Chapter 5:

Concluding discussion

Currently, the first ever Phase III clinical trials are being conducted on a vaccine against malaria. Unfortunately, it has already been established that its efficacy will not be 100% [255] but the important fact is that it would contribute to a decrease in the number of new malaria cases. Malaria prevention and eradication requires the concerted efforts of various disease-control factors and a constant vigilance of each of these would hopefully, one day, lead to a malaria-free world. A reduction in malaria prevalence requires the control of its transmission with methods such as IRS and ITNs, while prevention of malaria infection requires prophylaxis and once infected we need to be able to effectively treat the disease to prevent further transmission.

Disease-controlling factors require the joint cooperation of not only the individuals who are at risk of contracting the disease but also the government, health authorities, aid organisations, funding agencies, public awareness campaigns and the scientists in the field of malaria research. Extensive malaria research has been made possible by funding from large organisations and has become a highly competitive field in the race towards finding a novel, effective and cheap antimalarial agent. As a result, scientists are making groundbreaking findings in both the understanding of parasite pathogenesis as well as strategies to curb the infection of humans. Malaria research has also become highly attractive from a scientific point of view due to the many unique characteristics of the malarial parasite, its complicated life cycle, successful immune evasion, advanced gene transcription and translation mechanisms as well as general metabolism, which contains characteristics of both plants and prokaryotes alike. Understanding this complexity has become a major challenge for scientists, which has resulted in the development of sophisticated methods and technologies. Ultimately, we can only hope that our research findings would contribute to the understanding of the malarial disease, which forms the foundation in an attempt to relieve the burden of not only malaria but other debilitating diseases including HIV-AIDS and tuberculosis.

To date, progress towards novel drug targets and antimalarials has been hampered by the incomplete knowledge of the parasite's biochemistry, particularly the enzymes of parasite origin that could represent useful drug targets. However, this knowledge has increased substantially through the application of biochemical and molecular biology studies that compare the biochemical aspects of the malaria parasite to that of the host cell. A rational approach to achieve

selective chemotherapy requires a thorough understanding of the metabolic and biochemical differences between the parasite and its host cell. In this way, several parasite-specific and novel targets have been identified by their cloning and expression, biochemical characterisation and subsequent inhibitor screening followed by structure-function relationship studies. The success of such an approach relies on the highly specific and selective inhibition of a specific parasite biochemical process that is vital to parasite growth and survival without affecting the process of the host cell. In this regard the polyamine biosynthetic pathway of the plasmodial parasite is particularly attractive and represents a novel strategy to interfere with parasite viability. This is particularly relevant since polyamines themselves are essential for growth and differentiation of all cells, but has drawn little interest from investigators worldwide mainly due to the difficulty of working with this pathway's constituent enzymes.

Research of the polyamine biosynthetic pathway in *P. falciparum* as well as other parasites such as *T. brucei* has identified sufficiently unique properties that would allow for its selective targeting. Despite the availability of a considerable number of inhibitors and some structural data of the polyamine biosynthetic enzymes already published, little information is available for the *P. falciparum* polyamine biosynthetic rate-limiting enzymes. This may be due to the technical difficulties involved in obtaining sufficiently large amounts of these proteins that are required for structural studies. Although previous studies have reported the successful recombinant expression of active bifunctional *Pf*AdoMetDC/ODC as well as the monofunctional *Pf*AdoMetDC and *Pf*ODC proteins in *E.* coli, further optimisation is still needed to increase the level of expression and to overcome the poor solubility as well as instability of these expressed proteins before the structures can be solved through X-ray crystallography. In the absence of crystal structures, the modelling of the proteins has been useful to permit exploration of the predicted catalytic mechanism and effects of potential drug interactions [120,127,130]. However, a homology model is highly dependent on the degree of sequence homology, which is generally low for *P. falciparum* and can thus only provide guidance to the correct structure. Homology models nonetheless represent a powerful tool for the rational design of substrate analogues and screening for potential inhibitors.

