Chapter 1: Literature review

1.1 Malaria

1.1.1 General

Tropical diseases attributable to infection with parasitic protozoa lead to high mortality rates and reduced economic growth (Reguera *et al.*, 2005). Of these, the World Health Organisation (WHO) ranks malaria as the number one tropical disease worldwide. Malaria pathogenesis manifests as severe malaria, clinical malaria, or asymptomatic parasitaemia, as well as several pregnancy-associated effects and ultimately mortality. Severe malarial infection can also lead to long term neurocognitive sequelae such as visual, aural, language and cognitive impairment, epilepsy, learning difficulties and severe motor deficits (Breman, 2001; Breman *et al.*, 2004). Every year, more than 300 million clinical cases of malaria infection occur worldwide, resulting in more than a million deaths (Fig. 1.1). In Africa, these deaths occur mostly among children, and according to the WHO there is, on average, a malaria-induced death of a child every 30 seconds (http://www.rbm.who.int).

Malaria is caused by infection with unicellular, eukaryotic protozoan parasites of the genus *Plasmodium* that are transmitted to humans during the blood meal taken by female *Anopheles* mosquitoes. Traditionally, there are four different *Plasmodium* species that can infect humans, namely *Plasmodium falciparum*, which causes the most deaths, as well as *P. malariae, P. vivax* and *P. ovale* (Greenwood *et al.*, 2005) (Fig. 1.1). However, in 2004 it was found that the parasite responsible for simian malaria, *P. knowlesi*, can also infect humans (Jongwutiwes *et al.*, 2004; Singh *et al.*, 2004). In Africa, malaria is caused exclusively by *P. falciparum* in Swaziland, Botswana, Namibia, Dominican Republic, Cape Verde and São Tomé and Príncipe, while both *P. falciparum* and *P. vivax* are responsible for malaria cases in the rest of the continent (Feachem *et al.*, 2010).

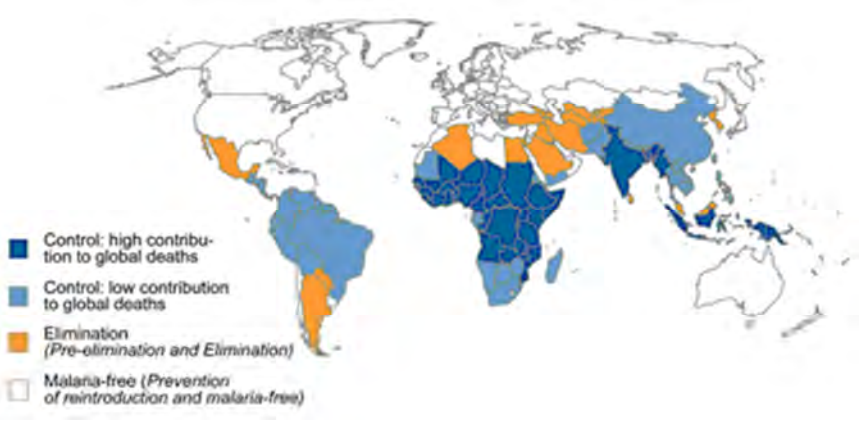

Figure 1.1: World-wide occurrence of malaria. Areas of high (dark blue) and low (light blue) malaria-induced mortality are indicated, as well as areas that are free of malaria (white) and areas where malaria is either eradicated or in the process of being eradicated**.** This image is freely available for use from http://www.rollbackmalaria.org.

1.1.2 Lifecycle of *P. falciparum*

The lifecycle of the *P. falciparum* parasite consists of a sexual developmental cycle inside the female members of the *Anopheline* species and an asexual developmental cycle inside the human host (Fig. 1.2) (Kappe *et al.*, 2010). When the mosquito takes a blood meal, sporozoites are injected subcutaneously into the human host with the saliva, and make their way to the liver through the bloodstream. Inside the liver, the sporozoites infect hepatocytes and develop into schizonts, which subsequently develop into thousands (10^4-10^5) of merozoites that are released into the blood. Here the merozoites proceed to infect red blood cells (RBCs) of the host. It is within a RBC that the subsequent asexual replication occurs where the parasite matures through various stages (ring-, trophozoite- and schizont-stage). Each mature schizont can then release up to 32 daughter merozoites that will proceed to infect healthy RBCs (Frevert, 2004; Ghosh *et al.*, 2002; Khan and Waters, 2004; Miller *et al.*, 2002). Ultimately, these iterative cycles of multiplication and infection causes a high parasitaemia of up to 10^9-10^{13} parasites circulating within human host (Kappe *et al.*, 2010).

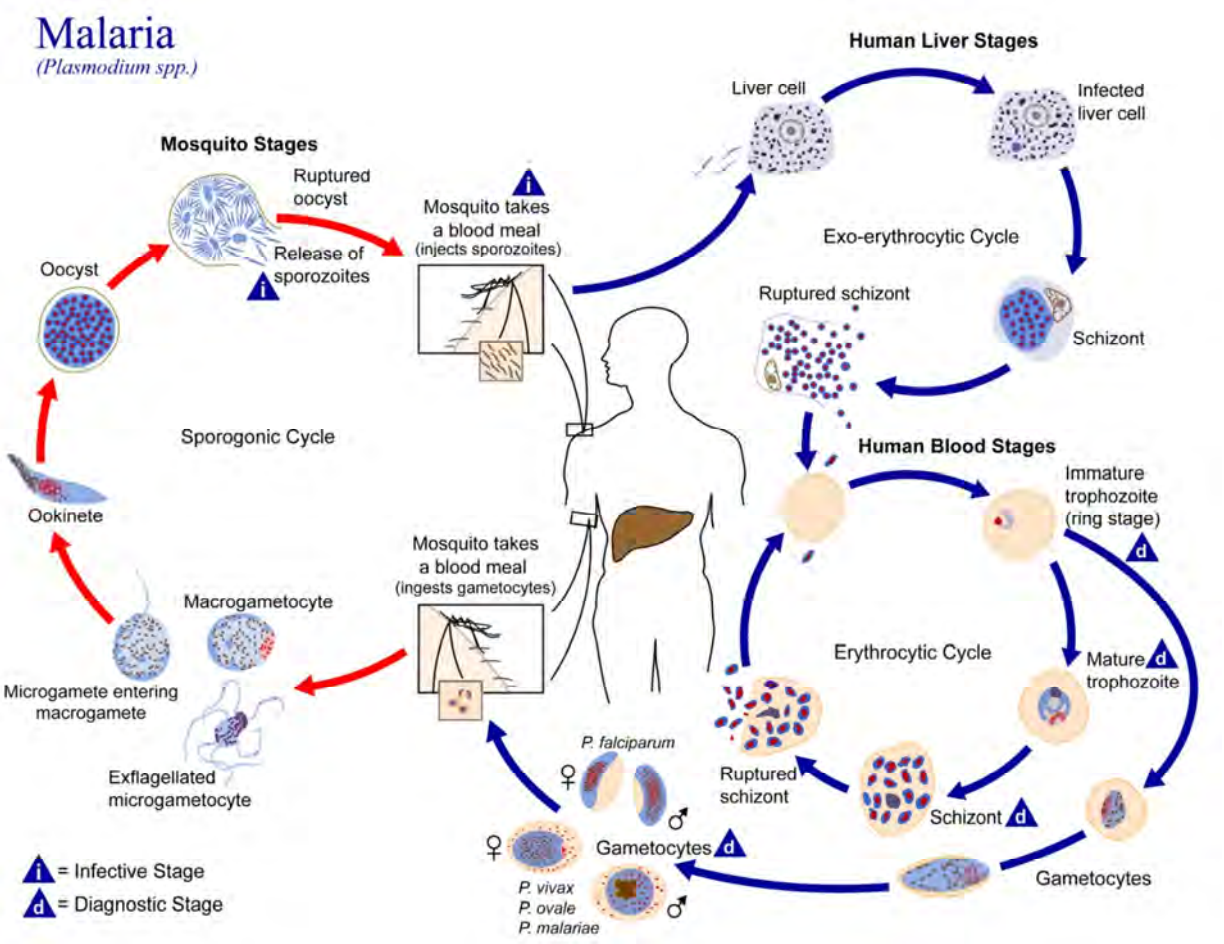

Figure 1.2: The lifecycle of the *Plasmodium* parasite. Sporozoites are transmitted to humans where they undergo liver stage and erythrocytic development before gametocytes are again taken up by feeding mosquitoes to initiate development within the insect vector. This image is in the public domain, credited to the CDC Public Health Image Library (http://phil.cdc.gov/phil/).

A few of the parasites do not undergo asexual replication, but develop into male and female gametocytes. These gametocytes are taken up by mosquitoes during a blood meal. Upon entering the mosquito, gametocytes develop into gametes in the mosquito midgut. A diploid zygote is then produced after fertilisation and develops into a motile ookinete that travels to the mosquito haemolymph, where it forms an oocyst. This in turn gives rise to thousands (10^3-10^4) of motile sporozoites that migrate to the salivary glands via the haemolymph (Frevert, 2004; Ghosh *et al.*, 2002; Khan and Waters, 2004; Miller *et al.*, 2002). This combination of asexual development in the human host and sexual development in the mosquito vector (Fig. 1.2) provides an optimal symbiotic progression for transmission of malaria parasites, but also allows various points that could be investigated to curb the disease transmission and development (Kappe *et al.*, 2010).

1.1.3 The control of malaria

Efforts to control malaria have employed a three-pronged approach involving drug development, vector control and vaccine development, applied separately or in combination (Bathurst and Hentschel, 2006). For example, by combining therapeutic strategies (e.g. the WHO recommends artemisinin-based combination treatments, ACT's) and widespread use of bed nets (vector control), the malaria cases in Rwanda decreased by more than 50% (Enserink, 2010b). It has also been postulated that pre-erythrocytic stage vaccines as well as transmission blocking vaccines could be extremely effective in reducing the disease burden (Kappe *et al.*, 2010). Although malarial infections can be reduced by the use of insecticide-treated bed nets and vector control, the absence of an efficient vaccine means that life-threatening infections, which can only be treated by drugs, still occur at an alarming rate (Bathurst and Hentschel, 2006). Although the RTS,S/AS01E vaccine, based on the circumsporozoite protein of *P. falciparum,* has had promising results, providing protection after 15 months following administration (Lemnge *et al.*, 2011), this vaccine is not yet in general use.

Several biological processes of the parasite have been targeted chemotherapeutically, but unfortunately, resistance has emerged against the majority of the available anti-malarial drugs, highlighting the urgent need for alternative therapies in the near future (Table 1.1) (Müller and Hyde, 2010). For example, resistance to the once widely-used anti-malarial drug chloroquine has become widespread. Chloroquine resistance arises as a result of mutations in the gene encoding PfCRT (the *P. falciparum* chloroquine-resistance transporter) (Martin *et al.*, 2009b). A lysine to threonine mutation at position 76 in the protein allows chloroquine, which usually accumulates in the parasite's acidic food vacuole, to exit the organelle and hence away from its primary site of action (Martin *et al.*, 2009b). Current efforts in the development and discovery of novel anti-malarials is being coordinated through several research networks e.g. European Union sponsored Antimal (FP6 network, www.antimal.eu) and the South African Malaria Initiative (www.sami.org.za), as well as international partnerships such as the Medicines for Malaria Venture (MMV, www.mmv.org). The stated goal of MMV is: '…to reduce the burden of malaria in disease-endemic countries by discovering, developing and facilitating delivery of new, effective and affordable anti-malarial drugs.' Several new drugs are under investigation within the MMV portfolio, including mini-portfolios where compound libraries are being screened from e.g. GlaxoSmithKline (Fig. 1.3).

