Novel Contributions to Understanding the Mechanism of Folate Transport and Anti-folate Resistance in *Plasmodium Falciparum*

Thesis submitted in accordance with the requirements of
the University of Liverpool for the degree of Doctor in
Philosophy

by

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September 2008

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DECLARATION

This thesis is the result of my own work. The material contained in the thesis has not been presented, nor is currently being presented, either wholly or in part for any other degree or other qualification.

The research work was carried out in The Liverpool School of Tropical Medicine, The University of Liverpool, United Kingdom.

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ACKNOWLEDGEMENTS

THESIS ABSTRACT

Title: Novel contributions to understanding the mechanism of folate transport and anti-folate resistance
Author: Edwin O. Ochong.

The primary focus of the PhD thesis has exclusively been on studies on *Plasmodium falciparum* with a remit to build a clear understanding on some of the critical and relevant questions relating to folates transport and antifolates resistance. The direction of this work has been constructed on the following themes of interest;

1. The Influence of folate pathway substrates on the In Vitro activities of antifolate drugs.

It has been demonstrated that it is possible to mitigate the activities of antifolates by the addition of folate substrates to the drug assay. This work involved the assessment of the specific folate substrate against a panel of commonly used antifolate compounds and in a number of well characterized laboratory reference clones. Substrate-specific differences were noted in their ability to abrogate antifolate activities. Likewise parasite responses to these substrates during drug inhibition also varied.

2. Functional characterization of heterologously expressed putative folate transporters in the *Xenopus laevis* oocyte.

Folates are highly charged molecules and hence membrane impermeable. We developed a hypothesis-driven research that *Plasmodium* expresses transporters that facilitate the uptake of folates which mitigate the activities of the antifolates as described in **section 1** above. A query on the *Plasmodium* genome has revealed the presence of putative folate transporter candidate genes. To more clearly elucidate this physiologically important process, we have heterologously expressed these putative candidate genes in the *Xenopus laevis* oocyte model and functionally demonstrated using radio-pulsing experiments that the genes are relevant in the mediation of the transport of the folates across the parasite membrane.

3. Transcriptional analysis of putative folate transporter genes

The biosynthesis of folic acid pathway in *Plasmodium* is now well defined but the inter-strain variability in response to folic acid levels during drug inhibition and differences in the survival ability in low levels of folate is of profound interest. We have extended the functionally characterised transporters described in **section 2** above to transcription studies. Our data reveal there are inter-strain differences in mRNA expression of these putative transporters. These differences could partly be linked to the reported inter-strain differences in the folate-linked phenotypes mentioned.

4. Validation of a Real-Time PCR method for the detection of the mutant DHFR 164 allele

The hallmark of antifolate resistance and a challenge to existing and novel antifolates in the pipeline is the mutant 164 allele. It is quite a puzzle that despite the heavy use of antifolates in Africa, the Leu-164 is rare unlike in South East Asia and South America. Part of the contribution to the low identification of Leu-164 in Africa has been attributed to the low sensitivity of the detection tools. We have developed a refined and validated Real-Time PCR method for the detection of the Leu-164 mutant allele. Using this method, we report the presence of the mutant allele in Thailand isolates but its absence in African isolates derived from Malawi and Zambia. This method can detect up to 5% level of the presence of the mutant allele in an individual patient parasite population.

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LIST OF ABBREVIATION

DNA   deoxyribonucleic acid

°C   degree celsius

mM   millimolar

mg/ml   milligram per millilitre

μg   microgram

%   percentage

μl   microlitre

hr   hour

min   minute

GTP   guanine triphosphate

CQ   chloroquine

DHNTP   dihydroneopterin triphosphate

DHN   dihydroneopterin

DHNA   dihydroneopterin aldolase

pABA   para-aminobenzoic acid

PTR1   pteridine reductase-1

DHF   dihydrofolate

FPGS   folylpolyglutamate synthase

ng/ml   nanogram per millilitre

SDX   sulfadoxine

PYR   pyrimethamine

CCG   chlocycloguanil

CPG	chloproguanil
DDS	dapsone
CDA	chloproguanil-dapsone-artesunate
FBT	folate-biopterin transporter
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthase
Ci	curie
Ci/mmol	curie per millimol
PCR	polymerase chain reaction
RT-	real time / reverse transcription
MBS	modified Barth's solution
CBS	complete Barth's solution
bp	base pair
5-MeTHF	5-methyltetrahydrofolate
5-FoTHF	5-formyltetrahydrofolate
MTX	methotrexate
FA	folic acid

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CHAPTER 1

Introduction

1.1 Malaria

Human malaria is an anopheline mosquito-borne, erythrocyte infection caused by the protozoan parasites of the genus *Plasmodium*. Until recently (Singh et al 2004), there were four well established species that cause human malaria; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale and Plasmodium malariae*. It is recognised that *Plasmodium* species have consistently shown great selectivity of their natural hosts. However, a fifth species, *Plasmodium knowlesi* known to infect the long-tailed macaque has potentially found its way into humans and is now widespread in Southeast Asia (Singh et al 2004). Of all these known species, *Plasmodium falciparum* is the most prevalent and virulent and is responsible for many of the clinical cases and majority of the deaths associated with the disease (Snow et al. 2005). *Plasmodium vivax* is the second most prevalent species and was recently shown to contribute to life threatening clinical cases of malaria (Kochar et al. 2005). This species is localised to South America, Asia and the Pacific but not found in Africa since africans lack the Duffy receptor necessary for its binding (Miller et al 1976).

1.1.1 The health and economic burden of malaria

More than a third of the world's population is at risk of malaria attack but the massive toll is borne by the poorest countries. It is estimated that approximately 500 million cases of new infections and 1-3 million deaths occur annually mostly amongst pregnant women and infants less than five years. Over 90% of these cases occur in the tropical and sub-tropical areas of the world (Snow et al. 2005). The climatic conditions in these areas appear to favour and support the anopheline vector that transmits the parasites. The entrenched malaria in these places also has a negative impact on the economy of these countries. It is linked to loss of man-hour through work absenteeism or decreased productivity, private investors' reluctance to pump funds/invest in projects in malaria prone regions, travellers/tourists reservations to visit malaria endemic sites, high costs of medical bills associated with the treatment of the severe case of the disease and a general effect on the population growth (Snow et al. 2005).

1.1.2 Life cycle of malaria parasite

The life cycle of the malaria parasite alternates between the human host (asexual phase) and the anopheline mosquito vector (sexual phase), **Figure 1.1**. Sporozoites released from the mosquito's salivary gland invade the liver cells (hepatocytes). In the hepatocytes, the parasites differentiate and undergo asexual multiplication resulting in thousands of merozoites which burst from the hepatoctye. Individual merozoites then

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invade red blood cells (erythrocytes) and undergo a series of ordered distinct developmental stages and additional round of multiplication producing schizonts which finally with a synchronous rupture of the infected erythrocyte releases merozoites. Not all of the merozoites divide into schizonts, some differentiate into sexual forms, male and female gametocytes. During a blood meal, the anopheline mosquito takes up the gametocytes which fertilize and develop further into sporozoites.
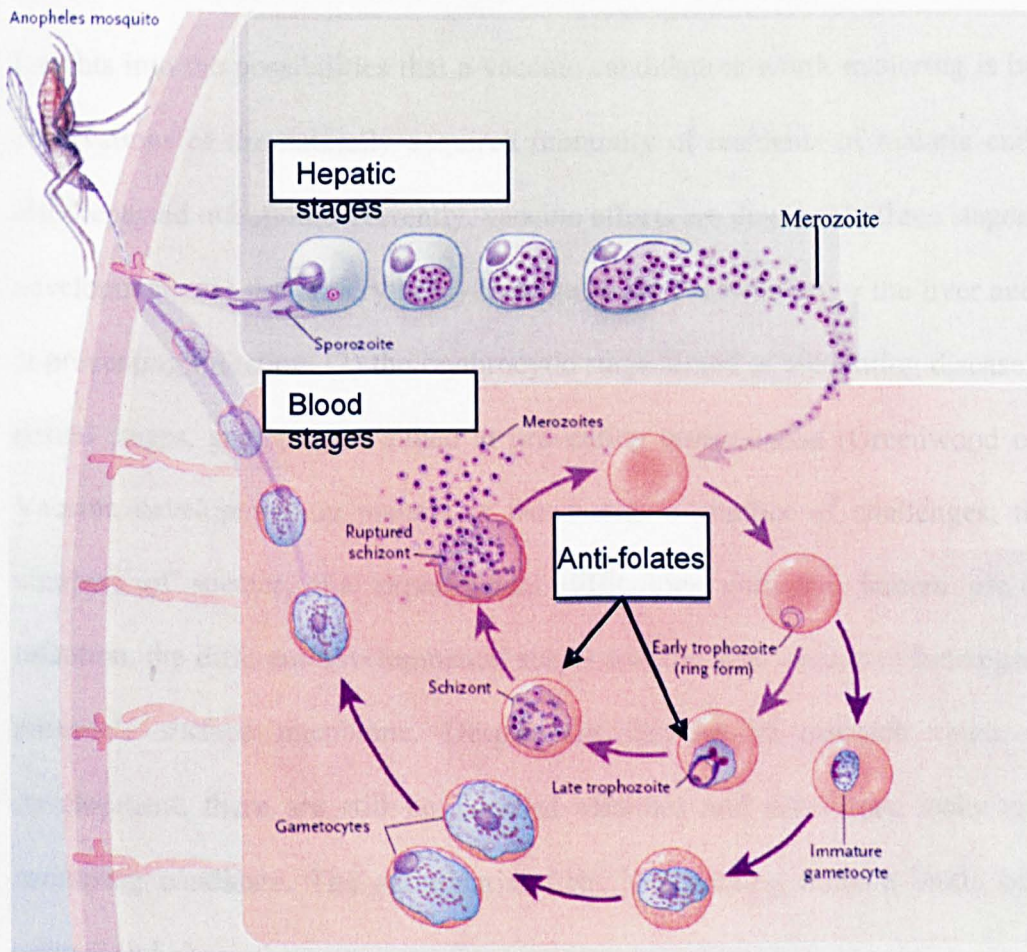
Figure 1.1 The life cycle of malaria parasite; Adapted from Daily, J.P and M.A. Waldron, *N Engl J Med 2003; 349: 287-95*

1.1.3 Intervention efforts in the fight against malaria

There are a number of intervention efforts geared towards the control and eradication of malaria. These are; vaccine development, vector control and antimalarial chemotherapy.

1.1.3.1 Vaccine development

Insights into the possibilities that a vaccine candidate is worth exploring is based on the observations of the naturally acquired immunity of residents of malaria endemic areas after repeated infections. Currently, vaccine efforts are directed at three stages of parasite development; (1) the pre-erythrocytic stage as the parasites enter the liver and are aimed at preventing infection, (2) the erythrocytic stage aimed at preventing disease and (3) the sexual stages, gametocytes aimed at preventing transmission (Greenwood et al. 2008). Vaccine development in malaria is faced with a number of challenges; the multiple numbers of species, the experimental difficulties with the human pre-erythrocytic infection, the different developmental stages and the high degree of heterogeneity of the parasite's surface membrane. Despite the decades of research spent on vaccine development, there are still no licensed vaccines and the future looks elusive for a promising candidate. The antimalarial fight is becoming more a battle of the vector control and chemotherapy.

1.1.3.2 Vector control

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Since the discovery that the mosquito is the vector for malaria, vector control has been used as a malaria intervention control strategy. The interruption of the breeding sources and antilarval measures contributed significantly to the reduction of malaria. The discovery of dichlorodiphenyltrichloethane (DDT) shifted the focus from the larva to the adults and its subsequent full implementation in malaria control had a significant impact in the eradication of malaria in the developed world. Despite the realised successes, sustained efforts in the developing world collapsed due to operational difficulties, lack of political will, socio-economic factors and anopheline mosquito vector resistance to DDT. A more focused approach targeting the most vulnerable category, pregnant women and children has recently been triggered. It's the use of Insecticide-treated bednets (ITNs). ITNs have been shown to have significantly lowered mortality in children, reduced low birth weights and minimised severe pregnancy-associated anemia (Muturi et al. 2008).

1.1.3.3 Antimalarial drugs

Chemotherapy is and still remains the main intervention strategy for fighting malaria. Efforts are being made in the development of new drugs as well as protecting the already existing ones. Antimalarial drugs are known to have played a critical role in the treatment and management of both severe and non-severe forms of malaria for a very long time.

1.1.3.3.1 Current antimalarial therapies and the status of global antimalarial resistance

(a) 4-aminoquinolines

The 4-aminoquinoline class of antimalarials are well represented by chloroquine (CQ) and its closest 'cousin' amodiaquine (AQ). Until resistance was noted, CQ was the most important antimalarial drug for decades. It was cheap, safe and potent to all known *Plasmodium* human species (Maitland et al 2004).

Although AQ is a comparatively superior molecule to CQ, initial concerns over its use were based on reported neutropenia and hepatitis (Taylor and White 2004). A comprehensive review (Olliaro and Mussano 2003) on the safety of the use of AQ revealed the drug to be safe and well tolerated. The compound is currently a potential alternative treatment regimen for uncomplicated malaria where it is recommended in combination with other drugs.

AQ and CQ target the erythrocytic forms of *Plasmodia*. It is widely accepted that the site of action of these compounds is the parasite's digestive vacuole but a huge controversy still exists on the mechanisms involved in the transport of the compounds across the digestive vacuole membrane and their mode of action, see (O'Neill et al 1998) for a comprehensive review.

The first cases of CQ resistance were reported in South America and Southeast Asia in the 1950s and gradually spreading across from these two foci with the first appearance in Africa seen at the East African coast in 1978. *P falciparum chloroquine resistance*

transporter gene (*Pfcrt*) particularly an alteration of lysine to threonine at position 76 is considered the major mediator of CQ resistance (Fiddock et al 2000) with the mutations and copy number amplifications of the *Plasmodium falciparum multi-drug resistance gene (Pfmdr1)* being implicated in modulating the levels of resistance. Based on amodiaquine's similarity in structure to CQ, these markers have been analysed in relation to AQ and the results show the two may contribute to AQ resistance in some cases (Ochong E et al 2003, Holmgren G et al 2006). The *Pfcrt* 76T is widespread but a study in Malawi has shown the reversal of the wild-type K76 and return to CQ sensitivity after the elimination of CQ use (Mita T et al 2003).

(b) **Quinoline methanols**

This includes quinine (QN) and mefloquine (MQ). Like the 4-aminoquinolines, these compounds act on the erythrocytic stages of the parasite. QN, a compound derived from the bark of the cinchona tree was one of the initial agents used in the treatment of malaria and still remains the mainstay for the treatment of severe malaria. However, in Southeast Asia, it is used in combination with other drugs for uncomplicated malaria. MQ is effective for both prophylaxis and treatment in low transmission areas.

QN resistance is still rare in Africa and it appears not to be of a major global clinical concern. With regard to MQ resistance, earlier studies on murine malaria (Peters and Robinson 1991) suggested a similarity between CQ- and MQ- resistance with fears of MQ-resistance expected in the field as the frequency of CQ-resistance increased.

