Chapter 1: Literature review

1.1 Malaria

Malaria in Africa kills a vast number of children every year. By the time you have read the next paragraph, two children will have died: in Africa, a child dies from malaria every 30 seconds, which translates to a massive 2880 children per day (Finkel, 2007).

Malaria, together with HIV/AIDS and tuberculosis, is one of the world's most vital public health challenges compromising development in poverty-stricken countries and accounting for up to an overwhelming 2.7 million deaths per annum (Gardner *et al.*, 2002). More than 3 billion people (~40%) reside in areas of the world where malaria is prevalent. As such, the disease is largely responsible for the poor economic growth of these areas, which further contributes to more cases of malaria (Figure 1.1) (Korenromp *et al.*, 2005). Malaria is a complicated disease and its spread may be attributable to a variety of factors such as ecological and socio-economic conditions, displacement of large population groups, agricultural malpractices causing an increase in vector breeding, parasite resistance to antimalarial drugs and vector resistance to insecticides. In 1998, The World Health Organisation (WHO) established a global partnership called Roll Back Malaria (RBM) in an attempt to halve the world's malaria frequency by 2010 (http://www.rbm.who.int). Apart from RBM, a number of promising antimalarial drug and vaccine discovery projects have also been launched. This includes the Medicines for Malaria Venture (MMV) funded by a number of organisations including The Bill and Melinda Gates Foundation, for the development of novel antimalarials. The latter has also contributed more than 300 million US dollars to the Malaria Vaccine Initiative (MVI).

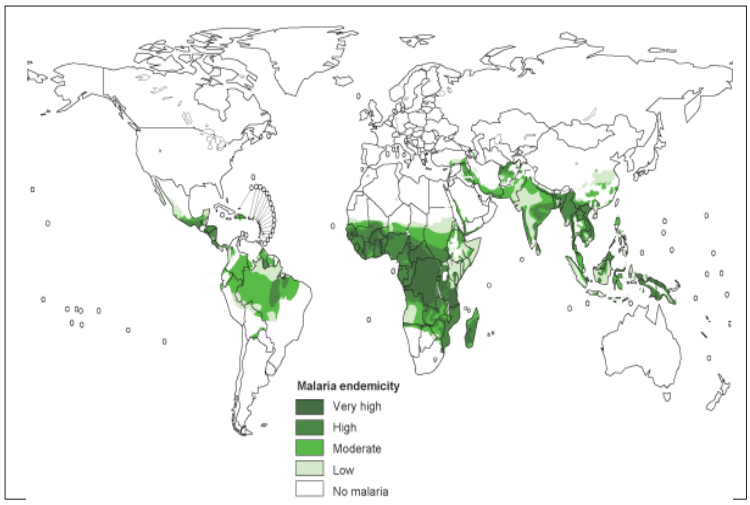


Figure 1.1: The worldwide distribution of malaria.
(http://www.rbm.who.int).

1.2 The *P. falciparum* life cycle

Malaria is caused by an infection from the intracellular Apicomplexan parasites of the *Plasmodium* genus. The genus consists of unicellular, eukaryotic protozoan parasites with a number of different species affecting humans including *P. falciparum* (the most severe form), *P. malariae, P. vivax* and *P. ovale* (Hoffman *et al.*, 2002). The parasites of the Apicomplexan phylum have complex life cycles and are all characterised by the presence of a special apical complex that is involved in host-cell invasion and which includes the microneme, dense granules and rhoptries (Figure 1.2) (Cowman and Crabb, 2006).

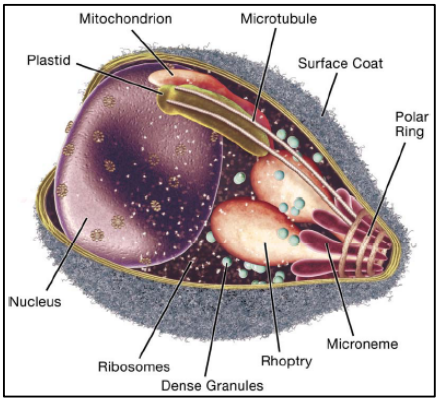


Figure 1.2: The *P. falciparum* merozoite showing the apical complex and other major cellular organelles and structures.
Taken from (Cowman and Crabb, 2006).

P. falciparum parasites invade host cells in order to acquire a rich source of nutrients. At the same time, these cells protect the parasites from host immune responses. The parasites are transmitted by the female *Anopheles gambiae* and *A. funestus* (Southern Africa) mosquitoes, which serve as vectors for the sexual reproduction of the parasites while the mammalian host provides the parasites with a niche for asexual development. The mosquitoes inject a sporozoite form of the parasites into the subcutaneous layer of the host skin during a blood meal. The sporozoites rapidly move to the liver where they infect the hepatocytes and differentiate into thousands of merozoites. These are subsequently released into the bloodstream where they invade erythrocytes. This invasion characterises the onset of the intra-erythrocytic asexual blood stage of the parasitic life cycle. The parasite cycles through ring, trophozoite and schizont stages and in so doing produce between 16 and 32 daughter merozoites per erythrocyte egression. This is accompanied by the characteristic bursts of fever and anaemia associated with the disease. The daughter merozoites repeat the asexual cycle by invading free erythrocytes (Figure 1.3 a). Some intra-erythrocytic stages, however, develop into male or female gametocytes that are ingested by the mosquito during its next blood meal. These develop into male and female gametes inside the mosquito's gut where they fuse to form diploid zygotes. The zygotes differentiate into ookinetes that subsequently

cross the midgut and develop into oocysts from which sporozoites are released. These sporozoites are stored in the salivary glands and are once again injected into the human host by the mosquito to repeat the parasitic life cycle, and thus increasing the number of malaria infectious cases (Figure 1.3 b) (Wirth, 2002).

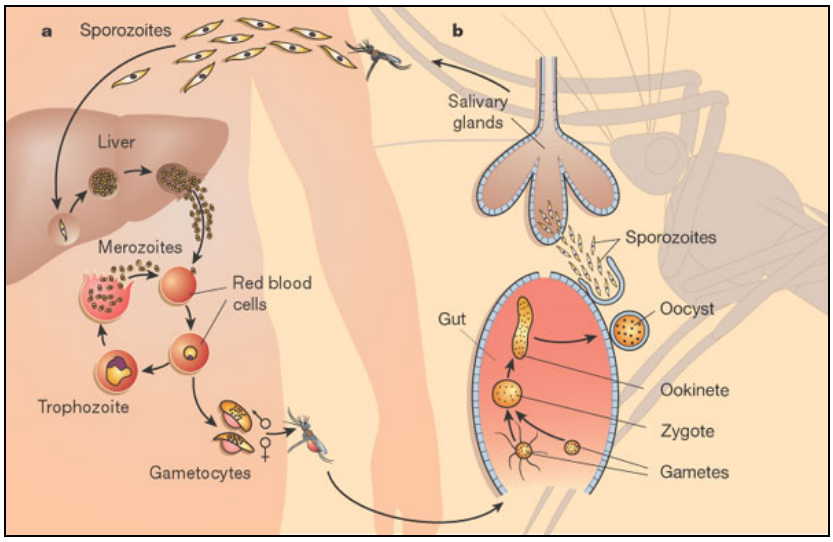


Figure 1.3: The life cycle of the *P. falciparum* parasite.
(a) The asexual stage of the *P. falciparum* life cycle within the human host, (b) the sexual stage within the mosquito host (Wirth, 2002).

1.3 Treating malaria

The development of a highly efficient, novel drug against any disease is in itself a challenging process but the development of an antimalarial is an exceptionally daunting task. The areas where malaria is prevalent at epidemic proportions are mostly devoid of trained physicians and health workers who possess the skills necessary for the early diagnosis of the disease as well as its efficient treatment. Novel antimalarials must be orally bioavailable, as the diseased individuals will most probably not have access to facilities such as hospitals and clinics. The treatment time period must also be less than a week to reduce the risks associated with the development of parasite resistance to the drugs, which may also be reduced with the use of combination-based therapy. Finally, the drugs must be cheap and have a relatively extended shelf life (Nwaka and Hudson, 2006).

1.3.1 Vector control

Alternative strategies to reduce the prevalence of malaria include the use of insecticide-treated bed nets and reduction of the vector population with insecticide spraying. Kingsley Holgate is a South African adventurer who took it upon himself to help fight Africa's largest killer. His epic yearlong journey, called the African Rainbow Expedition, started in 2005 and

was set off by Land Rover from Zululand in South Africa and continued along the African east coast to the Kenya-Somali border. Along the way, the expedition team visited remote villages to hand out insecticide-treated bed nets with the “One net, one life” motto in mind. A total of 10 000 nets were distributed, which is but a drop in the ocean, but the example set by this remarkable man was hoped to inspire others to reach out and help fight this devastating disease (http://www.kingsleyholgate.co.za). Since then, a partnership between Kingsley and the Nando’s fast food group has been established to continue the fight against malaria (Figure 1.4). With the support of Nando’s, the initiative aims to distribute thousands of insecticide-treated bed nets and malaria prevention information leaflets throughout Africa (http://www.nandos.co.za/Diary/AboutDiary.html).

ONE NET • ONE LIFE
MALARIA PREVENTION

Nando's life

Figure 1.4: Kingsley Holgate and his One net, one life anti-malaria compaign supported by Nando’s.
(http://www.kingsleyholgate.co.za/e_arainbow.html).

As far as indoor insecticide spraying is concerned, bis(4-chlorophenyl)-1,1,1-trichloroethane (better known as DDT) remains the most powerful and successful pesticide to date and is responsible for the eradication of malaria from the United States and Europe. In South Africa, the discontinued use of DDT in the 1990s resulted in the worst malaria epidemic the country has experienced since the introduction of indoor spraying in the 1950s (Figure 1.5). The reintroduction of DDT in 2000 resulted in an overall decrease in the number of malaria cases of approximately 50% in 2002 (Maharaj *et al.*, 2005). DDT is not only effective against malaria vectors but is equally potent at alleviating various other arthropod-borne diseases such as yellow fever, African sleeping sickness, dengue fever and typhus. However, DDT was also extensively used in agriculture where enormous quantities were aerially sprayed onto crops to curb pests. This widespread and uncontrolled use of DDT raised concerns in the 1960s amongst environmentalists who described possible catastrophic consequences for both the environment and humans leading to the ban of DDT use in the 1980s (Weissmann, 2006). Little scientific evidence, however, exists to support these concerns and no toxic effects caused by DDT in humans have been noted when used in low concentrations as required for the control of malaria vectors inside houses (Rogan and Chen, 2005). Since then, the efforts of several public health officials and malaria experts have resulted in the

restricted use of DDT in Africa for malaria vector control only and not in agriculture (Dugger, 2006).

