Biochemical characterisation of putrescine and spermidine uptake as a potential therapeutic target against the human malaria parasite, *Plasmodium falciparum*

by

Jandeli Niemand

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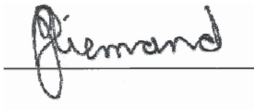
Signed: Jandeli Niemand 

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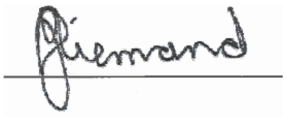
Signature:  Date: 24 August 2011

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Summary

Plasmodium falciparum causes the most severe form of human malaria, and the continual development of resistance of this parasite to current anti-malarial drugs underpins a pressing need for the discovery of novel chemotherapeutic approaches. Polyamines and their biosynthetic enzymes are present at high levels in rapidly proliferating cells, including cancer cells and protozoan parasites. Inhibition of the malaria parasite's polyamine biosynthesis pathway causes cytostatic arrest in the trophozoite stage, but does not cure infections *in vivo*. This may be due to the salvage of exogenous polyamines from the host, replenishing the intracellular polyamine pool; however the mechanism(s) of polyamine uptake by the intra-erythrocytic parasite are not well understood. In this study the uptake of the polyamines putrescine and spermidine into *P. falciparum*-infected erythrocytes (iRBC) well as into *P. falciparum* parasites functionally isolated from their host cell by saponin-permeabilisation of the erythrocyte membrane was investigated using radioisotope flux techniques. While the characteristics of transport of putrescine into infected erythrocytes were similar to those of transport into uninfected erythrocytes, spermidine entered iRBC in part via the 'new permeation pathways' induced by the parasite in the erythrocyte membrane. Both putrescine and spermidine were taken up across the plasma membrane of isolated parasites via a saturable, temperature-dependent process that showed competition between different polyamines as well as the polyamine precursor ornithine and basic amino acids. Inhibition of polyamine biosynthesis led to increased total uptake of both putrescine and spermidine. The influx of putrescine and spermidine into isolated parasites was independent of Na^+ but increased with increasing pH and showed a marked dependence on the membrane potential, decreasing with membrane depolarisation and increasing with membrane hyperpolarisation.

Both anthracene and polyamine derivatives have been shown to have anti-malarial activity. Anthracene-polyamine conjugates have been developed with the aim of utilising the polyamine uptake mechanisms of cancer cells to deliver the cytotoxic anthracene moieties to these cells. Here, several anthracene-polyamine conjugates showed promising anti-malarial activity. These compounds inhibited parasite proliferation with IC_{50} values in the nM range, and caused an arrest in the cell cycle, as well as a decrease in the mitochondrial membrane potential. Cytotoxicity could not be reversed by the addition of exogenous polyamines, nor did the conjugates have an effect on intracellular polyamine levels.

This doctoral study showed that *P. falciparum* parasites not only synthesise polyamines, but can also acquire putrescine and spermidine from the extracellular environment and paves the way for interfering with polyamine metabolism as an anti-parasitic strategy.

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$\Delta\psi$	Membrane potential
$\Delta\psi$m	Mitochondrial membrane potential
AdoMet	*S*-adenosylmethionine
AdoMetDC	*S*-adenosylmethionine decarboxylase
AMEL-3	Hamster melanoma cell line
APA	3-aminooxy-1-aminopropane
APAO	N^1 acetylpolyamine oxidase
APC	Amino acid/Polyamine/Organocation
BCBD	N^1,N^4-bis-(7-chloroquinoline-4-yl)butane-1,4-diamine
BCECF	2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein
BP	Bandpass
CCC	Cation-Cl^- cotransporter
CCCP	Carbonyl cyanide-*m*-chlorophenylhydrazone
CHO	Chinese hamster ovary
DAX	Diamine exporter
dcAdoMet	Decarboxylated *S*-adenosylmethionine
DCFDA	2'-7'-Dichlorodihydrofluorescein diacetate
DFMO	DL-α-difluoromethylornithine
DHFR	Dihydrofolate reductase
eIF5A	Eukaryotic initiation factor 5A
EPM	RBC plasma membrane
FACS	Fluorescence-activated cell sorting
Gpc-1	Glypican-1
HEK-293	Human embryonic kidney 293 cells
HEPES	*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid)
HL-60	Human leukaemia cell line
iRBC	*P. falciparum* (strain 3D7)-infected red blood cell
iRBCs	*P. falciparum* (strain 3D7)-infected red blood cells
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
L1210	Murine leukaemia cells
MDL27695	*N,N*-bis{3-(phenylmethyl)aminolpropyl}-1,7-diaminoheptane
MES	2-morpholinoethanesulfonic acid

MGBG	Methylglyoxal bis(guanylhydrazone)
MMV	Medicines for Malaria Venture
MR	Methionine recycling pathway
MTA	5'methylthioadenosine
NMDG	*N*-methyl-D-glucamine
NO	Nitric oxide
NOS2	Nitric oxide synthase
NPP	New Permeation Pathways
ODC	Ornithine decarboxylase
PAH	Polycyclic aromatic hydrocarbons
PAO	Polyamine oxidase
PBS	Phosphate-buffered saline
PfAdoMetDC/ODC	*P. falciparum S*-adenosylmethionine decarboxylase/ornithine decarboxylase
PfATP4	*P. falciparum* Ca^{2+}ATPase
PfCHA	Putative *P. falciparum* Ca^{2+}/H^{+} anti-porter
PfCRT	*P. falciparum* chloroquine-resistance transporter
PfENT1	*P. falciparum* Equilibrative Nucleoside/nucleobase Transporter 1
pH_i	Intracellular pH
pH_o	Extracellular pH
PPM	Parasite plasma membrane
PSAC	Plasmodial surface anion channel
PVM	Parasitophorous vacuolar membrane
RBC	Uninfected human red blood cell
RBCs	Uninfected human red blood cells
ROS	Reactive oxygen species
rpm	Revolutions per minute
S.E.	Standard error of the mean
SAM3	*S*-Adenosylmethionine transporter
SAMI	South African Malaria Initiative
-SH	Sulfhydryl
SI	Selectivity index
SMO	Spermine oxidase
SMR	Small multi-drug resistance

SpdSyn	Spermidine synthase
SpmSyn	Spermine synthase
Spp	Species
SSAT:	Spermidine/spermine N^1-acyltransferase
TPO	Transporter for polyamine
TS	Thymidylate synthethase
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labelling
WHO	World health organisation

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).

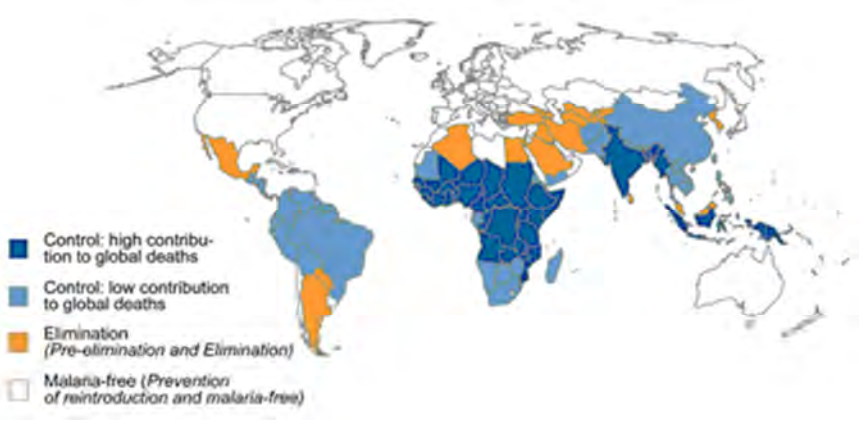


Figure 1.1: World-wide occurrence of malaria. Areas of high (dark blue) and low (light blue) malaria-induced mortality are indicated, as well as areas that are free of malaria (white) and areas where malaria is either eradicated or in the process of being eradicated**.** This image is freely available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

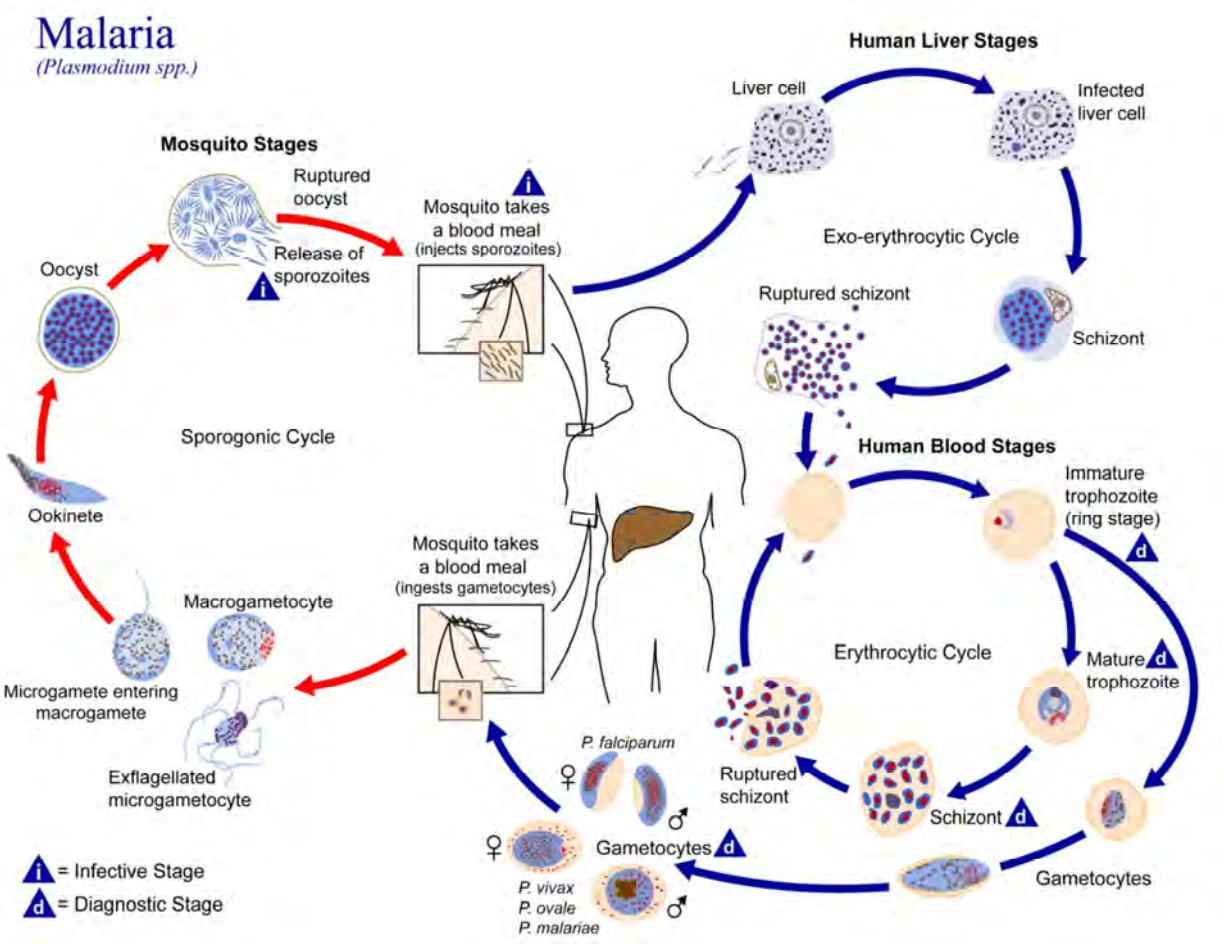


Figure 1.2: The lifecycle of the *Plasmodium* parasite. Sporozoites are transmitted to humans where they undergo liver stage and erythrocytic development before gametocytes are again taken up by feeding mosquitoes to initiate development within the insect vector. This image is in the public domain, credited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

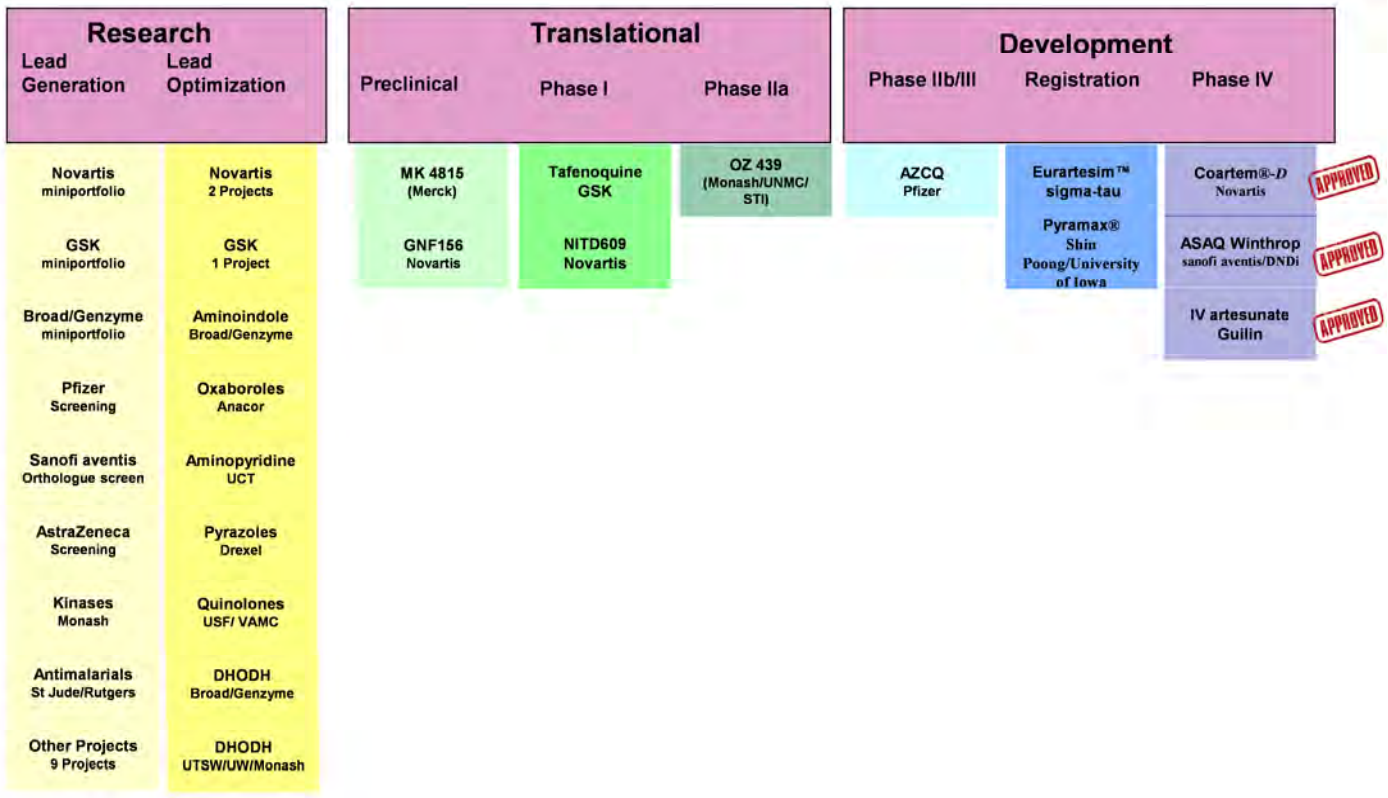


Figure 1.3: Current MMV portfolio (4th quarter 2010, www.mmv.org). DHODH: dihydroorotate dehydrogenase, NITD609: natural product selection project, OZ439: peroxides, AZCQ: azithromycin-chloroquine, Eurartesim: dihydroartemisinin-piperaquine, Pyramax: pyronaridine-artesunate, CoArtem: artemether-lumefantrine,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

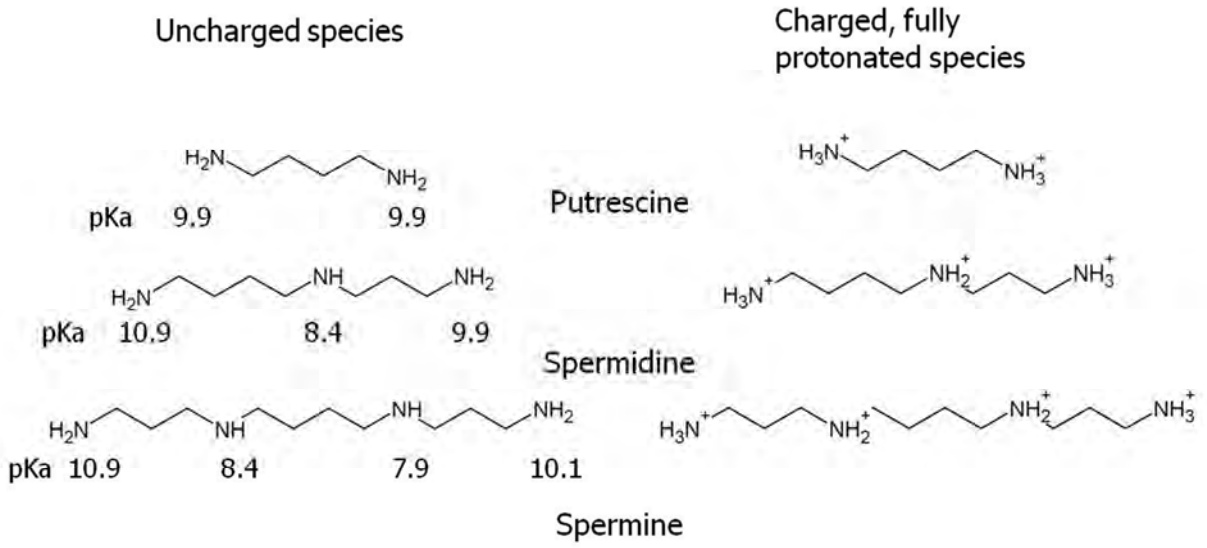


Figure 1.4: The structures of putrescine, spermidine and spermine. Due to the high pKa values, the polyamines are positively charged at physiological pH. pKa values from (Casero and Woster, 2009).

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).

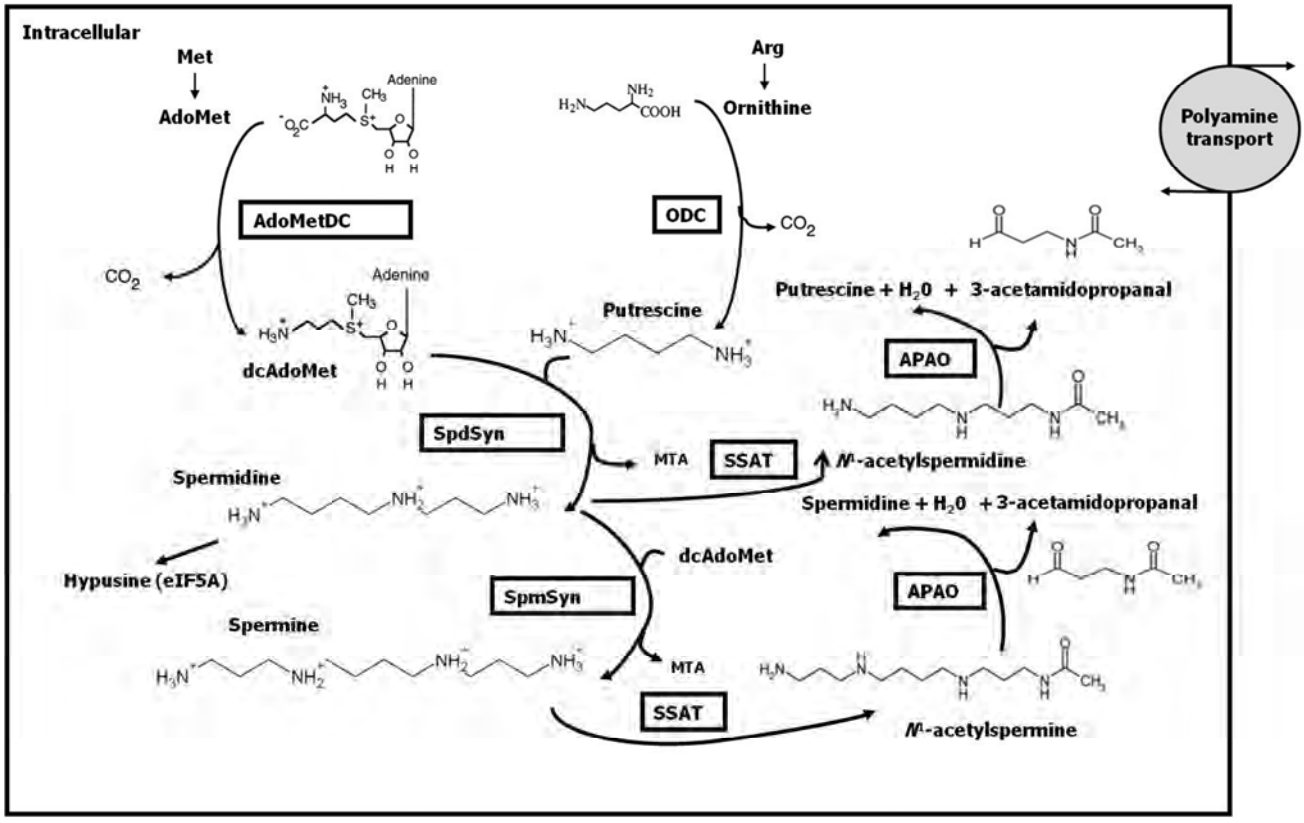


Figure 1.5: Polyamine metabolism in humans. Created from (Casero and Pegg, 2009). Putrescine, spermidine and spermine syntesis is indicated, catalysed by the enzymes ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine (SpdSyn) and spermine synthase (SpmSyn). The 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).

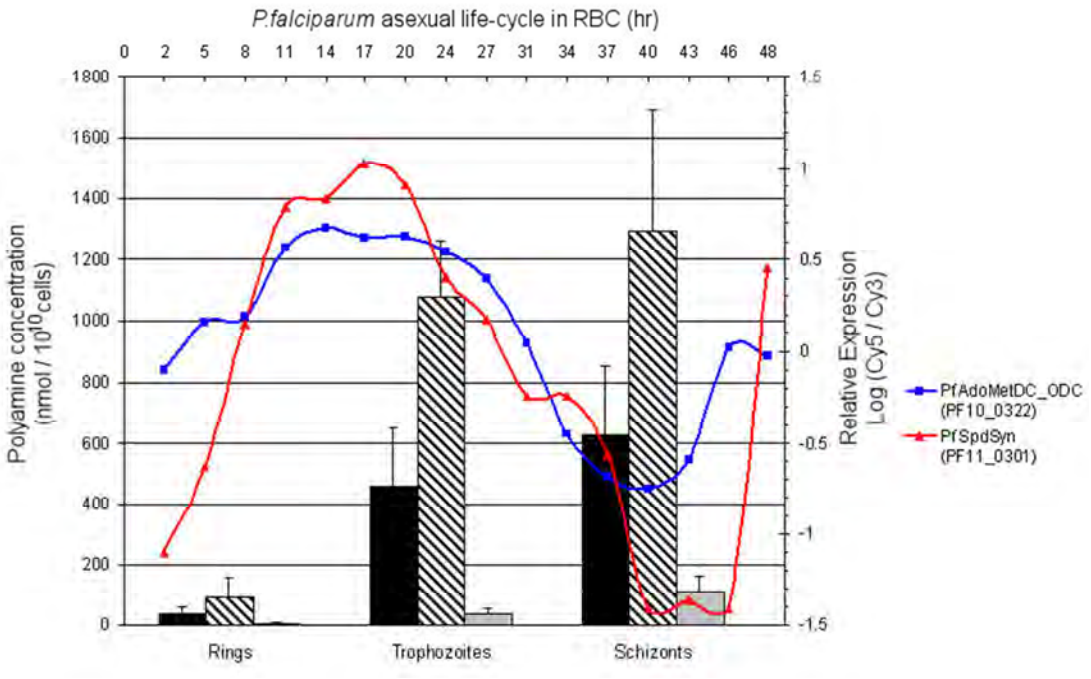


Figure 1.6: Composite of polyamine levels and polyamine biosynthetic enzyme levels (PfAdoMetDC/ODC and PfSpdSyn), during the intra-erythrocytic developmental cycle of *P. falciparum*. Compiled by Shaun Reeksting from (Assaraf *et al.*, 1984; Bozdech *et al.*, 2003; Das Gupta *et al.*, 2005). Bifunctional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

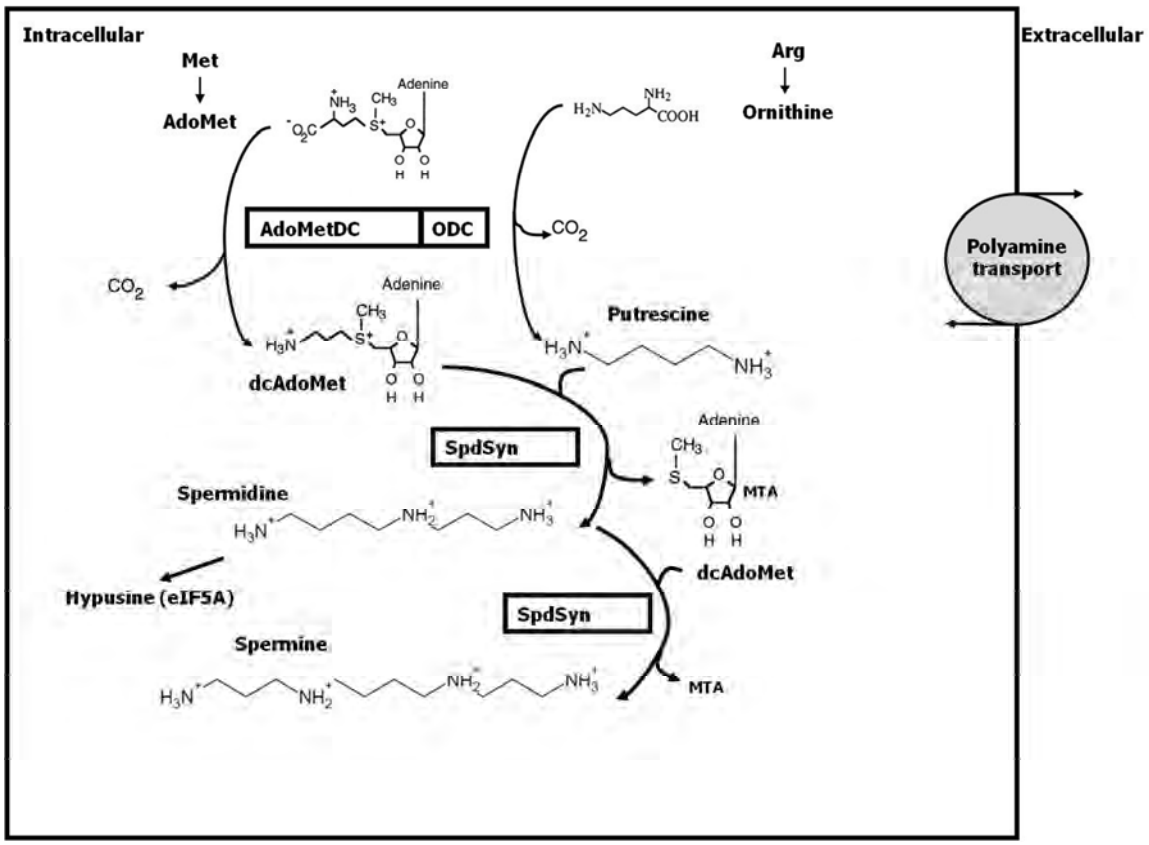


Figure 1.7: Schematic representation of polyamine metabolism in intra-erythrocytic *P. falciparum* parasites. Created from (Clark *et al.*, 2010). Bifunctional *P. falciparum S*-adenosylmethionine decarboxylase/ornithine decarboxylase (PfAdoMetDC/ODC) catalyses the production of putrescine and dcAdoMet, 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).

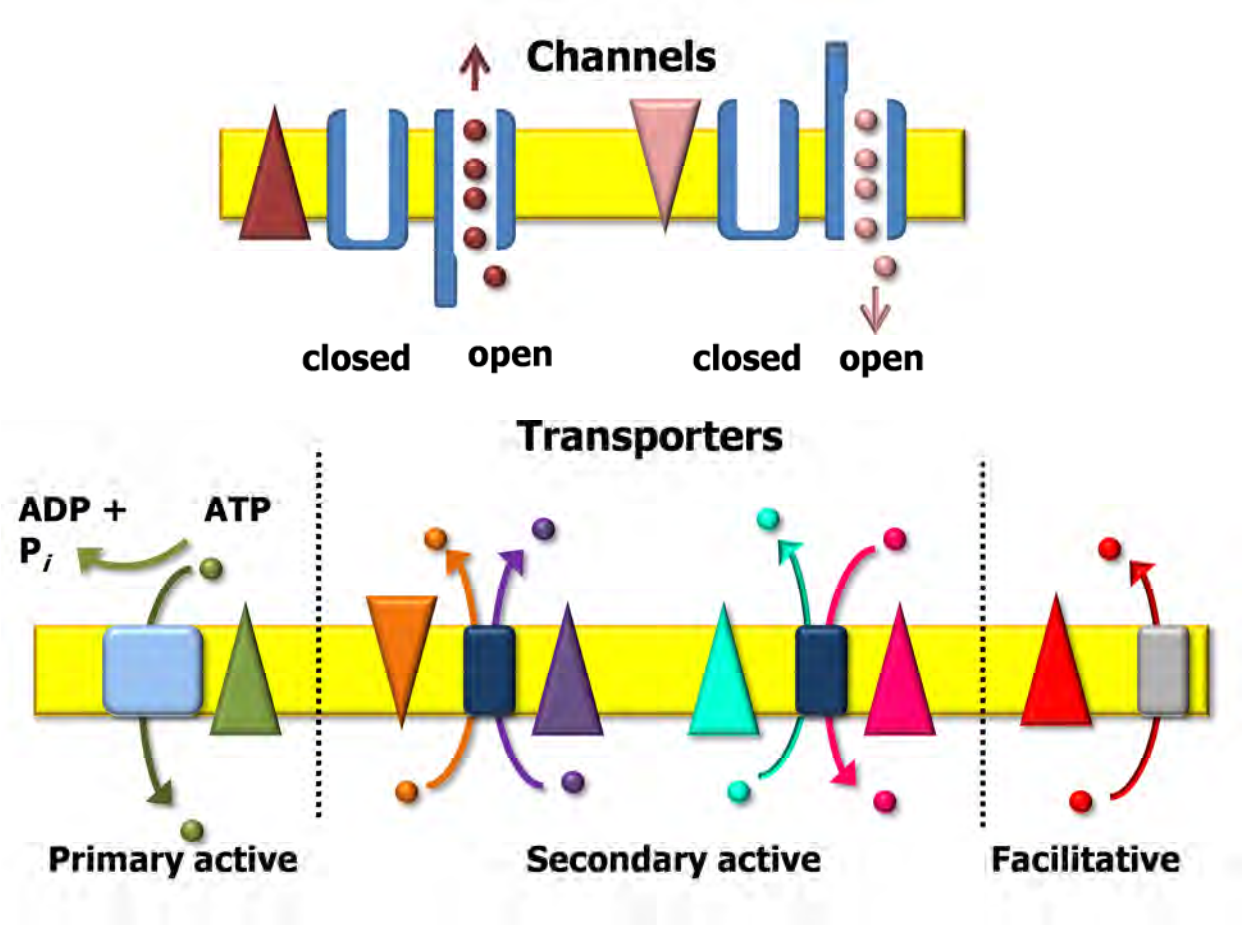


Figure 1.8: Schematic representation of membrane transport proteins. Channels facilitate the movement of molecules down their electrochemical gradient. Transporters consist of three classes, namely primary active transporters that couple the movement of molecules against their electrochemical gradient 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).