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However, in an *in vitro* study on CQ-resistant parasites under continuous CQ pressure, it was shown that the level of CQ-resistance increased but this increase was concomitantly associated with the loss of the *Pfmdr1* copy number and a decrease in MQ-resistance (Barnes et al 1992).

(c) Bisquinolines

A well known bisquinoline compound is piperaquine. This drug has been shown to have excellent activity against multidrug resistant *P. falciparum*. In China, the drug was widely used as a monotherapy to treat malaria and also as a prophylactic agent (Davis et al 2005). Based on its relationship with other 4-aminoquinoline agents and to the observed concentration in the parasite's digestive vacuole Warhurst et al 2007), it is assumed the drug is subject to CQ related resistance mechanism. Although resistance was reported in China in the backdrop of high CQ-resistance, *in vitro* tests from some African sites indicate excellent performance (Basco and Ringwald 2003). The drug is currently under development as a combination with dihydroartemisinin but there is still concern on the impact of high level CQ-resistance in the field.

(d) Sesquiturpene lactone endoperoxides

Artemisinins are the best known family of this class of compounds. The parent molecule, artemisinin, was first extracted from a sweet wormwood shrub of the species *Artemesia annua* and together with its derivatives; dihydroartemisinin, artemether, artesunate,

artemisone, arteether and artelinic acid are the leading partner drug of choices in antimalarial combination therapy. These are a well established group of compounds with a marked potency against *Plasmodium* both *in vitro and in vivo*. The molecules are known to act rapidly with a broad range of activity on the different erythrocytic stages of the parasite but like CQ, there is a considerable debate on their mode of action and the precise cellular target. See (Krishna et al 2008) for a comprehensive review on this subject. As compounds with a short half-life and only rolled out as a combination, resistance to artemisinin has not been of a concern but latest reports show resistance could be emerging (Alker et al 2007).

(e) Antifolates

Antifolates are the basis of this thesis and have therefore been extensively discussed into detail in **sections 1.6, 1.7 and 1.8.** In order to introduce the subject, we begin with an initial understanding on the discovery of folic acid, **section 1.2.**

1.2 Discovery of folic acid

The discovery of folic acid is credited to Dr Lucy Wills in her pioneering haematology work in the early 1930s to cure pernicious anaemia in pregnant women (Wills 1931). In a comparative study using monkeys fed on a diet of white rice and white bread, the same diet as her patients received, she was able to induce the same symptoms as her patients. She discovered that yeast extracts could correct the condition and inferred that yeast

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contain a factor (referred then as the *"Wills factor"*) which could cure the disease and prevent pernicious anaemia. Later on in the early 1940s, an independent group of investigators were able to isolate a similar factor in liver and spinach leaves (Mitchell et al. 1941; Stokstad 1941). They further demonstrated its role as a stimulant growth factor in certain bacteria and also confirmed that it possessed haematopoietic activity for animals as earlier described. The factor was then named *folic acid*, a term derived from the latin word *folia* meaning leaves. The isolation of this factor gave the impetus to study its structure. Using degradation reactions, Angier and colleagues (Angier et al. 1946) showed the compound as a dibasic acid containing a pteridine moiety, a p-aminobenzoic acid and a glutamic acid. Based on the evidence of the existing moieties, the workers postulated a structure in which a pteridine is attached to the para-aminobenzoic acid (**figure 1.2**) which is further linked via a peptide linkage to the glutamic acid (**figure 1.2**). In a substitution reaction in which a *p*-aminobenzoic acid was used instead of *p*-aminobenzoylglutamic acid, a compound with a distinct property was obtained. This compound was active for *S. faecalis R* but inactive for *L. casei*. The compounds formed from *p* -aminobenzoic acid and *p*-aminobenzoylglutamic acid were given the names *pteroic acid* (**figure 1.2**) and *pteroylglutamic acid* commonly refered to as **folic acid; figure 1.2.**

Folic acid (Pteroylglutamic acid) **5-MethylTetrahydrofolate**
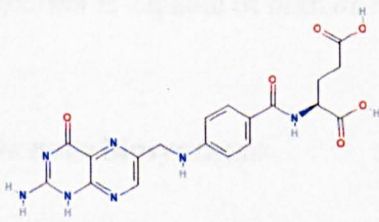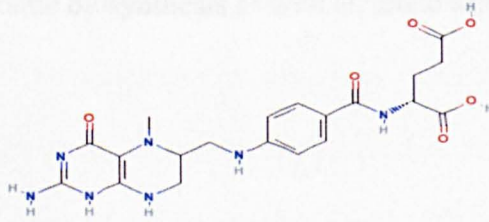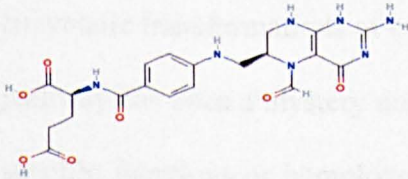
5-Formyltetrahydrofolate **para-aminobenzoic acid**
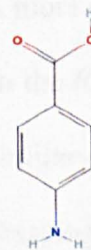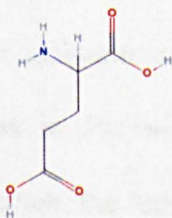
Glutamic acid **Pteroic acid**
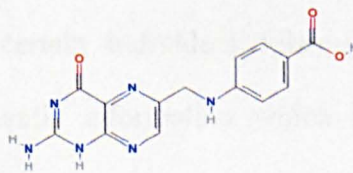
Figure 1.2 Structures of folic acid, 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, para-aminobenzoic acid, glutamic acid and Pteroic acid

1.3 Tetrahydrofolate *de novo* biosynthesis and salvage

P. falciparum is capable of both *de novo* folate biosynthesis as well as folate salvage.

1.3.1 *De novo* biosynthesis

Most of the knowledge about the biosynthesis of pteridines in *Plasmodium* has been generated through the comparative study of the enzymatic reactions involved in the formation of the pterin moiety of folic acid in bacteria. A more clear understanding of the enzymatic transformations of certain intermediates within the *Plasmodium de novo* folate pathway has been a mystery due to the failure to detect similar enzymes performing these specific functions or homologous genes. Nevertheless, existing satisfactory biochemical and molecular experimental evidence support the thesis that the *Plasmodium* route of *de novo* folate biosynthesis is quite similar to the conventional bacterial one.

The use of radioactive tracers in experiments, inhibitors of known mechanism of action and the isolation and characterisation of certain individual folate-linked enzymatic functions in *Plasmodium* has yielded authentic information which indicates that in *Plasmodium*, the 'conjugated' pterins are made from the same precursor in the same way they have been shown to be made in bacteria.

1.3.1.1 The pteridine branch

The first suggestion that purines might be biological precursors of pteridine compounds came from experiments performed by Albert (Albert 1954) who found that purines can be transformed chemically into pteridines under mild conditions. Later Weygand and colleagues (Weygand et al. 1961) extended this suggestion in a proposal of a hypothetical pathway of pteridine biosynthesis from guanosine or a guanine nucleotide. It has since been established that GTP was the most efficient of the guanine nucleotides. This first step is of special interest because it commits GTP (Guanine triphosphate) to the **pteridine branch** production (**figure 1.3 maroon colour**). The conversion of the GTP to dihydropterins involves the opening of the imidazole ring portion of the purine with the removal of carbon 8 of the purine ring system. GTP cyclohydrolase I (**GTPC EC 3.5.4.16**) is the enzyme that catalyzes the removal of carbon 8 from GTP resulting in the formation of 7, 8-dihydroneopterin triphosphate (DHNTP) and formate. The triphosphate side chain of DHNTP is further cleaved in a two step process to produce dihydroneopterin (DHN); the pyrophosphate group is removed by a specific nudix hydrolase followed by the action of a non-specific phosphatase. This is followed by the action of Dihydroneopterin aldolase (DHNA EC 4.1.2.25) which finally cleaves the lateral side chain of dihydroneopterin to release glycolaldehyde and produce 6-hydroxymethyl-7, 8-dihydropterin. So far, no evidence has been presented showing the identity of the candidate genes for the DHNTP nudix pyrophosphohydrolase and DHNA in *Plasmodium*. Recently, (Dittrich et al. 2008) it has been suggested that the activity is due to an unusual orthologue of 6-pyruvoyltetrahropterin synthase (PTPS) in *Plasmodium*. This enzyme gives rise to the substrate 6-hydroxymethyl-7, 8-dihydropterin (HMDP) and might thus provide the bypass for the missing DHNA activity. There is firm

evidence that the pyrophosphate ester of HMDP is the intermediate involved and not the parent substrate. 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase (**IIPPPK, EC 2.7.6.3**) catalyses the transfer of the pyrophosphate moiety from ATP to HMDP to form 6-hydroxymethyl-7, 8-dihydropterin pyrophosphate (DHPPP).

1.3.1.2 The pABA branch

The discovery of the plastid in human apicomplexan parasites (McFadden et al. 1996) raised the hypothesis that similar biosynthetic functions linked to this organelle in other organisms might be operative here. One such biochemical process is the shikimate pathway whose presence in *P. falciparum* has been demonstrated but since found to operate in the cytosol (Keeling et al. 1999). The shikimate pathway is an integration of a series of seven enzymes in which erythrose-4-phosphate from the pentose phosphate pathway and phosphoenol pyruvate derived from glycolysis are ultimately converted by chorismate synthase to chorismate, a precursor of pABA which is involved in folate, ubiquinone and tyrosine synthesis.

In *Escherichia coli,* the synthesis of pABA is a two step process (Basset et al. 2004). The first step is the replacement of the hydroxyl group of chorismate with an amino group of glutamine to yield the intermediate 4-amino-4-deoxychorismate (ADC). This reaction is mediated by a heterodimeric complex PabA and PabB collectively referred to as ADC synthase. PabA acts as a glutamine amido-transferase, supplying an amino group to PabB, which carries out the amination reaction. The second reaction catalysed by PabC (ADC lyase) involves the β-elimination of pyruvate and aromatization of the ADC ring to give pABA, (**pABA *de novo* branch, figure 2 blue colour shown**).

Genomic data show that *P. falciparum* has a bipartite protein with domains homologous to PabA and PabB (Triglia et al. 1999). Because there are no as yet identified orthologs to ADC lyase in *P falciparum*, there is an assumption that these proteins are wholly involved in pABA synthesis and are therefore commonly referred to as 'PABA synthase' (Paba S).

In certain organisms including *P. berghei*, p-amino-benzoylglutamate can be used as an alternative substrate to *p*ABA in the enzyme systems for the synthesis of dihydropteroate. Investigations have confirmed that p-amino-benzoylglutamate is a poor substrate since pABA is used ten times more effectively. In addition, no enzyme involved in the conversion of pABA and glutamic acid into p-aminobenzoylglutamic acid has been detected in human *Plasmodium*.

The synthesised *pABA* resulting from chorismate, a derivative of the shikimate pathway and DHPPP moiety of the **pteridine branch** are condensed together by Dihydropteroate synthase (DHPS, EC 2.5.1.15) to form dihydropteroate. The *Plasmodium* DHPS occurs as a bifunctional enzyme with the HPPPK, **(see section 1.5.2)**. Dihydrofolate synthase (DHFS EC 6.3.2.12) then catalyses the ATP-dependent reaction in which a glutamate residue is attached to the carboxyl moiety of pABA converting dihydropteorate to dihydrofolate. To this stage, DHFS and the preceding reactions are quite unique to *Plasmodium*, plants and certain microorganisms. The fact that humans are incapable of coupling the pteridine moiety to the PABA moiety and the subsequent addition of the

glutamic acid to dihydropteroic acid renders this pathway a plausible drug target. Dihydrofolate is further reduced to the biologically active tetrahydrofolate (THF) by Dihydrofolate reductase, DHFR in an NADPH dependent reaction, **(see section 1.5.1).** The formed tetrahydrofolate and other salvaged substituted tetrahydrofolate cofactors **(see section 1.3.2)** undergo a unique metabolism of polyglutamation. The enzyme Folyl-polyglutamate synthase (FPGS, EC 6.3.2.17) catalyses the MgATP-dependent sequential addition of single units of glutamate residues to the γ-carboxyl moiety of the tetrahydrofolate cofactors. The specificity of FPGS for the number of glutamate residues depends on the organism in which it is found. In *Plasmodium falciparum*, polyglutamate tails of around 5 residues in length have been found (Krungkrai et al. 1987). A substantial number of studies reviewed in (Assaraf 2006) have shown the significance of polyglutamation. First, polyglutamate conjugation increase the net anionic charge (polyanions) of THF cofactors making them impermeable to traverse through the lipid bilayers and are hence efficiently retained within cells. Second, most polyglutamate derivatives of reduced folates are better substrates than their parent monoglutamate forms for various folate-dependent enzymes and third folate synthesis is highly compartmentalised within the subcellular organelles like the mitochondria. Polyglutamation promotes the accumulation of folylpolyglutamate congeners in the mitochondria, a process crucial for other mitochondrion derived biosynthetic reactions.

1.3.1.3 Evidence for the existence of the *de novo* folate biosynthetic pathway in *Plasmodium falciparum*

a) Effectiveness of anti-folate therapies such as sulfa drugs

Following the elucidation of the structure of folic acid and the subsequent understanding of the sequential processes involved in its synthesis, a number of studies were dedicated to the identification of inhibitors of this pathway. A structural relationship and competitive interaction between sulfa drugs (sulphonamides and sulphones) and pABA were firmly established (Woods 1940) and satisfactorily confirmed the observation of inhibition of malaria parasites *in vivo* and *in vitro* (Ferone 1977). The DHPS enzyme found only in the parasite and absent in man was identified as the most sensitive locus of sulfa inhibition. Although the sulfa drugs (sulphadoxine and dapsone) have weak antimalarial property (Watkins et al. 1985), a wide therapeutic ratio between the effective dose of the drugs against the parasite and the relatively high toxic dose for humans exists.

b) Identification and detection of folate biosynthetic genes and proteins

From a doubtful point of view, even with the unravelling of the *Plasmodium* genome, the gene that codes for DHNA in the conventional *de novo* folate biosynthetic pathway outlined in figure 3 or of its related enzymatic activity has not been detected. This raised the question whether *Plasmodium* adopts this conventional route of folate biosynthesis. This doubt was challenged by the biochemical, genomic and molecular studies that have identified the other signature genes and proteins existing on this route. These key enzymatic functions as shown in figure 3 are required for folate biosynthesis and have been demonstrated in malaria parasites. Starting with GTP cyclohydrolase I (Krungkrai et

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al. 1985; Lee et al. 2001), DHPS-HPPK (Triglia et al. 1994), DHFS-FPGS (Salcedo et al. 2001) and DHFR-TS, (Bzik et al. 1987). These data are consistent with the view that *Plasmodium* synthesise folates *de novo*. Another relevant pathway known as the shikimate that has been described to feed into the folate synthesis via pABA has been identified in *Plasmodium* and its associated enzymes detected (Dieckmann et al. 1986; Fitzpatrick et al. 2001).

c) Incorporation of radio-labelled folate precursors into folate end products

A combination of radio-labelled techniques and HPLC assays has been used to trace the *de novo* folate pathway and to identify the end products. By the use of the radio-labelled folate precursors (GTP, pABA and Glutamate), in both parasitized RBCs and intact parasites, Krungkrai and colleagues (Krungkrai et al. 1989), were able to demonstrate the incorporation of the labelled compounds into a product identified by HPLC as pteroylpentaglutamate. These data are in line with the existence of the enzyme targets of these precursors and conclusively show that malaria parasites are capable of *de novo* folate synthesis.