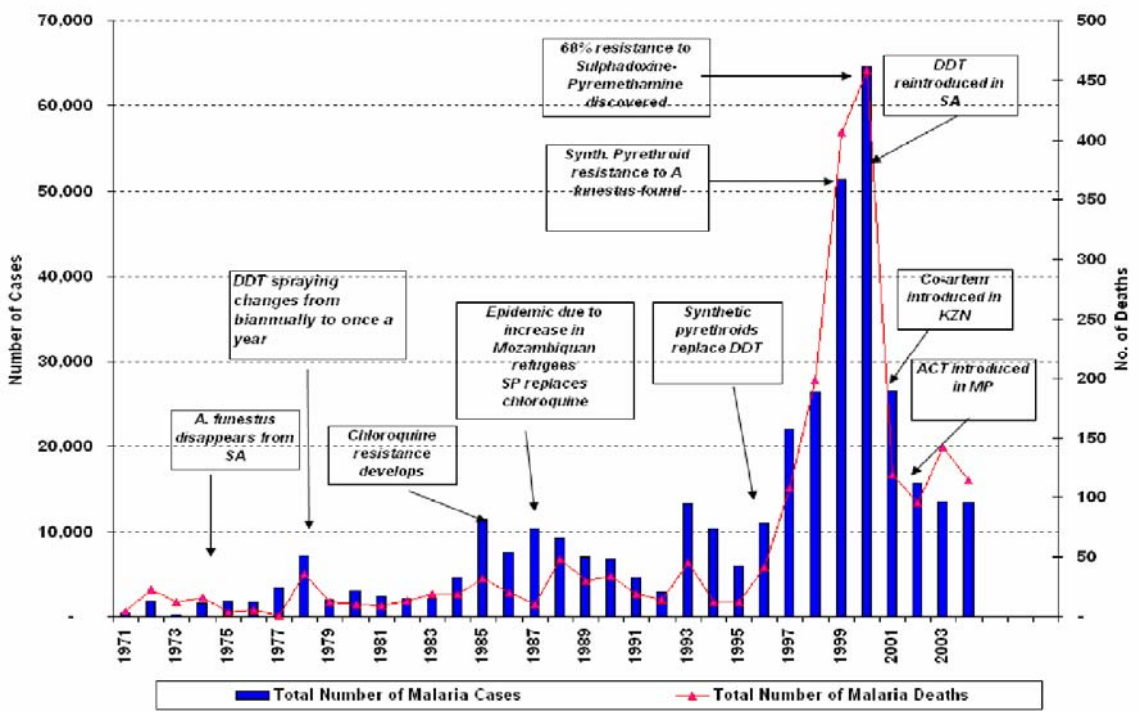


Figure 1.5: Annual recorded malaria cases and deaths in South Africa (1971-2004).
Taken from a report compiled by (Thiel, 2005).

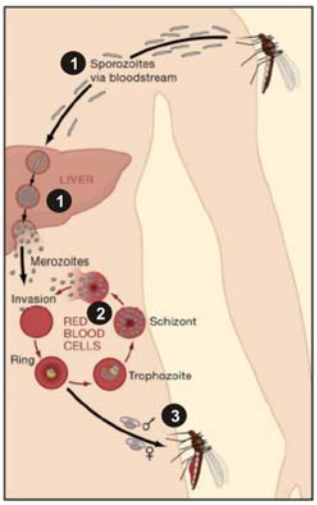
Malaria parasite transmission can additionally be prevented by the treatment of parasite vectors with a drug that blocks the sexual development of the parasites within the mosquito. Coleman tested the effect of 8-aminoquinolines on the sexual development of *P. berghei* and *P. falciparum* parasites in *A. stephensi* mosquitoes. The drug-fed mosquitoes produced fewer oocysts than the control-fed group, and in addition, the sporozoites that did manage to develop from the oocysts could not enter the salivary glands of the mosquito, and therefore prevented the parasites from being transmitted back to the mice (Coleman *et al.*, 1994). The antifolate drugs proguanil and pyrimethamine have also been shown to be sporontocidal. These drugs caused a reduction in oocysts in sensitive strains and pyrimethamine was also reported to directly damage ookinetes (Bray *et al.*, 1959). Gillet *et al*. showed that α-difluoromethylornithine (DFMO), a polyamine pathway inhibitor interferes with *P. berghei* sporozoite development in *A. stephensi* mosquitoes. Only one mouse out of 16 exposed to DFMO-treated mosquitoes contracted malaria. DFMO thus exerts its most deleterious effects on the stages where active cell division is taking place, namely the exo-erythrocytic schizogonous and sporogonous stages (Gillet *et al.*, 1983).

1.3.2 Vaccine development

The development of a safe and effective vaccine against infection represents an alternative method for treating parasitic diseases. Despite extensive efforts, not a single vaccine against any of the human parasitic diseases is currently available. Some malaria experts, however, remain adamant that vaccination may be the most valuable strategy for reducing mortality associated with malaria (Miller and Hoffman, 1998). People living in malaria endemic areas develop low levels of protective immunity against *P. falciparum* infection after five years of age but this immunity is never complete and seems to be specific for the parasite strain residing in a specific area. Protective immunity is therefore lost once the host moves into an area where a different strain resides and also once the host is no longer chronically infected (Bull and Marsh, 2002).

The complex life cycle of the malaria parasite, which allows it to co-exist with the host's immune response, is largely responsible for the absence of a successful vaccine (Todryk and Hill, 2007). Current vaccine development strategies focus on different protein antigens that are expressed during the different stages of the life cycle, namely the pre-erythrocytic (sporozoite and schizont-infected hepatic cells), the asexual intra-erythrocytic (merozoite-infected erythrocytes) and sexual exo-erythrocytic (gametocyte) stages (Figure 1.6) (Todryk and Hill, 2007). An ideal vaccine against malarial infection should therefore induce immune responses against every stage of the life cycle. Such a multistage, multivalent and multi-immune response vaccine presents the best strategy for a successful vaccine in the treatment of malaria (Doolan and Hoffman, 1997).

Antibodies directed against antigens on the surface of extracellular sporozoites (e.g. circumsporozoite protein, CSP) would result in the neutralisation of sporozoite infectivity in the bloodstream. Preliminary studies of the RTS,S/AS02 malaria vaccine (GlaxoSmithKline Biologicals) in African infants showed that the vaccine is safe, well-tolerated and reduces parasite infection and clinical illness due to malaria. The vaccine consists of two polypeptides; RTS corresponds to CSP amino acids 207-395 of *P. falciparum* 3D7 fused to the N-terminus of the hepatitis B surface antigen (HBsAg) and S consists of 226 amino acids of HBsAg (Stoute, 2007). A Phase II trail conducted in Mozambique reported that the vaccine is 65% effective against new infections over a three-month follow-up period after infants received three doses of the vaccine and reduced clinical malaria episodes by 35% over a six-month follow-up period starting after the first dose (Aponte *et al.*, 2007).



	Stage	Main target	Antigen	Vaccine
1	Pre-erythrocytic	Sporozoites	CSP	RTS,S/AS02A
		Hepatocytes	ME-TRAP	FP9/MVA, ME-TRAP
			LSA-1	LSA-1-AS02
			CSP	AdHu35
2	Asexual intra-erythrocytic	Merozoites	$MSP\text{-}1_{42}$	FMP-1-AS02
			AMA-1	AMA-1-AS02
			GLURP/MSP-3	GMZ2
			$AMA\text{-}1/MSP\text{-}1_{19}$	PfCp2.9
3	Sexual exo-erythrocytic	Gametocytes	Pfs25	-
			Pfs28	-
			Pfs48/45	-
			Pfs230	-

Figure 1.6: Selected malaria vaccines targeting different antigens in specific stages of the parasite life cycle.
(1) Pre-erythrocytic stage vaccines prevent host parasitic infection and disease development, (2) asexual erythrocytic stage vaccines block the multiplication of daughter merozoites, and (3) sexual stage vaccines prevent parasite transmission (Todryk and Hill, 2007). Abbreviations: CSP, circumsporozoite protein; ME-TRAP, multi-epitope thrombospondin-related adhesive protein; LSA-1, liver stage antigen 1; MSP, merozoite surface protein; AMA-1, apical membrane antigen 1; GLURP, glutamine-rich protein; Pfs, *P. falciparum* surface antigens; FP, fowl pox; MVA, modified vaccinia virus Ankara; AdHu35, human adenovirus serotype 35; FMP-1, Falciparum merozoite protein-1; PfCp2.9, *P. falciparum* chimeric protein 2.

Pre-erythrocytic stage vaccines focus on expressed parasite proteins on the sporozoite-infected hepatocyte surface, which elicit $CD8^+$ and $CD4^+$ T-cell responses resulting in reduced liver-stage malaria infection. These proteins include CSP, thrombospondin-related adhesive protein (TRAP), liver stage antigen 1 (LSA-1) and merozoite stage protein 1 (MSP-1). The FP9/MVA, ME-TRAP vaccine (University of Oxford), containing the T-cell-inducing vaccine vector (fowl pox strain FP9), followed by boosting with a second antigen-encoding vector modified vaccinia virus Ankara (MVA), which delivers TRAP, is fused to several liver-stage antigen T-cell epitopes (ME-TRAP). This prime-boost regimen with the concomitant delivery of the malaria antigens serves to extend the T-cell response against the infected hepatocytes (Gilbert *et al.*, 2006).

Extracellular merozoites released from the hepatocytes can be neutralised by antibodies directed against several surface antigens (MSP, apical membrane antigen 1 and glutamate-rich protein). Vaccines in this group that are currently undergoing clinical trials include the 42 kDa C-terminal $MSP\text{-}1_{42}$ vaccine as well as an apical membrane antigen 1 (AMA-1) vaccine consisting of the AMA-1 ectodomain. Both antigens are reconstituted in AS02A (Walter Reed Army Institute of Research) (Polhemus *et al.*, 2007).

In erythrocytic stage vaccines, antibodies raised to antigens on the erythrocyte plasma membrane (e.g. *P. falciparum* erythrocyte membrane protein 1, PfEMP1) would result in the

destruction of the infected erythrocyte or prevent the cytoadherence of these infected cells (Doolan and Hoffman, 1997). Blood-stage vaccines, however, are limited by the polymorphic character of the antigens, which creates diversity that restricts the efficacy of the vaccine representative of a particular genotype (Anders and Saul, 2000).