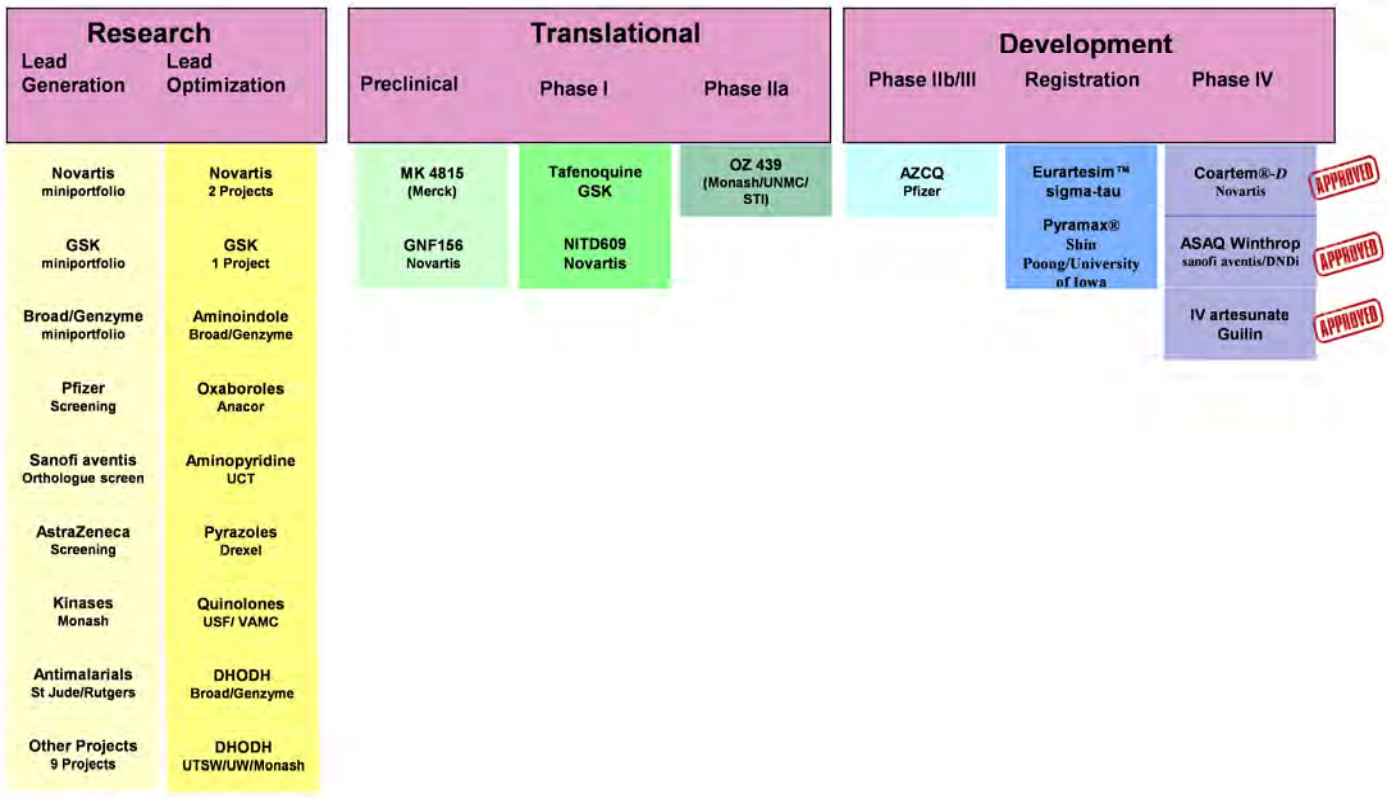

Figure 1.3: Current MMV portfolio (4th quarter 2010, www.mmv.org). DHODH: dihydroorotate dehydrogenase, NITD609: natural product selection project, OZ439: peroxides, AZCQ: azithromycin-chloroquine, Eurartesim: dihydroartemisinin-piperaquine, Pyramax: pyronaridine-artesunate, CoArtem: artemether-lumefantrine, ASAQ: artesunate-amodiaquine.

Despite these efforts and increasing funding investments, no chemically novel entity has entered phase IIb clinical trials or drug development stages, and it is only artemisinin-derivatives combination therapies (e.g. CoArtem: arthemether-lumefantrine and ASAQ: artesunate-amodiaquine) that are in phase IV trials (www.mmv.org). Nevertheless, several new chemotypes are under investigation, with peroxides (OZ439), carboxiimides (GSK MP), imidazolepyrazines and pyrrolindines (Novartis) in early stage development (Fig. 1.3). New drug targets are also being identified. Within the MMV portfolio, inhibitors for dihydroorotate dehydrogenase and the kinases are actively being investigated and there are nine other projects that may well deliver new leads in the near future (www.mmv.org).

Table 1.1: Currently used anti-malarial drugs.

Compiled from (Arav-Boger and Shapiro, 2005; Bathurst and Hentschel, 2006; Edwards and Biagini, 2006; Enserink, 2010a; Jambou *et al.*, 2005; Kappe *et al.*, 2010; Kremsner and Krishna, 2004; Luzzatto, 2010; Martin *et al.*, 2009b; Müller and Hyde, 2010; Nkrumah *et al.*, 2009; Petersen *et al.*, 2011; Ridley, 2002; Sanchez *et al.*, 2008b; Staines *et al.*, 2010)**.**

Anti-malarial drug	Year launched	Resistance emerged	Resistance mechanisms	Limitations	Location/target/life cycle stage
Quinolines and related antimalarials					
Quinine	19 th century	1910	Possibly via mutations in the *P. falciparum* chloroquine-resistance transporter, PfCRT, *P. falciparum* multi-drug resistance protein, PfMDR1 (Sanchez *et al.*, 2008a). Increases in DNNND repeat number in P. falciparum Na+/H+ exchanger 1 transported associated with quinine resistance (Henry *et al.*, 2009).	Compliance (3 times a day, 7 days) Safety	Food vacuole, haem crystallisation , blood stage
Chloroquine	1945	1957	PfCRT mutations (lysine to threonine at position 76) prevents the accumulation of chloroquine in the parasite's acidic food vacuole (charged drug leak hypothesis) (Martin *et al.*, 2009b)	-	Food vacuole, haem crystallisation, blood stage
Amodiaquine	1975	None	Cross-resistance with chloroquine, with mutations in PfMDR1 and PfCRT involved (Sá *et al.*, 2009).	Safety (occasional agranulocytosis, hepatotoxicity)	Food vacuole, haem crystallisation, blood stage
Mefloquine	1977	1982	Point mutations in PfMDR1 allows for export of drugs from food vacuole. Increased resistance with increased *pfmdr1* copy number (Preechapornkul *et al.*, 2009).	Safety (Neuropsychiatric disturbances) Cost	Food vacuole, haem crystallisation, blood stage
Halofantrine	1988	1992	Point mutations in PfMDR1 allows for export of drugs from food vacuole	Safety (contra-indicated for people with heart disease) Cost	-

Anti-malarial drug	Year launched	Resistance emerged	Resistance mechanisms	Limitations	Location/target/life cycle stage
Artemisinin					
Artemisinin derivatives	1970	*In vitro* to field isolates from French Guiana, signs of resistance in the Thai-Cambodian border	Initially suggested to be due to mutation of PfATP6 with a serine to asparagine mutation at position 769 (Jambou *et al.*, 2005). Increased resistance with increased *pfmdr1* copy number (Sidhu *et al.*, 2006). Modification in the intra-erythrocytic developmental cycle transcriptional cascade leads to early stages (ring stages) parasites entering a more resistant quiescent state as well as increased protein synthesis and turnover in schizont stage (Mok *et al.*, 2011).	Compliance (5-7 days of treatment) Cost Possible safety issues (neuronopathy in lab animals, sporadic allergic reactions)	Endoplasmic reticulum, generation of reactive intermediates due to Fe^{2+} mediated decomposition of artemisinin leads to protein alkylation, PfATP6 inhibition. Active against both blood stage and gametocytes
Other antimalarials					
Proguanil	1948	1949	Mutations in dihydrofolate reductase (DHFR) domain of the bifunctional DHFR-thymidylate synthethase (TS) protein	Cost	Parasite cytoplasm, folate metabolism, blood stage
Primaquine	1950	None		Safety, can lead to haemolytic anaemia	Target unknown. Active against liver stage and gametocytes
Sulfadoxine-pyrimethamine	1967	1967	Mutations in DHFR domain DHFR-TS		Parasite cytoplasm, folate metabolism (dihydropteroate synthase and dihydrofolate reductase), blood stage
Atovaquone	1996	1996	Point mutation in cytochrome c reductase l	High cost due to complexity of synthesis	Mitochondria, cytochrome bc_1 complex, blood and liver stage
Lapdap™ (combination of dapsone and chlorproguanil)	2003	None		Withdrawn in 2008 due to haemolytic effects	Parasite cytoplasm, folate metabolism, blood stage

PfCRT: *P. falciparum* chloroquine-resistance transporter; PfMDR1: *P. falciparum* multi-drug resistance protein, DHFR-TS: dihydrofolate reductase-thymidylate synthethase

Several other biological processes and metabolic pathways are being investigated as potential ant-malarial targets, with an emphasis on those that are unique to Plasmodia (Kappe *et al.*, 2010). The apicoplast (an ancient chloroplast relic) and associated biosynthetic activities (isoprenoid, fatty acid and haem synthesis) is of particular interest (Lim and McFadden, 2010), as is the parasite's atypical mitochondrion (van Dooren *et al.*, 2006). Folate metabolism, glycolysis, purine biosynthesis, shikimate biosynthesis and polyamine metabolism have been highlighted as potential targetable processes (Arav-Boger and Shapiro, 2005; Kappe *et al.*, 2010; Müller *et al.*, 2008; Ridley, 2002). Finally, the proteins involved in the transport of solutes into (and out of) the parasite have been highlighted as potential drug targets in their own right (Staines *et al.*, 2010), and are also recognised as playing a role in drug resistance mechanisms.

This thesis focuses on the transport and metabolism of polyamines in *P. falciparum* parasites. The following sections will introduce polyamines and polyamine metabolism in this parasite as well as other organisms, review the current knowledge of polyamine uptake mechanisms in other organisms and, lastly, focus on transport of solutes into *P. falciparum* parasites.

1.2 Polyamine metabolism

Polyamines are aliphatic, low-molecular weight nitrogenous bases consisting of methylene moieties separating 2-4 amine groups, which have pK values of ~10 and are therefore protonated at physiological pH (Moinard *et al.*, 2005; Reguera *et al.*, 2005; Romero-Calderón and Krantz, 2006; Wallace *et al.*, 2003). The four physiologically important polyamines are the primary diamines, cadaverine (1,5-diaminopropane,) and putrescine (1,4-diaminobutane or tetramethylenediamine), as well as the tri-amine spermidine (*N*-(3-aminopropyl)-1,4-diaminobutane or aminopropyl-tetramethylenediamine) and the tetra-amine spermine (*N*,*N*^{1}-bis (3-aminopropyl)-1,4-butanediamine or diaminopropyl-tetramethylenediamine) (Fig. 1.4) (Cohen, 1998; Moinard *et al.*, 2005). As organic cations, polyamines can interact with negatively charged macromolecules within cells, such as phospholipids and nucleic acids. However, there are two major differences between the polyamines and divalent inorganic cations such as $Ca^{2+}$and Mg^{2+}. Firstly, the positive charge on the polyamines is dispersed along the entire length of the flexible backbone chain in contrast to the point charges of the bivalent cations. Secondly, polyamine homeostasis is controlled by biosynthesis, catabolism, and transport to ensure tight control of the polyamine levels in cells (Jänne *et al.*, 2004; Wallace *et*

al., 2003). Polyamines and their biosynthetic enzymes occur at increased concentrations in rapidly proliferating cells, including cancer cells as well as parasitic organisms. As such, the exploitation of metabolic differences in polyamine metabolism is a rational approach to target proliferative diseases such as cancer and parasitic infections (Casero and Woster, 2009; Heby *et al.*, 2007).

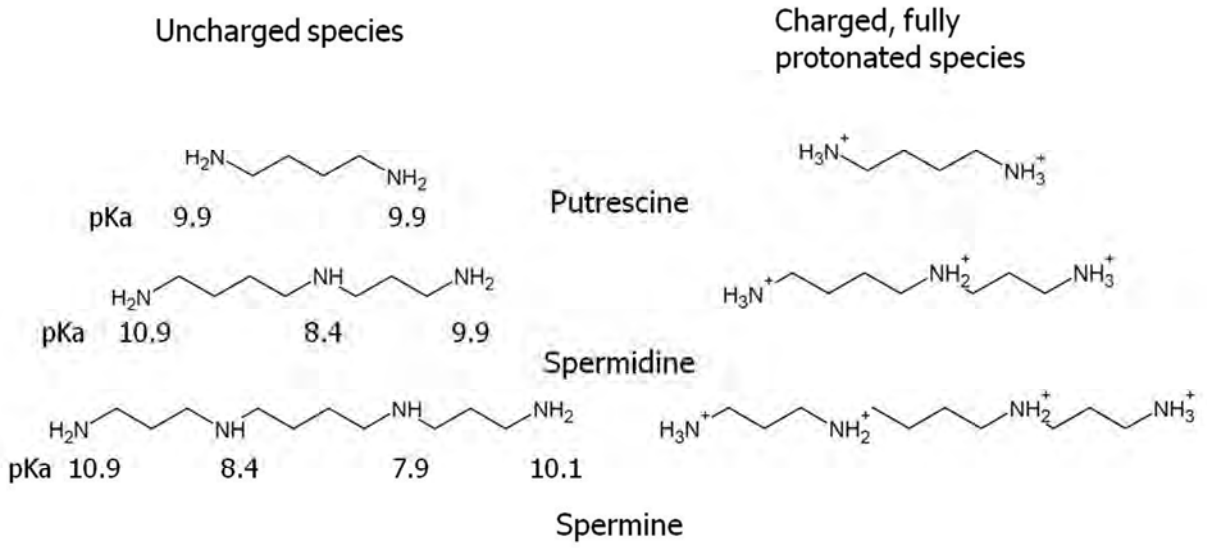

Figure 1.4: The structures of putrescine, spermidine and spermine. Due to the high pKa values, the polyamines are positively charged at physiological pH. pKa values from (Casero and Woster, 2009).