1.3.2 Salvage

Although compelling evidence demonstrates the existence of the *de novo* folic acid biosynthesis as indicated above, *Plasmodium* has been observed to have evolved an intricate and adaptively-derived salvage capability of scavenging folate precursors, intact

folic acid and C_1 substituted folate derivatives. Clues to this observation first emerged when it was noted that the antimalarial activity of sulphonamides in *Plasmodium gallinaceum* was greatly influenced by the presence of pABA in the culture medium. Since then many reports have shown exogenously supplied intact folic acid and folinic acid aid malarial growth and survival and also mitigate the activities of antimalarial antifolates (Ferone 1977).

Folate-related salvage pathways are of three kinds.

1.3.2.1 Intact folic acid

The intact folic acid salvage (**Figure 1.3, light purple**) facilitates the utilization of the exogenously supplied intact folic acid. This is an important route because it is critical that the pterin moiety of the salvaged intact folic acid be in a reduced form for it to primarily function as a cofactor of carrying single-carbon units. Only the reduced dihydro and tetrahydro forms are metabolically active. Following the entry into the cell, the intact folic acid must therefore be reduced to an active tetrahydro-form. The capacity to reduce oxidised pterins has been observed in two main NADPH-dependent enzymes; DHFR and Pterin reductase 1 (PTR1, EC 1.5.1.33), (Nare et al. 1997).

The substrate specificity of *Plasmodium* DHFR is known to be quite narrow and it's predominantly involved in the conversion of DHF to the biochemically active THF. It is still not yet clear whether it has a reductase activity on oxidised pterins. Whereas much

higher activity is observed with DHF, DHFR from other sources have been shown to exhibit a weak activity with folates (Webber et al. 1985; Smith et al. 1987).

PTR1 is found in *Leishmania* and other kinetoplastids but no homologs have been observed in *Plasmodium* (Ouellette et al. 2002). PTR1 is a broad spectrum enzyme belonging to the short chain dehydrogenase/ reductase (SDR) family and its ability to reduce pteridines such as biopterin and folate has been established by genetic and biochemical approaches (Nare *et al.* 1997).

It is capable of reducing folate to DHF and DHF to THF. If present in *Plasmodium*, the expression of PTR1 would offer a 'metabolic by-pass' of DHFR inhibition but wild type DHFR *Plasmodium* isolates demonstrate sensitivity to DHFR inhibitors. This observation in addition to the absence of PTR1 homologs suggests *Plasmodium* does not utilise this pathway for 'intact folate salvage'. This returns us back to the possibility of the *Plasmodium* DHFR-TS being involved in this function. Some evidence suggests so (Hyde 2005).

1.3.2.2 C_1 substituted Tetrahydrofolate salvage

Two well studied representatives of the C_1 substituted tetrahydrofolate cofactors are the 5-Methyl- and 5-formyl- tetrahydrofolate monoglutamates (5-MeTHF and 5-FoTHF respectively). Unlike the intact folic acid, these derivatives do not require the action of DHFR for their conversion and their function as THF cofactors are unaffected by the

inhibition of this enzyme. A presumed route for the salvage of the monoglutamate C_1 substituted THF is via FPGS (**Figure 1.3, maroon colour**) since polyglutamation is required for the intracellular retention of folates. There are a number of unique features associated with the incorporation of the 5-Methyl and 5-formyl THF into cellular metabolism as outlined below.

5-MeTHFn (polyglutamated) is the predominant folate species in mammalian cells (plasma concentration in the region of 3-30ng/ml) but has been described as a very poor substrate for folylpolyglutamate synthetase (Cichowicz et al. 1987). Following the entry into the cell, the incorporation of 5-MeTHF into cellular metabolism therefore requires the conversion of 5-MeTHF into THF or 5, 10-methyleneTHF$_1$ with subsequent conversion of 5, 10-methyleneTHF$_1$ into THF$_1$, DHF$_1$ or 10-formylTHF$_1$ which are excellent substrates for folylpolyglutamate synthetase (Green et al. 1988). Two enzymes known to react with 5-MeTHF; methylenetetrahydrofolate and cobalamin-dependent methionine synthase have been detected in *Plasmodium falciparum* and the parasite's trophozoite and schizont stages shown to take up exogenously supplied 5-MeTHF and incorporate the methyl substituent in the synthesis of methionine and regeneration of THF (Asawamahasakda et al. 1993).

5-FoTHF is the most chemically stable folate found in nature and unlike the other substituted derivatives of the tetrahydro forms, it does not serve as a single carbon unit donor in folate linked metabolism. *Plasmodium falciparum* has been known to take up exogenous 5-formylTHF$_1$ and the only enzyme known to participate in what has been

termed a salvage reaction of the 5-formylTHF$_1$ is 5, 10-methenylTHF synthetase (5-formylTHF cycloligase, EC 6.3.3.2). This enzyme prefers the polyglutamated form to the monoglutamate and catalyses the irreversible ATP-dependent conversion of 5-formylTHF$_1$ to methenylTHF (Roje et al. 2002).

1.3.2.3 pABA salvage

It has been established that certain lines of *Plasmodium falciparum* can grow at normal rates in media deplete of pABA (Milhous et al 1985). This observation combined with the detection of the *Plasmodial* enzymes for the synthesis of pABA via the shikimate pathway suggest that the parasites can synthesize sufficient pABA *de novo*. Despite the presence of this biosynthetic machinery to synthesize pABA, it has also been demonstrated that *Plasmodium falciparum* require exogenous dietary pABA for survival and that this pABA can be converted to folates. A potential salvage route for pABA is via the folate synthesising enzyme DHPS **(figure 1.3, light blue colour)** where it is incorporated into dihydropteroate in the normal biosynthetic pathway as described earlier. Roberts and colleagues, (Roberts et al. 1998) have shown that the *in vitro* growth of *Plasmodium falciparum* was inhibited by glyphosate, an inhibitor of the shikimate pathway enzyme, 5-enolpyruvyl shikimate 3-phosphate synthase but this activity was reversed in the presence of exogenous pABA suggesting that the parasite has the capability of scavenging the supplied pABA.

1.3.2.4 Evidence for the existence of the folate salvage in *Plasmodium falciparum*

a) *In vitro* antagonism of antifolates by folic acid/ 'folate' derivatives/PABA.

The *in vitro* effects of exogenous intact folic acid, C_1 substituted THF derivatives and pABA on the antifolate activities has been extensively studied. Antifolates currently in malaria use generally target the DHFR and DHPS enzymes. The addition of pABA or folic acid to the test medium significantly reduce the anti-DHPS action through competitive antagonism while the 5-formylTHF, a reduced form of folate, is capable of reversing readily the effects of anti-DHFR action. Watkins and co-workers (Watkins *et al.* 1985) observed at concentrations of 10^{-7}M, pABA and folic acid were potent antagonists of sulphadoxine, (SDX) producing 20 to 110- and 7,000 to 8000-fold increase in IC_{50} of SDX respectively in both M24 (SDX-sensitive) and K39 (SDX-resistant) isolates. Kinyanjui and co-workers, (Kinyanjui et al. 1999) reported that the activity of Pyrimethamine (PYR) against M24 (PYR-sensitive) and K39 (PYR-resistant) parasites was reduced 10-12 fold by 10^{-5} mol/L of folic acid, and virtually eliminated by 10^{-5} mol/L of folinic acid.

b) Uptake of radio-labeled folate derivatives supplemented in culture media

The confirmation of the salvage pathway in the malaria parasite has been established through the identity of the similarity of the radio-labelled final product, 5-methylTHFpentaglutamate generated from either *de novo* biosynthesis or via the

utilization of folates or pABA. An exposure of the parasite in a medium containing radio-label pABA or folate and tracking the fate of the compounds has shown the parasite incorporates both the ^{14}C and ^{3}H labels of pABA and folates into 5-methyTHFpentaglutamate (Krungkrai *et al.* 1989). The transport of exogenous folate has now been characterised in both intact and infected red blood cells (Wang et al. 2007).

c) Folate supplementation and malaria during pregnancy

Although folate supplementation appears to improve the haematological recovery (a significant boost in serum and red cell folate levels) of conditions like pregnancy associated megaloblastic anaemia and the reduction of incidences of low birth weight (Mahomed 2000). There are also signals that folate supplementation may compromise the effectiveness of SP particularly in the Intermittent Prevent Treatment programme. In a randomised control trial of SP treatment with folate supplementation during pregnancy, women who received 5mg per day of folate supplementation were more likely to fail treatment compared to those who received the recommended dose of 0.4mg per day or folic acid placebo (Ouma et al. 2006).

d) Folate deficiency and malaria protection

The absence of folates and pABA has been shown to have a significant reduction in parasite multiplication and a suppression in the erythrocyte schizont division (Ferone 1977). There are also reports that folate supplementation may enhance the progression

and severity of malaria. An 'acceleration phenomenon' in disease severity and pre-existing parasitemia was reported in rhesus monkey infected with *P. cynomolgi* that received 0.1 mg dose of folic acid daily (Das et al. 1992). In this work, folate deficiency was induced in rhesus monkey with a folate free diet and another control group fed the same diet enriched with 0.1mg of folate daily. Following an injection with *P. cyanomolgi,* none of the rhesus monkeys on a folate free diet had clinical malaria while all the monkeys on folate supplementation developed clinical malaria with subsequent deaths reported. There are suspicions that red cell folate deficiency partly contributes to the protection against malaria observed in hemoglobinopathies such as sickle cell disease, thalassemia and glucose-6-phosphate dehydrogenase (Herbert 1993).
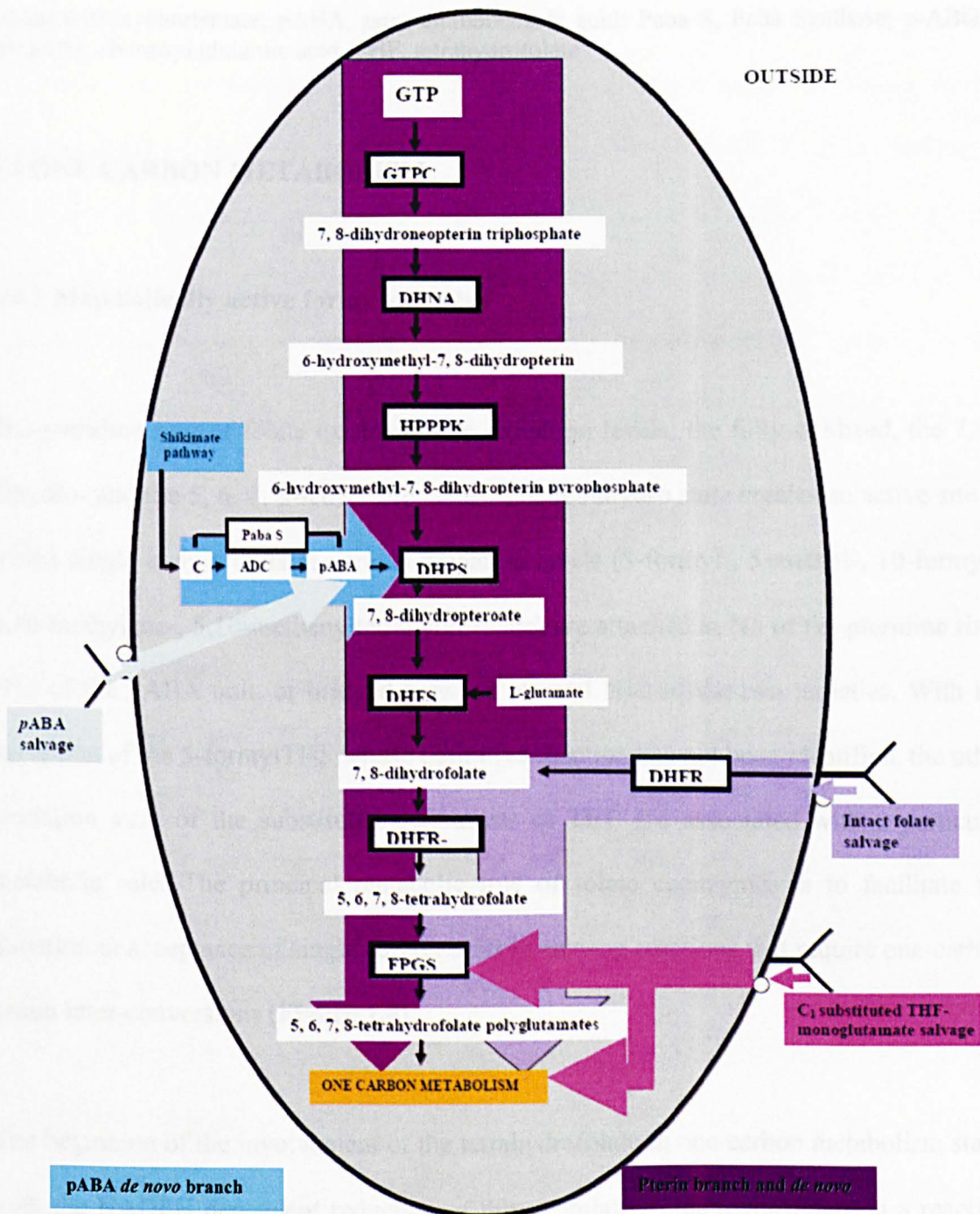
Figure 1.3 *Plasmodium falciparum* folate *de novo* synthesis and hypothetical a salvage pathway, Erythrocytic stage.
Enzymes are boxed while substrates and pathways are not boxed. **Pteridine and *de novo* branch:** GTP, Guanine triphosphate; GTPC, Guanine triphosphate cyclohydrolase I (EC 3.5.4.16); DHNA, dihydroneopterin aldolase **(EC 4.1.2.25)**; HPPK, hydroxymethyldihydropterin pyrophosphokinase **(EC 2.7.6.3)**; DHPS, dihydropteroate synthase (EC 2.5.1.15); DHFS, dihydrofolate synthase (EC 6.3.2.12); DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase (EC 6.3.2.17). **pABA branch:** C, chorismate; ADC, 4-

amino-4-deoxychorismate; pABA, *para*-aminobenzoic acid; Paba S, Paba Synthase; p-ABGA, *para*-aminobenzoyl glutamic acid. THF, tetrahydrofolate

1.4 ONE CARBON METABOLISM

1.4.1 Metabolically active forms of folates

The pteridine ring of folate exists at three oxidation levels; the fully oxidized, the 7, 8-dihydro- and the 5, 6, 7, 8-tetrahydro states. The tetrahydro state creates an active site to which single carbon units at various oxidation levels (5-formyl-, 5-methyl-, 10-formyl-, 5,10-methylene-, 5,10-methenyltetrahydrofolate) are attached at N5 of the pteridine ring, N10 of the pABA unit, or bridged between N5 and N10 of the two moieties. With the exception of the 5-formylTHF whose definitive function has not been identified, the other oxidation state of the substituted derivatives of THF are associated with a particular metabolic role. The principal metabolic role of folate coenzymes is to facilitate the donation or acceptance of single carbon units in enzyme reactions that require one-carbon group inter-conversions (**Figure 1.4**).