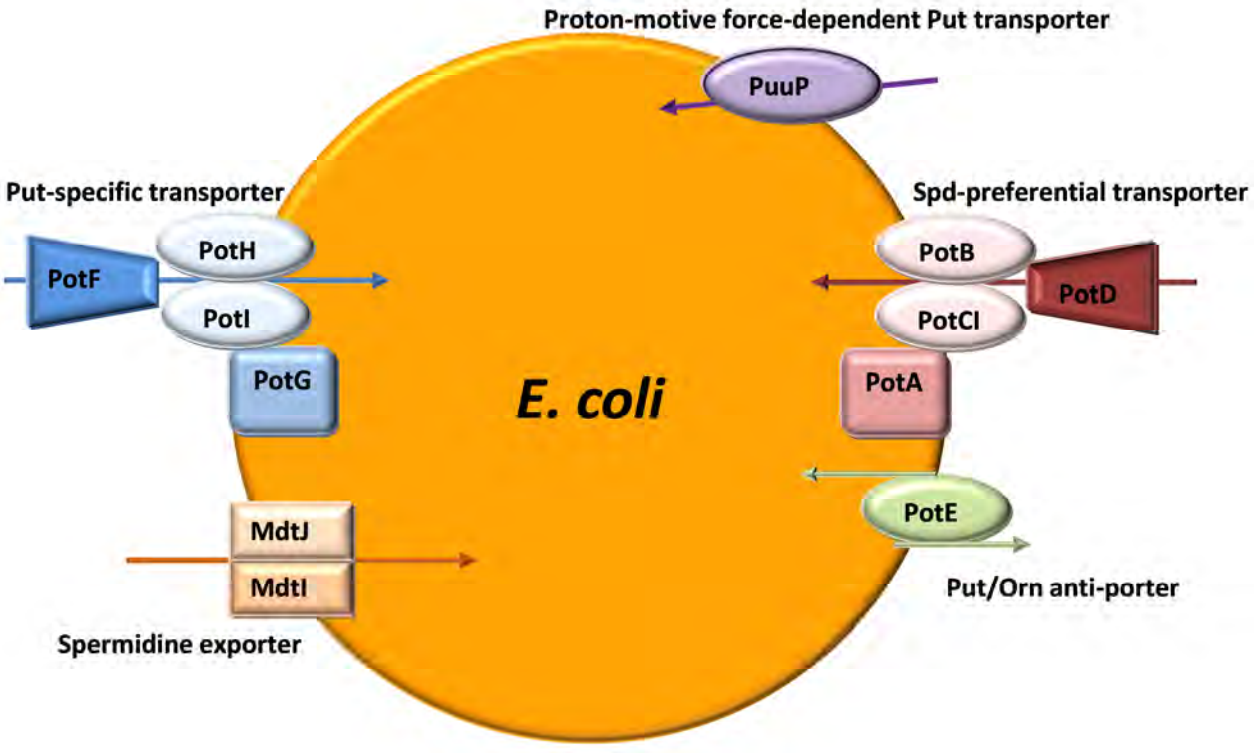


Figure 1.9: Schematic representation of the polyamine uptake and export systems in *E. coli*. Putrescine uptake is mediated by PotFGHI, PuuP and PotE, and putrescine export by PotE. Spermidine uptake is mediated by PotABCD, while the MdtJI complex is responsible for spermidine export. Compiled from (Higashi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

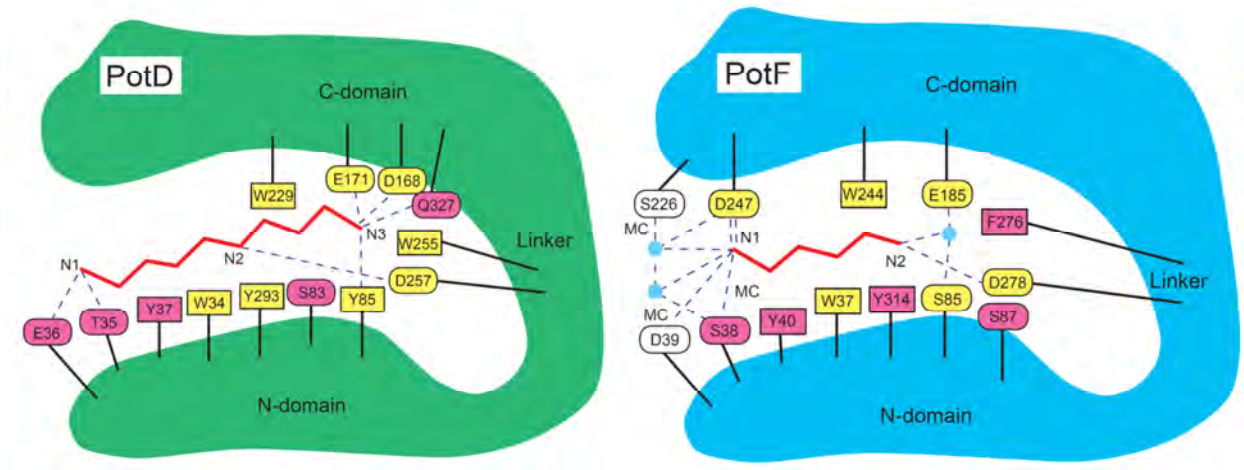


Figure 1.10: Schematic representation of the mechanism of polyamine recognition of PotD and PotF, taken from (Igarashi and Kashiwagi, 1999). The NH_2 and NH groups of polyamines are recognised by acidic Asp and Glu, while the propyl and butyl groups have interactions with Trp and Tyr. This figure is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

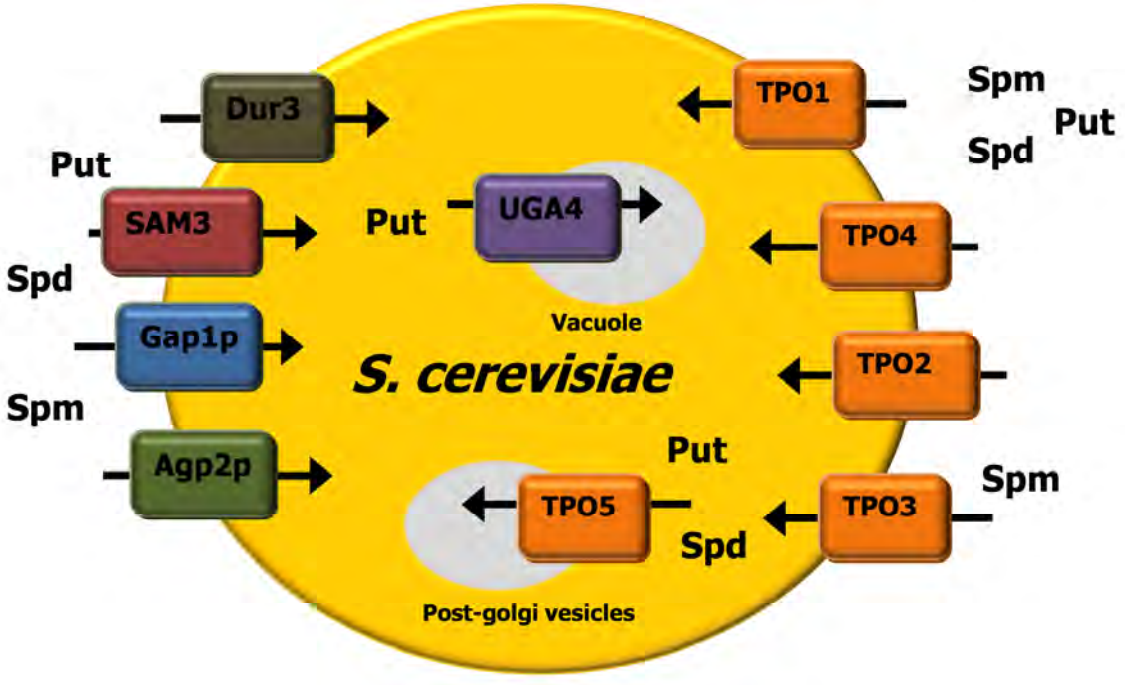


Figure 1.11: Polyamine import and export in *S. cerevisiae.* Polyamine (putrescine (put), spermidine (spd) and spermine (spm)) transport in *S. cerevisiae* is mediated by a variety of transporter proteins. DUR3 and SAM3, as well as Gap1p and Agp2p are responsible for polyamine uptake, while excretion 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.

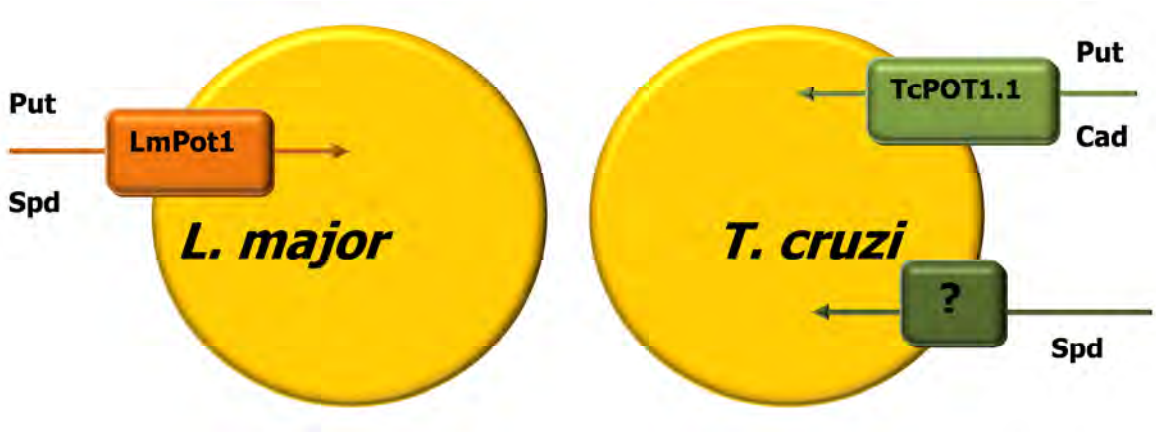


Figure 1.12: Polyamine transporters in parasitic protozoa. LmPot1 is responsible for putrescine (Put) and spermidine (Spd) uptake in *L. major*, while TcPot1.1 is responsible for putrescine and cadaverine (Cad) uptake in *T. cruzi*. The molecular identity of the spermidine transporter of *T. cruzi* is 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.

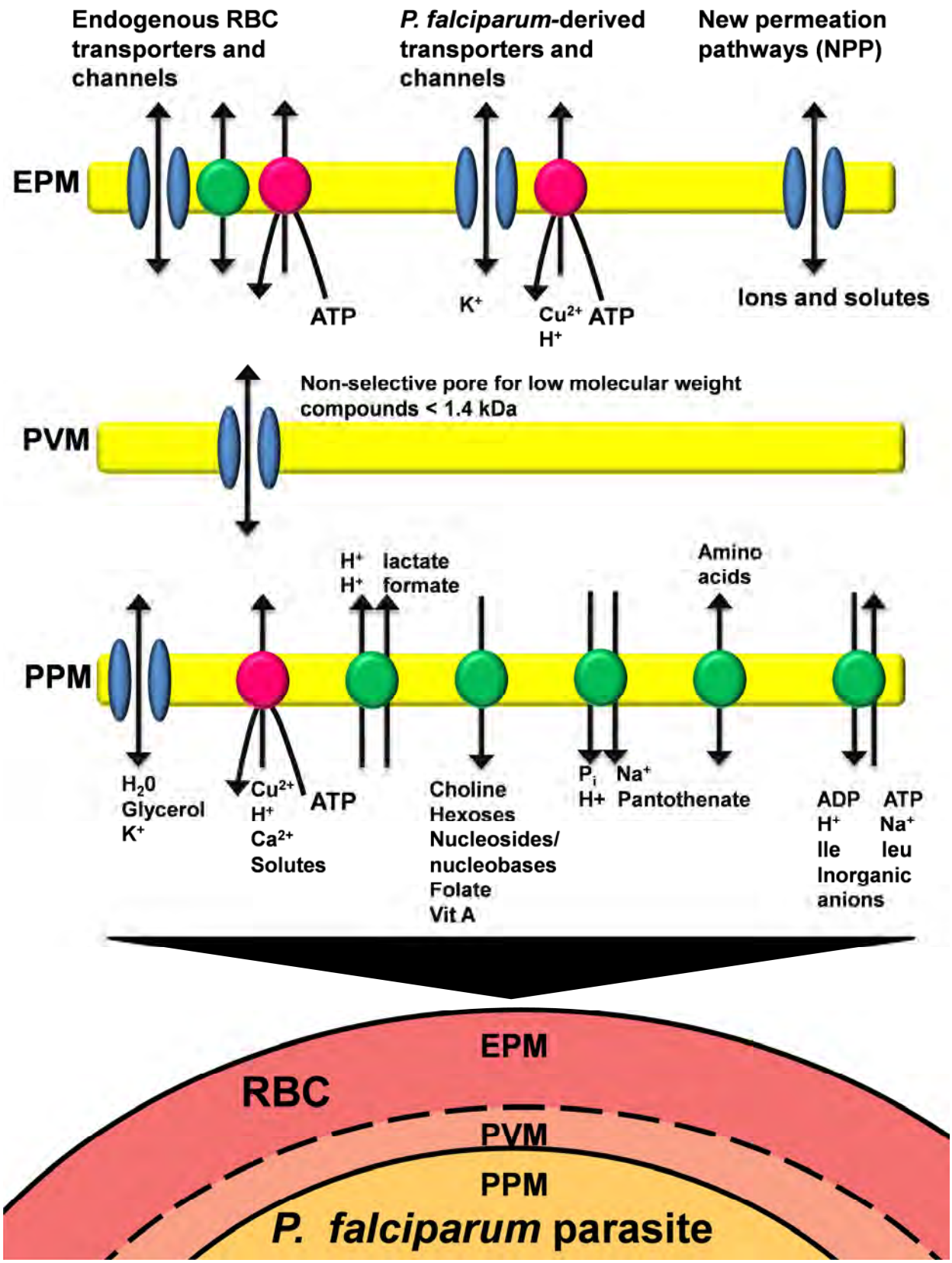


Figure 1.13: Schematic representation of transport processes in iRBCs. Channels (blue), primary active transporters (pink), secondary active or facilitative transporters (green) all occur in iRBCs. Abbreviations: erythrocyte plasma membrane (EPM), parasite plasma membrane (PPM), parasitophorous vacuolar 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.

Chapter 2: Polyamine uptake by the intra-erythrocytic malaria parasite, *P. falciparum*.

2.1 Introduction

Putrescine transport has been characterised in rhesus monkey RBCs infected with *P. knowlesi* (Singh *et al.*, 1997). Uptake was temperature dependent and there was a higher rate of influx in infected RBCs than in uninfected RBCs (infected RBCs K_m=37 μM and V_{max}=11.6 nmol/min/10^{10} RBCs; uninfected RBCs K_m=35 μM and V_{max}=4.21 nmol/min/10^{10} RBCs). Competition studies showed that various amino acids such as lysine, leucine, serine and aspartic acid had little effect on the rate of polyamine uptake (Table 2.1). In contrast, spermidine and spermine did affect the uptake of putrescine, which may imply a shared uptake system (Singh *et al.*, 1997).

The AdoMetDC inhibitor MGBG decreased putrescine uptake into *P. knowlesi*-infected RBCs to about 39% of the control; while the ODC inhibitor, DFMO, had no significant effect on uptake into *P. knowlesi*-infected RBCs even though it is structurally similar to putrescine (Table 2.1, A) (Singh *et al.*, 1997). *N*-ethyl maleimide and *p*-chloromercuric benzoic acid (as sulfhydryl blocking reagents) had the largest effect on putrescine uptake into *P. knowlesi*-infected RBCs, inhibiting it by more than 80%. This indicates that putrescine uptake is protein-mediated (Singh *et al.*, 1997).

There has been one previous investigation of polyamine uptake in *P. falciparum*-infected RBCs (Ramya *et al.*, 2006). The rate of putrescine uptake increased from the ring to early and late trophozoite with no further increase to schizont stages. In trophozoite infected RBCs, putrescine uptake was found to be highly temperature-dependent, with a significant difference in the activation energy between infected and uninfected RBCs. This indicated that either the existing transport pathways in the red blood cell membrane are altered following infection, or that new putrescine transport pathways are induced. Amino acids and the polyamines, spermidine and spermine (all at 1 mM), led to less than 20% reduction in uptake (Table 2.1, B). Uptake was suggested to be a carrier-mediated process and dependent on the membrane

potential, based on the action of –SH group blocker and ionophores (Table 2.1, B) (Ramya *et al.*, 2006).

Table 2.1: Effect of various polyamines, amino acids and metabolic inhibitors on putrescine uptake in A) *P. knowlesi* infected RBCs (Singh *et al.*, 1997) and B) *P. falciparum*-infected RBCs (Ramya *et al.*, 2006).

(A) Putrescine uptake in P. knowlesi-infected RBC Reagent (1mM)	**Putrescine uptake (% of control)**
Amino acids	
Serine (A type transporter)	84
Leucine (L type transporter)	86
Lysine (Ly' type transporer)	106
Aspartic acid (β type transporter)	92
SH-group intererference	
N-ethyl maleimide	15
p-Chloromercuricbenzoic acid	17
Dithiothreitol + *N*-ethyl maleimide	69
Dithiothreitol + *p*-Chloro Mercuric benzoate	91
Polyamine and related molecules	
Spermidine	62
Spermine	69
DFMO	106
MGBG	39

(B) Putrescine uptake in *P. falciparum*-infected RBCs Reagent (1 mM)	**Putrescine uptake (% of control).**
Amino acids	
D-Arginine	>80%
Poly-L-arginine	
Serine	
L-Arginine	
Leucine	
Aspartate	
Glutamine	
Lysine	
SH-group blockers	
Iodoacetate	39
N-Ethyl maleimide	78
p-Hydroximercuribenzoate	57
Polyamines and related molecules	
Ornithine	84
Putrescine	50
Spermidine	82
Agmatine	56
DFMO	84
Ionophores and metabolic inhibitors	
Cycloheximide	99
Valinomycin	26
Gramicidin	52
Calcium ionophore (unspecified by authors)	53
CCCP	20

P. falciparum-infected RBCs had a lower K_m and higher V_{max} (K_m=276 μM and V_{max}=4.69 nmol/30min/10^{10} RBCs) for putrescine uptake than did uninfected RBCs (K_m=546 μM and V_{max}=3.8 nmol/30 min/10^{10} RBCs), which suggested that infected RBCs have a higher affinity for putrescine than normal RBCs (Ramya *et al.*, 2006). By contrast with putrescine, spermidine transport did not appear to be greatly increased into RBCs following parasite infection. There were, however, differences in the activation energy for spermidine uptake in infected and uninfected cells, prompting the authors to speculate that there may be a new transporter present (Ramya *et al.*, 2006). The kinetics of spermidine transport were also determined and a large difference was found between the K_m values of uninfected and infected RBCs, indicating an increased affinity for spermidine in infected red blood cells (K_m=889 μM and V_{max}=58 nmol/30min/10^{10} RBCs) compared to uninfected RBCs (K_m=4992 μM and V_{max}=329 nmol/30min/10^{10} RBCs) (Ramya *et al.*, 2006).

The available evidence would suggest that there are significant differences between the polyamine transport characteristics of simian RBCs infected with *P. knowlesi* and human erythrocytes infected with *P. falciparum*. In *P. knowlesi*-infected RBCs, no significant difference was detected between the K_m values of infected and uninfected RBCs while the V_{max} values differed. By contrast, in *P. falciparum-infected* RBCs it was found that there were differences between the K_m values of infected and uninfected RBCs but not the V_{max} values. Whether this is due to species-specific differences or to technical differences between the two studies is unclear. By contrast, the effect of amino acids on the putrescine import between the two species appears to be similar (Ramya *et al.*, 2006; Singh *et al.*, 1997).

While the above studies dealt with polyamine uptake into *Plasmodium*-infected RBCs, the mechanism by which polyamines gain entry into the intracellular parasite has not been studied. In this study we have investigated the uptake of the polyamines putrescine and spermidine both into *P. falciparum*-infected human RBCs and into parasites functionally isolated from their host RBCs by saponin-permeabilisation of the host cell membrane.

2.2 Materials and methods

2.2.1 HEPES buffered solutions

A range of different HEPES (*N*-(2-hydroxyethyl)piperazine-*N*-(2-ethanesulphonic acid))-buffered salines was used in this study: solution A (125 mM NaCl, 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution B (135 mM NaCl, 5 mM KCl, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution C (130 mM NaCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution D (130 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); solution E (135 mM NMDG [*N*-methyl-D-glucamine], 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1); and solution F (125 mM NaCl, 5 mM KCl, 20 mM glucose,15 mM HEPES, 10 mM MES [2-morpholinoethanesulfonic acid] and 1 mM $MgCl_2$, pH 6.1, 7.1, 8.1). For competition assays, solutions of the compounds of interest (e.g. amino acids, polyamines) were prepared as 100 mM stocks in Solution A.

2.2.2 Cell culture and preparation

P. falciparum (3D7) parasites were maintained at 5% haematocrit and 10-20% parasitaemia in synchronous cultures in a gaseous environment of 90% N_2, 5% O_2, and 5% CO_2 in human RBCs (O^+ or A^+ obtained from the Canberra branch of the Australian Red Cross Blood service) in complete culture medium [RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 mM HEPES (Sigma-Aldrich), 20 mM D-glucose (Sigma-Aldrich), 200 μM hypoxanthine (Sigma-Aldrich), 0.2% sodium bicarbonate, 24 μg/ml Gentamycin (Invitrogen) and 0.5% Albumax II (Invitrogen)] (Trager and Jensen, 1976) in 75 cm^2 culture flasks. In order to avoid settling of the cells and to ensure maximal number of merozoite re-infection, the cultures were incubated at 37°C on a rotary platform (~60 rpm) (Allen and Kirk, 2004).

Parasites were synchronised every 48 hrs by the lysis of mature trophozoite-stage parasitised RBCs by suspension in an isosmotic sorbitol solution (Lambros and Vanderberg, 1979). Predominantly ring-stage cultures were transferred to 50 ml centrifuge tubes and pelleted (500xg, 5 min) before re-suspending the cell pellet in 5% w/v sorbitol solution and incubating at 37°C for 15 min. Afterwards, the cells were pelleted as above, washed with culture media and placed in culture.

Experiments were performed with either uninfected human red blood cells, predominantly type O^+ or A^+, mature trophozoite-stage parasites (30-36 h post-invasion) *P. falciparum* (strain 3D7)-infected red blood cells (iRBCs), or mature trophozoite-stage parasites that had been functionally isolated from their host blood cell by saponin-permeabilisation of the RBC and parasitophorous vacuole membranes with 0.05% w/v saponin (Saliba *et al.*, 1998). Saponin causes a disruption of cholesterol-containing membranes through the interaction with cholesterol, and is used to permeabilise the EPM and the PVM. This allows the study of transport processes of the PPM (Kirk, 2001). The PPM remains intact with an inward negative membrane potential (Allen & Kirk, 2004) and the ability to maintain ion gradients (Saliba and Kirk, 1999). The *P. falciparum* cultures (at 5% haematocrit and 10-20% parasitaemia) were transferred to 50 ml centrifuge tubes and saponin (containing ≥10% of the active compound sapogenin) was added to a final concentration of 0.05% w/v saponin, mixed and immediately centrifuged (1800xg, 8 min) before washing the now isolated *P. falciparum* parasites a minimum of three times to remove residual saponin and erythrocytic cytosolic compounds.

For experiments with iRBCs, the infected cells (*P. falciparum* cultures at 5% haematocrit and 10-20% parasitaemia) were purified to ≥95% parasitaemia using the magnetic properties of iRBCs and the magnetic field of a VarioMacs separation system (Miltenyi Biotec, North Ryde, NSW, Australia) with a CS column, as described previously (Teng *et al.*, 2009; Trang *et al.*, 2004). *P. falciparum* cultures were loaded onto the plastic-coated ferromagnetic fibres of the CS column in the VarioMacs separation unit's magnetic field and uninfected RBCs washed away using 5 column volumes of solution A. The column was removed from the magnetic field and the *P. falciparum-infected* RBCs were eluted using solution A (50 ml).

The different cell types (RBCs, iRBCs and functionally isolated parasites) were washed 3 times with the appropriate solution as required for each study prior to the initiation of the experiment and re-suspended to a final concentration of between $5x10^7$ and $1x10^8$ cells/ml using an improved Neubauer cell counting chamber. The cells were subsequently recovered in solution A for 10 min at 37°C before experimentation.

2.2.3 Radioisotope uptake measurements

2.2.3.1 Uptake measurements in intact RBCs and iRBCs

To measure the uptake of [^{3}H]putrescine (21.0 Ci/mmol, Amersham Biosciences) or [^{3}H]spermidine (16.6 Ci/mmol PerkinElmer) into RBCs and iRBCs, an appropriate volume of Solution A was supplemented with either 1 µCi/ml [^{3}H]putrescine or [^{3}H]spermidine. The uptake reaction was initiated by combining equal volumes of radiolabelled solution and cell suspension (thus 0.5 µCi/ml final concentration of either [^{3}H]putrescine or [^{3}H]spermidine). At predetermined time intervals (time courses) or after a fixed-time period, triplicate volumes (each 200 µl) were transferred to microcentrifuge tubes containing 300 µl of dibutyl phthalate (density 1.04 g/ml) to terminate the reactions by sedimenting the cells through the oil (17 000xg for 1 min). A 10 µl sample of the aqueous phase was transferred to a scintillation vial to determine the extracellular concentration of the radiolabel. The amount of radiolabel trapped in the extracellular space of the cell pellet was estimated by either extrapolating time-course data to t=0 or by taking replicate samples as quickly as possible after combining the cells and radiolabel and immediately centrifuging these through an oil layer as described above. The aqueous phase was aspirated and the tube and dibutyl phthalate layer rinsed three times with water to remove residual radioactivity before aspirating the dibutyl phthalate. The remaining cell pellet was lysed with 0.1% (v/v) Triton X-100 (0.5 ml) and the proteins precipitated with 5% w/v trichloroacetic acid (0.5 ml). The samples were centrifuged at 17 000xg for 10 min to clear the cellular debris before measuring the radioactivity present in the supernatant using a β-scintillation counter.

RBCs from a single donor were used for each experiment. Both uninfected and infected RBCs were cultured for 48 hours prior to experimentation. To determine initial uptake rates, incubations were performed at 22°C for putrescine and 4°C for spermidine.

2.2.3.2 Uptake measurements in isolated parasites

To investigate the uptake of [^{3}H]putrescine or [^{3}H]spermidine into functionally isolated *P. falciparum* parasites, an appropriate volume of Solution A-F (depending on the experiment) was supplemented with 1 µCi/ml of either [^{3}H]putrescine or [^{3}H]spermidine. The uptake reaction was initiated by combining equal volumes of radiolabelled solution and cell suspension. At pre-determined time intervals (time courses) or after a fixed-time period, the reaction was terminated by transferring 200 µl aliquots (in triplicate for putrescine uptake, quadruplicate for

spermidine uptake) to microcentrifuge tubes containing a dibutyl/dioctyl phthalate (5:4; 1.015 g/ml) blend and sedimenting the cells below the oil layer (17 000xg for 1 min). Initial experiments were performed with the dibutyl/dioctyl phthalate layered over 30% (v/v) perchloric acid (30 µl) to terminate the uptake reaction, lyse the cells and precipitate the protein (Martin and Kirk, 2007). In later experiment the perchloric acid layer was omitted and the pellets were processed as described for those from the intact RBC samples. Alternatively, the uptake reactions were terminated by transferring aliquots (600 µl) of the reaction to microcentrifuge tubes and centrifuging the samples at 8 000xg for 1 min to sediment the cells. The supernatant was immediately aspirated and the cells washed with an ice-cold 1 ml aliquot of the specific solution being used for each experiment (excluding the radiolabel) before sedimenting the cells at 17 000xg for 1 min. For the isolated parasites, the extracellular concentration of the radiolabel and the amount of radiolabel trapped in the extracellular space of the cell pellet was estimated as described for iRBCs (above). The wash solution was aspirated and the cell pellet lysed and processed as described above (Allen and Kirk, 2004). All control experiments were performed with cells suspended in Solution A. All reactions were performed at 37°C except where stated otherwise.

To determine the effect of temperature on [^{3}H]putrescine or [^{3}H]spermidine uptake, time-courses were performed at 22°C and 37°C. The energy dependence of the [^{3}H]putrescine or [^{3}H]spermidine uptake reactions were measured by washing isolated parasites three times with solution B to remove any glucose and incubating the cell suspension at 37°C for 30 min to deplete the cells of ATP (Saliba and Kirk, 1999), prior to the initiation of the reaction by the addition of reaction buffer (Solution B with 1 µCi/ml [^{3}H]putrescine or [^{3}H]spermidine) and measuring the uptake over time. The concentration dependence of the uptake of [^{3}H]putrescine and [^{3}H]spermidine was measured in solution A at 15 min over an extracellular concentration range of 1-15 mM and 0-500 µM for both putrescine and spermidine. To determine the specificity of the [^{3}H]putrescine or [^{3}H]spermidine uptake process, competing metabolites (5 mM) and the [^{3}H]putrescine or [^{3}H]spermidine were added simultaneously to the cell suspension and incubated for 30 min before terminating the reaction. In order to decrease the intracellular levels of polyamines, polyamine biosynthesis was inhibited by treating ring-stage parasites for 24 h with 1 mM DFMO prior to performing uptake experiments. The dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake on extracellular Na^+ was investigated by washing and re-suspending the cells in solution E or and performing the time-course at 37°C. In order to determine the effect of pH on polyamine uptake, functionally isolated parasites were re-suspendend in solution F and time-courses performed at 37°C. For

the experiments designed to investigate the effect of the membrane potential on [^{3}H]putrescine or [^{3}H]spermidine uptake the manipulation of the membrane potential was performed prior to the addition of the [^{3}H]putrescine or [^{3}H]spermidine. To hyperpolarize the membrane, the cell suspension were re-suspended in Solution C for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine reaction buffer in Solution C. A further hyperpolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM). To depolarize the membrane, the cell suspension were prepared in Solution D with and without valinomycin (1 µM), or in Solution A and D with the V-type ATPase inhibitor Concanamycin A (100 nM) and the cells were incubated for 30 min prior to the initiation of the reaction.

2.2.4 Creating RBCs with modified haemoglobin content

In one series of experiments the uptake of [^{3}H]putrescine was measured in RBCs in which the intracellular haemoglobin concentration was reduced, using a protocol modified from Krogstad *et al.*. (1985). Three different RBC cell sets were used: 1) an untreated control, 2) 'Resealed RBCs 1' in which the haemoglobin concentration was ~10% that in the control cells, and 3) 'Resealed RBCs 2' in which the haemoglobin concentration was ~40% that in the control cells. RBCs were lysed with hypotonic solutions, resulting in the release of the intracellular contents: 'Resealed RBCs 1' (1 part packed cells: 4.5 parts hypotonic solution 1, 5 mM HEPES, 11 mM glucose, and 2 mM ATP, pH 7.4 v/v) and 'Resealed RBCs 2' (1 part packed cells: 1.125 parts hypotonic solution 2, 2.5 mM HEPES, 5.5 mM glucose, and 1 mM ATP, pH 7.4 vol/vol). Following a 10 min incubation at 30°C a hypertonic solution was added to reseal the cells: 'Resealed RBCs 1' (equal volume of the hypertonic solution 1, 280 mM NaCl, 40 mM KCI, and 11 mM glucose pH 7.4) and 'Resealed RBCs 2' (0.4 volume of hypertonic solution 2, 1.4 M NaCl, 200 mM KCI, and 55 mM glucose, pH 7.4) (Krogstad *et al.*, 1985). The cells were washed three times with solution A and the resealed cells were then used to measure the uptake of [^{3}H]putrescine measured as described above.

2.2.5 Cytosolic pH measurements of isolated *P. falciparum* parasites

The effect of putrescine and spermidine import on the cytosolic pH (pH_i) was measured by preloading the isolated parasites with the fluorescent pH-sensitive dye, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) (Invitrogen). Isolated parasites were incubated

with 5 μM of the acetoxymethyl ester form (BCECF-AM) at 37°C for 10 min to allow diffusion of the non-fluorescent form into the cell. Once inside the isolated parasites, cellular esterases cleaved the ester moieties, leaving charged residues that was trapped within the cell (Wünsch *et al.*, 1997). The cells were washed three times (5 000xg, 1 min) and re-suspendend in solution A at 37°C for 10 min to allow for complete de-esterification. Prior to pH_i measurements, a 1 ml sample of these cells were centrifuged at 16 000xg, re-suspended in the appropriate solution, and transferred to a tapered polystyrene cuvette (Starna Pty Ltd). pH_i was calibrated using the K^+/H^+ ionophore nigericin/high K^+ method (Saliba and Kirk, 1999). By suspending cells in a high K^+ solution (130 mM KCl, 20 mM glucose, 20 mM Hepes, 20 mM MES, 1 mM $MgCl_2$ at pH 6.8, 7.1 or 7.8) in the presence of the K^+/H^+ ionophore nigericin, the K^+ gradient is abrogated and the pH_i equilibrates with the pH_o. The emission at 520 nm was recorded following excitation at both 490 nm (pH-dependent) and the isosbestic point of 440 nm (pH-independent) with a Perkin Elmer LS-50B spectrofluorimeter with the dual excitation Fast Filter accessory as described (Saliba and Kirk, 1999). By using the 495/440 nm ratio to monitor pH_i, pH independent artefacts can be eliminated. A linear regression line was calculated from the 495/440 nm fluorescence ratio at each of the three pH calibration values and used to convert the fluorescence data obtained with the FL WinLab program to pH_i.

The effect of putrescine import on the cytosolic pH (pH_i) at 37°C at different external pH was investigated by re-suspending the isolated, BCECF-loaded parasites in Solution A (pH 7.1) or Solution F (pH 8.1), obtaining a baseline pH_i, and monitoring pH_i following the addition of either 10 mM NH_4Cl or 10 mM putrescine dihydrochloride.

2.2.6 Data analysis

For the purpose of the figures the uptake of radiolabelled polyamines is represented as a 'distribution ratio' i.e. the concentration of radiolabelled polyamine inside the cell relative to that in the extracellular solution.

The concentration of radiolabel inside the cells was estimated from the amount of radiolabel in the cell pellet by first subtracting the radioactivity trapped in the extracellular space and adhering to the cell surface, determined as described in sections 2.2.3.1 and 2.2.3.2. This background measurement was subtracted from the data to obtain a measurement of the amount of [^{3}H]putrescine or [^{3}H]spermidine imported into the cells comprising the cell pellet. This was divided by the number of the cells in the pellet and by the intracellular water volume

to yield an estimate of the intracellular concentration. The intracellular water volume was taken as 75 fl for uninfected and *P. falciparum-infected* erythrocytes and 28 fl for isolated parasites (Saliba *et al.*, 1998).

Unless otherwise specified, the data are presented as the means from at least three independent experiments and the standard-error-of-the-mean (S.E.) is indicated. Statistical significance was determined by the two-tailed t-test using the Graphpad Instat (v 3.06) program, or by the Wilcoxon Matched Pairs Test using Statistica (v9). Non-linear regression was performed with SigmaPlot (v.11). The rates of [^{3}H]putrescine or [^{3}H]spermidine import were calculated by fitting the data to the first order equation $y=a \times (1-e^{-kt})$, where *a* is the maximum amount of [^{3}H]putrescine or [^{3}H]spermidine imported and *k* is the first order rate constant. The product of *a* and *k* gives the initial rate of [^{3}H]putrescine or [^{3}H]spermidine uptake.

For kinetics measurements, the K_m and V_{max} were determined by fitting the data to the Michaelis-Menten equation where $V_o=V_{max}[substrate]/(K_m+[substrate])$. In addition, the data were also represented using the Eadie-Hofstee plot ($V_0=-K_m.V_{max}/[substrate]+V_{max}$) as well as the Hanes-Woolf plot ($substrate]/V_0=[substrate]/V_{max}+K_m/V_{max}$).

