CHAPTER 6

Structure-based ligand binding and discovery of novel inhibitors against *P. falciparum* ODC.

6.1) INTRODUCTION

Early observations on inhibition of spermidine synthesis in a mammalian system by known anti-tumour agents encouraged the development of other compounds potentially inhibitory to specific steps in the polyamine biosynthetic pathway (Cohen, 1998). Due to its role as a rate-limiting enzyme of polyamine biosynthesis, ODC is an obvious target for chemical intervention (Janne and Alhonen-Hongisto, 1989b). However, inhibition of the enzyme triggers a series of compensatory reactions that conserve the intracellular polyamine pools including increased uptake, release of polyamines from intracellular pools, stabilisation of ODC in the case of competitive, reversible inhibitors and the secondary induction of AdoMetDC activity (Janne, *et al.*, 1985). Therefore, AdoMetDC has to be considered as the natural second target for chemotherapy.

The ODC catalysed reaction is inhibited by structural analogues of the cofactor, substrate or product (Janne and Alhonen-Hongisto, 1989a). The earliest inhibitors were structural analogues of ornithine or putrescine. The most successful of these reversible, competitive ODC inhibitors were α-methylornithine and α-hydrazinoornithine. However, these compounds were not sufficiently active *in vivo* and α-hydrazinoornithine also non-specifically binds PLP (Janne and Alhonen-Hongisto, 1989a; Pegg, 1989b). Co-factor analogues included compounds capable of reacting with PLP, such as L-canaline (α-amino-γ-aminooxybutyric acid) that inhibit ODC but also many other PLP-binding enzymes. Synthetic analogues of the Schiff-base intermediate were designed in the hope that it would act as multisubstrate adduct inhibitors of ODC (Pegg, 1989b). One such adduct, *N*-(5′-phosphopyridoxal)ornithine was a potent inhibitor of ODC but again also inhibited other PLP-dependent enzymes (Janne and Alhonen-Hongisto, 1989a). Unphysiological diamines that act as homologues of putrescine included 1,3-diaminopropane and its hydroxylated derivative, 1,3-diamino-2-propanol. These diamines are potent inhibitors of ODC but, because of the structural

homology to putrescine, may take over some of the physiological functions of the natural polyamines (Janne and Alhonen-Hongisto, 1989a). Reversible competitive inhibitors of ODC activity included 1-amino-oxy-3-aminopropane and its derivatives with their aminooxy group being isosteric with the aminomethylene of putrescine (Cohen, 1998). These are very powerful inhibitors of ODC with IC_{50}s in the nanomolar range (Mett, *et al.*, 1993; Standek, *et al.*, 1992). Fig. 6.1 indicates the structures of some of the ODC inhibitors.

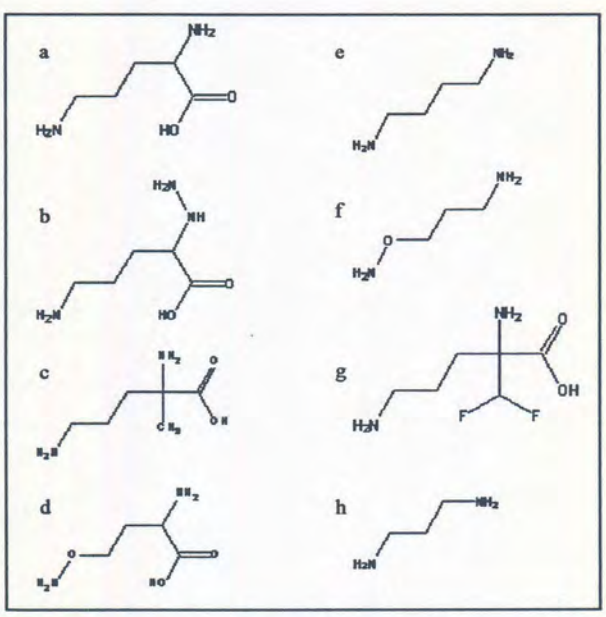


Figure 6.1: Structures of the natural substrates and reversible and irreversible inhibitors of ODC. A: ornithine, b: α-hydrazino ornithine, c: α-methyl ornithine, d: canaline, e: putrescine, f: 1-aminooxy-3-aminopropane, g: DFMO and h: 1,3-diaminopropane. Adapted from (Cohen, 1998).

It was not until the introduction of the concept of mechanism-based irreversible inhibitors, or suicide inhibitors, that more powerful and specific inhibitors of ODC were designed. Mechanism-based enzyme inactivators are unreactive compounds that bear a structural similarity to a substrate or product of a specific enzyme. Once at the active site the target enzyme converts the inactivator into a product that usually forms a covalent bond with the enzyme, generally via its normal catalytic mechanism (Silverman, 1988). The most potent of these, DL-α-difluoromethyl ornithine (DFMO), described in 1978 mimics ornithine but remains covalently bound to ODC after activation (Cohen, 1998), Fig. 6.2). A compound capable of competing favourably with DFMO is (2R,5R)-6-heptyne-2,5-diamine (or methylacetylenicputrescine, RR-MAP) which is at least ten times more potent than DFMO (Cohen, 1998; Janne and Alhonen-Hongisto, 1989a).

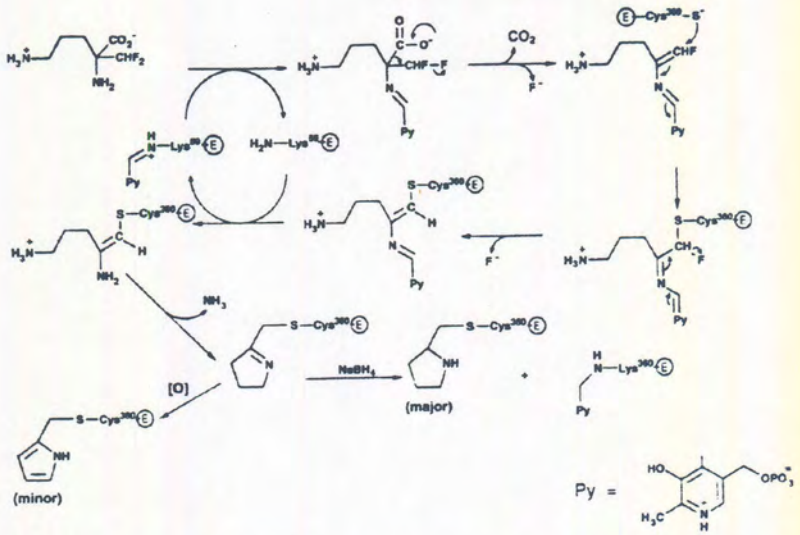


Figure 6.2: Proposed mechanism of inactivation of ODC with DFMO. Adapted from (Cohen, 1998).

The only known inhibitors of AdoMetDC of any physiological importance are derivatives of bis(guanylhydrazone) (Janne, *et al.*, 1985). The discovery that the antileukemic agent, methylglyoxal bis(guanylhydrazone) (MGBG) is an extremely potent inhibitor of eukaryotic, putrescine-activated AdoMetDC singled this compound out as a standard inhibitor for this enzyme (Janne and Alhonen-Hongisto, 1989b; Janne, *et al.*, 1985; Pegg, 1989b). Moreover, most of the bis(guanylhydrazone) derivatives, when combined with inhibitors of ODC produce a synergistic antiproliferative effect. Further alkylation at the glyoxal portion of these molecules produces even more potent inhibitors such as ethylmethylglyoxal bis(guanylhydrazone) (Cohen, 1998). Fig. 6.3 indicates the structural similarity between MGBG and spermidine as well as the substrate, adenosylmethionine (Janne, *et al.*, 1985).

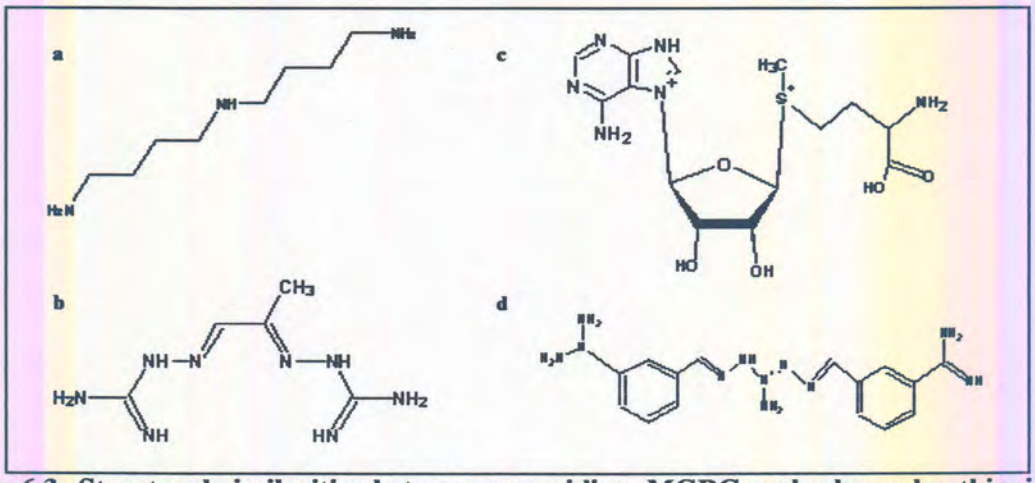


Figure 6.3: Structural similarities between spermidine, MGBG and adenosylmethionine. Adapted from (Janne, *et al.*, 1985; Müller, *et al.*, 2001). a: Spermidine, b: MGBG, c: AdoMet and d: CGP40215.

A number of other non-nucleoside substances have been described as inhibitors of AdoMetDC. Some of these, including Berenil and Pentamidine, resemble MGBG but their pharmacological actions are not well understood (Cohen, 1998; Pegg, 1989b). Other nucleoside analogues include the irreversible inhibitors 5′-[(3-aminooxypropyl)methylamino]-5′-deoxyadenosine (MAOPA) which are extremely potent inhibitors of mammalian AdoMetDC (Cohen, 1998; Pegg, 1989b).

