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Chapter 1

Literature Review

"Our vision is a world in which affordable drugs will help eliminate the devastating effects of malaria and help protect the children, pregnant women and vulnerable workers of developing countries from this terrible disease."
-Medicines for Malaria Venture, 2003

1.1 The burden of malaria

In Africa, a child under the age of five dies of malaria every 30 seconds of every day (2003 Africa Malaria Report by the World Health Organisation (http://mosquito.who.int/amd2003). The total malaria mortality in sub-Saharan Africa constitutes about 90% of nearly 3 million deaths caused by the disease per year. Adults living in endemic areas have acquired some immunity to malaria and therefore its impact is mostly observed in children (Marsh *et al.*, 1995). Low birth weight, improper nutrition, low school attendance rates (Brooker *et al.*, 2000), learning disabilities, behavioural disorders and loss of motor functions are disease-related factors that impede proper education and thus the development of the population in general (Holding and Snow, 2001). Apart from the social burden, other repercussions of the disease are immense poverty and lack of development in endemic countries, brought about by escalating public health costs and loss of labour (Gallup and Sachs, 2001). The economic burden of malaria is evident through the direct geographic correlation between disease severity and poverty and resultantly the percentage total income of an endemic country is reduced by up to a half of its potential (Sachs and Malaney, 2002).

1.2 Malaria pathogenesis

Plasmodium falciparum is the parasite species responsible for the most severe form of the disease in humans (Miller *et al.,* 2002). Humans are the hosts for the asexual phase of the parasite life cycle and contract the illness from the bite of an infected female *Anopheles* mosquito, the host for the sexual development phase (Figure 1.1). The exo-erythrocytic cycle starts with the infection of liver cells by sporozoites where they develop into schizonts. Schizonts rupture to release merozoites into the blood stream to re-invade red blood cells. During this process the red blood cells become deformed as the merozoites develop into trophozoites (Miller *et al.,* 2002). Most damage occurs during sequestration when infected red blood cells adhere to capillaries and restricts blood flow to the organs, resulting in organ failure (Miller *et al.,* 2002). Trophozoites develop into gametocytes, which are released through red blood cell lysis, ending the erythrocytic cycle. As the mosquito takes up blood from the human host, the sexual (sporogenic) cycle starts by fusion of the gametes in the mosquito gut. The main clinical symptom responsible for mortality is respiratory distress due to decreased oxygen supply to tissues. Lactic acidosis, severe anaemia and coma are other symptoms resulting ultimately in damage to the cerebral- and central nervous systems, and also the kidneys and lungs (Miller *et al.*, 2002).

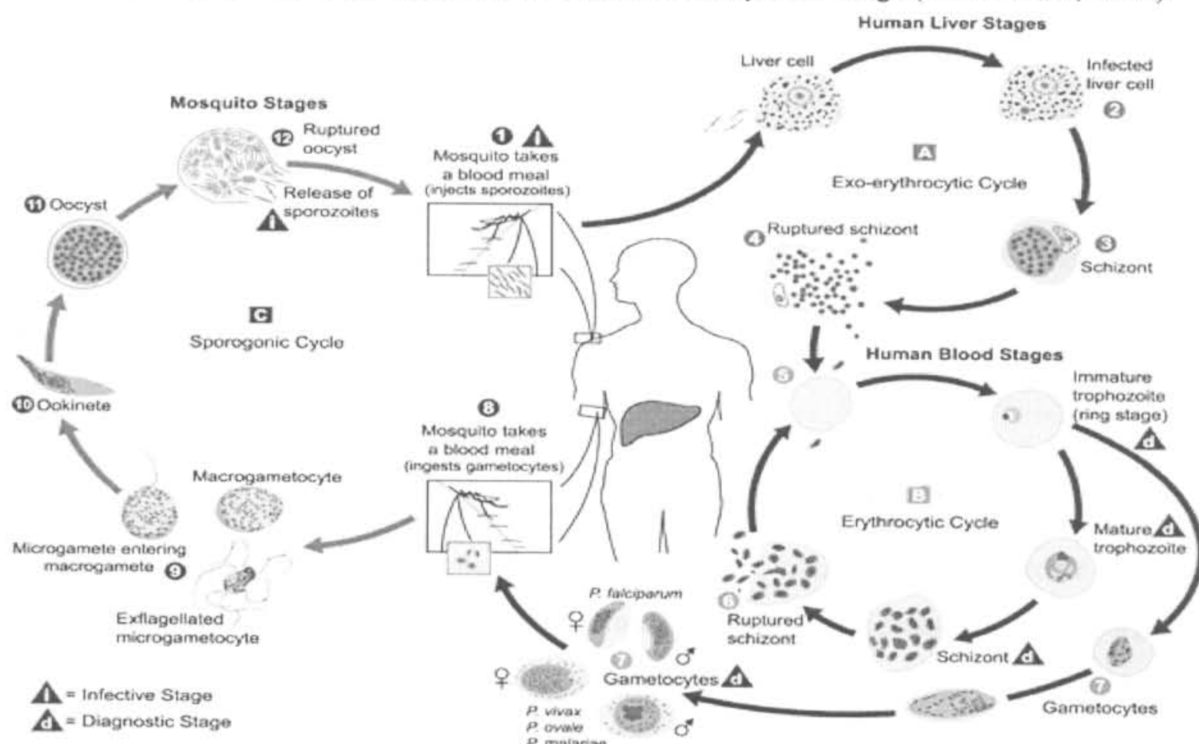

Figure 1.1: The life cycle of the human malaria parasite *P. falciparum*. (www.dpd.cdc. gov/dpdx/HTML/Malaria)

