Chapter 1

Introduction

1.1.Malaria

The first decade of the 21st century has been met with many successes as well as disappointments in the area of malaria control. From a scientific research perspective the achievements have been extraordinary and include developments such as 1) the sequencing of the *Plasmodium falciparum* (causative parasite) [1] and *Anopheles gambiae* (insect vector) [2] genomes; which has resulted in 2) the development of vast, freely available databases such as PlasmoDB [3]; 3) the release of the transcriptomic [4-7], proteomic [8,9] and metabolomic [10] profiles of the intra-erythrocytic infectious stages of the parasite within the human host; and 4) the promising results of the RTS,S/AS vaccine against falciparum malaria, which is currently in phase III clinical trials [11]. In terms of vector control, the WHO has revised the use of DDT (bis(4-chlorophenyl)-1,1,1-trichloroethane) in 2006 as a means to control the transmission of malaria by mosquitoes (http://www.who.int/whopes/), despite the resistance met from environmental protection agencies [12]. The creation of transgenic mosquitoes has also received attention in the scientific community to reduce the capacity of parasites to infect humans [13].

The 2010 World Malaria Report (WHO 2010) stated that nearly 289 million insecticide-treated mosquito nets (ITNs) were delivered to sub-Saharan Africa between 2008 and 2010, which conferred malaria transmission protection to 578 million people, including children and pregnant women (http://www.who.int/malaria/world_malaria_report_2010/). In 2009, 75 million Africans were also protected by indoor residual spraying (IRS) and these preventative efforts have resulted in measurable effects on public health as follows: 1) the number of malaria cases decreased from 244 million in 2005 to 225 million in 2009 (~7%); 2) the number of deaths decreased from 985 000 in 2000 to 781 000 in 2009 (~20%); 3) the number of countries that have reduced their malaria burden by 50% over the past decade continues to rise resulting in fewer countries that are endemic for malaria; and 4) in 2009 not a single case of cerebral malaria was reported in the WHO European Region. The decrease in malaria deaths can be attributed to improved access to treatment, vector control measures and diagnostic testing, which is reflected in the fact that most cases of fever in Africa are no longer due to malaria infection and the availability of inexpensive, easy-to-use, quality-assured rapid diagnostic tests for this disease (WHO 2010). Despite these successes, malaria resurgence is still observed in some African countries and even though funding for malaria control has increased dramatically in recent years

(from $592 million in 2006 to over $1 billion in 2008, and $1.7 billion in 2009). The Roll Back Malaria Partnership estimates that $5.2-6 billion is required per annum in order to achieve the targets by 2015 that have been set by the Global Malaria Action Plan. Furthermore, the current global economic recession is likely to decrease aid as reflected by the 5-10% cut in the USA science and technology budget for 2011 and 2012, which makes malaria funding uncertain.

The chief disappointment with regards to malaria control remains the ongoing development of parasite resistance, which has rendered several antimalarial medicines ineffective especially in the parts of the world where malaria remains cataclysmic. The most dreadful being the resistance threats of the most promising and highly effective artemisinin derivatives, which was confirmed at the Cambodia-Thailand border in 2009 [14]. However, despite the observed changes in parasite sensitivity to artemisinins, ACT (artemisinin-based combination therapy) remains in effect and has been combined with efforts to limit the spread of resistant parasites. Another alarming event observed in the last decade was the inclusion of *P. knowlesi*, common in macaque monkeys, as the fifth species than can cause malaria in humans [15].

More than 40% of the world's population reside in areas where they are at risk of malaria transmission (Figure 1.1, upper panel). Most deaths due to malaria occur in Africa, which is also one of the poorest regions of the world (Figure 1.1, lower panel). The disease contributes to poor economic growth, which has a further negative impact on malaria treatment and prevention. 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, global warming, parasite resistance to antimalarial drugs and vector resistance to insecticides.

A number of promising antimalarial drug and vaccine discovery projects have been launched. This includes the Medicines for Malaria Venture (MMV, http://www.mmv.org/) funded by a number of organisations including the Bill and Melinda Gates Foundation (http://www.gatesfoundatiojn.org/) for the development of novel antimalarials. The identification of new drug targets for malaria chemotherapeutic development is an ongoing process and is dependent on the study of disease pathology, parasite invasion and immune defence strategies, parasite transmission as well as parasite growth and development.

Total Population

MOST AND FEWEST PEOPLE

Rank	Territory	Value	Rank	Territory	Value
1	China	1295	191	Saint Kitts & Nevis	42
2	India	1050	192	Monaco	34
3	United States	291	193	Liechtenstein	33
4	Indonesia	217	194	San Marino	27
5	Brazil	176	195	Palau	20
6	Pakistan	150	196	Cook Islands	18
7	Russian Federation	144	197	Nauru	13
8	Bangladesh	144	198	Tuvalu	10
9	Japan	128	199	Niue	2
10	Nigeria	121	200	Holy See	1

millions *thousands*

Malaria Cases

HIGHEST MALARIA RATES

Rank	Territory	Value	Rank	Territory	Value
1	Uganda	49	11	Ghana	17
2	Sao Tome and Principe	32	12	Guinea-Bissau	14
3	United Republic of Tanzania	30	13	Madagascar	13
4	Mozambique	28	16	Benin	12
5	Burundi	27	17	Burkina Faso	12
6	Liberia	24	18	Senegal	11
7	Malawi	24	19	Angola	11
8	Namibia	22	20	Guinea	11
9	Zambia	19	21	Rwanda	10
10	Solomon Islands	18	22	Zimbabwe	10

*malaria cases per 100 people, 2003**

Malaria Deaths

HIGHEST MALARIA DEATH RATES

Rank	Territory	Value	Rank	Territory	Value
1	Angola	86	14	Dem Republic Congo	32
2	Sao Tome and Principe	77	15	Mozambique	19
3	Malawi	59	16	Togo	16
4	Guinea-Bissau	56	17	Ghana	16
5	Namibia	55	18	Solomon Islands	14
6	Zambia	54	19	Swaziland	14
10	United Republic of Tanzania	39	20	Senegal	14
11	Burkina Faso	35	25	Central African Republic	13
12	Uganda	34	26	Chad	12
13	Rwanda	32	27	Mali	10

*malaria deaths per 100,000 people per year in 2003**

National Income

HIGHEST AND LOWEST GROSS NATIONAL INCOMES

Rank	Territory	Value	Rank	Territory	Value
1	Norway	44.0	190	Rwanda	0.22
2	Switzerland	41.5	192	Tajikistan	0.21
3	United States	37.8	193	Niger	0.21
4	Japan	34.2	194	Sierra Leone	0.17
5	Denmark	33.5	195	Malawi	0.15
6	Greenland	29.3	196	Guinea-Bissau	0.14
7	Bahamas	29.3	197	Liberia	0.12
8	Sweden	29.1	198	Democratic Republic of Congo	0.11
9	United Kingdom	28.4	199	Ethiopia	0.09
10	Ireland	27.7	200	Burundi	0.09

*Gross National Income in thousands of US$ per person per year, 2003**

Figure 1.1: The worldwide distribution of malaria and its association with economic growth. Maps were obtained from (http://www.worldmapper.org/). The world's population data is from 2002, malaria cases are from data reported between 2000 and 2003 and malaria deaths are from data reported between 1998 and 2003. Gross national income (all income and profits received in a territory) was derived from the World Bank's 2003 World Development Indicators given as data in USD using an average exchange rate over three years.

1.1.1. 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 four major species (and one minor, *P. knowlesi*) affecting humans including *P. falciparum* (the most severe form), *P. malariae*, *P. vivax* and *P. ovale* [16]. The parasites of the apicomplexan phylum have complex life cycles and are characterised by the presence of a special apical complex, which is involved in host-cell invasion and includes the microneme, dense granules and rhoptries (Figure 1.2) [17].

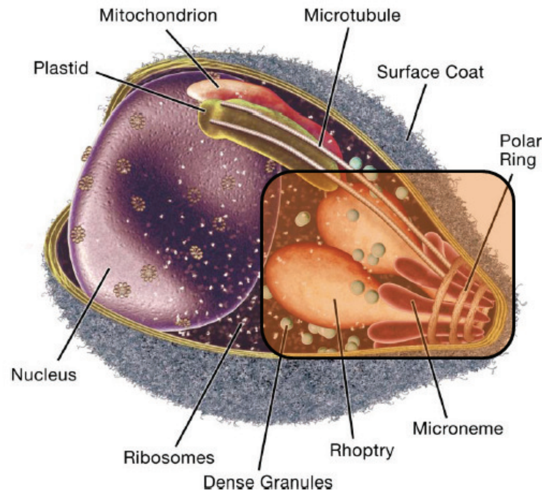


Figure 1.2: A *P. falciparum* merozoite showing the apical complex and other major cellular organelles and structures.
The apical complex is shaded. Adapted from [17].

P. falciparum invades host cells to acquire a rich source of nutrients and at the same time, these cells protect the parasites from host immune responses. The parasites are transmitted by the female *A. 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. During a blood meal the mosquitoes inject a sporozoite form of the parasites into the subcutaneous layer of the host skin. The sporozoites rapidly move to the liver where they infect the hepatocytes and differentiate into thousands of merozoites. *P. vivax* and *P. ovale* have a dormant stage which persists in the liver and cause relapses by invading the bloodstream sometime thereafter (Figure 1.3A). Merozoites 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, which is accompanied by the characteristic bursts of fever and anaemia associated with the disease, occurring every 24 hours. The daughter merozoites repeat the asexual cycle by invading free erythrocytes (Figure 1.3B) [18].

Some ring stage parasites 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 injected into the human host by the mosquito to repeat the parasitic life cycle resulting in its successful transmission (Figure 1.3C) [18].

