PfAdoMetDC/ODC* activity an increase in polyamines can be observed during infection of an erythrocyte (Das Gupta *et al.*, 2005) (Figure 1.10).

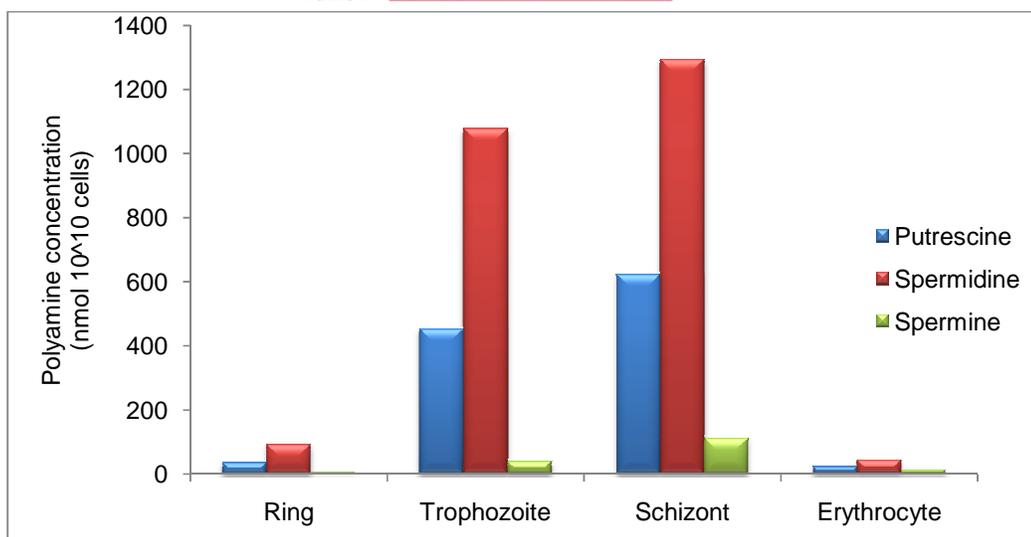


Figure 1.10: Polyamine content of erythrocytes. Adapted from (Das Gupta *et al.*, 2005)

A graph depicting the increase in polyamine levels when a erythrocyte is infected with *P. falciparum*. The metabolite levels of spermine, spermidine and putrescine all increase more than a 1000-fold upon infection of an erythrocyte.

Mice infected with *T. brucei* were treated with the AdoMetDC inhibitor 5'-[(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine (MDL73811) (Figure 1.11) and were subsequently cured from infection (Bitonti *et al.*, 1990). MDL73811 is a potent irreversible inhibitor of AdoMetDC and were effective against *T. brucei rhodesiense* infected mice (Bacchi *et al.*, 1992b). Similarly to the effectiveness of polyamine depletion with MDL73811 in Trypanosomes, polyamine depletion in *Leishmania* resulted in parasite death (Singh *et al.*, 2007) and the polyamine biosynthetic enzymes were subsequently validated as drug targets in *L. donovani* (Boitz *et al.*, 2009).

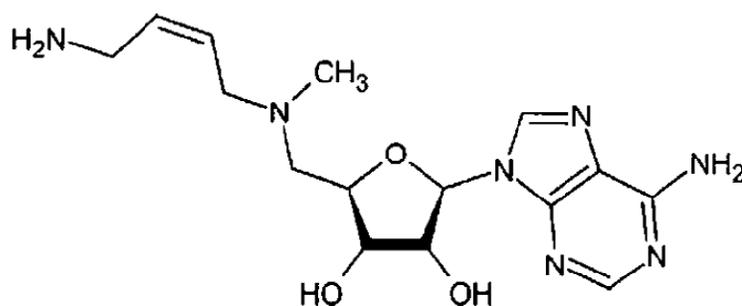


Figure 1.11: Structure of MDL73811

The bifunctional *PfAdoMetDC/ODC* is considered one of the top 20 drug targets and is highly druggable with a druggable index of 0.8 (max 1) according to the TDR database (Table 1.2). It is therefore of utmost importance that these unique features of *PfAdoMetDC/ODC* are exploited with the aim to validate this protein as a drug target.