Finally, neutralising antibodies to pre-fertilization (e.g. Pfs48/45 and Pfs230) or post-fertilization (Pfs25 and Pfs28) gametocyte surface antigens could ensure that any remaining infection will not be transmitted back to the mosquito vector (Doolan and Hoffman, 1997).

1.3.3 Current antimalarials

Various drugs have been developed and used in the fight against malaria. As with the vaccines, antimalarials target different stages of the parasite life cycle within the human host and specifically interfere with processes that are essential to parasite survival. Eradication of malaria with the use of antimalarials is, however, continuously compromised by the increased prevalence of parasite resistance to the small amount of available commercial drugs. Figure 1.7 shows the different stages of the parasite life cycle and drugs that specifically target these stages of parasite development.

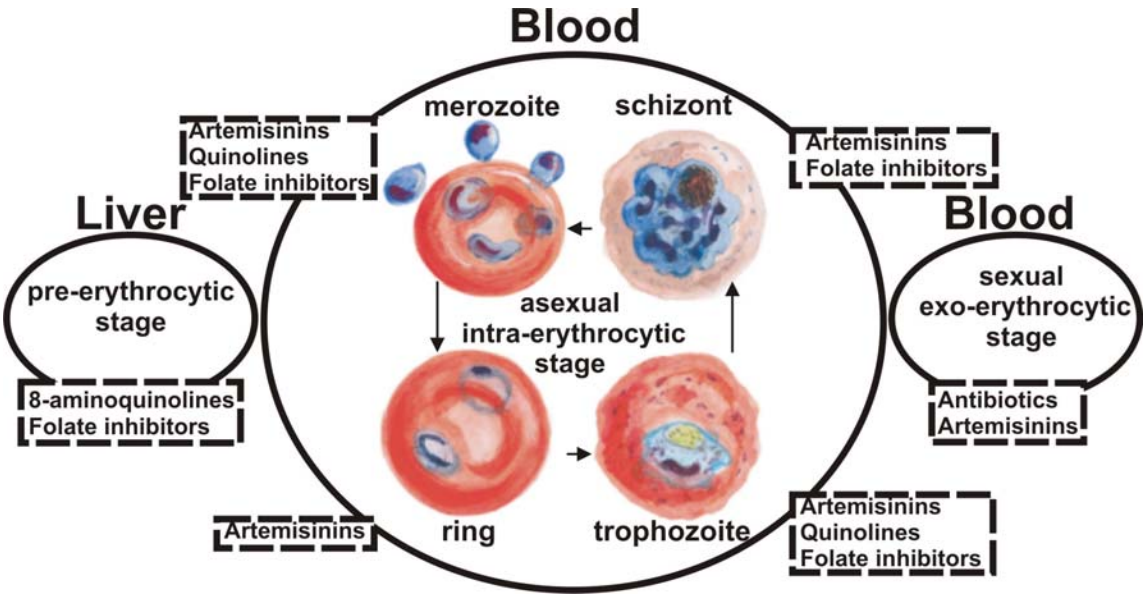


Figure 1.7: A schematic diagram of the parasite life cycle within the human host showing the targets of different antimalarials during the developmental stages.
The pre-erythrocytic, asexual intra-erythrocytic and sexual exo-erythrocytic stages are shown. The different intra-erythrocytic phases of malaria parasite development are also given. Finally, drugs that have been used at each stage are shown in the dashed boxes (Chauhan and Srivastava, 2001; Olliaro, 2001; Korenromp *et al.*, 2005).

A list of some antimalarials is given in Table 1.1. The proposed mechanisms of action of these different drug classes are also listed.

Table 1.1: Different antimalarial drug classes together with their mechanisms of action Compiled from (Chauhan and Srivastava, 2001; Olliaro, 2001; Korenromp *et al.*, 2005).

Stage	Drug class	Drug compounds	Mechanism of action
Pre-erythrocytic	Aminoquinolines	Primaquine (also gametocytocidal)	Unknown
	Hydroxynaphthoquinone	Atovaquone (also sporontocidal)	Interferes with cytochrome electron transport
Asexual intra-erythrocytic	Aminoquinolines	Chloroquine (also gametocytocidal)	Inhibits haem detoxification
		Quinine (also gametocytocidal)	Inhibits haem detoxification
	Sulfonamides	Sulfadoxine	Inhibits DHPS
	Sulfones	Dapsone	Inhibits DHPS
	Amidines	Proguanil (active as cycloguanil, also active against pre-erythrocytic forms and sporontocidal)	Inhibits DHFR
	Pyrimidines	Pyrimethamine (also sporontocidal and interferes with sexual reproduction)	Inhibits DHFR (used in combination with sulfadoxine or dapsone)
	4-Methanolquinoline	Mefloquine	Inhibits haem detoxification
	Sesquiterpene lactone	Artemisinin and derivatives (also gametocytocidal)	Inhibits calcium adenosine triphosphatase
Exo-erythrocytic	Antibiotics	Tetracycline (also active against intra-erythrocytic forms)	Inhibitors of aminoacyl-tRNA binding during protein synthesis
		Doxycycline (also active against intra-erythrocytic forms)	Inhibitors of aminoacyl-tRNA binding during protein synthesis

1.3.3.1 Quinolines

The bark of the *Cinchona* tree has been used for centuries to treat fever associated with malarial infection from which the active ingredient is quinine. It remained the antimalarial of choice until the 1940s, when other antimalarials such as its chloroquine derivative replaced quinine. Quinine, however, is still used today to treat clinical malaria and not as a prophylaxis due to its side effects and poor tolerability. Chloroquine is a 4-aminoquinoline derivative of quinine and for many years it was the main antimalarial drug used in the treatment of malaria until parasite resistance developed in the 1950s. It remains the most popular antimalarial developed to date due to its safety, low cost and efficacy. (Bjorkman and Phillips-Howard, 1990; Yeh and Altman, 2006). Today, the widespread resistance to the drug has rendered its use as a therapeutic agent useless, even though it still shows some efficacy in the treatment of the other human malaria parasites (Korenromp *et al.*, 2005).

Despite more than three decades of research, the exact molecular mechanism of chloroquine action remains a controversial topic. It is believed that the weak-base drug accumulates in the acidic food vacuole of the parasite where it prevents haem detoxification

(Bray *et al.*, 2005). Chloroquine resistance in malaria parasites has been attributed to reduced concentrations of the drug in the food vacuole possibly due to drug efflux, pH modification in the vacuole, the role of a Na^+/H^+ exchanger and transporters (Foley and Tilley, 1998; Bray *et al.*, 2005). In *P. falciparum*, two genes have been implicated in resistance, namely *Pfmdr1* and *Pfcrt*, which encodes Pgh1 and PfCRT, respectively. Both these proteins are localised to the food vacuole membrane. Mutations in these genes could lead to small increases in the food vacuole pH thus reducing the amount of chloroquine that can accumulate, rendering the drug ineffective (Spiller *et al.*, 2002). Alternatively, PfCRT may increase the efflux of chloroquine by directly interacting with the drug (Cooper *et al.*, 2007). Resistance is associated with several mutations in the PfCRT protein, while the loss of a Lys residue at position 76 has been shown as the critical mutation rendering the *P. falciparum* parasites resistant to the drug. This residue is located within the first transmembrane segment of PfCRT and may therefore play an important role in the properties of the channel or transporter (Cooper *et al.*, 2005). Mutations in the *Pfmdr1* gene is associated with resistance to mefloquine, quinine and halofantrine (Reed *et al.*, 2000).

A number of related aminoquinolines have since been developed and applied, including:

- Amodiaquine: effective against chloroquine-resistant strains, possible cross-resistance with chloroquine, safety limitation (Korenromp *et al.*, 2005; Bathurst and Hentschel, 2006)
- Atovaquone: usually used in combination with proguanil (Malarone®), resistance reported in 1996, cost limitation (Korenromp *et al.*, 2005; Bathurst and Hentschel, 2006)
- Lumefantrine: usually coformulated with artemether (Co-Artem™) and is highly effective against multi-drug resistant *P. falciparum* (Korenromp *et al.*, 2005)
- Halofantrine (Halfan): resistance reported in 1992, cost and safety limitations (Bathurst and Hentschel, 2006)
- Mefloquine (Lariam®): resistance reported in 1982, cost and safety limitations (Bathurst and Hentschel, 2006)
- Primaquine: used for its gametocytocidal effect (*P. falciparum*) and its efficacy against intra-hepatic forms of all types of malaria, no resistance, safety limitations (Chauhan and Srivastava, 2001; Bathurst and Hentschel, 2006)

1.3.3.2 Antifolates

The antifolates are some of the most widely used antimalarials. However, their role in malaria prevention is hampered by the rapid emergence of resistance once the parasites are placed under drug pressure. The direct effect of folate biosynthesis inhibition is a reduction in the synthesis of the amino acids serine and methionine as well as in pyrimidines, which leads to

decreased synthesis of DNA. The antifolate drugs target the intra-erythrocytic stages as well as the gametocytes of *P. falciparum* (Olliaro, 2001).

The antifolates can generally be divided into two classes; the type-1 antifolates mimic the *p*-aminobenzoic acid (pABA) substrate of dihydropteroate synthase (DHPS) and include the sulfonamides (sulfadoxine) and sulfones (dapsone), while the type-2 antifolates (pyrimethamine and proguanil) inhibit dihydrofolate reductase (DHFR) (Olliaro, 2001). Interestingly both of these classes of target proteins are arranged on separate bifunctional enzymes (hydroxymethyldihydropterin pyrophosphokinase/dihydropteroate synthase or PPPK/DHPS and dihydrofolate reductase/thymidylate synthase or DHFR/TS) (Ivanetich and Santi, 1990). In addition, malaria parasites are capable of *in vivo* folate salvage from the extracellular environment as well as synthesising folate derivatives from simple precursors. The mechanism of exogenous folate uptake by a carrier-mediated process has important implications in increasing the sensitivity of the antifolate inhibitors and is being investigated as a novel drug target (Wang *et al.*, 2007).

Pyrimethamine is a diaminopyrimidine and is mostly used in combination with sulfadoxine (Fansidar™) or dapsone leading to the simultaneous inhibition of DHFR and DHPS. Pyrimethamine crosses the blood-brain barrier as well as the placenta. Resistance to sulfadoxine-pyrimethamine combination therapy, however, emerged rapidly due to the appearance of point mutations in the active sites of the target enzymes resulting in reduced drug binding capacity (Cowman and Lew, 1990; Plowe *et al.*, 1997).

