Chapter 5
Concluding Discussion

5.1 'Discover, Develop, Deliver'

In the light of the decreasing effectivity of mosquito control and the absence of a safe and effective vaccine, antimalarial chemotherapy remains the first line of defence against malaria. Of the different antimalarial control strategies, the use of drugs is furthermore the only one that functions on two levels: prevention and cure. Over the past few decades, malaria has become predominantly an African problem due to neglected malaria control strategies and poor infrastructure, emphasising the dire need for cheap, effective and safe drugs that are readily available (www.rbm.int). The Medicines for Malaria Venture (MMV) slogan: '*discover, develop, deliver*' aptly describes the key factors involved in achieving this. The scope of this study lies within the 'discover' aspect: the evaluation of newly identified potential drug targets.

5.2 Antifolates: New targets, old pathway

In this study the 'discover' aspect is applied to the folate pathway, since it is a verified antimalarial drug target and current antifolates are losing their effectivity due to parasite resistance. Since the presence of folate cofactors is essential in all organisms, the antifolates are also widely used in cancer- and bacterial chemotherapy and thus forms a class of well-studied compounds (Moran, 1999). Antifolates also were among the first rationally designed chemotherapeutic compounds (Costi and Ferrari, 2001). The potential of this pathway for drug inhibition of *P. falciparum* has, however, been largely underexploited since only two enzymes of the pathway, DHPS and DHFR are proven drug targets. Resistance caused by mutations in both these drug targets greatly compromises the effectivity of current antifolates. It would be possible to rationally design novel antifolates against these well studied drug targets to improve the inhibitor efficiency, but it has been shown in the past that novel drugs and drug combinations against DHFR and DHPS only select for more dangerous and more

resistant strains carrying multiple mutations (Sibley *et al.*, 2001). Considering that certain proteins are more prone to obtaining resistance-causing mutations, the development of novel drugs targeted to other folate enzymes would circumvent this problem. The identification of three new *P. falciparum* genes encoding the folate biosynthetic proteins GTP-CH, SHMT and the bifunctional DHFS-FPGS, was thus an important event that laid the foundation for future drug target validation and drug discovery (Lee *et al.* 2001).

5.3 *P. falciparum* DHFS-FPGS: an attractive drug target

Of the different novel genes identified for folate-metabolism, the gene encoding DHFS-FPGS activity seems to be the most promising drug target. When compared to human FPGS, the DHFS-FPGS enzyme is an attractive drug target for selective inhibition due to the additional DHFS activity, low similarity and its bifunctionality, which is unique to the malaria parasite. Furthermore, these two enzymes function in different routes of folate metabolism; *de novo* folate synthesis (which can be bypassed in certain *P. falciparum* isolates by high serum folate concentrations) and folate salvage (refer to section 1.5.4). Furthermore it was shown in a functional complementation study that the current antifolates, which are structural analogs of the natural substrates don't select for resistant phenotypes of DHFS-FPGS as measured by mutations in the corresponding gene (Salcedo *et al.*, 2001). As shown in Chapter 4, the *P. falciparum* enzyme furthermore has characteristic features such as a parasite specific N-terminal extension, a divergent C-terminal domain and possibly different C-terminal catalytic residues, which might account for structural and functional differences and thus play an important role in the selective inhibition of the parasite enzyme. To properly evaluate the potential of DHFS-FPGS as a drug target, however, enzyme kinetics and structural studies need to be done, which require large amounts of active enzyme.

5.4 The aims of this study: obtaining sufficient amounts of active *P. falciparum* DHFS-FPGS

Malaria parasite genes are notorious for the problems involved in heterologous expression, brought about by the high A+T content and codon bias of the malaria genome, which results in low levels of gene expression (Baca and Hol, 2000). To address this problem, *P.*

falciparum dhfs-fpgs was synthesised as described in Chapter 2 with preferred *E. coli* codons and lower A+T content for high-level recombinant expression in *E. coli*. The method used for synthesis, the overlap-extension PCR of partially complementary oligonucleotides, was optimised and correct sequences could be obtained from as little as 30 total PCR cycles (assembly and amplification) of 1 pmole of partially overlapping oligonucleotides, in comparison with previous reports using 125 pmoles of complete overlapping oligonucleotides for a total of 50 PCR cycles (Carpenter *et al.*, 1999). The method used in this study therefore not only cut the costs of the gene synthesis with ~20% but also increased the efficiency of the PCR method. The optimisation revealed that crucial factors for obtaining the correct sequences were the quality of the oligonucleotides, the use of additional Mg^{2+} during the assembly step for efficient annealing of the overlaps, the use of a proofreading DNA polymerase as well as the use of a two-step PCR protocol; consisting of a limited number of assembly PCR cycles followed by dilution of the assembly mixture and primer addition for the specific amplification of the gene fragment. One of every three clones isolated contained the correct sequence. The full-length gene was also successfully constructed from ligation and further PCR of the correct subfragments.