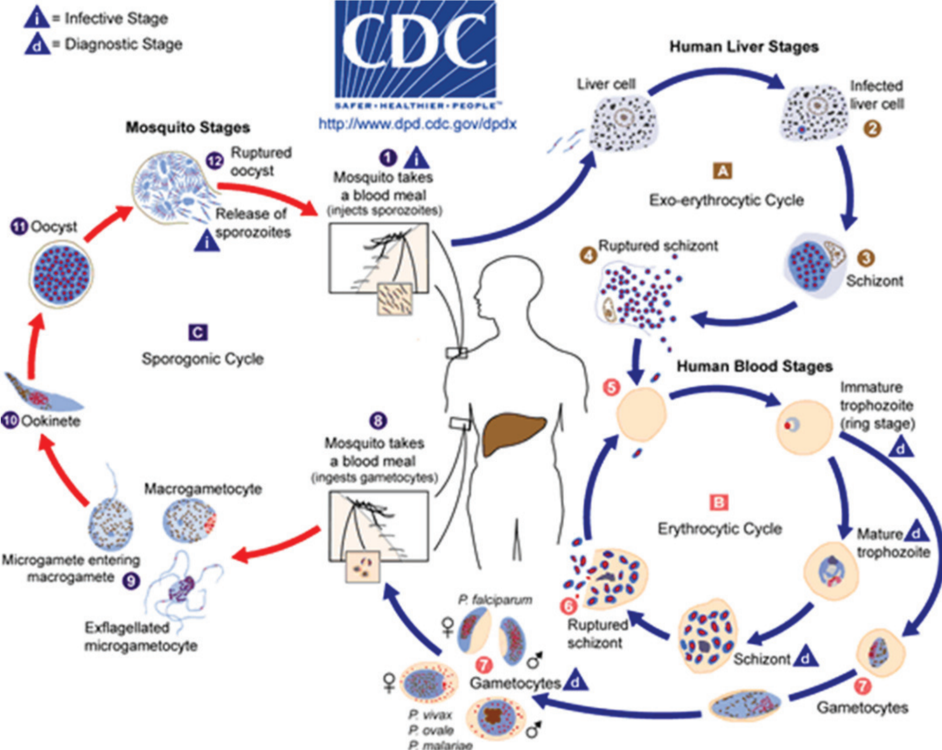


Figure 1.3: The asexual and sexual life cycles of the malaria parasite.
(1A) During a blood meal the malaria-infected female *Anopheles* mosquito injects sporozoites into the human host where they are transported to the liver cells (2A) and mature into schizonts (3A). The schizonts rupture and release merozoites (4A), which infect red blood cells (intra-erythrocytic asexual blood stage) (5B). The trophozoites mature into schizonts, which once again rupture to release merozoites (6B). Some parasites differentiate into sexual erythrocytic stages (7B) or gametocytes, which are ingested by another mosquito during a blood meal (8C). The male and female gametocytes fuse to form zygotes (9C), which differentiate into motile and elongated ookinetes (10C) that invade the midgut wall where they develop into oocysts (11C). These then grow and rupture to release sporozoites (12C), which move to the mosquito's salivary glands in order to be injected into a new human host during the next blood meal (1A). Obtained from http://www.cdc.gov/malaria/about/biology/.

1.2. Treating malaria

The areas where malaria prevalence is 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 therefore adhere to several pre-requisites such as oral bio-availability, since diseased individuals mostly do not have access to healthcare facilities, a short treatment period to reduce the risks associated with parasite resistance development and the drugs must be inexpensive with extended shelf lives [19].

1.2.1. Vector control

Strategies to reduce the prevalence of malaria include the use of ITNs and reduction of the vector population with IRS. DDT remains the most powerful and successful pesticide to date and is responsible for the eradication of malaria from both the North American and European continents. In South Africa, the discontinued use of DDT in the 1990s resulted in the worst

malaria epidemic this country has experienced since the introduction of IRS in the 1950s. The subsequent re-introduction of DDT spraying in 2000 once again resulted in an overall decrease in the number of malaria cases by approximately 50% [20]. 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 used extensively in agriculture during which enormous quantities were aerially sprayed onto crops to curb pests. This widespread and uncontrolled use of DDT raised concerns amongst environmentalists in the 1960s who described possible catastrophic consequences for both the environment and humans, ultimately leading to the ban of DDT in the 1980s [21]. However, the controlled use of DDT at the low concentrations required for malaria vector control [22] as well as the combined efforts of several public health officials and malaria experts, have resulted in the approval of restricted use of DDT for malaria control by the WHO Pesticide Evaluation Scheme (WHOPES) (http://www.who.int/whopes/).

Malaria parasite transmission can also be prevented by blockage of the sexual development of the parasites within the mosquito host. Coleman *et al*. tested the effect of 8-aminoquinolines on the sexual development of *P. berghei* and *P. falciparum* parasites in *A. stephensi* mosquitoes and showed that the drug-fed mosquitoes produced fewer oocysts than the control-fed group, and the sporozoites that did manage to develop from the oocysts could not enter the salivary glands [23]. The antifolate drugs proguanil and pyrimethamine have also been shown to be sporontocidal by causing a reduction in oocysts in drug sensitive strains while pyrimethamine directly damages ookinetes [24]. DL-α-difluoromethylornithine (DFMO), a polyamine pathway inhibitor, also interferes with *P. berghei* sporozoite development in *A. stephensi* mosquitoes [25].

A more recent development to control malaria transmission is the radical concept of rendering mosquitoes refractory to *Plasmodium* infection by creating transgenic mosquitoes. This can be obtained by either altering the lifespan of the female mosquitoes so that they cannot transmit the parasite or to introduce an agent into the mosquito that kills the parasite and thereafter becomes hereditary. Malaria transmitting mosquitoes are harmless for the first two weeks and only a small proportion of the female population actually live long enough to transmit parasites. Additionally, a problem that contributed to the rapid development of insecticide resistance was the instantaneous killing of the mosquitoes, which placed large resistance pressure on the mosquitoes to combat the insecticide. If the lifespan of the females could therefore be shortened by a few days, the transmission capacity would be reduced tremendously while the development of insecticide resistance would also be delayed [26]. Transgenic mosquitoes can be created by

using an antimalarial fungus, such as *Metarhizium anisopliae* that naturally infects mosquitoes, and inserting a gene for e.g. a human antibody into it, which is then transferred to the mosquito during the fungal infection. The mosquitoes are then sprayed with the transgenic fungus soon after being infected by the malaria parasite [27]. In addition, it has also been shown that fungus infection actually increases the susceptibility of resistant mosquitoes to the insecticide for which they have developed resistance [28].

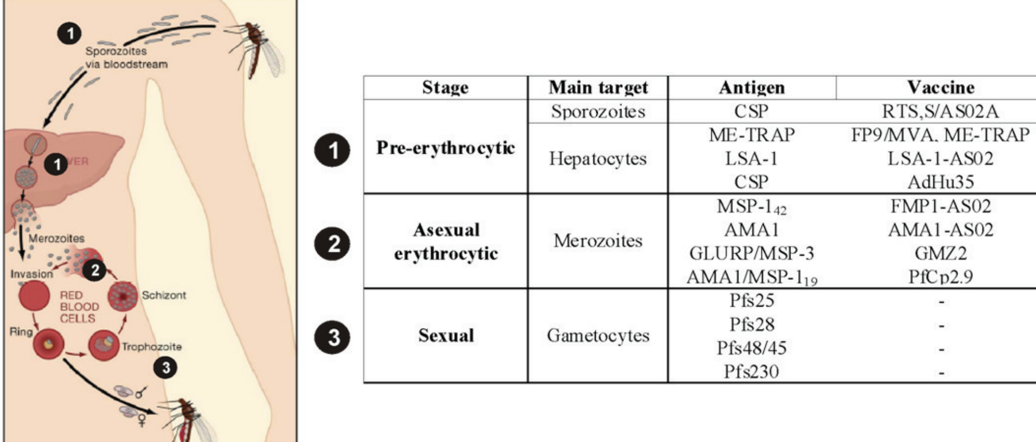
1.2.2. Vaccine development

Some malaria experts are of the opinion that vaccination represents the most valuable strategy to reduce the mortality associated with malaria [29]. This is due to the fact that people residing in malaria endemic areas do eventually develop low levels of protective immunity against *P. falciparum* infection 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 [30].

The complex life cycle of the malaria parasite, which allows it to co-exist with the host immune response, is largely responsible for the lack of a successful vaccine [31]. Current vaccine development strategies focus on different protein antigens that are expressed during particular 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 1.4) [31]. An ideal vaccine against plasmodial infection should therefore induce a multistage, multivalent and multi-immune response for it to be successful in the treatment of malaria [32].

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/AS malaria vaccine (GlaxoSmithKline Biologicals) in African infants showed that the vaccine is safe, well-tolerated and reduces parasite infection and clinical illness related to malaria. The vaccine consists of two polypeptides; RTS corresponds to CSP residues 207-395 of *P. falciparum* 3D7 fused to the N-terminus of the hepatitis B surface antigen (HBsAg) and S consists of 226 residues of HBsAg [33]. Testing of the vaccine in Phase II, or mid-stage, clinical trials showed a 53% reduction of clinical malarial episodes in young children administered over a period of eight months. A

success rate of 80% is expected and combined with vector control strategies and antimalarials the vaccine is predicted to be extremely effective in reducing malaria infections. Currently, the vaccine has entered pivotal Phase III trials and, if approved, is expected to be available by 2015 [11].