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1.3 The multifaceted problem of malaria

As described in section 1.2, the chain of malaria transmission can thus be grouped into three interconnected links. Current malaria control strategies aims at the disruption of either of these links or disruption of contact points between the links (Figure 1.2). Apart from strategies targeting each link separately, transmission control focuses at the contact points between the mosquito and parasite and mosquito and human, whereas disease control focus on the contact points between the parasite and humans. The main problems of malaria control strategies are the resistance of the parasite to chemotherapeutic drugs, insecticide resistance of the mosquito and social and environmental changes brought about by human migration (Muentener *et al.*, 1999). Each of these control strategies will be discussed in more detail in the following sections.

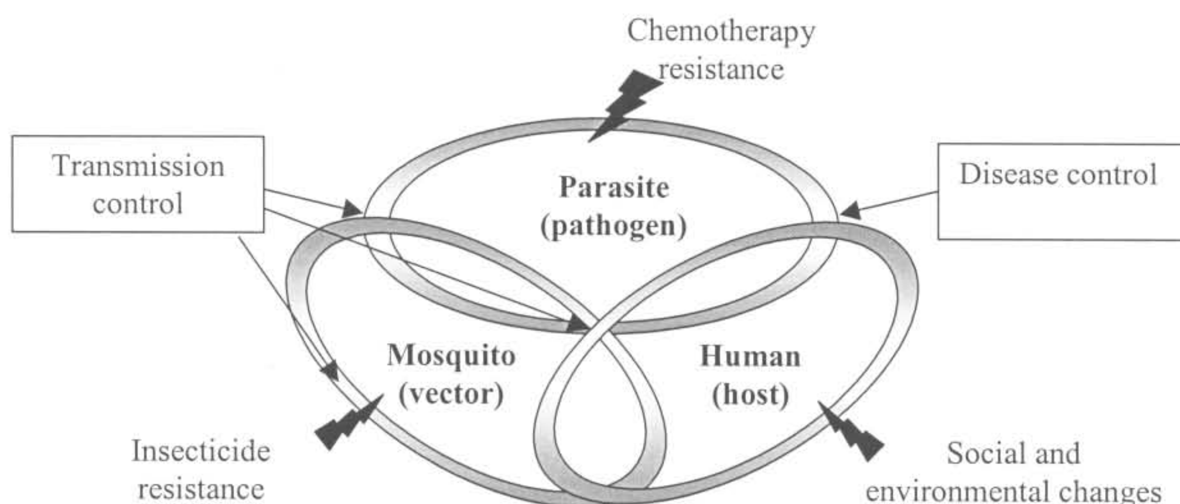

Figure 1.2: The three main links in the cycle of malaria transmission.

1.3.1 Vector control

Transmission control strategies aimed at the main mosquito vector, *Anopheles gambiae,* involves primarily the use of insecticide-treated bed nets to minimise human-vector contact (Armstrong-Schellenberg *et al.*, 2001). Pyrethroid resistance of the mosquito vector largely hampers the use of such insecticides (Kristan *et al.*, 2003). Genetic strategies aimed at genotyping different mosquito species for determination of transmission patterns are curbed by the diverse genotypes of the *Anopheles* species (Gentile *et al.*, 2001). The

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availability of the *Anopheles gambiae* genome however, is a recent advance that would provide a platform for better vector characterisation, understanding of the mechanisms of insecticide resistance and pave the way for the development of new effective insecticides (Hoffman *et al.,* 2002). At the contact point between the mosquito and parasite, attempts to develop transgenic mosquitoes incapable of sustaining the parasite, might result in the reduction of parasite transmission (James, 2003). At the contact point between the mosquito and human, satellite imagery is employed to predict the variation in *A. gambiae* distribution and density that is caused by climatic and environmental changes and thus high transmission risk areas (Rogers *et al.*, 2002).

1.3.2 Host genetic factors

Individual genetic traits such as the human leukocyte antigen (HLA)-, haemoglobin- and red blood cell type are responsible for different host susceptibilities (Miller, 1999). Single nucleotide polymorphisms (SNPs) within various immune response genes of infants were analysed by means of the amplification refractory mutation system PCR (ARMS PCR), which showed that a CD36 T1264G mutation was responsible for the hosts' parasite clearing phenotype (Djimde *et al.*, 2002). In another independent study it was also observed that resistance to malaria was linked to glucose-6-phosphate dehydrogenase (G6PD) and CD40 ligand variants containing protective mutations (Sabeti *et al.*, 2002). Since human susceptibility is mostly an individual characteristic, this information though useful, is limited by the patients' genotype.