This codon-optimised gene was then expressed in a variety of different vector systems and *E. coli* expression hosts to obtain sufficient amounts of active protein as described in Chapter 3. High-level gene expression in fast-growing BL21 Star (DE3) cells became toxic to the cells and the protein was produced in inclusion bodies. DHFS-FPGS is normally expressed in very low native levels, ~4% of the total proteins expressed by *E. coli* (Bognar *et al.*, 1985) and overexpression of human FPGS results in cancer (Osborne *et al.*, 1993), which might explain the insolubility observed with the high level expression of the synthetic gene. Lower expression levels in slower growing BL21 (DE3) pLysS cells produced lower amounts of total protein but more soluble protein. It was also found that the removal of a tag decreased total expression but increased soluble expression. By means of functional complementation it was shown that all the different synthetic DHFS-FPGS constructs contained DHFS and FPGS activity and complemented the deficient *E. coli* cell line to a greater extent than the native *P. falciparum* DHFS-FPGS.

The primary aims of the thesis was thus achieved by:

- the successful synthesis of the codon-optimised *P. falciparum dhfs-fpgs* and

- expression of active DHFS-FPGS from the synthetic gene at higher levels than the native gene.

Furthermore, functional complementation indicated that the tagless and C-terminal His_6-tagged DHFS-FPGS had the most DHFS and FPGS activity and preliminary purification consisting of affinity chromatography and size exclusion HPLC of the C-terminal His_6-tagged DHFS-FPGS proteins was performed as groundwork for future purification strategies.

The *in silico* analysis of DHFS-FPGS also identified possible parasite specific features, such as a 40 amino acid N-terminal extension consisting of low complexity sequence and a low conserved C-terminal domain. Mutagenesis strategies aimed at the cassette replacement of these domains could provide valuable structural information in future.

5.5 A look into the near and distant future

Due to the complexity and cost of the enzyme assay as well as the instability of the substrates, the purification of the DHFS-FPGS enzyme will first have to be optimised so that activity assays can be done on the enzyme obtained after different purification steps at the same time. Guided by the results from functional complementation, it seems that tagless DHFS-FPGS would be the preferred enzyme for activity assays. The purification of this enzyme however is complicated through the absence of a tag. Using the C-terminal His_6-tagged DHFS-FPGS, however for affinity purification, combined with detection of the tagged enzyme with anti-His antibodies; the size exclusion and anion exchange HPLC purification of the C-terminal His_6-tagged enzyme could be optimised and used to purify the tagless protein. Once sufficient amounts of protein are obtained, activity assays could then be performed. Polyclonal, monospecific antibodies raised against epitopes of tagless DHFS-FPGS could furthermore be used for the large-scale purification of active, correctly folded enzyme. This could then be used for the X-ray crystallography or NMR analysis of the enzyme and thus determination of its three dimensional structure. This information would be invaluable for the rational design and prediction of inhibitors as well as to provide insights on the role of DHFS-FPGS enzyme in the folate metabolism of the malaria parasite and thus provide new options for selective inhibition. In concluding the 'discover' aspect of antimalarial drug strategies, the development of novel antifolates targeted to this novel folate enzyme, which is potentially

less prone to resistance development could revolutionise the current antifolate status, by replacing drugs of declining efficiency with highly effective, novel inhibitors against which the parasite has no defence mechanism from previous exposure. Alternatively, the novel antifolates could be used in combination with current antifolates and thus control the parasite by means of synergistic inhibition of different points within the same pathway. This would mean that the 'develop' aspect has been successfully completed. If the cheap, effective drugs with short half-lives are widely distributed through large-scale control strategies, the 'deliver' aspect of antimalarial drug treatments could achieve stringent malaria control to relieve the burden caused by malaria on the developing countries of Africa.