1.2.1 Polyamine biosynthesis in mammals

Polyamine levels in cells are regulated by several pathways including *de novo* synthesis, uptake mechanisms that recover polyamines from the external environment, catabolism and export (Fig. 1.5) (Casero and Pegg, 2009; Pegg, 2009). The primary precursors of polyamines are the amino acids L-ornithine and L-methionine. L-ornithine is obtained from the diet, or produced from L-arginine by mitochondrial arginase II and then decarboxylated by the rate-limiting enzyme ornithine decarboxylase (ODC) to yield putrescine. L-methionine is converted to *S*-adenosyl-L-methionine (AdoMet) and then decarboxylated by another rate-limiting enzyme, *S*-adenosylmethionine decarboxylase (AdoMetDC) to form decarboxylated *S*-adenosylmethionine (dcAdoMet). dcAdoMet subsequently functions as an aminopropyl donor and donates its aminopropyl moiety to putrescine to form spermidine and 5′ methylthioadenosine (MTA) in a reaction catalysed by spermidine synthase. Another aminopropyltransferase reaction transfers a second aminopropyl moiety to spermidine to form spermine and MTA in a reaction catalysed by spermine synthase. During inter-conversion and degradation, spermidine and spermine are

first acetylated by cytosolic spermidine/spermine N^1-acetyltransferase (SSAT) using acetyl-CoA as a source of the acetyl group, to form N^1-acetylspermidine and spermine which is either exported or oxidized by N^1-acetylpolyamine oxidase (APAO) to spermidine and putrescine in a reaction that also yields H_2O_2 and 3-acetamidopropanal. Spermine can also be converted back to spermidine by spermine oxidase (SMO) that also produces *N*-acetyl-3aminopropanaldehyde and H_2O_2 (Casero and Pegg, 2009; Pegg, 2009).

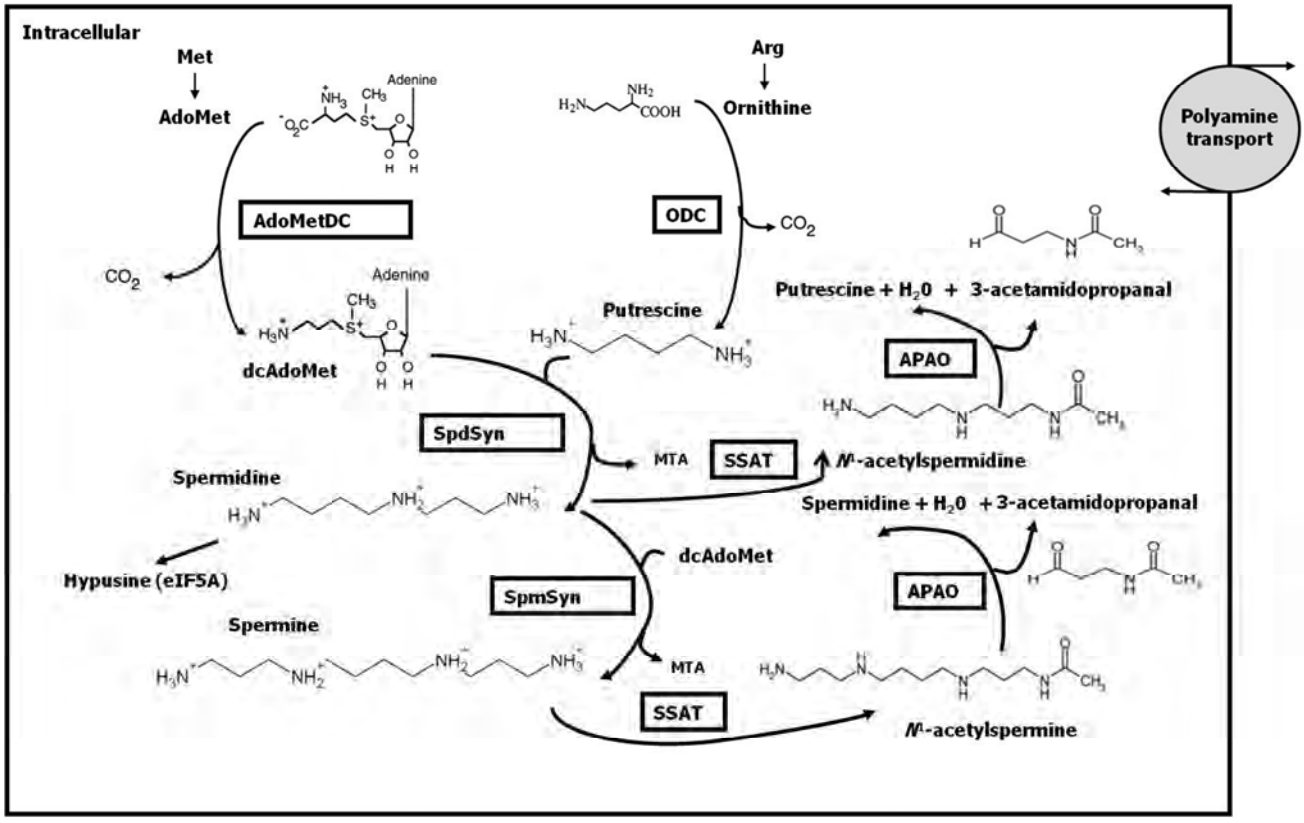

Figure 1.5: Polyamine metabolism in humans. Created from (Casero and Pegg, 2009). Putrescine, spermidine and spermine syntesis is indicated, catalysed by the enzymes ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine (SpdSyn) and spermine synthase (SpmSyn). The inter-conversion to oxidised polyamines as well as catabolic processes to aminopropylated forms of the polyamines and export is also indicated. *S*-adenosylmethionine (AdoMet), N^1-acetylpolyamine oxidase (APAO), spermidine/spermine N^1-acetyltransferase (SSAT) and 5' methylthioadenosine (MTA).

Additionally, spermidine also serves as a precursor for the amino acid hypusine [N^8-(4-amino-2-hydroxybutyl)lysine] that is derived from the aminobutyl moiety of spermidine and forms an integral part of the eukaryotic initiation factor 5A (eIF5A) (Pegg, 2009). The aminobutyl moiety of spermidine is attached to an internal lysine residue of eIF5a by deoxyhyposine synthase and hyposine is then formed by deoxyhyposine hydroxylase. Although the exact function of eIF5A has not yet been elucidated, it has been implicated in ribosomal protein synthesis, RNA transport and mRNA stability (Pegg, 2009).

1.2.2 *P. falciparum* parasites' polyamine metabolism

Polyamines have been shown to be a major metabolite present within malaria parasites, highlighting the importance of the polyamines to these rapidly dividing parasites (Teng *et al.*, 2009). There is a significant increase in polyamine levels in *P. falciparum*-infected RBCs (iRBCs) during the development of the parasite from trophozoite to schizont stages, reflecting the increased activities of the polyamine biosynthetic enzymes as well as increased macromolecular biosynthesis and replication (Fig. 1.6) (Assaraf *et al.*, 1984; Das Gupta *et al.*, 2005). At a total concentration of 10 mM inside the parasites, polyamines make up some 14% of the total metabolome of the intra-erythrocytic trophozoite-stage parasite. Spermidine is a major metabolite present in the trophozoite stage, present at an estimated 6 mM, followed closely by its precursor, putrescine (3 mM), with low amounts of spermine (0.5 mM) (Das Gupta *et al.*, 2005; Teng *et al.*, 2009). In contrast, human RBCs contain only trace amounts (5–50 μM) of polyamines (Gugliucci, 2004; Olszewski *et al.*, 2009) while serum polyamine levels only amount to 0.1 μM (Gugliucci, 2004).

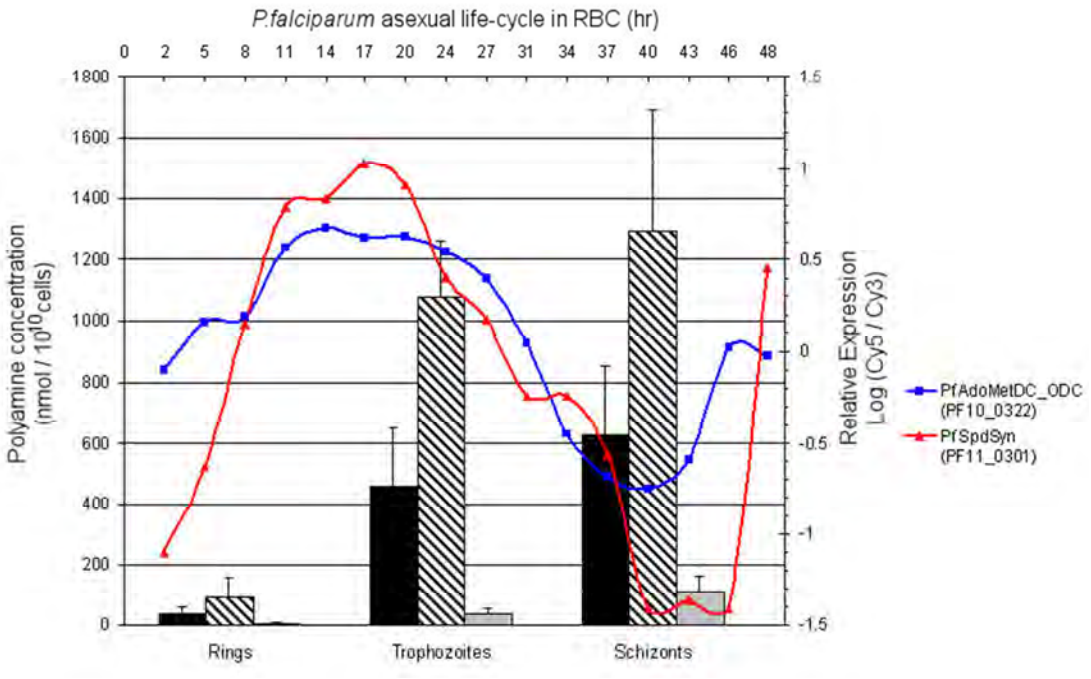

Figure 1.6: Composite of polyamine levels and polyamine biosynthetic enzyme levels (PfAdoMetDC/ODC and PfSpdSyn), during the intra-erythrocytic developmental cycle of *P. falciparum*. Compiled by Shaun Reeksting from (Assaraf *et al.*, 1984; Bozdech *et al.*, 2003; Das Gupta *et al.*, 2005). Bifunctional *P. falciparum* AdoMetDC and ODC (PfAdoMetDC/ODC), *P. falciparum* spermidine synthase (PfSpdSyn).

Polyamine synthesis in the human malaria parasite is much simpler than that of the human host and differs in several ways (Fig. 1.7). A single open reading frame encodes both ODC and AdoMetDC in a bifunctional protein consisting of 1419 amino acids with three domains:

residues 1-529 (N-terminal region) encode AdoMetDC, residues 530-804 form a linker region and residues 805-1419 (C-terminal) are homologous to known ODC sequences (Müller *et al.*, 2000). This bifunctional PfAdoMetDC/ODC decarboxylates both ornithine and AdoMet to form putrescine and dcAdoMet, from which spermidine is synthesised by spermidine synthase (PfSpdSyn) (Haider *et al.*, 2005). It has been suggested that the low levels of spermine present in the parasite are due to the fact that this enzyme can also transfer an aminopropyl moiety to spermidine to form spermine (Burger *et al.*, 2007; Haider *et al.*, 2005).

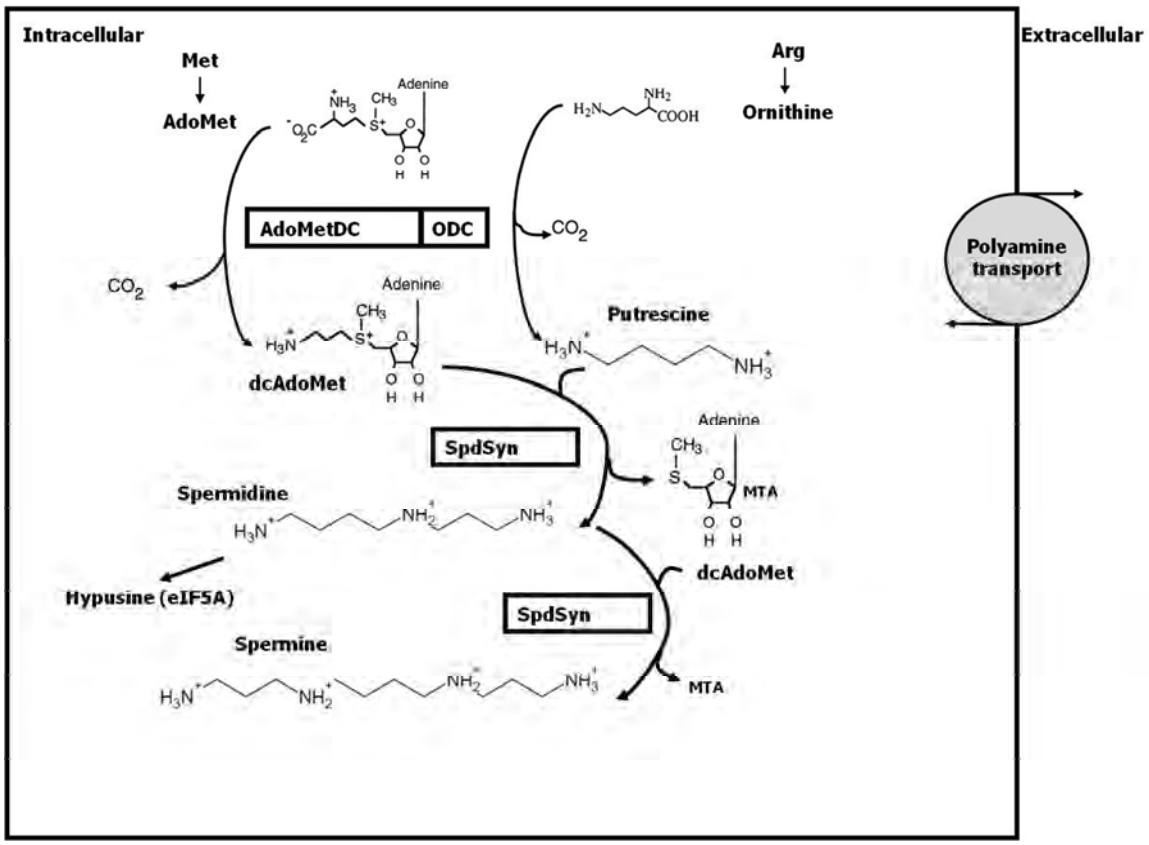

Figure 1.7: Schematic representation of polyamine metabolism in intra-erythrocytic *P. falciparum* parasites. Created from (Clark *et al.*, 2010). Bifunctional *P. falciparum S*-adenosylmethionine decarboxylase/ornithine decarboxylase (PfAdoMetDC/ODC) catalyses the production of putrescine and dcAdoMet, from which the production of both spermidine and spermine is catalysed by *P. falciparum* spermidine synthase (PfSpdSyn). The 5' methylthioadenosine (MTA) that is also formed by this reaction enters the methionine recycling pathway. Arginine (Arg), decarboxylated *S*-adenosylmethionine (dcAdoMet), eukaryotic initiation factor 5A (eIF5A), methionine (Met) and *S*-adenosylmethionine (AdoMet).