Various ODC and AdoMetDC inhibitors have been tested for antimalarial activity. Five ODC inhibitors, DFMO (> 3 mM) and three of its α-monofluoromethyl derivatives as well as RR-MAP have been shown to inhibit *P. falciparum* schizogony *in vitro*. DFMO was also effective in limiting *P. berghei* schizogony *in vivo* (Assaraf, *et al.*, 1987a; Assaraf, *et al.*, 1986; Assaraf, *et al.*, 1987b; Bitoni, *et al.*, 1987; Whaun and Brown, 1985). Two 3-amino-oxy-1-propanamine analogues, CGP52622A and CGP54169A, were also shown to inhibit malarial ODC with K_i values in the nanomolar range (Krause, *et al.*, 2000; Wrenger, *et al.*, 2001). Irreversible inhibition of *P. falciparum* AdoMetDC with MGBG analogues prevented growth of the parasite *in vitro* (Wright, *et al.*, 1991). The most potent of these, MDL 73811, inhibited growth with an IC_{50} in the low μM range. The diamidine, CGP40215 synthesised as an anticancer compound, showed inhibition against both recombinant *P. falciparum* AdoMetDC and *P. berghei* infection in mice (Müller, *et al.*, 2001).

The relatively poor pharmacokinetics of inhibition of polyamine biosynthesis of *in vivo* *P. falciparum* infections prompts investigations to develop novel inhibitors. Structure-

based approaches for designing novel inhibitors of protein function have been used to generate clinically useful drugs against a number of diseases. These include antibacterial agents (DHFR as target), anticoagulants (thrombin inhibition), antiviral agents (neuramidase inhibition), anti-AIDS agents (HIV protease inhibition), anticancer agents (thymidylate synthase and purine nucleoside phosphorylase as targets), and an anti-inflammatory agent (phospholipase A_2) (Blundell, 1996; Bohm and Klebe, 1996; Whittle and Blundell, 1994). Specifically focussing on parasitic protozoa, success has been obtained in the discovery of novel lead inhibitors against trypanosomal glyceraldehyde-3-phosphate dehydrogenase (Bohm and Klebe, 1996) and the antimalarial targets, DHFR (Lemcke, *et al.*, 1999; Toyoda, *et al.*, 1997; Warhurst, 1998) and two major protease families, the serine and cysteine proteases using this approach (Ring, *et al.*, 1993).

Structure-based or rational inhibitor design methods identify favourable and unfavourable interactions between a potential ligand and the target receptor and maximise the beneficial interactions to increase binding affinity (Blundell, 1996; Whittle and Blundell, 1994). The central assumption is that good inhibitors must possess significant structural and chemical complementarity to their target receptor (Kuntz, 1992). Molecular recognition in ligand-protein complexes is responsible for the selective binding. Interactions are normally noncovalent in nature and experimentally determined inhibition constants K_i are typically in the range of 10^{-2} to 10^{-12} M (Bohm and Klebe, 1996). Hydrogen bonds are the most important stabilising force in protein interactions and define the specificity of protein-ligand interactions. Furthermore, oppositely charged functional groups of the protein and ligand are paired in electrostatic interactions. In addition, apolar groups of ligands are found in hydrophobic pockets in the active sites (Bohm and Klebe, 1996; Zubay, 1993). Structure-based approaches employ either novel ligand discovery by searching three-dimensional databases of known chemical structures (docking), modifications of existing inhibitors (linking) or attempts to *de novo* assemble compound structures from chemical fragments (building) as summarised in Fig. 6.4.

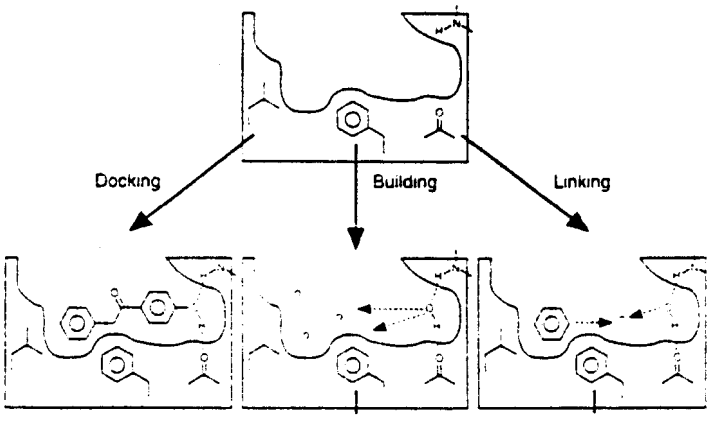


Figure 6.4: Strategies for the discovery of novel lead structures by ligand docking. Adapted from (Bohm and Klebe, 1996).

Computational methods for structure-based ligand discovery require a three-dimensional structure of the target protein. In the absence of an accurate X-ray or NMR structure, a homology model of the protein can be used if the errors of the prediction cluster away from the enzyme active site (Blundell, 1996; Kuntz, 1992; Ring, *et al.*, 1993; Walters, *et al.*, 1998). The most direct way to find a novel ligand is then to search a database of three-dimensional chemical structures. The pioneering program DOCK searches these databases based on shape and electronic complementarity (by means of a molecular mechanics force field) between the protein and ligands (Kuntz, 1992; Whittle and Blundell, 1994). The Cambridge Structure Database (CSD) contains experimentally determined crystal structures whereas other databases such as the Fine Chemicals Directory or Available Chemical Directory (ACD) can be converted to three-dimensional databases with programs such as Corina or Concord (Kuntz, 1992; Whittle and Blundell, 1994).

LUDI is a fragment-based *de novo* design program that can be used both for searching of three-dimensional databases and for the automatic construction of novel ligands (Bohm and Klebe, 1996). The program positions molecules into clefts of a protein such that hydrogen bonds can be formed and hydrophobic pockets are filled with apolar groups. The program calculates interaction sites derived from composite crystal-field environments. An important aspect of LUDI is its ability to tolerate small uncertainties in the determined protein structure. Any positioning of ligands is completely based on geometric parameters. A fast and error-tolerant empirical scoring function is used based on the assumption that noncovalent interactions are additive and the function then takes into account hydrogen bonds, ionic interactions, apolar protein-ligand contact surface,

interactions between aromatic rings, the replacement of water molecules and the number of rotatable bonds in the ligand. The capacity of the LUDI programme to discover novel ligands is supported by the identification of a novel inhibitor of human phospholipase A2, compound 23 for the immunosuppressant FK506 binding protein-12, inhibitors of trypsin, HIV-protease, purine nucleoside phosphorylase and streptavidin (Bohm and Klebe, 1996)

In this chapter, the active site of PfODC revealed by the homology model (Chapter 5) is used to explain the experimental inhibition of PfODC seen with DFMO and the CGP-series of inhibitors described above. Furthermore, novel ligands that selectively bind to PfODC are identified in a structure-based approach.

Results obtained in this chapter have been presented as a talk at the second polyamine conference: 'Polyamine metabolism as a drug target in parasitic protozoa and worms', Texas, USA (Birkholtz, 2002c).

6.2) MATERIALS AND METHODS

6.2.1) Docking of known inhibitors into the active site of dimeric PfODC

6.2.1.1) Generation of three-dimensional structures for known inhibitors.

Structures for PLP and DFMO (used as substrate analogue) were generated with the Builder module of the InsightII package and subjected to energy minimization with the Discover3 module of the InsightII package (cff91 force field for 10 000 iterations with a conjugate gradient) as described in Chapter 5. Structures for the two competitive inhibitors CGP52622A and CGP54169A from Novartis Pharma (Krause, *et al.*, 2000; Mett, *et al.*, 1993; Standek, *et al.*, 1992) were generated as described above.

6.2.1.2) Docking of the known inhibitors into the active site pocket of PfODC.

Binding of PLP and DFMO requires the formation of a Schiff-base between the two ligands with DFMO then also forming a covalent bond to the S_γ atom of Cys_{360} (*T. brucei* numbering). In order to dock this transition state complex of PLP-DFMO into PfODC, the structure for the linked PLP-DFMO was created and formed a covalent link with Cys_{1355} of the dimeric form of PfODC described in Chapter 5, section 5.2.3. The

ligand-ODC complexes were then minimized as described above. Possible interactions between the ligands and residues in PfODC were analysed with LigPlot (Wallace, *et al.*, 1995). The structures were analysed for accuracy with the WHAT IF program (Vriend, 1990).

The two competitive inhibitors, CGP52622A and CGP54169A (Novartis Pharma), are 3-amino-oxy-1-propanamine homologues of the polyamines (Krause, *et al.*, 2000; Mett, *et al.*, 1993; Standek, *et al.*, 1992). To analyse the possible interactions of these inhibitors in the active site pocket of PfODC, the structures were superimposed on the coordinates of the PLP-ornithine complex minimized in the PfODC active site pocket (Chapter 5, section 5.2.4). Energy minimization was repeated for the ligand-ODC complexes as described above. The ligands were also analysed for any bumps occurring between van der Waals radii. Possible interactions between the ligands and residues in ODC were analysed with LigPlot (Wallace, *et al.*, 1995). The structures were analysed for accuracy with the WHAT IF program (Vriend, 1990). The same procedure was followed for docking of these inhibitors in the human ODC structure to explain the selective inhibition.

6.2.2) Discovery of novel ligands for PfODC.