## 1.10 The “omics” era

Research is currently dominated by the “omics” boom, and the wealth of information that are being made available. With the completion of the *Plasmodium* genome (Gardner *et al.*, 2002), as well as the *Anopheles* genome (Holt R.A., 2002), the hope has been on finding a vaccine for malaria or finding a novel drug target. The *P. falciparum* 3D7 nuclear genome is composed of 22.8 megabases (Mb) that are distributed among 14 chromosomes ranging in size from approximately 0.643 to 3.29 Mb, with an overall A+T composition of 80.6% (Gardner *et al.*, 2002). The availability of the genome sequence has opened the way for application of functional genomics. Functional genomics attempts to answer questions on the function of genes and proteins by a genome wide approach using high-throughput methods like transcriptomics, proteomics and metabolomics.

### 1.10.1 Transcriptomics

Microarray data for *P. falciparum* has been published on the IDC (Bozdech *et al.*, 2003) sexual gametocytes (Young J.A. *et al.*, 2005), as well as the comparative gene expression profiles of the IDC for 3D7, Dd2 and HB3 (Llinas *et al.*, 2006). The IDC transcript profile was established by monitoring transcripts every hour over the complete 48 hour life cycle of the parasite. This transcriptional profile revealed that 60% of the transcriptome is transcriptionally active during the IDC with a unique “just-in-time” manufacturing process by which the genes are only transcribed once they are needed (Bozdech *et al.*, 2003). Therefore, a transcript is generally only expressed for a period of 0.75 to 1.5 cycles over the 48 hour life period of the parasite (Bozdech *et al.*, 2003). Only a few transcripts are expressed throughout the life cycle of the parasite. Cross comparison of 3 Plasmodial strains revealed that the transcripts between 3D7, Dd2 and HB3 share more than 80% similarity (Llinas *et al.*, 2006). The transcripts of all 3 strains are also expressed and regulated remarkably similar to each other (Figure 1.12). The *in vivo* transcriptome derived from *P. falciparum* infected patients revealed similarities to the *in vitro* Pf3D7 ring stage transcriptome, with a major difference being the over expression of surface proteins in the *in vivo* data (Daily *et al.*, 2004). Various malarial drug perturbation studies have been investigated on a global transcriptome level and will be discussed in more detail in Chapter 4.

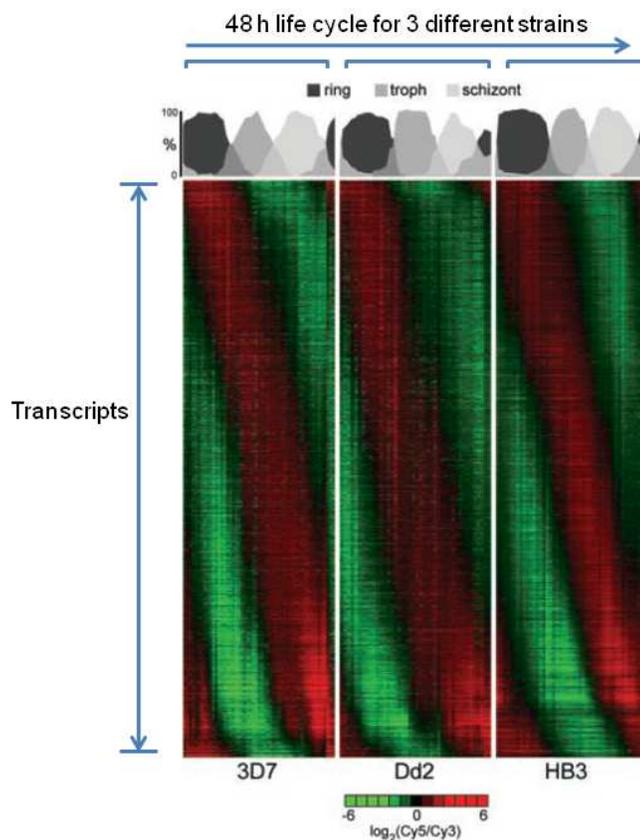


Figure 1.12: The phaseograms of the IDC of 3 Plasmodial strains depicted over a 48 hour period (Llinas *et al.*, 2006).