The beginning of the involvement of the tetrahydrofolate in one carbon metabolism starts with the NADPH dependent reduction of dihydrofolate to tetrahydrofolate in a reaction catalysed by DHFR (**reaction 1**). In this state, tetrahydrofolate has the potential to carry a single carbon moiety.

The key source of the single carbon unit in folate dependent one carbon metabolism is the β-carbon of the serine released during the inter-conversion of serine to glycine. In this reaction catalysed by Serine hydroxymethyltransferase, SHMT, EC 2.1.2.1 (reaction 2) tetrahydrofolate acts as an acceptor molecule of the carbon unit of serine and it's converted to 5, 10-methylenetetrahydrofolate. This single carbon unit exists at the formaldehyde level of oxidation and strategically positions the 5, 10-methylenetetrahydrofolate as a crucial intermediate in a number of folate linked single carbon metabolsim. The methylene unit of 5, 10-methylenetetrahydrofolate can be converted to the formate level for purine synthesis or the methanol level for methionine synthesis or be used directly for thymidylate synthesis. On the path committing 5, 10-methylenetetrahydrofolate to methionine synthesis, it undergoes a transformation to 5-methyltetrahydrofolate in a reaction catalysed by 5, 10-methylenetetrahydrofolate reductase EC 1.5.1.20 (reaction 3) while *en route* to purine synthesis, it is converted first to 5, 10-methenyltetrahydrofolate in a reaction catalysed by 5, 10-methylenetetrahydrofolate dehydrogenase (reaction 4). The synthesised 5, 10-methenyltetrahydrofolate sits at a branching point of committing the product either to purine synthesis via the 10-formyltetrahydrofolate in a reaction catalysed by the enzyme methenyltetrahydrofolate cyclohydrolase (reaction 5) or undergoing an irreversible hydrolysis to a metabolically inactive 5-formyltetrahydrofolate, a reaction catalysed by SHMT (reaction 9).

1.4.1.1 Role of folates

a) Thymidylate cycle

The functional role of folates in supplying single carbon units in enzyme reactions involved in DNA synthesis has been well documented and perhaps the most important (Ferone 1977). An uninterruptible flow of folates must be steady in *Plasmodium falciparum* since folate scarcity precipitates a disordered DNA synthesis leading to a thymineless death. Folates are not directly involved in the synthesis of the pyrimidine ring but participate in the biogenesis of the methyl functional group of thymine. The critical folate intermediate is 5, 10-methylenetetrahydrofolate which acts as a source of the single-carbon to methylate dUMP and form dTMP. The enzyme responsible is thymidylate synthase, EC 2.1.1.45 (**reaction 6**). In this reaction, the reduction of the methylene to a methyl group is accompanied by the oxidation of tetrahydrofolate to dihydrofolate. The resulting dihydrofolate must further be reduced in an NADPH-dependent reduction to tetrahydrofolate before it can participate again in the thymidilate synthesis making this stage the rate limiting step. It is this recycling process through dihydrofolate that accounts for the high sensitivity of *Plasmodium falciparum* to the anti-DHFR inhibitors. Anti-DHFR affect thymine synthesis by inhibiting the reduction of dihydrofolate formed during thymidylate synthesis.

b) Methionine cycle

5-methyltetrahydrofolate serves as the chief donor of the single carbon unit involved in methionine synthesis. The cobalamin-dependent methionine synthase EC 1.16.1.8

(reaction 7) catalyses the demethylation of 5-methyltetrahydrofolate to produce tetrahydrofolate and in the process homocysteine is converted to methionine. The enzymatic reactions involved in the synthesis of methionine and the contribution of 5-methyltetrahydrofolate have been demonstrated in *Plasmodium falciparum* (Krungkrai et al. 1989; Asawamahasakda and Yuthavong 1993). This is the route through which the methyl group of the salvaged 5-methyltetrahydrofolate is used for methionine synthesis and the tetrahydrofolate joins the folate pool.

c) Purine cycle

Unlike in pyrimidine synthesis where folate does not participate in the pyrimidine ring formation, here the 10-formylTHF is directly concerned with the introduction of the 2- and 8-carbons of the purine ring in a two step process. The first step involves the introduction of the 8-carbon by formylation of the glycinamide ribonucleotide (GAR) to give formylglycinamide ribonucleotide. This reaction is catalysed by 5'-phosphoribosylglycinamide transformylase, GART, EC 2.1.2.2 (reaction 8a). The second reaction involves the formylation of the aminoimidazole-4-carboxamide ribonucleotide (AICAR) to give formylaminoimidazole-4-carboxamide ribonucleotide. This reaction is catalysed by 5'-phosphoribosyl-5-amino-4-imidazole- carboxamide formyltransferase, AICARFT, EC 2.1.2.3 (reaction 8b). *Plasmodium* lacks these enzymes and therefore cannot synthesise purines *de novo* (Nzila et al. 2005). It relies on the salvage system to scavenge for purines from the environment.

d) 5-formyltetrahydrofolate cycle

The biological origin of 5-formylTHF was first observed in an *in vitro* enzymatic systems involved in the conversion of formate to serine, reviewed in (Stover et al. 1993) where it was shown formate was incorporated into serine in the presence of reducing equivalents. However, when the reducing equivalents were depleted, it was found that formate was incorporated into 5-formyltetrahydrofolate. This reaction was described as an irreversible hydrolysis of 5, 10-methenylTHF catalysed by a minor activity of SHMT, **(reaction 9)** the same enzyme involved reaction 2 of figure 4. A second unidirectional enzyme involved in the 5-formylTHF cycle is methenylTHF synthetase or 5-formylTHF cycloligase EC 6.3.3.2 **(reaction 10)**. This is the only enzyme known to utilise 5-formylTHF in an ATP-dependent irreversible conversion to 5, 10-methenylTHF. The definitive metabolic role of 5-formylTHF is not yet known and therefore together, SHMT and methenylTHF synthetase constitute a futile cycle.

5-FoTHF or leucovorin is used as a rescue agent following a methotrexate dose in cancer chemotherapy. Its usefulness is based on the salvage of the 5-formylTHF by methenylTHF synthetase to 5, 10-methenylTHF which undergoes a rapid conversion to 10-formylTHF and the tetrahydrofolate (Stover and Schirch 1993). *Plasmodium falciparum* is known to salvage 5-formylTHF and its impact in mitigating the antifolates activity has been reported (Kinyanjui *et al.* 1999). However, this salvage enzyme, methenylTHF synthetase has not been detected or homologous genes identified in

Plasmodium (Nzila et al. 2005). The mechanism involved in the utilisation of salvaged 5-formylTHF by the parasite is yet to be determined.
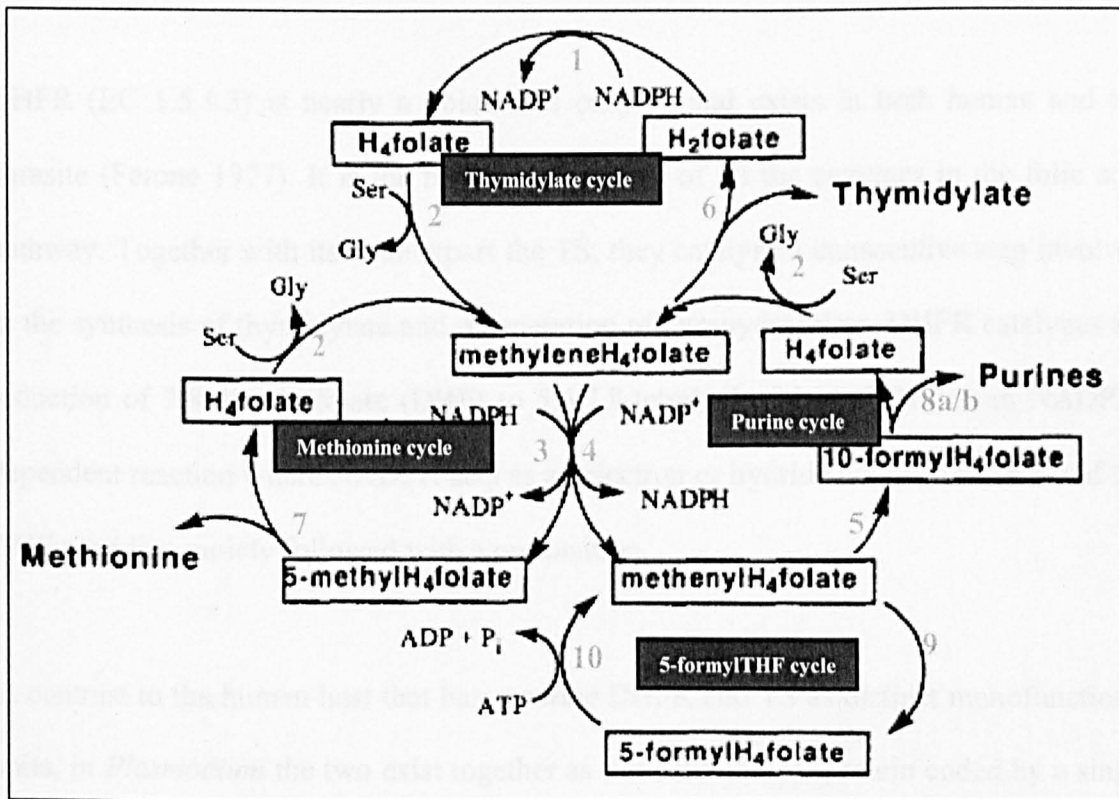
Figure 1.4: Metabolic pathways associated with one-carbon metabolism.

1, Dihydrofolate reductase **(EC 1.5.1.3)**; 2, Serine hydroxymethyltransferase **(EC 2.1.2.1)**; 3, 5, 10-methylenetetrahydrofolate reductase **(EC 1.5.1.20)**; 4, 5, 10-methylenetetrahydrofolate dehydrogensae **(EC 1.5.1.5)**; 5, methenyltetrahydrofolate cyclohydrolase; 6, Thymidylate synthase **(EC 2.1.1.45)**; 7, Methionine synthase **(EC 1.16.1.8)**; 8a, 5'-phosphoribosylglycinamide transformylase **(EC 2.1.2.2)**; 8b, 5'-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase **(EC 2.1.2.3)**; 9, Serine hydroxymethyltransferase **(EC 2.1.2.1)**; 10, methenylTHF synthetase/ 5-formylTHF cycloligase **(EC 6.3.3.2)**

1.5 Key therapeutic targets of the *Plasmodium falciparum* folic acid pathway

1.5.1 *P. falciparum* dihydrofolate reductase-thymidylate synthase (*Pf*DIIFR-TS)

DHFR (EC 1.5.1.3) is nearly a ubiquitous enzyme and exists in both human and the parasite (Ferone 1977). It is the most characterised of all the enzymes in the folic acid pathway. Together with its counterpart the TS, they catalyse a consecutive step involved in the synthesis of thymidylate and regeneration of tetrahydrofolate. DHFR catalyzes the reduction of 7,8-dihydrofolate (DHF) to 5,6,7,8-tetrahydrofolate (THF) in an NADPH-dependent reaction where NADPH acts as an electron or hydride donor to carbon 6 of the DHF pteridine moiety followed with a protonation.

In contrast to the human host that has separate DHFR and TS as distinct monofunctional units, in *Plasmodium* the two exist together as one bifunctional protein coded by a single gene located on chromosome 4 (Bzik *et al.* 1987). The *Pf* DHFR-TS is made up of 608 amino acids. The DHFR domain is located on the N-terminus and comprises of amino acids 1-228. It is linked by a junction peptide (amino acids 229-322) to the C-terminus which makes up the TS domain (amino acids 323-608) (Inselburg et al. 1989). It is the existence of the bifunctional structure that has led some workers suggest the phenomenon of 'electrostatic or substrate' channelling where the DHF product from the TS domain is channelled to the DHFR active site (Rathod et al. 2003).

The similarity of amino acid sequence of PfDHFR to DHFR of other species ranges from 24% to 42%. Of the 228 amino acids that make up the DHFR domain, the parasite only shares 61 of them with the human DHFR. Differences between the PfDHFR and the human DHFR have made this enzyme a chemotherapeutic target where selective inhibition has been exploited.

The PfDHFR-TS activity has been shown to be very low at the ring stage but a significant increase is noted at the trophozoite and schizont stage (Inselburg et al. 1989), the phase where active DNA synthesis takes place. In fact, the inhibition of DHFR is markedly felt at the level of the DNA synthesis compared to other folate associated metabolic functions.

1.5.2 P. falciparum dihydropteroate synthase (PfDHPS)

The enzyme PfDHPS catalyses the synthesis of 7,8-dihydropteroate from the condensation of pABA with 6-hydroxymethyl-7,8-dihydropterin. The basis for the utilisation of the DHPS as a pharmacological target emanates from the fact that humans lack the enzyme and are incapable of the folate de novo biosynthetic process. It is through the competitive inhibition of this enzyme that sulphonamides and sulphones exert their chemotherapeutic influence.

Like in the Pf DHFR, the DHPS exists as a bifunctional protein in conjunction with 6-hydroxymethyl-7,8-dihydropterin pyrophosphokinase (HPPPK). HPPPK catalyses the

immediate reaction preceding the one for DHPS. In terms of domain orientation, the DHPS domain occupies the C-terminus and the HPPPK the N-terminus. This protein is coded for by a single gene located on chromosome 8. The bifunctional protein has been shown to be expressed through out the parasite's asexual life cycle but more abundantly at the trophozoite stage which is characterised with intense DNA synthesis (Triglia and Cowman 1994).

1.5.3 *P. falciparum* Thymidate synthase (*Pf*TS)

As discussed in **section 1.5.1**, *Pf*TS is part of the bifunctional DHFR-TS enzyme where the two domains are established to participate in the catalysis of the consecutive steps involved in dTMP synthesis (section **1.4.1.1 (a))**. An inhibition of either enzyme leads to the disruption of DNA synthesis and ultimate cell death. As highlighted in **section 1.5.1**, the comparatively well studied *Pf*DHFR domain is highly divergent to the human DHFR. This is not the case with the TS domain which shows significant homology to its counterpart human TS. Despite this close homology, 5-fluoroorotate, a potent and selective inhibitor of the *Pf*TS has been identified with activity demonstrated both *in vitro* and *in vivo* (Rathod and Gomez 1991; Rathod et al 1989). There are two mechanistic approaches by which 5-fluoroorotate can inhibit the parasite, either through the direct inactivation of the TS domain or through its conversion to 5-fluorouridine and subsequent incorporation into RNA molecules. Rathod and colleagues (Rathod et al 1992) investigated the two mechanisms and conclusively established the antimalarial activity of 5-fluoroorotate is directly linked to its selective and tight action on the *Pf*TS.