	Stage	Main target	Antigen	Vaccine
1	Pre-erythrocytic	Sporozoites	CSP	RTS,S/AS02A
		Hepatocytes	ME-TRAP LSA-1 CSP	FP9/MVA, ME-TRAP LSA-1-AS02 AdHu35
2	Asexual erythrocytic	Merozoites	$MSP\text{-}1_{42}$ AMA1 GLURP/MSP-3 $AMA1/MSP\text{-}1_{19}$	FMP1-AS02 AMA1-AS02 GMZ2 PfCp2.9
3	Sexual	Gametocytes	Pfs25 Pfs28 Pfs48/45 Pfs230	- - - -

Figure 1.4: 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 [12,31]. Abbreviations: AdHu35, human adenovirus serotype 35; AMA-1, apical membrane antigen 1; CSP, circumsporozoite protein; FMP-1, *falciparum* merozoite protein-1; FP, fowl pox; GLURP, glutamine-rich protein; LSA-1, liver stage antigen 1; ME-TRAP, multi-epitope thrombospondin-related adhesive protein; MSP, merozoite surface protein; MVA, modified vaccinia virus Ankara; PfCp2.9, *P. falciparum* chimeric protein 2.9; Pfs, *P. falciparum* surface antigens. Figure adapted from [17].

Extensive research is also being conducted on antibodies raised to antigens on the erythrocyte plasma membrane (e.g. *P. falciparum* erythrocyte membrane protein 1, *Pf*EMP1) as this would result in the destruction of the infected erythrocyte or prevent the cytoadherence of these infected cells [32,33] . Blood-stage vaccines are, however, limited by the polymorphic character of the antigens, which creates diversity and restricts the efficacy of the vaccine representative of a particular genotype [34].

1.2.3. Current antimalarials

Various drugs have been developed and used in the fight against malaria. As with malaria 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. Figure 1.5 shows the different stages of the parasite life cycle and current drugs that specifically target these stages of parasite development. Eradication of malaria with the use of antimalarials is continuously compromised by the increased prevalence of parasite resistance to the small number of available commercial drugs.

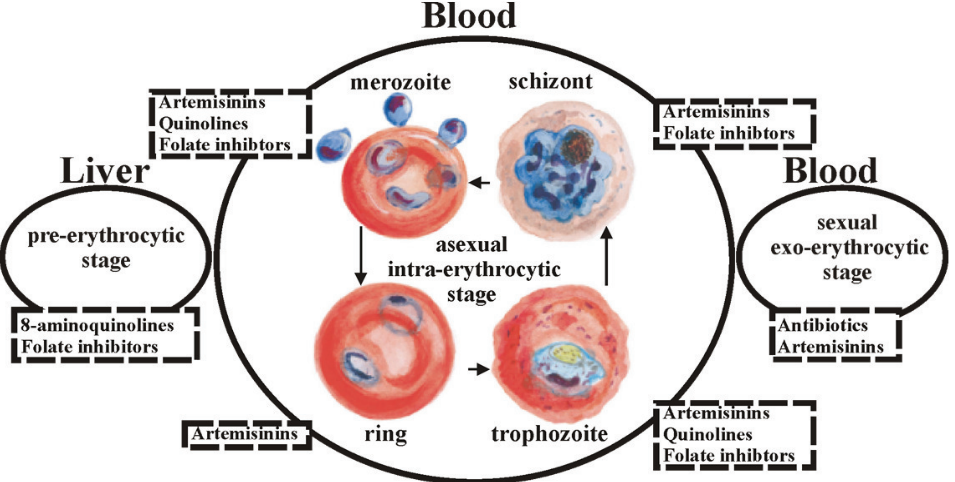


Figure 1.5: A schematic diagram of the *P. falciparum* life cycle within the human host showing the targets of different antimalarials during the developmental stages.
The pre-erythrocytic, asexual intra-erythrocytic and sexual exo-erythrocytic stages as well as the different intra-erythrocytic phases of malaria parasite development are shown. Examples of drugs that have been used at each stage are listed in the dashed boxes [35-37].

1.2.3.1. Quinolines

The bark of the Cinchona tree has been used for centuries to treat fever associated with malaria from which the active ingredient is quinine [38]. It remained the antimalarial of choice until the 1940s, where after it was replaced by the chloroquine derivative. Quinine is, however, still used today to treat clinical malaria. Chloroquine is a 4-aminoquinoline derivative of quinine and for many years it was the main antimalarial drug used in malaria treatment caused by *P. falciparum* until parasite resistance developed in the 1950s (Table 1.1). However, it remains the most popular antimalarial developed to date due to its safety, low cost and efficacy [39,40]. Currently, the widespread resistance to the drug has rendered its use as a therapeutic agent useless, but it is still used to treat falciparum malaria in certain critical situations and shows some efficacy against the other *Plasmodium* spp (WHO 2010) [41].

Despite more than three decades of research, the exact molecular mechanism of chloroquine action remains controversial. It is believed that the weak-base drug accumulates in the acidic food vacuole of the parasite where it prevents haem detoxification [43]. 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 [43-45]. Two genes have been implicated in this resistance, namely *Pfmdr1* and *Pfcrt*, which encode P-glycoprotein homologue 1 (Pgh1) and *P. falciparum* chloroquine transporter (*Pf*CRT), respectively [45,46]. 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 chloroquine accumulation [47]. Alternatively, *Pf*CRT may increase the efflux of

chloroquine by directly interacting with the drug [48]. Resistance is associated with several mutations in the *Pf*CRT protein, while the loss of Lys76 has been shown as the critical mutation that renders the *P. falciparum* parasites resistant to the drug [49].

Table 1.1: Antimalarial drug classes

Stage	Drug class	Drug compounds	Mechanism of action
Pre-erythrocytic	Aminoquinolines	Primaquine (and gametocytocidal)	Unknown
	Hydroxynaphthoquinone	Atovaquone (and sporontocidal)	Interferes with cytochrome electron transport
Asexual intra-erythrocytic	Aminoquinolines	Chloroquine (and gametocytocidal) Quinine (and gametocytocidal)	Inhibits haem detoxification
	Sulphonamides	Sulphadoxine	Inhibits DHPS
	Sulphones	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 sulphadoxine or dapsone)
	4-Methanolquinoline	Mefloquine	Inhibits haem detoxification
	Sesquiterpene lactone	Artemisinin and derivatives (and gametocytocidal)	Unknown
Exo-erythrocytic	Antibiotics	Tetracycline (and active against intra-erythrocytic forms) Doxycycline (and active against intra-erythrocytic forms)	Inhibitors of aminoacyl-tRNA binding during protein synthesis

Abbreviations: DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase. Compiled from WHO 2005 and [35,37].

A number of related aminoquinolines have been developed (Table 1.1) and are clinically applied including: Amodiaquine, Atovaquone (used in combination with proguanil, Malarone®), Lumefantrine (highly effective against multi-drug resistant *P. falciparum* when co-formulated with artemether, Co-Artem™), Halofantrine (Halfan), Mefloquine (Lariam®), and Primaquine (WHO 2005 and [42]). Mutations in the *Pfmdr1* gene have also been associated with resistance to these derivatives including quinine, Mefloquine and Halofantrine [43].

1.2.3.2. Antifolates

The antifolates are some of the most widely used antimalarials but their role in malaria prevention is increasingly 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 serine, methionine and pyrimidines, which leads to decreased DNA synthesis (Table 1.1) [37].

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 sulphonamides (sulphadoxine) and sulphones (dapsone), while the type-2 antifolates (pyrimethamine and cycloguanil, the active metabolite of the prodrug proguanil) inhibit dihydrofolate reductase (DHFR) (Table 1.1) [37]. Interestingly both of these classes of target proteins are arranged on separate bifunctional enzymes; hydroxymethyldihydropterin pyrophosphokinase/DHPS (PPPK/DHPS) and DHFR/thymidylate synthase (DHFR/TS) [44]. In addition, malaria parasites are capable of *in vivo* folate salvage from the extracellular environment as well as *de novo* synthesis of folate derivatives from simple precursors. The mechanism of exogenous folate uptake by a carrier-mediated process has important implications in the sensitivity of the antifolate inhibitors and is being investigated as a novel drug target [45].

Pyrimethamine is a diaminopyrimidine and is mostly used in combination with sulphadoxine (Fansidar™) or dapsone leading to the simultaneous inhibition of DHFR and DHPS (Table 1.1). Pyrimethamine crosses the blood-brain barrier and the placenta. Resistance to sulphadoxine-pyrimethamine combination therapy emerged rapidly due to the appearance of point mutations in the active sites of the target enzymes resulting in reduced drug binding capacity [46,47]. The Ser108 (AGC) to Asn (AAC) mutation is present in all pyrimethamine-resistant parasites and mutations of Asn51 to Ile, Cys59 to Arg and Ile164 to Leu confer additional resistance [48-50]. In addition, the DHFR and TS activities were found to be up-regulated upon challenge with antifolate drugs, independent of the mutational status of the gene [51]. Quantitative trait locus analysis on the rodent parasite *P. chabaudi*, of a genetic cross between clones with different resistance patterns to pyrimethamine, sulphadoxine and a combination thereof also showed the influence of one or more genes other than *dhfr* and *dhps* on the observed levels of resistance in the cross progeny [52]. A new combination of antifolates, chlorproguanil and dapsone (LapDap™), with shorter half-lives than pyrimethamine and sulphadoxine, were subsequently investigated as a means to delay drug resistance and was shown to clear Fansidar™-resistant parasites [53], but was later discontinued (see below) [54].