1.3.3 Pathogen control

1.3.3.1 Cutting-edge technologies

In 2002 the full genome sequence of *P. falciparum* (strain 3D7) was completed (Gardner *et al.,* 2002). This 30 million basepair genome is divided into 14 chromosomes, a mitochondrial genome and a circular plastid genome, which all have an extraordinary high A+T content of approximately 80% (Gardner *et al.*, 2002). Roughly 5400 protein-encoding genes were identified, 60% of which have unknown functions (Gardner *et al.*, 2002). The availability of the sequence data has thus enabled the following functional genomics studies of the parasite:

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- Knockouts for the assignment of unknown gene functions, such the functional assignment of knob-formation to the HRP1 gene (Crabb *et al.*, 1997)
- Comparative genomics between different *Plasmodium* species, which led to the construction of the *Plasmodium* genome database (http://PlasmoDB.org).
- Comparative genomics between *Plasmodium* genes and their homologues (Wirth, 2002).
- Assignment of variant antigen families (Carlton *et al.*, 2002)
- Determination of drug resistance loci (Wirth, 2002).
- Microsattelite typing to identify areas under selection pressure (Li *et al.*, 2002)

For the first time it is possible through these cutting-edge technologies to obtain a global view of the functioning of the parasite in terms of the regulation of mRNA production (transcriptome) as well as protein expression (proteome). A better understanding and prediction of the response of the parasite when exposed to various external conditions would enhance the current pathogen control strategies.

1.3.3.2 Vaccines

Malaria is prevalent in countries that lack the financial and social infrastructure to effectively prevent such an illness by the distribution of bed nets and expensive prophylaxis. Compliance by the community is also a serious problem. An effective and safe vaccine would address these issues by means of a single-dosage treatment for sustained pathogen control. Most effective vaccines in the past (e.g. against rabies) have been directed towards infectious agents that elicit immune responses from their host and are then eliminated by the body's own defence system. The malaria parasite, however, has elaborate mechanisms to evade the human immune system and so establish a chronic infection, which is beneficial for the continuance of the parasite life cycle by means of transmission to the mosquito (Saul, 1999). In cases where evasion by the parasite fails, the human immune response manages to control the parasite infection but the exact mechanism by which this happens is not known (Richie and Saul, 2002). A second problem is the immense variability of parasite antigens, which depend on the specific stage of the life cycle of the parasite as well as the polymorphism of such genes (Saul, 1999). Lastly, humans also vary greatly in terms of their immune response towards the parasite,

which depends on their haemoglobin type and HLA (human leukocyte antigen) locus (Richie and Saul, 2002).

On the other hand, the fact that individuals repeatedly infected with malaria develop a natural acquired immunity that prevents the fatal symptoms involved in the disease (as mentioned in 1.3.2), combined with the success of vaccines in animal models, raises the hope that a vaccine is a realistic expectation (Stowers *et al.*, 2001). Current vaccine strategies are aimed at the pre-erythrocytic stage (Kester *et al.*, 2001), the red blood cell stages (merozoite surface protein 1 and 3) (Stowers *et al.*, 2001) of the parasite life cycle or a combination of stages through the use of multiple epitopes (Doolan and Hoffman, 2001). The MVI (Malaria Vaccine Initiative, Maryland, USA) launched clinical stage one trials in children under five years of age for a sporozoite vaccine candidate; RTS,S (CS circumsporozoite protein containing a hepatitis B virus core and adjuvant for enhanced response) in July 2003 and the results will be available at the end of 2005 (Kester *et al.*, 2001). Despite much time, cost and effort, an effective vaccine still remains elusive and will therefore not be available in the near future.

1.3.3.3 Drugs

In contrast to vaccines, drugs have been available for over fifty years and are currently the primary aspect in the parasite control strategy (www.rbm.int). The main problem with the use of drugs is the rapid evolution of drug-resistant parasite strains (Sibley *et al.*, 2001). This is mainly the result of non-compliance by the population where the parasites are not totally eradicated, but remain in undetectable levels in the blood and so develop resistance. This is also influenced by the biological half-life of the drugs. The longer the half-life, the higher the chances of resistance development since the drugs then take longer to be cleared from the body (Ridley, 2002).

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Table 1.1: Problems associated with antimalarial drugs (adapted from Ridley 2002).

Antimalarial drug	Main limitations
Prophylaxis	
• Chloroquine	• Resistance
• Amodiaquine	• Safety, resistance
• Mefloquine	• Safety, resistance, cost
• Halofantrine	• Safety, resistance, cost
• Doxycycline	• Low effectivity
Treatments	
• Sulfadoxine-pyrimethamine (treatment)	• Resistance
• Quinine	• Compliance, safety, resistance
• Atovaquone-proguanil (malarone)	• Potential resistance, cost
• Dapsone-proguanil (lapdap)	• Potential resistance, cost
• Lumefantrine-artemether	• Compliance, potential resistance, cost
• Artemisinins (artemether, erteether, artesunate)	• Compliance, safety, cost

For drugs to be a viable control strategy in Africa, they have to meet the criteria of low costs (to enable large scale distribution), short half-lives or single dosage options (to lessen the effect of weak compliance), effectivity and safety (Ridley, 2002).