The three-dimensional active site pocket of the PfODC model was defined in Chapter 5, section 5.3.6. The coordinates of the defined active site pocket was used for the identification of novel ligands that could selectively bind residues involved in catalysis of PfODC. The active site was specified for the forced inclusion of residues Cys_{1355}, Asp_{1356}, Arg_{1117}, Tyr_{966}, Arg_{955} and His_{998}. The receptor atoms specified were Cys_{1355} and Lys_{868} to allow the generation of a virtual atom as the central point of the search domain. The LUDI module of the Insight II package was then used to screen a three-dimensional chemical structure library to identify novel ligands that bind to PfODC. LUDI suggests novel ligands by selecting from a library of small molecules due to the appropriate spatial orientation of hydrogen-bonding and hydrophobic contacts of the molecules with the target receptor. The LUDI parameters included a 7 Å search domain around the specified centre atom with cut-off limits on the size of the identified ligands as maximum 50 atoms and minimum 5. The USA National Cancer Institute (NCI) Open database of chemical compounds was screened. These compounds are all publicly and freely available from NCIs' Developmental Therapeutics Program and were generated originally as compounds with anticancer potential. The three-dimensional version of the

database was generated with Corina v 1.7 (Gasteiger, *et al.*, 1990) and included structures for 249 017 compounds.

The NCI Open database was screened against PfODC using LUDI. The same procedure was performed for the human ODC active site. Predicted K_i values for the compounds were obtained with the formula:

Score = -100log K_i where score is the LUDI score given for binding of each compound (Bohm and Klebe, 1996).

Compound information was obtained from the Enhanced NCI database browser. Interaction maps of the compounds with either PfODC or the human structure were obtained with LigPlot (Wallace, *et al.*, 1995). PASS predictions (Prediction of the biological activity spectra of substances) were performed for the top ten compounds identified (www.ibmh.msk.su/PASS).

6.3) RESULTS.

6.3.1) Docking of known inhibitors in the active site pocket of PfODC.

In order to explain the mechanism of competitive inhibition of PfODC (either on its own or in the bifunctional enzyme complex) by DFMO and the CGP-series of inhibitors, these compounds were docked into the proposed active site pocket.

6.3.1.1) Docking of DFMO in PfODC.

In order to simulate the binding of the irreversible competitive inhibitor DFMO, it was covalently linked to Cys_{1355} (atom S_γ) as well as coupled to PLP via a Schiff-base and minimized to an energy of –15 307 kcal/mol. Analyses of the minimized interacting site of PfODC with DFMO are shown in Fig. 6.5. The majority of the residues are also involved in binding to the substrate with the exception of Asn_{1393} and Phe_{1392} (Chapter 5, section 5.3.6). The only PfODC-specific residues involved in the binding of DFMO are Arg_{1117} and Tyr_{966} compared to the residues involved in the human enzyme. Both of these residues make contact with the ε-amino group of DFMO.

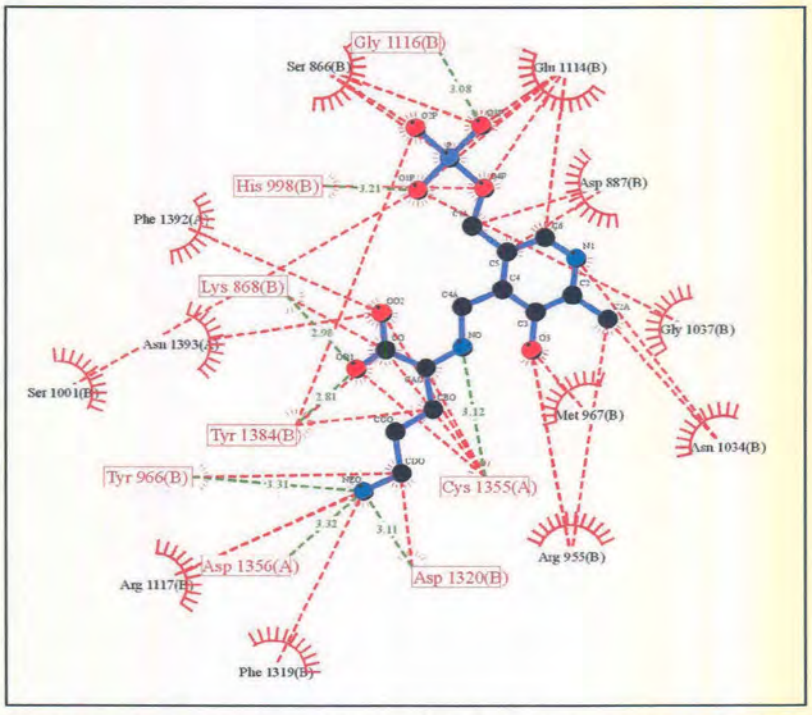


Figure 6.5: Interactions between the cofactor (PLP) and competitive inhibitor DFMO in the active site pocket of PfODC. Hydrogen bonds and their lengths are indicated in green, non-ligand residues involved in hydrophobic contacts with the ligand in red arcs with spokes.

6.3.1.2) Docking of the reversible competitive CGP inhibitors in PfODC.

To analyse the possible interactions of two reversible competitive inhibitors, CGP54169A and CGP52622A in the active site pocket of PfODC, the structures were superimposed on the coordinates of the corresponding ligands minimized in the PfODC active site pocket. Stable complexes had minimised energies of –15 343.94 kcal/mol for the CGP52622A-PfODC complex and a corresponding –15 346.68 kcal/mol for the CGP54169A-PfODC complex. Analogous procedures were followed for docking of the CGP-inhibitors against the human ODC structure and stable complexes showed minimised energies of –14 821 kcal/mol (CGP52622A-human ODC complex) and –14 807.75 kcal/mol for the CGP54169A-human ODC complex. This predicts a slightly lower ability of the human ODC active site pocket to accommodate these compounds.

Fig. 6.6 indicates the predicted Ligplot interaction sites between the CGP-inhibitors with PfODC. In the case of CGP52622A, inhibition can be explained due to the ability of this molecule to interact with essential residues involved in binding of PLP (Asp_{887}, Arg_{955}, Thr_{993}, Ser_{1001}, Asn_{1034} and Cys_{1355},) as well as substrate (Lys_{868}, Tyr_{1384} and Cys_{1355}). Two unique interactions with Ala_{912} and Phe_{997} are also suggested. The calculated total free energy of interaction of this inhibitor with PfODC was –32.1

kcal/mol. As expected for an substrate analogue, CGP54169A shows major interactions with residues predicted to bind to ornithine (Arg_{1117}, Tyr_{966}, Phe_{1319}, Asp_{1320}, Asp_{1356}, Cys_{1355} and Phe_{1392}) and also to two PLP-binding residues (His_{998} and Tyr_{1384}) with a total free energy of interaction of –27.67 kcal/mol. This is lower than the binding free energy of –84.2 kcal/mol obtained for the interactions with Schiff-base linked PLP-ornithine as natural ligands. These proposed interactions of the inhibitors with the essential active site binding residues would therefore prevent or interfere with any subsequent binding of the substrate/co-factor and lead to enzyme inhibition.

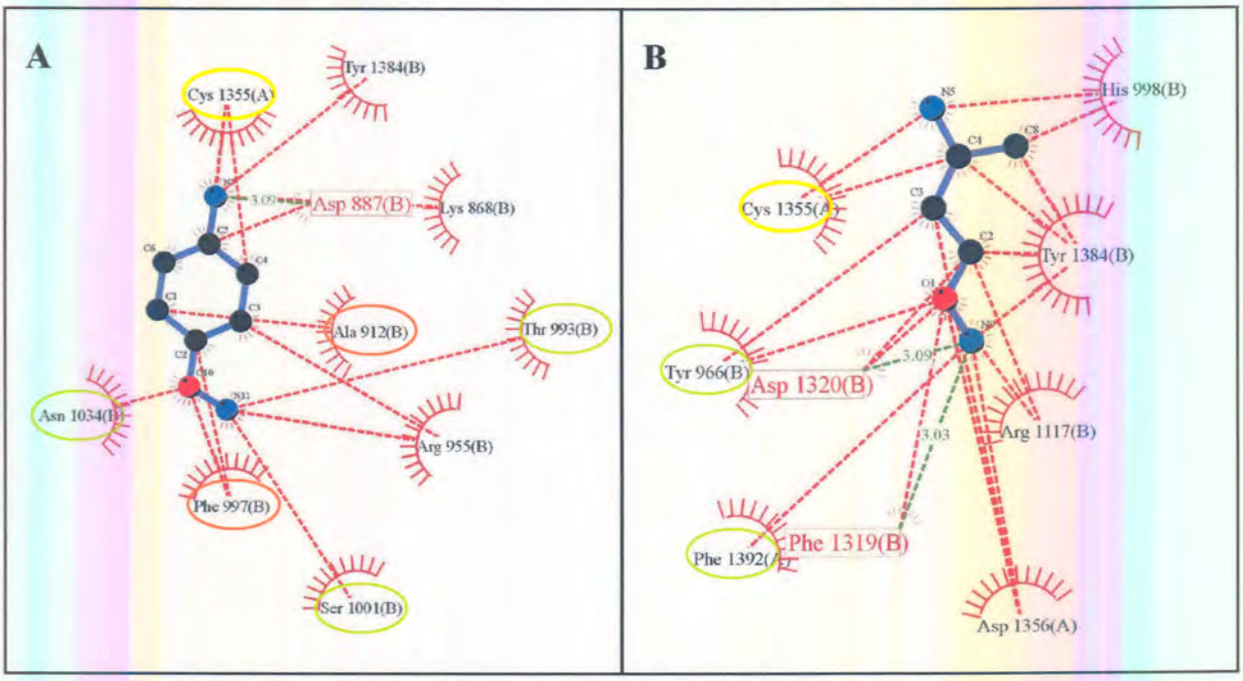


Figure 6.6: Ligplot analyses of the interactions between two competitive inhibitors and PfODC. (A) CGP52622A and (B) CGP54169A. Hydrogen bonds and their lengths are indicated in green, non-ligand residues involved in hydrophobic contacts with the ligand in red arcs with spokes. Green and yellow ellipses indicated residues in PfODC that were uniquely involved in binding to this enzyme. The catalytic Cys_{1355} is indicated in the yellow ellipse. The unique residues involved in CGP52622A binding but not in substrate or co-factor binding are shown in orange ellipses.