1.3.3.3 Artemisinins

Artemisinin is a sesquiterpene lactone extracted from the leaves of *Artemisia annua*. It is a potent, fast acting blood schizontocide that shows efficacy against all *Plasmodium* species. Its efficacy is especially broad and shows activity against all the asexual stages of the parasites including the gametocytes (Figure 1.7) (Korenromp *et al.*, 2005). The latter makes this class of antimalarials especially important as they reduce the transmission potential through its gametocytocidal activity.

Originally the mechanism of action of artemisinin was thought to be mediated by the peroxide ring of the drug, which is cleaved and activated by ferrous iron in the heme stores into toxic free radicals that can subsequently damage intracellular targets via alkylation (Meshnick *et al.*, 1991). Recently, however, this theory was challenged by evidence that artemisinin exerts its inhibitory effects on the malarial sarcoplasmic-endoplasmic reticulum calcium ATPase (SERCA) resulting in an alteration of intracellular calcium levels (Eckstein-Ludwig *et al.*,

2003). The exact mechanism of action, however, remains elusive and different studies have produced contradicting results [reviewed in (Krishna *et al.*, 2004)].

Several derivatives of artemisinin have been developed since artemisinin itself is poorly absorbed and include dihydroartemisinin, artesunate (sodium salt of the hemisuccinate ester of artemisinin), artemether (methyl ether of dihydroartemisinin) and artemether (ethyl ether of artemisinin) (Korenromp *et al.*, 2005). Currently, the WHO recommends artemisinin-based combination therapy (ACT) as the first-line treatment against malaria infections where resistance to other antimalarials is prevalent. One of the obvious disadvantages of using ACT for malaria case-management in Africa is the increased cost involved in combining therapies. Even so, several reasons exist for combining antimalarials with an artemisinin derivative, namely: 1) an increase in the efficacy of the antimalarials; 2) a decrease in the treatment time period; and 3) a reduced risk of resistant parasites arising through mutation (Kremsner and Krishna, 2004).

Several ACTs that have been developed and may have entered clinical trails are listed below (Gelb, 2007):

- Pyramax: artesunate and the 4-aminoquinoline pyronaridine
- Co-Artem™: artemether and lumefantrine
- Artekin™: dihydroartemisinin and the quinoline-based drug piperaquine
- Lapdap™: artesunate, chlorproguanil and dapsone
- ASAQ: artesunate and amodiaquine

There are several reasons why the appearance of parasite resistance to artemisinin was originally thought to be unlikely or at least delayed: 1) parasites are not exposed to the drug for prolonged periods due to the short half-life of the drug; 2) artemisinin is gametocytocidal, which reduces the transmission potential and spread of the parasite; and 3) the frequent use of artemisinin combined with other antimalarials (ACT) was specifically introduced to delay the onset of resistance (Meshnick, 2002). Evidence for *in vitro* resistance to an artemisinin derivative, however, appeared in field isolates from French Guiana in 2005. The increased artemether IC_{50}s were ascribed to the presence of a mutation in the SERCA *PfATPase6* gene and was attributed to inappropriate drug use that exerted selection pressures, favouring the emergence of parasites with an artemether-resistant *in vitro* profile. Even though reduced *in vitro* drug susceptibility is not tantamount to diminished therapeutic effectiveness, it could lead to complete resistance and thus called for the rapid deployment of drug combinations (Jambou *et al.*, 2005). Lapdap™, a combination of chlorproguanil (targets DHFR), dapsone (targets DHPS) and the artemisinin derivative artesunate, was introduced in 2003 as malaria

therapeutic to replace sulfadoxine-pyrimethamine treatment in Africa (Edwards and Biagini, 2006). No resistance has been detected to date (Bathurst and Hentschel, 2006).

1.3.3.4 Antibiotics

Several antibiotics such as tetracycline, doxycycline and minocycline are active against the exo-erythrocytic as well as the asexual blood stages of the *P. falciparum* parasite. The tetracyclins are antibiotics that were originally derived from *Streptomyces* species, but are usually synthetically prepared. They interfere with aminoacyl-tRNA binding and therefore inhibit protein synthesis in the parasite's apicoplast or mitochondrion (Korenromp *et al.*, 2005). This is due to the presence of genomes in the mitochondrion and apicoplast that encode prokaryote-like ribosomal RNAs, tRNAs and various proteins (Wilson *et al.*, 1996). Doxycycline is a synthetic tetracycline derivative with a longer half-life than tetracycline. A disadvantage of these type of antibiotics as antimalarials is the development of photosensitivity during treatment, which is an obvious drawback for tourists entering malaria areas (Korenromp *et al.*, 2005).

1.3.4 Novel antimalarial targets

Despite the availability of various antimalarials and attempts aimed at preventing parasite infection with the use of suitable vaccines, high malaria mortality continues to persist in endemic areas while the deadline of the RBM endeavour is rapidly approaching. The identification of novel drug targets that can possibly reduce the prevalence of malaria without inducing rapid resistance thus remains imperative and a major challenge for researchers in the field of infectious diseases. A good starting point for the identification of drug targets is to pinpoint differences between host and parasite essential metabolic pathways that are more easily identified once the parasite physiology and host-parasite relationships are better understood. The presence or absence of specific essential pathway enzymes and special features thereof can subsequently be identified and investigated for possible chemotherapeutic intervention strategies.

1.3.4.1 Type II fatty acid biosynthesis

The Apicomplexan plastid, or apicoplast, originated from an endosymbiotic cyanobacterium, which once lived within the host, and the original acquisition of the plastid presumably provided the host with various anabolic products. In addition, several biosynthetic pathways similar to those in plant and algal chloroplasts have been characterised in apicoplasts, including a malaria specific type II fatty acid synthesis pathway. This pathway has major differences compared to the type I fatty acid synthesis pathway found in humans and thus allows for good drug target discovery opportunities (Ralph *et al.*, 2001).

It has been suggested that fatty acids are synthesised for the formation of the parasitophorous vacuole during erythrocyte invasion of the *P. falciparum* parasites and is thus essential for successful infection of the host cells (McFadden and Roos, 1999). Acetyl-CoA carboxylase (ACC) catalyses the first committed step of the *de novo* fatty acid synthesis pathway and provides the sole source of malonyl-CoA (Gornicki, 2003). This rate-limiting enzyme is targeted by the arloxyphenoxyproprionate class of Gram-negative herbicides (Golz *et al.*, 1994). Current research is focussing on the interaction between the herbicides and ACC for the development of more potent ACC inhibitors. Enoyl-ACP-reductase (FabI) catalyses and controls the rate of the final step of fatty acid elongation and is inhibited *in vitro* and *in vivo* by the broad spectrum antibiotic and antifungal, triclosan (Surolia and Surolia, 2001). Despite the *in vivo* efficacy of triclosan, it is too early to apply it in the field since pharmacokinetic and toxicological evaluations remain absent. The molecular mechanism of the inhibitors are known and the 3-dimensional structure of FabI has also been solved, which provides a basis for further rational design of novel inhibitors against type II fatty acid biosynthesis in *P. falciparum* (Gornicki, 2003).

1.3.4.2 DNA topoisomerases

DNA topoisomerases are complex enzymes that control the topological state of DNA and include the type I (transiently cleaves and then reseals one DNA strand) and type II (cleaves and reseals both DNA strands) topoisomerases. The type II topoisomerase, or DNA gyrase, also catalyses DNA supercoiling (Chauhan and Srivastava, 2001).

Recently, the presence of topoisomerases in the protozoan-infected erythrocytes has been observed, which encouraged studies on these enzymes as possible antimalarial targets. Camptothecin has been shown to kill parasites through its inhibitory effects on topoisomerase I (Bodley *et al.*, 1998) while specific antisense oligonucleotides to topoisomerase II decreases the growth of the parasites in culture (Noonpakdee *et al.*, 2003). The future challenge of drug discovery with the DNA topoisomerases as targets is the structural determination of these proteins. This will aid the design of chemical entities, which can ascertain the actual worth of targeting these sites in malaria chemotherapy.

1.3.4.3 Polyamine biosynthesis

Several studies have investigated the importance of polyamines and their involvement in various processes within the cell. In most organisms, the polyamine pathway has been fully elucidated and a decade of research has resulted in major advances in our understanding of polyamine biosynthesis in the malaria-causing parasite. Wang and Assaraf have respectively shown that interruption of polyamine biosynthesis hampers the development of disease-causing *Trypanosoma brucei gambiense* and *P. falciparum* parasites (Assaraf *et al.*, 1984;

Wang, 1995). Further studies have identified unique parasite-specific properties in the *P. falciparum* polyamine pathway, which present possible targets for chemotherapeutic intervention (Müller *et al.*, 2000; Wrenger *et al.*, 2001; Birkholtz *et al.*, 2004). A sensible approach is thus the biochemical characterisation (both structural and functional) of the pathway's constituent enzymes for rational drug development strategies (Birkholtz *et al.*, 2004). The polyamine biosynthesis pathway as drug target in *P. falciparum* will thus be the main focus of this study.

1.4 Polyamines

The physiologically important polyamines putrescine, spermidine and spermine are found in all living organisms except the Methano- and Halobacteriales (Hamana and Matsuzaki, 1992). The widespread presence of these polyamines signifies its considerable contribution to the survival of living eukaryotic and prokaryotic cells. They have been implicated in many growth processes such as cell differentiation and proliferation (Cohen, 1971; Bachrach, 1973; Tabor and Tabor, 1976; Heby, 1981), which is reflected by the general abundance of polyamines and increased activity of its biosynthetic enzymes during stages of rapid growth (Assaraf *et al.*, 1984; Russell, 1985). Polyamine levels are thus closely regulated as their depletion may lead to growth arrest and aberrant embryonic development (Johnson, 1994) while their accumulation may result in apoptosis (Poulin *et al.*, 1995; Tobias and Kahana, 1995; Xie *et al.*, 1997). The intracellular polyamine levels are maintained at optimal levels by tight regulation of its synthesis, degradation as well as their uptake and secretion from and into the extracellular compartment. The importance of the naturally occurring polyamines as well as their regulation by various biosynthetic and catabolic enzymes has led to the identification of various enzymes in the polyamine pathway as drug targets for the treatment of cancer and parasitic diseases.