1.5.4 *P. falciparum* shikimate pathway

As discussed in **section 1.3.1.2**, the shikimate pathway is the driving force for the *de novo* synthesis of pABA and that a functioning system is necessary for parasite growth as the inhibition of 5-enolpyruvylshikimate 3-phosphate synthase by glyphosate is reversed in the presence of pABA. This pathway is very unique to the parasite. Apart from a selected few of the seven upstream series of enzymes leading to chorismate that have been explored as candidate drug targets, there exists a huge potential of other downstream pathways after chorismate synthesis that still remain unexplored and these include aromatic amino acids, ubiquinone and vitamin K synthesis. In fact, of all the known downstream pathways, it is the folate synthesis via pABA that is well studied. With the concept of synergy in antifolates and the position of the shikimate pathway in feeding into this system, McRobert and colleagues (McRobert et al 2005) have identified shikimate analogues that potentiate the activity of other antimalarial drugs.

1.6 Antimalarial Antifolates

Antimalarial antifolates or folic acid antagonists are compounds that are known to act by interfering with specific enzymatic reactions involved in the biosynthesis of folate coenzymes. Thus far, in the entire spectrum of the folic acid pathway, only two enzymes, DHFR and DHPS have been utilised as pharmacological targets and hence the inhibitors are classified as anti-DHFR or anti-DHPS. Pyrimethamine and chlorcycloguanil (the active metabolite of chlorproguanil) are examples of anti-DHFR while sulfadoxine and

dapsone are examples of anti-DHPS. The target asexual erythrocytic stages of antimalarial antifolates are shown in figure 1.

1.6.1 The history, pharmacology and mechanism of action of selected anti-DHFR and anti-DHPS inhibitors

1.6.1.1 Chlorproguanil

1.6.1.1.1 History

Chlorproguanil is an off-shoot of proguanil and it is generated through the chlorination of the phenyl ring of the proguanil molecule. The compound was discovered in the 1940s by Curd and colleagues at Imperial Chemical Industries (Curd et al. 1945) in a collaborative work with the Liverpool School of Tropical Medicine. In their antimalarial exploratory research away from the quinolines and acridines, they noted activity in certain pyrimdine substances and this work subsequently led them to the biguanides. Investigations on the direct *in vitro* effects of the compound on the exoerythrocytic form of avian and monkey malaria showed it had no action upon the parasites at higher concentrations whereas serum from animals dosed with the drug had outstanding antimalarial activity. This observation led to the conclusion that the compound is converted into a molecule possessing antimalarial property.

1.6.1.1.2 Pharmacology

Chlorproguanil, (1E)-1-[amino-[(3, 4-dichlorophenyl)amino]methylidene]-2-propan-2-ylguanidine, CPG (figure 1.5) like its congener proguanil (PG) belongs to the chemical family of aryl biguanides that target the folic acid pathway. The drug is considered a pro-drug and it undergoes a metabolic activation to the active dihydrotriazine derivative, chlorcycloguanil (CCG) via hepatic cytochrome P450-dependent process. Inter-individual variation in the metabolic bio-transformation of CPG has been demonstrated (Helsby et al. 1991). The drug has a pronounced action on the late trophozoite stage of asexual erythrocytic cycle. It's used in combination with dapsone and has been shown to possess both a causal prophylactic and therapeutic effect.

1.6.1.2 Pyrimethamine

1.6.1.2.1 History

The discovery of pyrimethamine (daraprim) stems from work directed at the synthesis of 2, 4-diaminopteridines whose structures were noted to simulate the pteridine portion of the folate molecule (Hitchings 1952). This was based on an understanding that the rate of nucleic acid synthesis in reproducing parasites within hosts was more rapid. This comparatively pronounced nucleic acid synthesis could be the Achilles heel of the parasite as an antagonist could target the parasite more than the host. A test of these compounds on different species of organisms revealed the 2, 4-diamino derivatives were relatively potent species-specific inhibitors than the closely related 2-amino-4-

hydroxypteridines. A structural resemblance noted between a certain 2, 4-amino derivative and proguanil, later cycloguanil the metabolite (whose anti-malarial activity was known at the time) prompted the trial of pyrimidines as anti-malarial and the results were startling. The choice of daraprim was based on the consistent demonstration of activity against several species of *Plasmodia* including human malaria. Although introduced as a monotherapy, its synergy with other antifolate molecules such as SDX and the emergence of drug resistance promoted the use of this compound as a combination, fansidar (PYR/SDX).

1.6.1.2.2 Pharmacology

Pyrimethamine (2, 4-diamino-5-(p-chlorophenyl)-6-ethylpyrimidine, PYR), **(figure1.5)** is structurally related to trimethoprim and belongs to the chemical group of diaminopyrimidine that target the DHFR enzyme in the parasite's nucleic acid synthesis pathway. The drug affects the asexual erythrocytic stage of the parasite. It specifically targets the stage of chromatin division at the late trophozoite and schizont stages of the parasite. PYR is rapidly absorbed from the gastrointestinal tract and it's predominantly hepatically metabolised.

1.6.1.2.3 Proposed mechanisms of action of CCG and PYR

a) Inhibition of DHFR

Earlier experiments that showed the inhibitory activity of CCG and PYR could be reversed by folinic and folic acid in analogous fashion to aminopterin undoubtedly provided the proof of concept that these compounds are antifolates and could be targeting the same site as aminopterin (Hitchings et al. 1980). The biochemical basis for the pronounced activity of CCG and PYR against *Plasmodia* species is due to their strong intrinsic selective effects on the parasite's DHFR in comparison to the human host's DHFR. These compounds are structural analogues to the natural substrate, dihydrofolate and bind tightly to the *Plasmodial* DHFR thus blocking the reduction catalysis of dihydrofolate to tetrahydrofolate. This results in significantly diminished levels of recycled tetrahydrofolate hence disruption of the folate dependent metabolism system.

In addition to the definitive evidence on the role of mutations in mediating resistance to PYR and CCG based on IC 50, the successful heterologous expression of the wild-type and mutants *Pf*DHFR domain or bifunctional PfDHFR-TS enzymes in *Escherichia coli* and yeast (Sirawaraporn et al 1997; Sirawaraporn et al 1993; Wooden et al 1997) have provided an excellent platform for elucidating the kinetics and molecular properties of the enzymes and inhibition by these compounds. These expression systems have been adopted in order circumvent the difficulties associated with obtaining significant amounts of the parasite protein. Discrepancies in the kinetic parameters of the enzymes have been noted and this has been attributed to either the expression of *Pf*DHFR or the complex *Pf*DHFR-TS. In one study using the *Pf*DHFR (Sirawaraporn et al 1997), the naturally occurring mutant forms of the enzyme (**see section 1.7.1**) showed reductions in the relative efficiency, Kcat/Km compared to the wild-type form, similar Km values for the

dihydrofolate substrate and higher Km values for the NADPH. In contrast, another study utilising the *Pf*DHFR-TS (Sandefur et al 2007) suggest the mutant forms are more efficient than the wild-type i.e higher Kcat/Km, lower Km values for the substrate dihydrofolate and higher Km values for the NADPH.

The degree of binding of PYR and cycloguanil as a measure of free energies were also explored in the *Pf*DHFR study (Sirawaraporn et al 1997). The results showed lower free energies for PYR binding for the mutant forms (with an exception of the A16V + S108T) than the sum of the corresponding individual mutations while in relation to cycloguanil, the A16V + S108T had the largest. Analysis of the Ki indicated the mutant forms had higher values than the wild-type. These data are consistent with the observations made *in vivo* and *in vitro* where the roles of these mutations have been established (**see section 1.7.1).**

b) Site-specific uncoupling and inhibition of oxidative phosphorylation

A proposed activity of the biguanide derivatives on the mitochondrial respiration has been suggested (Schafer 1969). Biguanides were shown to demonstrate site specificity in oxidative phosphorylation and that their inhibition of energy transfer required an energised state of the mitochondria. However, this uncoupling effect is seen in the millimolar range rendering it pharmacologically irrelevant but the enhanced synergy between CPG and atovaquone is of profound interest.

1.6.1.3 Sulfadoxine

1.6.1.3.1 History

The starting point for sulfadoxine is tightly linked to the discovery of protonsil in 1935 by Gerhard Domark (Stokstad et al. 1987). In applying Ehrlich's testing procedure, he discovered that a red dye (protonsil) protected mice against haemolytic *Streptococci*. It was later shown that this activity was not due to the red dye but a metabolite identified as sulfanilamide. This gave rise to the synthesis of sulfanilamide and its chemical derivatives and the subsequent testing in many infectious diseases. The discovery of the antimalarial action of sulfanilamide compound was reported by Díaz de León (Díaz de León 1937). Following the bactericidal properties of the dyes Rubiazol Roussel, he decided to try it in the treatment of malaria and the results were satisfactory. Other tests followed and revealed the activity was generally of a higher order compared to the other existing antimalarial drugs. Although demonstrating antimalarial activities, their structure was distinct from the well known quinoline and acridine derivatives and hence the question of their mode of action arose. The demonstration that the antimalarial activities of sulphonamides are inhibited by pABA provided the definitive proof on the link between the structural relationship and the antimalarial mode of action.

1.6.1.3.2 Pharmacology

Sulfadoxine also spelled as sulphadoxine (N^1-(5, 6-dimethoxy-4-pyrimidinyl)-sulfanilamide, SDX) is a member of the sulphonamide family with the functional group, -$S(=O)_2$-NH_2, a sulfonyl group connected to an amine group, **figure 1.5**. The drug is active against the asexual erythrocytic phase of the parasite with a particularly profound effect on the late trophozoite and schizont stages. The drug is often used in combination with PYR. Following absorption, the drug is metabolised to the major N-acetylated derivative and a minor glucoronide form.

1.6.1.4 Dapsone

1.6.1.4.1 History

The first description of the synthesis of dapsone was from *p*-nitrothiophenol in 1908 in a research work that had no clinical or therapeutic meaning. This was a triumph discovery in the field of chemistry aimed at identifying a molecule of producing azo dyes. In 1941, Coggeshall and co-workers investigated the effectiveness of disodium *p, p'*-diaminodiphenylsulfone N, N'-diglucose sulfonate (promin) against avian, monkey and human malaria species and noted its anti-malarial activity. However, this compound could not be considered for anti-malarial adoption because there were other more active established drugs available and the worry about the toxicity which had been observed in certain clinical applications. Renewed attention to this compound was awakened after about 15 years when a possible suppressive activity against malaria was noted by the observation that there was no malaria among lepers who had been treated with DDS in a

holoendemic area. Recently, there has been rekindled interest in DDS because of increasing worries of other anti-malarial drug resistance, its low costs and its potent synergising activity with other anti-DHFR compounds.

1.6.1.4.2 Pharmacology

Dapsone (4, 4-sulfonylbisbenzenamine or 4, 4-diaminodiphenylsulfone, DDS) is a sulfone drug. It is structurally the simplest of the sulfones, all of which share the characteristic structure: a sulphur atom linked to two carbon atoms (**figure 1.5**). DDS possesses schizontocidal activity against the asexual erythrocytic stage of the parasite. It is rapidly absorbed through the gastrointestinal tract and transported through the portal circulation to the liver, where it is metabolised via two routes; the N-acetylation produced by the N-acetyltransferase or the N-hydroxylation produced by the cytochrome P450 enzymes. The major metabolite is the monoacetyldapsone.

1.6.1.4.3 Proposed mechanisms of action of dapsone and sulfadoxine

a) Inhibition of dihydropteroate synthase

Strictly speaking, of the proposed mechanisms of action of DDS and SDX, the inhibition of the DHPS is the most studied and well established. As mentioned earlier, *Plasmodium* is dependent on the endogenous folic acid synthesis. These drugs are structural analogues of pABA and compete for the active site of the enzyme thus blocking pABA

incorporation into dihydrofolic acid and consequently impairing the synthesis of the metabolically active folate coenzymes. The structural analogy between the SO_2 group of DDS and SDX and CO_2^- ion of pABA were initially pointed out and the negativity of the SO_2 group was partly linked to pABA antagonism. However, the anti-malarial superiority of DDS over SDX cannot be explained by the SO_2 group negativity and it's thought to be due to steric factors. The inhibitory effect of both drugs can be reversed by the exogenous supply of pABA and thus confirming their role in interfering with the parasite's *de novo* pathway. Another fact which indicates the similarity in their mode of action is the cross-resistance observed between the two drugs.

Some two totally different proposed possibilities for the mode of action of SDX and DDS which have so far failed to attract intense research interest are the sulfa-adduct formation and glucose utilisation.

b) Sulfa-adduct formation

There has been speculation about the parasite's ability to convert sulfa based compounds (which mimic pABA) to sulfa-pterin adducts such as sulfa-dihydropteroate (sulfa-DHP) by the target enzyme DHPS (Chulay et al. 1984). Sulfa-DHP has been identified in *Escherichia coli* and *Saccharomyces cereviae*. In *S. cerevisiae* sulfa-DHP was inhibitory to growth through competition with dihydrofolate. This suggests an additional mechanism of action for SDX other than direct inhibition of DHPS. Following the administration of radiolabelled sulfadoxine to *P. falciparum*, 50% of the drug was

converted to an adduct of high molecular weight, hypothesised to be sulfadoxine-DHP. Following purification of this compound, the adduct had superior *in vitro* antimalarial activity compared to sulfadoxine, and furthermore, efficacy was not antagonised by physiological levels of folate or point mutations in the DHFR and DHPS domains. The *in vivo* target of sulfa-DHP is yet to be elucidated (Patel et al. 2004).

c) Inhibition of glucose utilisation

Another mechanism of anti-malarial action for DDS involving the inhibition of glucose utilization has been suggested (Cenedella et al. 1970). Work with *P. berghei* in rats demonstrated that this inhibition originates through interference with glucose transport at the level of the host's red-cell membrane (where DDS accumulates) in contrast to a direct effect upon the intracellular parasite, since the inhibition is antagonized by raising medium glucose concentrations. The addition of pABA had no apparent effect upon the DDS inhibition of intraerythrocytic parasite glycolysis. This work has been corroborated by a study in which a single oral dose of DDS (100 mg/kg) had less effect on depressing malarial infections in chronically hyperglycaemic mice than it did in normoglycemic mice. In addition, parasitised blood cells removed from normoglycemic rats 6 hours following an oral dose of 100 mg/kg DDS consumed less glucose than identically parasitised cells from untreated rats (Cenedella et al. 1971).

Pyrimethamine
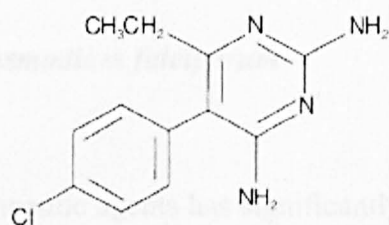
Sulfadoxine
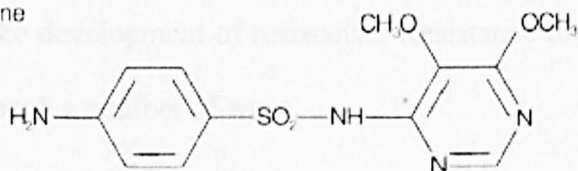
Chlorproguanil
(Lapudrine)

Chlorcycloguanil
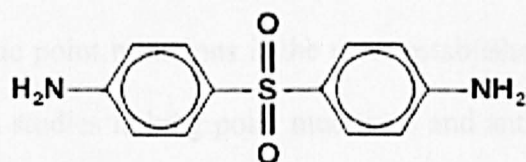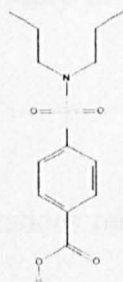
Dapsone

Probenecid

Figures 1.5 Structures of pyrimethamine, sulfadoxine, chlorproguanil, chlorcycloguanil, dapsone and probenecid

1.7 Antifolate resistance in *Plasmodium falciparum*

The development of chemotherapeutic agents has significantly improved the therapy and management of malaria. However, a potential concern for antimalarial drug treatment including antifolates is the development of resistance. Resistance to antifolates has been proposed to occur as through a number of ways.

