Functional consequences of perturbing polyamine metabolism in the malaria parasite, *Plasmodium falciparum*

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Running title: Polyamines in plasmodia

Abstract

Inhibition of polyamine biosynthesis and/or the perturbation of polyamine functionality have been exploited with success against parasitic diseases such as *Trypanosoma* infections. However, when the classical polyamine biosynthesis inhibitor, α-difluoromethylornithine, is used against the human malaria parasite, *Plasmodium falciparum*, it results in only a cytostatic growth arrest. Polyamine metabolism in this parasite has unique properties not shared by any other organism. These include the bifunctional arrangement of the catalytic decarboxylases and an apparent absence of the typical polyamine interconversion pathways implying different mechanisms for the regulation of polyamine homeostasis that includes the uptake of exogenous polyamines at least *in vitro*. These properties make polyamine metabolism an enticing drug target in *P. falciparum* provided that the physiological and functional consequences of polyamine metabolism perturbation are understood. This review highlights our current understanding of the biological consequences of inhibition of the biosynthetic enzymes in the polyamine pathway in *P. falciparum* as revealed by several global analytical approaches. Ultimately, evidence suggests that polyamine metabolism in *P. falciparum* is a validated drug target worth exploiting.

Keywords:
Polyamines, malaria, *Plasmodium*, parasites, functional genomics, difluoromethylornithine

Abbreviations:
ADA, adenosine deaminase; AdoDATO, S-adenosyl-1,8-diamino-3-thiooctane; AdoHC, S-adenosylhomocysteine; AdoMet, S-adenosylmethionine; AdoMetDC, S-adenosylmethionine decarboxylase; AdoMetSyn, S-adenosylmethionine synthase; AHC, S-adenosyl homocysteinase; APA, 3-aminooxy-1-aminopropane; APE, 5-amino-1-pentene; CHA, cyclohexylamine; CQ, chloroquine; dcAdoMet, decarboxylated S-adenosylmethionine; DFMO, α-difluoromethylornithine; eIF5A, eukaryotic translation initiation factor 5A; EMP, erythrocyte plasma membrane; HC: homocysteine, HS: homospermidine, LDC, lysine decarboxylase; 4-MCHA, trans-1,4-methylcyclohexylamine; MDL73811/AbeAdo, 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine; MDL27695, N,N'-bis{3-[(phenylmethyl)aminolpropyl}-1,7-diaminoheptane; MetSyn, methionine synthase; MGBG, methylglyoxal bis(guanylhydrazone); MTA, 5'-methylthioadenosine; MTI, 5'-methylthioinosine; MTRP, 5'-methylthio-D-ribose-1-phosphate; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; PNP, uridine phosphorylase; PPM, parasite plasma membrane; PVM, parasite vacuolar membrane; SpdSyn, spermidine synthase,

Polyamines as antiproliferative targets.

Various constituents control the fate of a cell as it progresses through cellular development amongst which low-molecular weight nitrogenous bases, known as the polyamines, are essential (and near ubiquitous) metabolites enabling well regulated cell growth and development. Due to their polycationic nature, the polyamines, including the diamines putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopropane) as well as the triamine putrescine derivative spermidine (N-(3-aminopropyl)-1,4-diaminobutane) and the tetra-amine derivative spermine (N,N1-bis(3-aminopropyl)-1,4-butanediamine), 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 (reviewed in e.g. (Heby et al., 2003; Wallace et al., 2003). As a consequence, increased concentrations of the polyamines and their biosynthetic enzymes are observed in highly proliferating cells such as cancerous cells and parasitic organisms (Heby et al., 2003). This has led to proposing polyamine metabolism as a drug target by either preventing intracellular polyamine biosynthesis or by competitively displacing the functional polyamine pool within cells with non-functional polyamine analogues (Seiler, 2003a; Seiler, 2003b; Casero et al., 2007; Wallace et al., 2007; Müller et al., 2008). In the search for anti-tumour therapeutic intervention, inhibitors to almost all the biosynthetic enzymes have been successfully designed and tested (Marton et al., 1995; Casero et al., 2007). The vast numbers of anti-tumour drugs thus produced are a rich source of potential drugs or drug leads that could be applied against other etiologic agents like rapidly proliferating parasites (Müller et al., 2001; Heby et al., 2003; Heby et al., 2007). Malaria (caused by *Plasmodium* species), west African sleeping sickness (*Trypanosoma brucei gambiense*), and leishmaniasis (*Leishmania donovani*) amongst others, are global parasitic diseases and in all of these has polyamine metabolism been identified as a drug target (Müller et al., 2001; Heby et al., 2007). Importantly, the biosynthesis inhibitor α-difluoromethylornithine (DFMO, Eflornithine, Ornidyl^{TM}) is currently the only therapeutically and clinically useful drug shown to potently prevent proliferation of *T. brucei gambiense*.

Polyamine metabolism in *Plasmodium*.