2.3 Results

Polyamines carry multiple positive charges at physiological pH, and these can lead to electrostatic interactions with the negatively charged components of membranes (Schuber, 1989). This complicates polyamine uptake studies since a significant proportion of the cell-associated polyamines may be adhering to the cell surface (Pistocchi *et al.*, 1988). In *E. coli,* it was shown that this absorbed component increased with increasing valency of the polyamine (Tabor and Tabor, 1966). In initial polyamine uptake experiments carried out as part of this study it was found that for both intact parasitised erythrocytes, and parasites isolated from their host cells by saponin-permeabilisation of the erythrocyte membrane, there was, on addition of radiolabelled polyamine, a rapid association of radiolabel with the cells, followed by a slower, progressive increase. A representative experiment (uptake of [^{3}H]spermidine into isolated parasites) is shown in Fig. 2.1. The rapid initial association of radiolabel is most likely due to polyamines trapped in the extracellular space as well as adherence of the radiolabelled polyamines to the cell surface (Pistocchi *et al.*, 1988; Schuber, 1989; Tabor and Tabor, 1966). For all uptake experiments this 'background' radioactivity was estimated (as described in section 2.2.3.1 and 2.2.3.2) and subtracted from the total measured cpm to obtain an estimate of the intracellular concentration of radiolabelled polyamine, from which the 'distribution ratio' was calculated.

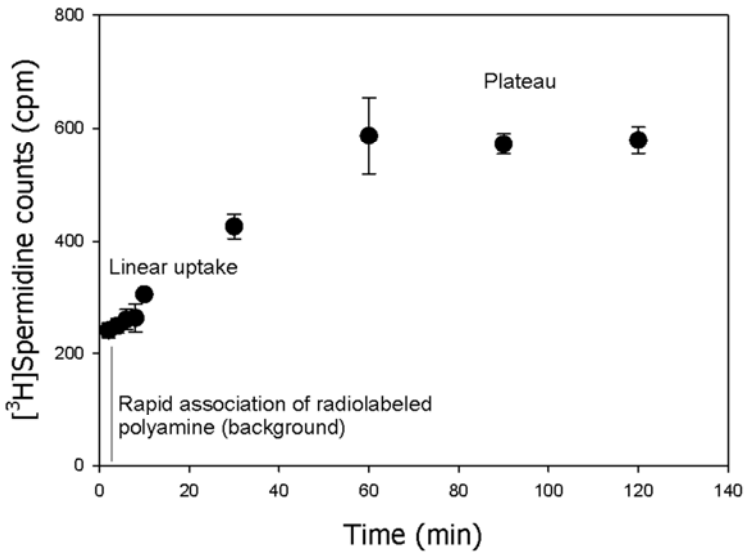


Figure 2.1: Typical time course for the uptake of a radioactive polyamine (in this case [^{3}H]spermidine) by isolated parasites. The background radioactivity due to the rapid association of the radiolabel with the cells, followed by the linear increase due to uptake into the cells and the subsequent plateau of the radiolabel, are indicated. Uptake was measured at 37°C over 120 min. The data are from a single representative experiment, carried out in triplicate and shown ± standard deviation. Where not shown, the error bars fall within the symbols.

For all time-course experiments the estimated distribution ratio was plotted as a function of time. The initial rate of uptake was determined from the initial slope of the uptake time course. The final 'equilibrium' distribution ratio was estimated from the distribution ratio measured after the time course had 'plateaued' (See Fig. 2.1).

In some later experiments the initial rate of uptake into the cells of interest was estimated from the amount of radiolabel taken up within a fixed incubation period. Ideally such experiments are carried out using an incubation period that falls within the initial approximately linear portion of the uptake time course. In the case of the polyamines (as compared to less 'sticky' substrates) the high degree of association of radiolabelled compound with the cell surface gives rise to a high 'background count' against which the time-dependent uptake of radiolabel into the cells has to be measured. For this reason it was, in some experiments necessary to use longer incubations than would ideally be the case, in order to ensure that the measured uptake of radioactivity was significantly above the high background level.

2.3.1 [^{3}H]putrescine or [^{3}H]spermidine uptake into *P. falciparum*-infected RBCs

2.3.1.1 Uptake of [^{3}H]putrescine or [^{3}H]spermidine into RBCs and iRBCs

The uptake of [^{3}H]putrescine (extracellular concentration of 5 nmol/l) or [^{3}H]spermidine (extracellular concentration of 6 nmol/l) into intact iRBCs was compared with that into RBCs over 60 min at 37°C (Fig. 2.2). Consistent with published results on polyamine uptake into RBCs (Fukumoto and Byus, 1996), both [^{3}H]putrescine and [^{3}H]spermidine were taken up by the RBCs. By the end of the 60 min incubation, [^{3}H]putrescine uptake had reached a distribution ratio of 1.9±0.3 (n=6) and [^{3}H]spermidine uptake had reached a higher distribution ratio of 7±2 (n=5). Thus, both putrescine and spermidine are accumulated by RBCs. In iRBCs, the total accumulation of [^{3}H]putrescine and [^{3}H]spermidine measured at the end of the 60 min incubation was much lower than that seen in uninfected RBCs ($P \leq 0.05$). Uptake of [^{3}H]putrescine or [^{3}H]spermidine into iRBCs reached a distribution ratio of 0.8±0.4 (n=6) or 1.4±0.4 respectively, following 60 min incubation.

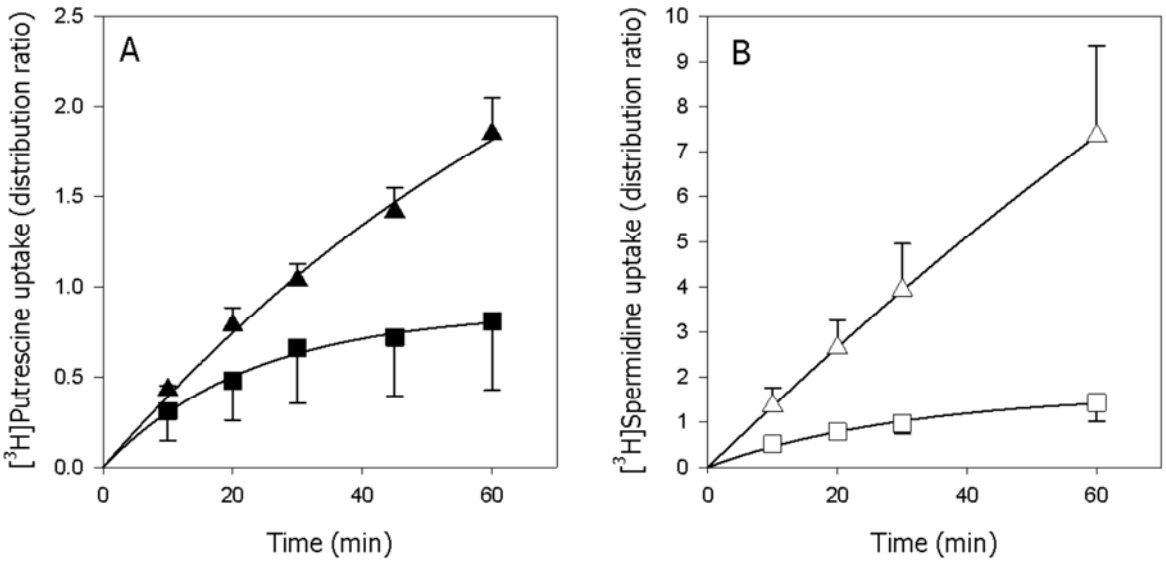


Figure 2.2: Uptake of [^{3}H]putrescine and [^{3}H]spermidine by RBCs and iRBCs. Time courses for the uptake of (A) [^{3}H]putrescine uptake into RBCs (▲) and iRBCs (■) at 37°C measured over 60 min. The data are averaged from six independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake into RBCs (△) and iRBCs (□) at 37°C over 60 min averaged from five independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM. Where not shown, the error bars fall within the symbols.

The initial rates of [^{3}H]putrescine or [^{3}H]spermidine uptake were calculated by expressing the uptake as pmol/10^{10} cells as a function of time and fitting the data to the first order equation $y=a \times (1-e^{-kt})$, where *a* is the maximum amount of [^{3}H]putrescine or [^{3}H]spermidine imported and *k* is the first order rate constant. Since [^{3}H]spermidine uptake into RBCs reached a distribution ratio of more than 1 (indicating that the radiolabelled polyamine has accumulated to levels higher than the extracellular levels) by t=10 min (Fig. 2.2), the incubation temperature was decreased (putrescine uptake to 22°C and spermidine uptake to 4°C) to slow the uptake sufficiently to obtain initial rate measurements over an initial period during which the distribution ratio was below 1 (Cobbold *et al.*, 2011). The results are shown in Fig. 2.3.

At 22°C, there was no statistically significant difference in the initial rate of [^{3}H]putrescine uptake into RBCs compared to iRBCs (36±7 fmol [^{3}H]putrescine/10^{10}cells/min vs. 38±7 fmol [^{3}H]putrescine/10^{10}cells/min, n=4, $P \geq 0.05$). Likewise, at 4°C, there was no statistically significant difference in the initial rate of spermidine uptake into RBC compared to iRBC (15±5 fmol [^{3}H]spermidine /10^{10}cells/min vs. 13.3±2.6 fmol [^{3}H]spermidine /10^{10}cells/min, n=4, $P \geq 0.05$). These results show that although the total accumulation of [^{3}H]putrescine or [^{3}H]spermidine in iRBCs are less than for RBCs following 60 min incubation, the initial rate of uptake is the same for both iRBCs and RBCs.

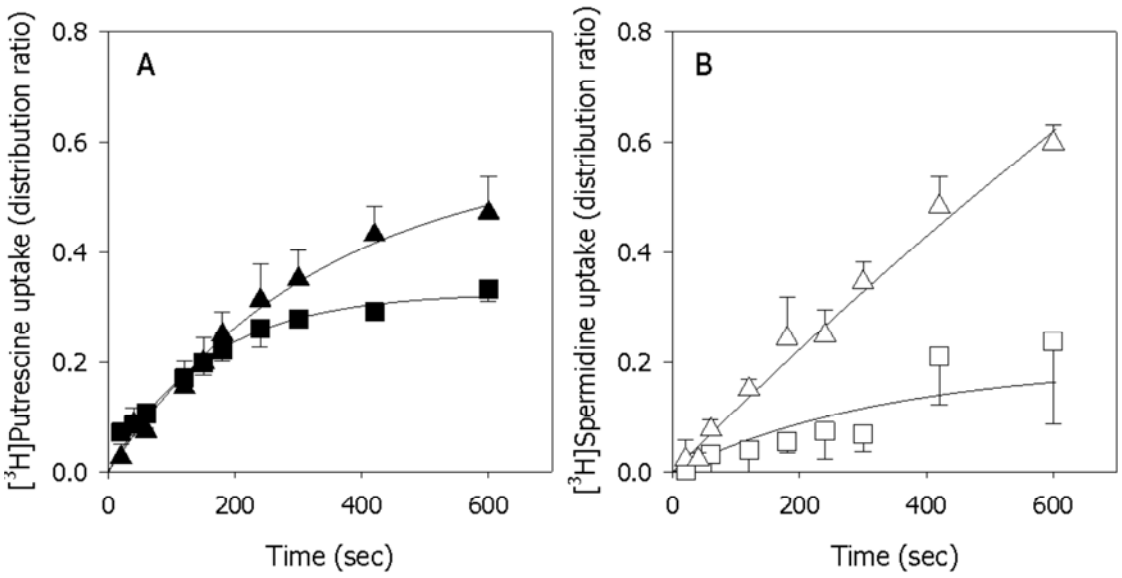


Figure 2.3: Uptake of [^{3}H]putrescine and [^{3}H]spermidine by RBCs and iRBCs. RBCs from a single donor were used for each experiment. Both uninfected and infected RBCs were cultured for 48 hours prior to experimentation. Time courses for the uptake of (A) [^{3}H]putrescine at 22°C over 10 min averaged from four separate experiments and shown ± S.E. and (B) [^{3}H]spermidine at 4°C over 10 min averaged from three separate experiments and shown ± S.E. into RBCs (A ▲, B△) and iRBCs (A ■, B □). For both polyamines the extracellular concentration was approximately 5 nM.

2.3.1.2 Effect of haemoglobin on polyamine uptake into RBCs

The observed decrease in [^{3}H]putrescine or [^{3}H]spermidine uptake in RBCs following infection with *P. falciparum* parasites (Fig. 2.2) may have a number of possible explanations. There is a ~2-fold increase in putrescine levels and ~3-fold increase in spermidine levels in the RBC cell compartment of iRBCs compared to co-cultured, uninfected RBCs (Das Gupta *et al.*, 2005). This increase in polyamines in iRBCs compared to RBCs may contribute to the reduced accumulation of radiolabelled polyamines taken up from the external medium; e.g. the endogenous polyamines may be occupying intracellular binding sites, thereby excluding the radiolabelled polyamines from these. Alternatively, the accumulation of the two polyamines to high intracellular concentrations in the RBC could be due to the binding of the polyamines to cytosolic RBC proteins (Kakhniashviki *et al.*, 2004), with the decreased uptake of polyamines in iRBCs resulting from the parasite having digested a significant fraction of these proteins (Loria *et al.*, 1999). The most abundant protein present in the RBC cytosol is haemoglobin (Chakrabarti *et al.*, 2011). The effect of intracellular haemoglobin concentration on the accumulation of polyamines by uninfected erythrocytes was therefore investigated.

A lysis-and-reseal method used previously to load fluorescent markers into RBCs (Krogstad *et al.*, 1985) was adapted to obtain resealed RBCs with different internal concentrations of

haemoglobin. The amount of haemoglobin present following resealing was estimated from volumes of the hypotonic and hypertonic solutions used in preparing the resealed RBCs; e.g. if 1 ml RBCs were lysed and resealed in solutions to a final volume of 10 ml, each resealed RBC would have 10% of the initial haemoglobin concentration. [^{3}H]putrescine uptake was measured into control RBCs, RBCs containing ~40% of the initial haemoglobin concentration and RBCs containing ~10% of the initial haemoglobin concentration (Fig. 2.4). The decrease in haemoglobin concentration caused a pronounced decrease in [^{3}H]putrescine accumulation. This data indicate that (i) the accumulation of polyamines to high levels within RBCs may be attributed to a large extent to the interaction of the polyamines with haemoglobin (and/or possibly other, less abundant protein components within the RBCs cytosol); (ii) the reduced accumulation of polyamines within iRBCs may be due, at least in part, to the reduced haemoglobin content of these cells.

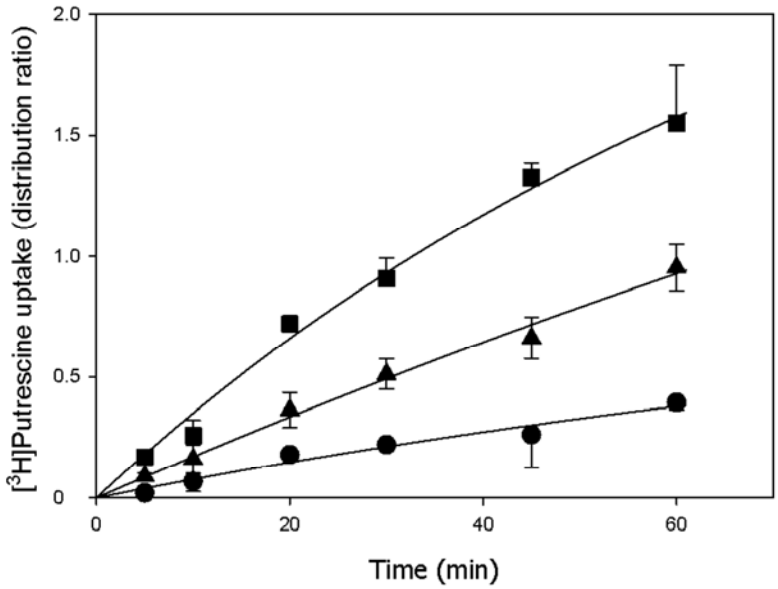


Figure 2.4: Uptake of [^{3}H]putrescine by RBCs with different haemoglobin concentrations. Time courses for the uptake of [^{3}H]putrescine at 37°C over 60 min from one experiment for RBC control (100% haemoglobin) (■), 'Resealed RBCs 1' (~40% haemoglobin) (▲) and 'Resealed RBCs 2' (~10% haemoglobin) (●), shown ± standard deviation. The extracellular putrescine concentration was approximately 5 nM. Where not shown, the error bars fall within the symbols.

2.3.1.3 Effect of the NPP-inhibitor furosemide on [^{3}H]putrescine and [^{3}H]spermidine uptake into RBCs and iRBCs

In iRBCs, uptake of metabolites occurs via a combination of endogenous RBC transporters, the parasite-induced broad-specificity NPP, and, possibly also via parasite-derived substrate-specific transporters that are directed to the EPM (Kirk, 2001). To investigate the involvement of the

NPP in the uptake of polyamines into iRBCs, [^{3}H]putrescine or [^{3}H]spermidine uptake was measured in the presence or absence of the NPP-inhibitor furosemide. In the case of [^{3}H]putrescine, uptake was not influenced by the presence of furosemide (Fig. 2.5) ($P \geq 0.05$), indicating that the NPP are not involved in [^{3}H]putrescine uptake into iRBCs. By contrast, the uptake of [^{3}H]spermidine did have a furosemide-sensitive component with a ~60% decrease in spermidine uptake in the presence of furosemide (distribution ratio decreased from 0.60±0.11 to 0.253±0.005, n=4, $P \leq 0.05$). The partial furosemide sensitivity of the uptake of spermidine into iRBCs contrasted with the lack of furosemide sensitivity of the uptake of spermidine into uninfected RBCs (Fig. 2.5, inset; $P \geq 0.05$).

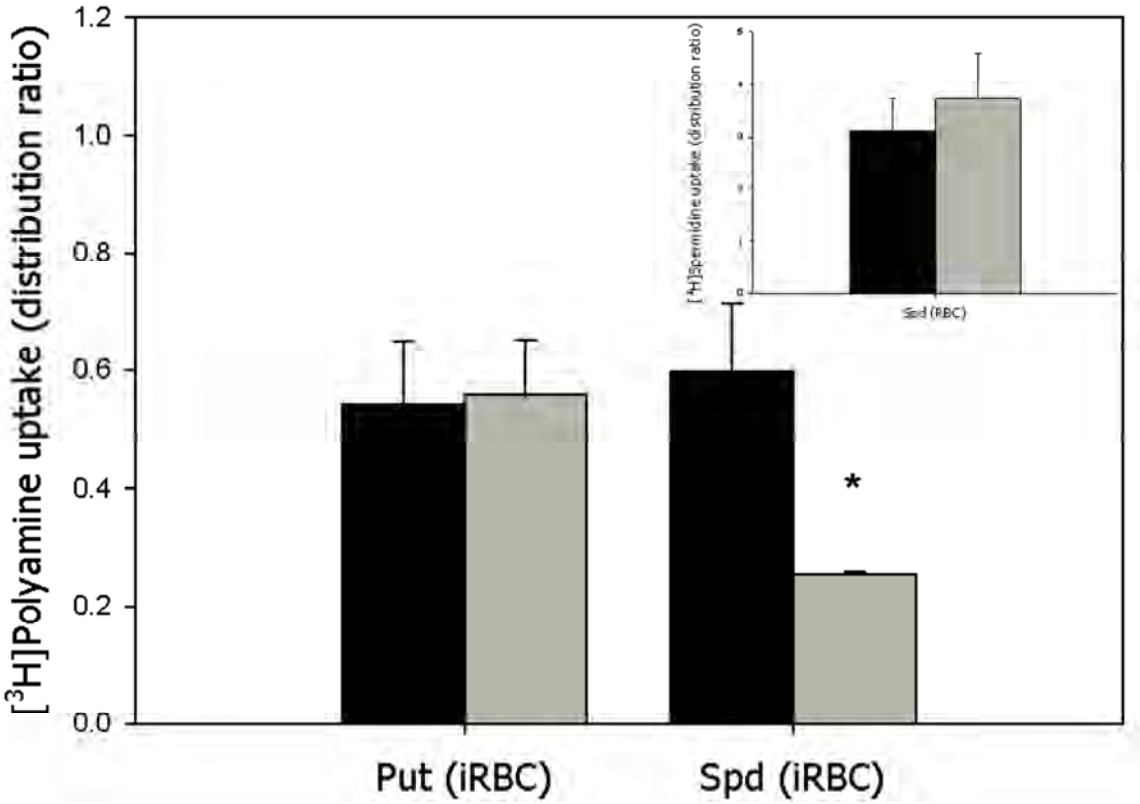


Figure 2.5: Effect of furosemide on uptake of [^{3}H]putrescine or [^{3}H]spermidine into iRBCs. [^{3}H]putrescine (Put), or [^{3}H]spermidine (Spd) uptake into iRBCs in the presence of furosemide (100 µM) (grey bars) vs. the control (black bars) over 10 min at 37°C, averaged from four independent experiments and shown ± S.E., * $P<0.05$. For both polyamines the extracellular concentration was approximately 5 nM. **Inset:** [^{3}H]spermidine uptake into RBCs in the presence of furosemide (100 µM) (grey bars) vs. the control (black bars) over 10 min at 37°C, averaged from seven independent experiments and shown ± S.E.

The data are consistent with the furosemide-sensitive NPP playing some role in [^{3}H]spermidine uptake into iRBC, despite the overall rate of uptake of [^{3}H]spermidine remaining the same as that in uninfected RBCs.

These results confirmed a previous report that both [^{3}H]putrescine and [^{3}H]spermidine uptake occurs in the *P. falciparum-infected* RBCs (Ramya *et al.*, 2006). Subsequent investigations focused on the uptake of [^{3}H]putrescine or [^{3}H]spermidine into functionally isolated parasites.

2.3.2 [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

2.3.2.1 Comparison of [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

The ability of the intracellular *P. falciparum* parasite to take up [^{3}H]putrescine or [^{3}H]spermidine across its plasma membrane was investigated in parasites functionally released from their host RBCs by saponin-permeabilisation of the erythrocyte and parasitophorous vacuolar membranes (Saliba *et al.*, 1998). Both [^{3}H]putrescine and [^{3}H]spermidine were taken up by isolated, trophozoite-stage *P. falciparum* parasites (Fig. 2.6). [^{3}H]spermidine uptake occurred both at a faster initial rate compared to [^{3}H]putrescine uptake (0.99±0.40 pmol [^{3}H]spermidine/10^{10}cells/min vs. 0.41±0.13 pmol [^{3}H]putrescine/10^{10}cells/min, n=7, $P \leq 0.1$), as well as to a significantly higher total accumulation level (Fig. 2.6), with [^{3}H]spermidine total uptake ~2-fold higher than [^{3}H]putrescine uptake after 2 hrs (distribution ratio [^{3}H]putrescine=1.33±0.22 vs. [^{3}H]spermidine 2.4±0.5, n=7, $P \leq 0.05$).

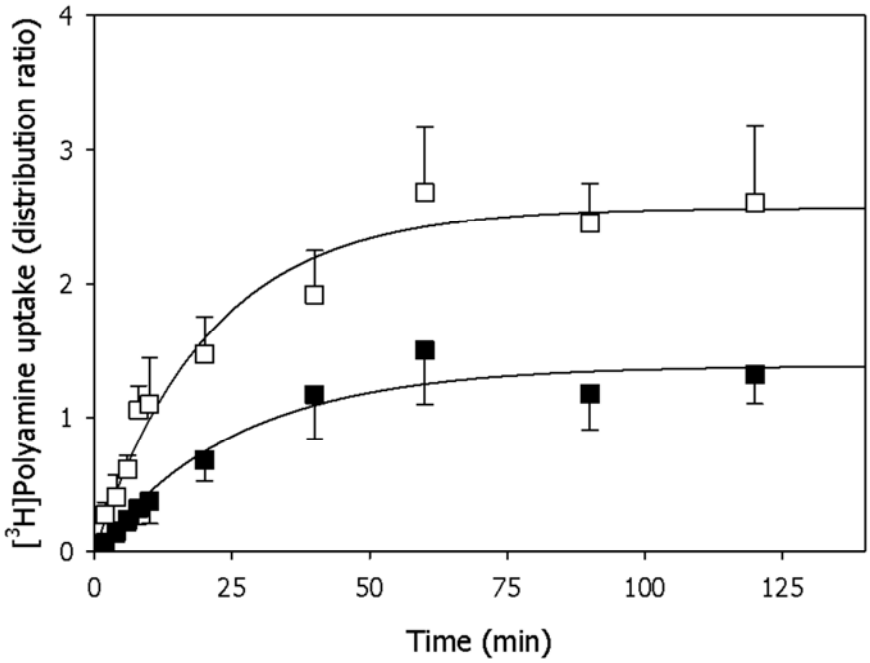


Figure 2.6: Time courses for the uptake of [^{3}H]putrescine (■) and [^{3}H]spermidine (□) by isolated *P. falciparum* trophozoites at 37°C. For both polyamines the extracellular concentration was approximately 5 nM. The data are averaged from seven independent experiments and shown ± S.E.

2.3.2.2 Temperature dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

Polyamine uptake has been shown to be temperature dependent in a variety of cells (Basselin *et al.*, 2000; Fukumoto and Byus, 1996; Romero-Calderón and Krantz, 2006; Soulet *et al.*, 2002). The same was found to be true here in isolated *P. falciparum* parasites. As shown in Fig. 2.7, reduction of the temperature from 37°C to 22°C led to a significant decrease in the initial rate of uptake of [^{3}H]putrescine (188±21 fmol [^{3}H]putrescine /10^{10} cells/min at 37°C vs. 99±25 fmol [^{3}H]putrescine /10^{10} cells/min at 22°C, n=6, $P \leq 0.05$). There was also a decrease in initial uptake rates of [^{3}H]spermidine with a decrease in temperature (1.38±0.16 pmol [^{3}H]spermidine/10^{10} cells/min at 37°C vs. 0.56±0.12 pmol [^{3}H]spermidine/10^{10} cells/min at 22°C, n=6, $P \leq 0.05$).

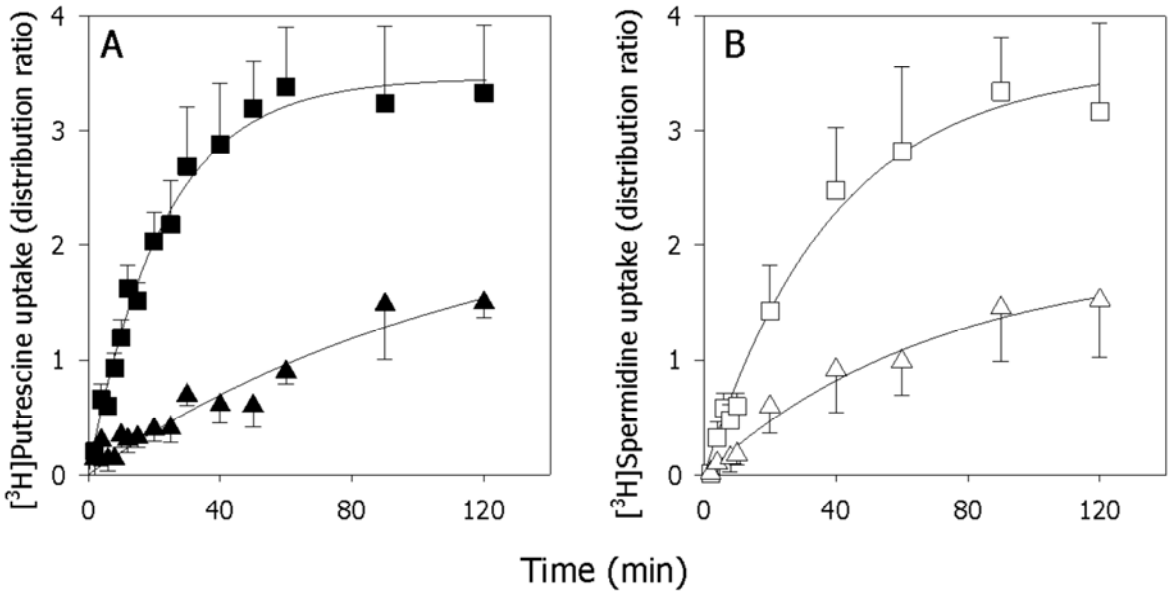


Figure 2.7: Temperature dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites. To determine the effect of temperature on [^{3}H]putrescine or [^{3}H]spermidine uptake, time-courses were performed at 22°C and 37°C. (A) [^{3}H]putrescine uptake at 37°C (■) and 22°C (▲) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake at 37°C (□) and 22°C (△) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

2.3.2.3 Energy dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites

The energy dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* trophozoites was measured by washing and equilibrating isolated parasites in a glucose-free medium (solution B) at 37°C for 30 min to deplete the cells of ATP, prior to the initiation of the

reaction. The malaria parasite is wholly dependent on glycolysis for the production of ATP (van Dooren *et al.*, 2006) and suspension of isolated parasites in glucose-free medium leads to a rapid depletion of the parasite's ATP (Saliba and Kirk, 1999).

The initial rate of uptake of [^{3}H]putrescine in glucose-deprived parasites was significantly reduced from that of the control cells (119±15 fmol [^{3}H]putrescine/10^{10} cells/min in glucose-replete cells vs. 53±15 fmol [^{3}H]putrescine/10^{10} cells/min in glucose-depleted cells, n=6, $P \leq 0.05$). By contrast, the total uptake of [^{3}H]putrescine as measured at 90 min was not significantly influenced by glucose depletion (distribution ratio of 2.4±0.4 in glucose-replete cells vs. 2.2±0.2 under glucose-depleted conditions, n=6, $P \geq 0.05$). Moreover, accumulation still occurred as a distribution ratio of more than 1 was reached even in the absence of glucose (Fig. 2.8, A).

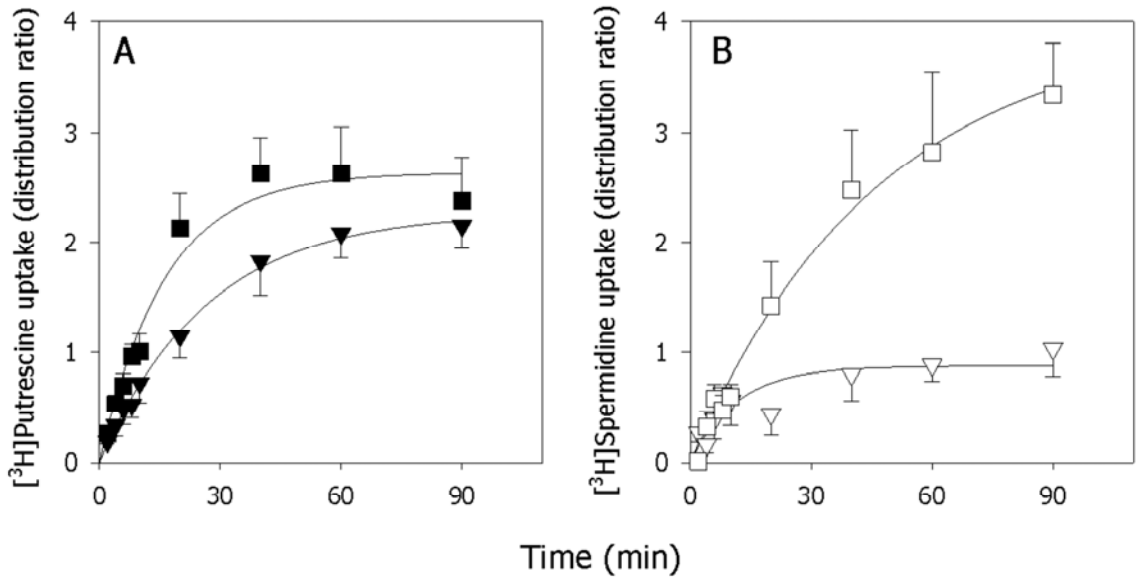


Figure 2.8: Glucose dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites. (A) [^{3}H]putrescine uptake in normal glucose-replete conditions (Solution A) (■) and in parasites suspended in glucose-free saline (Solution B) (▼) at 37°C, averaged from six independent experiments and shown ± S.E . (B) [^{3}H]spermidine uptake in normal glucose replete conditions (Solution A) (□) and glucose free saline (Solution B) (▽) at 37°C, averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

In contrast to the slight, if any, effect of glucose depletion on the total uptake of putrescine into isolated *P. falciparum* parasites following 90 min, there was a very striking effect of glucose depletion on the total uptake of [^{3}H]spermidine into isolated parasites following the 90 min incubation period (Fig. 2.8, B). In glucose-replete cells, [^{3}H]spermidine uptake increased progressively over the 90 min incubation period, reaching a distribution ratio of 3.3±0.5, n=6 after 90 min (and still increasing at this point). By contrast, in glucose-depleted cells, the

distribution ratio levelled off at a value of approximately 1 at around 30 min and remained at this level for the remainder of the time-course. A comparison of the initial rates of [^{3}H]spermidine uptake into isolated parasites under glucose-replete and glucose-depleted conditions revealed a modest but not statistically significant decrease in the initial uptake rate of glucose-depleted cells (138±16 fmol [^{3}H]spermidine/10^{10} cells/min in glucose-replete cells vs. 80±30 fmol [^{3}H]spermidine/10^{10} cells/min in glucose-depleted cells, n=6, $P \geq 0.05$).

2.3.2.4 Kinetics of [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites

[^{3}H]putrescine or [^{3}H]spermidine uptake into isolated *P. falciparum* parasites was measured over a range of concentrations of putrescine and spermidine, respectively, in order to investigate the kinetics of uptake. Uptake was measured over a 15 min period in order to obtain measurements that were (approximately) within the initial linear part of uptake, as well as having a large part of the measurement due to uptake and not background radiation as discussed in section 2.3. In the case of putrescine, there was a non-linear concentration dependence of uptake over the 0-15 mM range, whereas in the case of spermidine, uptake increased in a linear manner with spermidine concentration over this range (Fig. 2.9 A and B). A Michaelis-Menten equation could be fitted to the putrescine data resulting in a K_m=9.1±1.2 mM and a V_{max}=9.7±2.2 µmol putrescine/10^{10} cells/h (n=5). The uptake of [^{3}H]putrescine or [^{3}H]spermidine was subsequently measured over an extracellular concentration range of 0-500 µM putrescine and spermidine (Fig. 2.9 , C and D). Over this concentration range, putrescine uptake was linear, while spermidine uptake followed apparent Michaelis-Menten kinetics with a calculated K_m=0.42±0.12 mM and V_{max}=0.14±0.02 µmol spermidine/10^{10} cells/h (n=5).