The positive charges residing on the amine groups of the polyamines at physiological pH are responsible for the specialised functions of these ubiquitous organic cations. Polyamines are, simply put, low molecular weight (MW), long-chain aliphatic carbon compounds derived from amino acids with multiple amine groups (Figure 1.8) (Whaun and Brown, 1983). The positive charges allow for electrostatic interactions with anionic molecules such as nucleic acids, proteins and lipids (Tabor, 1962).

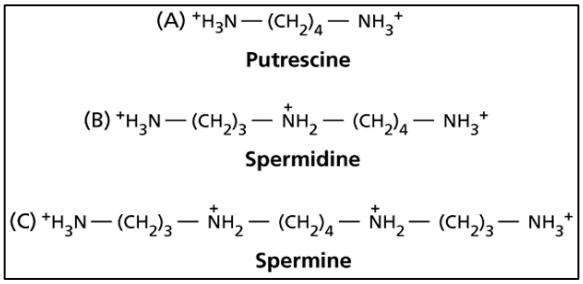


Figure 1.8: Structures of the positively charged polyamines: the divalent putrescine, trivalent spermidine and tetravalent spermine.

Electrostatic associations between polyamines and DNA result in the stabilisation of these molecules, which often promotes DNA bending. This subsequently facilitates the binding of gene regulatory elements and thus indirectly influences transcription (Tabor, 1962; Feuerstein *et al.*, 1986; Feuerstein *et al.*, 1989; Kerppola, 1998). Polyamines additionally influence transcription by modifying chromatin structure via the stimulation of histone acetyltransferase (Hobbs *et al.*, 2002). One of the most unique post-translational protein modifications is the spermidine-dependent hypusinilation of eukaryotic translation initiation factor, eIF5A. The function of eIF5A is not entirely understood but it appears to be essential for cell proliferation as its depletion arrests yeast cells in the G1 stage of the cell cycle (Kang and Hershey, 1994). Cells maintain optimal levels of polyamines as they play paradoxical roles in the prevention as well as in the stimulation of cell death. Increased levels of polyamines protect cells by steering them into the proliferative pathway and away from cell death (Thomas and Thomas, 2001). However, the accumulation of excess intracellular putrescine triggers apoptosis possibly due to an imbalance in intracellular positive and negative charges and the decreased formation of modified eIF5A (Tome *et al.*, 1997; Xie *et al.*, 1997).

1.4.1 Polyamine metabolism in *P. falciparum*

Polyamines are essential components in all living cells where they execute multiple functions, such as cell proliferation and macromolecular synthesis. Interference with the biosynthesis of these polycationic molecules has been applied in the search for antitumour drugs [for review see (Seiler, 2003)]. Inhibitors to almost all of the biosynthetic enzymes have been successfully designed and tested for application in antitumour therapeutic intervention (Marton and Pegg, 1995). Although success in finding antitumour drugs that target the polyamine pathway has been limited, it does suggest that these kinds of drugs may be useful against rapidly proliferating parasites. The parasite's pathway is distinctly different from that of the human's, which means that interference with the parasite's polyamine biosynthetic pathway could have more severe consequences on the parasite than its mammalian host (Figure 1.9) (Müller *et al.*, 2001). Obvious differences between the main polyamine

biosynthetic enzymes in the two organisms may provide possible drug target development opportunities for the treatment of parasitic infectious diseases.

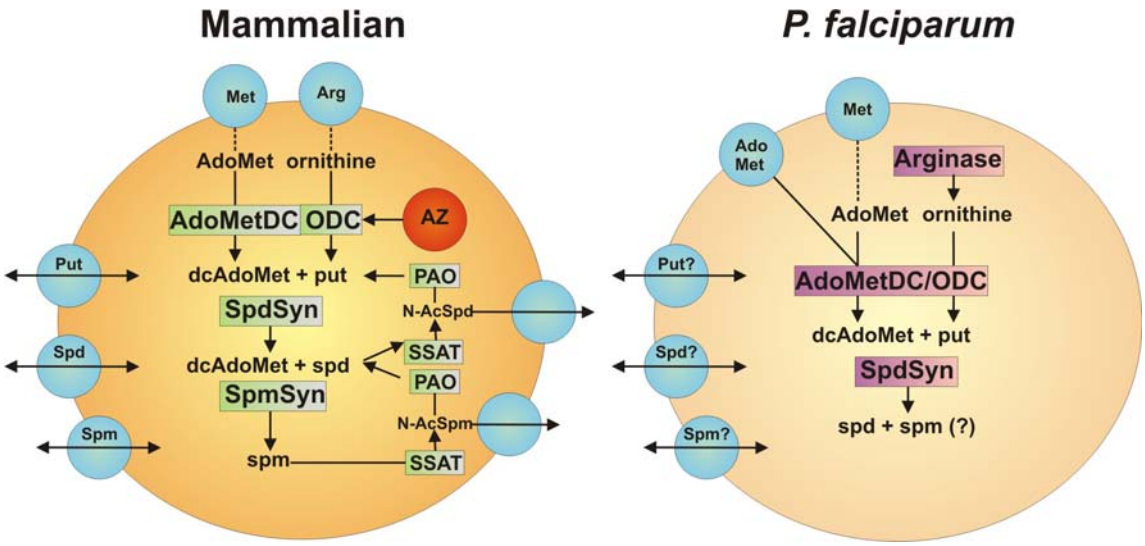


Figure 1.9: Outlines of the polyamine metabolic pathways in the mammalian cell and the *P. falciparum* parasite.
Transporters are shown as blue circles. Intermediates and reaction products are written in plain text while the enzymes producing these are given in green (mammalian cell) and purple (parasite) boxes. Abbreviations: AdoMetDC, S-adenosylmethionine decarboxylase; ODC, ornithine decarboxylase; dcAdoMet, decarboxylated S-adenosylmethionine; put, putrescine; SpdSyn, spermidine synthase; SpmSyn, spermine synthase; spd, spermidine; spm, spermine; SSAT, spermidine/spermine-N^1-acetyltransferase; PAO, polyamine oxidase; N-AcSpd and N–AcSpm, N^1-acetylated spermidine and spermine; AZ, antizyme; Met, methionine; AdoMet, S-adenosylmethionine; Arg, arginine.

Polyamines are synthesised via the decarboxylation of ornithine to putrescine by the enzyme ornithine decarboxylase (ODC*).* The diamine putrescine then acts as the precursor of spermidine and spermine. Another decarboxylation enzyme, S-adenosylmethionine decarboxylase (AdoMetDC), synthesises decarboxylated S-adenosylmethionine (dcAdoMet), which serves as a donor of propylamine moieties for the synthesis of spermidine and spermine. These last two reactions are catalysed by spermidine synthase (SpdSyn) and spermine synthase (SpmSyn), respectively (Müller *et al.*, 2000). SpmSyn has, however, not been identified in *P. falciparum* although PfSpdSyn is capable of synthesising low levels of spermine (Haider *et al.*, 2005).

A more detailed diagram of the *P. falciparum* pathway for polyamine biosynthesis and its side reactions is given in Figure 1.10.

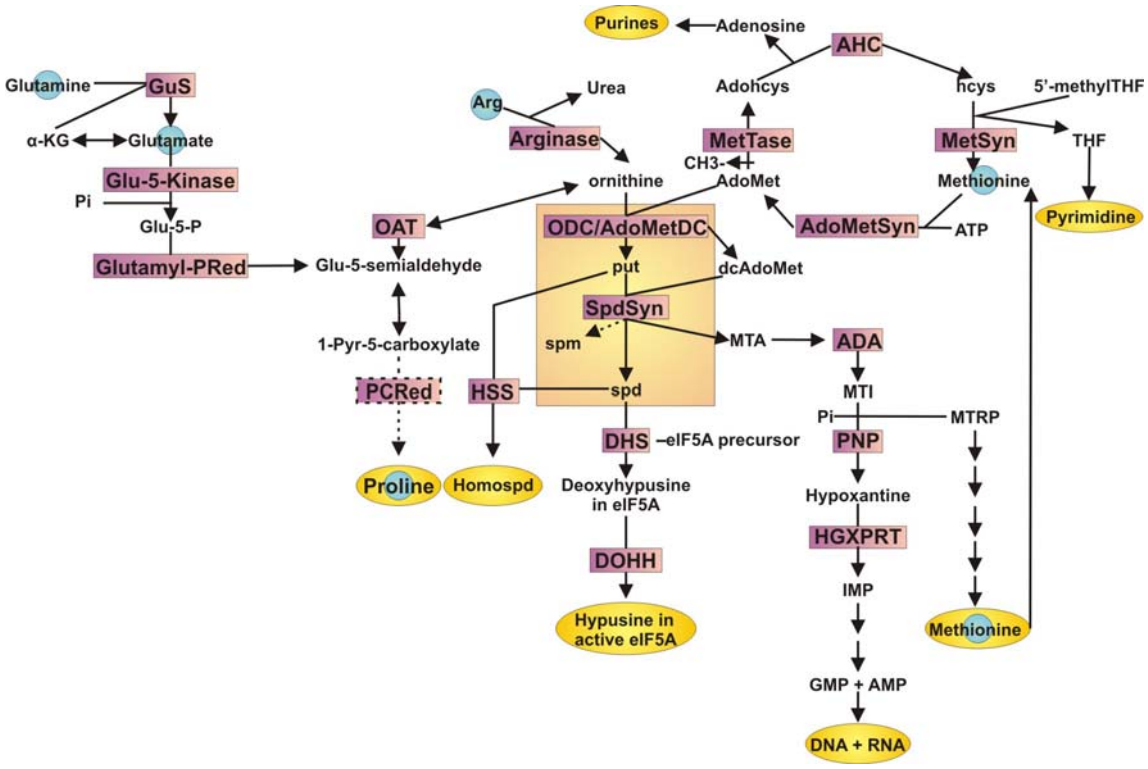


Figure 1.10: The polyamine biosynthesis pathway in *P. falciparum*.
The polyamine biosynthesis pathway is given in the central orange block. Substrates and products for which transporters have been characterised are shown as blue circles. Yellow circles represent products of some of the side reactions of the polyamine pathway. Abbreviations: ODC/AdoMetDC, ornithine decarboxylase/S-adenosylmethionine decarboxylase; put, putrescine; SpdSyn, spermidine synthase; spm, spermine; spd, spermidine; AdoMet, S-adenosylmethionine; MetTase, methionine transferase; CH3-, methyl group; Adohcys, S-adenosylhomocysteine; AHC, adenosylhomocysteinase; hcys, homocysteine; 5'-methylTHF, 5'-methyltetrahydrofolate; THF, tetrahydrofolate; MetSyn, methionine synthase; AdoMetSyn, S-adenosylmethionine synthetase; dcAdoMet, decarboxylated S-adenosylmethionine; MTA, 5'-deoxy-5'-(methylthio) adenosine; ADA, adenosine deaminase; MTI, 5'-methylthioinosine; Pi, inorganic phosphate; PNP, purine nucleoside phosphorylase; HGXPRT, hypoxantine-guanine-xanthine-phosphoribosyl transferase; IMP, inosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; AMP, adenosine 5'-monophosphate; ATP, adenosine 5'-triphosphate; MTRP, 5'-deoxy-5'-(methylthio) ribose-1-phosphate; Arg, arginine; OAT, ornithine aminotransferase; Glu-5-semialdehyde, glutamate-5-semialdehyde; 1-Pyr-5-carboxylate, 1-pyrroline-5-carboxylase; PCRed, 1-Pyr-5-carboxylase reductase; Glutamyl-PRed, glutamylphosphate reductase; Glu-5-P, glutamate-5-phosphate; α-KG, α-ketoglutarate; GluS, glutamate synthase; HSS, homospermidine synthase; Homospd, homospermidine; eIF5A, eukaryotic initiation factor 5A; DHS, deoxyhypusine synthase; DOHH, deoxyhypusine hydroxylase.