In mammalian cells, polyamine homeostasis is tightly controlled by biosynthesis, transport, and inter-conversion, which converts spermidine and spermine into putrescine via their acetylated forms (section 1.2.1) (Pegg, 2009). It has been proposed that the bifunctional nature of PfAdoMetDC/ODC in *P. falciparum* parasites is to allow tight control of polyamine biosynthesis

in the absence of other more obvious regulatory mechanisms such as those present in mammalian cells (Birkholtz *et al.*, 2004). The advantage of having the bifunctional PfAdoMetDC/ODC is that polyamine synthesis can be controlled by the regulation of a single protein (Wrenger *et al.*, 2001), since there is synchronised transcription and translation of the rate-limiting enzymes of the polyamine metabolic pathway (Müller *et al.*, 2001). While mammalian ODC is barely inhibited by putrescine (Kitani and Fujisawa, 1983), the ODC activity of the bifunctional malarial protein is susceptible to feedback inhibition by putrescine (Krause *et al.*, 2000). In addition, PfAdoMetDC/ODC has a half-life of more than two hours, which contrasts with the exceptionally short half-lives of the mammalian AdoMetDC and ODC (Müller *et al.*, 2001). Because of this unique bifunctional nature and implied unique regulatory systems PfAdoMetDC/ODC is considered to be a highly druggable protein (index of 0.8 out of 1) and ranks as one of the top 20 novel anti-malarial targets according to the World Health Organisation's Tropical Diseases Research Targets Database (http://tdrtargets.org) (Clark *et al.*, 2010). PfAdoMetDC/ODC seems to be essential to the parasite since numerous genetic validation strategies have indicated that null mutants are not viable (Müller *et al.*, 2008).

Merrell Dow synthesized the putrescine analogue DL-α-difluoromethylornithine (DFMO) in the late 1970s. DFMO acts as a 'suicide inhibitor' of ODC (Metcalf *et al.*, 1978), causing irreversible alkylation of the enzyme near or at the active site (Krause *et al.*, 2000). The fact that DFMO is effective in the treatment of West African trypanosomiasis (Marton and Pegg, 1995) led to it being tested as a possible chemotherapeutic agent for malarial infection. *In vitro*, DFMO prevented the progression of trophozoites to schizonts, although the development to ring form and merozoite invasion was not affected (Assaraf *et al.*, 1984). In addition, DFMO treatment led to the decreased synthesis of some proteins, partial inhibition of RNA synthesis and completely abrogated DNA synthesis (Assaraf *et al.*, 1987a). However, the effect of DFMO treatment on the parasite was only cytostatic arrest; exogenously added polyamines could reverse the DFMO inhibition, suggesting the presence of a polyamine uptake mechanism in the parasite (Assaraf *et al.*, 1987b). Although there are some preliminary data on polyamine uptake into iRBCs available (Ramya *et al.*, 2006), the mechanism of polyamine uptake by the parasite itself is unknown.

Table 1.2: Reversal of *in vitro* intra-erythrocytic *P. falciparum* parasites' growth inhibition with exogenous polyamines.

Drug	*In vitro* IC_{50}	Drug concentration	Inhibition reversal with polyamines	Parasite	Ref
AdoMetDC inhibitors					
5'-{[(*Z*)-4-amino-2-butenyl]methylamino}-50-deoxyadenosine (MDL73811 or AbeAdo)	1-3 μM	0-10 μM	Reversal with spermidine and spermine, not putrescine	*P. falciparum*	(Wright *et al.*, 1991)
ODC inhibitors					
3-aminooxy-1-aminopropane (APA)	1 μM	2 μM	Complete reversal of inhibition with 500 μM putrescine, but not spermidine	*P. falciparum* (3D7)	(Das Gupta *et al.*, 2005)
CGP52622A	2.7μM	5 μM	Complete reversal of inhibition with 500 μM putrescine, but not spermidine	*P. falciparum* (3D7)	(Das Gupta *et al.*, 2005)
CGP54169A	7.9 μM	5 μM	Complete reversal of inhibition with 500 μM putrescine, but not spermidine	*P. falciparum* (3D7)	(Das Gupta *et al.*, 2005)
DFMO	1.250 mM	10 mM	Reversal of inhibition with 100 μM putrescine, 400 μM cadaverine, 250 μM spermidine even in presence of inhibitor. 400 μM spermine and the diamine 1,3-diaminoproane had no effect.	*P. falciparum* (FCR-3)	(Assaraf *et al.*, 1987b) (Das Gupta *et al.*, 2005)
Dicyclohexylamine	97μM	20-200 μM	Partial reversal of inhibition with 800 μM spermidine within first 24h of treatment	*P. falciparum* NF54	(Kaiser *et al.*, 2001)

The inhibitory effect of other ODC inhibitors, which leads to decreased levels of putrescine, can also be reversed by the addition of exogenous putrescine and in certain cases spermidine (Table 1.2). Of these, the ODC inhibitors 3-aminooxy-1-aminopropane (APA), CGP52622A and CGP54169A are between 500-1300-fold more potent against *P. falciparum* cultures than the classical ODC inhibitor DFMO (Das Gupta *et al.*, 2005). Spermine usually has no antagonistic effect on ODC inhibition (Table 1.2) (Assaraf *et al.*, 1987b; Das Gupta *et al.*, 2005). As expected, AdoMetDC inhibition could not be reversed with exogenous putrescine but

spermidine and spermine could rescue parasites after 5'-{[(Z)-4-amino-2-butenyl]methylamino}-50-deoxyadenosine (MDL73811) inhibition (Das *et al.*, 1997; Das Gupta *et al.*, 2005; Kaiser *et al.*, 2003; Wright *et al.*, 1991). The inhibition of SpdSyn could also not be reversed with putrescine and spermidine (Table 1.2) (Haider *et al.*, 2005; Kaiser *et al.*, 2001).

A bis(benzyl) polyamine analogue, *N,N'*-bis{3-(phenylmethyl)aminolpropyl}-1,7-diaminoheptane (MDL27695), rapidly inhibits growth in both chloroquine-sensitive and chloroquine-resistant *P. falciparum* strains *in vitro* (Bitonti *et al.*, 1989). The administration of MDL27695 in combination with DFMO cures murine malaria caused by *P. berghei* and these mice were immune to re-challenge with sporozoites (Bitonti *et al.*, 1989). Bis(benzyl) polyamine analogues have been shown to inhibit putrescine and spermidine uptake in the filarial worm *Brugia pahangi* (Müller *et al.*, 1991). It is thus conceivable that the curative effect of DFMO in combination with MDL27695 against *P. berghei* is due to combined inhibition of biosynthesis and uptake. Furthermore, in *P. knowlesi*-infected RBCs, a putrescine conjugate N^1,N^4-bis-(7-chloroquinoline-4-yl)butane-1,4-diamine (BCBD) prevented nucleic acid synthesis and inhibited *in vitro* growth of these parasites (Singh *et al.*, 1997). This conjugate also prevented putrescine uptake in a dose-dependent manner. It was suggested that apart from displacing intracellular polyamines from their binding sites, this compound also acts as a putrescine uptake inhibitor (Singh *et al.*, 1997). The available data are consistent with the intra-erythrocytic *P. falciparum* parasite having the ability to take up polyamines; however, the exact mechanism by which the parasite achieves this is as yet uncharacterised.

1.3 Membrane transport

The translocation of metabolites across biological membranes is mediated by two classes of integral membrane proteins that are functionally distinct from one another, namely channels and transporters (Fig. 1.8) (Lodish *et al.*, 2000). Channels function as water-filled pores and create a membrane spanning diffusion pathway that allows appropriately sized and charged metabolites to travel down their electrochemical gradient. The opening and closing of the channels are regulated by physiological signals. Transporter proteins (also referred to as carriers or permeases) have highly specific substrate binding sites. Following binding of the substrate, the protein undergoes a conformational change that transports the bound substrate from one side of the membrane to the other. Transporters have the capacity to move solutes

against their electrochemical gradient whereas channels do not. There are various classes of transporters (Fig. 1.8). These include 1) primary active transporters, that couple the transport of a solute to the hydrolysis of a phosphodiester bond; 2) secondary active transporters, that couples the transport of solutes against their electrochemical gradient to the movement of one or more other solutes (typically Na^+ or H^+) down their electrochemical gradient; and 3) facilitative transporters that mediate the transport of substrates down their electrochemical gradient (facilitative diffusion) (Kirk, 2004; Staines *et al.*, 2010).

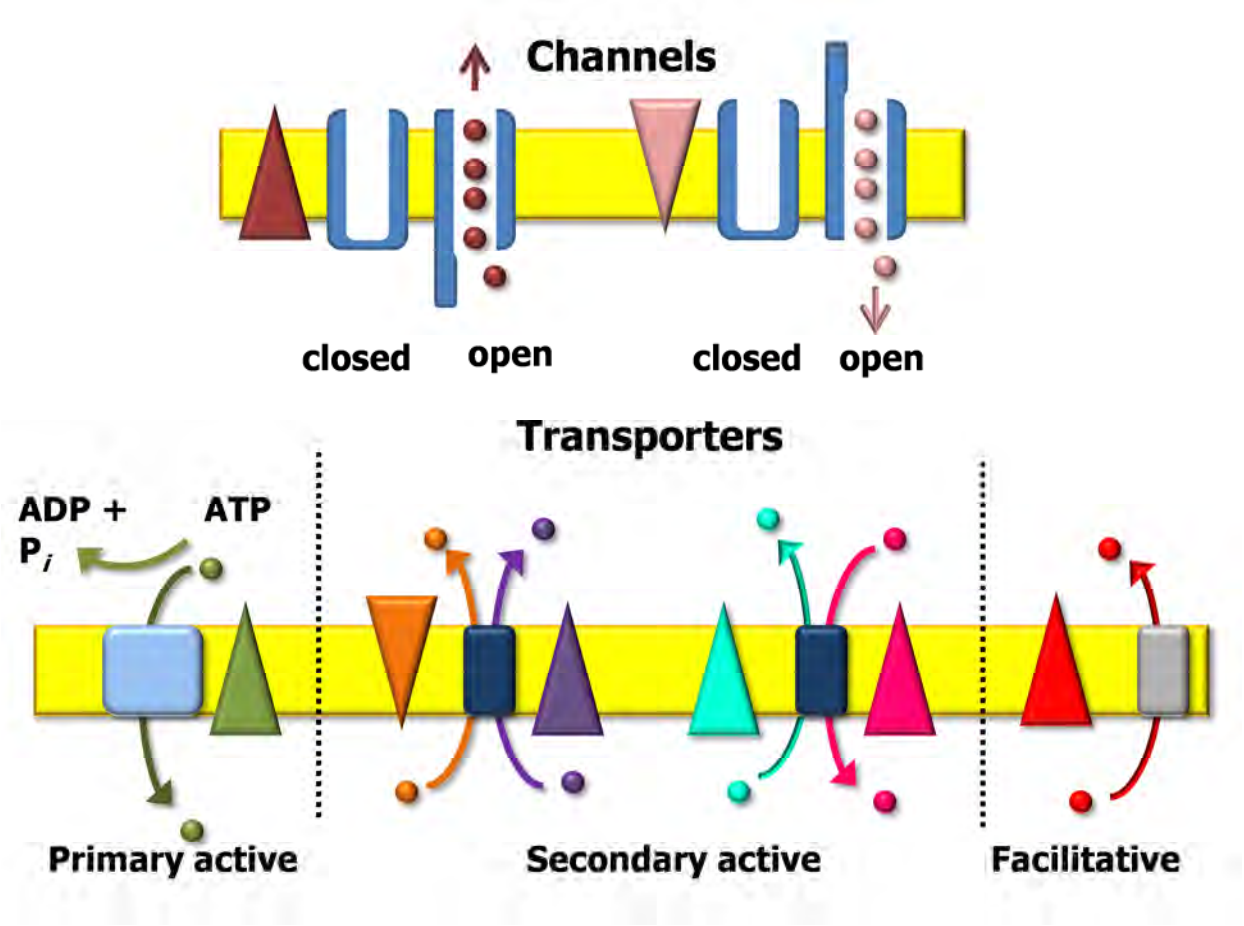

Figure 1.8: Schematic representation of membrane transport proteins. Channels facilitate the movement of molecules down their electrochemical gradient. Transporters consist of three classes, namely primary active transporters that couple the movement of molecules against their electrochemical gradient to the hydrolysis of a phosphodiester bond; secondary active transporters that couple the movement of a molecule against its electrochemical gradient to the movement of another molecule down its electrochemical gradient and facilitative transporters that allow the movement of a molecule across the membrane down its electrochemical gradient. The triangles indicate the direction of the concentration gradient, electrical potential or both. Compiled from (Lodish *et al.*, 2000).