The genome sequences of the parasite, plastid and mitochondria provide a virtually unlimited source for the discovery of novel drug targets and screening of possible inhibitors. The next section will describe in more detail the focus areas of current drug development, the functions of the mentioned antimalarial drugs and future prospects.

1.4 Drug targets

The effectivity and safety (as determined by parasite specificity) of drugs depend mainly on differences between the parasite and its hosts' metabolism. The molecular targets are not known for all drugs and therefore drugs can be broadly classified according to their subcellular locations (Figure 1.3). The figure below indicates the subcellular compartments

where current antifolates are active as well as explores the options for future drug targets. Current antimalarial drugs are either targeted to the lysosomal food vacuole (quinolines), mitochondrion (atovaquone), apicoplast (doxycycline) or the cytosol (antifolates). For each of these compartments, many other potential drug targets exist such as proteases (lysosomal food vacuole), dihydroorotate dehydrogenase (mitochondrion), fatty acid type II biosynthesis (apicoplast), enzymes involved in folate and nucleotide biosynthesis (cytosol) as well as nutrient transport mediated by the plasma membrane proteins.

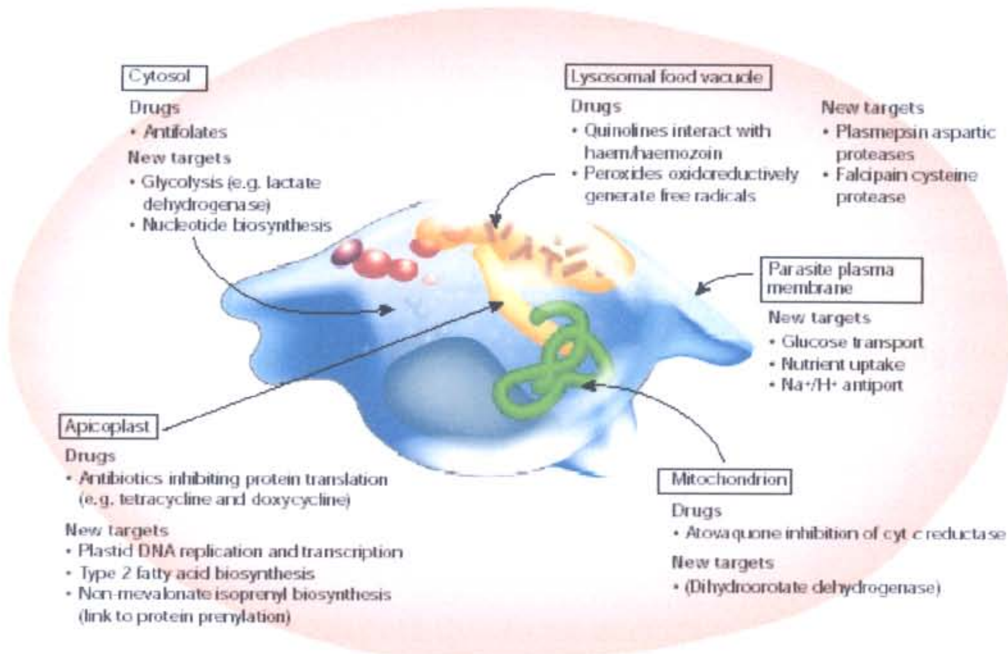

Figure 1.3: The subcellular locations of past, present and future drug targets. A schematic representation of a trophozoite in a red blood cell (Ridley, 2002).

1.4.1 Haemoglobin degradation in the lysosomal food vacuole

Quinoline and artemisinin antimalarial drugs are concentrated in the food vacuole (Figure 1.3). These drugs are expected to interfere with the breakdown of toxic haem, Fe (II) to the oxidised form hematin, Fe (III) and the subsequent formation of hemozoin (Francis *et al.*, 1997). The molecular target of artemisinin drugs was determined as PfATP6, which is a sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase (SERCA) ortholog but the molecular targets of the quinolines are unknown (Krishna *et al.*, 2003). Novel targets involved in haem metabolism are studied as putative drug targets e.g. the aspartic proteases or plasmepsins, the cysteine protease falcipain, and the metallopeptidases (Coombs *et al.*, 2001) (Shenai *et al.*, 2000) and (Eggleson *et al.*, 1999).

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1.4.2 Apicoplast metabolism.

The apicoplast is an organelle unique to the malaria parasite, which is similar to a plant's chloroplast. It also contains prokaryotic transcription and translation elements on its circular chromosome that could possibly explain the antimalarial action of bacteriostatic agents such as doxycycline (Fichera and Roos, 1997). The apicoplast proteins comprise 10% of the total parasite proteins and were identified by means of a signal signature motif shared between these proteins (Foth *et al.*, 2003). This expands the drug target possibilities immensely and opens new possibilities for herbicides as drugs. The herbicide fosmidomycin targeted to 1-deoxy-D-xylulose-5-phosphate synthase, was for instance shown to have anti-malarial activity (Lell *et al.*, 2003). The farnesyltransferase proteins which link up with the deoxyxylulose phosphate (DOXP) pathway through the farnesylation of isopentenyl diphosphate might also be potent drug targets (Ohkanda *et al.*, 2001). The apicoplast is furthermore the centre for type II fatty acid synthesis (Figure 1.3). In humans the fatty acid synthase II system (FASII) is absent. One of the drug discovery projects launched by the Medicines for Malaria Venture (MMV) is the development of inhibitors against enoyl ACP reductase, a key enzyme in this metabolic pathway (MMV Annual report 2002).