Thus, for both putrescine and spermidine there was a low-affinity component for uptake into isolated parasites. In the case of spermidine there was, in addition a high-affinity component, whereas the same was not the case for putrescine. There are several assumptions inherent in the Michaelis-Menten equation, including the involvement of a single enzyme (or here, transport protein) (Palmer, 2001). If a single uptake mechanism is present, the representation of the data using linear data analyses with either the Eadie-Hofstee equation or the Hanes-Woolf equation should result in a straight line (Coons *et al.*, 1995). An absence of linearity indicates that a multi-component system is present, as is the case with glucose uptake into *S. cerevisiae* (Coons *et al.*, 1995).

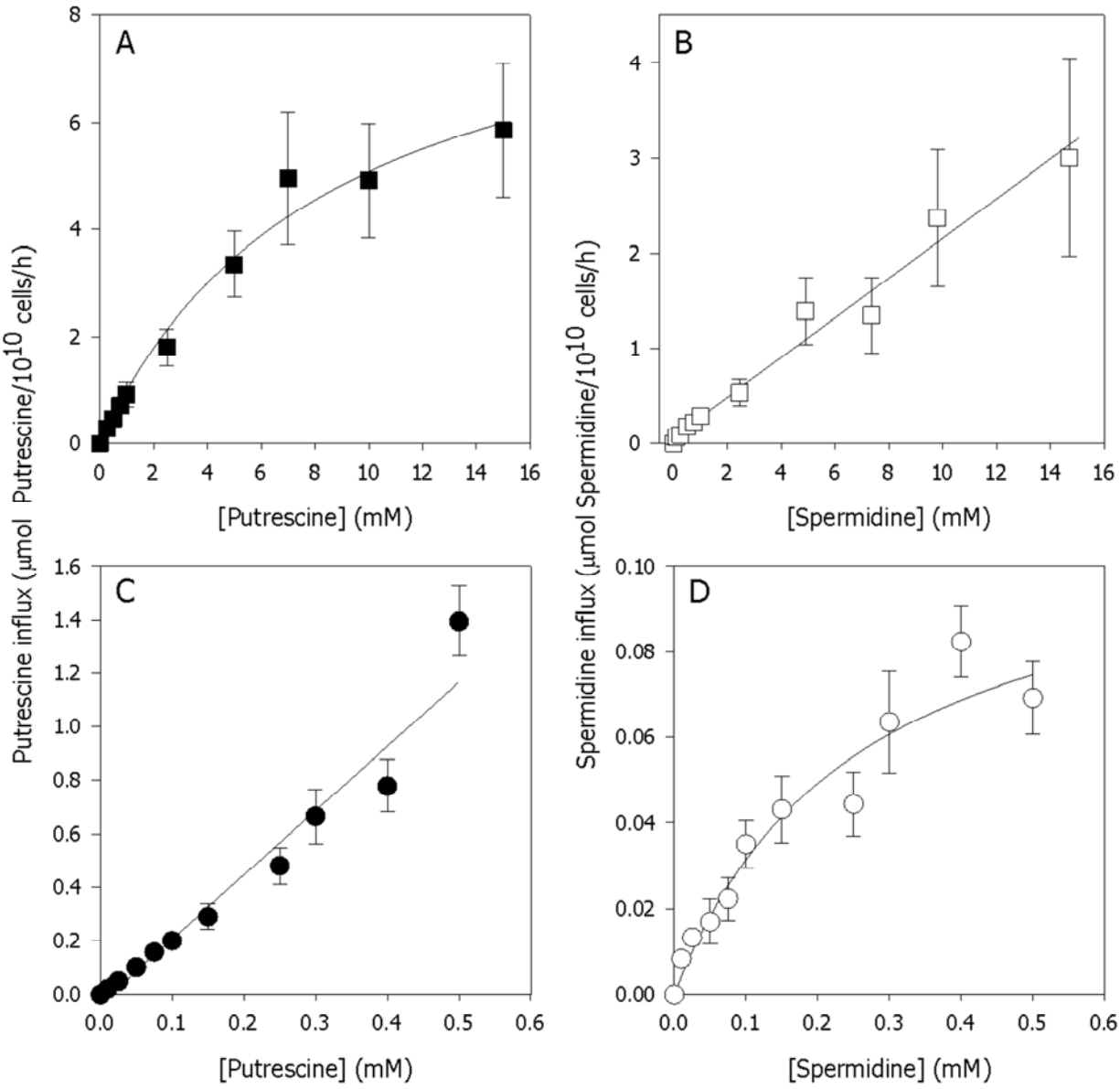


Figure 2.9: Kinetics of [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites at 37°C. The concentration dependence of the uptake of [^{3}H]putrescine or [^{3}H]spermidine was measured in solution A at 15 min over an extracellular concentration range of 1-15 mM (A) putrescine and (B) spermidine and of 0-500 µM (C) putrescine and (D) spermidine. In each case the data were fitted to the Michaelis-Menten equation: polyamine influx=V_{max} [polyamine]/(K_m + [polyamine]). Data were averaged from 5 independent experiments and are shown ± S.E.

Further analyses of the kinetic data was performed using the Eadie-Hofstee equation as well as the Hanes-Woolf equation (Palmer, 2001). Neither the Eadie-Hofstee equation nor the Hanes-Woolf equation applied to the measurements of [^{3}H]putrescine uptake or [^{3}H]spermidine uptake into isolated parasites resulted in a plot to which a linear line could be fitted (Fig. 2.10). These results imply that there are multiple components to the uptake of putrescine as well as spermidine into isolated *P. falciparum* trophozoites as measured over 15 min.

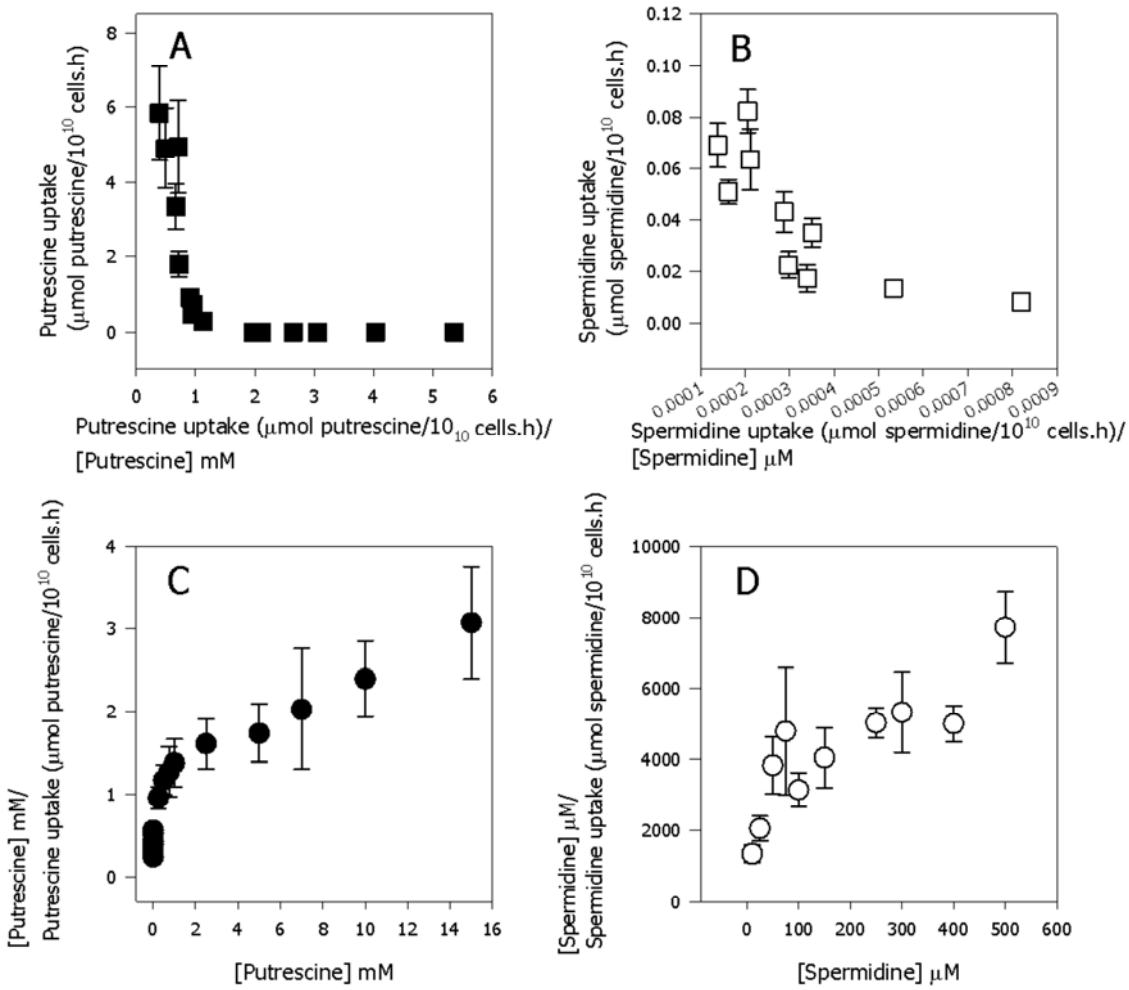


Figure 2.10: Characterisation of kinetics of [^{3}H]putrescine or [^{3}H]spermidine transport into isolated *P. falciparum* parasites at 37°C. The data given in Fig. 2.9 A (putrescine uptake at extracellular putrescine concentrations of 0-15 mM) and D (spermidine uptake at extracellular spermidine concentrations of 1-500 µM), are represented using the Eadie-Hofstee equation: V_0=-K_M.V_{max}/[substrate] +V_{max}, for (A) putrescine and (B) spermidine or Hanes-Woolf equation: [substrate]/V_0=[substrate]/V_{max} + K_m/V_{max}, for (C) putrescine and (D) spermidine. Data were averaged from 5 independent experiments and shown ± S.E.

2.3.2.5 Specificity of [^{3}H]putrescine or [^{3}H]spermidine import by *P. falciparum* parasites

To investigate the specificity of the uptake mechanism by which the parasites take up polyamines, competition assays were performed with a range of polyamines and amino acids, each introduced at an external concentration of 5 mM in the presence of 5 nM [^{3}H]putrescine or 6 nM [^{3}H]spermidine. The polyamines putrescine, spermidine and spermine inhibited both [^{3}H]putrescine and [^{3}H]spermidine uptake into isolated *P. falciparum* parasites ($P \leq 0.05$), with inhibition of uptake increasing with the increase in size and charge of the polyamine (putrescine<spermidine<spermine) (Fig. 2.11). Spermidine and spermine had the greatest

effect (>80% competition) on [^{3}H]spermidine uptake. Ornithine, the precursor of putrescine, had an inhibitory effect on [^{3}H]putrescine uptake comparable to that of unlabelled putrescine itself (i.e. an approximately 50% inhibition), while causing only a ~20% decrease in [^{3}H]spermidine uptake.

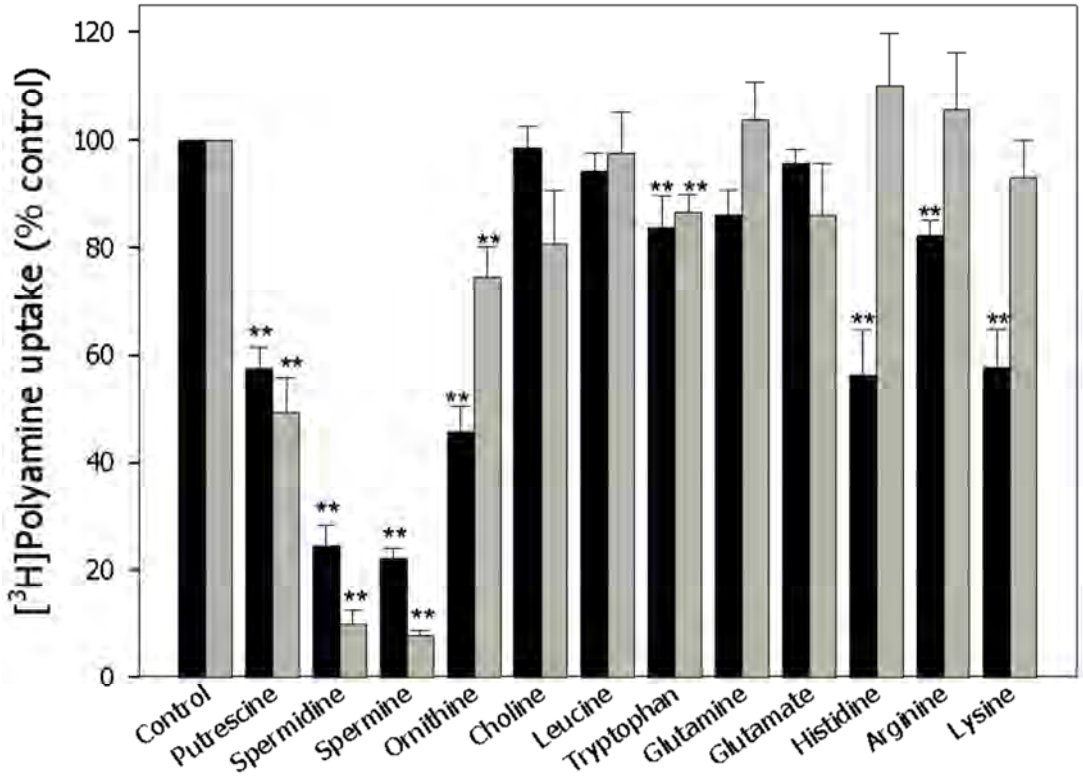


Figure 2.11: Inhibition of [^{3}H]putrescine or [^{3}H]spermidine uptake by *P. falciparum* trophozoites by various metabolites (5 mM) at 37°C. The data are expressed as percentage of control (i.e. uptake relative to the control, measured under conditions in which no competing substrate was added). Statistical significance was determined with a Wilcoxon Matched Pairs test, * $P<0.01$, ** $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars averaged from 5 independent experiments and shown ± S.E. and [^{3}H]spermidine uptake by grey bars averaged from 6 independent experiments and shown ± S.E.

It has been suggested that putrescine may be transported by the same carrier as choline (Staines and Kirk, 1998); however unlabelled choline had no significant effect on putrescine uptake ($P\geq0.05$). Neither the neutral amino acid leucine, nor the acidic amino acid glutamate affected polyamine uptake ($P\geq0.1$). The basic amino acids histidine, lysine and arginine did not affect spermidine uptake, but did decrease [^{3}H]putrescine uptake. In contrast, tryptophan inhibited both [^{3}H]putrescine and [^{3}H]spermidine uptake. These results suggest two possible scenarios. One is that there is a single transport system for the polyamines putrescine, spermidine and spermine, as well as the putrescine precursor ornithine, with an additional uptake mechanism for putrescine that is shared with the basic amino acids. The other is that

there are different putrescine-specific and spermidine-specific uptake mechanisms in *P. falciparum*, albeit with some cross-reactivity. The putrescine-specific uptake mechanism may be more promiscuous and allow for the uptake of unrelated but structurally similar metabolites (including the polyamines, ornithine and the basic amino acids). Furthermore, the occurrence of [^{3}H]putrescine and [^{3}H]spermidine uptake even in the presence of a large excess of unlabelled substrate (5 mM) might suggest the presence of an additional 'non-specific' uptake component, such as diffusion.

2.3.2.6 Effect of biosynthesis inhibition on subsequent [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites

Polyamines are present at an estimated total concentration of 10 mM inside *P. falciparum* trophozoites (Teng *et al.*, 2009) and the uptake of both [^{3}H]putrescine and [^{3}H]spermidine therefore occurs against this concentration gradient. The high concentration of polyamines inside *P. falciparum* trophozoites can be reduced by polyamine biosynthesis inhibitors, with DFMO leading to a ~20-fold decrease in putrescine and a ~10-fold decrease in spermidine levels in iRBCs (Assaraf *et al.*, 1987b). The effect of polyamine biosynthesis inhibition, with the resulting decrease in polyamine levels in intra-erythrocytic *P. falciparum* cultures, on [^{3}H]putrescine or [^{3}H]spermidine uptake into isolated parasites was investigated. In this study, treatment of parasites with DFMO (1xIC_{50}) was initiated in ring-stage cultures, prior to the expression of ODC (Fig. 1.6), to ensure that the inhibitor was present from the onset of expression of the enzyme. Uptake experiments with isolated (DFMO-treated and untreated) parasites showed an significant ~2-fold increased accumulation of [^{3}H]putrescine (18±4 pmol putrescine/10^{10} untreated cells vs. 36±9 pmol putrescine/10^{10} DFMO-treated cells , n=7, $P \leq 0.1$) and ~4-fold increased accumulation of [^{3}H]spermidine (17±5 pmol spermidine/10^{10} untreated cells vs. 63±15 pmol spermidine/10^{10} DFMO-treated cells , n=5, $P \leq 0.05$) in the DFMO-treated parasites compared to untreated parasites (Fig. 2.12) after a 1 hr incubation. DFMO was not present during the actual uptake experiments as these measurements were performed using washed, isolated parasites. While there was no statistical difference in the initial rate of [^{3}H]putrescine uptake (1.12±0.27 pmol [^{3}H]putrescine/10^{10}cells/min vs. 1.38±0.29 pmol [^{3}H]putrescine/10^{10}cells/min, n=7, $P \geq 0.05$) the initial rate of [^{3}H]spermidine was affected by DFMO pre-treatment (0.98±0.09 pmol [^{3}H]spermidine/10^{10}cells/min vs. 3.4±0.8 pmol [^{3}H]spermidine/10^{10}cells/min, n=5, $P \leq 0.05$) (Fig. 2.12). The increased total uptake following biosynthesis inhibition is postulated to be due to decreased intracellular polyamine levels caused by DFMO inhibition of ODC.

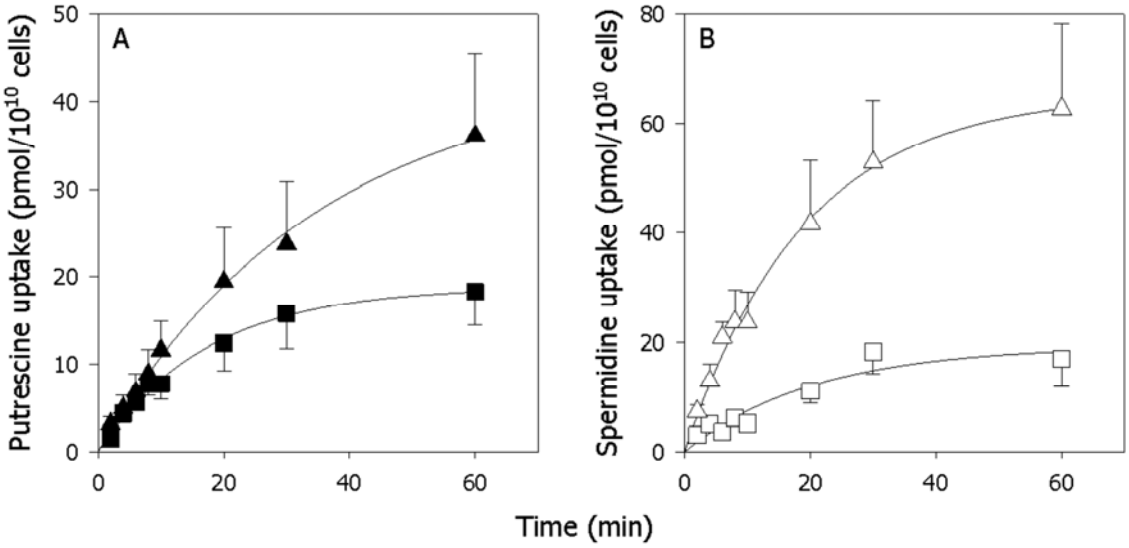


Figure 2.12: Effect of *in vitro* polyamine depletion on the uptake of [^{3}H]putrescine and [^{3}H]spermidine by *P. falciparum* trophozoites at 37°C. In order to decrease the intracellular levels of polyamines, polyamine biosynthesis was inhibited by treating ring-stage parasites for 24 h with 2 mM DFMO prior to performing uptake experiments. (A) [^{3}H]putrescine uptake by control (■) and DFMO-treated (▲) saponin-isolated *P. falciparum* trophozoites was measured at 37°C and the data were averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake by control (□) and DFMO-treated (△) saponin-isolated *P. falciparum* trophozoites was measured at 37°C and the data averaged from five independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM. Data are represented as the amount of [^{3}H]polyamine uptake per cell and not distribution ratio to account for any possible cell volume changes due to DFMO treatment.

2.3.2.7 Dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake on external Na^+

The accumulation of polyamines against their concentration gradient, may involve uptake being coupled to a Na^+or H^+ co- or anti-port (section 1.3). The ionic composition of the extracellular medium was varied to determine whether polyamine uptake into isolated *P. falciparum* trophozoites is coupled to Na^+ transport. In the case of putrescine, replacement of Na^+ with the Na^+ substitute NMDG in the extracellular medium had no significant effect on the initial rate of uptake of this polyamine (152±50 fmol [^{3}H]putrescine/10^{10}cells/min in the presence of Na^+, vs. 137±80 fmol [^{3}H]putrescine/10^{10}cells/min under Na^+-free conditions, n=7, P≥0.05). In the case of spermidine, the initial rate of uptake did appear to be slightly increased in medium containing NMDG in place of Na^+. However, this apparent increase in the initial uptake rate was not statistically significant (0.59±0.11 fmol [^{3}H]spermidine/10^{10}cells/min in the presence of Na^+ vs. 0.81±0.17 pmol [^{3}H]spermidine/10^{10}cells/min for the Na^+-free conditions, n=6, P≥0.05).

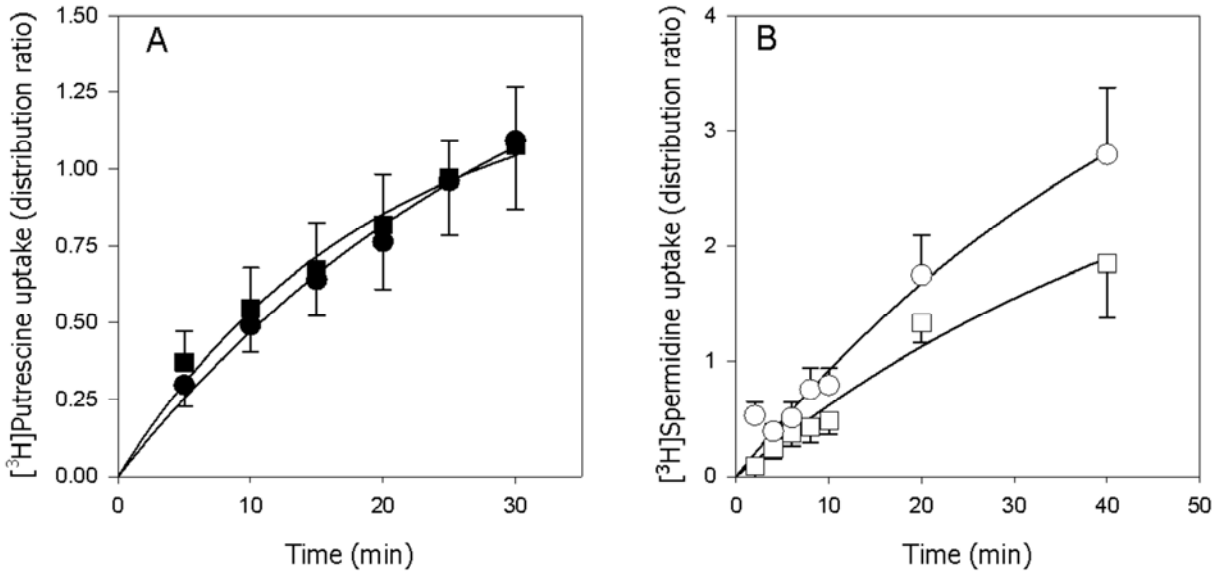


Figure 2.13: Na^+ dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by *P. falciparum* trophozoites at 37°C. (A) [^{3}H]putrescine uptake under control (Solution A) (■) and Na^+ free conditions (Solution E) (●) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake under control (Solution A) (□) and Na^+ free conditions (Solution E) (○) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

2.3.2.8 pH dependence of [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated *P. falciparum* parasites

2.3.2.8.1 Effect of pH on the uptake of [^{3}H]putrescine or [^{3}H]spermidine

The effect of extracellular pH (and thus extracellular [H^+]) on [^{3}H]putrescine or [^{3}H]spermidine uptake by isolated parasites was determined by isolating and washing the parasites in the strongly buffered solution F at pH 6.1, 7.1 or 8.1 and performing the uptake measurements in solution F at the appropriate pH. Varying the extracellular pH had a substantial effect on both [^{3}H]putrescine and [^{3}H]spermidine influx time-courses (Fig. 2.14).

Total [^{3}H]spermidine uptake following 60 min incubation increased ~2-fold (albeit non-significantly) on increasing the extracellular pH from pH 7.1 to pH 8.1 (distribution ratio 3.5±0.9 at pH 8.1 vs. 1.77±0.19 at pH 7.1, n=6, $P \geq 0.05$) and decreased by ~1.5-fold on decreasing the pH from pH 7.1 to pH 6.1 (distribution ratio 1.77±0.19 at pH 7.1 vs. 1.14±0.19 at pH 6.1, n=6, $P \leq 0.1$). The decrease in pH from pH 7.1 to pH 6.1 did not however have an

effect on the initial [^{3}H]spermidine uptake rate (0.71±0.15 pmol [^{3}H]spermidine/10^{10}cells/min at pH 7.1 vs. 0.91±0.24 pmol [^{3}H]spermidine/10^{10}cells/min at pH 6.1, n=6, P≥0.05).

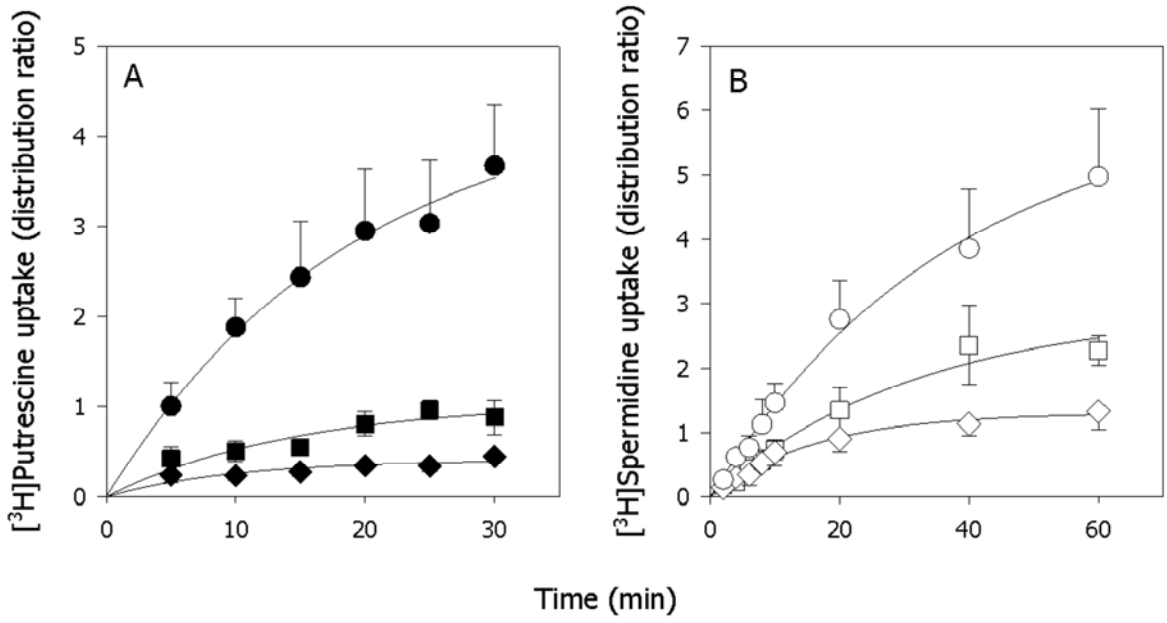


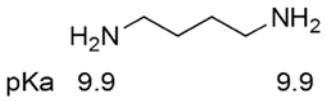
Figure 2.14: pH Dependence of [^{3}H]putrescine and [^{3}H]spermidine uptake by isolated *P. falciparum* trophozoites at 37°C. In order to determine the effect of pH on polyamine uptake, functionally isolated parasites were re-suspendend in solution F and time-courses performed at 37°C. (A) [^{3}H]putrescine uptake at pH_o of pH 6.1 (◆), pH 7.1 (■) and pH 8.1 (●) averaged from seven independent experiments and shown ± S.E. (B) [^{3}H]spermidine uptake at pH_o of pH 6.1 (◇), pH 7.1 (□) and pH 8.1 (○) averaged from six independent experiments and shown ± S.E. For both polyamines the extracellular concentration was approximately 5 nM.

In contrast, there was a statistically significant increase in the initial rate of [^{3}H]spermidine uptake at pH 8.1 vs. pH 7.1 (0.71±0.15 pmol [^{3}H]spermidine/10^{10}cells/min at pH 7.1 vs. 1.50±0.28 pmol [^{3}H]spermidine/10^{10}cells/min at pH 8.1, n=6, P≤0.05). Total [^{3}H]putrescine uptake following 30 min incubation increased ~3-fold on increasing the extracellular pH from pH 7.1 to pH 8.1 (distribution ratio 3.4±0.8 at pH 8.1 vs. 1.0±0.2 at pH 7.1, n=7, P≤0.05) and decreased ~2-fold from pH 7.1 to pH 6.1 (distribution ratio 1.0±0.2 at pH 7.1, vs. 0.49 ±0.05 at pH 6.1, n=6, P≤0.05) (Fig. 2.14). As was seen for [^{3}H]spermidine uptake, although there was a decrease in initial rate of [^{3}H]putrescine uptake on decreasing the extracellular pH from pH 7.1 to pH 6.1 (166±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 7.1 vs. 71±23 fmol [^{3}H]putrescine/10^{10}cells/min, pH 6.1, n=7, P≥0.05), this decrease was not statistically significant. However, there was a statistically significant increase in the initial rate of [^{3}H]putrescine uptake on increasing the extracellular pH from pH 7.1 to pH 8.1 (166±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 7.1 vs. 377±70 fmol [^{3}H]putrescine/10^{10}cells/min, pH 8.1, n=7, P≤0.1).

The increased uptake of polyamines at higher extracellular pH values might indicate the presence in the parasite of an H^+-coupled transport system. One possibility is a polyamine/H^+ exchange system whereby extracellular polyamines are exchanged for intracellular H^+. At increased extracellular pH, the extracellular concentration of H^+ is reduced, thereby inducing an outward H^+ gradient which might facilitate polyamine uptake. In contrast, at lower extracellular pH, there is an inward H^+ gradient (and therefore a decreased driving force for H^+ efflux).

Another possibility is that the pH dependence of polyamine uptake is due to changes in the protonation state of the putrescine or spermidine. The proportion of uncharged polyamines increases as the pH decreases (Table 2.2) with a larger proportion of polyamines in the uncharged form at pH 8.1 than is the case at pH 7.1 or pH 6.1. The pH dependence of [^{3}H]putrescine or [^{3}H]spermidine may reflect a preference of one or more carrier protein/s for polyamines that don't carry the full complement of positive charge.

Table 2.2: Effect of pH on the protonation status of putrescine. The proportion of single or double protonated putrescine changes as the extracellular pH changes.



			A + H=(HA^+)		(HA^+) + H =($H2A^{++}$)	
pH	**Proportion protonated @ position 1**	**Proportion protonated @ position 2**	**% of A (Put)**	**% of HA^+ ($PutH^+$)**	**% $H2A^{++}$ ($Put2H^{++}$)**	**Total**
5.1	0.99	0.99	0.00	0.00	99.99	100
6.1	0.99	0.99	0.00	0.03	99.96	100
7.1	0.99	0.99	0.00	0.31	99.68	100
8.1	0.98	0.98	0.02	3.07	96.90	100
9.1	0.86	0.86	1.87	23.61	74.51	100

It is also possible that the increased uptake of polyamines at higher extracellular pH is due to a larger proportion of the molecules being present in the unprotonated form (Table 2.2), capable of crossing the parasite's plasma membrane by simple diffusion (Fukumoto and Byus, 1996; Yohannes *et al.*, 2005). This possibility was investigated using a method that entailed measuring the intracellular pH.

2.3.2.8.2 Effect of putrescine import on cytosolic pH

If polyamines enter the parasite by diffusion of the uncharged species, a transient alkalinisation of the cell cytosol might be expected, as the uncharged species is protonated (i.e. takes up protons) upon entering the cell. The effect of putrescine (10 mM, putrescine dihydrochloride) on the cytosolic pH (pH_i) of *P. falciparum* trophozoites at different extracelluar pH (7.1 and 8.1) was investigated by preloading the isolated parasites with the pH-sensitive fluorescent indicator dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) and monitoring the fluorescence (and thereby pH_i) with a spectrofluorometer (Saliba and Kirk, 1999).

The initial resting pH_i of the cells suspended at an external pH (pH_o) of 7.1 was 7.28 ±0.03 (n=4) and at pH_o=8.1 was 7.690 ±0.002 (n=4) (Fig. 2.15). Parasites treated with 10 mM NH_4Cl at pH_o=7.1 showed in accordance with published results (Saliba and Kirk, 1999) an immediate transient alkalinisation, (from pH 7.233±0.025 to pH 7.305±0.027, Δ pH_i=0.072±0.004, n=4, $P \geq 0.05$), which was significantly increased at pH_o=8.1 (from pH 7.694±0.009 to pH 7.938±0.015, Δ pH_i=0.24±0.01, n=4, $P \leq 0.05$) (Fig. 2.15, A).