Various similarities can be drawn between the bifunctional *P. falciparum* DHFR/TS enzyme and that of bifunctional *Pf*AdoMetDC/ODC. Like *Pf*AdoMetDC/ODC, *Pf*DHFR/TS, is also a validated target for the inhibition of *de novo* folate biosynthesis in the parasite and antimalarials such as pyrimethamine and sulphadoxine have been used as successful antimalarial strategies for a considerable period of time. The emergence of resistance has rendered these antimalarials

largely ineffective but they are still being used today in combination therapies and in certain malaria treatment cases (WHO 2010). The biochemical and structural characterisation of these two enzymes as well as the other folate biosynthetic enzymes has allowed researchers to pinpoint the mechanism of resistance development, methods to delay this and the identification of novel inhibitors that could in future be used to replace the current antifolates, either as single drugs or in combinations [256]. *Pf*DHFR/TS is a dimeric enzyme with extensive interdomain interactions significantly mediated by the junction region (known as the hinge region in *Pf*AdoMetDC/ODC) as well as additional parasite-specific inserts in the *Pf*DHFR domain. The activity of the *Pf*TS domain depends on the integrity of the N-terminal *Pf*DHFR as well as the junction region [122,257] and therefore represents a unique feature that may be exploited in the development of *Pf*TS-specific inhibitors. Interestingly, it was shown that deletion of only five residues at the N-terminus of *Pf*DHFR/TS resulted in significant impairment of DHFR function, and further deletion of 15 residues resulted in an inactive bifunctional enzyme [257]. These results indicated that the N-terminal residues play an important role in both activities of the bifunctional complex, even if the start of the *Pf*TS domain is 320 residues away from the N-terminus. The crystal structure of *C. hominis* DHFR/TS showed that, while parasite-specific insert 1 extends away from the domain surface and does not interact with the core *Ch*DHFR structure, it forms an interaction with the *Ch*TS domain and thereby contributes to the stabilisation of the interdomain attachment [258]. The junction region, notably the "donated helix", also forms extensive interactions with the other *Ch*DHFR domain and includes part of insert 2.

In *P. falciparum*, AdoMetDC/ODC also exists as a dimer of the two *Pf*AdoMetDC/ODC polypeptides, which are connected by a hinge region. Autocatalytic cleavage within the *Pf*AdoMetDC domain for its activation as well as α- and β-subunit formation, results in the formation of the heterotetrameric protein [70]. Biochemical studies have previously shown that the hinge region as well as the *Pf*AdoMetDC domain is important for the C-terminal *Pf*ODC activity and that this domain is more refractory to change [69,103]. Like *Pf*DHFR, *Pf*AdoMetDC is not dependent on the C-terminal domain and removal of this domain actually improves the enzyme efficiency of *Pf*AdoMetDC [71]. However, various interdomain interactions are formed [69] and their interaction sites have been predicted with the help of homology models [120] and *in silico* protein-protein docking experiments [152]. Delineation of these exact sites awaits the crystal structure of the bifunctional complex, which could be identified for *Pf*DHFR/TS and *Ch*DHFR/TS once their structures were solved. Further studies have also identified various parasite-specific inserts within *Pf*AdoMetDC/ODC that are important for protein activities and are predicted to mediate interactions within the two domains [69] as has been observed for

DHFR/TS from both *P. falciparum* and *C. hominis* [122,258]. However, and in contrast to *Pf*DHFR/TS, the activities of *Pf*AdoMetDC/ODC are not involved in substrate channelling and the basis for the bifunctional arrangement is largely unknown. It has been shown that *Pf*ODC is feedback regulated by its product putrescine, which has no effect on *Pf*AdoMetDC activity [71]. Furthermore, while *Pf*AdoMetDC can function independently, monofunctional *Pf*ODC is inactive [71,103].

The evolutionary role of such a large bifunctional arrangement has extensively been questioned. Possible reasons include the regulated biosynthesis of polyamines in *Plasmodium* spp [70] via the interference of a single domain, which then communicates the change to the adjacent domain. In addition to the enormous 5'-UTR of *PfAdometdc/Odc* consisting of nearly 3000 nucleotides, the hinge region has been suggested to represent the remnants of the 5'-UTR of *PfOdc* as it is not predicted to have any conformational role but is within the reading frame of translation from *PfAdometdc*. Further studies are needed to identify specific areas within the UTRs that may mediate transcriptional and translational control as well as possible sites that could bind polyamines as a feedback control mechanism as seen for the plant and human transcripts [211,212,259]. In addition, while antizyme [102] is absent in *P. falciparum* and the presence of a prozyme [213] seems improbable, *Pf*ODC may behave in an analogous fashion to the *Pf*DHFR/TS association, and have adopted a role as regulator of the activity of *Pf*AdoMetDC (and *vice versa*) in the bifunctional complex. This is evidenced by the improved enzyme kinetics of the recombinantly expressed, monofunctional *Pf*AdoMetDC protein compared to its activity in complex with *Pf*ODC [71]. This has led to the postulation that the bifunctional arrangement could mediate the co-regulation of both activities of the polyamine rate-limiting enzymes and that these are made possible by the various interdomain activities, which can regulate the domain activities. Comparison between *Pf*DHFR/TS and *Pf*AdoMetDC/ODC has thus revealed that the parasite may employ the bifunctional arrangement of enzymes for 1) the coordinated regulation of enzyme activities in essential metabolic pathways; 2) to allow for the dependence of the activity of the C-terminal domain on the N-terminal domain within the complex; 3) in the case of *Pf*DHFR/TS to allow for substrate channelling; and 4) to allow for additional interdomain protein-protein interactions for the metabolic regulation of important metabolites such as folates and polyamines.