1.2.3.3. Artemisinins

Artemisinin is a sesquiterpene lactone extracted from the leaves of *Artemisia annua* and is a

potent, fast acting blood schizontocide that shows efficacy against all *Plasmodium* spp. Its efficacy is especially broad and shows activity against all the asexual stages of the parasites including the gametocytes, which results in reduced transmission potential (Figure 1.5) [55]. The exact mechanism of action of artemisinin remains vague and different studies have produced contradicting results (reviewed in [56,57]). Evidence to suggest that the primary activator of artemisinin is an iron source and protein alkylation due to artemisinin treatment is well established but a single molecular target that has a direct role in cell death due to artemisinin has not been identified. The multi-faceted nature of the plasmodial cellular response to artemisinin may explain the use of this drug against multi-drug resistant strains of *P. falciparum* and its effect on practically all stages of the parasite life cycle (Figure 1.5) [56].

The low aqueous solubility of artemisinin resulting in poor absorption upon oral administration has led to the development of several artemisinin derivatives including dihydroartemisinin, artesunate and artemether [58]. Despite the appearance of artemisinin resistance [14], the WHO still recommends ACTs as the first-line treatment against malaria infections where resistance to other antimalarials is prevalent (WHO 2010). One of the obvious disadvantages of using ACT for malaria case management in Africa is the increased costs involved in combining therapies, but several reasons exist for combining antimalarials with an artemisinin derivative, namely: 1) the increase in the efficacy of the antimalarials involved; 2) the decrease in the duration of treatment; and 3) the reduced risk of resistant parasites arising through mutation [59].

Originally, the appearance of parasite resistance to artemisinin was thought to be unlikely or at least delayed for several reasons, including 1) the short exposure of the parasites to the drug due its short half-life; 2) the gametocytocidal effect of artemisinin, which reduces the transmission potential and therefore spread of the parasite; and 3) the frequent use of ACTs was specifically introduced to delay the onset of resistance [60]. The appearance of artemether resistance in field isolates from French Guiana in 2005 resulted in increased inhibitory concentrations and was attributed to inappropriate drug use that exerted selection pressures, favouring the emergence of parasites with an artemether-resistant *in vitro* profile [61]. 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 [61]. Lapdap™, a combination of chlorproguanil (targeting DHFR), dapsone (targeting DHPS) and the artemisinin derivative artesunate (Table 1.1), was introduced in 2003 as malaria therapeutic to replace sulphadoxine-pyrimethamine treatment in Africa [62]. However, resistance to artesunate monotherapy appeared on the Thai-Cambodian border in 2009 and it was also discontinued due

to significant haemoglobin reductions in patients with glucose 6-phosphate dehydrogenase (G6PD) deficiency [63].

Currently the WHO recommends the following ACTs for malaria treatment, which should be combined with a single dose of primaquine as gametocytocidal (provided the risks of haemolysis in patients with G6PD deficiency have been established) and should be combined with knowledge on the efficacy of the specific combination therapy in the area of use: 1) artemether + lumefantrine (Co-Artem™); 2) artesunate + amodiaquine (ASAQ); 3) artesunate + mefloquine; 4) artesunate + sulphadoxine-pyrimethamine; and 5) dihydroartemisinin + the quinoline-based drug piperaquine (Artekin™) (WHO 2010 and [64]).

1.2.3.4. 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. Tetracycline was originally derived from *Streptomyces* species, but is now synthetically prepared. They interfere with aminoacyl-tRNA binding and therefore inhibit protein synthesis in the parasite's apicoplast and additionally have been shown to block apicoplast genome replication [65]. This is due to the presence of a genome in the apicoplast that encodes prokaryote-like ribosomal RNAs, tRNAs and various proteins [66]. Doxycycline is a synthetic tetracycline derivative with a longer half-life than tetracycline, but shows a disadvantageous property in that it causes photosensitivity, which is an obvious drawback for tourists entering malaria areas (WHO 2005).

1.3.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. The identification of novel drug targets that can 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 essential metabolic pathways of the host and parasite, which 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 in possible chemotherapeutic intervention strategies.

1.3.1. Polyamine biosynthesis as a drug target

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 extensive research has resulted in major advances in our understanding of polyamine biosynthesis in the malaria-causing parasite. Previous studies have shown that interruption of polyamine biosynthesis hampers the development of disease-causing *Trypanosoma brucei gambiense* and *P. falciparum* parasites [67,68]. Further studies have identified unique parasite-specific properties in the *P. falciparum* polyamine pathway, which present possible targets for chemotherapeutic intervention [69-71]. A sensible approach is thus the structural and functional characterisation of the pathway's constituent enzymes for rational drug development strategies. The polyamine biosynthesis pathway as drug target in *P. falciparum* will thus be the main focus of this study.

1.3.2. Polyamines

The physiologically important polyamines putrescine, spermidine and spermine are found in all living organisms except the Methano- and Halobacteriales [72]. The widespread prevalence of these polyamines signifies its considerable contribution to the survival of living cells and as such they have been implicated in many growth processes such as cell differentiation and proliferation [73-76]. This is reflected by the general abundance of polyamines and increased activities of its biosynthetic enzymes during stages of rapid growth [67,77]. Polyamine levels are thus controlled by tight regulation of its synthesis, degradation, uptake and secretion as their depletion may lead to growth arrest and aberrant embryonic development while their accumulation may cause apoptosis [78-80].

Electrostatic associations between polyamines and DNA result in the stabilisation of these nucleic acids, which often promotes DNA bending and facilitates binding of gene regulatory elements thereby indirectly influencing DNA transcription [81-84]. Polyamines additionally influence transcription by modifying chromatin structure via the stimulation of histone acetyltransferase [85]. One of the most unique post-translational protein modifications is the spermidine-dependent hypusinilation of eukaryotic translation initiation factor (eIF-5A) of which the function is not entirely understood but it appears to be essential for cell proliferation since its depletion arrests yeast cells in the G1 stage of the cell cycle [86,87]. Cells therefore maintain optimal levels of polyamines as they play paradoxical roles in the prevention as well as in the stimulation of cell death; increased levels protect cells by steering them into the proliferative

pathway and away from cell death [88]. However, the accumulation of excess intracellular putrescine has been shown to trigger apoptosis possibly as a result of an imbalance in intracellular positive and negative charges as well as decreased formation of modified eIF-5A [80,89].

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 [90-92]. The limited success, however, in finding an anti-tumour drug specifically targeting the polyamine pathway in humans has opened new possibilities in finding a drug against rapidly proliferating parasites [93] such as *P. falciparum* (malaria), *T. brucei* (African trypanosomiasis), *T. cruzi* (Chagas' disease) and *Leishmania donovani* (leishmaniasis) [91,92]. An overview of the polyamine biosynthetic pathways within these organisms as compared to the human host is shown in Figure 1.6 (Birkholtz *et al*., Biochemical Journal, in press).

Polyamines are synthesised via the decarboxylation of L-ornithine to putrescine by the enzyme ornithine decarboxylase (ODC). This enzyme catalyses the first and rate-limiting step of the polyamine biosynthetic pathway and an increased growth rate of rapidly proliferating cells is observed when this enzyme is over-expressed [84,101]. The diamine putrescine then acts as the precursor of spermidine and spermine synthesis. Another decarboxylation enzyme, *S*-adenosylmethionine decarboxylase (AdoMetDC), synthesises decarboxylated *S*-adenosyl-L-methionine (dcAdoMet), which serves as a donor of aminopropyl moieties to putrescine for the synthesis of spermidine and spermine (Figure 1.6). The latter reactions are catalysed by spermidine synthase (SpdS) and spermine synthase (SpmS), respectively [77].