1.4 Polyamine transport

1.4.1 General

Polyamine transport plays a major role in the regulation of intracellular polyamine levels in mammalian and yeast cells (Moinard *et al.*, 2005; Uemura *et al.*, 2007; Wallace *et al.*, 2003). It has been characterised at a biochemical level in various cells, but for most organisms the molecular identification of the proteins involved has not yet been achieved. Polyamine transporter proteins have been identified in bacterial cells and yeast (Igarashi and Kashiwagi, 2010) and for the parasites *Leishmania major* (Hasne and Ullman, 2005) and *Trypanosoma cruzi* (Hasne *et al.*, 2010). In mammalian cells, two possible polyamine transporters have been identified. One of these, SLC3A2, is thought to export putrescine and mono-acetylated spermidine in exchange for arginine uptake in colon epithelial cells (Uemura *et al.*, 2008), as well as being responsible for putrescine uptake in the gastrointestinal tract when tissue polyamine levels are low (Uemura *et al.*, 2010). The human cation-Cl^- co-transporter, CC9A has also been shown to promote polyamine transport in human embryonic kidney 293 (HEK-293) cells (Daigle *et al.*, 2009). The positive charge these organic cations carry at physiological pH prevents passive sequestration by cells and as such the process is generally carrier-mediated, saturable and energy-dependent (Igarashi and Kashiwagi, 2010). In addition, polyamines can also be transported against concentration gradients (Reguera *et al.*, 2005; Seiler *et al.*, 1996). Although some cells have a single transporter for putrescine, spermidine and spermine, such as the hamster melanoma cell line AMEL-3 cells (García-Fernández *et al.*, 2005), other cells such as Chinese hamster ovary (CHO) cells (Xie *et al.*, 1997) have separate transporters for putrescine on the one hand and spermidine and spermine on the other (Marton and Pegg, 1995; Reguera *et al.*, 2005; Seiler *et al.*, 1996; Wallace *et al.*, 2003).

1.4.2 Polyamine transport in multi-cellular organisms

1.4.2.1 Biochemical characterisation of polyamine transport in cells from multi-cellular organisms

The biochemical characteristics of a diamine exporter (DAX) from CHO cells were investigated by Xie and co-workers using inverted membrane vesicles (Xie *et al.*, 1997). Putrescine export via this system was shown to be saturable and pH dependent (Xie *et al.*, 1997). Since export

increased when the vesicular pH was lower than the medium pH, it is possible that putrescine transport occurs via a proton anti-port mechanism in these cells. ATP did not have an effect on putrescine export in this inverted membrane vesicles system. Charge was postulated to play a major role in substrate recognition by DAX, since spermidine (+3), spermine (+4) and acetylated putrescine (+1) had no effect on putrescine transport, while N^1-acetylspermidine (+2) and various diamines (1,3- and 1,5-diaminopentane; 1,6-diaminohexane; 1,7-diaminoheptane) inhibited putrescine export (Xie *et al.*, 1997). Potassium ionophores had no effect on putrescine export, leading the authors to postulate that DAX catalyses putrescine export through a non-electrogenic mechanism (Xie *et al.*, 1997). Additionally, there is an apparent functional relationship between polyamine transporters in CHO and multi-drug-resistance transporters (Aziz *et al.*, 1998).

Various studies on polyamine transport have been performed using *Xenopus laevis* oocytes. Polyamine levels were found to be ~900 μM for putrescine, ~800 μM for spermidine and ~200-400 μM for spermine (Fukumoto and Byus, 1997). By injecting radiolabelled spermidine into the oocytes, Sha *et al.* showed that spermidine export is dependent on the membrane potential, with increased efflux observed upon membrane depolarisation. This electro-diffusive efflux of spermidine was blocked by Ba^{2+} and Ca^{2+} ions, consistent with the involvement of a simple membrane cation channel. No Na^+ or K^+ co- or counter-transport was observed (Sha *et al.*, 1996). Kinetic analyses showed that putrescine uptake into *X. laevis* oocytes had two distinct components, but the authors were unable to distinguish between a saturable system with two different affinities or a saturable system with a non-saturable component (Fukumoto and Byus, 1997). Putrescine export was energy dependent and could occur against the concentration gradient (Fukumoto and Byus, 1997).

Polyamine transport in RBCs has been studied using radiolabelled polyamines (Fukumoto and Byus, 1996). Putrescine uptake (K_m=21 μM) had both non-saturable (indicative of diffusion) as well as saturable components and was pH and temperature dependent. Spermidine uptake was shown to be saturable (K_m=13 μM), with little dependence on pH, although a strong temperature dependence was observed. Extracellular polyamines present at 10x the spermidine concentration had no statistically significant effect on spermidine uptake (Fukumoto and Byus, 1996). These results suggest that there are different transport mechanisms for putrescine and spermidine in RBCs, with a diffusive process mediating a portion of putrescine uptake (Fukumoto and Byus, 1996).

Polyamine transport was also determined in the melanoma cell line AMEL-3 derived from hamsters (García-Fernández *et al.*, 2005). The uptake of putrescine (K_m=3 μM), spermidine (K_m=4 μM) and spermine (K_m=5 μM) was found to be saturable, temperature, time and pH dependent, with an optimum extracellular pH of 7.4. Metabolic inhibitors severely reduced putrescine uptake, indicating that import is an energy-requiring process. Sodium ionophores inhibited putrescine uptake, but whether this was due to Na^+ dependence or the disruption of the membrane potential was unclear. It was found that putrescine uptake was inhibited by some 90% in the presence of the oxidising agent *N*-ethylmaleimide. This inhibition was overcome by the addition of reducing agents, indicating the possible need for sulfhydryl groups for polyamine transport. Putrescine transport was inhibited both by spermidine and spermine, as well as a variety of diamines, and extracellular putrescine inhibited spermidine and spermine uptake. This indicates a shared polyamine transport system in AMEL-3 cells that is able to recognise a wide variety of polyamine-like compounds (García-Fernández *et al.*, 2005).

Spermidine (K_m=6 μM) and spermine (K_m=4 μM) uptake in *Drosophila* S2 cells was found to be temperature dependent, saturable and pH dependent with higher uptake occurring at high pH (Romero-Calderón and Krantz, 2006). Neither putrescine nor cadaverine had a significant effect on spermidine or spermine uptake, which may suggest the presence of separate transporters for putrescine and spermidine/spermine these cells. Neither Na^+, K^+, Cl^- nor Ca^{2+} was necessary for spermidine and spermine uptake in S2 cells. An H^+-specific ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), which disrupted the H^+ gradient across the plasma membrane while maintaining the extracellular pH, immediately decreased spermine uptake. Depolarisation of the mitochondrial plasma membrane and inhibition of ATP synthesis had a negative effect on spermine uptake. Spermine uptake was proposed to occur via a polyamine/H^+ anti-porter activity as it was dependent on the H^+ gradient across the plasma membrane and increased in an alkaline environment (Romero-Calderón and Krantz, 2006).

1.4.2.2 Molecular identification of polyamine transport proteins in multi-cellular organisms

Very little is known regarding the proteins mediating the uptake of polyamines into the cells of multi-cellular organisms. SLC3A2 (a member of the solute carrier family) is highly expressed in putrescine tolerant CHO cells (Uemura *et al.*, 2008). In a human colon cancer cell line, SLC3A2 mediates putrescine and acetylpolyamine efflux in exchange for arginine. SLC3A2 co-precipitates with SSAT and it is possible that this interaction facilitates the export of acetylated

polyamines (Uemura *et al.*, 2008). Under conditions of high extracellular and low intracellular putrescine, SLC3A2 can also function as a polyamine importer (Uemura *et al.*, 2010).

In HEK-293 cell, CCC9a (or SLC12A8), a human splice variant of the CCC9 cation-Cl^- cotransporter (CCC), was found to facilitate the transport of polyamines and amino acids (Daigle *et al.*, 2009). CCC9 is expressed both at the surface and in intracellular compartments. Spermidine and spermine uptake was higher than that of putrescine, and uptake was independent of Na^+, K^+ Cl^- or Ca^{2+}, inhibited by pentamidine and the CCC inhibitor furosemide (Daigle *et al.*, 2009).

1.4.2.3 An alternative mechanism for polyamine uptake into cells: possible role of endocytosis

Multi-cellular organisms display a variety of proteins on their cell surfaces and these allow interaction with other cells, ligands or the extracellular matrix (Belting *et al.*, 2003). Proteoglycans such as the surface protein glypican-1 (Gpc-1) may be receptors for polyamine uptake in mammalian cells, with polyamines binding to cell-surface glypicans through electrostatic interactions with the heparin sulfate side chains (Belting *et al.*, 2003). The receptor-polyamine complex then undergoes endocytosis via the non-classical caveolae-dependent pathway. Once inside the vesicle, nitric oxide (NO) is released from Gpc-1–*S*-nitroso groups and disrupts the interaction between Gpc-1 and the transported polyamines, which are subsequently released into the cytosol while the Gpc-1 receptor is recycled to the plasma membrane (Belting *et al.*, 2003). Caveolin-1 stabilizes caveolae structures, thereby negatively regulating cavelolae-dependent endocytosis, while NO is produced by nitric oxide synthase (NOS2) (Uemura *et al.*, 2010). Caveolin-1 (a negative regulator of caveolae-dependent endocytosis) knock-down and the knock-out of NOS2 in mice led to increased caveolae-dependent endocytosis and increased intracellular putrescine accumulation, while the lack of NO production diminished putrescine uptake (Uemura *et al.*, 2010). However, independent investigations of role of heparin sulphate in polyamine uptake could not conclusively validate an interaction between spermine and heparin sulphate under physiological conditions (Hoshino *et al.*, 2005). Furthermore, while Spd-C_2-BODIPY {*N*-(4,4-difluoro-5-7-dimethyl-4-bora-3a,4a,-diaza-s-indacene-3-propionyl)-*N*-(*S*-(spermidine-(N^4-ethyl)thioacetyl)ethylenediamine), a fluorescent spermidine derivative (Soulet *et al.*, 2004), co-localised with fluorescent probes of recycling endosomes, CHO mutant cells, defective in receptor-mediated endocytosis, were still able to accumulate these fluorescent probes,

indicating that endocytosis is not responsible for polyamine uptake into these cells (Soulet *et al.*, 2002).

1.4.3 Polyamine transport in bacterial cells

Polyamine transport systems have been best characterised in *E. coli*. *E. coli* polyamine transport occurs through five mechanisms (Fig. 1.9). A spermidine-preferring mechanism (PotABCD) and a putrescine-specific mechanism (PotFGHI) both consist of 4 proteins: an inner plasma membrane membrane-associated ATPase that provides energy for the transfer, two channel-forming transmembrane proteins (porins) and a substrate-binding protein that is located in the periplasm (Fig. 1.9). A third transport mechanism (PotE), catalyse both the membrane potential-dependent uptake of putrescine and the excretion of putrescine, which is dependent on the exchange reaction between intracellular putrescine and extracellular ornithine (Igarashi and Kashiwagi, 1999, 2001). A fourth mechanism, PuuP is responsible for putrescine uptake and is dependent on the proton motive force (Kurihara *et al.*, 2009). A fifth mechanism consists of a protein complex MdtJI, formed by MdtJ and MdtI, and is responsible for spermidine excretion (Higashi *et al.*, 2008) (Fig. 1.9).