In this study, the possibility of interdomain regulation between *Pf*AdoMetDC and *Pf*ODC in the bifunctional complex was studied via the delineation of specifically the O1 parasite-specific insert of the *Pf*ODC domain and its role in interdomain interactions as well as with the

biochemical and structural characterisation of monofunctional *Pf*AdoMetDC. Peptides as probes to determine the possible roles of the O1 insert in interdomain interactions showed that these peptides that are identical to the insert itself could displace the binding sites of this insert and resulted in an increase in *Pf*AdoMetDC activity that was comparable to the activity of this enzyme when it exists in its monofunctional form [71]. These peptides therefore mimic the monofunctional arrangement of the *Pf*AdoMetDC domain and a peptide that specifically targeted the O1 insert reduced *Pf*ODC activity, thereby mimicking the effect of expressing *Pf*ODC in its monofunctional form [103]. These results alluded to the possibility that this insert represents the delineating site that could mediate the activities within the bifunctional *Pf*AdoMetDC/ODC complex. In addition, future studies involving the crystallisation of the *Pf*AdoMetDC domain could utilise the stabilising effects of the peptides on *Pf*AdoMetDC activity to increase its crystallisation in the absence of its protein partner. It was subsequently necessary to characterise the enzyme kinetics of the monofunctional *Pf*AdoMetDC domain in order to determine whether the kinetics of this domain are improved in the presence of *Pf*ODC and would therefore corroborate the O1 insert peptide results. In addition, the biochemical characterisation of *Pf*AdoMetDC represents novel results as only limited information is available on this protein in its monofunctional form.

Expression of monofunctional *Pf*AdoMetDC (without the majority of the hinge region) showed that while the specific activity of this enzyme is higher in this form, the substrate affinity decreased compared to the K_m in the bifunctional complex. In addition, comparison of the *Pf*ODC kinetics showed that this monofunctional enzyme is inactive while the presence of *Pf*AdoMetDC increases its specific activity as well as substrate binding affinity [71,103]. Furthermore, analyses of the turnover numbers of monofunctional and bifunctional *Pf*AdoMetDC or *Pf*ODC showed that the bifunctional arrangement resulted in matched rates, which would allow for balanced synthesis of the products of the decarboxylase reactions. The results therefore show that the rate-limiting enzymes of the polyamine pathway in *P. falciparum* are co-regulated within the bifunctional complex such that their rates can concurrently be controlled for the subsequent synthesis of the metabolically relevant polyamines via the reaction of *Pf*SpdS [98]. We have thus shown for the first time that polyamine biosynthesis in *P. falciparum* is regulated via the arrangement of activities in a bifunctional protein. Future studies should focus on the crystallisation of the monofunctional proteins as well as bifunctional complex such that rational drug design can be facilitated. The studies on monofunctional *Pf*AdoMetDC provide important starting points for crystallisation in which a stable protein should be used. These results also prove the relevance of targeting the already validated drug

target, *Pf*AdoMetDC/ODC, with a compound that could simultaneously affect both enzyme activities and thereby inhibit the synthesis of downstream polyamines. The latter studies will have to be performed in combination with the characterisation of polyamine transport in *P. falciparum* since the uptake of polyamines as a means for the parasite to overcome polyamine biosynthesis inhibition will have to be taken into account.