Around 40% of the world's population in 107 countries live under the constant risk of malaria infection, with an estimated 350-500 million clinical malaria episodes occurring annually. The scale of the malaria problem serves to emphasise the fragile nature of prevailing control programmes and the importance of developing more effective methods for the eradication of malaria. Malaria pathogenesis is exhibited during the asexual intraerythrocytic cycle of *Plasmodium falciparum* in the human host where the parasite replicates during schizogony from ring and trophozoite stages to mature merozoite-releasing schizonts.

Polyamines have been shown to be the major metabolite present within malaria parasites, highlighting the importance of these molecules to these rapidly dividing parasites (Teng et al., 2008). At a total concentration of 10 mM inside the parasites, free, unbound polyamines make up 14% of the total *Plasmodium* metabolome. In contrast, human erythrocytes contain only trace amounts (5-50 µM) of polyamines (Gugliucci, 2004; Olszewski et al., 2009) and lack the necessary polyamine biosynthetic enzymes. Serum polyamine levels only amount to 0.1 µM. However, there is a significant increase in polyamine levels in *P. falciparum*-infected erythrocytes during the development of the parasite from trophozoite to schizont

stages, proportional to increased parasitaemia, the increased activities of the polyamine biosynthetic enzymes as well as increased macromolecular biosynthesis and replication (Assaraf et al., 1984; Das Gupta et al., 2005). Spermidine is the major metabolite present in all stages of the parasite at maximally 6 mM, followed closely by its precursor, putrescine (3 mM) with only trace amounts of spermine (0.5 mM) (Das Gupta et al., 2005; Teng et al., 2008) (Fig. 1). Polyamine metabolism has also been closely linked to malaria pathogenesis and host-parasite interactions during a severe *P. falciparum* infection resulting in hypoarginineamia (Olszewski et al., 2009).

Systematic investigations of the polyamine metabolic pathway in *P. falciparum* and characterization of the involved proteins (Müller et al., 2000; Wrenger et al., 2001; Birkholtz et al., 2003; Birkholtz et al., 2004; Müller et al., 2005; Wells et al., 2006; Burger et al., 2007) have indicated that polyamine biosynthesis in this parasite exhibits several unique aspects (Müller et al., 2001; Müller et al., 2008). Polyamine metabolism in *P. falciparum* is less complex compared to the scenario in the human host (Fig. 1). The synthesis of polyamines is initiated by the production of the precursor, ornithine, from arginine through the sole activity of arginase. Subsequent synthesis of putrescine and spermidine in the parasite is respectively dependent on the activities of ornithine decarboxylase (ODC, EC 4.1.1.17) and spermidine synthase (SpdSyn, EC 2.5.1.16). S-adenosylmethionine decarboxylase (AdoMetDC, EC 4.1.1.50) provides activated aminopropyl donor, decarboxylated S-adenosylmethionine (dcAdoMet), for the synthesis of spermidine. No spermine synthase activity has been described in plasmodia, but uniquely, SpdSyn is able to produce low levels of spermine (Haider et al., 2005). Most importantly and interestingly, the two rate-limiting decarboxylase activities are found in a bifunctional arrangement in a single protein complex (AdoMetDC/ODC), a property unique to plasmodia (Müller et al., 2000). No evidence for interconversion of polyamines to their acetylated intermediates for catabolism could be shown thus far compared to other organisms (Seiler, 2004) and regulatory mechanisms described for ODC and AdoMetDC activities in other organisms (including antizyme and prozyme, respectively), are absent in *P. falciparum*. However, putrescine has regulatory functions resulting in the feedback inhibition of ODC with no stimulation of AdoMetDC activity (Wrenger et al., 2001; Wells et al., 2006). It has therefore been proposed that the control of polyamine metabolism in this parasite is mediated through the coordinated expression of the bifunctional AdoMetDC/ODC enzyme and regulation of the associated activities. However, metabolic flux appears to be dependent on the bifunctional decarboxylase, SpdSyn as well as polyamine transport mechanisms.

Beyond these obvious enticing differences between polyamine metabolism of humans and *P. falciparum*, polyamine depletion has not met with obvious therapeutic success in malaria. In contrast to the situation in trypanosomes, polyamine depletion in *P. falciparum* only results in cytostatic arrest of the parasite, reversible by the exogenous *in vitro* provision of some polyamines (Assaraf et al., 1987). However, while essential trypanothione synthesis as well as methylation potential in trypanosomes seems influenced by polyamines, this relationship seems to be absent in plasmodia and has been proposed as a possible reason for the therapeutic failure of polyamine depletion in malaria parasites (van Brummelen et al., 2009a). Details of the functional consequences of polyamine metabolism perturbation in the malaria parasite, and the unique regulatory mechanisms that exists to exquisitely control polyamine

homeostasis in *P. falciparum*, warrant further clarification and will be discussed below.

Inhibition of AdoMetDC/ODC in *P. falciparum*

The bifunctional AdoMetDC/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 antimalarial targets according to the World Health Organisation's Tropical Diseases Research Targets Database (http://tdrtargets.org). Most inhibitory studies thus far, targeted the decarboxylase activities individually. Inhibition of either ODC or AdoMetDC of *P. falciparum* alone causes cytostatic arrest *in vitro* in the trophozoite stage of the parasite schizogony in erythrocytes but does not cure *P. berghei*-infected mice *in vivo* (Assaraf et al., 1987; Bitonti et al., 1987; Wright et al., 1991).