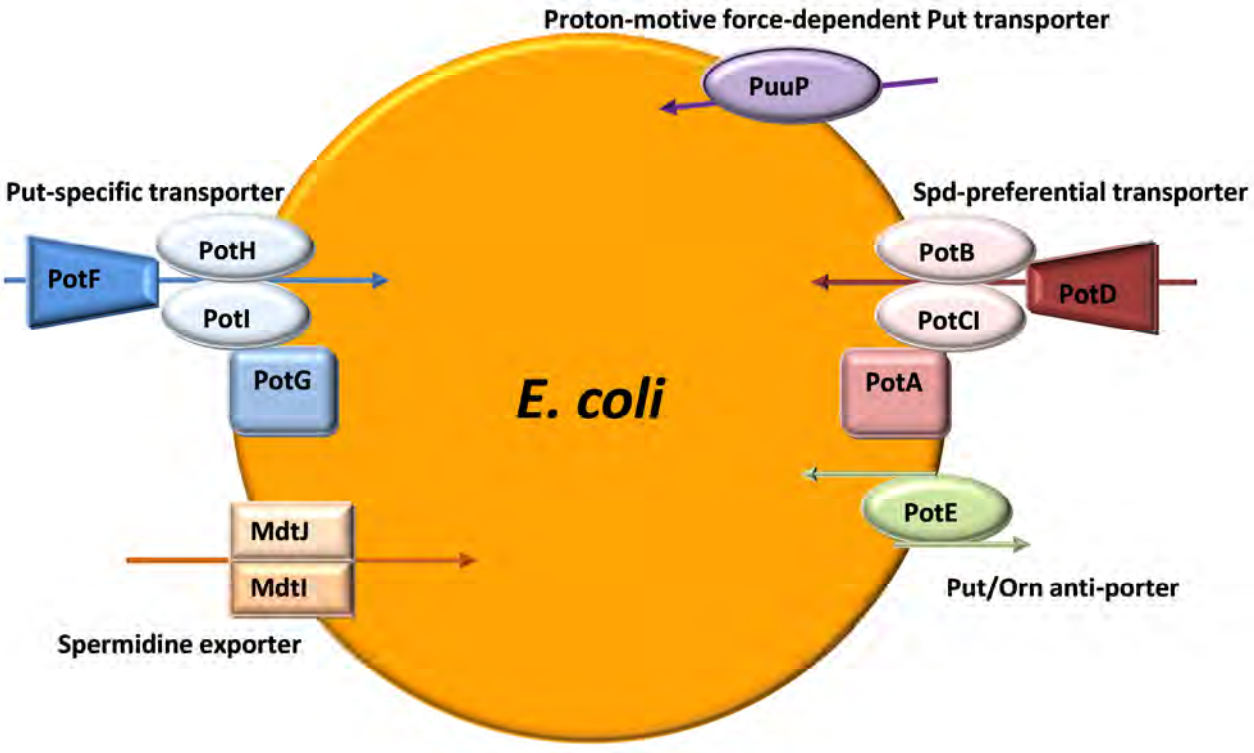

Figure 1.9: Schematic representation of the polyamine uptake and export systems in *E. coli*. Putrescine uptake is mediated by PotFGHI, PuuP and PotE, and putrescine export by PotE. Spermidine uptake is mediated by PotABCD, while the MdtJI complex is responsible for spermidine export. Compiled from (Higashi *et al.*, 2008; Igarashi and Kashiwagi, 2010; Kurihara *et al.*, 2009).

In the spermidine-preferring mechanism (PotABCD), PotA (43kDa) encodes the ATPase, PotB (31 kDa) and PotC (29 kDa) are the porins and PotD (39 kDa) is responsible for substrate recognition, with K_m values of 0.1 μM for spermidine and 2 μM for putrescine (Igarashi and Kashiwagi, 2001). Six transmembrane segments have been predicted for PotB and PotC, which are also associated with PotA. The X-ray structure of a PotD-spermidine complex indicates that there is only one polyamine-binding site on PotD (Igarashi and Kashiwagi, 2001). In addition, the substrate-binding site is situated in a cleft between two domains with β-α-β topology that enables an open-close movement upon substrate binding. In the polyamine-binding site, the carbon-hydrogen backbone of the substrate is anchored by van der Waals interactions, while the protonated nitrogen atoms are recognised by acidic residues (Fig. 1.10) (Igarashi and Kashiwagi, 1999). Polyamine uptake by this system is regulated by both PotD and spermidine that inhibits transcription of the *potABCD* operon (Igarashi and Kashiwagi, 1999, 2001).

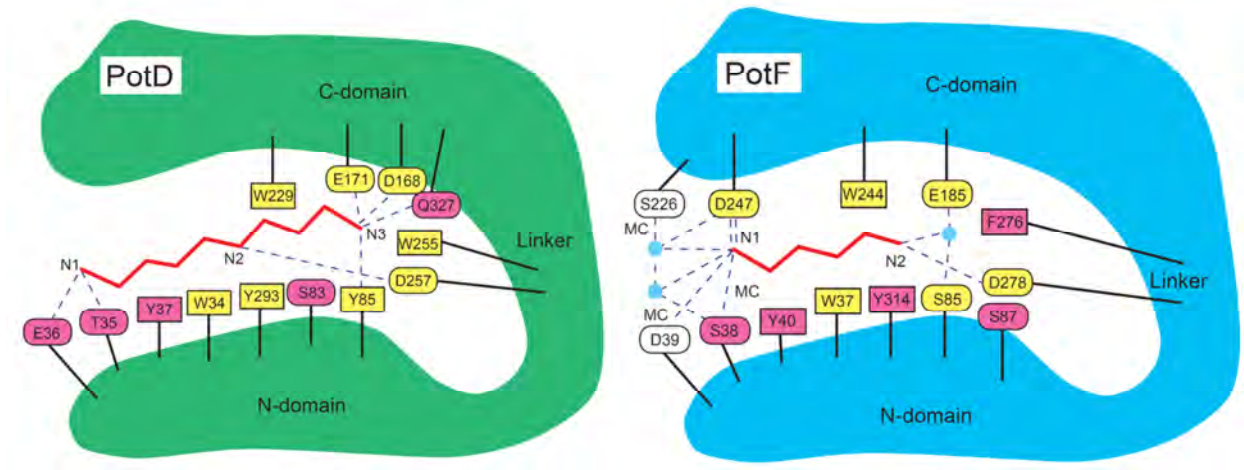

Figure 1.10: Schematic representation of the mechanism of polyamine recognition of PotD and PotF, taken from (Igarashi and Kashiwagi, 1999). The NH_2 and NH groups of polyamines are recognised by acidic Asp and Glu, while the propyl and butyl groups have interactions with Trp and Tyr. This figure is freely available for academic purposes from the journal.

The putrescine-specific system (PotFGHI) is formed by the 45 kDa PotG (membrane-associated ATPase), the 31-35 kDa porins, PotH and PotI and the 370 amino acid, 38 kDa putrescine-specific PotF (Igarashi and Kashiwagi, 1999). Both PotD and PotF are periplasmic binding proteins, but while the PotABCD system can recognise both spermidine and putrescine, this system is putrescine specific (K_m=0.5 μM), possibly due to the unique hydrogen-bond network formed with the substrate that is mediated by water molecules (Fig. 1.10) (Igarashi and Kashiwagi, 1999). PotF has similar domain architecture to PotD, with α-helices flanking the five-stranded mixed β-sheet domains. The putrescine-binding site is formed by the interface of

the two domains, with multiple residues from both domains involved in the polar and van der Waals interactions necessary for putrescine binding. The open-closed movement mediated by substrate binding is thought to be due to substrate binding first to the one domain, followed by stabilisation of the closed conformation through interactions with the second domain (Igarashi and Kashiwagi, 1999, 2001; Vassylyev *et al.*, 1998).

The third polyamine transporter, PotE, is a member of the amino acid/polyamine/organocation (APC) superfamily (Igarashi and Kashiwagi, 1999; Kashiwagi *et al.*, 2000). Both the N- and C-termini of this 46 kDa protein is located in the cytoplasm and its 12 transmembrane helices are connected by hydrophilic segments. This protein is responsible for both putrescine uptake, which is dependent on the membrane potential (K_m=2 μM), and putrescine excretion through the putrescine/ornithine anti-porter activity (K_m=73 μM) (Kashiwagi *et al.*, 2000). The uptake of putrescine is very specific, with no inhibition by spermidine, spermine, ornithine or cadaverine. Glu residues in cytoplasmic, hydrophilic loops might be involved in putrescine binding (Igarashi and Kashiwagi, 1999; Kashiwagi *et al.*, 2000). Further analyses indicated that amino acids from transmembrane helices 3, 6, 7 and 12, as well as from loops between helices 2-3, 6-7 and 8-9 in the cytoplasm are located near each other in the tertiary structure and are involved in substrate binding (Kashiwagi *et al.*, 2000). Loop 7-8 in the periplasm consists of a predicted α-helix, surrounded by hydrophobic regions and it also involved in PotE function. It has been suggested that this loop, like loops 2-3 and 6-7, are involved in the formation of the binding site by entering the channel of the transporter (Minchin and McCoubrie, 2004).

The fourth transporter, PuuP, is a proton motive force dependent putrescine transporter, and has similar kinetic parameters to PotFGHI and PotABCD, with a K_m of 4 μM (Fig. 1.9) (Kurihara *et al.*, 2009). PuuP was identified as part of the Puu putrescine degradation pathway and the expression of PuuP can be induced by putrescine (Kurihara *et al.*, 2009).

The fifth transporter, the MdtJI protein complex formed by MdtJ and MdtI (Fig. 1.9), is part of the small multi-drug resistance (SMR) family and is responsible for the export of spermidine at neutral pH (Higashi *et al.*, 2008). MdtJI mRNA is increased by high levels of exogenous spermidine, presumably to off-set the higher uptake of spermidine under these circumstances. As part of the SMR family, MdtJI is predicted to have four transmembrane regions. Spermidine recognition occurs via interaction between Asp ans Glu residues with the NH_2 and NH groups of

spermidine, while Trp and Tyr residues interact with the butyt and propyl groups of spermidine, similarly to what was seen for PotD and PotF (Fig. 1.11) (Higashi *et al.*, 2008).

Although prokaryotic polyamine transport has been mostly characterised in *E. coli*, other bacterial species' polyamine uptake has also been investigated. Functional genomic analyses of *Streptococcus pneumoniae* revealed genes with homology to polyamine transporters (Hoskins *et al.*, 2001). Further studies indicated that these genes encode products similar to the *E. coli* PotABCD system and these gene products were also vital for the overall pathogenesis of the microorganism (Ware *et al.*, 2006). A spermidine-preferential ABC transporter system has also been found in *Pseudomonas aeruginosa* (Lu *et al.*, 2002).

1.4.4 Polyamine transport in yeast cells

Multiple polyamine transport systems have been extensively characterised in the yeast *Saccharomyces cerevisiae* (Igarashi and Kashiwagi, 1999). Several proteins have been localised to yeast vacuolar membranes, where it is thought that they allow excretion of polyamines from the yeast cytoplasm into vacuoles in order to protect the cells from polyamine toxicity (Tomitori *et al.*, 2001) (Fig. 1.11). *S. cerevisiae* polyamine uptake has K_m values of 5-100 μM for both spermidine and spermine and is energy dependent (Igarashi and Kashiwagi, 1999). TPO1 (**t**ransporter for **po**lyamine **1**) is a 586 amino acid protein that has 12 transmembrane helices and is a member of the yeast major facilitator superfamily. It contains 3 glutamine residues (Glu-207, Glu-323/324 and Glu-574) that have been shown to correlate with polyamine binding residues in PotE (Igarashi and Kashiwagi, 1999). Using TPO1 as query sequence, three more yeast vacuolar polyamine transporters were identified (TPO2, TPO3, and TPO4) and are also members of the major facilitator superfamily (Tomitori *et al.*, 2001). These proteins all contain 12 transmembrane helices, a high percentage of threonine and serine residues (indicating possible kinase regulation) and a long N-terminus (Tomitori *et al.*, 2001). It was shown that all 4 transporter proteins are induced by the presence of polyamines in the culturing medium and are able to recognise and provide resistance to the polyamine analogue and biosynthesis inhibitor, methylglyoxal bis(guanylhydrazone) (MGBG), possibly by transporting the analogue into polyamine containing vesicles. Uptake studies indicated that TPO1 and TPO4 transport both spermidine and spermine, while TPO2 and TPO3 recognise spermine (Igarashi and Kashiwagi, 1999; Tomitori *et al.*, 2001).

Further studies have indicated that these transporter proteins are located on the plasma membrane when expressed from a single copy vector or the chromosome, but localise to both plasma and vacuolar membranes upon over-expression of the proteins from multi-copy expression vectors (Uemura *et al.*, 2005b). Additionally, polyamine analogues like paraquat are exported from yeast cells expressing these proteins. These results indicate that TPO1 may have functional similarity to PotE, since it can catalyse both the uptake of polyamines at pH>7 and the excretion of polyamines at acidic pH. Protein kinases 1 and 2 are needed for the localisation of TPO1 to the plasma membrane (Uemura *et al.*, 2005b).

In addition to the TPO proteins, several other polyamine transport mechanisms have been described in *S. cerevisiae* (Fig. 1.11). *AGP2* encodes a 12 transmembrane, plasma membrane protein of the APC superfamily, Agp2p (Aouida *et al.*, 2005). It was initially classified as a carnitine transporter, but it was found that *S. cerevisiae* cells defective in this gene was resistant to polyamine analogue toxicity, implying that Agp2p may be involved in polyamine import into yeast cells (Aouida *et al.*, 2005). Agp2p is able to transport spermidine (K_m=15 μM) and to a lesser extent putrescine into yeast cells and is vitally important for the maintenance of intracellular polyamine levels upon biosynthesis inhibition (Aouida *et al.*, 2005).