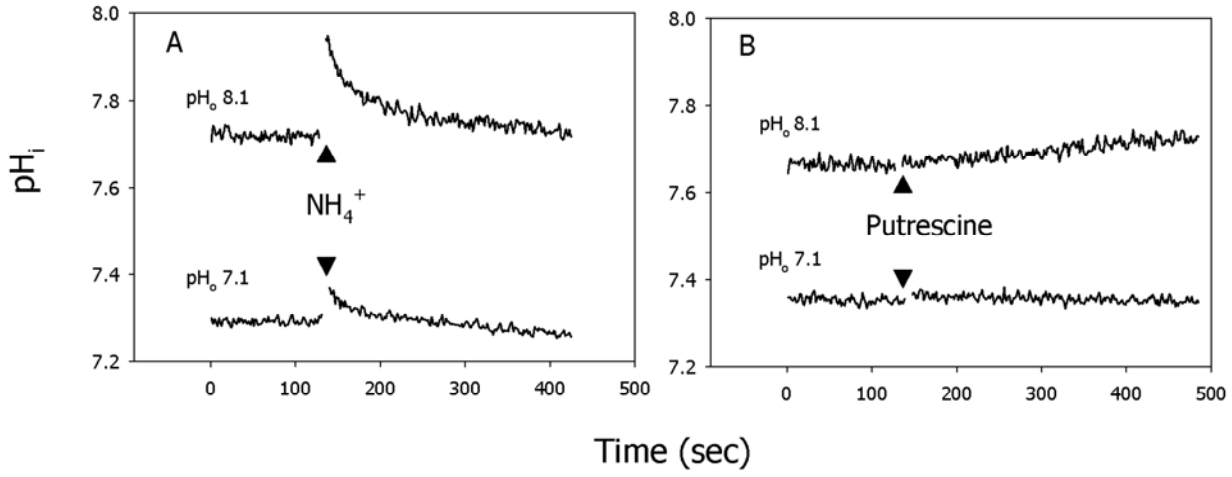


Figure 2.15: pH_i response of isolated *P. falciparum* trophozoites following extracellular exposure to 10 mM NH_4Cl or 10 mM putrescine dihydrochloride at 37°C. Isolated parasites were re-suspendend in Solution A, pH_o 7.1, lower traces or Solution F, pH_o 8.1, upper traces before tracing the change in pH_i following the addition of (A) 10 mM NH_4Cl or (B) 10 mM putrescine dihydrochloride. The traces are representative of those obtained from four independent experiments.

In contrast to the alkalinisation seen on the addition of NH_4Cl, there was no significant alkalinisation observed following the addition of putrescine at either pH_o=7.1 (from pH 7.331±0.011 to pH 7.341±0.014, Δ pH_i=0.01±0.003, n=4, $P \geq 0.05$), or pH_o=8.1 (from pH 7.689±0.017 to pH 7.705±0.022, Δ pH_i=0.016±0.008, n=4, $P \geq 0.05$) (Fig. 2.15, B). This lends support to the view that the increased uptake of [^{3}H]putrescine (and [^{3}H]spermidine) at higher

extracellular pH (Fig. 2.14) is *not* due to increased diffusion. However, it can be seen from Fig. 2.15 that *P. falciparum* parasites has pH regulatory mechanisms with which it counters the ammonium-induced alkalinisation. The possibility cannot be excluded that a fraction of the polyamines do enter via diffusion, more slowly than NH_3/NH_4^+ and that the parasite effectively counters (and therefore masks) the potential pH changes.

2.3.2.9 Effect of plasma membrane potential perturbation on [^{3}H]putrescine or [^{3}H]spermidine import by isolated *P. falciparum* parasites

An increase in the extracellular pH induces a plasma membrane hyperpolarisation, and vice versa (Allen and Kirk, 2004). The pH dependence of the membrane potential might, at least in part, contribute to the observed pH dependence of polyamine uptake. A variety of membrane potential manipulations were therefore performed to determine the effect of the plasma membrane potential on [^{3}H]putrescine and [^{3}H]spermidine uptake.

In intra-erythrocytic *P. falciparum* parasites, the plasma membrane potential (Δψ) is generated by the electrogenic export of protons via the V-type H^+ pump on the PPM. This is offset by the electro-diffusion of K^+ into the parasite (Allen and Kirk, 2004). On removal of K^+ from the extracellular solution, the Δψ is hyperpolarized since there is no influx of K^+ ions to offset the H^+ pump-generated Δψ. This hyperpolarisation can be increased by adding the K^+ ionophore valinomycin, which increases the efflux of K^+ ions down their concentration gradient, out of the cell (Allen and Kirk, 2004). Conversely, the Δψ can be depolarized by re-suspending the cells in a high K^+ saline solution, resulting in an increased influx of K^+ ions (and hence positive charge) into the parasite, increasing the extent to which the H^+ pump-generated Δψ is offset. This depolarisation can be increased by adding valinomycin, which further increases the influx of K^+ ions (and hence positive charge) into the parasite. The plasma membrane of the parasite may also be depolarised through the inhibition of the V-type ATPase using the inhibitor Concanamycin A (Allen and Kirk, 2004). The combination of Concanamycin A together with a high extracellular K^+ concentration effectively eliminates the Δψ completely (Allen and Kirk, 2004).

Under conditions in which the parasite plasma membrane was hyperpolarised, the total uptake (measured over 15 min) for both [^{3}H]putrescine and [^{3}H]spermidine increased in comparison to

controls ($P \leq 0.1$ and $P \leq 0.05$, respectively) (Fig. 2.16). By contrast, under conditions in which the parasite plasma membrane was depolarised, the total uptake of both [^{3}H]putrescine and [^{3}H]spermidine were significantly decreased ($P \leq 0.05$) (Fig. 2.16). These results indicate that polyamine uptake in *P. falciparum* trophozoites is plasma membrane potential dependent, with increased uptake with membrane hyperpolarisation and a decreased uptake with membrane depolarisation.

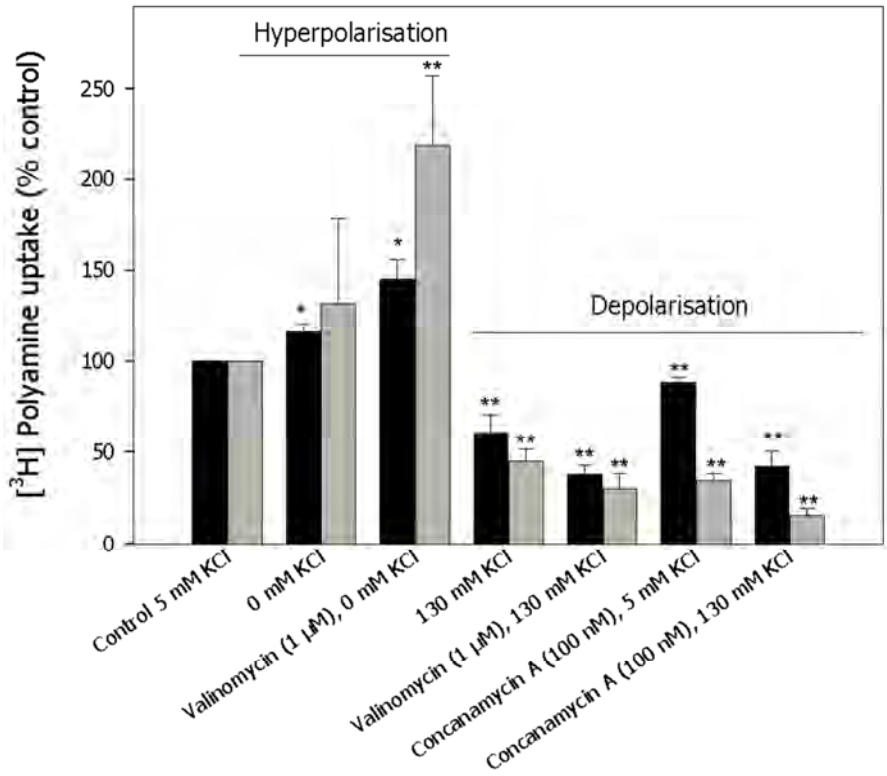


Figure 2.16: Effect of membrane potential perturbations on [^{3}H]putrescine and [^{3}H]spermidine uptake by *P. falciparum* trophozoites. Uptake was measured over 15 min at 37°C. The cells were hyperpolarised by being suspended in Solution C (containing 0 K^+) for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine. A further hyperpolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM, added to cells pre-incubated for 30 min in Solution C) concurrently with the [^{3}H]putrescine or [^{3}H]spermidine. The cells were depolarised by being suspended in Solution D (containing 150 mM K^+) for 30 min prior to adding the [^{3}H]putrescine or [^{3}H]spermidine. A further depolarisation was achieved by adding the K^+ ionophore valinomycin (1 µM, added to cells pre-incubated for 30 min in Solution D) concurrently with the [^{3}H]putrescine or [^{3}H]spermidine. Cells were also depolarised using the V-type ATPase inhibitor Concanamycin A (100 nM). Maximum depolarisation was achieved by adding Concanamycin A to cells suspended in a high-K^+ medium (solution D). All results are given as percentage of control. Statistical significance was determined with a Wilcoxon Matched Pairs test, * $P<0.01$, ** $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars and [^{3}H]spermidine uptake by grey bars. For both polyamines the extracellular concentration was approximately 5 nM.

2.4 Discussion

The total uptake of a metabolite, as measured in experiments such as those presented here, is dependent on the initial transport across the plasma membrane and the subsequent fate of the metabolite (in particular metabolism and binding) within the cell (Kirk *et al.*, 2009). In contrast to other metabolites taken up by the parasite such as amino acids (Cobbold *et al.*, 2011; Martin and Kirk, 2007), which are incorporated into proteins, and choline (Lehane *et al.*, 2004) which is incorporated into lipids, the majority of polyamines such as putrescine and spermidine are not metabolised but, rather, interact electrostatically with negatively charged macromolecules such as phospholipids, proteins and nucleic acids within cells (Wallace *et al.*, 2003). A small fraction of spermidine can be converted to the unique amino acid hypusine, present in eukaryotic initiation factor-5A (eIF-5A) (Molitor *et al.*, 2004). While the relative levels of spermidine and hypusine in intra-erythrocytic *P. falciparum* is not known, it has been shown that in *S. cerevisiae* the level of spermidine is ~70-fold higher than that of hypusine (Chattopadhyay *et al.*, 2008). As the exact fate of the polyamines are unclear, the traditional methods of separating transport and uptake, such as performing the experiments in cells depleted of ATP and the use of metabolic inhibitors (Kirk *et al.*, 2009), could not be applied to separate transport and 'metabolism' (or sequestering in macromolecule binding sites) of polyamines in *P. falciparum* parasites. As such, the results presented herein reflect a combination of transport and the subsequent fate of the polyamines within the infected cell and the intracellular parasite.

It was previously shown that iRBCs are capable of taking up polyamines (Ramya *et al.*, 2006) and that in *P. knowlesi*-infected erythrocytes, the K_m of putrescine uptake is similar to that of putrescine uptake into uninfected erythrocytes (Singh *et al.*, 1997). Polyamine uptake into iRBCs may occur via any of a number of different routes or a combination thereof: the endogenous RBC uptake mechanism can remain active following invasion, substrate-specific *P. falciparum* transporter proteins may be targeted to the EPM or polyamine uptake may occur via the parasite-induced, broad-specificity NPP (Kirk, 2001). Whilst putrescine uptake does not occur via the NPP, spermidine uptake did have an additional parasite-derived furosemide-sensitive component, suggesting a possible multi-component uptake mechanism (Fig. 2.5). The NPP are in general anion selective (Kirk *et al.*, 2005), with the basic amino acids arginine and lysine barely permeable through the NPP (Ginsburg *et al.*, 1985). Other cations such as inorganic K^+ (Kirk *et al.*, 1994) and Rb^+(Kirk and Horner, 1995) and the organic cations choline

(Kirk *et al.*, 1994) and a range of quaternary ammonium cations (Staines *et al.*, 2000) did exhibit NPP permeability. It is important to note that while furosemide inhibits the NPP, it is a broad-specificity anion transport inhibitor and is not solely specific for the NPP. It has recently been shown that the human cation-Cl^- transporter, CCC9A, which mediates polyamine uptake in HEK-293 cells, is furosemide sensitive (Daigle *et al.*, 2009). The furosemide-sensitivity of spermidine uptake into iRBCs (Fig. 2.5) raises the possibility that a similar transporter may be involved in the uptake of spermidine into the infected cells, but this is difficult to reconcile with the complete lack of effect of furosemide on the uptake of spermidine into uninfected cells (Fig. 2.5, inset). Additionally, since spermidine is both larger and contains more positive charges than putrescine (with 3 positive charges as opposed to the 2 of putrescine) the NPP component of spermidine uptake and not putrescine uptake is in contrast to the previous findings on the NPP where the permeability of a range of quaternary ammonium cations was dependent on both the size and the hydrophobicity of the cation, with smaller and more hydrophobic cations exhibiting higher permeability than the larger and more hydrophilic compounds (Staines *et al.*, 2000).

Both [^{3}H]putrescine and [^{3}H]spermidine were taken up by the isolated *P. falciparum* parasites, with [^{3}H]spermidine uptake occurring at a faster initial rate, as well as to a higher total accumulation level, compared to [^{3}H]putrescine (Fig. 2.6). The higher rate of uptake of the polyamine with the higher valency (spermidine>putrescine) has previously been seen for the uptake of polyamines into rat liver mitochondria (Toninello *et al.*, 1992). As has been seen in other organisms such as *Leishmania* spp (Basselin *et al.*, 2000), import of both [^{3}H]putrescine and [^{3}H]spermidine was temperature dependent in isolated *P. falciparum* parasites with a higher rate of uptake at 37°C than at 22°C (Fig. 2.7).

The uptake of both [^{3}H]putrescine and [^{3}H]spermidine by isolated *P. falciparum* parasites appeared to involve saturable components but these components showed vastly different affinities. Both putrescine and spermidine uptake occurred via an apparent low affinity process, while kinetic analyses of spermidine uptake indicated the operation of an additional high affinity mechanism (Fig. 2.9). It is however unclear whether this additional high-affinity component of spermidine uptake is due to transport across the membrane, accumulation of spermidine or an alternative (as yet unknown) mechanism. The involvement of a high capacity system in the uptake of spermidine was observed in later experiments in which a 5 mM extracellular concentration of spermidine caused a ~90% inhibition of the uptake of

radiolabelled spermidine, whereas a 5 mM concentration of putrescine inhibited putrescine uptake by only ~50% (Fig. 2.11).

Using the Michaelis-Menten equation to analyse kinetic data can lead to an underestimation of the number of transport systems, as it attributes more weight to the data points derived from the low-affinity component of transport (Coons *et al.*, 1995). Further analyses of the data using the Eadie-Hofstee and Hanes-Woolf equations indicated the involvement of multiple components in both putrescine and spermidine uptake (Fig. 2.10). These might include a combination of free diffusion and carrier-mediated transport, multiple transport proteins or a single transport system with variable K_m depending on cellular conditions (Coons *et al.*, 1995). In *X. laevis* oocytes, kinetic analyses showed that putrescine uptake into *X. laevis* oocytes had two distinct components, but the analyses 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 uptake in RBCs had both a non-saturable (perhaps indicative of diffusion) as well as a saturable component (Fukumoto and Byus, 1996). It is thus not unusual for polyamine uptake to have more than one component. Further investigation is required to determine the identity and contribution of the various polyamine transport mechanisms in the isolated *P. falciparum* parasite's plasma membrane, as well as to clarify whether putrescine and spermidine transport have shared or separate uptake mechanisms.

The inhibitory effect of polyamines and the precursor ornithine on the uptake of both putrescine and spermidine into isolated *P. falciparum* parasites (Fig. 2.11) may suggest that polyamines and polyamine precursors compete for uptake via a single broad specificity polyamine transport system present in the parasite plasma membrane that accommodates the polyamines putrescine, spermidine and spermine, as well as the polyamine precursor ornithine. However, since the basic amino acids inhibited putrescine uptake, whilst having little effect on spermidine uptake, it is possible that there is a second mechanism for putrescine uptake into isolated *P. falciparum* parasites that is shared with the basic amino acids. Alternatively, the inhibitory effect on putrescine uptake caused by the basic amino acids may merely be due to the inhibition of the uptake mechanism(s) due to structural similarity with the polyamines, without utilising this uptake mechanism for cellular entry. For instance, the basic amino-acids may have sufficient structural similarity to putrescine to interfere with the recognition of putrescine by the putrescine binding site on the transporter protein, but this similarity may not be sufficient for transport. On the other hand, the lack of effect of the basic amino acids on spermidine uptake (in contrast to putrescine uptake) may indicate that the transporter protein

contains separate binding sites for putrescine and spermidine, and that the structural requirements of the spermidine binding site does not lend to inhibition by the basic amino acids. Certain cell types, such as murine leukaemia L1210 cells (Porter *et al.*, 1985) or AMEL-3 cells (García-Fernández *et al.*, 2005) do have a single, shared polyamine transporter, while others have separate polyamine transport systems for putrescine, and spermidine and spermine e.g. human leukaemia HL-60 cells (Palmer and Wallace, 2010), CHO cells (Xie *et al.*, 1997), or *T. cruzi* parasites (Hasne *et al.*, 2010), and the distinction between separate or shared polyamine transporters for putrescine and spermidine uptake by the *P. falciparum* trophozoite cannot be determined on the basis of the data obtained.

Polyamine depletion in intra-erythrocytic *P. falciparum* parasites results in cytostatic arrest of the parasite, reversible by the exogenous addition of some polyamines to *in vitro P. falciparum* cultures (Assaraf *et al.*, 1987a). In this study both putrescine and spermidine uptake was shown to increase following polyamine depletion, induced through DFMO inhibition of polyamine biosynthesis (Fig. 2.12). DFMO is not known to have any 'off-target' effects in the parasites (van Brummelen *et al.*, 2009). Such increased polyamine uptake following DFMO treatment has also been observed in AMEL-3, L1210 and HL-60 cells (Alhonen-Hongisto *et al.*, 1980; García-Fernández *et al.*, 2005; Walters and Wojcik, 1994). It is possible that the increased accumulation of the radiolabelled polyamines is a compensatory, homeostatic response in order to maintain the intracellular polyamine levels. Since these experiments only measure unidirectional uptake and not net uptake it is, however, unclear whether this observed increased uptake of radiolabelled polyamines leads to a net increase in polyamine levels in the DFMO-treated cells. It is possible that the observed increased uptake of the radiolabelled polyamines is due to the reduced concentration of polyamines within the parasite, causing more binding sites being available for the radiolabelled polyamines in the DFMO-treated cells, and that it is this availability of binding sites that leads to the increased uptake. It is also possible that the increased accumulation is due to reduced export of the polyamines (including the radiolabelled polyamines). Another alternative is that the increased uptake observed is due to differential expression of polyamine transporters in response to the polyamine depletion. Functional genomics analyses of the transcriptional profile of intra-erythrocytic *P. falciparum* following polyamine depletion did not however identify any up-or down-regulated genes that have similarity to known polyamine transporters (van Brummelen *et al.*, 2009).

The importance of the uptake of polyamines and consequent reversal of DFMO-mediated inhibition of *P. falciparum* proliferation in the *in vivo* context is unclear, since the polyamine

concentrations in serum are orders of magnitude less than those found necessary to reverse inhibition in *in vitro* experiments (Table 1.2) (Das *et al.*, 1997; Das Gupta *et al.*, 2005; Haider *et al.*, 2005). However, it is important to note that in humans, the polyamine pool is continually replenished by biosynthesis, production by the intestinal microorganisms as well as from the diet (Thomas and Thomas, 2001) and that this constant supply might well be sufficient to ensure the survival of intra-erythrocytic *P. falciparum* parasites in which the biosynthesis of polyamines is impaired.

Putrescine or spermidine uptake into the intra-erythrocytic *P. falciparum* parasite may occur in either the uncharged or the charged form. The uncharged forms of the molecules (present as an extremely low proportion of the total at physiological pH values, Table 2.2) may enter via a process of simple diffusion across the lipid phase of the membrane (Yohannes *et al.*, 2005). The charged forms may enter via protein-mediated 'facilitated diffusion' down the electrical gradient, with the large inward negative membrane potential of intra-erythrocytic *P. falciparum* parasites (Allen and Kirk, 2004) responsible for the uptake of the positively charged polyamines. The charged forms may also enter via either primary active transporters or secondary active transporters. Consistent with previous findings (Nadler and Takahashi, 1985; Romero-Calderón and Krantz, 2006; Sha *et al.*, 1996), iso-osmotic replacement experiments showed that neither [^{3}H]putrescine nor [^{3}H]spermidine uptake by *P. falciparum* parasites are coupled to Na^+ transport (Fig. 2.13). By contrast, changes in pH did have an effect on [^{3}H]putrescine and [^{3}H]spermidine uptake, with increased uptake at higher pH and vice versa. A similar trend has been reported for the uptake of putrescine in human RBCs (Fukumoto and Byus, 1996). However it is unclear whether this was due to an involvement of H^+ (or OH^-) ions in the transport process (either via co-or anti-port), a general effect of pH on one or more transport systems, the pH dependence of the relative concentrations of the different polyamine species, or the effect of the extracellular pH on the parasite's membrane potential.

It is possible that the increased uptake of polyamines observed at higher pH is due to a larger proportion of the molecules being present in the deprotonated form, capable of crossing the parasite's plasma membrane as membrane permeable weak bases (Fukumoto and Byus, 1996; Yohannes *et al.*, 2005). The extent to which this occurs was investigated using cytosolic pH measurements to assess the level to which the neutral polyamine species were taken up. On entering the parasite, the neutral polyamine would be expected to become protonated, thus removing protons from, and thereby alkalinising, the cytosol. The fact that no alkalinisation was observed (Fig. 2.15) when parasites were exposed to a very high (10 mM) concentration

of putrescine, is consistent with the neutral species not entering the parasite very rapidly. As it is known that a fraction of polyamine uptake in certain cells is due to diffusion (Fukumoto and Byus, 1996, 1997), and since it appears that polyamine uptake in *P. falciparum* have multiple components (section2.3.2.4), the possibility that a small portion of polyamine uptake in *P. falciparum* parasites is due to diffusion cannot be completely excluded. Furthermore, no acidification was observed (Fig. 2.15) when parasites were exposed to a very high (10 mM) concentration of putrescine, which might suggest that the influx of positive charge due to the uptake of the charged is offset in some way. One possibility is a polyamine/H^+ exchange system whereby extracellular polyamines are exchanged for intracellular H^+, which is consistent with the pH dependence of polyamine uptake observed here. Such polyamine/H^+ exchange systems have been suggested for Drosophila S2 cells (Romero-Calderón and Krantz, 2006) as well as CHO cells (Xie *et al.*, 1997).

An imposed plasma membrane hyperpolarisation led to increased putrescine and spermidine uptake, and an imposed plasma membrane depolarisation led to decreased putrescine and spermidine uptake (Fig. 2.16). Membrane potential dependence is also likely to contribute to the reduced initial uptake rates for both [^{3}H]putrescine and [^{3}H]spermidine following extended incubation of isolated *P. falciparum* parasites in glucose-free saline (Fig. 2.8). The parasite's $\Delta\psi$ is generated by the electrogenic export of protons via the V-type ATPase on the PPM; consequently, depletion of ATP results in membrane depolarisation. A similar membrane potential dependence for putrescine and spermidine uptake has been found in several other organisms cells such as *Leishmania* spp. (Basselin *et al.*, 2000), *X. laevis* oocytes (Sha *et al.*, 1996) CHO cells (Soulet *et al.*, 2004) and *E. coli* cells (notably via PotE) (Igarashi and Kashiwagi, 1999). The data are consistent with the entry of the polyamines into the parasite involving an influx of positive charge, under the influence of the inward negative membrane potential.

In conclusion, both putrescine and spermidine are taken up by iRBCs and specifically, both of these polyamines are taken up by the intra-erythrocytic *P. falciparum* parasite via a membrane potential dependent process. While there is circumstantial evidence for the existence of a polyamine carrier (namely the saturation kinetics) and that this carrier is responsible for the uptake of both putrescine and spermidine (competition by other polyamines), this is yet to be confirmed by the identification of such a transporter protein. Several of the known polyamine transporters, such as the bacterial Pot E (Kashiwagi *et al.*, 2000); *S. cerevisiae* Agp2p (Aouida *et al.*, 2005), UGA4 (Uemura *et al.*, 2004) and *L. major* LmPot1 (Hasne and Ullman, 2005) have

been shown to be members of the amino acid/polyamine/organocation (APC) superfamily of transporters. While preliminary investigations show that the *P. falciparum* genome encodes at least one APC superfamily transporter protein, the substrate specificity of this protein has yet to be established. Nevertheless, regardless of the identity of the protein/s involved in the polyamine uptake mechanism, this study proved that *P. falciparum* parasites are capable of polyamine uptake.

Recently, a 'Trojan horse' approach for drug delivery in cancer cells has been described (Palmer and Wallace, 2010). In this approach cytotoxic drugs are attached to polyamine moieties for selective targeting of cancer cells, based on the increased polyamine uptake of these cells in comparison to normal mammalian cells (Palmer and Wallace, 2010). The uptake processes of intra-erythrocytic *P. falciparum* parasites, notably that for choline, have been suggested as drug delivery systems (Biagini *et al.*, 2005). The effect of a range of anthracene-polyamine conjugates, specifically designed to utilise the polyamine uptake mechanism(s) of cells (Phanstiel *et al.*, 2007; Wang *et al.*, 2003a; Wang *et al.*, 2003b), on intra-erythrocytic *P. falciparum* parasites was investigated in the following chapter.

Chapter 3: The effect of anthracene-polyamine conjugates on intra-erythrocytic *P. falciparum* parasites.

3.1 Introduction

Polyamine metabolism has been proposed as a target for the treatment of proliferative diseases such as cancer, as well as microbial and parasitic infections (Heby *et al.*, 2003). However, DFMO, used to treat West African sleeping sickness (caused by *T. brucei gambiense*) (Bacchi *et al.*, 1990; Casero and Woster, 2009), is the only polyamine metabolism antagonist that has shown clinical usefulness. The clinical failure of most of the polyamine biosynthesis inhibitors (Paridaens *et al.*, 2000; Seiler *et al.*, 1998) is often attributed to compensatory mechanisms that maintain the intracellular polyamine pool (Müller *et al.*, 2001; Wallace *et al.*, 2003). This includes the up-regulation of polyamine uptake mechanisms (Alhonen-Hongisto *et al.*, 1980; Boncher *et al.*, 2007). DFMO causes cytostatic growth arrest in most cancerous cells (Wallace *et al.*, 2003) as well as in parasitic organisms such as intra-erythrocytic *P. falciparum* parasites (Assaraf *et al.*, 1987b). However, in polyamine transport deficient cancerous cell lines, DFMO elicits a cytotoxic response since these cells are unable to overcome the polyamine-depletion by the uptake of extracellular polyamines (Palmer *et al.*, 2009; Seiler, 1991; Wallace and Fraser, 2004).

The disappointing results obtained with polyamine biosynthesis inhibitors alone has prompted a shift in focus from developing compounds that target the biosynthetic enzymes, to producing polyamine-like molecules (polyamine analogues) (Casero and Woster, 2009; Palmer and Wallace, 2010). These compounds have sufficient similarity in structure to the polyamines to gain entry into the cell via polyamine uptake pathways, but once inside cells, act as polyamine antagonists (Palmer and Wallace, 2010; Wallace and Niiranen, 2007; Wallace and Fraser, 2003). 'Polyamine mimetics' are defined as polyamine analogues that decrease cellular growth without causing polyamine depletion, by occupying normal polyamine binding/effector sites (e.g. DNA) but failing to produce the normal biological actions of the endogenous polyamines (Wallace and Fraser, 2004; Wallace *et al.*, 2003).

'Polyamine anti-metabolites', in contrast, result in cell growth inhibition and a reduction in polyamine levels through either biosynthesis inhibition and/or induction of polyamine catabolism and export (Wallace and Fraser, 2004; Wallace *et al.*, 2003). Polyamine analogues have been synthesised with modifications including simple conjugation of additional moieties (Phanstiel *et al.*, 2000), both symmetrical (Bergeron *et al.*, 1988) and unsymmetrical (Saab *et al.*, 1993) terminally alkylated polyamines and structural analogues of the polyamines (reviewed extensively elsewhere (Casero and Woster, 2001, 2009; Palmer and Wallace, 2010).

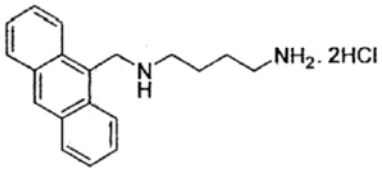
Polyamine analogues have also been designed to inhibit polyamine uptake itself (Palmer and Wallace, 2010). For instance, the polyamine analogue, *trans*-1,4-diamino-2-butene, inhibits *E. coli* putrescine transporters PotE and PotFGHI by 50%, (200 and 50 µM, respectively) (Kashiwagi *et al.*, 2000). Spermine conjugated to lysine is an effective polyamine transport inhibitor in MDA-MB-231 breast carcinoma cells (Graminski *et al.*, 2002), whereas spermine heterocyclically conjugated to benzazepine effectively inhibits polyamine transport (Ki 0.12 µM) in L1210 cells (Tomasi *et al.*, 2010). A polyamine analogue, 1,4-diamino-2-butanone (DAB), exhibited dose-dependent inhibition of *in vitro* proliferation in *Giardia lamblia* (Maia *et al.*, 2008), *Tritrichomonas foetus* (Reis *et al.*, 1999), *Trichomonas vaginalis* (Alvarez-Sanchez *et al.*, 2008) as well as *T. cruzi* epimastigotes (Menezes *et al.*, 2006). For the latter, it was postulated that DAB binds to a putrescine transporter at the cell surface, thus leading to putrescine depletion as well as oxidative stress and cell death (Menezes *et al.*, 2006).

The broad specificity of certain polyamine uptake systems allows the uptake of polyamine analogues (Palmer *et al.*, 2009; Phanstiel *et al.*, 2007; Wang *et al.*, 2003a). This promiscuity has led to strategies being proposed whereby polyamine moieties conjugated to e.g. toxic cargo would enhance the delivery of this cargo into cells through the polyamine uptake system (Palmer and Wallace, 2010; Phanstiel *et al.*, 2007). Several terminally alkylated polyamine analogues have been investigated where the cargo-polyamine spacer, the size of the N^1-substituent cargo and the degree of N^1-substitution all influenced the uptake of these analogues (Breitbeil *et al.*, 2006; Kaur *et al.*, 2008). A range of anthracene-polyamine conjugates has been designed to utilise polyamine uptake mechanisms in mammalian cells (L1210 cells and CHO cells) to deliver the toxic anthracene cargo (as DNA intercalating agent) to these cells (Kaur *et al.*, 2008; Phanstiel *et al.*, 2007; Wang *et al.*, 2003a). Anthracene-conjugated polyamines had an 150-fold higher cytotoxicity in CHO cells

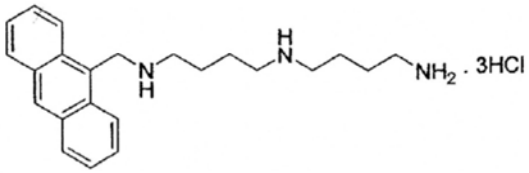
compared to alkylated (e.g. propyl or butyl conjugated for solubility) anthracene derivatives (Cullis *et al.*, 1999; Tsen *et al.*, 2008; Wang *et al.*, 2003a). Additionally, these anthracene-polyamine conjugates have shown selectivity for the mammalian polyamine transport system(s) with enhanced toxicity in CHO cells compared to polyamine transport deficient CHO-MG mutant cells (Covassin *et al.*, 1999; Delcros *et al.*, 2002; Phanstiel *et al.*, 2000; Wang *et al.*, 2001). Recently, an anthracene-putrescine conjugate was shown to enter HL-60 cells through the polyamine uptake mechanism, followed by apoptotic cell death caused by the polyamine moiety as well as the attached anthracene cargo (Palmer *et al.*, 2009).

The work described in this chapter investigates the effect of selected anthracene-polyamine conjugates (Fig. 3.1) on intra-erythrocytic *P. falciparum* parasites. Anthracene was conjugated to various polyamines, all with different combinations of carbon-backbones. As such, Ant-4 was produced as a putrescine conjugate (Ant-CH_2-NH-$(CH_2)_4$-NH_2); Ant-44 as a homospermidine conjugate (Ant-CH_2-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2); Ant-444 as a homospermine conjugate (Ant-CH_2-NH-$(CH_2)_3$-NH-$(CH_2)_4$-NH-$(CH_2)_3$-NH_2); and 44-Ant-44 as a bis-homospermidine conjugate (NH_2-$(CH_2)_4$-NH-$(CH_2)_4$-NH-CH_2-Ant-CH_2-NH-$(CH_2)_4$-NH-$(CH_2)_4$-NH_2) (Liao *et al.*, 2009; Phanstiel *et al.*, 2000).

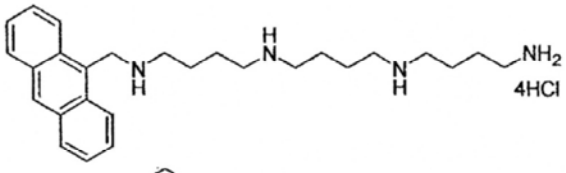
Ant-4



Ant-44



Ant-444



44-Ant-44

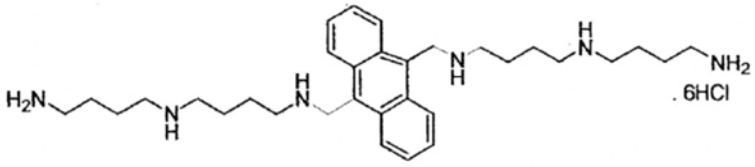


Figure 3.1: Structures of anthracene-polyamine conjugates. Adapted from (Liao *et al.*, 2009). Ant-4 is a putrescine conjugate; Ant-44 is a homospermidine conjugate; Ant-444 is a homospermine conjugate; and 44-Ant-44 is a bi-conjugated homospermidine conjugate.