1.7.1 Proposed mechanisms of resistance to anti-malarial anti-folates

a) Synthesis of altered DHFR/DHPS (point mutations)

Of all the proposed mechanisms of antifolate resistance, the synthesis of altered DHFR and DHPS due to genetic point mutations is the most established and well defined. The literature is replete with studies linking point mutations and antifolate resistance both *in vitro* and *in vivo*. The molecular basis of resistance due to point mutations is the introduction of successive amino acid substitutions near the active site of the target enzymes thus reducing the binding affinity of the drugs while not compromising the utilisation of the substrate.

A unique observation on the point mutations relevant to anti-DHFR and anti-DHPS has been noted. Accumulation of point mutations on the DHFR gene is known to follow an ordered staircase fashion with the level of resistance rising as the number of point mutations increase. CCG resistance is strongly linked to the presence of the rare dual

mutations Ala16Val + Ser108Thr on the *dhfr* gene while PYR is linked to the acquisition

of the following point mutations: Ser108Asn; Asn51Ile/Cys59Arg + Ser108Asn;

Asn51Ile + Cys59Arg + Ser108Asn; or Asn51Ile + Cys59Arg + Ser108Asn + Ile164Leu

(Sirawaraporn et al. 1997). Molecular genetic cross analysis of *P. falciparum* DHFR-TS

has shown that the Ser 108 substitution to Thr or Asn is the prerequisite mutation leading

to a low level resistance to CCG or PYR respectively (Peterson et al. 1988). Cross-

resistance between these two compounds is known to occur and the culprit allelic

combination is the Asn51Ile + Cys59Arg + Ser108Asn + Ile164Leu.

Resistance to SDX and DDS is associated with a combination of point mutations on the

dhps gene. SDX and DDS are cross-resistant to one another. Different combination of

point mutations at positions Ser436Ala/Phe, Ala437Gly, Lys540Glu, Ala581Gly and

Ala613Thr/Ser appear to play a critical role in the development of resistance to these

compounds (Triglia et al. 1997).

Epidemiological surveillance data show some difference in the pattern of the nature of

antifolate resistant alleles at both inter-continental and intra-continental level. In Bolivia,

South America a novel Cys50Arg and a repeat termed 'Bolivia repeat' have been

detected (Plowe et al 1997). The Cys50Arg and Bolivia repeat were consistently found in

the presence of the Asn51Ile and the entire Bolivia repeat harboured the I164L. In that

site it appears these two forms have replaced the Cys59Arg which is common in some

parts of Africa. Also the Ile164Leu which is common in Bolivia and South East Asia is a

rare occurrence in Africa and has not been consistently detected.

In relation to the DHPS, it is the Ser436Ala together with Lys540Glu or Ala581Gly that were found in Bolivia. Unlike the scenario in Bolivia, molecular data in Africa strongly indicate the point mutation at position 437 is a prerequisite and in most cases it pairs with either a mutation at position 540 or 581 which augment the level of resistance, see (Sibley et al. 2001; Gregson and Plowe 2005) for comprehensive review.

The independent contribution of the mutations in either *dhfr* or *dhps* to the clinical failure of antifolates is still a matter of conjecture since different *dhfr* and *dhps* mutation combination has been observed in different treatment or parasitological outcomes. However, the triple *dhfr* [Asn51Ile + Cys59Arg + Ser108Asn] in conjunction with the double *dhps* [Ala437Gly + Lys540Glu] has been strongly linked to both treatment and parasitological failure associated with PYR/SDX, fansidar (Sibley, Hyde et al. 2001).

b) Over-expression of the target protein

Over-expression of the drug target is one of the earliest proposed mechanisms of resistance to the antifolates. In one study, Inselburg and co-workers (Inselburg et al. 1987) investigated the DHFR-TS enzyme content of a PYR mutant, FCR3-D7. This mutant was isolated after progressively increasing the PYR levels in a parasite population of the parent sensitive strain, FCR3. They compared the [³H] FdUMP-labeled DHFR-TS levels prepared from FCR3 and FCR3-D7 parasite extracts. Their work showed PYR resistance in the mutant FCR3-D7was connected to a demonstrable increase of 5-10 times

in the DHFR-TS protein. The increased DHFR-TS protein level was also shown to correspondingly bind higher levels of PYR. This increase in DHFR-TS is thought of to be achieved through gene duplication.

c) Synthesis of pteroate-analog

The speculation that sulfonamides condense with the pool of 7, 8-dihydropterin by reacting as a pABA substrate analog to form a pteroate adduct responsible for a novel mechanism of action (Patel *et al.* 2004) has equally been proposed as a possible mechanism of resistance. An investigation on the significance of the formation of sulfadoxine-analog product to the mechanism of resistance has been studied (Dieckmann and Jung 1986). Uninfected red cells were unable to convert sulfadoxine to a pteroate-analog but the synthesis of this sulfa-adduct was significantly reduced in sulfadoxine-resistant strain compared to the sensitive one. The sulfa-adduct has been presumed to be the toxic agent targeting other downstream folate enzymes and a reduction in its formation by the resistant strain could be linked to their better survival when challenged with the drug (Dieckmann and Jung 1986).

d) Altered transport of antifolate

Antifolates are generally simple lipophilic compounds that easily traverse the membrane bilayer. Dieckmann and Jung studied the uptake of [^{35}S] sulfadoxine in uninfected red cells and in both sensitive (FCBR) and resistant (W2) strains of *Plasmodium falciparum*

(Dieckmann and Jung 1986). Their work showed that infected cells accumulated more of the radio-labeled sulfadoxine than the uninfected cells. When the drug accumulation was compared between the sensitive and resistant strain, the sensitive strain accumulated the drug six times more than the resistant ones. It is not clear whether this relatively increased drug accumulation is responsible for the enhanced 'killing effect' in the sensitive strain.

e) Intracellular expansion of tetrahydrofolate cofactors through salvage

The impact of alterations in cellular THF-cofactors through the incorporation of salvaged folates has been demonstrated. Salvaged folates are thought to augment the cellular folate pool or increase the level of specific substrates that effectively compete with the antifolates at the stage of their targets and hence potentially diminishing the activity of these antifolates.

The importance of this resistance phenomenon has recently gained intense interest as research currently is being directed towards the identification and characterization of the membrane transporters involved and isolating the mechanisms underlying the observed inter-strain variability in the utilization of the exogenous folate sources.

1.8 Strategies to overcome anti-folate Resistance

The elucidation of the biochemical processes involved in both *de novo* synthesis and salvage of folate, the molecular characterisation of the key targets in the folate pathway

and an improved understanding on the mode of action of antifolates and resistance mechanisms has tremendously allowed the development of strategies to overcome antifolate resistance.

a) Novel antifolates

The crystal structures of the wild type and mutant DHFR-TS complexed with the anti-DHFR inhibitors PYR and the dihydrotriazine WR99210 has unravelled insightful molecular information on the role of the mutated residues in conferring resistance and providing useful clues as a guide on the development of novel anti-DHFR inhibitors (Yuthavong et al. 2005). Unlike PYR, WR99210; 4,6-diamino-1,2-dihydro-2,2-dimethyl-1-(2,4,5-trichlophenoxypropyloxy)-1,3,5-triazine belongs to the second generation of antifolates. Since its first reported *in vitro* antimalarial activity in 1973 (Rieckmann 1973), the compound has demonstrated consistent potency against both drug-sensitive and drug-resistant malaria parasites (Edstein et al 1997) with a superior activity compared to the first generation of antifolates described in **section 1.6.1.** It also lacks cross-resistance to the DHFR inhibitors, PYR, CPG and PG (Edstein et al 1997). These excellent properties prompted a clinical trial for the safety and tolerance in healthy volunteers (Canfield and Rozman RS 1974) but the outcome was not promising. Following oral administration, volunteers experienced gastrointestinal irritations with a poor drug bioavailability reported and as a consequence, further development of the drug was abandoned. To circumvent these problems and exploit the observed drug's advantages, a synthetic approach similar to the bioconversion of proguanil to cycloguanil

was employed and a precursor compound of WR9910 known as PS-15 developed (Canfield et al 1993). PS-15 (N-(3-(2,4,5-trichlorophenoxy)propyloxy)-N'-(1-methyethyl)-imidocarbonimidicdiamide has a good bioavailability property and like WR99210 retains intrinsic significant potency against both sensitive and resistant parasites and no cross-resistancce to the DHFR inhibitors (Edstein et al 1997). Although PS-15 turned out a huge success in overcoming the problems associated with WR99210, it has experienced severe obstacles in its development process (Jensen et al 2001). There are very tight government restrictions in the manufacture of PS-15 starting material, 2,4,5-trichlophenol and the disposal of wastes associated with its manufacture (Jensen et al 2001). Due to this, synthesis of structural analogues of PS-15 that avoid the use of the regulated starting material is in the pipeline (Jensen et al 2001). So far, a number of these analogues for example JPC-2067-B (Jacobus Pharmaceutical Company, Inc., New Jersey, USA) have shown great promise with potent *in vitro* activity against drug resistant parasites and in *in vivo* tests in mice (Mui et al 2008).

b) Antifolate combination

Although chloproguanil /dapsone-artesunate (CDA) has been withdrawn from the market based on concerns regarding the prevalence of glucose-6-phosphate dehydrogenase deficiency (G6PD) in african populations, the principle behind the drug formulation was an excellent strategy of protecting and overcoming antifolate resistance. In their own respect, CCG and DDS are individually more potent inhibitors than PYR and SDX (Winstanley et al. 1995). As a combination, they derive their potent synergistic activity

through the sequential blockade of enzymes in the folate pathway in a similar way to PYR/SDX combination. Unlike PYR and SDX, the comparative rapid elimination of these compounds from the body exerts less selection pressure and lower resistance development (Winstanley et al. 1995). The incorporation of the artesunate component would slow resistance development or effectively enhance its activity in the eventuality of a Ile164Leu mutant establishing itself in Africa. This triple combination has another added advantage because it demonstrates varying effects on the different stages of the malaria parasite's life cycle offering a complementary effect by reducing the parasite biomass and slowing the development from one stage to another. This antifolate combination has since been recalled due to concerns of toxicity in glucose-6-phosphate-dehydrogenase deficiency cases.

c) Antifolate resistance reversal

The strategy of antifolate chemosensitization in *Plasmodium* gathered momentum with the pioneering work on the effect of probenecid (PBN; **figure 1.5**) on antimalarial antifolates (Nzila et al. 2003). PBN was shown to reverse the resistance of antifolates in the presence of physiological levels of folic acid. The effect of PBN was also associated with a reduction in the uptake of radio-labelled folic acid in infected cells.

This concept of resistance reversal has been used in antifolate cancer chemotherapy. As described by Kamen and colleagues (Kamen et al 1991), the receptor-coupled transmembrane transport of folate ligand proceeds via a four step process (i) binding of ligand to receptor (ii) translocation of ligand-receptor complex to the cell interior (iii)

ligand receptor dissociation (iv) ligand movement through an organic anion carrier into the cytoplasm. PBN inhibits the movement of folates through the interaction with the organic anion carrier and does not affect the receptor binding process or the internalization steps.

It is hypothesized that PBN may inhibit the transport of folates that are capable of boosting the intracellular levels of folate derivatives which compete with the antifolates at their target site (Nzila et al. 2003).

An open randomized clinical trial has been undertaken to compare the effectiveness of PYR/SDX, fansidar alone or in combination by the addition of PBN in the treatment regimen (Sowunmi et al 2004). It was observed that PYR/SDX-PBN combination improved treatment efficacy.

1.9 Challenges facing the development and deployment of novel therapies and future role of antifolates

As discussed in **section 1.1.1,** the global malaria toll is huge but this market does not necessarily translate into an appealing venture for the pharmaceutical industry as the profitability margin is very low since a majority of the patients cannot afford treatment. In fact, most of the work on anti-malarial drug discovery is done within the academia but further development of the compound ultimately requires the input of the pharmaceutical industry but with lack of interest, very few molecules are bound to reach the level of human clinical trials. Performing clinical trials to the expected standard is resource

intensive. It requires trained personnel, trial drugs have to meet Good Manufacturing Practices standard (GMP), excellent laboratory facilities and proper monitoring of patients.

Apart from the parasite's ability to develop resistance to virtually every molecule it is introduced to, most of these compounds target specific stages of the parasite. With specific reference to antifolates, we have pointed out in **section 1.6** that the antifolates principally target the trophozoite and schizont stages (stages of active DNA synthesis) of the parasite yet gametocytes are an important stage in resistance dissemination. Tackling the parasite's potential to develop resistance and slowing dissemination by targeting the gametocytes require an antifolate combination with a partner drug. Co-formulation or co-packaging of drugs is an additional cost that may hamper the patients' ability to purchase the drugs.

Drug safety is a critical consideration in the development of drug molecules. We have cited the case of CDA in **section 1.8 (b)** as one drug combination of great promise that suffered withdrawal due to the haemolytic effect of the dapsone component on G6PD deficient individuals.

Other barriers that compound the deployment of novel therapies include misdiagnosis, distance from health facilities and the increased chances of fake drugs circulating in the market, see (Whitty et al 2008; Craft 2008) for comprehensive review.

Despite the overwhelming challenges faced by antifolates in development, the position of the existing antifolate SDX-PYR in Intermittent Preventive Treatment of infants, children and pregnant women appears very strong and is responsible for a significant reduction in cases of mortality and morbidity associated malaria.

1.10 Molecular and Cellular transport of folates in *Plasmodium falciparum*

The molecular biology of folate transport in *Plasmodium falciparum* is poorly understood and is presently the focus of considerable attention. The current status of cellular folate transport system in organisms appears to be of two forms; (1) organisms completely incapable of synthesizing the pterin moiety from GTP (*de novo* folate biosynthesis) including mammals and thus exclusively fulfil the need for folates by relying on the salvage mechanism of preformed folates. (2) Organisms capable of *de novo* folate synthesis but also with the capacity to acquire folates from the host's environment. This phenomenon has been identified in intracellular parasites such as *P. falciparum* and *T. gondii*. Although having the biosynthetic machinery, intracellular parasites enjoy the luxury life within the host cells rich in nutrients. It appears the parasites have over time adaptively evolved the ability to obtain complex nutrients such as the membrane impermeable folates from the host while retaining the *de novo* system. The relative contribution of each route in meeting the folate need has not been established. It will be interesting also to see whether the acquisition of the salvage system can lead to certain strains of *P. falciparum* loosing completely the *de novo* capability.