DFMO has proved to be the most promising ODC inhibitor since its development in 1978 (Metcalf et al., 1978), and as a mechanism-based suicide analogue of ornithine, effectively inhibits this enzyme in *P. falciparum* with a Ki of 87.6 µM, more than double that of the mammalian ODC (Krause et al., 2000). However, with an *in vitro* IC_{50} of ~1 mM (Krause et al., 2000; Das Gupta et al., 2005) (Table 1), DFMO treatment of *P. falciparum* only results in a cytostatic block in the transformation from trophozoites to schizonts (Assaraf et al., 1987) and effectively synchronises the parasites (Assaraf et al., 1986). Moreover, DFMO in combination with the common antimalarial, chloroquine (CQ), did not show an additive effect on CQ sensitive *P. falciparum* strains (Das et al., 1995).

The functional consequence of DFMO-mediated inhibition of *P. falciparum* and the proposed mechanism-of-action has been the depletion of putrescine levels (>98.5% decrease) and a >80% decrease in spermidine levels (Assaraf et al., 1987; Wright et al., 1991; Das Gupta et al., 2005). Changes in spermine levels range from largely unchanged (Assaraf et al., 1987; Wright et al., 1991) to a 2-fold increase (Das Gupta et al., 2005). This increase supports the finding that SpdSyn can use spermidine as substrate, and in the absence of putrescine, SpdSyn seems to be stimulated towards spermine synthesis. However, alternative mechanisms including an enhanced spermine uptake mechanism or release of spermine from endogenously sequestered forms may exist.

The cytostatic (as opposed to cytotoxic) nature of ODC inhibition by DFMO in *in vitro P. falciparum* has been proposed to be due to the ability of the parasite to take up exogenously provided polyamines to replenish intracellular pools. Intraerythrocytic *P. falciparum* is enclosed by the parasite plasma membrane (PPM), the parasitophorous vacuolar membrane (PVM) and the erythrocyte plasma membrane (EPM), all of which have to be transversed to access the parasite (Fig. 1). Evidence to date suggests that putrescine enters the infected erythrocyte by exploiting the endogenous polyamine uptake mechanism of erythrocytes. Once inside the parasitophorous vacuole, putrescine is taken up by *P. falciparum* against the concentration gradient in an electrogenic process that is mediated by the membrane potential (Fig. 1). DFMO-mediated inhibition of polyamine biosynthesis results in a decreased concentration gradient for putrescine and stimulates putrescine uptake (J. Niemand, unpublished data). A polyamine transporter has not yet been identified in *P. falciparum*, but putrescine transport in *P. knowlesi* has been characterized (Singh et al., 1997). With this transport ability in mind, evidence suggests that the inhibition of ODC activity can be reversed with exogenous putrescine (0.5-1 mM) (Das Gupta et al., 2005) with

some evidence for spermidine (0.25 mM) (Assaraf et al., 1987) as well as against spermidine reversal (Bitonti et al., 1987), but no reversal by spermine. Moreover, the diamine cadaverine (0.4 mM) could also elicit partial reversal of DFMO inhibition of ODC (Assaraf et al., 1987). The physiological importance of the uptake of putrescine (and conflicting reversal by the other polyamines) and consequent reversal of DFMO-mediated inhibition of *P. falciparum* remains questionable since the available polyamines in serum is orders less than the *in vitro* concentrations (~1 mM) used to reverse inhibition. However, as polyamine metabolism and homeostasis is more complex in other organisms than in plasmodia, the increase in polyamine transport under conditions of biosynthesis inhibition, or the release of sequestered polyamines from intracellular systems like vacuoles, probably plays a significant role in regulating homeostasis in these parasites.

Hepatic schizogony of *P. falciparum* is effectively blocked by DFMO (Hollingdale et al., 1985). Moreover, DFMO protected mice from malaria sporozoite infection by blocking exoerythrocytic schizogony (Hollingdale et al., 1985). The sporogonous cycle of *P. berghei* is also interrupted by DFMO in *Anopheles* mosquito vectors (Gillet et al., 1983). However, the treatment of mice already infected by blood stage *P. berghei* with DFMO alone had little effect on *P. berghei* erythrocytic schizogony and does not increase the survival time of infected mice (Bitonti et al., 1987). These conflicting results obtained with DFMO as only a cytostatic agent against erythrocytic stages of plasmodia but effective inhibitor of hepatic stages and sporozoites, implicate either differences in the uptake of the drug in different life cycle stages of these parasites or otherwise unknown regulatory mechanisms.