3.2 Materials and methods

3.2.1 *In vitro* cultivation of intra-erythrocytic *P. falciparum* parasites

P. falciparum (3D7) parasites were maintained as described in Chapter 2, section 2.3.

3.2.2 Parasite proliferation assays

The *in vitro* anti-plasmodial activity of the compounds were determined by using the malaria SYBR Green I fluorescence assay as has been described (Bennet *et al.*, 2004; Smilkstein *et al.*, 2004) with minor modifications. The SYBR green I fluorescence assay relies on the fluorescent dye (SYBR Green I) intercalating into DNA, which correlates DNA levels directly with parasite growth. *In vitro* ring-stage *P. falciparum*-infected RBC cultures (1% haematocrit, 1% parasitaemia) were incubated with Ant-4, Ant-44, Ant-444 and 44-Ant-44 (Wang *et al.*, 2003b), all dissolved in 1xPBS and diluted to specific concentrations in complete *P. falciparum* culture medium, with chloroquine disulphate used as a positive control (0.5 µM) or vehicle (1xPBS) as negative control. iRBC cultures (200 µl) were grown statically at 37˚C for 96 hrs in 96-well plates, after which the cells were re-suspended and combined in equal volumes (100 µl each) with SYBR Green I lysis buffer (0.2 µl/ml 10 000x SYBR Green I Invitrogen, Inc; 20 mM Tris, pH 7.5; 5 mM EDTA; 0.008% (w/v) saponin; 0.08% (v/v) Triton X-100) and fluorescence measured after an 1 hr incubation at 37˚C using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The data, after subtraction of background (chloroquine disulphate treated iRBCs, no parasite growth) were expressed as percentage of untreated control to determine cell proliferation and averaged from at least 3 independent experiments (± S.E.), unless where otherwise stated.

3.2.3 Determination of anthracene-polyamine uptake into iRBCs

Deconvolution fluorescent microscopy was used to visualise uptake of Ant-4 into iRBCs. *In vitro* ring-stage *P. falciparum* (3D7) cultures at a 5% haematocrit and ~5% parasitaemia were incubated for 24 hrs to allow the parasites to develop to the trophozoite stage before

addition of Ant-4 (100 µM) for 1 hr at 37°C. Subsequently, 200 µl of these iRBC cultures were centrifuged at 500xg, washed in 1xPBS and re-suspended in 200 µl of a 1/1000-dilution SYBR Green I (10 000x SYBR Green I Invitrogen, Inc) in 1xPBS at 37°C for 30 min. Images were captured with a Zeiss Axio Observer Z.1, with an Andor iXon EMCCD camera and Andor iQ imaging software (Andor Technology, Ireland) or a Nikon Eclipse Ti with an Andor iXon EM+EMCCD camera (Andor Technology) using µManager open source imaging software (http://valelab.ucsf.edu/) and analysed with ImageJ (Abramoff *et al.*, 2004). Nuclei were visualised using a FITC filter (excitation band pass (BP) 470/40 and emission BP 525/50; or excitation BP 500/24 and emission BP 542/27, respectively) and the anthracene moiety was visualised with a DAPI filter (excitation 365 nm and emission BP 445/50 or excitation BP 377/50 and emission BP 447/60). Three independent drug treatments were performed.

Uptake of Ant-4 into RBCs, iRBCs and isolated *P. falciparum* trophozoites was also investigated using an alternative approach. RBCs (5% haematocrit) or ring stage iRBCs cultures (5% haematocrit and ~5% parasitaemia) were pre-treated with DFMO (1 mM) for 24 hrs to deplete intracellular polyamine levels (Assaraf *et al.*, 1987b) before incubating 200 µl of either RBCs cultures or now trophozoite-stage iRBCs cultures with Ant-4 (400 µM) for 60 min at 37°C. Isolated *P. falciparum* trophozoites were obtained with saponin lysis as described in section 2.2.2 (Saliba *et al.*, 1998). The RBCs, iRBCs or isolated parasites described above were subsequently centrifuged, washed and re-suspendend in 1xPBS (750 µl). Fluorescence was measured with a DAPI filter (BP 450/40 emission) using a BD FACS ARIA (BD Biosciences) equipped with a violet laser with excitation at 407 nM. Data from at least 10^5 cells from three independent experiments were analysed with Cyflogic v1.2.1 (CyFlo Ltd).

3.2.4 Determination of the effects of polyamine conjugates on putrescine uptake into isolated *P. falciparum* trophozoites

The effects of Ant-4, Ant-44, Ant-444 and 44-Ant-44 on [^{3}H]putrescine uptake into isolated *P. falciparum* trophozoites were investigated as described in sections 2.2.2 and 2.2.3. The reaction buffer (125 mM NaCl, 5 mM KCl, 20 mM glucose, 25 mM HEPES and 1 mM $MgCl_2$, pH 7.1) was supplemented with 1 µCi/ml [^{3}H]putrescine (Amersham) and anthracene-polyamine conjugates (1 mM). The uptake reactions were initiated by the addition of equal volumes of reaction buffer and the isolated trophozoite cell suspension to reach final

concentrations of 0.5 µCi/ml [^{3}H]putrescine and 500 µM each of Ant-4, Ant-44, Ant-444 and 44-Ant-44. These concentrations are 10-fold lower than was used for the metabolite inhibition assays (section 2.3.2.5) due to limited compound availability. Following a 30 min incubation at 37°C, the uptake reactions were terminated and the uptake of the radiolabel determined as described in section 2.2.3. Data were represented as percentage of control (± S.E.) averaged from four independent experiments.

3.2.5 Determination of the effect of polyamines on Ant-4 uptake into intra-erythrocytic *P. falciparum* trophozoites

The effects of putrescine and spermidine on Ant-4 uptake into isolated *P. falciparum* trophozoites were investigated as follows: ring-stage iRBC cultures (5% haematocrit and ~5% parasitaemia) were pre-treated with DFMO (1 mM) as described in section 3.2.3 for 24 hrs to deplete intracellular polyamine levels (Assaraf *et al.*, 1987b) before washing the cells, followed by re-suspending the cells to (5% haematocrit and ~5% parasitaemia), and incubating 200 µl of the now trophozoite-stage iRBCs cultures with Ant-4 (400 µM) with and without putrescine dihydrochloride or spermidine trihydrochloride (500 µM) for 60 min at 37°C. A 50 µl aliquot of the iRBC culture (5% haematocrit and ~5% parasitaemia), was sampled and the cells were washed and re-suspended in 1xPBS (750 µl). Fluorescence was measured with a DAPI filter (BP 450/40 emission) using a BD FACS ARIA (BD Biosciences) equipped with a violet laser with excitation at 407 nM. Data from at least 10^5 cells from three independent experiments were analysed with Cyflogic v1.2.1 (CyFlo Ltd). Gating was performed based on uninfected RBCs to obtain the iRBC population.

3.2.6 Measurement of intracellular polyamine levels in intra-erythrocytic *P. falciparum* trophozoites

Intracellular polyamine levels following Ant-4 treatment were measured as has been described by Becker *et al.* (2010). In brief, synchronised *P. falciparum* (3D7) ring-stage cultures (5% haematocrit, 15% parasitaemia) were incubated with Ant-4 (2xIC_{50}) for 24 hrs before the cells were harvested and washed four times in 1xPBS. The polyamines were extracted from the cell pellet with an equal volume of 5% (v/v) perchloric acid (PCA), followed by 12 hrs incubation at 4°C. The acid-insoluble components were removed by centrifugation at 16 000xg for 10 min at 4°C. NaOH (2 M, 1 ml) was added to the polyamine-PCA extracts, followed by benzoylation with 1:100 v/v benzoyl chloride (Sigma-

Aldrich) for 30 min at 37°C. The benzoylated polyamines were extracted with chloroform, dried under a nitrogen stream and re-constituted in 500 µl of 60% MeOH. HPLC analysis was performed using a 250×4.0 mm Luna C18 5 µm reverse-phase column and a WATERS System under isocratic solvent conditions (60% MeOH:ddH_2O) (Becker *et al.*, 2010). The Empower 2 Software (2006 ed., Waters Corporation) with Apex™ Trac functionality was used for peak integration. Data were expressed as a percentage of control (± S.E.) and averaged from four independent experiments.

3.2.7 Cytotoxicity and cell viability measurements

Cytotoxicity assays were performed using the CellTiter 96® Aq_{ueous} One Solution Cell Proliferation assay (Promega, USA) according to the manufacturer's instructions. Cell viability is indicated by the reduction of the tetrazolium compound MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to a coloured formazan product by metabolically active cells (Cory *et al.*, 1991). Ring-stage iRBC cultures (0.5% haematocrit, 25% parasitaemia) were exposed to Ant-4 (1x and 5xIC_{50}), chloroquine disulphate (0.5 µM, positive control) or vehicle (1xPBS, negative control) at 37°C for 24 hrs. The relatively high parasitaemia of 25% was needed to ensure sufficient parasite cells for the cytotoxicity assay. By reducing the haematocrit, the overall cell-density was reduced to ensure pH maintenance and a sufficient supply of nutrients. iRBCs were subsequently washed and re-suspended in culture media to 1% haematocrit and 25% parasitaemia to ensure that sufficient cells were present to obtain detectable signals. CellTiter 96® Aq_{ueous} One Solution (20 µl) was added to 100 µl of the above trophozoite cell suspension and incubated at 37°C for 4 hrs in a gaseous environment of 90% N_2, 5% O_2, and 5% CO_2 before measuring the absorbance at 492 nm with a Multiskan Ascent scanner (Thermo labsystems). Cell counts were performed using an improved Neubauer cell counting chamber. Data were expressed as a percentage of control and averaged (± S.E) for four independent experiments. Additionally, parasite proliferation was monitored over 96 hrs following treatment of ring-stage parasites (1% haematocrit, 1.5% parasitaemia) with Ant-4 (3xIC_{50}) in the presence and absence of 1 mM putrescine dihydrochloride using Giemsa-stained smears. These were prepared every 24 hrs and visualised with a light microscope to determine the parasitaemia by counting at least 10 fields of >100 cells each.

3.2.8 Determination of oxidative stress

Intracellular glutathione was measured using the GSH-Glo™ Glutathione Assay (Promega). Luciferin is produced in the presence of reduced glutathione and a luminescence signal is generated via a coupled luciferase reaction. Intra-erythrocytic ring-stage cultures (0.5% haematocrit, 25% parasitaemia) were incubated with Ant-4 (1xIC_{50}), or vehicle (1xPBS, positive control) at 37˚C for 24 hrs. The iRBC cultures were then centrifuged, washed and re-suspended in 1xPBS to 1% haematocrit and 25% parasitaemia. GSH-Glo™ reagent (100 µl) was added to 100 µl of the above cell suspension, which was incubated for 30 min at room temperature, before adding equal volumes of the cell/GSH-Glo™ reagent mix to the Luciferin Detection reagent. Following a 10 min incubation, the luminescence was measured using an Infinite® F500 multimode microplate reader (Tecan Group Ltd.) with an integration time of 1000 ms. A range of glutathione standards were prepared and measured as above to generate a standard curve for the determination of the amount of reduced glutathione in the samples. Data were averaged (± S.E.) from four independent experiments.

In addition, DCFDA (2'-7'-dichloro-dihydrofluorescein diacetate) was used to measure the production of reactive oxygen species (ROS), particularly H_2O_2 levels, in intra-erythrocytic *P. falciparum* cultures treated with Ant-4. DCFDA is non-fluorescent, but upon oxidation by ROS and peroxides converts to the fluorescent derivative DCF. Intra-erythrocytic ring-stage cultures (5% haematocrit, 5% parasitaemia) were incubated for 24 hrs with Ant-4 (1xIC_{50}) at 37˚C or with H_2O_2 as a positive control for ROS for 30 min. The treated and untreated iRBC cultures (5% haematocrit, 5% parasitaemia, 100 µl) were subsequently incubated with 20 µM DCFDA for 20 min at 37°C. Additionally, iRBC cultures (5% haematocrit, 5% parasitaemia, 100 µl) were incubated with 20 µM H_2O_2 immediately prior to the DCFDA staining described above. Fluorescence was measured in the FITC channel (FL1) on a FACS FC500 System (Beckman Coulter) equipped with an air-cooled argon laser with 488 nm excitation. Data from at least 10^6 cells were analysed with FlowJo v9.1 (Tree Star).

3.2.9 Investigation of DNA levels and DNA replication

DNA levels were monitored over 96 hrs following treatment of ring-stage iRBC cultures (1% haematocrit, 1.5% parasitaemia) with Ant-4 (3xIC_{50}) using an adapted SYBR green I Fluorescence assay. SYBR Green I fluoresce due to the intercalation of the dye into DNA thereby allowing the measurement of relative DNA levels (Bennet *et al.*, 2004). Equal

volumes (100 µl) of these iRBC cultures were combined with the SYBR Green I lysis buffer as described above (section 3.2.2) and the fluorescence was measured after a 1 hr incubation at 37˚C, using a Fluoroskan Ascent FL microplate fluorometer (Thermo Scientific, excitation at 485 nm and emission at 538 nm). The background fluorescence was subtracted and the data averaged ± S.E. from three independent experiments.

To monitor DNA replication as well as nuclear division, ring-stage iRBCs (1% haematocrit, ~5% parasitaemia) were incubated with Ant-4 (1xIC_{50} and 5xIC_{50}), or vehicle (1xPBS, negative control) at 37˚C for 24 hrs. iRBC cultures (100 µl) were used either fresh or fixed with 1 ml of 0.025% glutaraldehyde for 45 min and kept at 4°C until assayed. These cells were washed, and re-suspended in 20 µl 1:1000 SYBR Green I for 30 min in the dark at room temperature to stain the parasite's DNA, washed four times with 1xPBS and analysed on a FACS FC500 System (Beckman Coulter) equipped with an air-cooled argon laser with 488 nm excitation. At least 10^5 cells were analysed for each sample with fluorescence emission collected in the FL-1 channel (FITC signal). FlowJo v9.1 (Tree Star) was used to analyze the data. Experiments were performed in duplicate for three independent experiments. Gating was performed based on uninfected RBCs to obtain the iRBC population. Within this population, gating was subsequently also performed for peaks corresponding to iRBCs containing parasites with 1 nucleus (1N, either rings or trophozoite forms), or multiple nuclei (2N, 3N, 4N or >4N) corresponding to late-stage schizont parasites.

3.2.10 Determination of mitochondrial membrane potential

The effect of Ant-4 treatment of intra-erythrocytic *P. falciparum* parasites on the mitochondrial membrane potential ($\Delta\psi m$) was determined with the dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide, Biotium). JC-1 fluoresces red (FL2 signal, DAPI filter) in normal cells and green (FL1 signal, FITC filter) in cells that have undergone a loss of $\Delta\psi m$. Ring-stage *P. falciparum* (3D7) cultures (1% haematocrit, ~5% parasitaemia) were incubated with Ant-4 (1xIC_{50}) at 37˚C for 24 hrs. Subsequently, 10 µl of the untreated and treated iRBC cultures were washed in 1xPBS and the cells re-suspended in 1xJC-1 solution as per the manufacturer's instructions, incubated at 37˚C for 15 min in the dark, washed twice in 1xPBS and analysed by flow cytometry as described above (section 3.2.8).

3.2.11 Statistical analysis

Statistical significance was determined with a two-tailed t-test using the Graphpad Instat (v 3.06) program. Non-linear regression was performed with SigmaPlot (v.11). Unless otherwise specified, data were presented as the means from at least three independent experiments performed in triplicate with S.E. indicated.

3.3 Results

3.3.1 Effect of anthracene-polyamine conjugates on iRBC proliferation

The effect of the anthracene-polyamine conjugates on intra-erythrocytic *P. falciparum* (3D7) parasite proliferation *in vitro* was determined using the SYBR Green I fluorescence assay. Dose-response curves indicated that the compounds have IC_{50} values in the nM range (Fig. 3.2). However, culture media has previously been shown to contain serum amine oxidases that could artificially enhance the growth inhibitory effect of polyamines and their analogues in cell cultures *in vitro* (Gahl and Pitot, 1978). Amine oxidases convert spermidine and spermine (but not the diamine putrescine) into their aldehyde forms with the resultant production of toxic NH_3 and H_2O_2, which is toxic to intra-erythrocytic *P. falciparum* parasites (Lee and Sayre, 1998; Morgan *et al.*, 1986). This can be prevented by the addition of the amine oxidase inhibitor aminoguanidine (Stjernborg and Persson, 1993). Therefore, in order to restrict the anthracene-polyamine conjugates to their polyamine form, non-toxic concentrations of aminoguanidine (0.5 mM; (O'Connell-Jones, 2009)) were included in the SYBR Green I fluorescence assays. In the presence of aminoguanidine, the IC_{50} values of the homospermidine (Ant-44 and bi-conjugated 44-Ant-44) and homospermine (Ant-444) conjugates increased at least 5-fold ($P \leq 0.05$) (Fig. 3.2 and Table 3.1), indicating that their oxidation products contributed to the low IC_{50} values observed in the initial experiments. In contrast, and as expected, the IC_{50} values of the putrescine-conjugate, Ant-4, were similar in the absence or presence of aminoguanidine (640±9 nM vs. 622±40 nM, n=9, $P \geq 0.05$, Table 3.1).

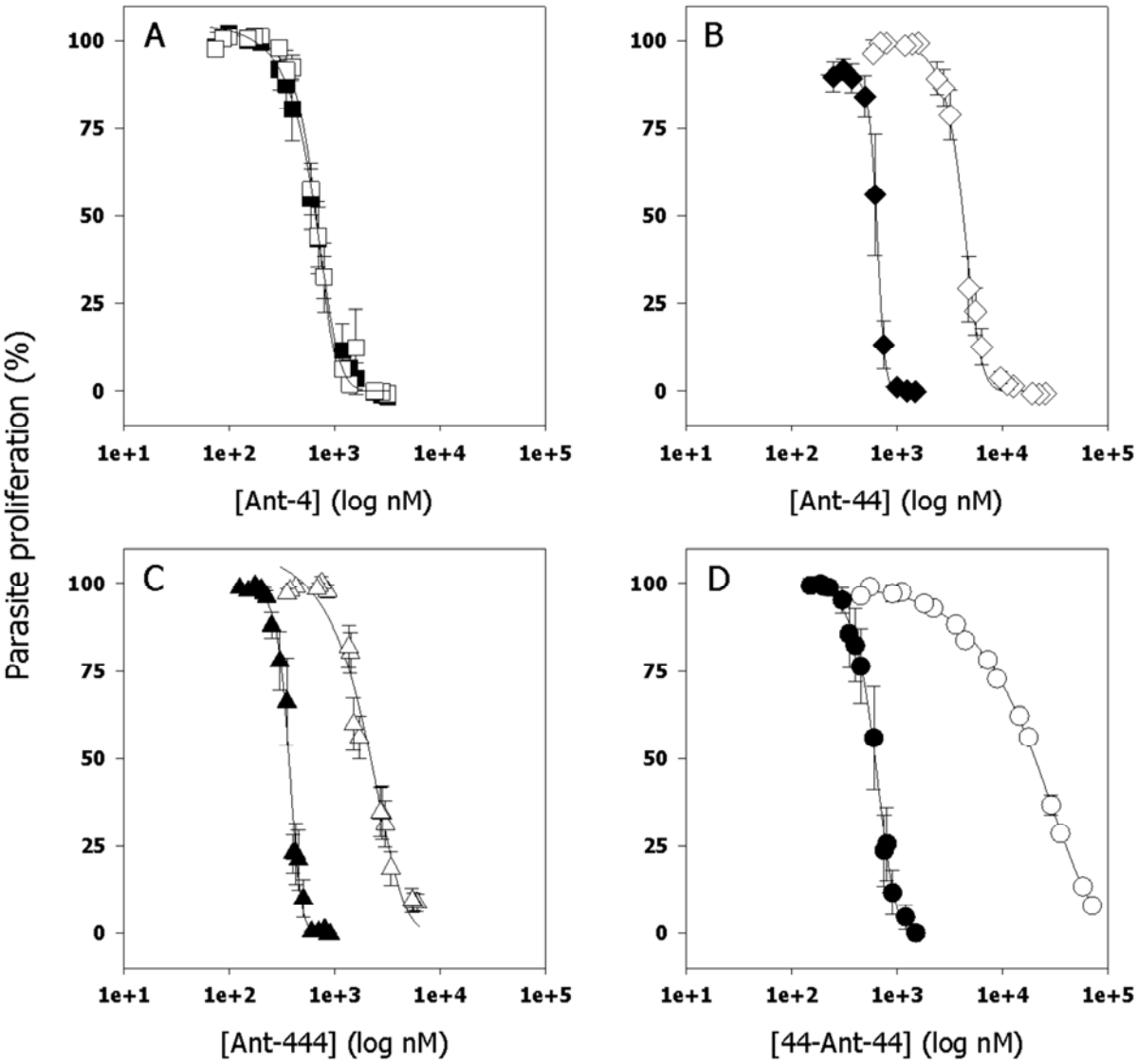


Figure 3.2: Dose-response curves showing the inhibitory effect of anthracene-polyamine conjugates on the proliferation of intra-erythrocytic *P. falciparum* parasites *in vitro* over 96 hrs (initiated with ring-stage parasites) at 37°C in the absence (filled symbols) or presence (empty symbols) of 0.5 mM aminoguanidine. The data are averaged from n experiments and are shown ± S.E. (A) Ant-4; n=9, (B) Ant-44; n=5, (C) Ant-444; n=5 and 6, respectively for the presence and absence of aminoguanidine, (D) 44-Ant-44; n=7 and 3, respectively for the presence and absence of aminoguanidine. Where not shown, error bars fall within the symbols.

To date, CHO cells are the only non-cancerous cell line for which inhibition data (IC_{50}) are available for the anthracene-polyamine conjugates (Phanstiel *et al.*, 2007). This published data were used here, together with published data for cancer cell lines (HL-60 and L1210) (Kaur *et al.*, 2008; Palmer *et al.*, 2009) to compare the selectivity of Ant-4 for intra-erythrocytic *P. falciparum* parasites relative to cancerous and non-cancerous mammalian cells. Selectivity was calculated using the selectivity index (SI) of Ant-4, which is the IC_{50}

ratio of Ant-4 against e.g. CHO cells, compared to intra-erythrocytic *P. falciparum* parasites (i.e. SI=IC_{50} CHO/IC_{50} *P. falciparum*). The SI of Ant-4 for CHO compared to intra-erythrocytic *P. falciparum* parasites was 12, indicating that Ant-4 is more toxic to intra-erythrocytic *P. falciparum* parasites than to CHO cells. This selectivity becomes more pronounced with a SI of 31 compared to cancerous HL-60 cells (Table 3.1). This selectivity of Ant-4 for intra-erythrocytic *P. falciparum* parasites compared to mammalian cells is within the accepted range of >10 for anti-malarial lead compounds (www.mmv.org). The SI values for all the other anthracene-polyamine conjugates were below 10 (Table 3.1).

Table 3.1: IC_{50} values of anthracene-polyamine conjugates against the iRBCs, CHO cells and human (HL-60) and murine (L1210) leukaemia cell lines. Intra-erythrocytic *P. falciparum* parasites were incubated with anthracene-polyamine conjugates at different concentrations at 37°C for 96 hrs (initiated with ring-stage iRBCs) and the IC_{50} values determined using the malaria SYBR Green I fluorescence assay. The rest of the IC_{50} values are from published work as indicated.

	Polyamine conjugated group[a]	IC_{50} *Pf* (nM)	IC_{50} (+AG) *Pf* (μM)	IC_{50} CHO (μM)[b]	IC_{50} HL-60 (μM)[c]	IC_{50} L1210 (μM)[d]	SI (CHO/Pf)	SI (HL-60/Pf)	SI (L1210/Pf)
Ant-4	R-putrescine	640±9 (n=9)	0.66±0.04 (n=9)	7.7	20	-	12	31	-
Ant-44	R-homospermidine	700±3 (n=5)	4.3±0.4 (n=5)	0.45	-	0.3	0.1	-	0.07
Ant-444	R-homospermine	367±19 (n=5)	1.71±0.24 (n=6)	10.6	-	7.5	6.23	-	4.4
44-Ant-44	Bi-conjugated homospermidine	627±70 (n=7)	21±5 (n=3)	1.1	-	1.5	0.05	-	0.075

[a] R indicates the anthracene conjugated moiety (Liao *et al.*, 2009)
[b] (Phanstiel *et al.*, 2007)
[c] (Palmer *et al.*, 2009)
[d] (Kaur *et al.*, 2008)
AG: aminoguanidine; L1210: mouse leukaemia cells; HL-60: human leukaemia cells; *P. falciparum* parasites: *Pf*; SI: selectivity index=ratio of the IC_{50} of Ant-4 against CHO, HL-60 or L121 cells and the IC_{50} of Ant-4 against *P. falciparum* parasites.

3.3.2 Cytostatic vs. cytotoxic effects of Ant-4

Anti-proliferative agents can be either cytotoxic, causing cell death, or cytostatic, which prevents cell proliferation and thus disease progression without actually causing cell death (Rixe and Fojo, 2007). For example, DFMO is cytostatic against intra-erythrocytic *P. falciparum* parasites, thus allowing the cells to remain viable and thereby capable of proliferation if the DFMO pressure is removed or if exogenous putrescine is provided (Assaraf *et al.*, 1987b). The malaria SYBR Green I fluorescence assay measures parasite

proliferation based on changes in DNA levels (Smilkstein *et al.*, 2004). While this assay is useful for measurement of parasite proliferation over time for IC_{50} measurements, it only measures relative DNA levels and does not distinguish between a dead cell and a viable cell capable of proliferation. The CellTiter 96® Aq_{ueous} One Solution Cell Proliferation assay (Promega, USA) is used for measuring the number of viable cells in cytotoxicity assays (Gauduchon *et al.*, 2005). This assay uses the reduction of a tetrazolium compound to a coloured formazan product by dehydrogenases to measure whether cells are viable (Cory *et al.*, 1991) and was thus used to measure the viability of Ant-4 treated intra-erythrocytic *P. falciparum* parasites (Fig. 3.3).

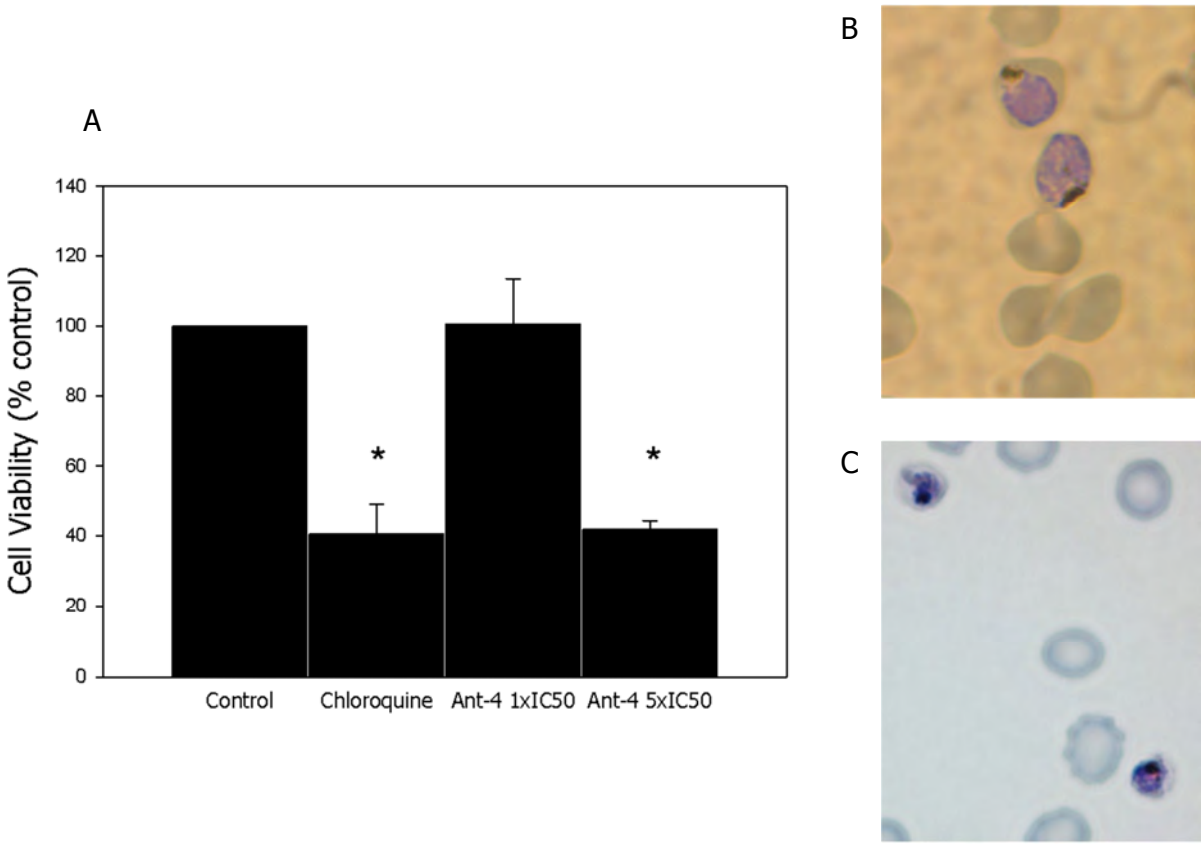


Figure 3.3: Effect of Ant-4 on the viability of intra-erythrocytic *P. falciparum* trophozoites. (A) Cell viability of trophozoite parasites was measured using the production of a coloured formazan product during 4 hrs incubation, following 24 hrs incubation of initial ring-stage iRBC cultures with different concentrations of Ant-4 at 37°C. As a positive control, chloroquine at >10xIC_{50} was used. Viability is expressed as a percentage of the untreated control cells, averaged from four independent experiments ± S.E. * $P<0.05$. (B) Untreated, trophozoite-stage Giemsa-stained intra-erythrocytic *P. falciparum* parasites. (C) Giemsa-stained 3xIC_{50} Ant-4 treated *P. falciparum* trophozoites following 24 hrs treatment at 37°C.

Ant-4 treatment caused a dose-dependent decrease in cell viability of iRBCs within 24 hrs of treatment. There was no statistically significant difference in the viability of untreated iRBCs and iRBCs treated at 1xIC_{50} ($P\geq0.05$). Treatment at 5xIC_{50}, however, showed a significant

decrease ($P \leq 0.05$) in cell viability. This decrease in cell viability was similar to that seen after treatment of iRBCs with chloroquine at a concentration of >10xIC_{50}. The Ant-4 treated trophozoites were also visibly affected, displaying the pyknotic morphology characteristic of stress-forms of the parasite (Fig. 3.3 C) (Deponte and Becker, 2004).

Parasitaemia was furthermore monitored in the presence of Ant-4 over 96 hrs using Giemasa-stained smears to determine if the cell number remains constant, indicative of a cytostatic response, or decreased due to a cytotoxic response to Ant-4 treatment of iRBCs (Fig. 3.4) (Rixe and Fojo, 2007). From 24 hrs onwards, there was a statistically significant increase in the parasitaemia of the control iRBCs compared to the Ant-4 treated iRBCs at the same time-points ($P \leq 0.05$), suggesting reduced viability of the Ant-4 treated iRBCs. Furthermore, at the end of the two lifecycles monitored, the parasitaemia of Ant-4 treated iRBCs was significantly reduced in comparison to the starting parasitaemia ($P \leq 0.05$), confirming the cytotoxic (as opposed to cytostatic) effect of Ant-4 on the parasites. In contrast to what was seen with DFMO treatment (Assaraf *et al.*, 1987b), the presence of ~500-fold excess of putrescine (compared to Ant-4) was not able to reverse the cytotoxic phenotype and restore growth of the parasites (Fig. 3.4).

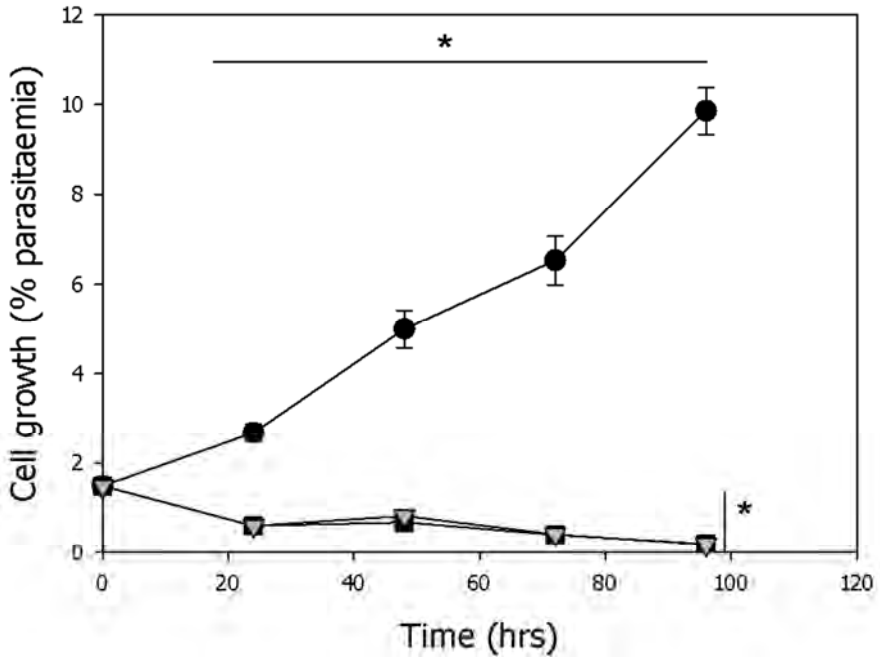


Figure 3.4: Cytotoxicity of Ant-4 against intra-erythrocytic *P. falciparum* parasites in the absence and presence of putrescine. iRBC cultures were treated with vehicle (1xPBS) for negative control (●), 3xIC_{50} Ant-4 (■) and 3xIC_{50} Ant-4 with 1 mM putrescine (▼) at 37°C and the parasitaemia monitored for 96 hrs using Giemsa-stained smears. The experiment was initiated with ring-stage parasites. Data are averaged from three independent experiments and given ± S.E. * $P<0.05$. Error bars fall within the symbols where not shown.

3.3.3 Uptake of anthracene-polyamine conjugates by iRBCs

The uptake of the fluorescent anthracene-polyamine conjugates into trophozoite-stage iRBCs was visualised with deconvolution fluorescence microscopy, using the inherent fluorescence of the anthracene moiety (excitation 364 nm, emission 416 nm) (Wang *et al.*, 2003b). Intra-erythrocytic *P. falciparum* parasites took up Ant-4, with the fluorescence localised to the parasite cytosol and nucleus (Fig. 3.5). No fluorescence was observed for Ant-4 in the RBC compartment, suggesting that there was no significant accumulation of the compound in this compartment. However, the possibility that the haemoglobin quenched the fluorescence in the RBC cannot be excluded.