1.4.2 Polyamine transporters

The presence of a specific polyamine transport system in malarial parasite-infected erythrocytes remains a controversial subject. Transport systems have, however, been suggested to exist based on three specific observations. It is a well-studied observation that parasites induce numerous biochemical, structural and functional changes in infected erythrocytes resulting in the membrane becoming more permeable to various solutes via new permeability pathways (Ginsberg, 1990; Deitsch and Wellems, 1996). Evidence also suggests that the replenishment of intracellular polyamine pools in parasites treated with

polyamine biosynthesis enzyme inhibitors is due to an influx of polyamines across the membrane (Seiler *et al.*, 1996; Singh *et al.*, 1997). Finally, the exogenous addition of putrescine rescues DFMO-treated *P. falciparum* cultures, which suggests that the parasites are able to internalise and metabolise putrescine for growth and macromolecular synthesis. This last observation might explain why *in vitro* DFMO treatment has a mere cytostatic effect on parasites as reduced intracellular polyamine pools are replenished with exogenous polyamines (Assaraf *et al.*, 1984; Assaraf *et al.*, 1987).

To date, the only polyamine transporter that has been characterised in *Plasmodia* is the *P. knowlesi*-induced putrescine-specific transporter (Singh *et al.*, 1997). Haider *et al*. used *trans*-4-methylcyclohexylamine (4MCHA) to inhibit PfSpdSyn. The addition of spermidine did not result in the resumption of parasite growth, which indicates inefficient uptake of spermidine by the infected erythrocytes and an apparent absence of a spermidine-specific transporter in *P. falciparum*-infected erythrocytes (Haider *et al.*, 2005). The direct targets of this inhibitor are, however, unknown and it is possible that additional sites may be effected in *P. falciparum* (Haider *et al.*, 2005). However, PfAdoMetDC inhibition could not be rescued by the addition of putrescine or spermidine in *P. falciparum* parasites (Das Gupta *et al.*, 2005). The effects of ODC inhibitors were, on the other hand, completely abolished by putrescine supplementation, suggesting the presence of a putrescine transporter system (Das Gupta *et al.*, 2005), which is true for both *P. falciparum* and *P. berghei* parasites (Hollingdale *et al.*, 1985; Assaraf *et al.*, 1987).

In the process of drug discovery it is empirical to take note of strategies that parasites employ to counteract the depletion of a specific essential compound. The most effective drug will be one that interferes with the biosynthesis of a specific essential compound, such as putrescine, and at the same time obstructs its uptake into the *P. falciparum*-infected erythrocyte as a competitive inhibitor of a putrescine-specific transporter.

1.4.3 Polyamine levels during malarial infection

Human erythrocytes only contain trace amounts of polyamines and lack the enzymes necessary for active polyamine biosynthesis (Das Gupta *et al.*, 2005). *P. falciparum* invasion of these erythrocytes leads to rapid proliferation within the host cells and this rapid growth and differentiation of the parasites coincide with high intracellular concentrations of putrescine, spermidine and spermine in the parasites (Assaraf *et al.*, 1984). Spermidine is the major polyamine during all stages of growth (Figure 1.11) (Das Gupta *et al.*, 2005).

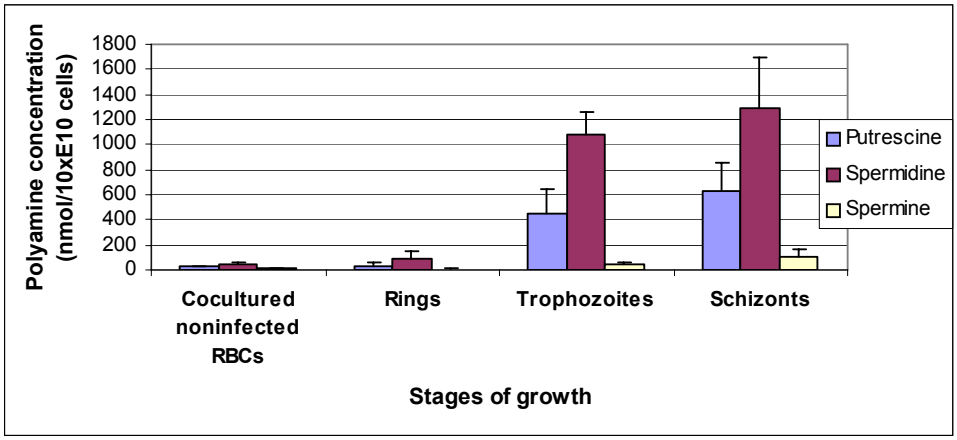


Figure 1.11: Stage-specific polyamine content of *P. falciparum*-infected erythrocytes. Adapted from (Das Gupta *et al.*, 2005).

Polyamines are significantly increased during the trophozoite and schizont stages of parasitic growth compared to that of the normal uninfected erythrocytes. The difference in polyamine levels between the various stages is most pronounced when putrescine and spermidine are compared. Spermine levels are only slightly elevated in the parasitized cells (Figure 1.11). These increases in polyamine content are proportional to the parasitaemia, the activities of the polyamine biosynthetic enzymes, and also the biosynthesis of proteins and nucleic acids (Assaraf *et al.*, 1984; Das Gupta *et al.*, 2005).

1.4.4 Polyamine biosynthetic enzymes as drug targets

The importance of polyamines in parasitic growth suggests that the inhibition of the polyamine pathway will interfere with the proliferation of the parasites (Assaraf *et al.*, 1984). This inhibition can be approached by three general routes: 1) with the application of active site-based inhibitors targeting the pathway's essential biosynthetic enzymes; 2) by interfering with polyamine transport; and 3) by using non-functional polyamine structural analogues to replace functional polyamines resulting in an altered intracellular polyamine homeostasis (Müller *et al.*, 2001). Biochemical studies on the polyamine pathway enzymes have highlighted important differences between the host and parasite enzymes such as size, substrate specificities and unique structural features. Differences between the mammalian and *P. falciparum* AdoMetDC and ODC enzymes are listed in Table 1.2.

Table 1.2: Mammalian ODC and AdoMetDC *versus P. falciparum* AdoMetDC/ODC
Compiled from (Krause *et al.*, 2000; Müller *et al.*, 2001; Wrenger *et al.*, 2001; Wells *et al.*, 2006).

Mammalian AdoMetDC, ODC	PfAdoMetDC/ODC
1. Separate monofunctional proteins	1. Arranged on bifunctional protein
2. Limited ODC inhibition by putrescine	2. PfODC more strongly feedback regulated by putrescine
3. AdoMetDC stimulated by putrescine	3. PfAdoMetDC not stimulated by putrescine
4. AdoMetDC inhibition by Tris	4. No PfAdoMetDC inhibition by Tris
5. Inserts absent	5. Presence of parasite-specific inserts
6. Half-lives between 15 and 35 min	6. Half-life of more than 2 hrs

The ability of substrate analogues to interfere with polyamine enzyme activity as well as their effects on parasite growth has been investigated. DFMO is a well-known enzyme-activated irreversible inhibitor of ODC and causes the alkylation of the enzyme's active site. Even though its effect on *P. falciparum* growth is only cytostatic, it has been successfully applied in the treatment of West African sleeping sickness caused by *T. brucei gambiense* (Assaraf *et al.*, 1984; Wang, 1995). The success rate in DFMO treatment of the latter infections may be attributed to several aspects: 1) rapid division of parasitic cells results in a higher polyamine requirement than in the host cells; 2) trypanosomes also use spermidine to produce trypanothione, which maintains the intracellular redox state (Fairlamb *et al.*, 1987); 3) the trypanosomal ODC is more stable and has a longer half-life than that of the host's (Phillips *et al.*, 1987); and 4) DFMO may be effectively transported into the trypanosomal parasites as the drug does not have to cross several membranes as is the case for the intracellular malaria parasites (Wrenger *et al.*, 2001).

The ODC inhibitor 3-aminooxy-1-aminopropane (APA) and its derivatives CGP52622A and CGP54169A as well as the AdoMetDC inhibitors CGP40215A and CGP48664A severely effect PfAdoMetDC/ODC activity and results in reduced intracellular polyamine concentrations (Das Gupta *et al.*, 2005). MDL73811 irreversibly inhibits PfAdoMetDC and is roughly a 1000-fold more effective than DFMO treatment (Wright *et al.*, 1990). Bitonti's group also showed that the bis(benzyl)-polyamine analogue, MDL27695, rapidly inhibits the *in vitro* growth of both chloroquine-sensitive and resistant *P. falciparum* strains, and if administered in combination with DFMO, cures malaria in *P. berghei*-infected mice (Bitonti *et al.*, 1989).

Treatment of *P. falciparum* with the potent PfSpdSyn inhibitor, dicyclohexylamine, completely arrests parasite growth of both chloroquine-sensitive and resistant strains (Kaiser *et al.*, 2001) and its derivative 4MCHA, results in up to 85% growth arrest within 48 hrs when used in micromolar quantities (Haider *et al.*, 2005).