The phaseograms depicts the “just-in-time” expression of transcripts only when they are needed. The picture is representative of *P. falciparum* strain 3D7, *P. falciparum* strain Dd2 and *P. falciparum* strain HB3. Red is indicative of transcripts with increased abundance (“switched on”), while green is indicative of transcripts with decreased abundance (“switched off”).

### 1.10.2 Proteomics

Integration of microarray data with proteomic data will further our understanding of molecular mechanisms and regulation. Proteomic data is of utmost importance since it is able to provide a blue print of the functional units within a cell at any given moment in time. Similar to microarray data, the whole proteome of the different stages of *Plasmodium* has been characterised (Lasonder *et al.*, 2002), with additional Plasmodial life stages classified that include trophozoites, merozoites, sporozoites and gametocytes (Florens *et al.*, 2002). Proteome data for *P. berghei* and *P. chabaudi*, (Hall *et al.*, 2005) as well as *P. falciparum* ItG, A4, C24 and 3D7 strains are available (Wu Y. & Craig A., 2006). Plasmodial proteomic advances and Plasmodial perturbation studies investigated with proteomics will be discussed in more detail in Chapters 2 and 3.

### 1.10.3 The Metabolome, kinome and interactome

The Plasmodial interactome has been created and can be accessed at PlasmoMAP for interactive information regarding the interactome (Date & Stoeckert, 2006). Recently, clusters within the interactome has also been determined and revealed the importance of especially the ring and schizont stages (Wuchty *et al.*, 2009). The Plasmodial kinome identified a total of 65 genes encoding the protein kinase family within Plasmodial parasites (Ward *et al.*, 2004). The most interesting observation was the identification of the FIKK family of kinases which consists of 20 unique enzymes that are only found in apicomplexa. All these FIKK kinases contain a unique PEXEL sequence targeting proteins carrying this for transport to the erythrocyte membrane. Plasmodial parasites contain about 85-100 protein kinases which accounts for 1.1-1.6% of the total Plasmodial proteome. In contrast to Plasmodial parasites, humans have about 2% protein kinases (Doerig *et al.*, 2008). The Plasmodial metabolome is still relatively unknown with only 2 metabolome investigations to date. The Plasmodial metabolome was investigated using two-dimensional nuclear magnetic resonance (2-D NMR) (Teng *et al.*, 2009). Various extraction methods were investigated with more than 50 metabolites that were quantitated. Another metabolome investigation used LC-MS to determine metabolite levels of the Plasmodial parasite (Olszewski *et al.*, 2009).

### 1.11 The use of functional genomics to validate drug targets

With the completion of the genome for *P. falciparum* it sparked renewed hope for a novel drug target, although it was soon realised that the gene sequence alone cannot predict the gene activity and ultimately the gene and protein function (Chanda & Caldwell, 2003). Target validation entails identification of all the parasite proteins and processes that are affected and related to the efficacy of the particular drug in question (Figure 1.13). Targets that are unique to parasites and differ from host proteins are ideal, but parasite metabolism, drug binding to the target, and drug uptake should also be considered. A drug target can only be validated if the target is essential to growth with two validation strategies that can be followed. The first is genetically, by knock-out or knock-down or chemically, by inhibition of a specific protein (Cowman & Crabb, 2003). A gene can only be regarded as essential when the organism cannot survive without it (Freiberg & Brotz-Oesterhelt, 2005). The “omics” technologies alone does not provide sufficient information and for a complete understanding of the physiology and pathogenicity of organisms, integration between all the components of “omics” technologies are needed to gain maximal understanding of an particular organism (Hegde *et al.*, 2003, Birkholtz *et al.*, 2008b).