New generation ODC inhibitors, 3-aminooxy-1-aminopropane (APA) and derivatives CGP 54169A and CGP 52622A have also been shown to arrest *P. falciparum* at the trophozoite stage *in vitro* (Das Gupta et al., 2005) (Table 1). These structural putrescine analogues all have Ki values in the low nM range due to parasite-specific interactions with ODC (Birkholtz et al., 2003) and are >600 times more potent than DFMO *in vitro* (Das Gupta et al., 2005). All of these inhibitors decreased putrescine and spermidine levels but again result in increased spermine concentrations and could be antagonised with exogenous addition of putrescine (0.5 mM) (Assaraf et al., 1987). As putrescine analogues, these inhibitors might exploit the putrescine uptake mechanism to gain entry into *P. falciparum* and in this manner be more effective than the ornithine analogue, DFMO.

AdoMetDC catalyses a chokepoint reaction due to its production of decarboxylated S-adenosylmethionine (dcAdoMet) (Yeh et al., 2004). One of the first AdoMetDC inhibitors, methylglyoxal bis(guanylhydrazone) (MGBG) prevented *P. falciparum* growth *in vitro* (Heidrich et al., 1983). Second generation inhibitors, resembling the substrate molecule AdoMet, include the irreversible inhibitor 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine (MDL73811 or AbeAdo), which arrests both CQ sensitive and resistant *P. falciparum* in the trophozoite stage of development (Wright et al., 1991). At a Ki of 1.6 µM and with an *in vitro* IC_{50} of 1-3 µM between CQ sensitive and resistant parasites, MDL73811 is 1000-fold more potent than DFMO (Wright et al., 1991; Das Gupta et al., 2005) (Table 1). However, the use of MDL73811 alone was not effective in curing mice infected with *P. berghei* (Wright et al., 1991), possibly due to rapid clearance of the drug (plasma half-life of only 10 min). Further inhibitory studies, based on the bis(benzylhydrazone) pharmacophore, identified CGP40215A as the most potent with an IC_{50} value of 1.8 µM (Das Gupta et

al., 2005) (Table 1). This compound was shown to have widespread antiprotozoal activity against trypanosomes and *Leishmania*, possibly due to its structural similarity to diamidines (Das Gupta et al., 2005).

The functional consequences of AdoMetDC inhibition have been diverse and inhibitor specific. CGP40215A, although potent, did not perturb the intracellular polyamine concentrations in *P. falciparum*, suggesting off-target effects beyond AdoMetDC inhibition (Das Gupta et al., 2005). Polyamine-specific targeting was observed with MDL73811 treatment, where a 3-fold upregulation of putrescine concentrations were observed. Moreover, spermidine concentrations were ~85% decreased and spermine levels were ~93% decreased after extended drug pressure (Wright et al., 1991; Das Gupta et al., 2005). MDL73811 inhibition cannot be reversed by the exogenous addition of putrescine or adenosine (Wright et al., 1991; Das Gupta et al., 2005), again with contrasting results for reversal with 250 µM of spermidine or spermine (Wright et al., 1991) as well as against (Das Gupta et al., 2005), leading to speculations of poor uptake mechanisms for spermidine in the parasite. However, recent data indicate that the parasites are able to take up spermidine at both a higher rate than putrescine, as well as to higher levels (J. Niemand, unpublished data). The mechanism of MDL73811 has also been proposed to be related to the accumulation of AdoMet in addition to polyamine depletion, leading to a hypermethylated state in trypanosomes as the major functional explanation of the therapeutic success of MDL73811 in these parasites (Byers et al., 1991; Bacchi et al., 1992). However, MDL73811 treatment of *P. falciparum* did not lead to increased levels of AdoMet and neither could a hypermethylation status be detected in these parasites after AdoMetDC inhibition (Reeksting, 2009; van Brummelen, 2009b).

Most studies focussed solely on the inhibition of the individual decarboxylase domains of the bifunctional AdoMetDC/ODC. However, even though both activities can function independently of each other (Wrenger et al., 2001), experimental evidence suggests allosteric regulation of the individual domains by interdomain interactions, most likely influenced by the prevailing metabolite concentrations (Birkholtz et al., 2004). Since both of these activities reside on a single bifunctional polypeptide, it may be an important contributor towards the regulation of polyamine metabolism in malaria. Co-inhibition of the bifunctional protein with DFMO and MDL73811 indicated a non-synergistic but additive effect due to the bifunctional nature of the protein (Assaraf et al., 1987; Wright et al., 1991; van Brummelen, 2009b) whereas the co-treatment of trypanosomes results in a synergistic action (Bacchi et al., 1992). Both decarboxylase activities of AdoMetDC/ODC in *P. falciparum* were depleted after the simultaneous addition of these drugs, indicating their complete and irreversible action (van Brummelen, 2009b). The functional consequence of the co-inhibition of both decarboxylases is the depletion of putrescine and spermidine (10% residual for the latter) in the parasite with no significant change in spermine levels (Reeksting, 2009; van Brummelen et al., 2009a). Downstream metabolites dependent on the presence of polyamines as precursor, 5'-methylthioadenosine (MTA) and 5'-methylthioinosine (MTI), were also depleted (Reeksting, 2009; van Brummelen et al., 2009a). The co-inhibition of both of these activities is therefore more interesting from a therapeutic point of view.

Inhibition of SpdSyn in *P. falciparum*.