Crystal structures and homology models of the polyamine metabolic enzymes are helpful to visualise the interaction between the inhibitor and the active site residues, providing a physical glimpse into a formerly unknown chemical space. These structures are particularly helpful in the identification and *in silico* testing of a specific set of lead chemical compounds, which would have been painstaking to test experimentally (Mehlin, 2005). Homology models provide an excellent alternative to obtaining structural information due to the challenges involved in expressing pure and sufficient amounts of *P. falciparum* proteins required for crystallisation studies. Homology models of three *P. falciparum* polyamine biosynthetic proteins have been solved and published, i.e. PfAdoMetDC (Wells *et al.*, 2006), PfODC (Birkholtz *et al.*, 2003) and PfSpdSyn (Burger *et al.*, 2007) [also crystallised, PDB entry 2HTE and (Dufe *et al.*, 2007)].

A structure for the PfAdoMetDC domain of the bifunctional PfAdoMetDC/ODC was produced by homology modeling, which revealed a number of novel properties. In comparison to the monofunctional human AdoMetDC, PfAdoMetDC undergoes more protein–protein interactions within the bifunctional arrangement. This more protein rich environment results in lower sequence identity to the template for those regions that are involved in these new interactions, which was found to correspond to the one face of PfAdoMetDC. The model showed that the replacement of a single residue within the active site of PfAdoMetDC is responsible for the lack of Tris inhibition. Basic or non-polar residues also replace certain residues and prevent any interaction with putrescine due to repulsion between these residues and the amine ends of putrescine. These differences in human and parasite active sites, as revealed with homology modeling, can be exploited in the design of potential parasite-specific inhibitors (Wells *et al.*, 2006).

A homology model has also been created for PfODC bound to DFMO, as well as the substrate analogues CGP52622A and CGP54169A. Possible interactions between these ligands and the PfODC active site residues were identified. The DFMO interacting site is conserved amongst different species, which explains its non-specific binding to ODC from different organisms. Both the substrate analogues interact with parasite-specific residues that are unique to PfODC explaining the competitive inhibition of PfODC without affecting the human counterpart. The model thus provides support for further studies aimed at exploring these compounds as possible antimalarial agents (Birkholtz *et al.*, 2003).

A PfSpdSyn homology model could also explain its effective inhibition with 4MCHA. This inhibitor was shown, with the help of molecular dynamics, to bind in the putrescine-binding cavity via its amine group while its hydrophobic region protrudes into an adjacent hydrophobic cavity. Information on inhibitor interactions obtained from the PfSpdSyn model is

useful for further research in the development of parasite-specific inhibitors based on structural information and different computational methods (Burger *et al.*, 2007). Recently, the crystal structure of PfSpdSyn in complex with dcAdoMet, 4MCHA and another inhibitor S-adenosyl-1,8-diamino-3-thio-octane (AdoDATO) became available. The structure with bound dcAdoMet showed that this substrate stabilises the active site gatekeeper as well as changes the active site conformation for the subsequent binding of the second substrate, putrescine. The mode of interactions with the inhibitors also revealed important binding sites that may be modified for the synthesis of future, more potent inhibitors (Dufe *et al.*, 2007).

Apart from the characteristics already mentioned here, there are unique properties of the rate-limiting bifunctional PfAdoMetDC/ODC that can be exploited during the design of antimalarial drugs as will be highlighted in the subsequent section.

1.4.4.1 The bifunctional PfAdoMetDC/ODC protein

The bifunctional PfAdoMetDC/ODC protein has a predicted molecular mass of ~330 kDa. The PfAdoMetDC domain is located at the N-terminus and consists of residues 1-529, the C-terminal PfODC domain consists of residues 805-1419 while the hinge region connecting these two domains spans residues 530-804 (Figure 1.12) (Müller *et al.*, 2000). PfAdoMetDC is post-translationally cleaved into two low (9 kDa) and two high (60 kDa) molecular weight subunits The PfAdoMetDC and PfODC domains combine to form an enzymatically active heterotetrameric complex, consisting of a PfODC homodimeric component and a heterotetrameric PfAdoMetDC component, two bifunctional heterodimers of 166 kDa each thus associate to form the active bifunctional heterotetrameric protein of ~330 kDa (Figure 1.12) (Müller *et al.*, 2000).

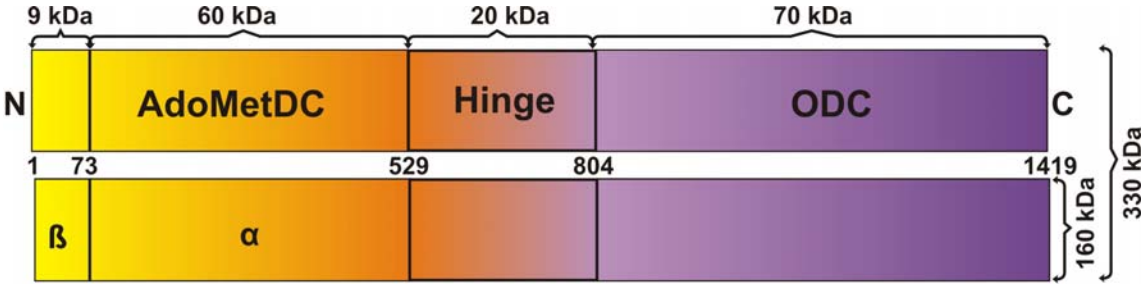


Figure 1.12: Schematic diagram of the bifunctional *P. falciparum* AdoMetDC/ODC protein. The N-terminal PfAdoMetDC domain consists of α and β chains and is connected to the C-terminal PfODC domain via a hinge region. The sizes of the individual domains, dimeric components and the heterotetrameric complex are also given (Müller *et al.*, 2000).

The individual domains participate in direct protein-protein interactions (Birkholtz *et al.*, 2004), while their active sites have been shown to function independently (Wrenger *et al.*, 2001). A possible explanation for the bifunctional arrangement could be that controlling the

abundance and activity of a single protein regulates polyamine biosynthesis (Müller *et al.*, 2001).

1.4.4.2 Ornithine decarboxylase

The vitamin B6-derived cofactor pyridoxal-5'-phosphate (PLP) is absolutely essential for the proper functioning of the ODC enzyme during the transimination of ornithine. Two active sites are formed at the interface of the homodimeric protein as a consequence of the dimerisation of the PfODC monomers and consist of residues that are donated by both of these. The homology model showed that the two PfODC monomers are arranged in a head-to-tail manner where the N-terminal of one monomer faces the C-terminal of the other and *vice versa* (Figure 1.13) (Birkholtz *et al.*, 2003).

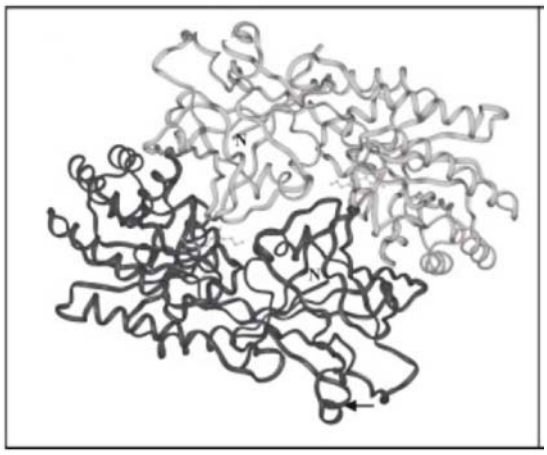


Figure 1.13: The head-to-tail organisation of the two monofunctional *P. falciparum* ODC monomers.
The two monomers are shown in different shades of grey and viewed from the bottom. Taken from (Birkholtz *et al.*, 2003).

The specific activity of PfODC for its substrate ornithine is 93.2 pmol/min/mg with a *K*m value of 42.4 µM (Müller *et al.*, 2000). The monofunctional homodimeric PfODC domain has a molecular mass of approximately 140 kDa (Birkholtz *et al.*, 2004). The expression and catalytic properties of two different recombinant constructs of PfODC have been investigated. The first construct is a recombinant PfODC protein (rPfODC) and the second one included 144 amino acids of the hinge region (rPfHinge-ODC). The *K*m value of rPfHinge-ODC for ornithine is approximately four times lower than that of rPfODC indicating that the hinge region is involved in substrate binding while the presence of the hinge region also increased the specific activity of the PfODC enzyme (Krause *et al.*, 2000).

The hinge region connecting the two domains of the bifunctional protein is involved in the folding of PfODC and causes a 10-fold increase in its specific activity. These results led to

further investigations of possible protein-protein interactions between the bifunctional domains (Krause *et al.*, 2000; Wrenger *et al.*, 2001). Domain-domain interactions have been reported to play a role in the Plasmodial bifunctional DHFR/TS where the catalytic activity of TS is dependent on its interaction with DHFR (Shallom *et al.*, 1999). These interactions are, however, not mirrored in PfAdoMetDC/ODC. The specific activities of the respective enzymes are not effected when the neighbouring enzyme is inhibited or when its substrate is omitted suggesting that the active sites of the two domain enzymes act independently (Wrenger *et al.*, 2001).

Several amino acids essential for catalytic activity (cofactor and DFMO binding) and dimerisation are conserved in the PfODC sequence even though the overall homology of PfAdoMetDC/ODC to the mammalian monofunctional counterparts is low (Müller *et al.*, 2000). Mammalian ODC is relatively unstable with a half-life of several minutes due to the action of antizyme and the presence of a C-terminal PEST region involved in the recruitment of the 26S proteasome (Russell, 1985; Hayashi and Murakami, 1995). ODC in *Crithidia fasciculata* (a non-pathogenic trypanosomatid) is also a metabolically unstable protein and is rapidly degraded in mammalian systems, whereas the closely related *Leishmania donovani* ODC is not. The degradation of *C. fasciculata* ODC in the mammalian systems was shown to be dependent on a functional 26S proteasome and, in contrast to the degradation of mammalian ODC, does not involve antizyme but instead appears to be associated with the poly-ubiquitination of the enzyme (Nasizadeh *et al.*, 2003; Persson *et al.*, 2003).

Closer inspection of the PfODC sequence also revealed a high abundance of Pro, Glu and Ser residues that may constitute a PEST sequence for degradative signalling. This, however, was thought not to be the case since PfODC has been shown to be relatively stable (Assaraf *et al.*, 1988). This theory is also supported by the differential host-parasite responses to the irreversible inhibitor, DFMO (Müller *et al.*, 2001).

Apart from the bifunctional nature of the PfAdoMetDC/ODC protein, PfODC also has another unique characteristic compared to the mammalian counterpart. This domain contains two parasite-specific inserts (namely O1 and O2) that have no homology to other proteins in other organisms (Birkholtz *et al.*, 2003) and are discussed in more detail in section 1.4.4.4.