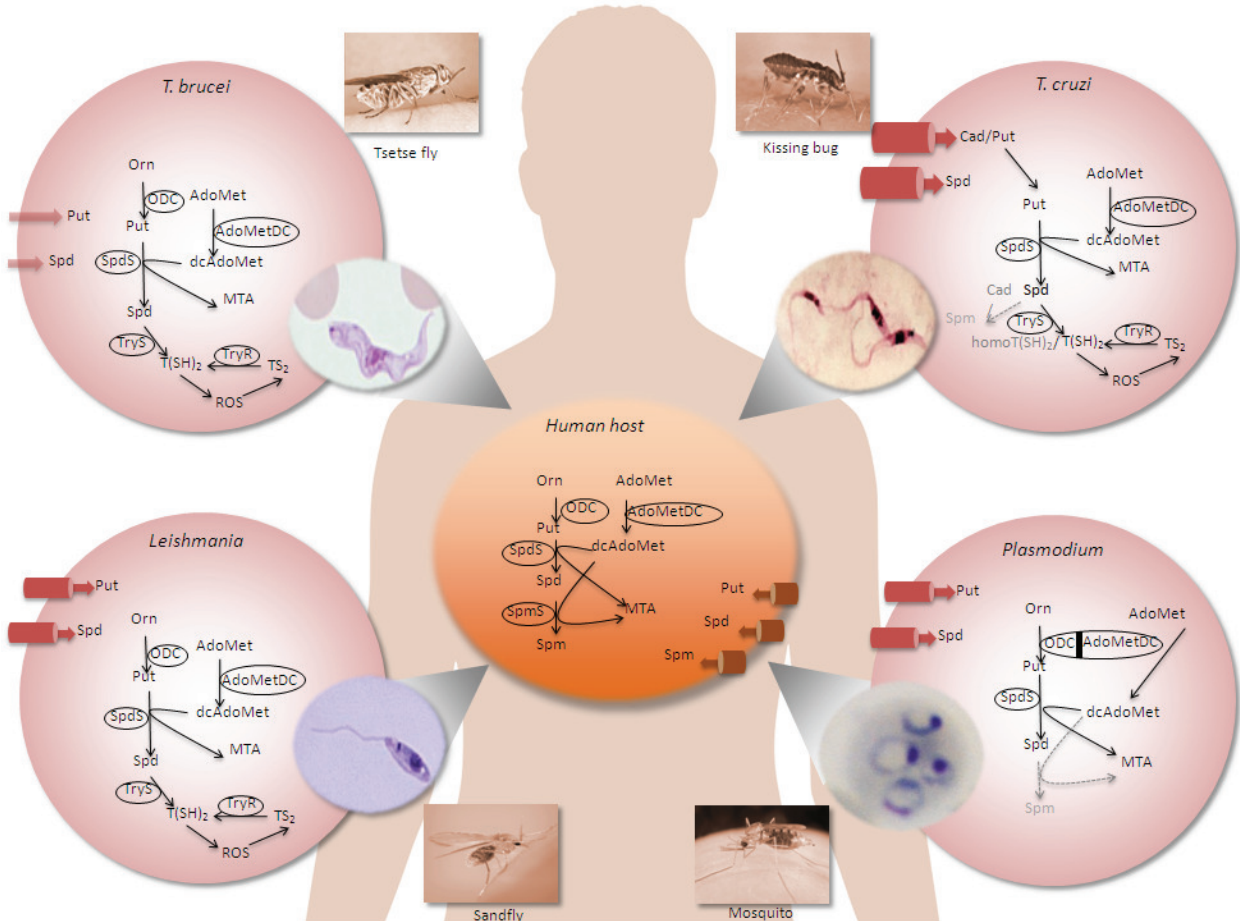


Figure 1.6: Polyamine biosynthetic pathways of various parasites compared with that of the human host. The parasites and their vectors are shown. *T. brucei* is transmitted by tsetse flies while *T. cruzi* is transmitted by kissing bugs resulting in sleeping sickness and Chagas' disease within the human host, respectively. *Leishmania* spp are transmitted by sand flies and malaria-causing *Plasmodium* parasites are transmitted by *Anopheles* mosquitoes. Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, AdoMet decarboxylase; cad, cadaverine; dcAdoMet, decarboxylated AdoMet; homoT(SH)$_2$, homotrypanothione; MTA, 5'-methylthioadenosine; ODC, L-ornithine decarboxylase; put, putrescine; ROS, reactive oxygen species; spd, spermidine; SpdS, spermidine synthase; spm, spermine; TryS, trypanothione synthetase; TryR, trypanothione reductase; TS$_2$, oxidised trypanothione; T(SH)$_2$, reduced trypanothione. Taken from Birkholtz *et al.* (Biochemical Journal, in press).

Mammalian cells can also interconvert polyamines for the production of spermidine from spermine and putrescine from spermidine, which is successively catabolised by spermidine/spermine-N^1-acetyltransferase and polyamine oxidase [94]. *T. brucei* and other trypanosomatids are uniquely capable of synthesising a conjugate between glutathione and spermidine called trypanothione [N^1,N^8-bis(glutathionyl)spermidine] by trypanothione synthetase, which is involved in the parasite's redox metabolism (Figure 1.6) [95]. *T. cruzi* lacks ODC and is therefore auxotrophic for putrescine, which is taken up from the host and converted into spermidine by AdoMetDC and SpdS [96]. Furthermore, similar to *Thermotoga maritima* SpdS, it appears that *T. cruzi* SpdS activity may be promiscuous since the active site can accommodate both putrescine and spermidine to synthesise spermidine and spermine, respectively [97]. *Leishmania* parasites possess a complete intact polyamine biosynthetic

pathway and are capable of synthesising putrescine and spermidine as well as trypanothione for redox control. As in prokaryotes, SpmS is absent in *Trypanosoma* spp, *L. donovani* and *P. falciparum* (Figure 1.6) [98,99].

1.3.3. Polyamine metabolism in *P. falciparum*

Human erythrocytes contain trace amounts of polyamines and lack the necessary enzymes for active polyamine biosynthesis. However, in *P. falciparum*-infected erythrocytes there is a significant increase in polyamine levels during the trophozoite and schizonts stages of parasitic infection, with large variation in the spermidine and to a lesser extent putrescine levels. In contrast, it was found that spermine levels are only slightly elevated in the parasitised cells (Figure 1.7). In general, polyamine synthesis increases from the ring to the schizont stages during intra-erythrocytic parasite infection with spermidine being the major polyamine present at all stages. These increases in polyamine levels were found to be proportional to the parasitaemia, the activities of the polyamine biosynthetic enzymes as well as the biosynthetic activities of the parasite such as macromolecular synthesis and replication (Figure 1.7) [67,100].

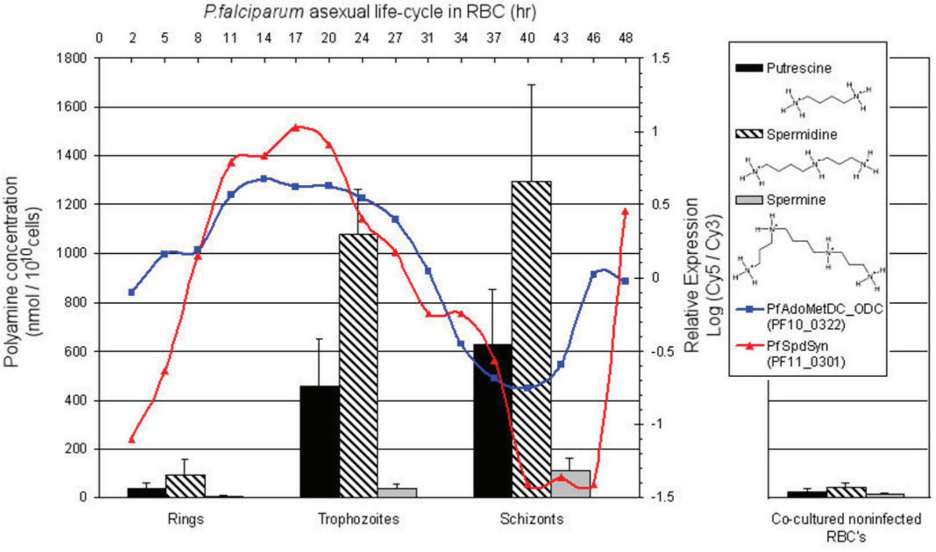


Figure 1.7: Polyamine levels during the intra-erythrocytic developmental cycle of *P. falciparum*.
The levels of the three polyamines (structures on the right) are shown together with the transcript abundance of the polyamine biosynthetic genes (*PfAdometdc/Odc* and *PfSpds*) [4] during the asexual intra-erythrocytic stages of *P. falciparum*. The polyamine levels within uninfected erythrocytes are also shown. Taken from [101].

The *P. falciparum* parasite polyamine pathway is distinctly different from that of the human host, which means that interference with the parasite's polyamine biosynthetic pathway could have more severe consequences on the parasite than its host [92]. Obvious differences between the pathways and the main polyamine biosynthetic enzymes between the two organisms are highlighted in Figure 1.8.

In *P. falciparum*, a single open reading frame encoding a bifunctional protein with both *Pf*AdoMetDC and *Pf*ODC activities uniquely facilitates polyamine synthesis [70]. In contrast to the short half-lives (~15 min) of the monofunctional mammalian AdoMetDC and ODC enzymes, *Pf*AdoMetDC/ODC has a half-life of more than two hours [92]. The short half-live of human ODC is due to the polyamine-dependant effect of antizyme and recruitment of the 26S proteasome [77,102]. While mammalian ODC is barely inhibited by putrescine, *Pf*ODC activity is susceptible to feedback inhibition by putrescine [103] and *Pf*AdoMetDC activity is not stimulated by putrescine [71]. In contrast to the mammalian pathway, the SpmS enzyme [98] and a retro-conversion pathway [92] have not been identified in *P. falciparum*. In the absence of SpmS, *Pf*SpdS has been shown to be capable of synthesising low levels of spermine [98]. Mammalian cells are not only capable of synthesising and interconverting polyamines, but can also take up polyamines from their environment via a poorly understood transport system [104]. These differences may provide possible drug target development opportunities for the treatment of parasitic infectious diseases.

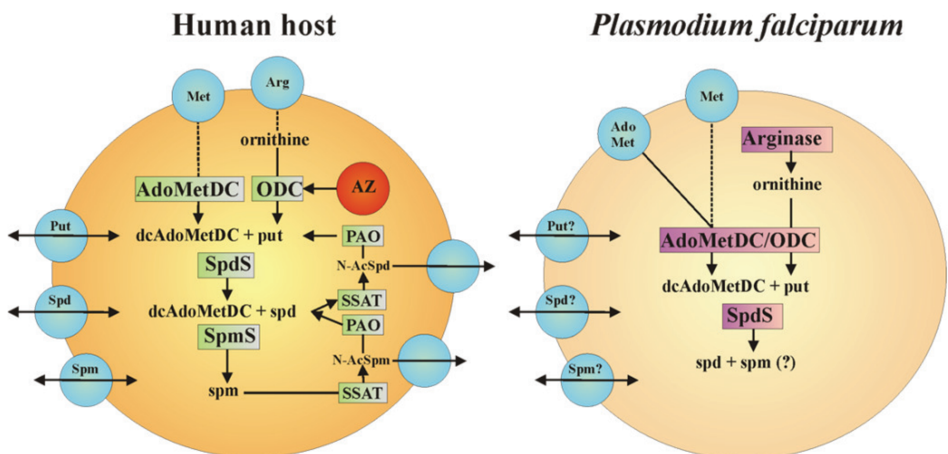


Figure 1.8: Summary of the polyamine metabolic pathways in the human host and *P. falciparum* parasite. Transporters or channels are shown as blue circles. Intermediates and reaction products are written in plain text while the enzymes producing these are given in green (human host) and purple (parasite) boxes. Abbreviations: AdoMet, *S*-adenosyl-L-methionine; AdoMetDC, AdoMet decarboxylase; Arg, arginine; AZ, antizyme; dcAdoMet, decarboxylated AdoMet; Met, methionine; *N*-AcSpd and *N*-AcSpm, N^1-acetylated spermidine and spermine; ODC, ornithine decarboxylase; PAO, polyamine oxidase; put, putrescine; spd, spermidine; SpdS, spermidine synthase; spm, spermine; SpmS, spermine synthase; SSAT, spermidine/spermine-N^1-acetyltransferase. Adapted from [92].