1.4.3 Limited electron transport due to an acrystate mitochondrion

The malaria parasite lives in an oxygen poor environment and relies on glycolysis as the main source of ATP. There is thus no real oxidative phosphorylation and enzymes normally associated with electron transport perform other functions. An example of this is cytochrome *c* reductase, which is linked to dihydroorotate dehydrogenase, an enzyme involved in nucleotide biosynthesis (Fry and Beesly, 1991).

1.4.4 Cytosolic and membrane bound drug targets

The dependence of the parasite on glycolysis for energy production (as described in 1.4.3 above) also makes the cytosolic enzyme lactate dehydrogenase (LDH), which is involved in anaerobic glycolysis, an important target (MMV Annual Report 2002). Although humans have LDH, they are less dependent on this enzyme for energy production since the main source of ATP production is the citric acid cycle and electron transport systems (Koukourakis *et al.*, 2003). The primary cytosolic target is the folate pathway. Folate

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metabolism is a validated drug target since it differs significantly between the human host and parasite (Ferone, 1977). Past successes with the use of the antifolates furthermore underscores the importance of folate metabolism as a drug target. The current resistance of the malaria parasite to the available antifolates however calls for new drug targets to be identified within this pathway. Section 1.5 will deal with folate metabolism of the malaria parasite, its current status, targets and future possibilities.

Apart from the above-mentioned targets, a variety of membrane proteins involved in e.g. the transport of glucose, other nutrients and essential ions are also potential important points of drug inhibition (Manning *et al.*, 2002) and (Desai *et al.*, 2000).

1.5 The folate pathway

1.5.1 Folate metabolic organisation

Folates are cofactors used in essential reactions of prokaryotic and eukaryotic cells. Tetrahydrofolate (vitamin B9) is used in one-carbon transfer reactions that take place during DNA synthesis (conversion of dUMP to dTMP and purine synthesis), formylmethionine tRNA formation and amino acid biosynthesis (conversion of serine to glycine) (Ferone, 1977). Antifolates have a wide range of applications, not only as antimalarials but also as anti-cancer agents such as methotrexate (Edelman and Gandara, 1996). Through targeting specific enzymes of the folate pathway, DNA base and amino acid synthesis involving a few 1-carbon transfer reactions, are disrupted resulting in cell death (Edelman and Gandara, 1996). Despite the absolute need for folates, different organisms have different ways of obtaining folates. Humans and mammals obtain preformed, reduced folates from their diet whereas prokaryotes, plants, fungi, yeast and the malaria parasite are able to synthesise folates. The *de novo* folate synthesis from guanosine 5'-triphosphate (GTP), p-aminobenzoic acid (PABA) and L-glutamate as well as polyglutamylation of p-aminobenzoylglutamate and pterin-aldehyde (host serum folate degradation products) indicated a unique biosynthetic pathway in the human malaria parasite, as shown in figure 1.4 (Krungkrai *et al.* 1989). Folate metabolism and the enzymes involved thus greatly differ between the parasite and its human host and thus make this metabolic pathway a validated antimalarial drug target (Hitchings, 1971). As shown in figure 1.4, folate biosynthesis can be divided into two main routes, namely *de*

novo synthesis and folate salvage. *De novo* synthesis uses GTP as starting metabolite and ends with the production of dihydrofolate (H_2folate) where it links up with the thymidylate cycle, which is the recycling of dihydrofolate through various steps to provide the essential cofactors needed in metabolism (Figure 1.4).