Folates are hydrophilic charged molecules that require transporters to cross the lipid membrane bilayer. Therefore organisms capable of salvaging folates must develop transporters/carrier or receptor systems.

The biochemical mechanisms involved in the membrane folate transport in mammalian cells have been extensively characterised (Matherly et al. 2003). It comprises of (1) the glycosylphosphatidylinositol-linked folate receptor; a specific, high-affinity, externally-oriented, membrane associated folate binding protein on the surface of the cells. The receptor has been shown to mediate a unidirectional internalization of folate through a process referred to as 'potocytocis'. (2) An integral membrane protein reduced folate carrier; it is the best studied and mediates the bidirectional transport of both reduced folates and antifolates such as methotrexate. (3) The organic anion carrier; a member of the facilitative carriers that can transport folates and other organic anions. (4) The low pH folate transporters; operate at low pH and is the mechanism of folate transport in intestinal cells.

Unlike the mammalian cells, folate transport in *P. falciparum* is slightly complex. Since the parasite is intraerythrocytic, folates must cross the red cell membrane, the parasite cytoplasmic membrane and the parasitophorous vacuolar membrane. Transport of folates in *P. falciparum* has been biochemically characterised (Wang *et al.* 2007). Uptake measurements confirmed that *P. falciparum* have the ability to transport folates but the corresponding genes are the subject of investigation. It is unlikely these genes could be related to any of the mammalian transport systems described above since these systems

lack homologs among protists, bacteria and plants. High affinity folate and biopterin transporters are known to occur in the auxotrophic trypanosomatid protist, *Leishmania* and have been shown to confer the ability to transport folates and antifolates (Ouellette *et al.* 2002). The folate-biopterin transporters (FBT) family (TC #2.A.71) is a member of the major facilitator superfamily of secondary carriers and homologs of this protein have been identified in cyanobacteria and arabidopsis (Klaus et al. 2005). The average hydropathy plot indicate that the FBT family proteins exhibit 12 (10-14) putative TMSs (Chang et al. 2004). It is hypothesised proteins of similar function exist in *P. falciparum* and could be responsible for the mediation uptake process of folates across membranes. Investigations of the putative homolog genes in *P. falciparum* that might encode members of the FBT family will be discussed.

1.11 Aims of thesis

The primary focus of the thesis has exclusively been on studies on *Plasmodium falciparum* with a remit to build a clear understanding on some of the critical and relevant questions relating to folates transport and antifolates resistance. The direction of this work has been constructed on the following themes of interest;

1. Biochemical studies on the mechanism of action of antifolates and resistance reversal

2. Identification and sequence analysis of putative folate transporters genes

3. Functional characterization of heterologously expressed putative folate transporters in the *Xenopus laevis* oocyte.

4. Transcriptional regulation of putative folate transporter genes.

5. The detection of a rare anti-folate drug resistant allele, the mutant DHFR 164 using the Real-Time Polymerase Chain Reaction

CHAPTER 2

General materials and methods

2.1 Washing of Red blood cells

Human red blood cells (type O[+]) for malaria culture were obtained from National Blood

Service, West Derby Street, Liverpool, UK after testing negative for HIV, HCV, HBsAg,

syphilis and assumed to be free from malaria parasite and traces of any anti-malarial

drugs. Aseptic conditions were maintained through out the washing process. Appropriate

volume of blood was distributed in 50-ml centrifuge tubes and centrifuged (MSE,

SANYO) at 3000g for 5 min. The plasma and much of the buffy coat were discarded. For

normal routine culture work, the cells were re-suspended in cold folate supplemented

Roswell Park Memorial Institute, RPMI 1640 medium buffered with 25mM HEPES/0.02

mg/ml gentamycin (wash media + folate) to the 50-ml mark and re-centrifuged as above.

The supernatant was drawn off and the washing repeated two more times. If cells were to

be used in assays that do not require 'folates', the washing was done in folate-free RPMI

1640 buffered with HEPES (wash media - folate). To minimise contamination of the

cultures with the white blood cells, cells were always drawn from the bottom of the

sedimented mass. When not in use, the cells were kept at 4°C and not used if more than 5

days old.

2.2.1 Preparation of non-dialysed human serum

Varying volumes of blood supplied in blood bags with no anticoagulant (Baxter, UK) were obtained as a kind gift from the Haematology and Gastro-intestinal units of the The Royal Liverpool Hospital, UK. The blood was allowed to stand overnight at room temperature in the original bags and the plasma squeezed into 50ml sterile non-pyrogenic tubes (Greinerbio-one, Germany) under aseptic conditions. Remnant clots were "rimmed" with sterile pippets, the tubes centrifuged for 5 minutes at 3,000g and the supernatant serum aspirated. Non-dialysed human sera from approximately 20 different people were pooled together, distributed into 50-ml tubes and frozen at -80°C for later use. On the day of assay, the serum was thawed by placing the tubes in a 37°C water bath.

2.2.2 Preparation of human dialysed serum

Normal human serum contains freely dialyzable pABA at 250-300 µg/L and total folate at ~ 36nM. (Tan-ariya et al. 1983; Pfeiffer et al. 2004). Therefore, the serum was extensively dialysed to minimise the presence of these exogenous folate sources in assays that require folate-free conditions. The serum was dialysed in wet dialysis membrane tubing with a molecular weight cut-off of 3,500 (Medicell International Ltd, London UK) at 4°C against a hundred volume of sterile 1X Phosphate-buffered saline (PBS), pH 7.4 for 3 days with buffer changes every 8 hours. Usually these changes are sufficient to

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deplete serum total folate and pABA to undetectable levels (limit 1fg/ml). The serum was then filter-sterilised and frozen at -80°C.

2.3 Slide Preparation, Giemsa staining and parasitemia determination

The growth of the parasites and the specific forms/stage was monitored daily through the observation under a light microscope (Zeiss, Germany). One drop of culture material was placed on a microscope slide (76 X26 mm, VWR International) and a thin blood smear prepared, fixed for 5 s with absolute methanol and stained in 1% Giemsa for 10 min. Infected RBC in a total of 1000 RBC were counted using the laboratory counter (Clay Adams, Parsippany, NJ) and parasitemia determined as; number of parasitized RBC/total RBC X 100.

2.4 Preparation of HEPES

The lactic acid produced by *P. falciparum* during *in vitro* cultivation tends to create an acidic condition for the culture. In order to regulate the pH shift, HEPES (N-[2-Hydroxythyl]piperazine-N'-[2-ethanesulfonic acid] has been found to offer a satisfactory buffering capacity. A 1M HEPES buffer (molecular weight, 283.3, SIGMA, Poole, UK) was prepared in double distilled water and pH calibrated to 7.4 using 5M NaOH. The solution was filter-sterilised and stored at 4°C until required for use.

2.5 Preparation of culture media; folate supplemented and folate-free

Media preparation was carried out under aseptic conditions in the Microbiological Safety Cabinet, Unidirectional Laminar flow (ENVAR, UK LTD, York Avenue, Hanslingden, UK). Two types of culture media were used. For general continuous culture, the standard RPMI-1640 medium (containing folic acid and pABA each at 1.0mg/L, Sigma, Poole, UK) supplemented with pooled 10% non-dialysed human serum, designated herein as folate rich media (FRM) was used while for assays that require a folate free condition, a specially ordered media, RPMI-1640 (Poole, Sigma, UK) without folate, L-glutamine and $NaHCO_3$ and supplemented with 10% dialysed human serum, designated herein as folate deficient media, (FDM) was employed. The FDM was reconstituted to contain 2mM L-glutamine and 23.8mM $NaHCO_3$ as in the standard medium. The two media were buffered with 25mM HEPES and 0.02 mg/ml gentamycin and kept at 4°C when not in use.

2.6 Cryopreservation of parasites

Parasite cultures made up mainly of the ring stage (>5%) were centrifuged at 2000g for 5 min and the supernatant discarded. The resultant pellet (~1ml) was resuspended in an equal volume of filter-sterilised cryoprotectant freezing solution (28% glycerol; 72% of 4.2% ^{w}/v D-sorbitol in 0.9% ^{w}/v NaCl). The parasite suspension was gently 'pipet mixed' and then 1ml transferred into well labelled cryogenic vials (non-pyrogenic polypropylene sterile, Corning Incorporated, Corning, NY 14831 Mexico). The samples were

immediately stored under liquid nitrogen (Union Carbide, UK) in storage tank Kseries cryostorage system, (JENCONS, Scientific LTD).

2.7 Retrieval of cryopreserved isolates

Desired vials of parasite strains were carefully removed from the liquid nitrogen storage tank and left to thaw at room temperature in the laminar flow hood. The contents were quickly transferred aseptically to the 15ml sterile non-pyrogenic tubes (Greinerbio-one, Germany) and centrifuged at 2000g for 5 min. The supernatant was discarded and the pellet resuspended in an equal volume of sterile 3.5% ^{w}/v NaCl followed with another centrifugation as indicated above. The supernatant was discarded and the pellet resuspended in 5 ml complete media (FRM) and then centrifuged at 2,000g for 5 min. Following centrifugation (2000g, 5 min), the supernatant was discarded and the parasite pellet resuspended in 15 ml complete media plus 0.5ml washed RBC. The cell suspension was transfered to a 40ml sterile culture flask (Nunc^{TM} Rosklide, Denmark), gassed and then transferred to the 37°C incubator.

2.8 Gassing of Parasites

Parasites were grown in an atmosphere of a gas mixture comprising of CO_2-3%, O_2-4% and N_2-93% (BOC, Guildford, UK). For routine growth of the parasites in culture flasks, a reasonable flow of the gas was passed through a 0.22µm millipore filter and the cultures flashed for 1 min following which the flasks were tightly closed. Cultures grown

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in 96-well plates were generally placed in a humidified gas-tight chamber and flushed for 3 min. After gassing, the flasks and the gas-tight chamber were incubated at 37°C.

2.9 Synchronisation of Parasite Cultures

P. falciparum in continuous culture exhibits a morphologically asynchronous asexual development. To achieve synchrony, the parasites were synchronised according to a modification of the procedure by Lambros and Vanderberg (Lambros et al. 1979). This technique allows the osmotic lysis of late stage trophozoites and schizonts but does not affect the early ring stage. A mixed stage culture predominantly of the early ring stage was centrifuged at 2000 g for 5 min, the supernatant discarded and the pellet (~1ml) re-suspended in 5ml of 5% aqueous D-sorbitol for 20 min at room temperature with intermittent gentle shaking after every 5 min. After centrifugation (2000g, 5 min), the supernatant was discarded and the pellet re-suspended again in 5ml of complete media followed with another centrifugation as above. The culture was finally re-suspended in the same media (FRM) for routine culture, transferred to sterile flasks, gassed and kept in the incubator at 37°C.

2.10 Folate compounds:

Folic acid (FA) [pteroylmonoglutamic acid (PteGlu) free acid], 5-methyltetrahydrofolate (5MeTHF) disodium salt [(6S)-5-CH_3-H_4PteGlu-Ca], 5-formyltetrahydrofolate (5FoTHF) calcium salt [(6S)-5-CHO-H_4PteGlu-Ca] and pABA were purchased from Sigma, UK.

2.10.1 Preparation of standard stock and working Solutions

All 'folate' compounds and pABA were prepared at 10mM stock concentration. Standard stocks of pABA and FA were prepared by dissolving the compounds in 20mM PBS (NaCl; KCl; K_2HPO_4; Na_2HPO_4, pH7.2) while 5MeTHF and 5FoTHF were prepared according to Sangwon Cha and Kim Hie-Joon (Cha et al. 2003). In brief, the appropriate quantity of the 'folate' compound was dissolved in 1ml of 0.1M sodium hydroxide and 35ml 0.1% TFA in water/acetonitrile mixture (2:1) containing 0.1% 2-mercaptoethanol. 93% Nitrogen gas was bubbled through these standards for 15sec.

The stock solutions were filtered through the 0.22µm millipore and protected from light using aluminium foil. Working standards were prepared by diluting the stock solution as necessary in the desired culture media. When not in use, the stock solutions were stored at -80°C. All folate-based assays were performed in the dark.

2.11 [^{3}H]-hypoxanthine Assay

2.11.1 Preparation of [^{3}H]-hypoxanthine working concentration and labelling of parasites

The effects of 'folate' substrates and antifolates were evaluated using the incorporation of radio-labeled [^{3}H]-hypoxanthine as a measure to assess the level of parasite growth. [^{3}H]-

hypoxanthine was purchased from Amersham Biosciences, UK (specific activity 814GBq/mmol, 22.0Ci/mmol, 5mCi, 185MBq). 1 ml of the stock solution was diluted in 19 ml of folate deplete media (FDM) to give a working concentration of 0.05mCi which was stored at -20°C. Following appropriate culture incubation times, 10 µl each of the 0.05mCi was added to each well and the plates gently agitated. The final concentration of the radio-labeled [³H]-hypoxanthine dilution per each well was 0.0045mCi. The plates were gassed and incubated at 37°C as required.

2.11.2 Harvesting of plates and sealing of the printed filter mat

At the end of the radio-labeled [³H]-hypoxanthine incubation, the plates were frozen at -20°C for at least 2 hr to terminate the test assay and then thawed to lyse the erythrocytes. The contents of the plates were transferred onto a printed filter mat A Glass fibre, (size 90 X 120 mm, Wallac Oy, Turku, Finland) through an extensive wash and aspirate process using the cell harvester (96® Mach III M Tomtec Harvester; Wallac Ltd, Turku, Finland). The mats were then dried in a microwave for 5 min and placed in transparent sample bags (90 X 120mm, PerkinElmer Life Sciences), overlaid with a melt-on scintillator sheet (Size 73 X 109mm, Meltilex ™ A, Wallac Oy, Turku, Finland) and the open ends sealed using a heat sealer (Wallac 1295-012, Wallac Oy, 20101, Turku, Finland). The sealed mats were incubated at 65°C for 5 min in an oven and then left to dry at room temperature for 10 min.

2.11.3 Scintillation counting of [³H]-hypoxanthine labelled plates

The parasite [^{3}H]-hypoxanthine incorporation was estimated using the Liquid Scintillation and Luminescence Counter (1450 Microbeta Trilux, Wallac Ltd). The printed mats were correspondingly fixed on specially designed 96-hole cassettes and then placed in the counter for radioactivity counting.

2.12 Cell culture

In vitro cultivation of the malaria parasites was carried out according to the method of Trager and Jensen (Trager et al. 1976) with minor modifications. Parasitized human red blood cells (type O$^+$) were grown continuously at 37°C as a suspension culture under an atmosphere of CO_2-3%, O_2-4% and N_2-93% in sterile 160 ml culture flasks (Nunc^{TM} Rosklide, Denmark) containing the routine folate rich media (FRM). The medium was removed daily, replaced with the same fresh medium and fresh RBCs added every 3 to 5 days depending on the vitality of the culture. Cultures were regularly monitored and the parasitemia and growth stage assessed before any assays initiated.