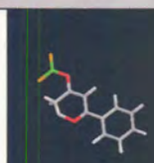
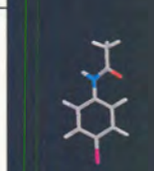
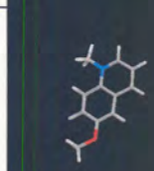
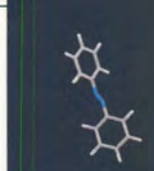
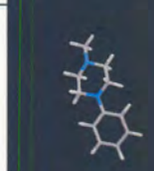
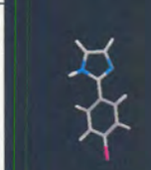
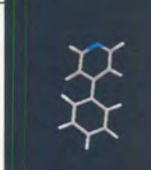
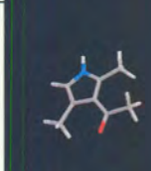
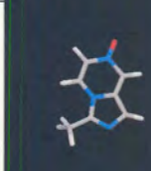
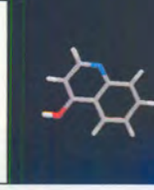
The CGP-series of compounds inhibit rat liver ODC with IC_{50} values in the nanomolar range and recombinantly expressed human ODC with IC_{50} values of 25 and 10 nM for CGP52622A and CGP54169A, respectively (R. Walter, personal communication). Stable complexes were minimized to energies of –14 821 kcal/mol for the CGP52622A-human ODC complex and –14 807.75 kcal/mol for the CGP54169A-human ODC complex. The predicted interaction sites of CGP52622A with the human ODC did not include Thr_{933}, Ser_{1001}, Asn_{1034}, Cys_{1355}, Tyr_{1384} and Ala_{912} (PfODC numbering) shown to be involved in its interaction with PfODC (Fig. 6.6). The total calculated free energy

of the interaction between CGP52622A and the human ODC was –27.07 kcal/mol. Furthermore, CGP54629A-human ODC interactions also excluded residues Tyr_{966}, Cys_{1355} and Phe_{1392} of PfODC (Fig. 6.6) with a calculated free energy for the complex of –26.6 kcal/mol. Again, the interaction of the natural ligands, PLP and ornithine with human ODC has a somewhat higher free binding energy of –71.9 kcal/mol. Importantly, Cys_{1355} is essential to ODC activity and both inhibitors bind this residue in PfODC but not the equivalent residue of the human enzyme. Furthermore, Thr_{933}, Phe_{997} and Tyr_{966} uniquely interact with the inhibitors only in the malarial enzyme.

6.3.2) Discovery of novel ligands for PfODC.

The NCI Open database of three-dimensional chemical structures was screened against the PfODC structure for the identification of novel ligands that bind selectively against only the malarial ODC. Approximately 96724 compounds were screened and 694 potential hits were found. The top ten scoring compounds were analysed. Table 6.1 summarise the ten compounds identified as potential ligands or lead inhibitors against PfODC. The compounds are all iso- or heterocyclic compounds. Predicted K_i values were in the low μM range for all ten compounds with the top scoring ligand having a K_i of 9.33 μM. PASS predictions of the biological activity spectra of the compound detected antiprotozoal activity for 9 of the 10 compounds. Six of these have predicted antimalarial activity.

Table 6.1: Summary of the identified novel ligands for PfO ligands identified are listed in order of highest to lowest scores. Biological activity spectra (PASS prediction) are given in order of activity.

Compound	Structure	LUDI Score	K_i (μM)	PASS prediction
1-methyl-3-oxo-3-phenyl-1-propenyl difluoridoborate		503	9.33	Antimalarial Antitrypanosomal
4-chloroacetanilide		488	13.2	Antimalarial Antiprotozoal
6-methoxy-1-methyl-1-quinoline		483	14.8	Antiprotozoal Antimalarial
Azobenzene (1,2-diphenyldiazene)		467	17.4	Antitryponosomal Antiprotozoal
1-methyl-4-phenylpiperazine		465	22.4	Antitrypanosomal
2-(4-chlorophenyl)-1H-imidazole		446	34.7	Antiprotozoal Antimalarial Antitrypanosomal
4-phenylpyridine		419	64.5	Antimalarial Antitrypanosomal Antiprotozoal
3-acetyl-2,4-dimethylpyrrole		419	64.5	None
3-methyl-7-imidaso[1,5-a]pyrazin-7-ol		417	67.6	Antiprotozoal
4-quinolinol		416	69.2	Antiprotozoal Antimalarial

LigPlot analysis of the interacting residues of the top scoring compound, 1-methyl-3-oxo-3-phenyl-1-propenyl difluoridoborate (mopp-DFB), indicated that the predicted binding of mopp-DFB to PfODC is mediated by residues involved in binding of the PLP co-factor or ornithine substrate (Fig. 6.7). Residues Ser_{1001}, Asp_{887}, Cys_{1355} and Tyr_{1384} also show interactions with CGP52622A while the majority of residues (Cys_{1355}, Tyr_{1384}, Arp_{1117}, Tyr_{966}, Asp_{1356} and His_{998}) were also involved in interactions with CGP54169A. Two of the five residues shown to be present exclusively in the active site pocket of PfODC have interactions with the novel compound. Tyr_{966} is involved in hydrophobic contacts whereas Arg_{1117} is hydrogen-bonded to the compound. Importantly, the catalytic Cys_{1355} is again involved in interactions with the novel ligand.

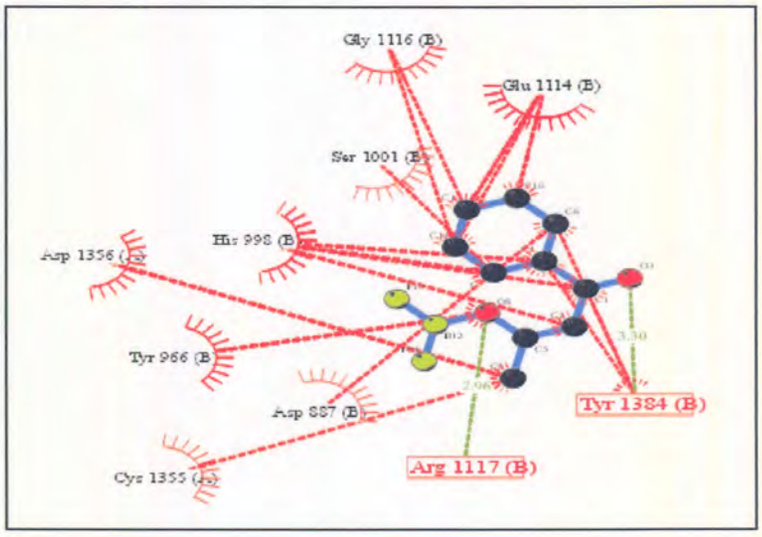
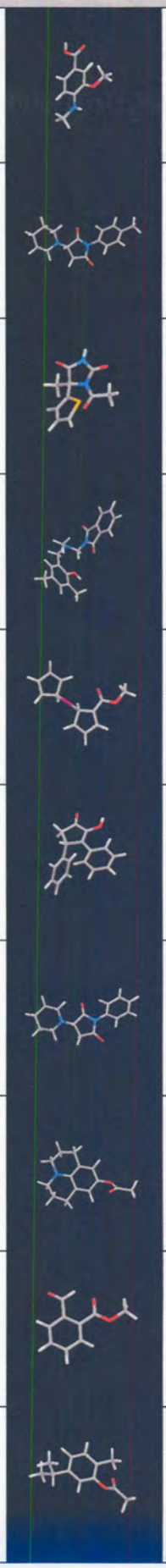


Figure 6.7: Interactions between the top scoring novel ligand and PfODC. Compound 1-methyl-3-oxo-3-phenyl-1-propenyl difluoridoborate was identified with LUDI and interactions determined with LigPlot. Hydrogen bonds and their lengths are indicated in green, non-ligand residues involved in hydrophobic contacts with the ligand in red arcs with spokes.

The specificity of the novel compounds identified as possible ligands of PfODC was validated by screening the identical NCI Open database of three-dimensional chemical structures against the human ODC structure. 1951 potential hits were found and the top ten scoring compounds are listed in Table 6.2. The predicted K_i values were lower than those found against the PfODC (between 2 and 10 μM). None of the top scoring hits identified by screening against PfODC was included in the results obtained with the human enzyme. This indicates sufficient differences between the active site pockets to allow structure-based discovery of novel ligands for PfODC.

Table 6.2: Summary of the comparative ligands of the human ODC. The top ten scoring ligands identified are listed in order of highest to lowest scores.

Compound	Structure	LUDI Score	K_i (μM)
3-methoxy-4-(methylamino)benzoic acid		564	2.29
1-(4-methylphennyl)-3-(1-piperidinyl)-2,5-pyrrolidinedione		552	3.02
1-actyl-5-methyl-5-(2-theinyl)-2,4-imidazolidinedione		551	3.09
2-(2-hydroxy-3,5-dimethylbenzyl-methyl-amino-methyl)-1H-isoindole-1,3(2H)-dione		545	3.55
2,4-cyclopentadien-1-yl(2-(methoxycarbonyl)-2,4-cyclopentadien-1-yl)iron		544	3.63
2-hydroxy-3,4-diphenyl-2-cyclopenten-1-one		530	5.01
1-phenyl-3-(1-piperidinyl)-2,5-pyrrolidinedione		502	9.55
2,3,6,7-tetrahydro-1H,5H-pyrido[3,2,1]quinolin-9-yl acetate		502	9.55
methyl 2-formylbenzoate		497	10.7
5-isopropyl-2-methylphenyl acetate		496	10.96

6.4) DISCUSSION.

6.4.1) Structural explanations for the inhibition of PfODC with known inhibitors.