1.3.4. Polyamine transport in *P. falciparum*

The presence of a specific polyamine transport system in malaria parasite-infected erythrocytes remains a controversial subject but evidence has suggested their presence based on three specific observations: 1) 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 [105,106]; 2) evidence 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 [107,108]; and 3) the exogenous addition of putrescine rescues DFMO-treated *P. falciparum* cultures, suggesting that the parasites are able to internalise and metabolise putrescine for growth and macromolecular synthesis [67,109].

To date, the only polyamine transporter that has been characterised in plasmodia is the *P. knowlesi*-induced putrescine-specific transporter [108], which was shown to be temperature-dependent and competed for by both spermidine and spermine. Haider *et al*. showed that parasites treated with the *Pf*SpdS inhibitor, *trans*-4-methylcyclohexylamine (4MCHA), could not be rescued with the exogenous addition of spermidine, which indicated inefficient uptake of this polyamine by the infected erythrocytes and an apparent absence of a spermidine-specific transporter in *P. falciparum*-infected erythrocytes [98]. However, since the exact targets of this inhibitor are unknown it is possible that additional sites may be affected in *P. falciparum* and thereby prevented parasite rescue [98]. *Pf*AdoMetDC inhibition could also not be rescued with the addition of putrescine or spermidine while the effects of *Pf*ODC inhibition with DFMO could be reversed by putrescine supplementation, suggesting the presence of a putrescine transporter system [100].

In a recent study it was shown that both putrescine and spermidine are indeed taken up across the membrane of viable isolated parasites with a saturable, temperature-dependent process that competed for different polyamines, L-ornithine and other basic amino acids [110]. Further inhibition of polyamine biosynthesis in the isolated parasites resulted in an increased uptake of these polyamines while the rate of uptake was shown to be independent of extracellular Na^+ and K^+. However, uptake was shown to be dependent on the extracellular pH, which was increased with an increase in pH; putrescine and spermidine uptake therefore decreased with membrane depolarisation and increased with membrane hyperpolarisation [110]. In contrast to *L. major* and *T. cruzi*, a molecular candidate of polyamine transport in *P. falciparum* remains to be identified.

In the process of drug discovery it is empirical to take into account the strategies that parasites employ to counteract the depletion of an essential metabolic compound. The most effective drug would be one that interferes with the biosynthesis of the compound, such as putrescine, and at the same time obstructs its uptake into the *P. falciparum*-infected erythrocyte. Alternatively, the putrescine and spermidine uptake systems may provide a mechanism for the selective delivery of antimalarials via their conjugation to polyamines, which might result in improved inhibitory activities of currently available antimalarials [111,112].

1.3.5. The *P. falciparum* polyamine biosynthetic enzymes as drug targets

The importance of polyamines in parasitic growth suggests that the inhibition of the polyamine pathway would interfere with the proliferation of the parasites [67], which can be approached by three general routes: 1) by 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 altered intracellular polyamine homeostasis [92].

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. b. gambiense* [67,68]. The success of DFMO treatment of the latter infection may be attributed to several factors including 1) the rapid division of parasitic cells resulting in a higher polyamine requirement than the host cells; 2) trypanosomes also use spermidine to produce trypanothione, which maintains the intracellular redox state (Figure 1.6) [113]; 3) the trypanosomal ODC is more stable and has a longer half-life than the host [114]; and 4) DFMO may be effectively transported into the trypanosomal parasites since the drug does not have to cross several membranes as is the case for the intracellular malaria parasites [71].

The ODC inhibitor 3-aminooxy-1-aminopropane (APA) and its derivatives CGP52622A and CGP54169A as well as the AdoMetDC inhibitors CGP40215A and CGP48664A (both analogues of methylglyoxal bis(guanylhydrazone), MGBG), severely affect *Pf*AdoMetDC and *Pf*ODC activities and result in reduced intracellular polyamine concentrations (Table 1.2) [100]. Additionally, 5'-([(Z)-4-amino-2-butenyl]methylamino)-5'-deoxyadenosine (MDL73811 or AbeAdo) irreversibly inhibits *Pf*AdoMetDC and is roughly a 1000-fold more effective than DFMO treatment [115]. Furthermore, Bitonti *et al*. 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 [116]. Treatment of *P. falciparum* with the *Pf*SpdS inhibitor, dicyclohexylamine, completely arrests parasite growth of both chloroquine-sensitive and resistant strains [117] and its derivative, 4MCHA, results in up to 85% growth arrest within 48 h when used in micromolar quantities (Table 1.2) [98].

Table 1.2: Selected inhibitors of *P. falciparum* ODC, AdoMetDC and SpdS
The *in vitro* inhibitory concentrations (IC_{50} in µM) of these drugs against *P. falciparum* parasites and recombinant enzyme (K_i in µM) are indicated.

	IC_{50}	K_i	Reference
ODC inhibitors			
DFMO	1250	87.6	[100,103,118]
APA	1	2.7	[100]
AdoMetDC inhibitors			
MDL73811	3	1.6	[100]
CGP48664A	8.8	3	[100]
CGP40215A	1.8	0.8	[100]
SpdS inhibitors			
AdoDATO	-	8.5	[98,119]
CHA	19.7	198	[98]
4MCHA	1.4	0.18	[98]
Dicyclohexylamine	>1 000	342	[98]
Polyamine analogue			
MDL27695	3	-	[116]

Adapted from Birkholtz *et al*. (Biochemical Journal, in press). Abbreviations: AdoDATO, *S*-adenosyl-1,8-diamino-3-thiooctane; APA, 3-aminooxy-1-aminopropane; CHA, cyclohexylamine; DFMO, DL-α-difluoromethylornithine; 4MCHA, *trans*-4-menthylcyclohexylamine.

The combined use of inhibitor treatment and protein X-ray crystallography of the polyamine metabolic enzymes allows the visualisation of the interactions 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 [128]. Homology models provide an alternative to protein crystal structures due to the challenges involved in expressing pure and sufficient amounts of *P. falciparum* proteins required for crystallisation studies [129,130]. Models of the three *P. falciparum* polyamine biosynthetic

enzymes have been solved, i.e. monofunctional *Pf*AdoMetDC [131], monofunctional *Pf*ODC [132] and *Pf*SpdS [133] (also crystallised [127]).

1.3.5.1. The bifunctional *P. falciparum* AdoMetDC/ODC complex

In *P. falciparum*, the *Pf*AdoMetDC and *Pf*ODC domains are uniquely assembled into a bifunctional complex of approximately 330 kDa (Figure 1.9) [70]. The N-terminal *Pf*AdoMetDC domain (residues 1-529) exists as a protomer that is post-translationally cleaved into a large ~55 kDa α-subunit, and a smaller β-subunit of approximately 9 kDa. This domain is covalently linked to *Pf*ODC at the C-terminus (residues 805-1419) via a hinge region that spans residues 530-804 [70,71]. The quaternary structure of the functional ~165 kDa heterodimeric polypeptide thus consists of two subunits, the ~155 kDa α-*Pf*AdoMetDC/ODC and the ~9 kDa post-translationally cleaved β-*Pf*AdoMetDC subunit. Two of these polypeptides have an obligatory association through the *Pf*ODC domain, to form the active ~330 kDa bifunctional complex (Figure 1.9) [70,71,120].

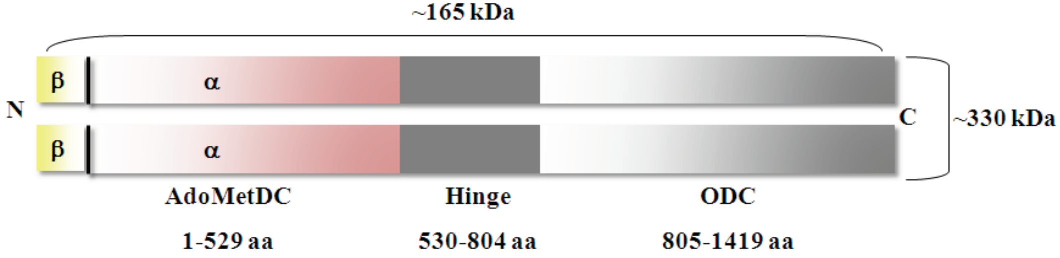


Figure 1.9: Schematic diagram of the bifunctional *P. falciparum* AdoMetDC/ODC protein.
The N-terminal *Pf*AdoMetDC domain consists of α- and β-subunits. This domain is connected to the C-terminal *Pf*ODC via a hinge region. The sizes of the heterodimeric and heterotetrameric complexes are shown [70].

The 275-residue hinge region connects the *Pf*AdoMetDC and *Pf*ODC domains (Figure 1.9) [70] and is involved in the conformational stability and quaternary structure formation of the *Pf*ODC domain [103]. Previous studies have shown that the hinge stabilises the heterotetrameric *Pf*AdoMetDC/ODC complex by mediating interdomain interactions [69]. Several secondary structures are present within this region, notably two α-helixes and a β-sheet that have been shown to have indirect effects on the catalytic activities of both domains due to contributions to interdomain interactions [121]. The importance of the hinge region in the activity of monofunctional *Pf*ODC (see below) has led to investigations of possible protein-protein interactions between the domains of the bifunctional protein [71,103]. Interdomain interactions have been reported to play a role in other bifunctional proteins of *P. falciparum* such as DHFR/TS where the catalytic activity of the TS domain is dependent on its interaction with the DHFR domain [122]. In *Pf*AdoMetDC/ODC it was shown that although the specific activities of

the respective enzymes (referred to here as monofunctional protein domains) are not affected upon inhibition or substrate removal of the neighbouring enzyme [71], interdomain interactions occur within the bifunctional complex that are essential for domain activities [69]. A possible explanation for the bifunctional arrangement could therefore be that the control of the abundance and activity of a single protein regulates polyamine biosynthesis within *P. falciparum* [92].