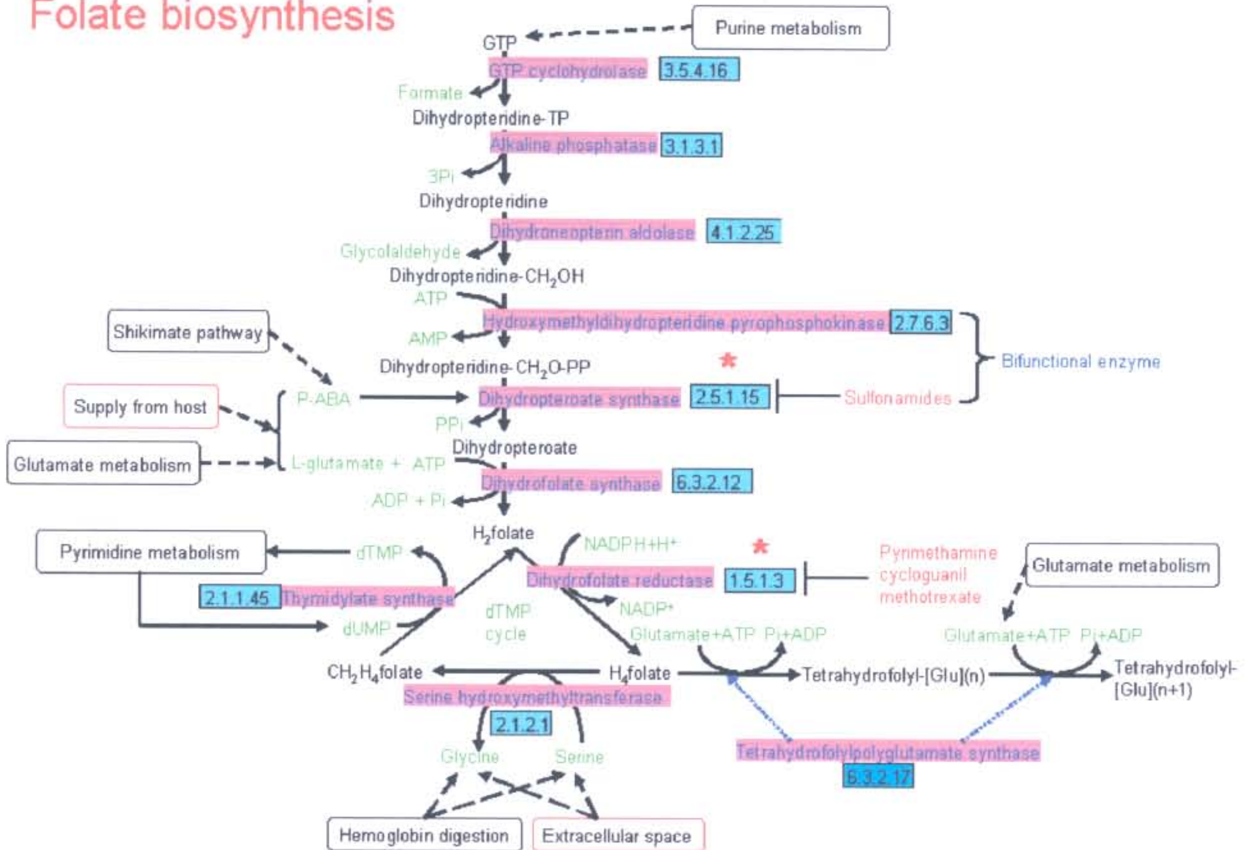

Figure 1.4: Folate metabolism in the human malaria parasite *P. falciparum*. (http://sites.huji.ac.il/ malaria/maps/ folatebiopath.html). Enzyme Commission (E.C.) numbers are indicated next to the enzyme names. Stars indicate enzymes targeted by current antifolates and the links with other metabolic pathways are shown in white boxes.

1.5.2 Current status of the antifolates

Antifolate targets are the dihydropteroate synthase activity of bifunctional hydroxymethylpterin pyrophosphokinase-dihydropteroate synthase (PPPK-DHPS) and the dihydrofolate reductase activity of dihydrofolate reductase-thymidylate synthetase (DHFR-TS) (Brooks *et al.*, 1994; Hyde, 1989). PPPK-DHPS is responsible for the formation of dihydropteroate in the *de novo* pathway. DHFR-TS is responsible for the cycling of dihydrofolate to tetrahydrofolate and *vice versa* in the thymidylate cycle (Ferone, 1977). The antifolates sulfadoxine (SDX) and pyrimethamine (PYR) are inhibitors of DHPS and DHFR, respectively (Brooks *et al.*, 1994; Hyde, 1989). Specific advantages of

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pyrimethamine are a much higher affinity for the parasite DHFR than the host homologue and that its' inhibition of the enzyme is late in the erythrocytic cycle when schizonts form and DNA synthesis reaches a maximum (Yuthavong, 2002). The antifolates are also structurally very similar to the respective DHPS and DHFR substrates. This mode of competitive inhibition unfortunately placed the enzymes' active sites under selection pressure, forcing the development of mutations necessary for survival. This resulted in resistant strains with point mutations in the coding sequences of target genes that limited the useful therapeutic life of the drugs (Table 1.2) (Sibley *et al.*, 2001). When comparing the characterized *dhfr-ts* gene (Hyde, 1989) as well as *pppk-dhps* (Brooks *et al.*, 1994) with various clinical isolates, it was evident that certain point mutations in *dhfr* (PYR resistant strains) and *dhps* (SDX resistant strains) were responsible for the reduced affinity of DHFR and DHPS for the drugs, i.e. resistance. It was also discovered in *dhps* that the number of mutations was directly and quantitatively related to sulfadoxine resistance and that a key residue, Glycine 437, was common to all mutants (Triglia *et al.*, 1998). The new resistant strains were subjected to allele-specific tests for the detection of point mutations. Comparison of the point mutations between different strains has led to the construction of maps of mutation patterns and worldwide distribution frequencies (Wang *et al.*, 1995).

Table 1.2: Primary mutations responsible for antifolate resistance (Sibley *et al.*, 2001). Point mutations responsible for resistance are underlined. One-letter amino acid abbreviations are given in brackets.