Investigations of SpdSyn as drug target in *P. falciparum* have not been as extensive as those of AdoMetDC/ODC. Cyclohexylamine analogues (CHA) have been

investigated as inhibitors of the aminopropyltransferase activity of SpdSyn by competitively binding to the putrescine binding pocket. Investigations of CHA derivatives 4-MCHA (trans-1,4-methylcyclohexylamine), dicyclohexylamine as well as the ODC inhibitor APA and its derivative, 5-amino-1-pentene (APE), revealed 4-MCHA to exhibit the best *in vitro* growth IC_{50} at 34 µM (Table 1). Moreover, 4-MCHA was also the most potent direct inhibitor of SpdSyn activity with a Ki of 0.18 µM (Haider et al., 2005). APA seems to be a more potent inhibitor of ODC activity and only inhibits SpdSyn at an IC_{50} of 84 µM. Unfortunately, administration of 25 mg/kg of 4-MCHA to mice infected with *P. berghei* had no effect on parasitic proliferation or survival of the mice (Müller et al., 2008), possibly due to assimilation of 4-MCHA in the host organism. Multi-substrate analogues, which resemble both the dcAdoMet and putrescine transition state, have remarkably good binding character. S-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) was reported to have potent inhibitory activity against SpdSyn and an IC_{50} of 8.5 µM (Dufe et al., 2007) (Table 1).

The functional consequence of SpdSyn inhibition by CHA is a significant decrease in both spermidine and spermine levels, accompanied by a 4-fold increase in putrescine levels (Kaiser et al., 2001; Becker et al., submitted). Reports on the antagonism of the inhibition of SpdSyn are controversial, with some (but not complete) reversibility of CHA affected with spermidine (Kaiser et al., 2001). Neither putrescine nor spermidine (500 µM) could antagonise APE and 4-MCHA inhibition of SpdSyn. However, the specific ability of 4-MCHA to target only SpdSyn has not been proven and off-target effects cannot be excluded.

Polyamine analogues active against *P. falciparum*.

The most promising and enticing evidence of polyamine metabolism as drug target in plasmodia is based on the use of not only direct enzyme-based inhibitors aimed at polyamine biosynthesis inhibition and polyamine depletion, but also from the effects of replacement of the functional polyamine pool within the parasites with polyamine analogues. Bitonti *et al*. proved that a bis(benzyl) polyamine analogue, N,N'-bis{3-[(phenylmethyl)aminolpropyl}-1,7-diaminoheptane (MDL27695), rapidly inhibits growth in both CQ sensitive and resistant *P. falciparum* strains *in vitro* (Bitonti et al., 1989). The combined use of MDL27695 and CQ on drug sensitive *P. falciparum in vitro* is additive and enhances CQ action (Das et al., 1995). As clear polyamine depletion was not observed and exogenously supplied polyamines could not rescue MDL27695-treated parasites, mechanistically different effects of this polyamine analogue beyond polyamine biosynthesis inhibition are implicated. The administration of MDL27695 in combination with DFMO however, cures murine malaria caused by *P. berghei* and these mice were immune to re-challenge with sporozoites (Bitonti et al., 1989). The polyamine analogue caused inhibition of DNA, RNA and at a later stage also protein synthesis and this disruption of macromolecular biosynthesis resulted in cell death. Unfortunately, although promising, this polyamine analogue was never further investigated due to possible hepatotoxic properties (L Marton, personal communication) but could be exploited as a pharmacophore for rational drug design (de Beer et al., 2009).

Recently, new polyamine analogues aimed specifically at antiparasitic actions have been synthesised. These include polyamine amides of N-methylanthranilic acid that mimic the charge perspective of natural polyamines and exploit the parasite's polyamine transport mechanism to gain entry into the cell (Geall et al., 2004). Although interesting from the perspective of a molecular tool to probe polyamine

transport, since the spermine analogues effectively accumulated within *P. falciparum*, the therapeutic use of these fluorescent compounds is not clear. Chemical library screening of diamine derivatives has proven some activity against plasmodia at IC_{50} values 0.68 µM provided these derivatives contained a benzyloxy group (Labadie et al., 2004) (Table 1). Selectivity could not be indicated but antiparasitic activity was confirmed in *Leishmania*. Several of a series of 1,3,5-triazine-substituted polyamines have also shown inhibitory activity in the low µM range, especially against CQ resistant *P. falciparum* (Klenke et al., 2003). In none of these studies were the functional consequences investigated and it remains to be seen if there is a direct perturbation of polyamine metabolism.

Functional consequences of *P. falciparum* polyamine depletion from a global perspective.

In a scenario such as that seen with the polyamines, where no single function can be ascribed to these molecules and diverse cellular processes including stabilisation of DNA, RNA, nucleoside triphosphates (e.g. ATP), phospholipids and proteins are affected by them, determining their exact function using reductionist biological approaches may be uninformative. On the other hand, the application of integrative global analyses systems like functional genomics investigations may yield insight into the basic biological processes governed by the polyamines, as well as provide global responses and mechanism-of-action predictions of inhibitors aimed at perturbing polyamine metabolism (Montanez et al., 2007; Birkholtz et al., 2008).