1.3.5.2. Monofunctional S-adenosylmethionine decarboxylase from *P. falciparum*

*Pf*AdoMetDC utilises pyruvoyl as a co-factor, which is formed from an internal autocatalytic processing event at Ser73 resulting in the formation of the α- and β-subunits (Figure 1.9). The native bifunctional protein isolated from the *P. falciparum* parasites showed a K_m of 33.5 µM for its substrate AdoMet and a specific activity of 14.8 pmol/min/mg [70]. In contrast to the human enzyme, *Pf*AdoMetDC activity is not stimulated by putrescine, indicating that *Pf*AdoMetDC lacks the regulatory mechanism proposed for mammalian cells to relate putrescine abundance with spermidine synthesis [71,120]. Similarly to the human protein, monofunctional *Pf*AdoMetDC exists as an $(\alpha\beta)_2$ dimer within the bifunctional complex [71,120,123] where each active site is located between the β-sheets of the monomeric αββα-sandwich fold (Figure 1.10) [120].

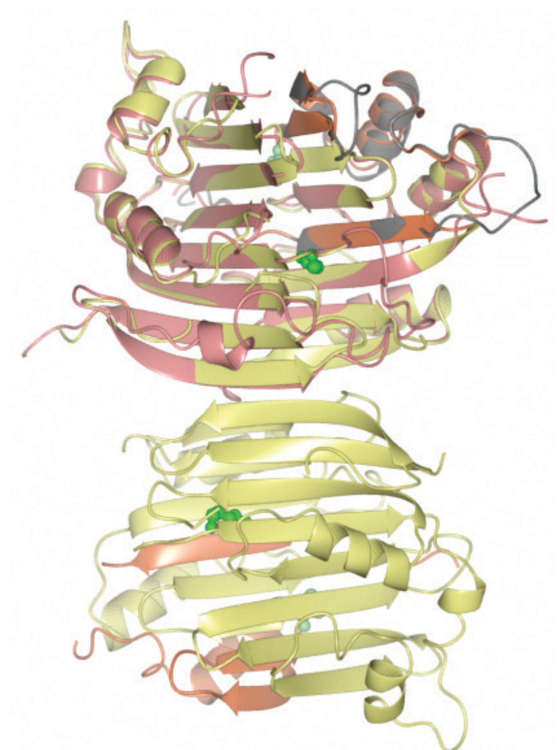


Figure 1.10: The αββα-sandwich fold of monofunctional, monomeric *P. falciparum* AdoMetDC superimposed with the dimeric human protein.
The crystal structure of dimeric human AdoMetDC (1JEN, α- and β-subunits in yellow and orange, respectively) [123] superimposed with the homology model of the monofunctional, monomeric *Pf*AdoMetDC (pink and grey for α- and β-subunits, respectively) [120]. Putrescine within the charged-buried site is shown in green.

The monofunctional *Pf*AdoMetDC homology model showed that the residues within the active site are in a similar orientation to those of the human protein with only four substitutions in the active site and surrounding surface of *Pf*AdoMetDC. Interactions with the substrate analogue MeAdoMet (methyl ester of AdoMet) are conserved where the adenine ring is hydrophobically stacked between residues Phe5 and Phe415 [120], which are contributed from both β-sheets. Mutagenesis studies confirmed the involvement of these aromatic residues in substrate and inhibitor binding of the human protein [124]. Glu438 forms two hydrogen bonds with the hydroxyl groups on the ribose moiety while a third hydrogen bond is also present between N^1 on the adenine ring and the amide nitrogen of Glu72. Lastly, the model showed that the pyruvoyl group in *Pf*AdoMetDC is more out-of-plane while the carbonyl group remains in plane for its purpose as an electron sink during the decarboxylation reaction [120]. The model could also explain the lack of *Pf*AdoMetDC activity stimulation by putrescine. The putrescine-binding site of the human protein is lined with acidic residues that can interact with the positive amines of putrescine [125]. In *Pf*AdoMetDC, these residues are substituted by the basic residues Arg11, Lys15 and Lys215. Subsequent mutagenesis of these residues to non-polar ones showed that especially Arg11 is essential for activity and therefore suggests that these residues assume the function of putrescine binding [120].

1.3.5.3. Monofunctional ornithine decarboxylase from *P. falciparum*

*Pf*ODC decarboxylates L-ornithine to form putrescine in a reaction that is dependent on the vitamin B_6-derived co-factor, pyridoxal-5'-phosphate (PLP) [126]. ODC exists as an obligate homodimer as a consequence of the two active sites that are formed at the dimer interface and consist of residues contributed from both monomers of ~70 kDa each. This interface is distinguished by an aromatic amino acid zipper, formed by the head-to-tail association of the two *Pf*ODC monomers, placing the C-terminus of one monomer vertical to the N-terminus of the other and *vice versa*. The *Pf*ODC monomer consists of two distinct structural domains, an N-terminal α/β triosephosphate isomerase (TIM)-barrel (typical of the alanine racemase-like family) and a C-terminal modified Greek-key β-barrel (Figure 1.11) [127].

Several differences exist between the human and *Pf*ODC enzymes including the feedback inhibition of *Pf*ODC activity by putrescine and the extended *Pf*ODC half-life of more than two h compared to the ~15 min half-life of the human protein [77]. The instability of the latter protein is due to the action of antizyme and the presence of a C-terminal PEST region involved in the recruitment of the 26S proteasome (Figure 1.8) [84,110]. This difference has also provided a rationale for the differential host-parasite responses to DFMO treatment [99].

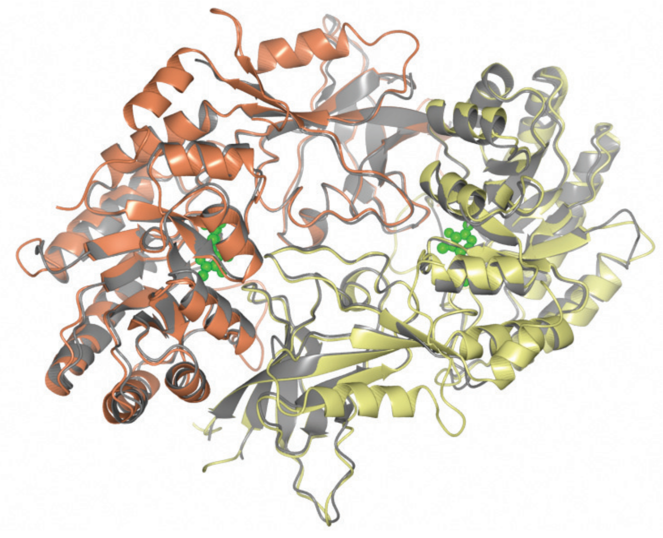


Figure 1.11: The head-to-tail organisation of *P. falciparum* ODC superimposed with the human protein. Crystal structure of homodimeric human ODC (1D7K, grey) [128] superimposed with the homology model of *Pf*ODC (monomers shown in yellow and orange) [127]. PLP within the active sites are shown in green.

The specific activity of the native bifunctional protein isolated from the *P. falciparum* parasites is 93.2 pmol/min/mg while it binds substrate with an affinity of 42.4 µM [70]. Investigations into the expression and catalytic properties of two recombinant constructs of monofunctional *Pf*ODC showed that the hinge region is involved in *Pf*ODC substrate binding while its presence also increases the specific activity of the enzyme [103]. Several residues that are essential for catalytic activity (co-factor and DFMO binding) and dimerisation are conserved in the *Pf*ODC sequence, with only three unique residue substitutions in the *Pf*ODC PLP-binding site [127]. The aromatic Phe1392, Tyr1305 and Phe1319 residues (numbering according to bifunctional protein) make hydrophobic contacts across the dimer interface resulting in an antiparallel-stacked interaction. Lys970 has in particular been predicted to interact with various residues surrounding the active site including Asp1356, Gly1352, Gly1357 and Asp1359. These residues also form part of the DFMO-binding region in the Gly1352-Gln-Ser-Cys-Asp-Gly-Leu-Asp1359 motif of *Pf*ODC [23,127,129].

1.3.5.4. Spermidine synthase from *P. falciparum*

*Pf*SpdS catalyses an aminopropyl transferase reaction to produce spermidine and MTA from dcAdoMet and putrescine. In addition, this enzyme is also responsible for the low levels of spermine within *P. falciparum* [98]. *Pf*SpdS consists of 321 residues with a monomeric molecular mass of ~37 kDa and associates to form a homodimer (Figure 1.12). The removal of 29 residues from an N-terminal extension allowed the recombinant expression in *Escherichia coli*. This extension is believed to have a signal peptide-like character and was also identified in plant SpdS [98]. Recombinant *Pf*SpdS catalyses spermidine synthesis with a k_{cat} of 0.48 s^{-1} and

substrate affinities of 52 µM and 35.3 µM for putrescine and dcAdoMet, respectively. MTA is produced as a stoichiometric by-product in this reaction and acts as a feedback inhibitor of the enzyme [98]. *Pf*SpdS is part of the aminopropyl transferase family of proteins that characteristically consists of a small N-terminal and a large C-terminal catalytic domain. The crystal structure showed that the N-terminal domain consists of a six-stranded β-sheet while the Rossmann-like C-terminal domain contains a seven-stranded β-sheet followed by nine α-helices (Figure 1.12).