The homology model created for PfODC (Chapter 5) allowed the determination of the interaction sites of DFMO and the CGP-compounds with the malarial enzyme to explain the experimental inhibition of activity. Inhibition of PfODC activity by DFMO has a K_i of 87.6±14.3 μM, almost double that of the murine enzyme (Krause, *et al.*, 2000). The majority of the residues that make contact with DFMO are usually involved in interactions with the substrate but two residues of PfODC were identified to be uniquely involved only in binding to DFMO and they are predicted to make the same contacts with DFMO as they make with ornithine. This information is useful in further mapping and validation of the PfODC active site pocket. DFMO does not show any selectivity towards experimental inhibition of ODCs from various origins (Cohen, 1998). The small differences in predicted DFMO-binding sites between PfODC and the human enzyme provide a partial explanation for the non-discriminatory inhibition. The K_i of *T. brucei* ODC for DFMO is 220 μM and can in part be explained by its long half-life (>6 hrs compared to 5-35 min of mammalian ODCs)(Ghoda, *et al.*, 1990). The slightly higher K_i of the PfODC can also be explained because this protein was also reported to be relatively stable (>2 hrs vs. 5-35 min)(Wrenger, *et al.*, 2001).

A series of potent ODC inhibitors were synthesized as analogues of 3-amino-oxy-1-propanamine (Mett, *et al.*, 1993; Standek, *et al.*, 1992). CGP52622A and CGP54169A, members of this series, were reported to inhibit rat liver ODC with an IC_{50} in the nanomolar range and are active against the growth of human T_{24} bladder carcinoma cells *in vitro* (Standek, *et al.*, 1992). They furthermore inhibit recombinantly expressed human ODC with IC_{50} values of 25 and 10 nM for CGP52622A and CGP54169A, respectively (R. Walter, personal communication) and inhibit PfODC with respective IC_{50} values of 63.5 nM and 25 nM (Krause, *et al.*, 2000). The results of Fig. 6.6 predict interaction of these compounds with essential residues for catalysis of PfODC, specifically with the catalytic Cys_{1355}, also proposed to be involved in the covalent interaction of DFMO with PfODC. The majority of the interactions were with residues normally involved in predicted interactions with the substrate (Table 5.2). This is in agreement with the original results of the activity of the CGP-compounds that indicated these inhibitors to be substrate-competitive inhibitors of mammalian ODC and do not interact non-specifically with PLP (Standek, *et al.*, 1992).

The proposed interactions of the CGP-compounds with the essential active site binding residues of PfODC would therefore prevent or interfere with any subsequent binding of the substrate/co-factor and lead to marked enzyme inhibition. Interestingly, two PfODC residues not previously implicated in ligand binding (Ala_{912} and Phe_{997}) were identified to interact with CGP52622A.

The higher free energies predicted for both CGP-human complexes compared to the energies predicted for the CGP-PfODC complexes suggest that the human ODC does not bind the CGP-inhibitors with the same affinity as PfODC. Importantly, Cys_{1355} is essential to ODC activity and both inhibitors bind this residue in PfODC but not the equivalent residue of the human enzyme. Furthermore, seven residues interacting with the PfODC does not have any corresponding residues in the CGP-human complexes. Of these parasite-specific residues Thr_{933}, Asn_{1034} and Tyr_{966}, were shown to be uniquely present in the active site pocket of PfODC (Chapter 5, Table 5.2). Taken together, the marked inhibition of PfODC seen with the CGP-compounds can be explained by their interaction with parasite-specific residues, including the essential Cys_{1355} with a higher affinity than the human ODC. Furthermore, the structures and interaction maps of these compounds could facilitate the design of even more potent, selective PfODC inhibitors in a rational drug design strategy (Bohm and Klebe, 1996).

6.4.2) Identification of novel compounds that selectively bind PfODC.

Approaches using modelled structures of malarial proteins provided promising results in other studies. Two compounds, triazinobenzimidazole and a pyridoindole were identified with experimentally determined Ki values in the low μM range (0.5-8 μM) against of DHFR (Toyoda, *et al.*, 1997; Warhurst, 1998). Novel lead inhibitors of the malarial cysteine proteases including bis[(2-hydroxy-1-naphthylmethylene)hydrazide] were identified with experimental IC_{50} values of ~6 μM (Ring, *et al.*, 1993). None of these compounds have been previously described as antimalarial agents and these compounds could act as scaffolds that can be modified to develop more potent inhibitors. The results obtained using the modelled active site pocket (and the predicted overall features of the substrate-binding site) of PfODC to explain experimental results obtained with various inhibitors lead to the further identification of novel and potential selective inhibitors of the enzyme by screening chemical structure libraries *in silico*.

The computational screening described in this chapter used the NCI Open database of chemical structures instead of more comprehensive sources of ligand structures like ACD for two reasons. Firstly, the former database contains compounds that were identified during drug development studies against cancer and includes comprehensive biological data on many of these compounds. It is therefore a chemical library that is focussed on a family of related targets rather than a targeted (only one single target) or more general library (broad interest targets). Since ODC inhibition has received a lot of attention in the antiproliferative field, using this database might provide a primary selection of ODC-specific ligands. Secondly, the database and all the compounds are publicly and freely available, making the synthesis of the compounds unnecessary if it is not commercially available.

The top ten scoring compounds obtained with LUDI (Table 6.1) were all iso- or heterocyclic compounds and were predicted to inhibit the enzyme with K_i values <100 μM (ranging between 9.33 and 69.2 μM). The two best compounds, 1-methyl-3-oxo-3-phenyl-1-propenyl difluoridoborate (abbreviated mopp-DFB) and 4-chloroacetanilide, were isocyclic. Analyses of databases of validated drugs revealed that only 32 frameworks are commonly found in drugs. These are all iso- or heterocyclic structures with benzene ring derivatives present in 606 out of 2548 drugs tested (Walters, *et al.*, 1998).

Interaction maps of the top scoring compound, mopp-DFB, indicated that this compound interact exclusively with residues involved with either co-factor or substrate binding in PfODC (Fig. 6.7). The specificity of binding of mopp-DFB is defined by two hydrogen bonds with Arg_{1117} and Tyr_{1384}. Interestingly, the majority of the PfODC residues predicted to be involved in binding with this compound is also predicted in the interaction map with the most potent experimentally observed inhibitor of PfODC, CGP54169A. Parasite-specific residues Arg_{1117} and Tyr_{966} of the PfODC active site pocket were predicted to interact with mopp-DFB. The binding to Cys_{1355} and His_{998} that are important for catalysis supports the potential inhibitory capacity of mopp-DFB against PfODC. This compound does not significantly bind to the human enzyme since it was not identified as one of the top 100 scoring compounds when the same library was screened against human ODC. This furthermore indicates the potential for mopp-DFB to selectively bind to PfODC.

Biological activity spectra predictions (PASS predictions) of the compounds indicated that the majority (9 out of 10) of the compounds might have antiprotozoal activity (Table 6.1). Furthermore, six of these compounds are further predicted to have antimalarial activity. mopp-DFB is a borate derivative and boric acid is classified under the astringent and antiseptic therapeutic categories (Windholz, 1983). Compound 2 contains an acetanilide backbone with antipyretic and analgesic therapeutic applications (Windholz, 1983). Compounds 3 and 10 are derivatives of the quinoline ring structure and could explain their predicted antimalarial activity (Gillman, *et al.*, 1985). It has been speculated that the action of chloroquine could be linked to polyamine inhibition after observations of the additive inhibitory effects of chloroquine and MGBG (Das, *et al.*, 1997) and that there was a marked induction of both ODC and AdoMetDC activities in chloroquine sensitive compared to resistant *P. berghei* (Mishra, *et al.*, 1997). However, both sensitive and resistant strains had similar polyamine profiles (Mishra, *et al.*, 1997). Compounds 4 and 5 are used in the veterinary industry as an acaricide and an antihelmintic, respectively (Windholz, 1983).

A parallel screen of the same database with the human ODC crystal structure was used to identify novel ligands that will potentially bind specifically and selectively only against human ODC. None of the first 100 compounds identified in the screen against PfODC were predicted to interact with the human enzyme. Furthermore, the top 10 scoring compounds identified against the human ODC were all more complex heterocyclic compounds compared to the top 10 scoring compounds identified against PfODC (Table 6.2 and 6.1 respectively). This furthermore indicates sufficient structural variability between the active site pockets of the human and malarial ODCs. None of the compounds identified as ligands of human ODC share structural similarities to known ODC inhibitors and therefore provide novel skeletal structures that could be further investigated.

Despite the inherent limitations of computer modelled 3D structures, these structures are helpful in defining potential lead compounds. The results presented here need further investigations to evaluate mopp-DFB and the other identified compounds as potential novel inhibitors of PfODC. This include *in vitro* determination of the Ki values of these compounds against monofunctional PfODC as well as in the bifunctional PfAdoMetDC/ODC. The parasite-specific nature of these drugs also needs to be established. The predicted novel inhibitors need to be further investigated for its *in*

vivo capability in inhibiting parasite growth in culture. Furthermore, the predicted interaction sites need to be confirmed with mutagenesis studies. Once validated, these chemical compounds can be used as starting points for the process of drug development.

The predicted structure of PfODC therefore contributed towards explaining experimental results obtained with known inhibitors (DFMO and the CGP-series of compounds) as well as defining novel putative inhibitory compounds. Goodman and Gilman define therapeutic drugs in the following manner: Many drugs stimulate or depress biochemical or physiological function in man in a sufficiently reproducible manner to provide relief of symptoms or, ideally, to alter favourably the course of disease. Conversely, chemotherapeutic agents are useful in therapy because they have only minimal effects on man but can destroy or eliminate pathogenic cells or organisms' (Gillman, *et al.*, 1985). Therefore, even after the identification and validation of a novel lead inhibitor, to ultimately describe such an inhibitor as a successful therapeutic drug necessitates major investments and investigations.

The results presented in this Chapter conclude the aims set for this study. The following Chapter will highlight the relevance of the results obtained and will focus on possible future investigations of the bifunctional PfAdoMetDC/ODC and polyamine metabolism in *P. falciparum*.

CHAPTER 7

Concluding Discussion.