Beyond polyamine depletion as a direct functional consequence of DFMO treatment, the involvement of polyamines in macromolecular synthesis was also implicated by the concomitant decrease observed in the synthesis of a subset of proteins, partial inhibition of RNA synthesis and total inhibition of DNA synthesis (Assaraf et al., 1987). Functional genomics investigations of polyamine metabolism perturbed *P. falciparum* have provided clear evidence of the functional consequences of inhibition of AdoMetDC/ODC and SpdSyn beyond the ascribed polyamine depletion. In the absence of polyamines, the cytostatic arrest seen in trophozoites in *P. falciparum* was demonstrated to be due to a massive transcriptional arrest in the parasite (van Brummelen et al., 2009a). This arrest results in a transcriptional signature that is characteristic of polyamine-specific perturbations due to inhibition of either ODC with DFMO (Clark et al., 2008) or AdoMetDC with MDL73811 (S Smit, unpublished data), their co-inhibition with both drugs (van Brummelen et al., 2009a) or the targeting of SpdSyn with CHA (Becker et al., submitted). Prior to the synthesis of these proteins (peaking in the early trophozoite stage parasites) and the onset of polyamine biosynthesis, morphological and functional genomics analyses of ring stage parasites did not indicate any off-target effects of these drugs. Analyses of the arrested transcriptomes of polyamine-depleted parasites compared to parasites perturbed metabolically or with other antimalarials, showed that polyamine-specific perturbations cluster tightly and provide a polyamine-specific signature profile not seen under any other conditions (van Brummelen, 2009b).

Inhibition of the individual decarboxylases of the complete bifunctional AdoMetDC/ODC results in the induction of presumably compensatory responses in the cytostatic, polyamine-depleted environment. A downregulation of the transcript for AdoMetDC/ODC itself is seen when the protein is inhibited (van Brummelen et al., 2009a) but not when SpdSyn is inhibited (Becker et al., submitted). These results suggest the presence of a molecular signal pertaining to the depletion of putrescine

and spermidine in the first situation, but an increase in putrescine and decrease in spermidine in the latter, for the auto-regulation of bifunctional protein production. This could be indicative of intricate feedback regulatory mechanisms to control the synthesis of the bifunctional decarboxylase. In contrast, it does not seem as if such regulatory mechanisms exist for the control of SpdSyn production, since SpdSyn transcript or protein levels are unchanged in a polyamine-depleted environment.

As a result of ODC or AdoMetDC/ODC inhibition, but seemingly not for AdoMetDC alone or SpdSyn inhibition, there is a marked increase in ornithine aminotransferase (OAT) transcript and protein levels (Fig. 1). It would be expected that inhibition of the ODC domain of AdoMetDC/ODC would result in an increase in the levels of its substrate, ornithine, that may be toxic to the parasite (Ueda et al., 1998). However, the increase in OAT levels could result in the catabolism of ornithine to glutamate-5'-semialdehyde to effectively ensure homeostatic control of ornithine levels (van Brummelen et al., 2009a). Moreover, the export of high levels of ornithine under conditions of polyamine depletion cannot be excluded (Olszewski et al., 2009).

Cadaverine, with functions similar to the other polyamines, has been shown to reverse the DFMO-mediated inhibition of ODC in *P. falciparum* (Assaraf et al., 1987), is able to sustain growth of ODC mutant *L. donovani* (Jiang et al., 1999) and has been described as a physiologically important polyamine in plants and bacteria (Gamarnik et al., 1991; Kim et al., 2006). Cadaverine is produced through the decarboxylation of lysine by lysine decarboxylase (LDC). In all instances of polyamine depletion in *P. falciparum* (targeting AdoMetDC/ODC or SpdSyn), a marked induction and upregulation of LDC transcripts was observed in the parasite (van Brummelen et al., 2009a; Becker et al., submitted). This protein has previously and erroneously been described as an alternative ODC essential to sporogony in *P. berghei*, which is not affected by DFMO. However, functional analyses of the *P. falciparum* ortholog indicated that this protein is a 280 kDa protein with specific activity against lysine, and would not metabolise arginine or ornithine (Müller et al., 2008). Neither the protein nor its activity is detectable under normal cellular conditions and the functional significance of the production of cadaverine under conditions of polyamine depletion in *P. falciparum* remains to be confirmed (van Brummelen, 2009b). AdoMetDC/ODC co-inhibition resulted in the first report of the potential role of LDC in the polyamine metabolism of protozoa (van Brummelen et al., 2009a).