AdoMetDC represents an important drug target due to various factors, including 1) its key responsibility in the synthesis of polyamines by downstream aminopropyl transferases; 2) the unique utilisation of a covalently bound pyruvate as co-factor synthesised by an autocatalytic cleavage event; 3) the maintenance of low steady-state levels of its product, dcAdoMet, as a result of the importance of AdoMet in methyl transfer reactions; and 4) the strict control of its activity in response to the requirement of polyamines by means of its transcription, translation, post-translational modification (pyruvoyl formation), enzyme activity as well as its degradation [258]. It therefore seems that AdoMetDC represents the most important enzyme in the polyamine pathway while SpdS is involved in flux control due to its dependence on the products of both the AdoMetDC and ODC reactions for the synthesis of spermidine. However, in *P. falciparum*, the association of AdoMetDC with ODC within a bifunctional complex allows for a unique opportunity to not only target AdoMetDC activity but also ODC. In addition, the identification of protein-protein interactions between these two domains, especially those mediated by the O1 parasite-specific insert, has allowed for additional strategies to target enzyme activities with the use of non-active site inhibitors. The use of such inhibitors are beneficial due to the limitations involved in targeting inhibitors to the enzyme active site when high structural conservation exists between the active sites of the host and the disease-causing parasite. The greater structural variability of protein-protein interfaces suggests that these interface contact sites may provide important target sites that are sufficiently different between the host and the parasite. The large *Pf*AdoMetDC/ODC complex possesses several protein-protein interaction regions that are absent in the host monofunctional counterparts and can thus be selectively targeted. Another advantage of targeting areas other than the active site is the reduced resistance pressure that is placed on the organism when a non-active site-based drug is used. Resistance to the drug via the introduction of mutations in the active site, as is seen for *Pf*DHFR/TS [146], will develop at a slower rate, which is extremely valuable in drug development against the multidrug resistant malaria-causing *P. falciparum* parasites [169]. Several other advantages of using peptides as therapeutic molecules include: 1) high activity and specificity; 2) unique 3D characteristics; 3) no accumulation in organs due to small size; 4) low toxicity; and 5) low immunogenicity [259].

Alternatively, the use of structure-based drug design was also evaluated by using the well-characterised *Pf*SpdS protein as drug target in a study that applied a dynamic, receptor-based pharmacophore model together with *in silico* chemical library screening. This approach identified important factors that should be taken into account to identify a promising lead compound and it specifically identified the importance of identifying a ligand that could bridge the catalytic centre of this two-substrate enzyme and thereby efficiently compete with both substrates. Two lead compounds were subsequently tested *in vitro* for their effects on enzyme inhibition and whole cell-based parasite cultures. The compounds also showed promising interactions and stabilisation within the active site as determined by the atomic resolution (1.9 Å) crystal structures. While the inhibitory results showed potential, these drugs still need to be effectively delivered into the parasites to obtain increased inhibition in the nanomolar range. However, in contrast to prior studies that focussed on ligand-based approaches, the dynamic, receptor-based pharmacophore model employed here identified a compound that could bind to a restricted site within the active site and cause inhibition. The co-crystallisation of these lead compounds with the purified *Pf*SpdS protein validated the *in silico* predictions and thus shows the relevance of applying such a study for prioritisation of potential inhibitors to be tested experimentally.

The results of this dissertation have thus completed a full circle in terms of identification of a novel drug target (polyamine biosynthesis), biochemical and structural characterisation thereof in order to identify potential unique properties that are exploitable for selective drug targeting (*Pf*AdoMetDC and *Pf*ODC, Chapters 2 and 3), followed by the identification of potential inhibitors via rational structure-based drug design and validation of the mechanism of inhibition with these novel compounds (*Pf*SpdS, Chapter 4). This study thus shows the importance of combining *in vitro* with *in silico* experiments to streamline the required studies that are needed to be performed in the laboratory and shows a successful interplay of three different, yet dependent scientific approaches, namely biochemistry, bioinformatics and medicinal chemistry. Future studies should focus on further structural characterisation of the bifunctional *Pf*AdoMetDC/ODC complex as well as validation of interdomain interactions, followed by the identification of novel inhibitory compounds. The efficiency of the inhibitory compounds indentified for *Pf*SpdS should also be improved in terms of delivery into the parasites and selectivity against host cells. Ultimately, the aim would be to apply a combination-based approach to target bifunctional *Pf*AdoMetDC/ODC with a non-active site based inhibitor as well as the flux-controlling activity of *Pf*SpdS with a highly selective, active site inhibitor that would result in a drastic reduction of polyamine levels within the parasite.