Malaria is still today one of the most devastating tropical infectious diseases of mankind. The emergence of multi-drug resistant parasites and the lack of a viable vaccine have stimulated the search for new chemotherapeutic targets. The traditional approach to drug discovery entails random screening of numerous compounds for parasite inhibition activity. Usually, no specific targets are identified thereby limiting understanding of the mechanisms of action of these drugs as well as the mechanisms involved in the development of resistance (as is the case for chloroquine). A more promising approach is the molecular and biochemical characterisation of a potential target protein prior to the design of specific inhibitors. This strategy of rational drug design allows the identification of particular characteristics of the target protein that could be exploited in first-line drug design (Blundell, 1996). By applying enhanced evolution techniques, the development of possible resistance mutations could be predicted and considered in the design of second-line drugs (Stemmer, 1994).

The global metabolome analyses of *P. falciparum* presented in Chapter 1 indicated several unique metabolic pathways that could be exploited as potential antimalarial drug targets (see Chapter 1, Fig. 1.6 and Table 1.2). At the start of this study in 1999, it was clear that polyamine metabolism in *P. falciparum* was unexplored and indeed that the biochemical reactions in the biosynthesis, catabolism and interconversion of these metabolites in respect to other pathways were largely unknown. However, based on the successful inhibition of polyamine metabolism in the treatment and prevention of diseases caused by other highly proliferative cells including cancer and infective diseases, the focus shifted to the elucidation of the polyamine metabolic pathway in other parasitic protozoa. The success achieved with the inhibition of polyamine biosynthesis on *T. brucei gambiense* propagation established polyamine biosynthesis as a novel antiparasitic target (Janne and Alhonen-Hongisto, 1989a; McCann and Pegg, 1992).

The study described here therefore focused on the biochemical characterisation of the polyamine metabolic pathway of *P. falciparum* in order to elucidate parasite-specific properties that can be exploited in the design of novel antimalarial therapies. The aims

set for this study included the identification and characterisation of the genes of the rate-limiting enzymes, AdoMetDC and ODC; followed by the structure and functional characterisation of the recombinantly expressed proteins and associated parasite-specific properties. The isolation and identification of the genes for *Adometdc* and *Odc*, as well as the recombinant expression of the monofunctional and bifunctional forms of the proteins are described in this thesis. The structure-activity relationships of the unique bifunctional PfAdoMetDC/ODC led to the identification of various parasite-specific properties. Ultimately, several novel and potentially specific inhibitors against the ODC component of PfAdoMetDC/ODC were identified using comparative modelling and *in silico* structure-based drug screening. The following sections highlight the significant aspects from each chapter.

The identification of genes of *P. falciparum* has always been a cumbersome task due to reasons explained in Chapter 2. An amplified, uncloned cDNA library for *P. falciparum* was constructed in our laboratory with suppression PCR technology to overcome most of the difficulties usually experienced with application of RACE procedures for this organism. We were able to use this elegant method to amplify the *Odc* cDNA as described in Chapter 2 using both 3′- and 5′-RACE techniques. This is to our knowledge one of the first examples where a *P. falciparum* cDNA was isolated and identified using degenerate primers in RACE protocols (Birkholtz, 1998c). However, this method failed to amplify the *Adometdc* cDNA of the expected size (~2900 bp). Two reasons for this result were identified. Firstly Müller *et al.* (2000) showed that *Adometdc* and *Odc* are located on the same transcript with an ORF of ~4 kDa. Secondly, the conserved amino acid sequence of AdoMetDCs of other organisms used for the design of a 3′-RACE degenerate primer was not as conserved in the *P. falciparum* sequence.

The large monocistronic transcript of 7 kbp for *Odc* and *Adometdc* was also confirmed in our laboratory using an *Odc*-specific probe on total RNA of *P. falciparum*. The 4.2 kbp cDNA was isolated by Müller *et al.* (2000) from a *P. falciparum* cDNA library that was screened with a *Odc*-specific probe amplified from genomic DNA with primers based on expressed sequence tags published by the *Plasmodium* genome project. Subsequently, the complete *PfAdometdc/Odc* cDNA was also amplified in our laboratory using the amplified, uncloned *P. falciparum* cDNA library. Preliminary predictions of the genomic structure and flanking regions of the *PfAdometdc/Odc* gene identified regulatory sequence elements flanking the gene. The transcript is proposed to

contain a large 5'-UTR of ~2600 bp although two transcription start sites were predicted (~300 bp apart) as a consequence of the high A+T content (>85%) of UTRs of *Plasmodial* genes (Lanzer, *et al.*, 1993). The size of the 5'-UTR is in agreement with the long UTR sizes observed for most malaria genes (Coppel and Black, 1998; Su and Wellems, 1998). *Adometdc* and *Odc* 5'-UTRs characterised in other organisms are also long and share certain features, including secondary ORFs and thermodynamically stable secondary structures, both of which are implicated in the regulation of translation of the proteins (Cohen, 1998; Heby and Persson, 1990). No secondary ORF was predicted for the putative 5'-UTR of *PfAdometdc/Odc* but significant secondary structures were observed. This suggests that the translation of PfAdoMetDC/ODC is not regulated in the same manner as the single proteins in other organisms through ribosome stalling (mediated by the second ORF product) but that feedback regulation through stabilisation of the secondary structures by polyamines could mediate unique translational regulatory mechanisms (Cohen, 1998).

The recombinant expression of both AdoMetDC and ODC demonstrated that the monofunctional forms of the proteins were active albeit with much lower activities compared to these activities in the natural bifunctional PfAdoMetDC/ODC state of the protein (Chapter 3 (Müller, *et al.*, 2000). All of the essential residues for catalysis of both decarboxylase functions shown for other organisms were conserved in the bifunctional PfAdoMetDC/ODC. The two decarboxylase domains of the bifunctional PfAdoMetDC/ODC were shown to group into the expected protein families of group IV decarboxylases (ODC) and S-adenosylmethionine decarboxylases (AdoMetDC). Furthermore, complete amino acid sequences were found for the bifunctional protein in two other murine *Plasmodia*, *P. berghei* and *P. yoellii* indicating an evolutionary advantage to the malaria parasites afforded by the bifunctional organisation of these enzymes. Certain parasite-specific areas were identified in the PfAdoMetDC/ODC sequence. These include the hinge region connecting the decarboxylase domains but also three additional parasite-specific insertions. Some of these areas are extremely large (>150 residues), have repetitive, polar sequences and were predicted to contain the majority of low-complexity regions present in the protein. The identification of the genes and characterisation of the recombinant proteins describes the first aims set in this study. From this, several parasite-specific properties were evident including the proposed genomic structure of the gene, the relationship of the bifunctional protein in

Plasmodia as well as parasite-specific inserts in the amino acid sequence. The structure-activity properties of the two enzymes were further investigated as described below.

In Chapter 4, the bifunctional organisation of PfAdoMetDC/ODC and the possible involvement of the parasite-specific inserts in the activities and physical interactions between the decarboxylase domains were investigated. All the inserts were shown to be important for catalytic activity of the respective domains. However, these inserts also influence the activity of the neighbouring domain to various degrees. Physical interactions between separately expressed, monofunctional AdoMetDC and ODC resulted in the reconstruction of the bifunctional complex *in vitro*. These interactions were mediated by the smallest insert in the ODC domain as well as by the hinge region. It was deduced from the results that part of the predicted hinge region also constituted a part of the ODC domain. The insert in the ODC domain was also predicted to be the most structured indicating its functional importance in the correct association of the bifunctional PfAdoMetDC/ODC complex. Protein-protein interactions between the monofunctional ODC and AdoMetDC domains seem to be more dependent on intact ODC than AdoMetDC. Unresolved aspects arising from this work include the possibility that deletion of large areas in proteins could lead to major conformational changes. The loss of activity should therefore be investigated by systematic analyses of more defined and smaller mutations. These could include removing the low-complexity areas inside the parasite-specific inserts and mutating the NND-rich areas to less polar residues to investigate the involvement of these areas in a polar zipper (Frontali, 1994; Perutz, *et al.*, 1994). However, the ODC domain seems to be more refractory to change since even single amino acid substitutions at the dimer interface result in inactive protein due to long-range coupling to the active site (Myers, *et al.*, 2001). Although the large parasite-specific inserts will be difficult to target in an antimalarial strategy, the importance of insert O_1 in mediating physical interactions in the bifunctional heterotetrameric complex could be used in strategies to prevent this association.

To exploit the bifunctional PfAdoMetDC/ODC as a potential antimalarial target, structural data is needed to aid rational drug design strategies. In Chapter 5, a homology model of the monofunctional ODC domain is described. The model predicted a global structure corresponding to those of homologous proteins in other organisms. However, certain parasite-specific properties were identified, including differences in the active site pocket and dimerisation interface. A significant degree of deviation was also

present in the solvent-accessible loops, most of which were elongated in the malarial protein. The smallest insert described in the ODC domain (Chapter 4) was predicted to be present on the surface of the protein. The predicted homology model is consistent with mutagenesis results and biochemical studies concerning some active site residues and areas involved in stabilising the dimeric state of the protein (Wrenger, *et al.*, 2001). The model described here presents the only structural data available for this protein in the absence of a crystal structure. The size of the bifunctional protein and the presence of the parasite-specific inserts as well as the low expression levels of the recombinant protein suggest that a crystal structure for this protein would be difficult to obtain. The large, nonglobular parasite-specific inserts are furthermore often removed to obtain useful crystal structures of malarial proteins (Dunn, *et al.*, 1996; Shi, *et al.*, 1999; Velanker, *et al.*, 1997). Attempts to crystalise a similar bifunctional protein, DHFR-TS, have not been successful and a similar strategy to obtain structural data using a homology model is currently the only useful alternative (Lemcke, *et al.*, 1999; Toyoda, *et al.*, 1997). Comparison of a homology model of TIM with the crystal structure revealed little deviation between the two and indicated the potential success of such an approach (Joubert, 2000; Velanker, *et al.*, 1997). Contentious aspects of the homology model include its accuracy in the absence of knowledge of the structure of the deleted parasite-specific inserts and of the AdoMetDC domain. However, the results obtained indicated a satisfactory correspondence of the core structure of the protein to those of other eukaryotic ODC enzymes. The correctness of the predicted active site is accurate in explaining the catalytic mechanism, is supported by mutagenesis results (Wrenger, *et al.*, 2001) and consistent with the experimental inhibition observed with various known ligands (Chapter 6).