Inhibition of either AdoMetDC/ODC or SpdSyn has revealed the downstream pathways that are dependent on the presence of polyamines. There is metabolic shut-down in the production of MTA and MTI due to the downregulation of the production of adenosyl deaminase as well as uridine phosphorylase (Reeksting, 2009; van Brummelen et al., 2009a; Becker et al., submitted) (Fig. 1). Ultimately, this contributes to a decreased production of methionine and thus interference with methionine recycling. A marked downregulation is observed in AdoMet synthetase transcript and protein levels when the AdoMetDC component of the bifunctional AdoMetDC/ODC is targeted specifically but not when SpdSyn is inhibited. Decreased AdoMet synthetase levels subsequently contribute to the homeostatic control of AdoMet levels in *P. falciparum* (Reeksting, 2009; van Brummelen et al., 2009a). Thus, in contrast to the situation in trypanosomes where increased AdoMet levels leads to hypermethylation, there appears to be no hypermethylated state in *P. falciparum* after inhibition of AdoMetDC. S-adenosylhomocysteine:AdoMet ratios are unchanged and no evidence for hypermethylated DNA is observed (Reeksting,

2009; van Brummelen, 2009b). There is also no evidence of a feedback inhibition of AdoMet synthetase activity by AdoMet itself (Müller et al., 2008), but it is clear that the levels of the protein are controlled by polyamine concentrations (decreased putrescine signals) and/or AdoMet levels. The lack of hypermethylation therefore could explain the therapeutic failure of MDL73811 in plasmodia, as this is proposed as the major cause of toxicity in trypanosomes (Bacchi et al., 1992). It therefore does not seem as if polyamines act epigenetically through hypermethylation in plasmodia.

Polyamine metabolism and the redox status of cells have been closely linked, particularly in Trypanosomatids due to the polyamine-dependent synthesis of unique trypanothione (Müller et al., 2003). In *P. falciparum*, the thioredoxin superfamily includes glutaredoxin, thioredoxin and plasmoredoxin and no evidence for trypanothione exists. It has recently been shown that OAT and AdoMet synthetase are among the binding partners of these three, while S-adenosyl homocysteinase (AHC) binds to thioredoxin and glutaredoxin, confirming a link between polyamine metabolism and thiol-based redox metabolism in *P. falciparum* (Sturm et al., 2009). It was further hypothesized that the tight control of polyamine biosynthesis is coupled to redox regulation of OAT and AdoMet synthetase and that this balances the flux of AdoMet between polyamine biosynthesis (generally utilising 2-5% of AdoMet levels) and reactions for transfer of methyl groups (Grillo et al., 2008). Depletion of polyamines by targeting AdoMetDC/ODC in *P. falciparum* has shown a marked decrease in 1-cys peroxiredoxin, thioredoxin, glutathione-S-transferase and glutathione reductase (K Clark, unpublished data) (van Brummelen et al., 2009a). It therefore seems as if there is an interplay between polyamine biosynthesis, control of AdoMet levels and the redox status in the parasite.

Lastly, the inhibition of spermidine synthesis could prevent the formation of the amino acid hypusine occurring in eukaryotic translation initiation factor (eIF-5A) and may be considered a major consequence of polyamine depletion (Kaiser et al., 2003). eIF5A is synthesised by deoxyhypusine synthase as well as deoxyhypusine hydroxylase, with both activities present in *P. falciparum*. The hypusinilation of eIF5A is wholly dependent on the presence of spermidine although the levels of hypusinilation seems to be independently regulated from polyamine depletion (Park, 2006). After inhibition of spermidine synthesis in *P. falciparum* by targeting AdoMetDC activity, a downregulation in the levels (and differential regulation in its isoforms) of eIF5A was observed (S. Smit, unpublished data). Therefore, beyond targeting the activating enzymes of eIF5A synthesis (as observed with the antifungal drug ciclopirox), polyamine-depletion in *P. falciparum* leads to an altered synthesis of eIF5A.

Conclusions:

Albeit being unique, polyamine metabolism in *P. falciparum* has not reached its full potential as a drug target against these parasites due to contrasting evidence of the effect of e.g. DFMO on *in vitro* parasites as opposed to *in vivo* malaria infections as well as intracellular vs. sporozoite forms of the parasite. Biosynthesis inhibition results in a massive transcriptional arrest in the parasite due to the absence of the stabilising function of the polyamines. Moreover, compensatory responses are induced in the parasite in this polyamine-depleted environment to possibly allow the parasite to survive and contribute to the cytostatic nature of the biosynthetic inhibitors. Since the parasite is able to take up both putrescine and spermidine under normal physiological conditions, the involvement of transport mechanisms seems

essential to the homeostatic control of polyamines in this parasite. The transport of at least putrescine is enhanced in conditions where the biosynthesis of this polyamine is inhibited, and the low levels of putrescine that this provides the parasite are presumably effectively converted to trace levels of the most functionally important polyamine, spermidine. In this way, SpdSyn activity seems to be a major component in controlling the overall flux in the system in *P. falciparum*. Inhibition of SpdSyn is seemingly not reversible by spermidine uptake, possibly due to a lack of a metabolic signal for its increased uptake when putrescine is still available. However, AdoMetDC/ODC still makes for an enticing drug target and seems to be essential to the parasite since numerous genetic validation strategies have indicated that null mutants are not viable (Müller et al., 2008). Ultimately, polyamine analogues (possibly based on bis(benzyl) containing pharmacophores) that act as transport as well as enzymatic inhibitors and in addition provide a pool of non-functional polyamines, may prove to be successful as novel antimalarials.