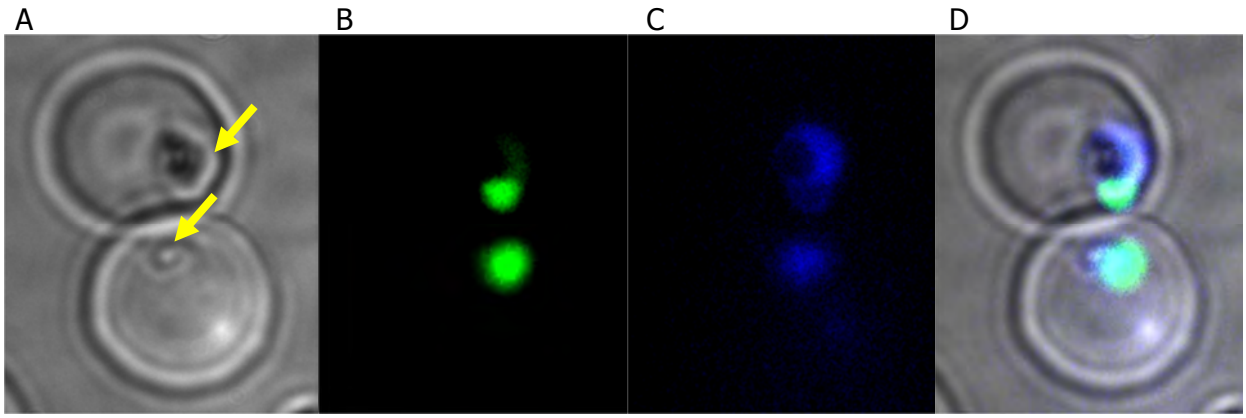


Figure 3.5: Deconvolution fluorescence microscopy of trophozoite-stage iRBCs incubated with Ant-4 (100 µM) at 37°C. (A) Trophozoite-stage parasites are visible inside RBCs in the bright-field image and are indicated with yellow arrows. Dark black haemozoin crystals are visible, particularly in the top parasite. (B) Intra-erythrocytic *P. falciparum* nuclei were visualised with SYBR Green I using a 1/1000 dilution of the commercially available stock solution and incubating the trophozoite-stage iRBCs for 1 hr at 37°C in the dark. (C) Ant-4 uptake into the trophozoite-stage iRBCs was visualised with a DAPI filter following incubation with Ant-4 (100 µM) for 1 hr at 37°C in the dark. (D) Co-localisation of Ant-4 with the *P. falciparum* parasite cytosol and nucleus.

This apparent preferential accumulation of Ant-4 in iRBCs, as opposed to accumulation throughout both iRBCs and RBCs, was confirmed with flow cytometric analyses of RBCs, iRBCs and isolated parasites incubated with Ant-4 (400 µM), with the emission measured in the DAPI channel (Fig. 3.6). There was a significant ~15-fold increase in the Ant-4 signal in iRBCs vs. RBCs (iRBCs 31.3±1.9 vs. RBC 2.1±0.2 relative fluorescence units, n=3, $P \leq 0.05$) (Fig. 3.6 A and B). Additionally, there was a strong fluorescent signal associated with the isolated *P. falciparum* trophozoites (112±9 relative fluorescence units, n=3, Fig. 3.6 C). These results are consistent with the observation made by deconvolution fluorescence microscopy: Ant-4 accumulates preferentially in iRBCs, with little uptake into RBCs.

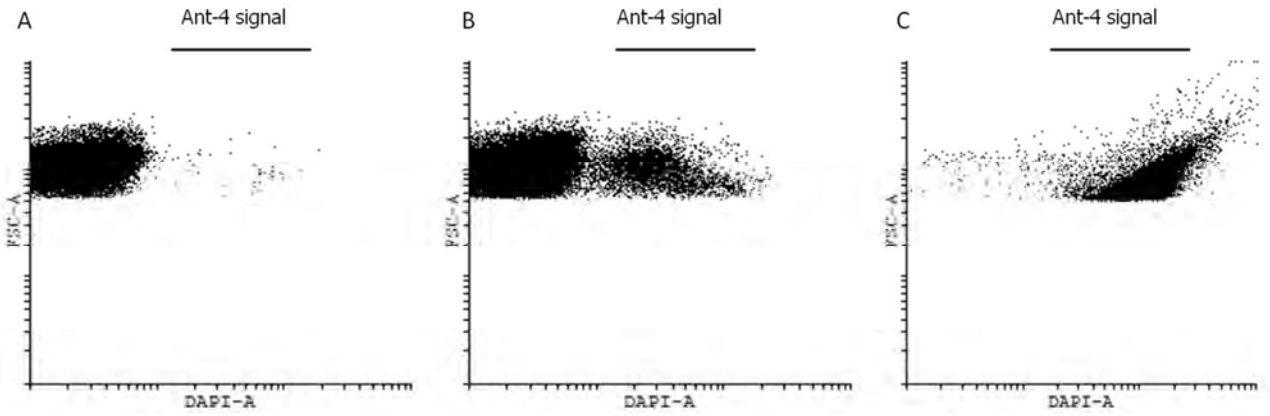


Figure 3.6: Flow cytometric scatter plots of Ant-4 signal (DAPI channel) in (A) RBCs, (B) iRBCs and (C) isolated *P. falciparum* trophozoites. The different cell suspensions were incubated with Ant-4 (400 µM) at 37°C for 1 hr before measuring the fluorescence in the DAPI channel. Results are representative of a single experiment, with data from at least 10^5 cells captured.

Pre-treatment of cancerous cells such as HL-60 and L1210 with DFMO increases subsequent polyamine uptake due to the inhibition of ODC, which causes a decrease in intracellular polyamine levels (Alhonen-Hongisto *et al.*, 1980; García-Fernández *et al.*, 2005; Walters and Wojcik, 1994). In these cells, DFMO treatment also led to a decrease in the IC_{50} of anthracene-polyamine conjugates, postulated to be due to increased uptake of the latter (Palmer *et al.*, 2009; Tomasi *et al.*, 2010). Treatment of iRBCs with the polyamine biosynthesis inhibitor DFMO results in decreased polyamine levels (Assaraf *et al.*, 1987a) and, as is the case in cancer cells, increased total putrescine uptake 2-fold and total spermidine uptake 4-fold (section 2.3.2.6). The IC_{50} of Ant-4 measured in cells pre-treated with DFMO was significantly lower than that measured in control cells (640±9 nM control cells vs. 545±17 nM DFMO-treated cells, n=3, $P \leq 0.05$) (Fig. 3.7).

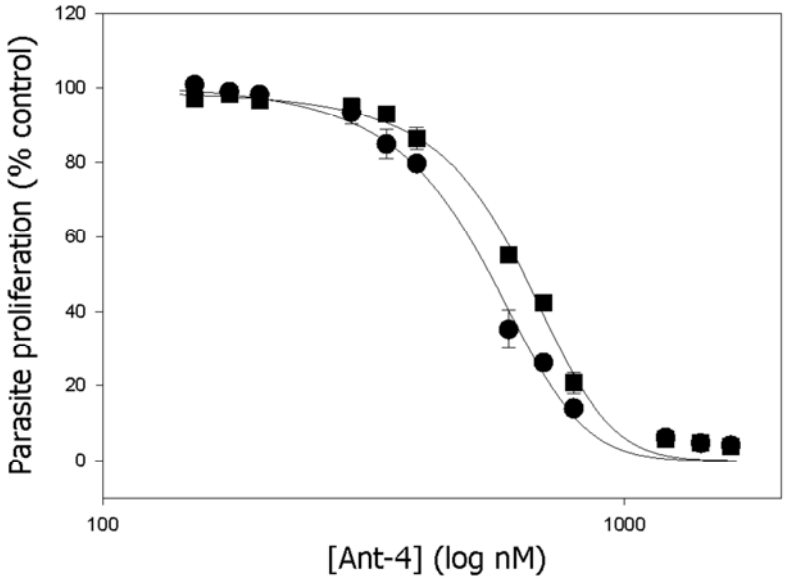


Figure 3.7: Effect of DFMO pre-treatment on the cytotoxicity of anthracene-polyamine conjugates against iRBCs, using Ant-4. Ring-stage iRBCs were incubated with (●) or without (■) 1 mM DFMO for 24 hrs, washed, and the now trophozoite-stage iRBCs incubated for a further 72 hrs with different concentrations of Ant-4 before determining the IC_{50} using the SYBR green I fluorescence assay. Data are averaged from three independent experiments and given ± S.E. Error bars fall within the symbols where not shown.

To investigate the effect of the anthracene-polyamine conjugates on the uptake of putrescine and spermidine, the inhibition of [^{3}H]putrescine and [^{3}H]spermidine uptake into functionally isolated *P. falciparum* trophozoites in the presence of the anthracene-polyamine conjugates was measured as described in section 2.2.1. The serum amine oxidases present in the culture media (present due to Albumax II) were washed away during the isolation process and thus the compounds were in their polyamine form without the presence of the oxidation products that led to the nM IC_{50} values obtained for the spermidine and spermine conjugates seen in the previous section. The putrescine conjugate Ant-4, the homospermidine conjugates Ant-44 and 44-Ant-44 and the homospermine conjugate Ant-444, all significantly ($P \leq 0.05$) reduced polyamine uptake into isolated *P. falciparum* trophozoites as measured over 30 min (Fig. 3.8). Putrescine uptake was reduced between 20% and 30% by the anthracene-polyamine conjugates (500 µM), whereas spermidine uptake was reduced by between 60% to 95%.

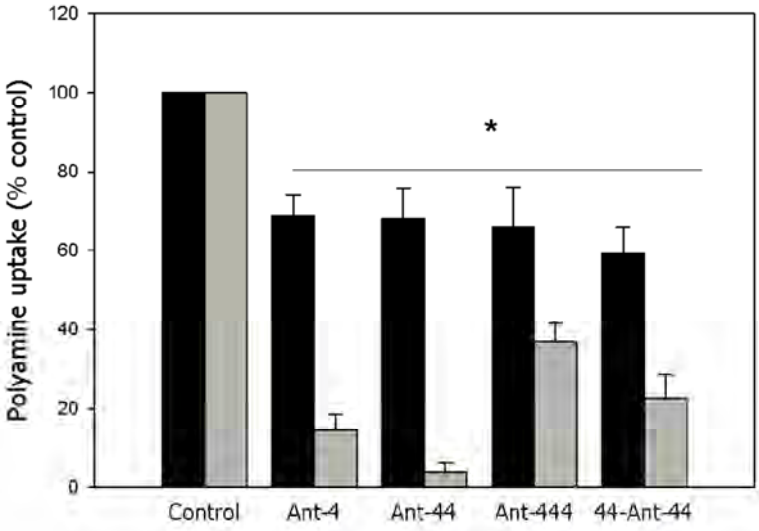


Figure 3.8: Inhibition of [^{3}H]putrescine (black bars) and [^{3}H]spermidine uptake (grey bars) into isolated *P. falciparum* trophozoites by anthracene-polyamine conjugates (500 µM) at 37°C during a 30 min incubation. For both polyamines the extracellular concentration was approximately 5 nM and the anthracene-polyamine conjugates were added to the cells simultaneously with the polyamines. All conditions are given as percentage of control (no competing substrate), averaged from four independent experiments, * $P<0.05$. [^{3}H]putrescine uptake is indicated by black bars and shown ± S.E. and [^{3}H]spermidine uptake by grey bars and shown ± S.E.

The previous result showed that the anthracene-polyamine conjugates inhibit polyamine uptake into isolated *P. falciparum* trophozoites. If these compounds enter the parasite via the polyamine uptake mechanism(s), the reverse, i.e. that polyamines reduce the uptake of the anthracene-polyamine conjugates, should also be true. The inherent fluorescence of the anthracene-polyamine conjugates, particularly Ant-4 was used with flow cytometric analyses to determine the effect of polyamines on Ant-4 uptake in iRBCs (Fig. 3.9).

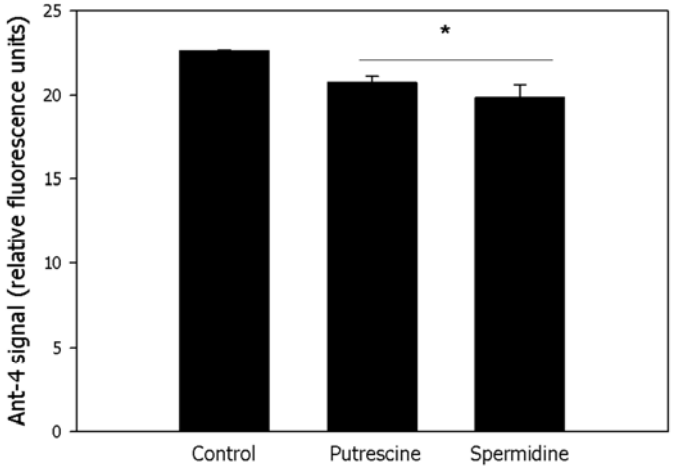


Figure 3.9: Inhibition of Ant-4 uptake (400 µM) into trophozoite-stage iRBCs by polyamines (500 µM) at 37°C following 1 hr incubation. The iRBC cultures were incubated with Ant-4 and polyamines (added simultaneously) at 37°C for 1 hr before flow cytometry fluorescence measurements from at least 10^5 cells were obtained. Results are given as relative fluorescence units, averaged from three independent experiments, * $P<0.05$, and shown ± S.E.

There was a statistically significant, albeit slight, decrease in the Ant-4 signal in trophozoite-stage iRBCs in the presence of near-equimolar concentrations of both putrescine (control 22.617±0.028 vs. putrescine 20.8±0.4, relative fluorescence units, n=3, $P \leq 0.05$) and spermidine (control 22.617±0.028 vs. spermidine 19.9±0.7, relative fluorescence units, n=3, $P \leq 0.05$).

3.3.4 Effect of Ant-4 treatment on intracellular polyamine levels in iRBCs

In contrast to what was observed with HL-60 cells (Palmer *et al.*, 2009), there were no statistically significant differences in the levels of putrescine, spermidine or spermine in iRBCs following 24 hrs of incubation with Ant-4 ($P \geq 0.05$) (Fig. 3.10), suggesting that Ant-4 treatment does not affect polyamine biosynthesis in intra-erythrocytic *P. falciparum* parasites. This lack of effect on polyamine levels of iRBCs treated with Ant-4 is supported by earlier experiments in which it was shown that Ant-4 treatment does not inhibit the activity of either ODC or AdoMetDC of *P. falciparum* (Verlinden, 2009). These results indicate that the major cytotoxic action of Ant-4 on intra-erythrocytic *P. falciparum* parasites is independent of changing the polyamine levels in the parasite.

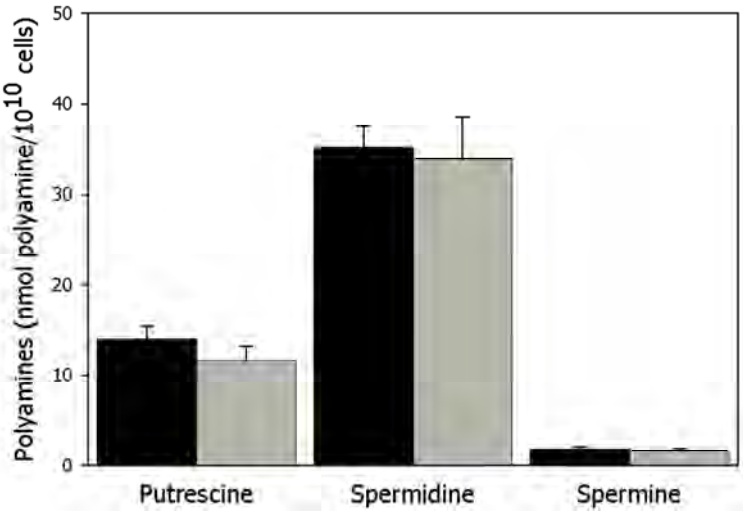


Figure 3.10: Polyamine levels of trophozoites-stage iRBCs following Ant-4 treatment. Ring-stage iRBC cultures were treated with vehicle for control (black bars) or 2xIC_{50} Ant-4 (grey bars) for 24 hrs at 37°C and the polyamines present in the now trophozoite-stage parasites extracted with PCA for 12 hrs at 4°C, followed by benzoylation for 30 min at 37°C to allow for HPLC measurement of the polyamines. Data are averaged from three independent experiments and given ± S.E.

3.3.5 Effect of Ant-4 on the reducing environment of iRBC

In HL-60 cells, Ant-4 induced cell death is postulated to be due in part to oxidative stress (Palmer *et al.*, 2009). The levels of ROS were determined with flow cytometric analyses using the fluorescent dye DCFDA that fluoresces when oxidised. In contrast to iRBCs treated with H_2O_2 as a positive control for ROS, there was no significant difference ($P \geq 0.05$) between the H_2O_2 levels in untreated intra-erythrocytic *P. falciparum* trophozoites and Ant-4 treated intra-erythrocytic *P. falciparum* trophozoites (Table 3.2).

Table 3.2: Measurement of H_2O_2 (DCFDA signal) and reduced glutathione levels as indicators for the reducing environment in Ant-4 treated intra-erythrocytic *P. falciparum* trophozoites. iRBCs were incubated with Ant-4 ($1xIC_{50}$) for 24 hrs or with H_2O_2 (20 µM) for 30 min at 37°C before incubating the untreated and treated trophozoite-stage iRBCs with 20 µM DCFDA (20 µM) for 20 min at 37°C. Flow cytometry was then used to measure the fluorescence from at least 10^5 cells. Alternatively, iRBCs were incubated with Ant-4 ($1xIC_{50}$) for 24 hrs at 37°C before using the luminescent GSH-Glo™ Glutathione Assay (Promega) to determine the concentration of reduced glutathione in the untreated and treated iRBCs. Data are averaged from n independent experiments and are shown ± S.E.

	DCFDA signal[a] (n=3)	Reduced glutathione[b] (n=4)
Untreated intra-erythrocytic *P. falciparum*	5.6±0.5	8.2±0.7
Ant-4 ($1xIC_{50}$) treated intra-erythrocytic *P. falciparum*	5.4±0.9	7.5±0.9
H_2O_2 (20 µM) treated intra-erythrocytic *P. falciparum*	86.0± 0.8	-

[a] DCFDA signal measures in relative fluorescent units,

[b] µM reduced glutathione/10^{10}cells

The presence of the reduced form of glutathione is vital for the maintenance of a reducing environment in the intra-erythrocytic *P. falciparum* cytosol (Becker *et al.*, 2003). To confirm the apparent lack of effect of Ant-4 on the reducing environment of intra-erythrocytic *P. falciparum* trophozoites, reduced glutathione levels were measured following Ant-4 treatment of intra-erythrocytic *P. falciparum* parasites. Ant-4 treatment of intra-erythrocytic *P. falciparum* parasites at $1xIC_{50}$ had no significant effect ($P \geq 0.05$) on the levels of reduced glutathione in intra-erythrocytic *P. falciparum* trophozoites (Table 3.2). This confirms that the cytotoxicity of Ant-4 to the parasite is not caused by oxidative stress.

3.3.6 Effect of Ant-4 on iRBC mitochondrial membrane potential (Δψm)

The Δψm of intra-erythrocytic *P. falciparum* trophozoites was monitored with the fluorescent, cationic dye JC-1 to determine if the cytotoxic effect of Ant-4 is due to depolarisation of the Δψm. In healthy cells with normal Δψm, JC-1 exhibits potential-dependent accumulation in mitochondria and give rise to a red fluorescence due to the concentration dependent aggregation of JC-1 (FL-2 signal, Fig. 3.11). In cells with a loss in Δψm e.g. apoptotic cells, the dye cannot enter the mitochondria and aggregate, and therefore fluoresces green (increased FL-1 signal, Fig. 3.11) in its cytosolic monomeric form.

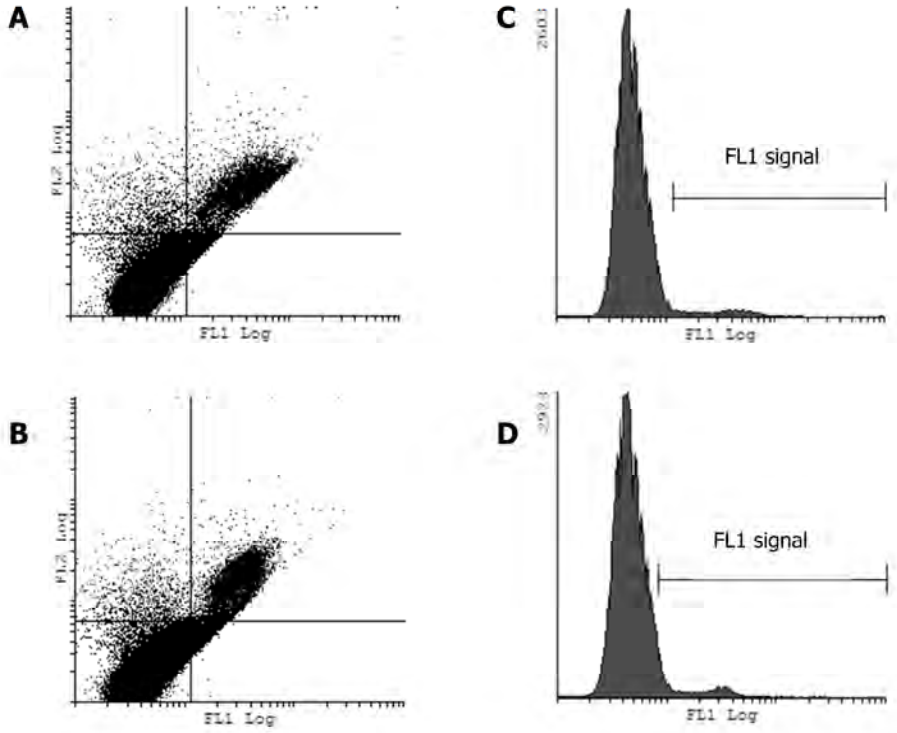


Figure 3.11: Flow cytometric profiles of JC-1 signal as indicator of changes in mitochondrial membrane potential due to Ant-4 treatment of iRBCs. Ring-stage iRBCs were incubated with Ant-4 (1xIC_{50}) at 37°C for 24 hrs before incubating the now trophozoite stage iRBCs in 1xJC-1 solution at 37°C for 15 min in the dark, and analysing 10^5 cells by flow cytometry. Scatter plots indicate the FL2 (red signal) and FL1 (green signal) channels of untreated (A) and Ant-4 treated parasites (B). Gating based on the increase in FL1 signal in the right bottom quadrants produced histograms (C) and (D), from which peak areas could be determined providing relative fluorescent units. Results are representative of a single experiment.

A 2-fold increase was observed in green fluorescence in Ant-4 (1xIC_{50}) treated intra-erythrocytic *P. falciparum* parasites compared to untreated parasites (1.24±0.12 in Ant-4

treated cells, vs. 0.63±0.03 in untreated cells, relative fluorescence units, n=2); however, this was not statistically significant ($P \geq 0.05$). This apparent loss in mitochondrial membrane potential in intra-erythrocytic *P. falciparum* following Ant-4 treatment may contribute to intra-erythrocytic *P. falciparum* cell death.

3.3.7 Ant-4 treatment affects DNA replication in iRBC

Polycyclic aromatic hydrocarbons (PAH) such as anthracene often induce DNA damage i.e. strand breaks, oxidative damage or PAH-DNA adduct formation (Cavallo *et al.*, 2008; Chakravarti *et al.*, 2008). To determine whether Ant-4 affects the intra-erythrocytic *P. falciparum* parasites' DNA replication, cells were incubated with Ant-4 ($3xIC_{50}$) and DNA levels were measured over 96 hrs using SYBR Green I fluorescence (Fig. 3.12). Following 24 hrs under Ant-4 pressure, the DNA levels in intra-erythrocytic *P. falciparum* parasites stayed constant over the two lifecycles analysed (comparison of DNA levels of Ant-4 treated parasites at t=24, 48, 72 and 96 hrs, $P \geq 0.05$), suggesting that no DNA replication occurred, compared to the significant increase in DNA levels of the control intra-erythrocytic *P. falciparum* parasites (comparison of DNA levels of untreated parasites at t=24, 48, 72 and 96 hrsas well as comparison of untreated to Ant-4 treated parasites' DNA levels at each individual time-point , $P \leq 0.05$).

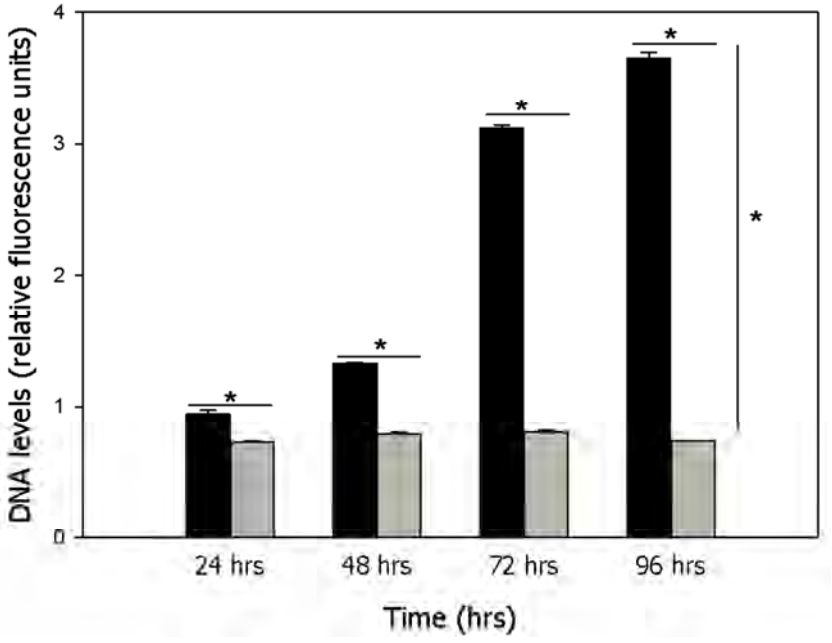


Figure 3.12: Intra-erythrocytic *P. falciparum* DNA levels following treatment with Ant-4. Intra-erythrocytic *P. falciparum* parasites were incubated with vehicle (1xPBS) for control (black bars) or $3xIC_{50}$ Ant-4 (grey bars) for the specified times at 37°C. At each time point, DNA levels were measured as relative fluorescence units following an 1 hr incubation of equal volumes of the iRBCs and SYBR Green I lysis buffer at room temperature in the dark. Data are averaged ± S.E. from three independent experiments. * $P<0.05$.

Every 48 hrs, the DNA of the intra-erythrocytic *P. falciparum* parasite replicates as the parasite develops from a single-nucleated (1N) ring stage (0 hr) and early trophozoite stage (24 hrs) to the multi-nucleated schizont stage (>2N, 36 hrs) and finally to the release of merozoites. The inhibition of DNA replication as a cause of the cytotoxic effect of Ant-4 against intra-erythrocytic *P. falciparum* was investigated by the flow cytometric profiles of the untreated and Ant-4 treated parasites over 48 hrs (Table 3.3). DNA replicated in untreated iRBCs and nuclear division occurred as expected, resulting in a reduction in the percentage of parasites with 1 nucleus (1N) and an increase in the percentage of parasites with >2 nuclei (2N) at >24 hrs. After 48 hrs, the untreated parasite again reflected a 1N ring-stage population starting their second lifecycle. Ant-4 treated intra-erythrocytic *P. falciparum* parasites exhibited a dose-dependent effect on DNA replication, with no DNA replication in parasites treated at 5xIC_{50}. In these cells, no nuclear division occurred and the parasites remained in the initial 1N stage, indicating that Ant-4 interferes with the parasite's DNA replication within the first 24 hrs of exposure.

Table 3.3: Flow cytometric analysis of nuclear division of intra-erythrocytic *P. falciparum* parasites. iRBCs (starting with ring-stage parasites) were exposed to Ant-4 (1xIC_{50} and 5xIC_{50}), or vehicle (1xPBS, negative control) at 37°C for 48 hrs and cell samples taken at 4 hrs, 24 hrs, and 48 hrs post treatment. iRBC DNA (originating from the *P. falciparum* parasite) was subsequently stained with 20 µl 1:1000 SYBR Green I for 30 min in the dark at room temperature, before measuring the fluorescence of at least 10^5 cells for each sample, with fluorescence emission collected in the FL-1 channel (FITC signal). Ring or early trophozoite-stage iRBCs contain 1 nucleus (1N), followed by nuclear division in late trophozoites (2N) and multi-nucleated shizonts (>3N). Data are averaged ± S.E. from three independent experiments**.**

		Relative percentage of cells/population				
Treatment	Hours post treatment	1N	2N	3N	4N	>4N
Control	4	88.4±0.2	3.60±0.04	0.21±0.02	0.07±0.01	0.08±0.01
	24	28.5±2.1	36.8±1.8	20.4±2.5	6.4±1.0	0.43±0.05
	48	60.5±0.5	22.0±0.6	9.9±0.5	2.7±0.1	2.6±0.1
Ant-4 (IC_{50})	4	88.7±0.4	3.2±0.1	0.12±0.01	0.06±0.01	0.05±0.01
	24	28.1±0.8	39.8±0.2	19.7±0.5	3.60±0.02	0.17±0.05
	48	54.7±0.8	18.4±0.3	7.5±0.2	3.9±0.2	12.4±1.0
Ant-4 (5xIC_{50})	4	88.7±0.1	3.5±0.1	0.1	0.03±0.03	0.03±0.01
	24	87.3±0.3	3.90±0.03	0.17±0.02	0.02±0.02	0.03
	48	74.1±0.7	8.5±1.2	0.4±0.1	0.05±0.01	0.02±0.01

3.4 Discussion

Several anthracene derivatives have been shown to have anti-malarial properties (Besley and Goldberg, 1954; Bruce-Chwatt and Archibald, 1953; Schmelzer and Gurib-Fakim, 2008; Traxler *et al.*, 1975), but in-depth analyses of the effect of these compounds on intra-erythrocytic *P. falciparum* parasites have not been performed. Additionally, a number of polyamine analogues have anti-plasmodial activity (Bitonti *et al.*, 1989; Edwards *et al.*, 1991; Klenke *et al.*, 2003). Previously, anthracene conjugated to a variety of polyamines were shown to be active against CHO (Phanstiel *et al.*, 2007; Wang *et al.*, 2003a), HL-60 (Palmer *et al.*, 2009) and L1210 cells (Kaur *et al.*, 2008). As both anthracene derivatives and polyamine analogues have shown anti-plasmodial activity, the effect of anthracene conjugated to polyamine moieties on intra-erythrocytic *P. falciparum* parasites was investigated.

An anthracene-putrescine conjugate (Ant-4) was shown to be highly active against intra-erythrocytic *P. falciparum* parasites in the nM range (Fig. 3.2), which makes it potentially interesting as a novel anti-malarial. International anti-malarial drug discovery consortia, including MMV, consider novel compounds to be attractive for further evaluation as lead inhibitors if they show activity below 1 µM (www.mmv.org). Anthracene conjugated to homospermidine and homospermine could not elicit an inhibition of parasite growth below 1 µM, making them less interesting regarding their development in therapeutic strategies against malaria. Since all four polyamine conjugates contain the same cytotoxic moiety, the differences in the inhibitory effect of these compounds on intra-erythrocytic *P. falciparum* parasites (Ant-4>Ant-444>Ant-44>44-Ant-44) can possibly be attributed to the different polyamine moieties. In the case of HL-60 cells, the putrescine moiety of Ant-4 acts as a DNA-targeting vector, delivering the anthracene moiety to DNA (Palmer *et al.*, 2009). Similarly, in intra-erythrocytic *P. falciparum* parasites, the polyamine moiety may play a crucial role in delivering the anthracene moiety to a particular effector site inside the cell, and this delivery may be polyamine-specific. Alternatively, this data may provide preliminary information on the polyamine requirements for cellular entry, with the reduced growth inhibition by homospermine and homospermidine conjugates indicating that these compounds are less able to enter the parasites.

In contrast to what was observed here for *P. falciparum* parasites, in the mammalian cell lines L1210 and CHO the anthracene-homospermidine conjugate Ant-44 was more effective in inhibiting cell proliferation than the anthracene-putrescine conjugate Ant-4 (Kaur *et al.*, 2008; Phanstiel *et al.*, 2007). This is reflected in the high SI of Ant-4 for intra-erythrocytic *P. falciparum* parasites (Table 3.1).

Ant-4 exhibited dose-dependent cytotoxicity (as opposed to a cytostatic effect) against intra-erythrocytic *P. falciparum* parasites, leading to decreased cell numbers over time (Fig. 3.3 and 3.4). Polyamine analogues may result in cell growth inhibition due to the reduction of polyamine levels through either biosynthesis inhibition or induction of polyamine catabolism and export (polyamine anti-metabolites). Alternatively, they may occupy normal polyamine binding/effector sites without performing the normal biological actions of the endogenous polyamines (polyamine mimetics) (Wallace and Fraser, 2004; Wallace *et al.*, 2003). In contrast to the situation in HL-60 cells (Palmer *et al.*, 2009), HPLC analyses showed that intra-erythrocytic *P. falciparum* parasites incubated with Ant-4 did not have decreased polyamine levels (Fig. 3.10). In addition, there is no evidence for polyamine biosynthesis inhibition in Ant-4 treated intra-erythrocytic *P. falciparum* parasites, as Ant-4 did not inhibit the activities of heterologously expressed PfAdoMetDC/ODC (Verlinden, 2009). In general, the anti-proliferative effect of polyamine biosynthesis inhibitors can be overcome with exogenous polyamines (summarised in Table 1.2). However, exogenous putrescine did not reverse the cytotoxic effect of Ant-4 on intra-erythrocytic *P. falciparum* parasites (Fig. 3.4).