DHFR gene	**codon 16**	**codon 51**	**codon 59**	**codon 108**	**codon 164**
Wild type	GCA (A)	AAT (N) AAC (N)	TGT (C)	AGC (S)	ATA (I)
Variants	GTA (V)	ATT (I)	CGT (R)	AAC (N) ACC (T)	TTA (L)
DHPS gene	**codon 436**	**codon 437**	**codon 540**	**codon 581**	**codon 613**
Wild type	TCT (S)	GCT (A)	AAA (K)	GCG (A)	GCC (A)
Variants	GCT (A) TTT (F)	GGT (G)	GAA (E)	GGG (G)	TCC (S) ACC (T)

Resistance to the antifolates is addressed through the development of novel inhibitors such as WR99210 that inhibits drug-resistant DHFR, through rational drug design (McKie *et al*, 1998). The recent determination of the *P. falciparum* DHFR-TS crystal structure has revealed the mode of action of this rationally designed inhibitor and will also be useful for the rational design of other antifolates targeted to DHFR (Yuvaniyama *et al.*, 2003).

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1.5.3 Drug synergy

In the past, the treatment of resistant strains was modified by the synergistic use of drugs that target different points in the pathway (Sibley *et al.*, 2001). Such drugs were Fansidar, a pyrimethamine/sulfadoxine combination or Maloprim, a pyrimethamine/dapsone combination. Inhibition occurred when the parasite was sensitive to either sulfadoxine or pyrimethamine and therefore the obstacle of resistance to the other drug was eliminated. The success of these drugs could be attributed to the fact that they blocked both possible routes of folate metabolism, namely, *de novo* synthesis and salvage, but the exact mechanism of the synergistic action is unknown and further knowledge of the folate metabolic pathway is thus required. The success of the synergistic application of pyrimethamine and sulfadoxine was however very short-lived (less than five years) as resistance developed to the drug combination (Sibley *et al.*, 2001). The exact mechanisms of resistance to the sulfadoxine-pyrimethamine combination is not yet known, but the minimum resistance requirements are a triply mutant DHFR (S108N, C59R and N51I) as well as a doubly mutant DHPS (Sibley *et al.*, 2001). Such strains are highly pathogenic and the use of combination therapy and synergistic drug treatments should thus be handled with great care since selection for more potent parasites could result from drug misuse (Sibley *et al.*, 2001).

1.5.4 Exogenous folate utilisation

The level of sulfadoxine resistance of parasites under monotherapy treatment fluctuates between various parasite strains depending on the serum concentration of folates of the host (Wang *et al.*, 1997). Additional mechanisms to alter the DHFR and DHPS parts of the bifunctional target enzymes were responsible for this, such as the ability of parasites to use exogenous folic acid (Wang *et al.*, 1999). Genetic crosses between sulfadoxine sensitive and –resistant parasites showed that the folate utilizer phenotype was gained by resistant parasites and was linked to the *dhfr* gene but not dependant on DHFR activity (Wang *et al.*, 1999). Furthermore it was observed that sulfadoxine resistant parasites reverted to sensitivity when a pyrimethamine-sulfadoxine combination was used. It was postulated that this so-called 'folate effect' was responsible for the bypassing of sulfadoxine inhibition of DHPS and the observed synergistic inhibition when pyrimethamine was added.

1.5.5 Putative drug targets within the folate pathway

To date, only two activities within the folate pathway have been targeted by the antifolates, DHFR and DHPS. When considering that almost half of the enzymes in the folate pathway have no human counterparts, the uncharacterised folate metabolising enzymes are promising drug targets. Three novel *P. falciparum* genes within the folate pathway have been identified but their respective enzymes have not yet been characterised (Lee *et al.*, 2001). The first of these is the gene encoding GTP cyclohydrolase (GTP-CH, E.C: 3.5.4.16), which catalyses the rate limiting step in *de novo* folate synthesis: the release of formic acid and formation of a triphosphate pteridine ring structure. In humans this enzyme has a different function, i.e. the formation of reduced biopterin since *de novo* folate synthesis is absent (Stokstad and Koch, 1967). Alignment between the deduced *P. falciparum* GTP-CH and its human homologue revealed a parasite specific N-terminal extension of 130 amino acids as well as 17% identity and 32% similarity in the aligned areas (Lee *et al.*, 2001). The second gene identified encoded serine hydroxymethyltransferase (SHMT, E.C: 2.1.2.1) that converts serine into glycine with the use of tetrahydrofolate as a 1-carbon acceptor (Lee *et al.*, 2001). Alignment with the human SHMT homologue indicates a higher amount of conservation, 43% identity and 63% similarity, which makes it a less favourable drug target when considering selective inhibition (Lee *et al.*, 2001). The last of the three genes identified encoded both dihydrofolate synthase and folylpolyglutamate synthase (DHFS-FPGS EC: 6.3.2.12 and EC: 6.3.2.17 respectively) activities as its bifunctionality was verified in yeast knockouts (Salcedo *et al.*, 2001). The next section will focus on this enzyme as the main topic of the thesis.