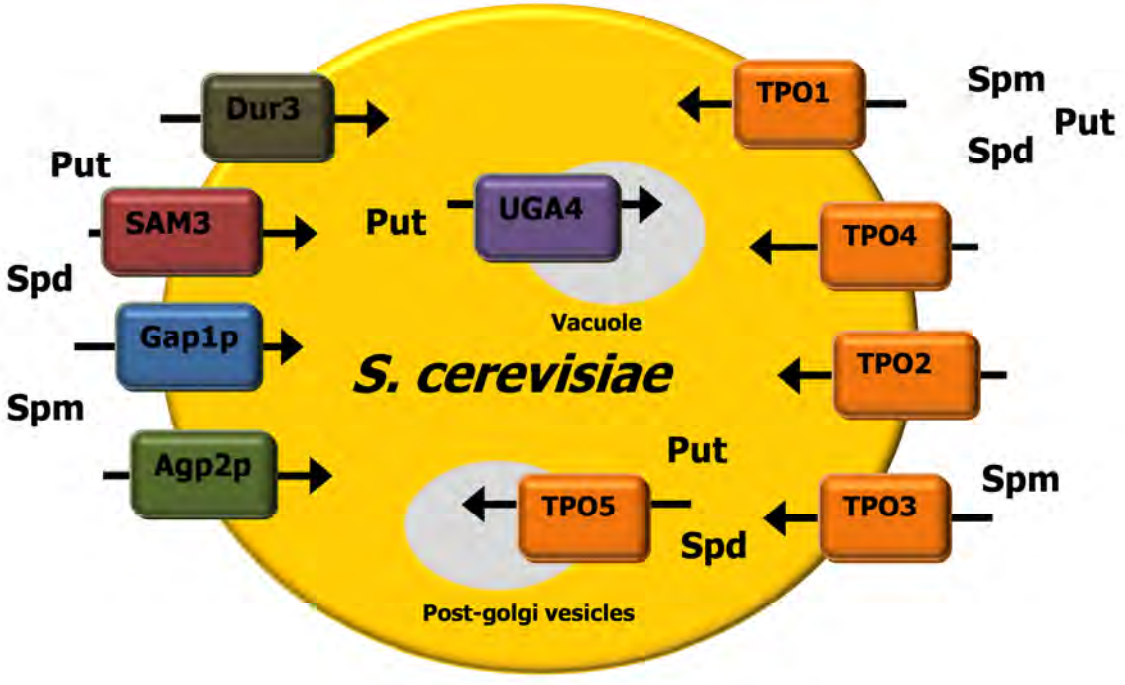

Figure 1.11: Polyamine import and export in *S. cerevisiae.* Polyamine (putrescine (put), spermidine (spd) and spermine (spm)) transport in *S. cerevisiae* is mediated by a variety of transporter proteins. DUR3 and SAM3, as well as Gap1p and Agp2p are responsible for polyamine uptake, while excretion is mediated by TPO1-4 on the plasma membrane and TPO5 on Golgi vesicles. UGA4 can also protect against toxic levels of polyamines by transporting putrescine into vacuoles (Uemura *et al.*, 2007). Compiled from (Uemura *et al.*, 2007).

The *S. cerevisiae* vacuolar γ-aminobutyric acid transporter, UGA4, is a member of the amino acid/polyamine/organocation family of transporters (Fig. 1.11) (Uemura *et al.*, 2004). Putrescine (K_m=700 μM), but not spermidine or spermine, can also be transported by this protein. Spermidine and spermine inhibit putrescine transport via this protein. It is postulated that the aminopropyl group of polyamines is recognised by UGA4 but due to steric hindrances, only putrescine can be transported by UGA4 (Uemura *et al.*, 2004). Additionally, due to the vacuolar location of this protein, it is possible that it ensures that stores of putrescine are available to *S. cerevisiae* during times of stress. Alternatively, the vacuolar segregation of putrescine may help protect yeast against polyamine toxicity (Uemura *et al.*, 2004).

A fifth *S. cerevisiae* polyamine transport protein, TPO5, was identified based on its similarity to UGA4 (Tachihara *et al.*, 2005). It excretes both putrescine and spermidine, but is not affected by basic amino acids such as lysine or by compounds such as ornithine. This protein is located on Golgi or post-Golgi secretory vesicles and may allow accumulation of polyamines in these vesicles prior to excretion (Tachihara *et al.*, 2005).

The general amino acid permease, Gap1p, is a member of the APC family of transporters and is located on the *S. cerevisiae* plasma membrane (Fig. 1.11) (Uemura *et al.*, 2005a). Apart from a range of amino acids, this protein can also transport structurally related compound such as putrescine (K_m=390 μM) and spermidine (K_m=21 μM) but not spermine, with similar affinities for spermidine and basic amino acids. Ornithine, GABA and spermine all inhibited putrescine uptake. Furthermore, *GAP1* mRNA levels are induced by putrescine, spermidine and spermine. As such, this protein, while not specific for polyamines, plays a part in maintaining optimal intracellular polyamine levels (Uemura *et al.*, 2005a).

An analysis of various transporter proteins with at least 12 transmembrane helices identified a further two plasma membrane proteins that preferentially transport polyamines (Fig. 1.11) (Uemura *et al.*, 2007). DUR3, a urea transporter, transports both putrescine (K_m=479 μM) and spermidine (K_m=21 μM) with a higher affinity than for urea, and is not influenced by amino acids. SAM3 (*S*-adenosylmethionine transporter) is a member of the APC superfamily of transporters and recognise both spermidine (K_m=21 μM) and AdoMet to the same extent, and putrescine uptake (K_m=433 μM) was inhibited by both glutamic acid and lysine. Additionally, both SAM3 and DUR3 led to higher polyamine uptake than Agp2p and GAP1 (Uemura *et al.*, 2007).

Putative polyamine transporter proteins have also been characterised for other yeast species. In *Candida albicans* the *GPT1* (**G**ABA/**p**olyamine **t**ransporter) gene was identified during a complementation experiment aimed at the identification of polyamine biosynthesis enzymes (McNemar *et al.*, 2001). This 553 amino acid protein contains 9 transmembrane helices and has homology to a GABA specific transporter. It was shown that in mutated cells, expression of GPT1 restores the transport of GABA (McNemar *et al.*, 2001). In addition, this protein also allowed the survival of mutant cells lacking polyamine biosynthesis. It was postulated that *GPT1* encodes a transporter that has the ability to both transport polyamines and GABA, based on its structural similarity to putrescine (McNemar *et al.*, 2001).

1.4.5 Polyamine transport in parasitic protozoa

Although polyamine transport has been characterised at a biochemical level in various parasitic organisms, the molecular identification of the proteins involved has been less successful than the molecular identification of polyamine transport proteins in bacteria and yeast. To date, only the *L. major* polyamine permease specific for putrescine and spermidine (LmPOT1) (Hasne and Ullman, 2005), and a *T. cruzi* putrescine/cadaverine transporter (TcPOT1.1) (Hasne *et al.*, 2010) (Fig. 1.12), have been identified.

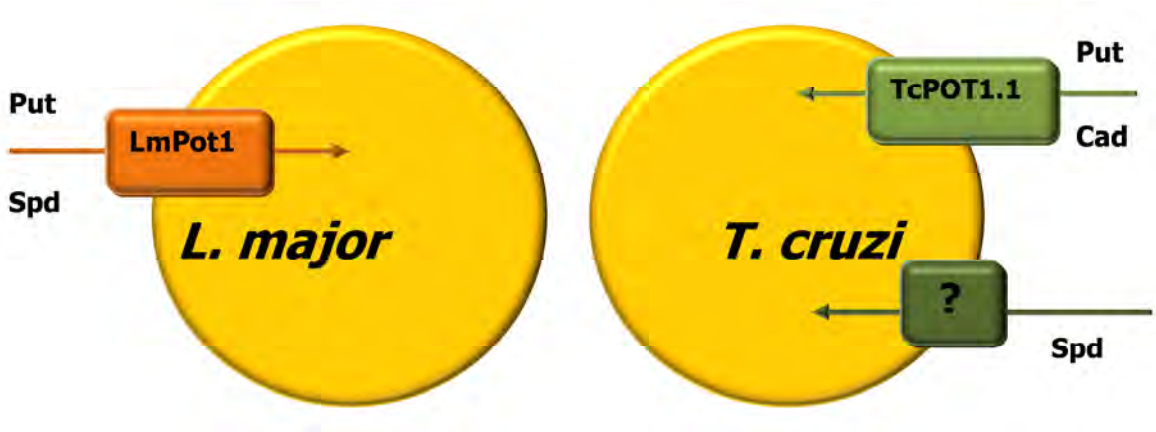

Figure 1.12: Polyamine transporters in parasitic protozoa. LmPot1 is responsible for putrescine (Put) and spermidine (Spd) uptake in *L. major*, while TcPot1.1 is responsible for putrescine and cadaverine (Cad) uptake in *T. cruzi*. The molecular identity of the spermidine transporter of *T. cruzi* is unknown. Compiled from (Hasne *et al.*, 2010; Hasne and Ullman, 2005).

In *Leishmania*, expression of the polyamine transport systems is often stage-specific, with different polyamine transport systems for the promastigote (extracellular infectious form in insect vector) and amastigote (intracellular form in human macrophages) stages (Basselin *et*

al., 2000). The promastigotes of *L. donovani* and *L. mexicana* and the mammalian stage amastigotes were investigated in terms of polyamine transport (Basselin *et al.*, 2000). Polyamine transport in both developmental stages is temperature and pH dependent (reflecting the proton motive force dependence) as well as saturable, and independent of Na^+ ions. Spermidine, spermine and cadaverine inhibited putrescine transport non-competitively, while putrescine and spermine inhibited spermidine transport also in a non-competitive manner. This, together with the fact that the K_i of spermidine for the putrescine transport process differed significantly from the K_m of spermidine, and vice versa, indicated that there are two different transporters with different substrate specificities (Basselin *et al.*, 2000).

LmPOT1 was identified in *Leishmania* on the basis of sequence homology with mammalian amino acid transporter sequences (Hasne and Ullman, 2005). It is an 803 amino acid protein with 9-12 transmembrane domains and a member of the APC superfamily with many of the characteristics of the subgroup L-type amino acid transporter family and a putative calmodulin-binding motif. Heterologous expression in *X. laevis* oocytes indicated that putrescine and spermidine (but not spermine, arginine, agmatine or pentamidine), are transported by LmPOT1 (Fig. 1.12) (Hasne and Ullman, 2005). Various ionophores, such as the proton ionophore CCCP, Na^+ ionophore monensin or Ca^{2+} ionophore A23187, had an inhibitory effect on polyamine uptake. The K^+ ionophore, valinomycin, had no significant inhibitory effect. Since Na^+ is not needed for polyamine transport by LmPOT1, the inhibitory effect of monensin is likely due to the disruption of the membrane potential. Immunofluorescence studies indicated that LmPOT1 localises to the cell surface of *L. major* promastigotes. LmPOT1 was the first eukaryotic surface polyamine transporter characterised (Hasne and Ullman, 2005).

T. cruzi is auxotrophic for putrescine and cadaverine uptake for survival since it lacks ODC and therefore cannot synthesize putrescine from ornithine. Putrescine and cadaverine uptake in *T. cruzi* as well as extracellular spermidine and spermine uptake have been characterised at a biochemical level (LeQuesne and Fairlamb, 1996). Initial data from the *T. cruzi* genome sequencing project was screened for amino acid/auxin permeases and this led to the identification of 60 possible amino acid transporter genes (Bouvier *et al.*, 2004). TcPAT12 had 55.3% identity to LmPOT1 and 13.4% amino acid identify with *E. coli* PotE. Functional expression of TcPAT12 in *X. laevis* oocytes indicated that it is a high-affinity spermidine transporter (K_m=14-26 μM) that also transports putrescine and L-arginine to a lesser extent (Carrillo *et al.*, 2006). TcPAT12 was recently re-annotated as TcPOT1.2, and along with another protein TcPOT1.1, are orthologues of LmPOT1 (Hasne *et al.*, 2010). Predicted

hydropathy profiles of these proteins (613 and 627 amino acids each for TcPOT1.1 and TcPOT1.2) indicated that both have 12 transmembrane domains with intracellular N- and C-termini (Hasne *et al.*, 2010). In contrast to previous findings (Carrillo *et al.*, 2006), Hasne *et al.* (2010) reported that TcPOT1.2 as well as TcPOT1.1 recognize putrescine and cadaverine but not spermidine or spermine (Fig. 1.12).

There is limited information available on polyamine transport mechanisms in other parasites. Flagellated, unicellular protozoa from the genus *Phytomonas* infect a wide range of plants and are similar to parasites that cause diseases in humans, such as the blood-stage forms of *T. brucei.* Characterisation of polyamine transport in *Phytomonas* cultures revealed that putrescine is transported at a lower rate than spermidine (0.4 pmol min^{-1} 10^{-7} cells vs. 3.4 pmol min^{-1} 10^{-7}cells) (Canepa *et al.*, 2007). This is similar to the situation found in *T. cruzi* (Carrillo *et al.*, 2006), perhaps reflecting similar polyamine requirements of the two organisms (Canepa *et al.*, 2007).