The transcriptome and proteome are both dynamic entities that changes rapidly in response to environmental changes, and therefore mining of both the transcriptome and the proteome may reveal valuable insight into the parasite response upon perturbation (Freiberg *et al.*, 2004). The application of functional genomics has proved successful in the elucidation of the mode-of-action of various anti-microbial agents (Scherl *et al.*, 2006, Pietiainen *et al.*, 2009). As such, functional genomics has proved indispensable in the mode-of-action determination of the drugs, isoniazid and ethionamide, against *Mycobacterium tuberculosis* (Wilson *et al.*, 1999, Fu & Shinnick, 2007, Boshoff *et al.*, 2004). Functional genomic investigations are currently contributing to the identification and validation of new drug targets to exploit in the fight against malaria (Birkholtz *et al.*, 2008b).

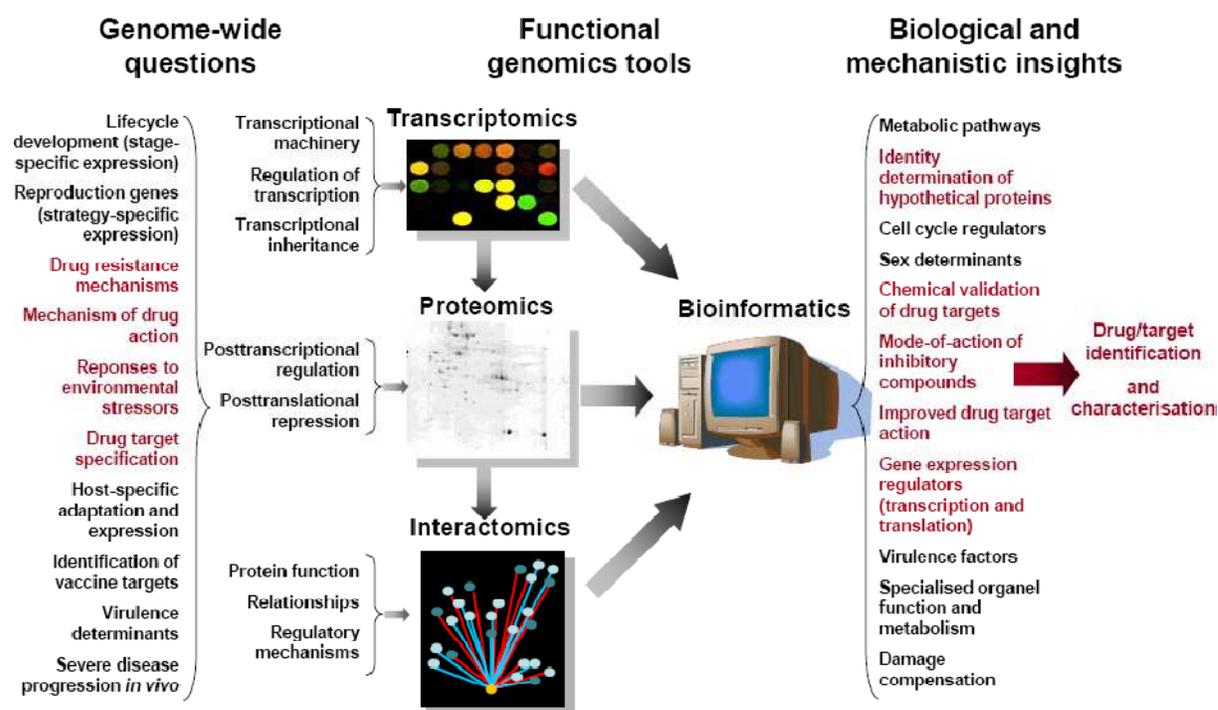


Figure 1.13: Functional genomics workflow (Birkholtz *et al.*, 2006).

The proposed workflow for Plasmodial functional genomics. Transcriptomics, proteomics and interactomics should be integrated to obtain biological and mechanistic insights into the functioning of Plasmodial parasites.

## 1.12 Objective

The objective of this study was the analysis of drug induced expression differences in the transcriptome and proteome of *P. falciparum* to allow the chemical validation of PfAdoMetDC as a drug target.