There is a complex relationship between polyamines and oxidative stress. In mammalian cells, high levels of polyamines, particularly spermidine and spermine, can be catabolised to their acetylated derivatives with the resultant production of H_2O_2 (Fig. 1.5), which leads to oxidative stress and ultimately cell death (Casero and Pegg, 2009; Wallace *et al.*, 2003). However, polyamines can also protect mammalian cells (mouse fibroblasts) from oxidative stress by directly scavenging radicals (Rider *et al.*, 2007) or, in prokaryotes, by the induction of ROS-responsive transcription factors needed for transcriptional activation cascades of anti-oxidant genes (Jung and Kim, 2003). Polyamine analogues have been shown to affect the oxidation status of cells, with Ant-4 treatment resulting in reduced polyamine levels as well as increased oxidative stress in HL-60 cells (Palmer *et al.*, 2009). However, in Ant-4 treated intra-erythrocytic *P. falciparum* parasites, where there was no change in the polyamine levels (Fig. 3.10), there was additionally no evidence of a change in reductive environment of the parasite or in the production of ROS (Table 3.2).

Therefore, Ant-4 does not seem to act as polyamine anti-metabolite in intra-erythrocytic *P. falciparum* parasites since: 1) it does not affect polyamine levels; 2) it does not affect polyamine biosynthesis enzyme activities; 3) it does not affect the reductive environment within the cell, and 4) cell growth inhibitory effects could not be reversed by exogenous polyamines. One possibility is that Ant-4, due to its polyamine moiety, occupies normal polyamine effector sites non-functionally and thereby acts as polyamine mimetic. In HL-60 and L1210 cancer cells, treatment with DFMO results in depletion of intracellular polyamine levels and a compensatory increase in polyamine uptake (Alhonen-Hongisto *et al.*, 1980; García-Fernández *et al.*, 2005; Walters and Wojcik, 1994). Additionally, for these mammalian cells, a decrease in anthracene-polyamine conjugate IC_{50} following DFMO treatment was attributed to increased transport of the anthracene-polyamine conjugates, similar to what was seen for polyamines themselves (Palmer *et al.*, 2009; Tomasi *et al.*, 2010). However, no data are available in these reports on the exact transport characteristics for polyamines or the anthracene-polyamine conjugates, and an alternative explanation may be that there is increased accumulation of polyamines/polyamine-conjugates in these cells due to increased availability of intracellular polyamine binding sites following biosynthesis inhibition. DFMO pre-treatment of intra-erythrocytic *P. falciparum* parasites led to an increase in total polyamine accumulation (section 2.3.2.6) and a decrease in IC_{50} of Ant-4 (Fig. 3.7). It is possible that this decrease is due to increased accumulation of the Ant-4 within intra-erythrocytic *P. falciparum* parasites as a result of increased availability of intracellular polyamine binding sites and that Ant-4 thereby acts as a polyamine mimetic. However, the possibility that the decrease in IC_{50} of Ant-4 against intra-erythrocytic *P. falciparum* parasites is due to other unknown deleterious effects caused by the DFMO pre-treatment of these parasites cannot be excluded.

Since polyamines bind to DNA as one of its effector sites (Casero and Pegg, 2009; Wallace *et al.*, 2003), the conjugation of polyamines to anthracene (as DNA intercalator) may be particularly effective in eliciting a cytotoxic effect in intra-erythrocytic *P. falciparum* parasites. The planar, polycyclic ring structure of anthracene intercalates tightly but reversibly between DNA base pairs (Rodger *et al.*, 1995), inhibits DNA synthesis and induces DNA damage, typically due to disruption of DNA topoisomerase activity (Phanstiel *et al.*, 2000; Wang *et al.*, 2001). Anthracene and anthracene-polyamine conjugates are known topoisomerase II inhibitors (Phanstiel *et al.*, 2000; Wang *et al.*, 2001). Furthermore, the polyamine moiety of anthracene-polyamine conjugates have been shown to enhance the intercalation of anthracene through the polyamine moiety locking intercalated anthracene

into DNA major grooves by associating with the phosphate backbone (Rodger *et al.*, 1995). In intra-erythrocytic *P. falciparum* parasites, Ant-4 treatment resulted in decreased DNA levels in the parasite (compared to untreated parasites) within 24 hrs (Fig. 3.12). Additionally, intra-erythrocytic *P. falciparum* parasites were unable to replicate their nuclei after Ant-4 treatment (Table 3.3). Ultimately, this means that Ant-4 treated intra-erythrocytic *P. falciparum* parasites are unable to replicate their DNA to allow for asynchronous nuclear division, as nuclear division precedes cytogenesis (Arnot *et al.*, 2011).

Interestingly, a series of 9-anilinoacridines has been shown to inhibit the *P. falciparum* parasite's topoisomerase II, possibly by the intercalation of the acridine moiety into DNA and an interaction between the topoisomerase II and the 9-anilino side chain (Auparakkitanon and Wilairat, 2000). Furthermore, these 9-anilinoacridine derivatives also inhibited β-haematin formation (Auparakkitanon *et al.*, 2003). β-haematin or haemozoin is a non-toxic crystalline form of haem which the parasite produces in order to rid itself of toxic levels of haem released during the degradation of haemoglobin (Egan, 2008). The quinoline family of anti-malarials, including chloroquine, are all proposed to prevent haemozoin formation particularly due to their planar aromatic constituents allowing for pi-pi stacking with haem, thus preventing haem detoxification through the formation of haemozoin and thereby causing parasite death (Egan, 2006). The nM IC_{50} of Ant-4 against *P. falciparum* compared to the μM IC_{50} against CHO en HL-60 cells (Table 3.1) may thus be due to simultaneous inhibition of both DNA replication, possibly due to topoisomerase II inhibition, and haemozoin formation. It will be interesting to investigate this further.

The observed pyknotic nature of intra-erythrocytic *P. falciparum* parasites incubated with Ant-4 (Fig. 3.3) suggests that programmed cell death events might be occurring (Deponte and Becker, 2004). In addition, there was a decrease in mitochondrial membrane potential in *P. falciparum* as a result of Ant-4 treatment (Fig. 3.11). Although temporary mitochondrial membrane depolarisation may occur under conditions which are unrelated to cell death, it is often an indicator of some programmed cell death event (Kepp *et al.*, 2011). Mitochondrial membrane potential is influenced during programmed cell death events and serves in particular, as an early apoptosis indicator (Kumar *et al.*, 2008) but also of autophagy-mediated damage of mitochondria (Nyakeriga *et al.*, 2006; Painter *et al.*, 2010). Loss in mitochondrial membrane potential has been observed in drug-treated intra-erythrocytic *P. falciparum* parasites even when the death is atypical of apoptosis (Nyakeriga *et al.*, 2006) since parasites can enter a reversible, static state (Painter *et al.*, 2010).

Further investigation would be needed to determine if Ant-4 treatment of intra-erythrocytic *P. falciparum* parasites causes programmed cell death.

The molecular mechanisms of programmed cell death is poorly characterised in Plasmodia (Deponte and Becker, 2004). There are reports of apoptosis-like signatures, including stress-like morphology of cells (pyknotic forms) and the presence of metacaspases (Arambage *et al.*, 2009); however there are other reports indicating autophagy (Totino *et al.*, 2008). Apoptosis is usually characterised by morphological changes such as DNA fragmentation, cell shrinkage, membrane blebbing and the formation of apoptotic bodies (Deponte and Becker, 2004), while autophagy is a physiological cell process that degrades damaged cell components via a lysosomal pathway, often induced by ROS, DNA damage or nutrient deprivation (Kroemer *et al.*, 2010). Intra-erythrocytic *P. falciparum* parasites are not amenable to traditional methods of studying DNA fragmentation developed for mammalian cells (Deponte and Becker, 2004). Methods such as DNA laddering, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end-labelling (TUNEL) assay, or single cell gel electrophoresis (Comet) assay, have inherent methodological pitfalls due to the small nuclear size (0.8-1.4 µM across) (Arnot *et al.*, 2011) and low DNA content of intra-erythrocytic *P. falciparum* parasites in comparison to the mammalian cells (Deponte and Becker, 2004). Furthermore, in contrast to mammalian cells, the entire blood stage development of *P. falciparum* parasites occurs with stage dependent DNA content. Even if synchronised cultures are used, inhibition of cell growth by drugs results in a DNA profile that is different from the control cells since, the latter parasites continue to progress through the subsequent developmental stages (Deponte and Becker, 2004).

As an example of these limitations, *P. falciparum* cultures treated with etoposide, a broad-spectrum DNA damaging agent, did not result in a positive TUNEL assay even though etoposide acts by inducing DNA damage (Nyakeriga *et al.*, 2006). Cognisant of these limitations, the Comet assay, which is based on differential migration of undamaged and nicked DNA in an electrical field (Olive and Bánath, 2006) was performed in an attempt to determine whether Ant-4 treatment of intra-erythrocytic *P. falciparum* parasites induce DNA damage, but the results were inconclusive. An alternative marker for apoptosis, phosphatidylinositol serine translocation to the outer plasma membrane is also difficult to determine in an intracellular parasite surrounded by three membrane systems (Deponte and Becker, 2004).

The fluorescent nature of Ant-4 allows its intracellular localisation to be monitored. The compound showed preferential accumulation in the parasite, with no significant fluorescence observed within the RBC cytoplasm of iRBCs or non-infected RBCs (Fig. 3.5 and 3.6). However, absence of detectable RBC fluorescence may be due to quenching of the Ant-4 fluorescence by haemoglobin. Polyamine analogues often have sufficient similarity in structure to the polyamines to enter mammalian cells via polyamine uptake mechanisms, since these uptake mechanisms are sufficiently promiscuous to recognise structurally similar compounds (Palmer and Wallace, 2010; Wallace and Niiranen, 2007; Wallace and Fraser, 2003). The anthracene-polyamine conjugates used in this study were initially designed to exploit this property of polyamine uptake mechanisms in mammalian cells, and it was found that these compounds are more active against cells with polyamine uptake mechanisms (Phanstiel *et al.*, 2007; Wang *et al.*, 2003a; Wang *et al.*, 2003b). The biochemical characterisation of the polyamine uptake mechanisms in *P. falciparum*-infected erythrocytes and in the intracellular parasite itself (Chapter 2) indicated that parasites take up both putrescine and spermidine. This polyamine uptake mechanism(s) was inhibited by a wide variety of structurally similar molecules, possibly due to competition for polyamine uptake (section 2.3.2.5). It is possible that these anthracene-polyamine conjugates are likewise taken up via the polyamine uptake mechanism in intra-erythrocytic *P. falciparum* parasites.

The anthracene-polyamine conjugates inhibit the uptake of putrescine and spermidine into iRBC by 30%-95% (Fig. 3.8). Additionally, putrescine and spermidine both reduced the uptake of Ant-4 (as measured over 1 hr) by ~10% (Fig. 3.9). There are several interpretations for these results. Firstly, these results may suggest that the anthracene-polyamine conjugates compete with the polyamines for uptake via the polyamine uptake mechanism to gain entry into intra-erythrocytic *P. falciparum* parasites, as was postulated for HL-60 cells (Palmer *et al.*, 2009). However, the data do not preclude the possibility that these conjugates inhibit the polyamine uptake mechanism of the isolated parasites without themselves utilising this mechanism for cellular entry, possibly due to structural similarity with the native polyamines. Secondly, the marginal effect of Ant-4 on putrescine uptake (and vice versa) may indicate that these compounds are not taken up via the same polyamine uptake mechanism, or that Ant-4 only partially utilise the same uptake mechanism as polyamines but may rely on additional uptake mechanisms into intra-erythrocytic *P. falciparum* parasites. Lastly, while a 1 hr incubation time was used to ensure that a signal was obtained using fluorescent cell sorting to measure Ant-4 uptake, Ant-4 uptake may already have largely equilibrated by this time, in which case any inhibition of the

initial rate of uptake of Ant-4 by the polyamines would not have been observed. It was shown that the uptake of putrescine reached a plateau within the first 15-30 min (section 2.3.2.1). Therefore, in order to confirm that Ant-4 utilise the polyamine uptake mechanism to enter intra-erythrocytic *P. falciparum* parasites, time-course analyses of polyamine uptake in the presence and absence of Ant-4 would have to be performed in order to determine whether Ant-4 affects the initial rate of putrescine or spermidine uptake (and thus transport across the membrane), and vice versa. For the study of Ant-4 inhibition of polyamine uptake using radiolabelled polyamines, as well as the study of polyamine inhibition of Ant-4 uptake, possibly based on the inherent fluorescence of Ant-4 with flow cytometric analysis, larger quantities of Ant-4 would be needed than are available at present.

In summary, this study indicates that anthracene-polyamine conjugates, particularly Ant-4, elicit a cytotoxic effect against intra-erythrocytic *P. falciparum* parasites in the nM range. This adverse effect was independent of the intracellular polyamine levels and could not be reversed by the addition of exogenous putrescine. While cytotoxicity of Ant-4 against intra-erythrocytic *P. falciparum* parasites is possibly due to the dual effect of both the polyamine moiety as well as the anthracene cargo resulting in interference with DNA replication, alternative modes of action, such as interference with haemozoin formation, remain to be investigated.

Chapter 4: Concluding Discussion

Malaria, caused by parasites of the genus *Plasmodium,* results in approximately 800 000 casualties per year (Kappe *et al.*, 2010) and is endemic in some 99 countries (Feachem *et al.*, 2010). Of these countries, 32 are actively pursuing malaria eradication, while in the rest malaria is controlled by a combination of infection prevention and treatment of clinical cases (Feachem *et al.*, 2010). Effective treatment of malaria infections is under threat due to the increase in resistance against the majority of anti-malarial drugs (Müller and Hyde, 2010).

Polyamines have the ability to interact electrostatically with the majority of polyanionic macromolecules in cells and thereby influence a variety of processes including cell differentiation and proliferation, embryonic development and apoptosis (Heby *et al.*, 2003; Wallace *et al.*, 2003). Increased concentrations and activities of the polyamines and their biosynthetic enzymes, respectively, are observed in highly proliferating cells such as cancerous cells and parasitic organisms (Heby *et al.*, 2003). As such, interference with polyamine metabolism has been proposed as a strategy for preventing cell proliferation, either by preventing intracellular polyamine biosynthesis or by competitively displacing the functional polyamine pool within cells with non-functional polyamine analogues (Casero and Marton, 2007; Müller *et al.*, 2008; Seiler, 2003; Wallace and Niiranen, 2007).

The putrescine biosynthesis inhibitor, DFMO (Eflornithine, Ornidyl^{TM}), is currently the only therapeutically and clinically useful drug shown to prevent proliferation of *T. brucei gambiense* (Bacchi *et al.*, 1990). Contrary to the situation in *T. brucei*, polyamine depletion via biosynthesis inhibition has not met with obvious therapeutic success in treating *P. falciparum* infections (Müller *et al.*, 2008). However, systematic investigations of the polyamine metabolic pathway in intra-erythrocytic *P. falciparum* parasites and characterisation of the proteins involved (Birkholtz *et al.*, 2003; Birkholtz *et al.*, 2004; Burger *et al.*, 2007; Müller *et al.*, 2000; Wells *et al.*, 2006; Wrenger *et al.*, 2001) showed that polyamine biosynthesis in this parasite exhibits several unique aspects, such as a bifunctional arrangement of the two rate-limiting enzymes in the pathway, AdoMetDC and ODC (Wrenger *et al.*, 2001). This bifunctional PfAdoMetDC/ODC enzyme 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). Additionally, intra-erythrocytic *P. falciparum's*

spermidine synthase is uniquely responsible for the production of both spermidine and spermine (Burger *et al.*, 2007; Haider *et al.*, 2005).

The lack of therapeutic success of polyamine biosynthesis inhibition with DFMO in *P. falciparum* parasites has been suggested to be due to the ability of these parasites to take up exogenous polyamines (Müller *et al.*, 2008). The antagonism of growth inhibition by exogenous polyamines (Table 1.2) has been taken as circumstantial evidence for polyamine transport as an alternative to *de novo* synthesis in supplying the polyamine needs of the parasite (Müller *et al.*, 2008). Polyamine uptake has only been studied in iRBCs (Ramya *et al.*, 2006) with limited information available about polyamine uptake by *P. falciparum* parasites themselves. This thesis focused on the characterisation of polyamine uptake by intra-erythrocytic *P. falciparum* trophozoite-stage parasites.

The results indicated for the first time that functionally isolated *P. falciparum* trophozoite-stage parasites are capable of both putrescine and spermidine uptake (section2.3.1). Previously, it was suggested that intra-erythrocytic *P. falciparum* parasites are not capable of spermidine uptake, since exogenously supplied putrescine, but not spermidine, was capable of overcoming biosynthesis inhibition caused by a variety of inhibitors (Das Gupta *et al.*, 2005). However, this work clearly indicates that isolated *P. falciparum* trophozoite-stage parasites not only take up spermidine, but that spermidine uptake occurs at a faster initial rate than putrescine uptake, and that spermidine accumulated to higher levels than putrescine over the time period investigated (section 2.3.1). It is possible that the inhibitors used by Das Gupta *et al.* (2005) were not polyamine enzyme-specific, but rather had additional effects on various enzymes and metabolic processes in the malaria parasite, which are not reversible by spermidine uptake alone.

P. falciparum trophozoites' polyamine uptake characteristics, such as temperature dependence (section 2.3.2.2) were similar to those that have been found for other parasitic organisms such as *Leishmania* spp (Basselin *et al.*, 2000) as well as for *P. falciparum* (Ramya *et al.*, 2006) and *P. knowlesi* (Singh *et al.*, 1997) iRBC. Putrescine, spermidine and spermine all inhibited both putrescine and spermidine uptake (section 2.3.2.5), but it is unclear if this inhibition is due to competition for the same transporter and thus indicates a shared polyamine uptake mechanism. Both single and shared polyamine transporters have been observed in other cells. For instance, AMEL-3 cells have a single transporter for putrescine, spermidine and spermine (García-Fernández *et al.*, 2005), while CHO cells (Xie *et*

al., 1997) and *T. cruzi* parasites (Hasne *et al.*, 2010) have separate transporters for putrescine on the one hand and spermidine and spermine on the other.

The pH dependence of polyamine uptake into isolated *P. falciparum* parasites (section 2.3.2.8), might indicate the presence of an H^+-coupled transport system whereby extracellular polyamines are exchanged for intracellular H^+ in the parasite, similar to what was seen for *Drosophila* S2 cells (Romero-Calderón and Krantz, 2006). Alternatively, since extracellular pH affects the membrane potential for which ATP (derived from glycolysis) is needed as energy source, the pH (section 2.3.2.8) and glucose (section 2.3.2.3) dependence of the initial rate of polyamine uptake might reflect the dependence of polyamine influx on the inward negative membrane potential (~-95 mV) of the intra-erythrocytic *P. falciparum* trophozoite (Allen and Kirk, 2004). The membrane potential dependence of polyamine uptake was confirmed in experiments in which the membrane potential of the parasite was manipulated directly; polyamine uptake in isolated *P. falciparum* parasites increased with membrane hyperpolarisation, and vice versa (section 2.3.2.9). The membrane potential dependence of polyamine uptake appears to be a universal feature for the positively charged polyamines, since in other cells such as *Leishmania* spp. (Basselin *et al.*, 2000), *X. laevis* oocytes (Sha *et al.*, 1996) CHO cells (Soulet *et al.*, 2004) and *E. coli* cells (notably via PotE) (Igarashi and Kashiwagi, 1999) polyamine uptake is also dependent on plasma membrane potential. In *P. falciparum* parasites, the uptake of other positively charged metabolites such as choline (Lehane *et al.*, 2004) and arginine (Cobbold, 2011) is also dependent on the isolated *P. falciparum* parasite's plasma membrane potential. The membrane potential dependence may thus reflect a general strategy for the uptake of positively charged compounds by the *P. falciparum* parasites (Biagini *et al.*, 2005).

One aspect of putrescine and spermidine uptake into *P. falciparum* parasites that was not addressed in this study is the molecular identity of the transporter protein involved. There is substantial heterogeneity between the various polyamine transporters and the mechanisms of polyamine uptake (Table 4.1). Nevertheless, several of the known transporters have been shown to be members of the amino acid/polyamine/organocation (APC) superfamily.

Table 4.1: Polyamine transporter proteins identified in various organisms

Transporter	Description	Species	Reference
Mammalian			
SLC3A2	Solute carrier family, arginine/putrescine exchange, putrescine export	Human	(Uemura *et al.*, 2010; Uemura *et al.*, 2008)
CCC9A	Cation-Cl- cotransporter	Human	(Daigle *et al.*, 2009)
Bacterial			
PotABCD	ABC transporter cassette, recognise spermidine and putrescine	*E. coli*	(Igarashi and Kashiwagi, 1999, 2001)
PotFGHI	ABC transporter cassette, putrescine import	*E. coli*	(Igarashi and Kashiwagi, 1999, 2001)
PotE	APC superfamily, putrescine/ornithine anti-porter, putrescine import	*E. coli*	(Kashiwagi *et al.*, 1986; Kashiwagi *et al.*, 2000)
MdtJI	Small multi-drug resistance (SMR) family	*E. coli*	(Higashi *et al.*, 2008)
Yeast			
TPO1-	Major facilitator superfamily, excretes spermidine and spermine via polyamine/H^+ anti-porter activity	*S. cerevisiae*	(Igarashi and Kashiwagi, 1999; Tomitori *et al.*, 2001)
TPO2	Major facilitator superfamily, excretes spermine	*S. cerevisiae*	(Igarashi and Kashiwagi, 1999; Tomitori *et al.*, 2001)
TPO3	Major facilitator superfamily, excretes spermine	*S. cerevisiae*	(Igarashi and Kashiwagi, 1999; Tomitori *et al.*, 2001)
TPO4	Major facilitator superfamily, excretes spermidine and spermine	*S. cerevisiae*	(Igarashi and Kashiwagi, 1999; Tomitori *et al.*, 2001)
TPO5	Similarity to APC superfamily, excretes putrescine and spermidine	*S. cerevisiae*	(Tachihara *et al.*, 2005)
Agp2p	APC superfamily, spermidine and putrescine import	*S. cerevisiae*	(Aouida *et al.*, 2005)
UGA4,	APC superfamily, putrescine transport	*S. cerevisiae*	(Uemura *et al.*, 2004)
Gap1p	APC superfamily, general amino acid permease, transports putrescine and spermidine	*S. cerevisiae*	(Uemura *et al.*, 2005a)
DUR3	APC superfamily, transports putrescine and spermidine	*S. cerevisiae*	(Uemura *et al.*, 2007)
Parasitic protozoa			
LmPOT1	APC superfamily, transports putrescine and spermidine	*L. major*	(Hasne and Ullman, 2005)
TcPOT1.2	APC superfamily, transports putrescine and spermidine	*T. cruzi*	(Hasne *et al.*, 2010)

The characteristics and signature sequences of the APC superfamily were used to screen the *L. major* genome and led to the identification of a polyamine permease for *L. major* (Hasne and Ullman, 2005) and subsequently, *T. cruzi* (Hasne *et al.*, 2010). We used these signature sequences, including those of the basic amino acid/polyamine anti-porter family (of which Pot E is an example) (Jack *et al.*, 2000), in addition to sequences of polyamine transporters from various organisms, to screen the *P. falciparum* genome in an attempt to

identify potential polyamine transporters of *P. falciparum* parasites. Two possible candidate proteins PFF1430c (Mal6P1.133), a putative amino acid transporter, and PFB0275w, a putative metabolite/drug transporter, were identified (results not shown). Using MEME as a motif discovery tool (Bailey and Elkan, 1994), conserved motifs that were found in TPO1-4 were also identified in these sequences (results not shown).

X. laevis oocytes have been used for the functional expression of various Plasmodial transporter proteins, such as a the hexose transporter, PfHT1 (Krishna *et al.*, 2000), *P. falciparum* equilibrative nucleoside transporter 1 (PfENT1) (Carter *et al.*, 2000; Parker *et al.*, 2000), PfMDR1 (Sanchez *et al.*, 2008a), the putative Ca^{2+}/H^{+} anti-porter PfCHA (Rotmann *et al.*, 2010), PfATP6 (Eckstein-Ludwig *et al.*, 2003) and PfCRT (Martin *et al.*, 2009b), to name a few. However, PFF1430c (S. Cobbold, personal communication) and PFB0275w (this study), identified bioinformatically as potential polyamine transport candidates, exhibited amino acid uptake but not polyamine uptake, after being expressed in *X. laevis* oocytes. The molecular identification of the intra-erythrocytic *P. falciparum* polyamine transport protein/s thus remains an exciting challenge.

P. falciparum parasites are wholly dependent on the uptake of certain metabolites, such as isoleucine (Martin and Kirk, 2007) in order to provide the parasite's metabolic needs. In contrast, although the relative contributions of polyamine uptake and biosynthesis in supplying the polyamine needs of the *P. falciparum* parasite are yet to be elucidated, *P. falciparum* parasites have a fully functional polyamine biosynthesis pathway (Müller *et al.*, 2008). It is thus possible that polyamine uptake may not play a significant role in parasite survival under conditions where there is no pressure on polyamine biosynthesis. In order to determine the contribution of *P. falciparum* parasite's polyamine uptake to parasite survival, the transporter responsible for polyamine uptake would have to be identified and a knock-out created to abolish polyamine uptake. Alternatively, a compound (for example a polyamine analogue) that solely and completely inhibits the uptake mechanism without affecting the activities of the biosynthesis enzymes or any other cellular processes would be needed. By measuring the intracellular polyamine levels (i.e. by HPLC), and parasite survival, following inhibition of polyamine uptake, the contribution of polyamine uptake to the intracellular polyamine pool and parasite survival may then be determined. In the absence of a molecular candidate responsible for polyamine uptake, rational design of such an inhibitor is not possible. Furthermore, as the possibility of some polyamine uptake into *P. falciparum* parasites via diffusion cannot be excluded, the determination of the contribution

of polyamine uptake to polyamine levels in intra-erythrocytic *P. falciparum* and parasite survival may prove to be difficult. Polyamine uptake did increase in isolated *P. falciparum* trophozoites when intracellular polyamine levels were decreased by DFMO inhibition of polyamine biosynthesis (section 2.3.2.6). It is thus possible that the clinical failure of polyamine biosynthesis inhibitors such as DFMO (Müller *et al.*, 2001) against intra-erythrocytic *P. falciparum* parasites is due to increased total polyamine uptake in response to the decreased intracellular polyamine levels during biosynthesis inhibition.

The current study characterised only the uptake component of polyamine transport into intra-erythrocytic *P. falciparum* parasites and thus the characteristics of polyamine export and the contribution (if any) of polyamine export to the maintenance of polyamine levels remains unclear. In mammalian cells, polyamine levels are tightly controlled by biosynthesis, transport, feedback regulation and inter-conversion (Casero and Pegg, 2009; Wallace and Fraser, 2003). In contrast, the pathways for polyamine catabolism and inter-conversion do not exist in *P. falciparum* parasites, as evidenced by the absence of the relevant genes in the genome (Müller *et al.*, 2008). While it has been proposed that the bifunctional nature of *Pf*AdoMetDC/ODC in *P. falciparum* parasites allows 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), it is further possible that the maintenance of polyamine levels in intra-erythrocytic *P. falciparum* parasites are more dependent on polyamine uptake and export than in other cells, making these processes viable anti-malarial targets. The investigation of polyamine export in the parasites is thus an extremely important avenue for further investigation of the regulation of polyamine levels in intra-erythrocytic *P. falciparum* parasites.

It has been suggested that the combined targeting of polyamine biosynthesis and uptake, possibly via a polyamine analogue that targets both, may be successful as a novel anti-malarial strategy (Clark *et al.*, 2010). In HL-60 cells, one such analogue, an anthracene-putrescine conjugate, was found to reduce both putrescine uptake and intracellular polyamine levels, thereby acting as a polyamine anti-metabolite (Palmer *et al.*, 2009). The effect of this and related anthracene-polyamine conjugates on intra-erythrocytic *P. falciparum* parasites was subsequently tested. In Chapter 3, it was shown that anthracene-polyamine conjugates caused inhibition of parasite proliferation with IC_{50} values in the nanomolar range (section 3.3.1). The anthracene-putrescine conjugate did not, however, act as a polyamine anti-metabolite as was observed in HL-60 cells (Palmer *et al.*, 2009),

since intracellular polyamine levels and the activities of the bifunctional enzymes were not affected (section 3.3.4). Rather, in intra-erythrocytic *P. falciparum* parasites, Ant-4 apparently acts as a polyamine mimetic, and the possibility that the polyamine moiety targets the anthracene moiety to its site of action cannot be excluded (Palmer *et al.*, 2009). The anthracene-polyamine conjugate interferes with affects the mitochondrial membrane potential, and interferes with DNA replication and prevents nuclear division (section 3.3.7). The exact mode of action, however, still has to be determined. Since intra-erythrocytic *P. falciparum* daughter cell formation (and progression through the cell cycle) is dependent on DNA replication as nuclear division precedes cytogenesis (Arnot *et al.*, 2011), interference with DNA replication is be a viable anti-malarial strategy. The promising anti-malarial effect of Ant-4 is confirmed by recent evidence of Ant-4 increasing the survival of mice infected with *P. berghei* (Anette Kaiser, personal communication).

The high sensitivity of intra-erythrocytic *P. falciparum* parasites to Ant-4, relative to that seen for other cell lines (including cancerous cells) (Table 3.1), may be more pronounced due to additional effects of Ant-4 in the parasite not seen in mammalian cells. In intra-erythrocytic *P. falciparum* parasites, a series of 9-anilinoacridine inhibitors has been shown to inhibit both *P. falciparum* topoisomerase II, (Auparakkitanon and Wilairat, 2000) as well as β-haematin formation (Auparakkitanon *et al.*, 2003). Anthracene-polyamine conjugates inhibit human topoisomerase II activity (Wang *et al.*, 2001) and Ant-4 may similarly inhibit *P. falciparum* topoisomerase II. Inhibition of topoisomerase II is a viable drug strategy, with the topoisomerase II inhibitor amsacrine (AMSA) used for the treatment of acute non-lymphocytic leukaemia (Phanstiel *et al.*, 2000). Further studies on the effect of Ant-4 on both haemozoin formation and topoisomerase II activity would be needed to confirm this hypothesis. As chloroquine also inhibits hemozoin formation, (Müller and Hyde, 2010) it would be interesting to compare Ant-4 IC_{50} values in chloroquine resistant and chloroquine CQ sensitive *P. falciparum* lines. Furthermore, quinoline-resistance reversal by compounds such as verapamil and primaquine have provided information on the characteristics of resistance reversers, namely planar rings, a positive charge at the pH of the digestive vacuole and the presence of a nitrogen atom (van Schalkwyk and Egan, 2006). Based on these characteristics, Ant-4 may also act as a quinoline resistance reverser as it satisfies these requirements. The comparison of combined treatment of Ant-4 and chloroquine against chloroquine resistant intra-erythrocytic *P. falciparum* parasites would be needed to test this hypothesis.

Polyamine transport systems have been shown, particularly in mammalian cells, to have a broad substrate specificity, allowing not only transport of the natural polyamines but also of related compounds This structural tolerance and a promiscuous nature of polyamine transport have also been described in several tumour cell lines (Palmer *et al.*, 2009; Phanstiel *et al.*, 2007; Wang *et al.*, 2003a). For instance, the anthracene-polyamine conjugates used in Chapter 3 were specifically designed to utilise the polyamine uptake mechanism(s) of cells for entry into cells (Phanstiel *et al.*, 2007; Wang *et al.*, 2003a; Wang *et al.*, 2003b). In Chapter 2 it was found that isolated *P. falciparum* parasites are capable of taking up both putrescine and spermidine, with uptake inhibited by (and possibly competed for) a variety of structurally similar compounds (section 2.3.2.5); this might indicate a structural tolerance similar to that seen in cancer cells (Palmer and Wallace, 2010). Preliminary data suggested that the anthracene-polyamine compounds may utilise the *P. falciparum* parasites' polyamine uptake mechanism for entry (section 3.3.4), but more in-depth uptake investigations would be needed to assess whether this is indeed the case. However, the exploitation of the polyamine uptake mechanism to deliver cytotoxic cargo to *P. falciparum* parasites remains enticing. A similar strategy has been proposed in *P. falciparum* parasites using choline uptake to mediate the uptake of the cytotoxic compounds (Biagini *et al.*, 2005). For example, the bisthiazolium choline analogue T16 is thought to enter the parasite via the parasite's choline transporter, and to cause parasite death by interfering with phosphatidylcholine metabolism (Ancelin *et al.*, 1998; Richier *et al.*, 2006). The increased polyamine uptake of cancerous cells compared to normal mammalian cells (Seiler *et al.*, 1996) provides a way to selectively target cancer cells through the use of polyamines conjugated to cytotoxic moieties such as anthracene (Palmer *et al.*, 2009). Similar selectivity for intra-erythrocytic *P. falciparum* parasites may be obtained through the choice of cytotoxic moiety. For instance, in intra-erythrocytic *P. falciparum* parasites, DNA unwinding occurs via both topoisomerase II, as well as a bacterial-derived, apicoplast located DNA gyrase (García-Estrada *et al.*, 2010). By conjugating inhibitors specific for *P. falciparum* DNA gyrase (such as the fluoroquinolones and amino coumarins effective against bacterial DNA gyrase (García-Estrada *et al.*, 2010)) to putrescine for possible intracellular targeting, cytotoxic effects similar to Ant-4 may be achieved, but without host toxicity. Furthermore, from other studies investigating polyamine conjugation as a possible method for cell delivery via the polyamine uptake mechanism, it seems as if the anti-malarial compounds needn't be constrained to those that interfere with DNA replication. For example, a range of artemisinin-spermidine conjugates, synthesized to exploit the polyamine

uptake mechanism of HL-60 cells and intra-erythrocytic *P. falciparum* parasites, exhibited ~10-40-fold lower IC_{50} values than artemisinin alone (Chadwick *et al.*, 2010).

In conclusion, this study showed that isolated *P. falciparum* parasites are capable of putrescine and spermidine uptake, and that this is dependent on the parasite's plasma membrane potential. Anthracene-polyamine conjugates (specifically designed to target polyamine uptake mechanism(s) of mammalian cells) inhibited intra-erythrocytic *P. falciparum* parasites' proliferation without affecting the intra-cellular polyamine levels, thus acting as polyamine mimetics.

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