Structure-based design of novel antimalarials is a strategy that is receiving widespread attention (Bohm and Klebe, 1996; Lemcke, *et al.*, 1999; Toyoda, *et al.*, 1997). This strategy was followed in Chapter 6 to explain the experimental inhibition of PfODC activity with three different known inhibitors and to identify their interaction sites with essential catalytic residues. Novel potential inhibitors were identified by the *in silico* screening of a chemical structure library. These potential inhibitors appear to be parasite-specific since they were not predicted to bind to human ODC. These compounds need further investigations e.g. inhibition of recombinantly expressed monofunctional ODC and bifunctional PfAdoMetDC/ODC as well as *in vitro* cultures of *P. falciparum*. Once confirmed, their structures could be furthermore used as

scaffolds in the design of more specific, higher affinity inhibitors. This approach has been employed to establish the essential characteristics of an inhibitor of drug-resistant *P. falciparum* DHFR (Yuthavong, 2002).

This thesis therefore described the analyses of the rate-controlling enzymes of polyamine metabolism in *P. falciparum* and resulted in the identification of parasite-specific properties. Certain of these properties were highlighted and proposed novel functions are explained. These properties include the bifunctional organisation of malarial proteins and the parasite-specific insertions. The thesis concludes with the exploitation of these parasite-specific properties in the design of potential novel antimalarials that could be developed as inhibitors of polyamine metabolism. However, these results raised important questions that are discussed in more detail in the following sections.

The bifunctional nature and origin of PfAdoMetDC/ODC.

The bifunctional nature of PfAdoMetDC/ODC is unique in several ways. Firstly, it is a property present only in *Plasmodia* and secondly, it is composed of two proteins found on different legs of the metabolic pathway. Other bifunctional proteins described in *P. falciparum* do have homologues in other protozoa (i.e. DHFR-TS found in *T. brucei* and *L. donovani*)(Bzik, *et al.*, 1987; Ivanetich and Santi, 1990). However, the majority of the described bifunctional proteins of *P. falciparum* participate in consecutive steps in the same metabolic pathways (Clarke, *et al.*, 2001; Triglia and Cowman, 1994).

Of the bifunctional nature of PfAdoMetDC/ODC there is little doubt. The bifunctional protein is translated from a single, monocistronic transcript. The deduced amino acid sequence contains homologies to protein families to which both AdoMetDC and ODC belong. Furthermore, all the necessary residues for catalysis are conserved in the primary sequence indicating that both proteins use the same mechanisms of catalysis as their eukaryotic homologues. Indeed, both decarboxylase activities were demonstrated in the recombinantly expressed PfAdoMetDC/ODC.

The origin of the bifunctional nature of the decarboxylases is unknown. An advantageous exon-shuffling event during the evolution of *Plasmodia* is one probability. Gene duplication can probably be excluded since the two decarboxylases belong to two distinct protein families. The original mutation that resulted in gene

linkage must have occurred before the divergence of the *Plasmodial* species, since the human and murine species that are more diverse than the human and primate species, both retained the bifunctional organisation of the enzymes. Therefore, the bifunctional organisation of these enzymes must confer some specific evolutionary advantage to *Plasmodia* species.

Several speculations have been forwarded to explain the advantages of the bifunctional nature of malarial proteins. These include substrate channelling, coordinated regulation of protein concentrations/activities or intramolecular communication and interaction (Ivanetich and Santi, 1990; Müller, *et al.*, 2000). Substrate channelling can in all probability be discounted as an explanation for the bifunctional nature of PfAdoMetDC/ODC since it could not be shown that this protein interacts with the subsequent enzyme in this pathway, spermidine synthase. However, alternative mechanisms must exist to regulate both the concentration and activity of the bifunctional protein in *P. falciparum* compared to the monofunctional homologues of other organisms. Firstly, both the decarboxylase activities are enhanced when present in the bifunctional complex compared to the monofunctional PfAdoMetDC and PfODC. Therefore, the bifunctional organisation appears to be beneficial for the activities probably through stabilisation of the protein conformations even though the decarboxylase activities are able to function independently from each other (Wrenger, *et al.*, 2001). Secondly, analyses of the single transcript of PfAdoMetDC/ODC indicated that translational regulation of the bifunctional protein differs from the mechanisms regulating translation of the monofunctional protein homologues of other organisms. Thirdly, the deduced amino acid sequence of PfAdoMetDC/ODC as well as the homology model of PfODC indicated that the regulation of the protein concentration via degradation is probably different to that found in other organisms. No evidence for possible antizyme-mediated regulation was observed as for other ODCs and the PfAdoMetDC is also not stimulated by putrescine as in other organisms.

Therefore, the bifunctional nature of PfAdoMetDC/ODC is proposed to allow a combination of coordinated regulation of the decarboxylase activities and protein concentrations as well as to allow beneficial intramolecular communications between the two domains.

Structure-activity properties of parasite-specific inserts in PfAdoMetDC/ODC.

One of the most intriguing and least investigated properties of a myriad of malarial proteins is the occurence of parasite-specific inserts that intersperse adjacent conserved areas shared with other protein homologues. Speculations on the functions and evolutionary advantages of these inserts include a mechanism to evade the host immune system, interaction sites with regulatory proteins in the parasite or human host or modular mediators of protein-protein interactions (Li and Baker, 1998; Perutz, *et al.*, 1994; Ramasamy, 1991; Schofield, 1991). These parasite-specific inserts in AdoMetDC/ODC are not unique to *P. falciparum* since it was shown to be also a characteristic of other *Plasmodial* species. Strong selective pressures must therefore exist to maintain and diversify these regions (Ramasamy, 1991). The conclusion drawn from the studies presented here is that the parasite-specific inserts in PfAdoMetDC/ODC are required to mediate certain protein-protein interactions in the bifunctional protein. This is supported by the following observations:

The bifunctional nature of PfAdoMetDC/ODC presented a unique opportunity to investigate the possible functions of the parasite-specific inserts in this protein. Firstly, the inserts are indeed present in the protein and must be correctly folded to allow the heterologous expression of the active form of the protein. Secondly, amino acid sequence analyses of these inserts showed a prevalence for Asn and Asp that could form polar zippers, a characteristic that have been proposed to behave as modular mediators in protein-protein interactions (Perutz, *et al.*, 1994). Thirdly, all of the parasite-specific inserts are required for effective functioning of both the decarboxylase activities. Fourthly, the large hydrophilic inserts contained low-complexity regions and, in the case of the ODC domain at least, structural analyses predicted that these areas form nonglobular domains most likely on the surface of the proteins. Finally, the hinge region as well as the structurally conserved and smallest insert in the ODC domain was essential in mediating protein-protein interactions between the two decarboxylase domains. The structured insert was predicted to be present on the surface of PfODC. It is furthermore bordered by highly flexible Gly residues, which are proposed to allow precise interaction with the PfAdoMetDC domain.

Therefore, at least in the bifunctional PfAdoMetDC/ODC, certain parasite-specific inserts mediate physical protein interactions. These results contribute towards a better understanding of the function and evolutionary advantage of the parasite-specific inserts

and is one of the first demonstrations of the essential roles of these inserts for function and protein-protein interactions.

Structural organisation of the bifunctional PfAdoMetDC/ODC.

Throughout this thesis, various structure-activity relationships were inferred for the bifunctional PfAdoMetDC/ODC from the multimeric states of the monofunctional proteins, homology modelling structural data of the PfODC component as well as mutagenesis results.

Fig. 7.1 proposes the structure of the bifunctional PfAdoMetDC/ODC and summarises the predicted protein-protein interactions. The ODC domain is depicted as a homodimer, since activity of this protein is dependent on the association between the two monomers. Furthermore, the ODC dimer has a head-to-tail association between the two monomers with the C-terminus of one monomer interacting with the N-terminus of the second as indicated by the homology model of PfODC. Both active sites are on the same side of the protein on the dimer interface.

Results from Chapter 3 showed that AdoMetDC of *P. falciparum* is a heterotetrameric protein. In the absence of structural data for the AdoMetDC domain, the association is extrapolated from the mammalian AdoMetDC in which an edge-on association of the α-chains of the proteins occurs (Ekstrom, *et al.*, 1999). All the inserts are important for the activities of the respective domains as indicated by the blue arrows in the schematic representation. However, the inserts also affect the activities of the neighbouring domains (red arrows). The large insert in the ODC domain (O_2) partly influences AdoMetDC activity (dashed red arrows) but more pronounced influences on enzyme activities are mediated between the domains by insert A_1 in the AdoMetDC domain and O_1 in the ODC domain (solid red arrows). Physical association occurs between the AdoMetDC and ODC domains as evidenced from formation of hybrid bifunctional complexes after co-incubation of separately expressed AdoMetDC and ODC. Only parasite-specific insert O_1 seems to be essential in mediating these intermolecular interactions (yellow arrows). The hinge region seems more important for ODC activity but also has, to a lesser extent, an influence on physical association with the AdoMetDC domain. Heterotetrameric complex formation of the bifunctional protein is due mostly to interactions between the AdoMetDC domains with no apparent contribution of a

mutated ODC domain. However, intramolecular interactions are more dependent on intact homodimeric ODC to associate with AdoMetDC.