### 1.12.1 Aims:

- a) Morphological assessment of *PfAdoMetDC* inhibited parasites over the complete life cycle to determine morphological time of parasite arrest.
- b) Proteome profile analysis of *PfAdoMetDC* inhibited parasites.
- c) Transcriptome profile analysis of *PfAdoMetDC* inhibited parasites.
- d) Determination of the effect of polyamine depletion on the methylation status in *PfAdoMetDC* inhibited parasites.
- e) Determination of the biological relevance of the drug-induced expression changes in *P. falciparum* as a result of AdoMetDC inhibition.

Chapter 2 provides a description of an optimised 2-DE proteomic approach and application of this optimised 2-DE proteomic approach to characterise proteins within the late ring and early trophozoite stages of *P. falciparum* strain 3D7.

Chapter 3 describes the application of the optimised 2-DE proteomic approach to determine the proteomic response of Plasmodial parasites upon inhibition of AdoMetDC.

Chapter 4 is an investigation into the transcriptomic response of *P. falciparum* using oligonucleotide microarrays after the inhibition of AdoMetDC with MDL73811.

In chapter 5, further characterisation of specific metabolic responses identified in the transcriptomic and proteomic investigations of AdoMetDC-inhibited *P. falciparum* is described. This chapter includes an investigation into specific metabolites as well as determination of the methylation status of the parasite upon AdoMetDC inhibition as well as possible synergistic interactions. Finally, comparisons are made between the transcript and proteome to determine possible regulatory mechanisms.

Chapter 6 is the concluding discussion which integrates the knowledge gained from the transcriptomic and proteomic investigations and highlights the scientific contribution made within this study.

### 1.12.2 Papers resulting from the work presented within this dissertation

- a) Smit, S., S. Stoychev, A. I. Louw & L. Birkholtz (2010) Proteomic profiling of *Plasmodium falciparum* through improved, semiquantitative two-dimensional gel electrophoresis. *J Proteome Res* **9**: 2170-2181.



- b) Clark, K., J. Niemand, S. Reeksting, S. Smit, A. C. van Brummelen, M. Williams, A. I. Louw & L. Birkholtz (2010) Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*. *Amino Acids* **38**: 633-644.
- c) Smit, S., Clark K., Louw A.I., Birkholtz L. Functional genomic investigations into inhibited Plasmodial AdoMetDC and ODC reveals polyamine specific regulatory mechanisms. (Manuscript in preparation)

### 1.12.3 Conferences attended

#### 1.12.3.1 Oral presentations

- a) Smit S., Louw A.I., Birkholtz L. (2010) Functional consequences of the inhibition of Plasmodial S-adenosylmethionine decarboxylase as a key regulator of polyamine metabolism. 6<sup>th</sup> Biennial Symposium on Polyamines in Parasites, 3-6 August 2010, Phalaborwa, South Africa.
- b) Smit S., Louw A.I., Birkholtz L. (2009) A functional genomic approach to investigate the effect of polyamine depletion induced by the inhibition of S-adenosylmethionine decarboxylase in the human malaria parasite. 5<sup>th</sup> Multilateral Initiative on Malaria (MIM) Pan-African Malaria Conference, 2-6 November 2009, Kenyatta International Conference Centre, Nairobi, Kenya.

#### 1.12.3.2 Posters

- a) Smit S., Louw A.I., Birkholtz L. (2009) An extensive proteomic view after inhibition of S-adenosylmethionine decarboxylase in *Plasmodium falciparum*. European Science Foundation Europe-Africa Frontier Research Conference Series Infectious Diseases: From Basic to Translational Research, 4 – 9 April 2009, The Cape Winelands, South Africa.
- b) Smit S., Louw A.I., Birkholtz L. (2008) Analysis of the malaria parasite proteome after inhibition of S-adenosylmethionine decarboxylase resulting in polyamine depletion. 2<sup>nd</sup> SA Proteomics & Genomics Conference, 03 - 05 March 2008, University of the Western Cape, South Africa.