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.