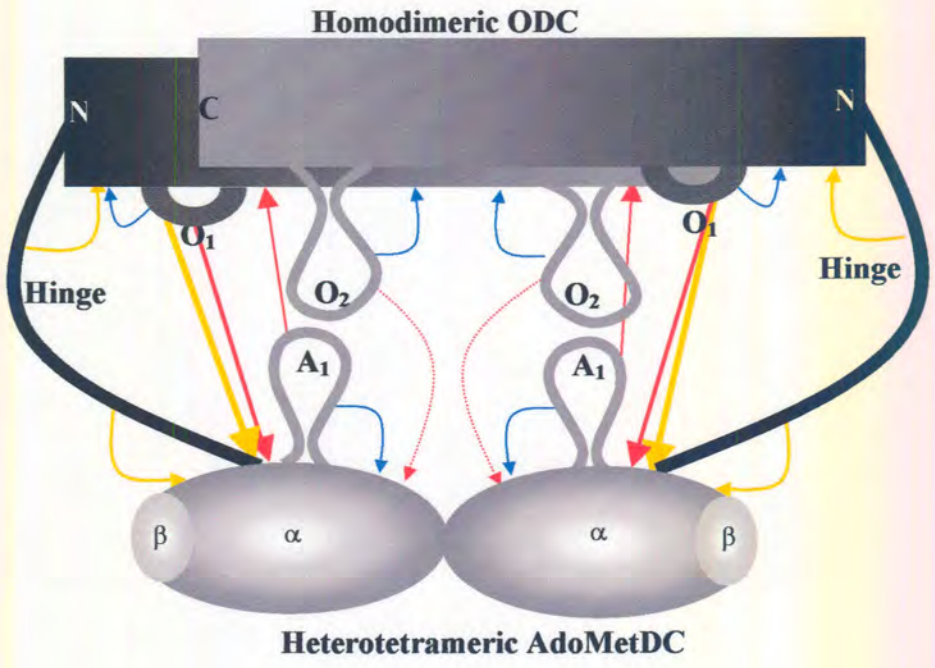


Figure 7.1: Schematic representation of the proposed structural arrangement of the bifunctional PfAdoMetDC/ODC. The obligate homodimeric ODC domain is indicated in dark blocks, and the heterotetrameric AdoMetDCs in ellipses. The hinge extends from the N-terminus of ODC to the AdoMetDC domain. The parasite-specific inserts are indicated as loops. The influences that the inserts had on the activity of the respective domains are indicated with blue arrows and influences on the neighbouring activity with red arrows. Dashed lines indicate minor roles and solid lines major influences. The contribution of the inserts to intermolecular protein-protein interactions between the domains is indicated with yellow arrows, the importance of the contribution indicated by the thickness of the arrow.

The formation of the heterotetrameric bifunctional protein is therefore due to various cumulative interactions in which the AdoMetDC domain association seems the most stable, and the ODC domain the most refractory to modifications. The parasite-specific inserts mediate some of the protein-protein interactions. In order to validate the proposed structural organisation of the bifunctional PfAdoMetDC/ODC, experimental structural data for the complete complex or, in the absence thereof, for the monofunctional domains are required. However, the crystallisation of various malarial proteins are complicated by the unstructured nature of inserted amino acid sequences containing mostly low-complexity regions. The low expression levels of the recombinant bifunctional PfAdoMetDC/ODC further impede such a strategy. Recent advances in the field of cryo-electron microscopy by which protein structures can be

determined to at least 3 Å could be employed to provide a clearer picture of the bifunctional arrangement of these proteins.

Polyamine biosynthesis of *P. falciparum* with reference to PfAdoMetDC/ODC as a viable drug target.

The ultimate aim of molecular and biochemical characterisation of metabolic processes of *P. falciparum* is to aid the design of novel antimalarial chemotherapies. However, before any metabolic pathway can be validated as a potential target for chemotherapy, it is imperative to understand the biochemistry of the processes involved. The identification of parasite-specific properties could be useful in the design of selective inhibitors.

The bifunctional nature of PfAdoMetDC/ODC and associated parasite-specific inserts present unique properties to investigate these as potential targets to inhibit polyamine metabolism. However, to date no drug could be shown to selectively inhibit either AdoMetDC or ODC activity of *P. falciparum* due to the shared conservation of the active sites of malarial and mammalian enzymes. Structural characterisation of the PfODC domain with homology modelling revealed parasite-specific residues in the active site pocket as well as at the entrance to the pocket. Additionally, the dimer interface revealed several interactions that were unique to PfODC and useful for the selective prevention of dimerisation. Novel putative inhibitors that are predicted to bind only to PfODC could be identified by *in silico* screening of chemical structure libraries.

Beyond active site targeting strategies, the bifunctional organisation of the malarial PfAdoMetDC/ODC compared to the monofunctional homologues of mammals presents another targeting strategy. A better understanding of the potential role of the parasite-specific inserts to mediate physical interactions between the domains in the bifunctional protein provides a distinct opportunity for selective chemotherapy against malaria. The greater structural variability of protein-protein interfaces, which in PfAdoMetDC/ODC also contain parasite-specific regions, suggests sufficient differentiation between such contact sites of the host and parasite enzymes. The use of small molecular weight peptidomimetics that disrupt protein-protein interactions has been explored for drug development in many systems including *Lactobacillus casei* thymidylate synthase (Prasanna, *et al.*, 1998), HIV-1 protease (Schramm, *et al.*, 1996; Zutshi, *et al.*, 1998), herpes simplex virus DNA polymerase and human glutathione reductase (Zutshi, *et al.*,

1998). Recently, synthetic peptides were also shown to prevent dimerisation and effectively inactivate *P. falciparum* TIM (Singh, *et al.*, 2001).

However, consideration of polyamine metabolism in *P. falciparum* as a viable target for chemotherapy requires demonstration of its relevance in parasite survival. Supporting evidence for validation of a metabolic pathway or specific enzyme as a chemotherapeutic target include 1) comparisons of profiles between enzyme inhibition and antiparasite action among a large number of chemical derivatives of the drug, 2) characterisation of the putative drug target from drug-resistant mutant parasites and 3) knockouts of the gene encoding the putative target accompanied by subsequent complementation of the missing gene (Wang, 1997). Of these, the latter may prove the most conclusive validation of a target and has been used to show that ODC from *T. brucei* is a *bona fide* target (Wang, 1997). Transfection is not as easily achieved in *P. falciparum* and results of knockout experiments for the bifunctional PfAdoMetDC/ODC are still lacking.

Inhibition of polyamine biosynthesis in *P. falciparum* is usually cytostatic rather than cytotoxic due to the inherent homeostatic mechanisms in place to control intracellular polyamine levels. This may include the transport of polyamines by the parasites from the extracelullar milieu or poor uptake of the drugs (Fukomoto and Byus, 1996; Müller, *et al.*, 2001). Folate metabolism is regarded as a validated chemotherapeutic target in protozoa, even in the presence of a folate transport system. The synergistic use of inhibitors against two enzymes in the pathway, DHPS and DHFR, overcomes resistance seen due to increased folate transport compared to when DHPS inhibitors are used separately. It was proposed that the DHFR inhibitor pyrimethamine counters the so-called folate effect probably through blockage of one or more steps in the uptake and utilisation of exogenous folate (Hyde, 2002). These same principles could be applied to the inhibition of polyamine biosynthesis in *P. falciparum*. Firstly, because of the bifunctional nature, inhibition of both decarboxylase activities would act synergistically in limiting polyamine biosynthesis. Furthermore, the combination with a polyamine transporter inhibitor would further contribute to foil the homeostatic mechanisms. Common features shared between the substrates/products of these enzymes and recognised by the polyamine transporter need however to be identified to exploit this route of drug design.

Future directions in the study of polyamine metabolism in *P. falciparum*.

This study raised several interesting questions that need to be further investigated. At the start of the study, the genomic structure of the *PfAdometdc/Odc* gene and transcript was proposed. However, the nature of the 5'-UTR and specific contribution this region do make to regulate translation of the transcript should be investigated through analyses of the complete nucleotide sequence of the transcript as well as *in vitro* translation studies to determine the various effectors needed during formation of active PfAdoMetDC/ODC protein. This will also aid in determining how the bifunctional protein is regulated post-translationally. Structure-activity investigations reported here indicated various parasite-specific properties including those of the parasite-specific inserts and their involvement in protein-protein interactions. Finer dissection of the proposed functions attributed to these inserts should be considered, including deletion of only the low-complexity regions in these inserts and mutation of the polar repeat sequences. The structure and organisation of the bifunctional PfAdoMetDC/ODC will be clarified once structural data for the AdoMetDC domain is available. The creation of a homology model of the AdoMetDC domain will greatly aid in the understanding of the interactions between the two decarboxylase domains. Ultimately, a complete three-dimensional structure of the bifunctional protein will have immense value in explaining the parasite-specific properties as well as aiding the design of novel inhibitors.

In Chapter 1, the metabolome of *P. falciparum* was represented. However, this did not include details on the possible interacting pathways and physiological functions of the polyamines in this parasite. In order to fully understand the metabolome and the effects that inhibition of polyamine biosynthesis will have on *P. falciparum*, additional data is required. The transcriptome (mRNA profile) of *P. falciparum* after polyamine depletion will identify upregulated transcripts and the corresponding proteome will identify the mechanisms utilised by the parasite to compensate for the lack of the essential metabolites. Furthermore, enzymes from related and interacting pathways (polyamine interconversion, methionine recycling and polyamine transport) could also be identified and collectively the new data will identify new potential drug targets as well as a more complete understanding of the polyamine metabolic profile of *P. falciparum*.

Albert L. Lehninger said the following on the challenge to Biochemists (Lehninger, 1975):

'Living things are composed of lifeless molecules. When these molecules are isolated and examined carefully, they conform to all the physical and chemical laws that describe the behaviour of inanimate matter. Yet living organisms possess extraordinary attributes not shown by collections of inanimate molecules.'

It therefore remains our responsibility as scientists not to become too absorbed in the spectacular peculiarities of single proteins or nucleic acids, but to try and unravel the mysteries of nature by completing the biochemical network from which cellular entities arises.