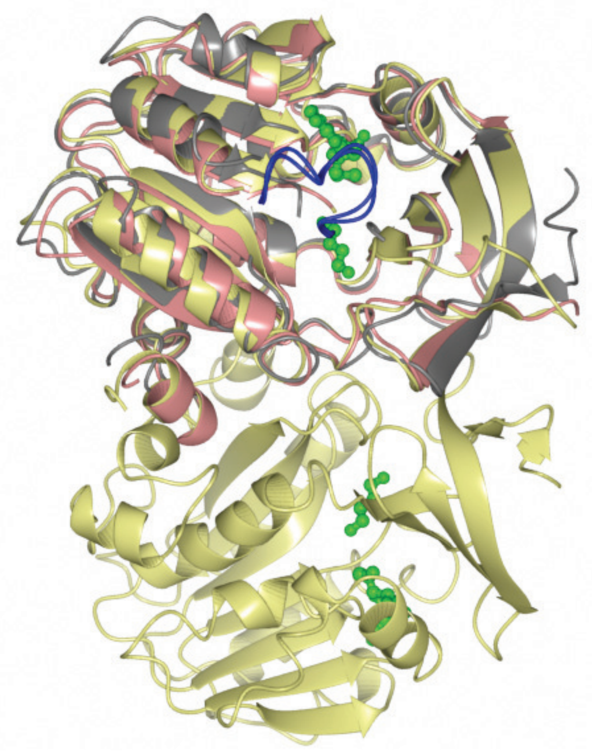


Figure 1.12: The structure of homodimeric SpdS from *P. falciparum* superimposed with the human protein.
The crystal structure of homodimeric human SpdS (2O06, yellow) [97] superimposed with the crystal structures of *T. cruzi* (3BWC, grey) (Bosch *et al*. unpublished results) and *P. falciparum* (2I7C, pink) [119]. MTA and putrescine within the active site are shown in green. The gate-keeping loops of human and *P. falciparum* SpdS are shown in blue.

A homology model of *Pf*SpdS, created with the *Arabidopsis thaliana* and *T. maritima* crystal structures as templates, identified essential features which were supported by mutagenesis studies [130]. The putrescine-binding cavity contains a hydrophobic region that is flanked by two negatively-charged regions that allow binding of the hydrophobic and positive termini of putrescine, respectively. Water molecules were predicted to form hydrogen bonds between the active site residues and the substrates to position and anchor them within the cavity. Several hydrogen bonds also form between dcAdoMet and the active site residues that are responsible for positioning of the aminopropyl chain for nucleophilic attack by putrescine [130]. In 2007, the model was superseded by the crystal structures of *Pf*SpdS in complex with dcAdoMet, 4MCHA

and the transition state analogue, *S*-adenosyl-1,8-diamino-3-thio-octane (AdoDATO). dcAdoMet binding was shown to be stabilised by an active site gate-keeping loop that controls access of the substrates into the active site pocket [131,132]. The flexible loop covers the entrance to the active site and opens to allow the exit of MTA followed by spermidine (Figure 1.12). The established interactions with the inhibitors also revealed important binding sites that may be modified for the synthesis of improved inhibitory compounds in the near future [119].

1.4. Research objectives

This study was aimed at the identification of novel aspects of the *P. falciparum* polyamine biosynthetic enzymes (both individual domains of the bifunctional *Pf*AdoMetDC/ODC as well as *Pf*SpdS) and ultimately forms part of a larger study to investigate possible antimalarial strategies via inhibition of polyamine synthesis within the malaria-causing parasite. The study involved the structural and functional characterisation of *Pf*AdoMetDC and *Pf*ODC in order to gain a better insight into their activities, protein-protein interactions as well as their arrangement within the bifunctional complex as a means to regulate catalytic activities. Lastly, and for the first time attempted by the Malaria Research group at the University of Pretoria, protein X-ray crystallisation was investigated as a means to validate the predicted binding sites of novel inhibitory compounds against *Pf*SpdS, which were identified by a pharmacophore-based approach.

The work involving the biophysical characterisation of *Pf*AdoMetDC as well as the crystallisation, diffraction data collection and components of the 3D structure solving of *Pf*SpdS were performed at Lund University (Sweden) as part of a South African-Swedish collaboration funded by the National Research Foundation-Swedish International Cooperation Development Agency (NRF-SIDA, Swedish Research Links Programme). The methodology and results of this study therefore forms part of a combination of work that was performed in South Africa and during research visits to Sweden.

Three distinct studies were thus undertaken:

- **Chapter 2: A conserved parasite-specific insert is a key regulator of the activities and interdomain interactions of *Plasmodium falciparum* AdoMetDC/ODC.**
  In this chapter the roles of a conserved parasite-specific insert within the *Pf*ODC domain in both activities of the bifunctional *Pf*AdoMetDC/ODC complex were investigated. The

native interaction sites of this insert were subsequently studied with the use of interface peptide probes. Novel insights were obtained that allowed us to better understand the unique arrangement of the decarboxylase domains within a bifunctional complex in the *Plasmodium* spp.

- **Chapter 3: Biochemical and structural characterisation of monofunctional *Plasmodium falciparum* AdoMetDC**.
  In this chapter the recombinant expression of the monofunctional *Pf*AdoMetDC domain is described that was subsequently used in a structure-function relationship study of this protein. The biochemical and biophysical characteristics of monofunctional *Pf*AdoMetDC are discussed, which provided insights into unique parasite-specific properties. The results of this study were also used to establish if the co-existence of the two domains in the bifunctional complex impacts on each other's properties and these were compared to that of the human protein to gain an understanding of the *in vitro* functional arrangement of the monofunctional protein.

- **Chapter 4: Validation of pharmacophore-identified inhibitors against *Plasmodium falciparum* SpdS with the use of X-ray crystallography.**
  This study focussed on novel drug development strategies of *Pf*SpdS, which resulted in the identification of promising inhibitory compounds by using a dynamic, receptor-based pharmacophore model. These compounds were tested *in vitro* and their interactions within the *Pf*SpdS active site were subsequently investigated with co-crystallisation studies of the enzyme-inhibitor complexes. These results validated the use of an *in silico* drug discovery approach to streamline the identification of compounds that could result in the parasite-specific inhibition of a drug target.

- **Chapter 5: Concluding discussion**

1.5.Outputs

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

Chapter 2:

1. Williams, M., Wells, G.A., Roux, S., Niemand, J., Rautenbach, M., Louw, A.I. and Birkholtz, L. "Insert-mediated regulation of the activities and interactions of the rate-limiting polyamine biosynthetic enzyme of *Plasmodium falciparum*. A

conserved parasite-specific insert is a key regulator of the activities and interdomain interactions of *Plasmodium falciparum* *S*-adenosylmethionine decarboxylase/ornithine decarboxylase." (Manuscript to be submitted to Experimental Parasitology)

2. Conference proceeding: "A conserved parasite-specific insert influences the activities and interdomain interactions of the malarial *S*-adenosylmethionine decarboxylase/ornithine decarboxylase." Invited oral presentation, 5th Symposium on Polyamines in Parasites, Detroit, USA in July 2008

Chapter 3:

1. Williams' M., Sprenger, J., Human, E., Al-Karadaghi, S. Persson, L., Louw, A.I. and Birkholtz, L. "Biochemical and structural characterisation of *S*-adenosylmethionine decarboxylase from *Plasmodium falciparum*." (Biochemical Journal, accepted with minor revision)
2. Conference proceeding: "Towards finding the structure of *Plasmodium falciparum* *S*-adenosylmethionine decarboxylase." Invited oral presentation, 6th Symposium on Polyamines in Parasites, Phalaborwa, South Africa in August 2010
3. Conference proceeding: "Malaria polyamine biosynthesis: The road from drug target validation to drug development." Oral presentation, Biology of Parasitism course at The Marine Biology Laboratories, Woods Hole, USA in June 2009
4. Conference proceeding: "Structural and functional characterisation of malarial *S*-adenosylmethionine decarboxylase." Poster presentation, 7th Protein Expression, Purification and Crystallisation course, Hamburg, Germany in August 2010

Chapter 4:

1. *Burger, P.B., *Williams, M., Reeksting, S.B., Al-Karadaghi, S., Briggs, J.M., Joubert, F., Birkholtz, L., Louw, A.I. "Design of novel inhibitors against *Plasmodium falciparum* Spermidine Synthase using structurally-derived binding descriptors." (Manuscript to be submitted to Journal of Medicinal Chemistry) *Authors contributed equally to this work.
2. Conference proceeding: "The development of a dynamic receptor-based pharmacophore model for *Plasmodium falciparum* spermidine synthase." Poster presentation, 6th Symposium on Polyamines in Parasites, Phalaborwa, South Africa in August 2010
3. Conference proceeding: "Crystal structure of *Plasmodium falciparum* spermidine synthase containing a novel inhibitor identified with a dynamic receptor-based pharmacophore model." Poster presentation, Gordon Research Conference: Polyamines, Waterville Valley Resort, USA in June 2011

Reviews:

1. Clark, K., Niemand, J., Reeksting, S., Smit, S., van Brummelen, A., Williams, M., Louw, A.I. and Birkholtz, L. (2010) "Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*." Amino Acids. **38**, 633-644
2. Birkholtz, L., Williams, M., Niemand, J., Louw, A.I., Persson, L. and Heby, O. "Polyamine homeostasis as a drug target in pathogenic protozoa: peculiarities and possibilities." (Biochemical Journal, in press)