1.5.6 Bifunctional dihydrofolate synthase-folylpolyglutamate synthase (DHFS-FPGS) as a possible drug target.

The bifunctional enzyme DHFS-FPGS is used in both *de novo* synthesis to form dihydrofolate by addition of L-glutamate to dihydropteroic acid, as well as in folate salvage to extend the chain of glutamate residues, which is important for the regulation of intracellular folate pools (Stokstad and Koch, 1967). The fact that all organisms have FPGS activity to synthesize folylpolyglutamate, regardless of whether they are capable of *de novo* folate synthesis or not, underlines the important function of FPGS.

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The polyglutamate tail addition is thus part of folate salvage and has the following important functions:

- It prevents efflux of cofactors from the cell, thus cellular folates are retained (McGuire and Bertino, 1981)
- It increases the affinity of folate synthesizing enzymes for folates. Most enzymes only use polyglutamated substrates (McGuire and Bertino, 1981)
- It increases the accumulation of folates in the mitochondria where it is used for glycine synthesis (Bognar *et al.*, 1985; Shane, 1980).

Much less is known of DHFS activity except that it is used for *de novo* folate biosynthesis in organisms that synthesise their own folates. DHFS and FPGS catalyse similar reactions in folate metabolism but function in two different routes of the pathway. If these two *Plasmodium* enzymes are co-linear in the bifunctional protein, as is the case in *E. coli* (Keshavjee *et al.*, 1991), then a single inhibitor would have a very powerful effect in blocking both routes of folate metabolism. This would achieve the same result as the synergistic application of drugs such as sulfadoxine and pyrimethamine that target separate points; each within another route of the folate metabolic pathway. On the other hand, it might be preferable to inhibit only the DHFS activity without affecting FPGS activity as it was shown in human cancer cells that FPGS inherently enhances the effect of antifolates (substrate analogues) (Gangjee *et al.*, 2002). This is accomplished by increasing the retention of the antifolates through polyglutamylation as well as the affinity of the folate utilising enzymes for the inhibitors (Gangjee *et al.*, 2002). Considering that both of these activities reside in the same protein it is thus important to determine the structural features necessary for FPGS activity in order to design drugs for the selective inhibition of DHFS.

What makes this enzyme furthermore an appealing drug target is that humans do not have a DHFS homologue. The extent of conservation (30% similarity and 17% identity) between human FPGS and *P. falciparum* DHFS-FPGS is also lower than between GTP-CH and SHMT and their respective homologues (Lee *et al.*, 2001). The whole DHFS-FPGS sequence is aligned against human FPGS, since there are no distinct DHFS and FPGS domains at the primary amino acid or DNA level in *P. falciparum*. The *dhfs-fpgs* gene identified in *P. falciparum*, is also unique since it is the first bifunctional gene

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identified in eukaryotes (Lee *et al.*, 2001). The effect of current antifolates on DHFS-FPGS was determined in a gene complementation system. This was based on the fact that DHFS-FPGS and DHFR both contain ATP- and folate binding sites. The *P. falciparum dhfs-fpgs* gene showed no mutations after growth at inhibitory concentrations of antifolates (Salcedo *et al.*, 2001). It seems promising that this enzyme would be less prone to the acquisition of resistance, an advantage for drug development.

To date, *P. falciparum* DHFS-FPGS has been characterized in terms of DNA sequence and bifunctionality verified by means of complementation of *E. coli* and yeast mutants (Lee *et al.*, 2001; Salcedo *et al.*, 2001). No other characteristics of this enzyme are known. To determine the potential value of this enzyme as a novel antifolate target, studies concerning the parasite-specific traits, kinetic parameters, activity and three-dimensional structure are needed. This requires sufficient amounts of correctly folded, soluble protein. The native parasite enzyme is only expressed at undetectable levels from gene-complemented *E. coli* or yeast systems and direct isolation from the parasite results in low yields (personal communication, J. Hyde). Expression of malaria genes in a heterologous system is greatly hampered by their A+T richness and rare codon usage (Baca and Hol, 2000). This study therefore concerns the synthesis of the *dhfs-fpgs* gene adapted to the codon preferences of *E. coli,* and the optimisation of its expression in *E. coli*.

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1.6 Research aims

The primary aim of the study is to obtain sufficient quantities of DHFS-FPGS for future kinetic characterisation and crystallisation or nuclear magnetic resonance (NMR) studies.

- Chapter 2 focuses on the PCR mediated synthesis of a synthetic *P. falciparum dhfs-fpgs* gene, which is modified for expression in *E. coli* by the use of preferred *E. coli* codons.
- Chapter 3 focuses on the heterologous expression of synthetic *P. falciparum dhfs-fpgs* in a variety of *E. coli* expression hosts and vector systems to obtain soluble expressed protein and verification of enzymatic activity through functional complementation.
- Chapter 4 describes the *in silico* analysis of the predicted primary amino acid sequence to determine its similarity with the other homologues and predict structural features.
- In Chapter 5 the relationship between the above chapters, their relevance to drug discovery and development and future prospects are discussed.