1.5 Transport of solutes in intra-erythrocytic P. falciparum *parasites*

Parasitic organisms are exposed to varying surroundings during the course of their development. Malaria parasites have to contend with a series of environmental changes as the parasite progresses through its lifecycle from the sexual stage in the Anopheline vector, through the bloodstream of the human host and various intracellular locations within the hepatocytes and RBCs of the human host. This has led to unique evolutionary adaptations that allow survival in these changing environments (Bouvier *et al.*, 2004). The intra-erythrocytic form of the malaria parasite has developed a range of strategies to obtain essential nutrients from the extracellular environment (Charpian and Przyborski, 2008) as well as to facilitate waste disposal (Staines *et al.*, 2010). The inner biochemical machinery of the intra-erythrocytic parasite is enclosed within by three membrane systems: the parasite plasma membrane (PPM), the parasitophorous vacuolar membrane (PVM) and the erythrocyte plasma membrane (EPM) (Fig. 1.13). The following sections aim to describe the nature of transport mechanisms into *P. falciparum* parasites, in relation to known transport mechanisms in other cell types.

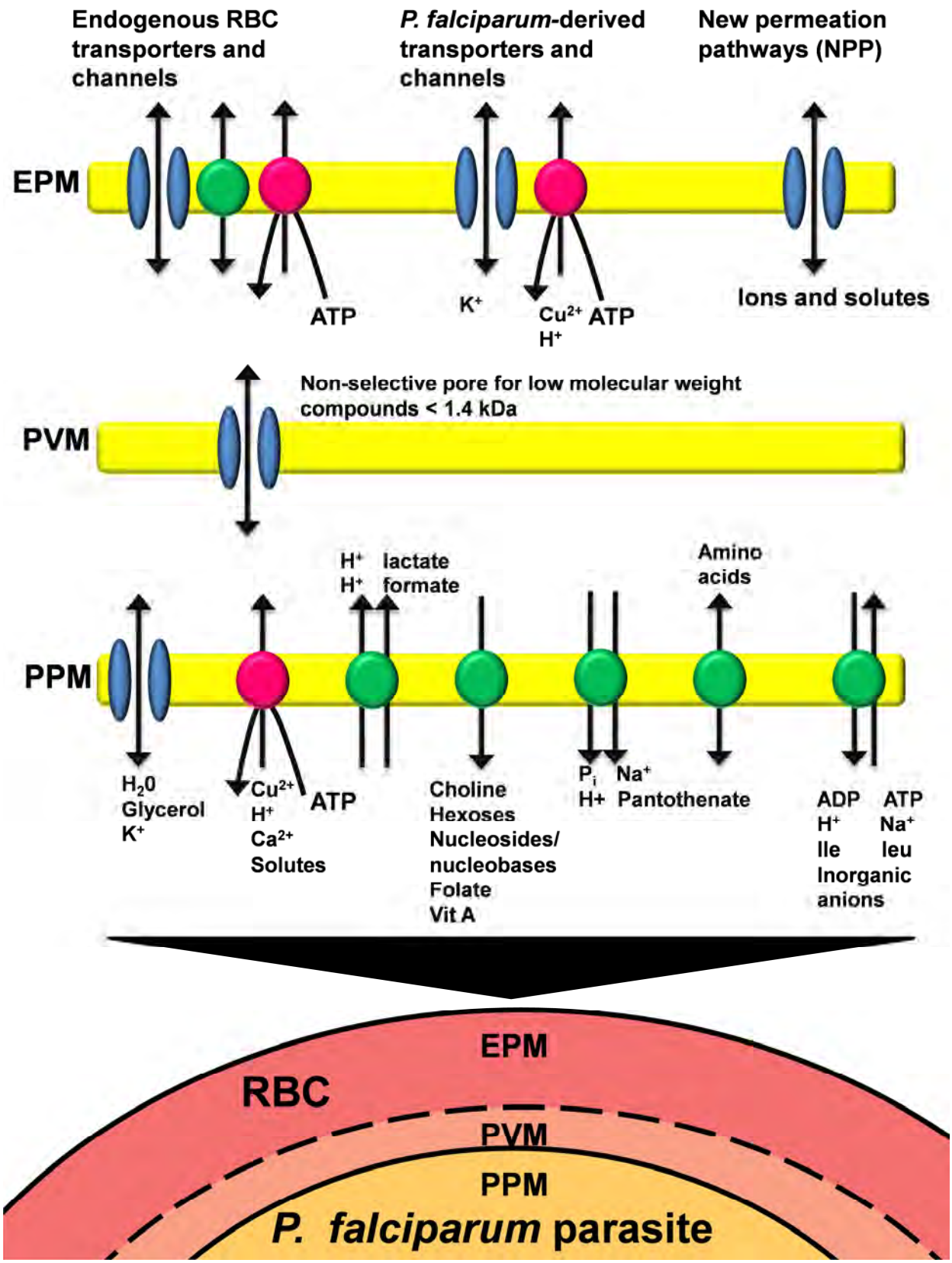

Figure 1.13: Schematic representation of transport processes in iRBCs. Channels (blue), primary active transporters (pink), secondary active or facilitative transporters (green) all occur in iRBCs. Abbreviations: erythrocyte plasma membrane (EPM), parasite plasma membrane (PPM), parasitophorous vacuolar membrane (PVM) New Permeation Pathways (NPP). Compiled from (Martin *et al.*, 2009a).

1.5.1 Transport across the EPM

Constitutively active transporters mediate the transport of metabolites across uninfected RBC membranes (Fig. 1.13). There is some evidence that following parasite infection, the transport characteristics of some endogenous RBC proteins are altered (Kirk, 2001). For example purine uptake into iRBCs occurs mainly via the human facilitative nucleobase transporter (hFNT1), with an increased rate of uptake in iRBCs compared to uninfected cells (Quashie *et al.*, 2010). There is also an increase in the V_{max} of tryptophan uptake (Ginsburg and Krugliak, 1983) and a two-fold increase in the activity of the Na^+-K^+ pump (Staines *et al.*, 2001).

Approximately 15 hrs post infection, the permeability of the EPM of iRBCs to small ions and molecules starts to increase (Staines *et al.*, 2001). The increased flux of low molecular weight metabolites across the infected erythrocyte membrane is mediated by parasite-induced 'New Permeation Pathways' (NPP) (Fig. 1.13). NPP have similar characteristics to anion-selective channels (Decherf *et al.*, 2004), have higher transport rates for anions than cations (Kirk *et al.*, 1994), prefer hydrophobic solutes to hydrophilic metabolites (Kirk *et al.*, 1994; Staines *et al.*, 2000), and are non-saturable (Kirk *et al.*, 1994; Saliba *et al.*, 1998). It is, however, unclear whether the NPP represent one or more parasite-encoded channels (the so-called plasmodial surface anion channel, PSAC) (Bokhari *et al.*, 2008; Hill and Desai, 2010), or are endogenous RBC proteins that are activated or modified by the malaria parasite, possibly as a result of oxidative stress (Huber *et al.*, 2005) or protein phosphorylation (Merckx *et al.*, 2008).

There is some evidence that parasite encoded transporters are targeted to the RBC membrane. Proteins for which this is proposed to occur include a putative K^+ channel (Waller *et al.*, 2008), a putative copper transporter (Rasoloson *et al.*, 2004) as well as vacuolar H^+ ATPase (Marchesini *et al.*, 2005).

1.5.2 Transport across the PVM

Solutes are thought to traverse the PVM from the host cell cytoplasm to the interior of the vacuole, through a high conductance 140 pS channel (Desai *et al.*, 1993) with a molecular cut-off of 1.4 kDa (Desai and Rosenberg, 1997). This channel has low selectivity and is permeable to cations, anions, and amino acids (Desai *et al.*, 1993). Since this channel is open more than 98% of the time and occurs at high density (Desai *et al.*, 1993), the PVM acts as a molecular sieve, allowing the diffusion of a range of substrates to and from the RBC cytosol (Kirk, 2001).

Furthermore, it has been suggested that the PVM extends into a tubovesicular membrane network that stretches to the periphery of the RBC thereby delivering solutes to the parasite itself (Lauer *et al.*, 1997), though this is controversial (Kirk, 2001).

1.5.3 Transport across the PPM

The PPM maintains substantial ion gradients and an inward negative membrane potential (Allen and Kirk, 2004) and the flux of solutes occurs via a range of transporters and channels (Fig. 1.13) (Martin *et al.*, 2009a). The predicted 'permeome' (proteins involved in membrane permeability) of malaria parasites includes both cell-surface proteins that are involved in the transport of solutes across the plasma membrane, as well as proteins located in membranes of intracellular organelles and compartments (Martin *et al.*, 2009a; Martin *et al.*, 2005). Bioinformatic analyses have revealed the presence of some 120 genes in the *P. falciparum* genome encoding proteins that are involved in the movement of solutes across membranes (Martin *et al.*, 2009a). These proteins are categorised into a variety of superfamilies including major facilitator superfamily and related transporters, drug/metabolite superfamily, ATP-binding cassette superfamily, P-type ATPases, H^+-translocating pyrophosphatases, mitrochondrial carrier family, F- or V-type ATPases and channels (Kirk *et al.*, 2005; Martin *et al.*, 2005). These transporters act in concert with the increased permeability of the EPM, and the high permeability of the PVM, to meet the substrate requirements of the malaria parasite (Martin *et al.*, 2009a). The uptake of polyamines by the *P. falciparum* parasite has, however, not been studied.

1.6 Objective

The primary objective of this study was to carry out a detailed biochemical characterisation of polyamine transport by the *P. falciparum* parasite, with subsequent investigations of the therapeutic potential thereof.

- Chapter 2 describes the outcome of experiments investigating the uptake of putrescine and spermidine by *P. falciparum*-infected erythrocytes, and by isolated *P. falciparum* parasites.
- Chapter 3 explores the effect of anthracene-polyamine conjugates on intra-erythrocytic *P. falciparum* parasites.
- Chapter 4 presents a concluding discussion

The following papers resulted from this study:

Published manuscripts:

1) L Birkholtz, AC van Brummelen, K Clark, J Niemand, E Maréchal, M Llinás and AI Louw (2008).·Exploring functional genomics for drug target and therapeutics discovery in *Plasmodia* Acta Tropica 105: 113-123
2) Clark K, Niemand J, Reeksting S, Smit S, van Brummelen AC, Williams M, Louw AI, Birkholtz L (2009): Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum* Amino Acids 38 :633-44
3) Birkholtz L, Williams M, Niemand J, Al-Karadaghi S, Louw A.I, Persson L and Heby O Polyamine homeostasis as a drug target in pathogenic protozoa: peculiarities and possibilities (accepted, Biochemical Journal)

Manuscripts in preparation

1) Polyamine uptake in the malaria parasite, *Plasmodium falciparum*, is dependent on the parasite's membrane potential. J Niemand, L Birkholtz, AI Louw and K Kirk
2) Effect of anthracene-polyamine conjugates on intra-erythrocytic *P. falciparum* parasites. J Niemand, BK Verlinden, O Phanstiel, AM Joubert, K Kirk, AI Louw and L Birkholtz
3) Anti-malarial activity of (bis)urea and (bis)thiourea polyamine analogues. BK Verlinden, J Niemand, AI Louw, P Woster and L Birkholtz. Submitted to Journal of Medicinal Chemistry

Research findings were also presented at the following international conferences:

J Niemand, BK Verlinden, O Phanstiel, AM Joubert, K Kirk, AI Louw and L Birkholtz. Anti-malarial activity of anthracene-polyamine conjugates. Invited presentation, 2nd International Conference on the Role of Polyamines and their Analogs in Cancer and other Diseases Rome, Italy (December 2010)

J Niemand, L Birkholtz, AI Louw and K Kirk. Characterisation of putrescine and spermidine uptake by the human malaria parasite, *Plasmodium falciparum*. Oral and poster presentation: OzBio2010 Conference incorporating the 12th IUBMB, 21st FAOBMB and ComBio2010 conferences with preceding YSF meeting Melbourne, Australia (October2010).

J Niemand, L Birkholtz, AI Louw and K Kirk. Polyamine uptake in the malaria parasite, *Plasmodium falciparum*, is dependent on the parasite's membrane potential. Presentation: Polyamines in Parasites 6th Biennial Symposium, Phalaborwa, South Africa (August 2010).

J Niemand, L Birkholtz, AI Louw and K Kirk: Characterisation of putrescine and spermidine uptake by the human malaria parasite, *Plasmodium falciparum*. Oral presentation: International Polyamine Conference, Gotemba, Japan (June 2010).

J Niemand, AI Louw, K Kirk and L Birkholtz: Characterisation of polyamine transport in the malaria parasite, *Plasmodium falciparum*. Poster and oral presentation: Polyamine Gordon Research Conference and graduate seminar, respectively, Waterville Valley, New Hampshire, USA, 2009.