1.4.4.3 S-adenosylmethionine decarboxylase

PfAdoMetDC activity is located on the N-terminus of the bifunctional PfAdoMetDC/ODC protein. This unique pyruvoyl-utilising decarboxylase has a *K*m value of 33.5 µM for its substrate AdoMet and a specific activity of 14.8 pmol/min/mg (Müller *et al.*, 2000). Experiments have shown that PfAdoMetDC lacks the regulatory mechanism proposed for

mammalian cells to relate putrescine abundance to spermidine synthesis as this enzyme is not stimulated by high levels of putrescine (Wrenger *et al.*, 2001; Wells *et al.*, 2006). The *P. falciparum* and eukaryotic AdoMetDC domains consist of an αββα-sandwich (Figure 1.14) (Wells *et al.*, 2006), which is formed from an autocatalytic cleavage event that produces the N-terminal β-chain (9 kDa) and the C-terminal α-chain (60 kDa). The dimerisation of PfAdoMetDC/ODC leads to the formation of the active site between the two β-sheets (Ekstrom *et al.*, 1999; Bennett *et al.*, 2002).

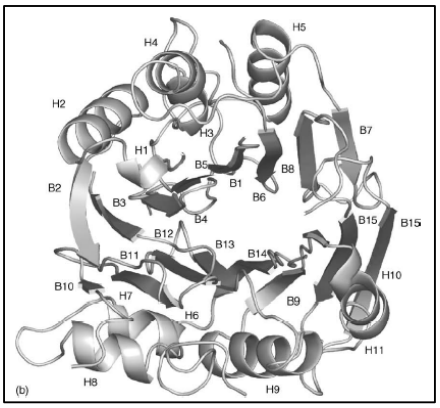


Figure 1.14: Diagram of the monofunctional *P. falciparum* AdoMetDC homology model showing the αββα structural arrangement.
Taken from (Wells *et al.*, 2006).

Unlike mammalian AdoMetDC, PfAdoMetDC is not inhibited by Tris, which may be due to four amino acid substitutions in the active site when compared to the mammalian counterpart. The lack of PfAdoMetDC stimulation with putrescine also seems to be due to the replacement of acidic residues with basic or non-polar residues (specifically Lys15 and Lys215) in the putrescine-binding site needed for the binding of the positive amine putrescine terminals. The positively charged PfAdoMetDC putrescine-binding pocket thus repels the positively charged putrescine molecules. The PfAdoMetDC domain also contains insertions that interrupt regions of homology to mammalian AdoMetDC (Wells *et al.*, 2006).

1.4.4.4 Parasite-specific inserts

An interesting feature of Plasmodial genomes, apart from the A+T richness, is the presence of rapidly diverging, species-specific, non-globular, possibly low-complexity containing regions that are either present in tandem repeats or sparsely distributed insertions, which are absent in orthologues. The insertions are mostly flexible, hydrophilic areas due to the presence of mainly Asn residues and therefore form loops that extrude from the protein core. The frequency of low-complexity regions (LCRs) in *P. falciparum* is particularly high and can be found in enzymes such as RNA polymerases (Bzik, 1991), glutamylcysteine synthetase (Lüersen *et al.*, 1998) and DNA topoisomerase (Cheesman *et al.*, 1994). The presence of

these regions in Plasmodial proteins raises questions about their origin and how they are maintained within the parasite genome (Pizzi and Frontali, 2000; Pizzi and Frontali, 2001). Compared to the size of the independent mammalian AdoMetDC and ODC enzymes, the PfAdoMetDC/ODC bifunctional protein size is much larger due to the presence of several parasite-specific inserts that interrupt sequence homology within both the domains (Birkholtz *et al.*, 2003; Birkholtz *et al.*, 2004; Wells *et al.*, 2006). The inserts range in size from 6 to 180 amino acids (Wells *et al.*, 2006). They have been shown to mediate physical interactions between the two domains by stabilising interdomain protein-protein interactions, thereby regulating the activity of both domains (Birkholtz *et al.*, 2004). The PfAdoMetDC domain contains inserts A1 (residues 57-63), A2 (residues 110-137) and A3 (residues 259-408) (Wells *et al.*, 2006). The hinge occupies residues 530-804 and is for all purposes also considered as an insert (Wells *et al.*, 2006), while the O1 (residues 1047-1085) and O2 (residues 1156-1302) inserts are present in the PfODC domain (Birkholtz *et al.*, 2003).

The O1 insert is particularly important for both the domain activities. Deletion of O1 reduces the PfAdoMetDC and PfODC activities by 77% and 94%, respectively (Birkholtz *et al.*, 2004). Furthermore when this 39 amino acid insert was deleted, the wild type PfAdoMetDC and the mutant PfODC domains failed to assemble into the heterotetrameric complex. The PfODC monomers also failed to dimerise, which could be due to a conformational change at the dimer interface upon deletion of the insert. The O1 insert is thus important for both inter- and intramolecular protein-protein interactions within the PfODC domain thereby facilitating bifunctional complex formation (Birkholtz *et al.*, 2004).

The O1 parasite-specific insert has considerable secondary structure. Original reports indicated the presence of four anti-parallel β-sheets but subsequent analysis improvements in the software now indicates the presence of a highly conserved α-helix. The insert appears to lie parallel to the protein core, looping out towards the C-terminus at the same side as the entrance to the active site (Birkholtz *et al.*, 2004). The insert is also flanked by mobile Gly residues (Gly1036-1038 and Gly1083) suggesting that insert O1 may be acting as a flexible gatekeeper for substrate entry into the active site pocket (Birkholtz *et al.*, 2004). Mutational analyses showed that the mobility of this insert is vital for the decarboxylase activities of both domains since the correct positioning of the α-helix may mediate physical contacts between the two domains (Roux, 2006). This may be communicated to the respective active sites via long-range interactions (Myers *et al.*, 2001). The smaller insert O1 is also better conserved between Plasmodial species (Birkholtz *et al.*, 2004).

The O2 insert forms no significant secondary structures and is more important for PfODC than for PfAdoMetDC activity since it is spatially removed from the N-terminus (Birkholtz *et*

al., 2004). However, this insert contains several (NND)x-repeats that could play an important role in the formation of the PfODC homodimer through the formation of a polar zipper (Perutz *et al.*, 1994; Birkholtz *et al.*, 2004; Roux, 2006). This is not unusual for a *P. falciparum* protein, as it has been shown that ~35% of these proteins contain amino acid repeats, with Asn being the most abundant (Singh *et al.*, 2004). However, deletion of this 23-residue region depleted the PfAdoMetDC activity by only 27% and the PfODC activity by 46% (Roux, 2006).

The 180 residue hinge region connects the PfAdoMetDC and PfODC regions (Müller *et al.*, 2000) and is involved in the conformational stability and the correct quaternary structure formation of the PfODC domain (Krause *et al.*, 2000). FPLC studies showed that the hinge stabilises the heterotetrameric bifunctional protein complex by mediating interdomain interactions between the two domains (Birkholtz *et al.*, 2004). Several secondary structures occur in this region, notably two α-helixes and a β-sheet (Roux, 2006). It is possible that the α-helices have an indirect effect on the catalytic activities of both enzyme domains due to contributions to interdomain interactions. Deletion of the β-sheet appeared to result in multiple conformations of the bifunctional enzyme, with variable catalytic activity, suggesting that this secondary structure is essential for the stabilisation of the entire protein (Roux, 2006).

Since the deletion mutagenesis studies done by Birkholtz *et al*. in 2004, the so-called A1 insert has been divided into inserts A2 and A3 when the homology model of PfAdoMetDC was solved (Birkholtz *et al.*, 2004; Wells *et al.*, 2006). This means that the activity depletion results of the A1 insert have been updated.

It is not yet known exactly how the domains of PfAdoMetDC/ODC interact and what specific residues play important roles in these interactions. The exact protein interaction areas need to be examined for their possible involvement in domain interactions and activities. In addition, the noteworthy effect that the deletion of the O1 parasite-specific insert has on the entire PfAdoMetDC/ODC protein indicates that this area may likely be important for protein-protein interactions involved in the dimerisation of PfODC. It is thus important to analyse the function of specific areas in this insert, which can then be used as a platform for the design and application of an interface interference peptide.

1.5 Research aims

This study aims to delineate functional areas within the PfAdoMetDC parasite-specific inserts as well as to determine the importance of the O1 insert to the bifunctional *P. falciparum* PfAdoMetDC/ODC. Three distinct studies were thus undertaken:

Chapter 2: An efficient method for the deletion of parasite-specific inserts in malarial genes. In this chapter, a novel inverse PCR method as particularly suitable for the deletion of large areas (>100 bp) in large malarial genes was investigated. The method successfully deleted the largest insert in the PfAdoMetDC domain.

Chapter 3: Delineation of structural features of the parasite-specific inserts in PfAdoMetDC. In this chapter, the roles of the parasite-specific inserts in the PfAdoMetDC domain as well as the secondary structures within these were investigated.

Chapter 4: Investigations of the conserved O1 parasite-specific insert. The role of the O1 parasite-specific insert in the dimerisation and activity of this domain was investigated. Specific synthetic peptides were subsequently designed and tested as possible inhibitors of protein activity via interference with protein-protein interactions.

Chapter 5: Concluding discussion

The results within this dissertation have been published and/or presented as follows:

Chapter 2: 1) Williams, M., A.I. Louw, L. Birkholtz (2007). "Deletion mutagenesis of large areas in *Plasmodium falciparum* genes: a comparative study." Malaria Journal **64**: 1-9

2) SASBMB XXth, University of Kwazulu-Natal, Pietermaritzburg, July 2006. Poster title: An improved method for the deletion of large areas in large genes. M. Williams, A.I. Louw, L. Birkholtz.

Chapter 3: 1) Molecular Cell Biology Group Symposium, University of Witwatersrand, Johannesburg, October 2006. Presentation title: Structure-function analysis of the O1 parasite-specific insert in the uniquely bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum*. M. Williams, A.I. Louw, L. Birkholtz.

2) Manuscript in preparation: Delineation of structural features within the parasite-specific inserts involved in enzymatic activities of the bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum*. Williams, M., S. Roux, G.A. Wells, L. Birkholtz, A.I. Louw.

Chapter 4: 1) Manuscript in preparation: A conserved parasite-specific insert influences the activities of the bifunctional S-adenosylmethionine decarboxylase/ornithine decarboxylase of *Plasmodium falciparum*. Williams, M., S. Roux, G.A. Wells, L. Birkholtz, A.I. Louw.