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Table 1. Summary of inhibitors aimed at the biosynthetic activities controlling polyamine biosynthesis in *P. falciparum*, as well as non-functional polyamine analogues active against the parasite. Direct enzyme inhibition values are given as Ki or IC_{50} (in some instances) and growth inhibition by *in vitro* IC_{50}. DFMO: α-difluoromethylornithine, APA: 3-aminooxy-1-aminopropane, MGBG: methylglyoxal bis(guanylhydrazone), MDL73811: 5'-{[(Z)-4-amino-2-butenyl]methylamino}-5'-deoxyadenosine, CHA; cyclohexylamine, 4-MCHA: 4-methylcyclohexylamine, APE: 5-amino-1-pentene, AdoDATO: S-adenosyl-1,8-diamino-3-thiooctane.

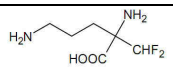
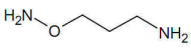
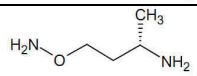
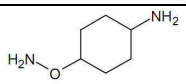
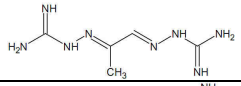
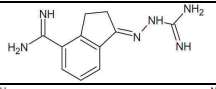
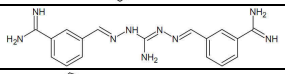
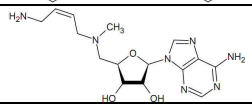
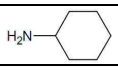
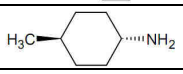
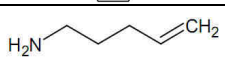
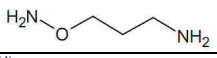
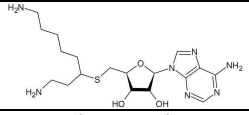
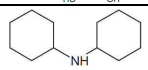
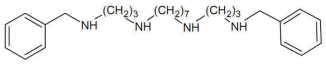
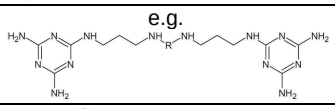
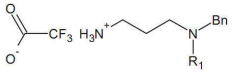
	Inhibitor	Structure	Ki (μM) or IC_{50}*	*In vitro* IC_{50} (μM)	Ref
ODC inhibitors					
	DFMO		87.6	1250	(Krause et al., 2000; Das Gupta et al., 2005)
	APA		0.0027	1	(Das Gupta et al., 2005)
	CGP54169A		0.0079	2	(Das Gupta et al., 2005)
	CGP52622A		0.002	2.7	(Das Gupta et al., 2005)
AdoMetDC inhibitors					
	MGBG			-	(Heidrich et al., 1983)
	CGP48664A		3	8.8	(Das Gupta et al., 2005)
	CGP40215A		1.3	1.8	(Das Gupta et al., 2005)
	MDL73811		1.6	3	(Das Gupta et al., 2005)
SpdSyn inhibitors					
	CHA		19.7*	198	(Haider et al., 2005)
	4-MCHA		0.18	34.2	(Haider et al., 2005)
	APE		6.5*	83.3	(Haider et al., 2005)
	APA		84	1	(Haider et al., 2005)
	AdoDATO		8.5	8.5	(Dufe et al., 2007)
	Dicyclohexyl amine		>1000	342	(Haider et al., 2005)
Polyamine analogues					
	Bis(benzyl) analogues (e.g. MDL27695)		-	0.2-14	(Bitonti et al., 1989)
	1,3,5-triazine analogues	e.g. 	-	0.0519->16	(Klenke et al., 2003)
	Diamine derivatives	 e.g.	-	2-6	(Labadie et al., 2004)

Fig. 1 Functional consequences of polyamine depletion in *P. falciparum*. The parasite is depicted in its three-membrane enclosed intraerythrocytic environment namely erythrocyte plasma membrane (EMP), parasitophorous vacuolar membrane (PVM) and parasite plasma membrane (PPM). Polyamine (Pa) concentrations are indicated. Thick red or green arrows indicate up- or downregulation of transcripts and proteins after inhibition of ODC, AdoMetDC or SpdSyn. Thin arrows of the same colour indicate metabolite level changes (left for AdoMetDC/ODC inhibition, right for SpdSyn inhibition). Unchanged metabolites are indicated with orange arrows. ODC: ornithine decarboxylase, AdoMetDC: S-adenosylmethionine decarboxylase, OAT: ornithine aminotransferase, SpdSyn: spermidine synthase, ADA: adenosine deaminase, PNP: uridine phosphorylase, MTA: 5'-methylthioadenosine, MTI: 5'-methylthioinosine, MTRP: 5'-methylthio-D-ribose-1-phosphate, AdoMet: S-adenosylmethionine, dcAdoMet: decarboxylated S-adenosylmethionine, AdoMetSyn: S-adenosylmethionine synthase, MetTfase: methionine transferase, AHC: S-adenosyl homocysteinase, AdoHC: S-adenosylhomocysteine, MetSyn: methionine synthase, HC: homocysteine, H-eIF5A: hypusine in eIF5A, HS: homospermidine, Met: methionine, Lys: lysine, Cad: cadaverine