2.13 *Plasmodium falciparum* genomic DNA isolation

Genomic DNA from infected cells (50ml with ~8% parasitemia) was isolated according to the protocol of Schlichtherle M. and M. Wahlgren (Schlichtherle et al. 2008) with modifications. Briefly; cells were centrifuged at 3,000g for 5 min, washed in 5 volumes cold PBS and saponised in 0.05% ^{v}/v (final saponin concentration in PBS). Following

centrifugation (3,000g, 5 min), 25µl lysis buffer (40mM Tris-HCl [pH8.0], 80mM EDTA [pH8.0], 2% SDS) plus 75µl double distilled H_2O plus 50µl proteinase K (0.1mg/ml) were added to the pellet and the suspension incubated at 37°C for ~3hr with intermittent mixing. At the end of incubation, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1; v/v/v) was added, the mixture vortexed vigorously and centrifuged (13,000g, 1 min, room temperature). The aqueous phase was transferred to a fresh tube and the genomic DNA precipitated by adding ^{1}/10 3M sodium acetate (pH5.2) and 2.5 volumes of ice-cold absolute ethanol with an overnight incubation at -20°C. The precipitate was then centrifuged (13,000g, 5 min, 4°C), washed once with 70% ^{v}/v ethanol and the pellet dried in the laminar flow hood. The dried pellet was resuspended in 100µl double-distilled H_2O, quantified and stored at -80°C.

2.14 *Plasmodium falciparum* total RNA isolation

All the reagents, glassware and plasticware used for RNA isolation were either purchased RNAse-free (Ambion, UK) or made RNAse-free by treating them with 0.05% diethylpyrocarbonate (DEPC) water.

Total RNA from ~10% parasitemia (late trophozoites/schizont stage, 2% hematocrit) per 50ml cell culture volume was isolated according to a modified protocol of Fiddock, D and others (Fidock et al. 2002) using Trizol reagent (Invitrogen). 150 ml total cell culture volume was centrifuged at 3,000g for 5 min, washed once in 5 volumes cold PBS and saponised in 0.05% ^{v}/v (final saponin concentration in PBS). The pellet was quickly

resuspended in 1 ml Trizol reagent and incubated for 10 min at room temperature or stored at -80°C for future isolation. Following complete dissociation, the suspension was mixed with 200μl absolute chloroform, vortexed vigorously and centrifuged (10,000g, 15min, 4°C). The top aqueous phase was transferred to a fresh tube, mixed with an equal volume of ice-cold isopropanol and stored at room temperature for 10 min. The mixture was centrifuged (10,000g, 15min, 4°C) and the RNA pellet washed once in cold 75% ethanol, dried in the laminar flow hood for 10min and then resuspended in 100μl DEPC-H_2O followed with a final incubation at 60°C/10min. RNA was quantified and stored as aliquots in either DEPC-H_2O or 100% formamide at -80°C.

2.15 cDNA synthesis

Prior to cDNA synthesis, total RNA (15-20μl) was mixed with 1μl Turbo DNase (Ambion, UK) and incubated at 37°C for 30 min to degrade any contaminating DNA. The sample was then heat inactivated at 70°C for 20min. cDNA synthesis was carried out using The ThermoScript™ RT-PCR System (Invitrogen) as follows; DNase treated total RNA (1.2μg) was incubated with 150 ng random-hexamer primers (50ng/μl) and 30 mM dNTP mix (10mM) for 5 min at 65°C. The cDNA synthesis mix prepared separately, (final concentration: 1X cDNA synthesis buffer; 0.005M DTT; 2U RNase OUT, 0.75 U ThermoScript™ RT) were added to a total volume of 20μl and the reaction left at room temperature for 10min. The reaction was further incubated at 50°C/1hr, 85°C/5min and then supplemented with 2 U RNase H and incubated for another 20 min at 37°C. The synthesised cDNA was stored in aliquots at -80°C.

2.16 Polymerase Chain Reaction (PCR)

The required DNA fragments were amplified *in vitro* using the PCR method. A typical amplification reaction was made up of the following recipe at the final concentration; 1X *Pfx* amplification buffer, 2X PCR_X enhancer solution, 2.5mM $MgSO_4$, 0.1mM dNTPs mix, 0.025 units Platinum® *Pfx* DNA polymerase (Invitrogen), 2µM of each primer (Sigma, Genosys), 40ng template DNA and PCR H_2O to a total volume of 20µl. PCR amplifications were carried out using Biometra's-T-Personal-Thermal-Cycler (Anachem, Luton, UK) under the following cycling conditions; initial denaturation at 94°C for 4 min followed with 35 cycles of denaturation, 94°C/15s, annealing 55°C/30s and extension 68°C/2 min. The primer combinations used to amplify the target DNA sequence are summarised in table 2.16. The size and integrity of the amplified product was visualised on a 1.5% agarose gel.

Oligo name	Sequence
PF7-forward primer	5'-AGATCTCCACCATGGAAGATGACGACTTC
PF7-reverse primer	5'-GGTAACCTTATTCCAAGGTTATGTC
Pf9-forward primer	5'-AGATCTCCACCATGATAGAAAGTCTAA
PF9-reverse primer	5'-GGTAACCTTATCCCTTGGATGTTTC

Table 2.1 Primer combination sequences used to amplify the target DNA

2.17 Agarose gel electrophoresis

A 1.5% ^{w}/v agarose gel (in 1X TBE) electrophoresis was used for the separation and identification of the DNA fragments after PCR amplifications and restriction digests. Samples were first mixed with orange G gel-loading buffer before loading and the TrackIt™ 1Kb plus DNA ladder used for as molecular weight markers. Electrophoresis was performed at 75-100V for 1-1^1/$_2$ hr and the gel incubated away from light in a solution of 0.5μg/ml ethidium bromide for 20min. The bands were analysed for the expected DNA fragment size by Gene Genius Bio-imaging Gel Documentation System (Syngene, Synoptics, Ltd. Cambridge, U.K.).

2.18 Purification of DNA fragments

The PCR products for each target DNA sequence were collected together to a final volume of 100μl and isopropanol-ethanol precipitated. Briefly, 2X volumes of isoporopanol was added to the collected PCR product and left at room temperature for 10min. The sample was then centrifuged at 13,000g/10min and the supernatant discarded. 2X volumes of 70%^{v}/v ethanol (after isopropanol addition) was added to the pellet followed with another round of centrifugation (13,000g/10min), the supernatant was discarded and the pellet left to dry in the laminar flow hood. The pellet was resuspended in 15ul PCR H$_2$O and incubated at room temperature for 5 min with intermittent gentle vortexing. The sample was finally incubated at 60°C for 5 min and then centrifuged at 13,000g/1min. An agarose gel electrophoresis of the precipitated DNA (13μl) was performed and the expected fragment size sliced under UV transilluminator (6 X 15 W –

312nM tube, Appligene, France) and gel-purified using the Clean-Up with Wizard® SV

for Gel and PCR (Promega, UK) according to the manufacturer's instructions.

2.19 deoxyadenosine (dA)-tailing

It is difficult to clone amplified DNA products from proofreading enzymes like *Pfx* DNA

polymerase into TOPO cloning vector because the proofreading activity removes the 3'

A-overhangs which are necessary for cloning. To address this challenge, 3' A-overhangs

were recreated through the process of dA-tailing. The reaction mixture contained the

following ingredients at final concentration; 1.5X Mg^{++}free buffer, 3.75 mM $MgCl_2$,

0.15mM dATP, 2.5 units Taq DNA polymerase (Promega, UK) and 10μl freshly purified

PCR product. The reaction was incubated at 68°C for 1 hr and the freshly synthesised

dA....tailed PCR product used for TOPO cloning directly.

2.20 Restriction digest

A restriction digest reaction was set up as follows; 1 μg DNA template was mixed with

the following ingredients at final concentration; 1X restriction buffer, 1X BSA, 10 U of

restriction enzyme (all as provided by the supplier) to a total volume of 20 μl. The

reaction was incubated for one hour at the appropriate temperatures after which the size

and integrity of the digested DNA was checked on agarose gel.

2.21 Dephosphorylation

Dephosphorylation of the 5'-cohesive ends was performed to prevent the religation of the double-digested vector as described in **section 2.20.** 20 µl of the digested vector was mixed with 0.1 U Antartic Phosphatase and 1X Antartic Phosphatase buffer (New England Biolabs, UK) to a total volume of 50 µl. The reaction was incubated at 37°C for 30min (heat inactivation) and then taken through a step of phenol extraction followed with a chloroform:isoamyl (24:1) treatment and ethanol precipitation.

2.22 Ligation

Ligation reaction was carried out using the T4 DNA ligase (Promega, UK) at a molar ratio of 3:1 (insert DNA:vector DNA) as follows; the double digested, dephosphorylated and purified vector DNA was mixed with the insert (obtained with the same restriction enzymes) in a reaction supplemented with T4 DNA ligase buffer at 1X final concentration and 3U of T4 DNA ligase. The reaction mixture was incubated at 18°C for $13\frac{1}{2}$ hours, chilled on ice and then used directly for transformation into TOP10 competent cells (Invitrogen).

2.23 TOPO cloning

A TOPO cloning reaction comprising of 4µl freshly synthesised deoxyadenosine tailed PCR product, 1µl salt solution and 1µl dual promoter pCR®II-TOPO® (Invitrogen) was set up according to the manufacturer's instructions.

2.24 Transformation into *E. coli*

3μl of the TOPO cloning reaction was added to a vial of TOP10 chemically competent cells and mixed gently. The bacterial suspension was incubated on ice for 5 min, followed by a 30s heat-shock at 42°C. The vial was immediately transferred to ice and 100μl of room temperature SOC medium added. After which the vial was tightly capped and agitated horizontally at 200g/37°C/1hr. A 25μl and 50μl volume from each transformation was spread on a pre-warmed LB plate (spread with 40μl of 40mg/ml X-gal) and incubated overnight at 37°C.

2.25 Mini preps plasmid preparation

A small scale plasmid DNA was isolated using the Wizard® Plus Minipreps DNA purification system (Promega, UK) for purposes of confirming the presence of the insert by restriction analysis or PCR screening or for use as templates for sequencing reactions. Briefly, individual colonies were picked and cultured overnight (37°C/200rev/min) in 5 ml LB medium containing 100μg/ml ampicillin. The mini prep plasmid DNA was obtained from a 3 ml bacterial suspension in accordance with the vacuum manifold (Diaphram Vacuum Pump, M³/h Vacuubrand GMBH+Co. Wertheim, Germany) purification method of the manufacturer's instructions.

2.26 Midi preps plasmid preparation

In order to obtain good quality plasmid DNA of high-purity and high-concentration for cRNA synthesis, a large scale isolation of plasmid DNA was carried out using the Wizard® Plus Midipreps DNA purification system (Promega, UK). To do this, a 50 ml LB medium with 100μg/ml ampicillin was inoculated with a 1 ml sub-culture of bacterial suspension containing the appropriate plasmid and cultured overnight (37°C/200rev/min). The plasmid DNA was then isolated and purified according to the vacuum manifold procedure as per the manufacturer's instructions.

2.27 Cryopreservation of bacteria stock culture

A 1 ml of an overnight midi prep volume of bacteria suspension was centrifuged at 13,000g for 1 min and 700μl of the supernatant discarded. To the remaining 300 μl bacteria suspension, 300 μl of 30% glycerol (final concentration, 15%) was added and the mixture transferred to -80°C.

2.28 Concentration and purity determination of RNA and genomic/plasmid DNA

The genomic/plasmid DNA and RNA purity (measured as the ratio of absorbance at 260 and 280 nm, $A_{260/280}$) and concentration (ng/μl) were determined using the NanoDrop Nd-1000 UV/Vis 1μl Spectrophotometer (Labtech International, UK). The sample integrity was assessed by loading 10μl each on a 1.5% agarose gel electrophoresis. RNA electrophoresis was done under denaturing conditions.

2.29 Sequence analysis of the DNA

Sequencing was done to verify the correct sequences of the amplified DNA of interest or of the insert in the plasmid DNA. PCR products and plasmid DNA material together with the specific primers were supplied to the Liverpool School of Tropical Medicine sequencing facilities and another similar batch sent to Lark Technologies DNA Sequencing (Essex, UK).

2.30 Parasite clones

Five well known laboratory reference parasite clones (HB3, 3D7, K1, Dd2 and VI/S) were used in this work. The relevant geographic origin, DHFR and DHPS genotype characteristics and antifolate drug-susceptibility pattern have previously been described (Wang et al. 1997) and are shown in **Table 2.2**

		DHFR, DHPS characteristics and susceptibility profiles												
		DHFR						DHPS						
Clone	Origin	16	51	59	108	164	PYR	436	437	540	581	613	SDX	F.U.
HB3	Honduras	Ala	Asn	Cyst	Asn	Ile	R	Ser	Ala	Lys	Ala	Ala	S	P
3D7	'Netherlands'	Ala	Asn	Cyst	**Ser**	Ile	S	Ser	**Gly**	Lys	Ala	Ala	R	E
K1	Thailand	Ala	Asn	**Arg**	Asn	Ile	R	Ser	**Gly**	Lys	**Gly**	Ala	R	E
DD2	Indochina	Ala	**Ile**	**Arg**	Asn	Ile	R	**Phe**	**Gly**	Lys	Ala	**Ser**	R	E
VI/S	Vietnam	Ala	**Ile**	**Arg**	Asn	**Leu**	R	**Phe**	**Gly**	Lys	Ala	**Ser**	R	P

Table 2.2 Parasite clone origin, DHFR and DHPS characteristics, susceptibility profiles and folate effect status. Bold characters= mutated amino, Regular=wild-type, S = susceptible, R = resistant, F.U= folate utilization, E=efficient utiliser, P=poor utiliser, the P for VI/S refers to a poor survivor in folate deprived condition

CHAPTER 3

The Influence of folate pathway substrates on the *In Vitro* activities of antifolate drugs

3.1 Introduction

As described in **chapter 1**, the antifolates represented by anti-DHFR (PYR and CCG) and anti-DHPS (SDX and DDS) inhibitors are an important class of antimalarial drug that have an established role in the treatment of uncomplicated malaria over decades. These are well studied drugs and we think we know a lot about their pharmacology, see (Sibley et al. 2001; Bukirwa et al. 2004; Gregson et al. 2005). We accept that the antimalarial activity of the anti-DHFR agent is markedly potentiated by the anti-DHPS and hence the drugs have been deployed as combinations. As described earlier, these compounds target the folic acid pathway and there is good evidence that pABA, folic acid and other related reduced folate substrates, which are critical to parasite survival, play an important role in the activity of these drugs **(see Chapter 1).**

The potential for folate substrates to influence antifolate potency arose following recommendations for folate supplements in malaria patients suffering from hematologic

complications such as anemia and leukopenia. There was concern that the proposed treatment with antifolates would make these conditions worse and logically it was suggested that administration of folic acid or related compounds would obviate this side effect (Tong et al. 1970). The foundation of this concept was based on the old assumption that plasmodia do not appear to utilize preformed folic acid and the simultaneous administration of the two compounds would not compromise the effectiveness of the antifolate. The pharmacological premise of this approach was that supplemented folic acid would circumvent the antifolate block at the level of the target enzymes and prevent hematologic toxicity.

It wasn't until the development of an assay for continuous culture of *P. falciparum* (Trager et al. 1976) that *in vitro* studies were first initiated to investigate antimalarial antifolate activities in the presence of exogenous pABA, folic acid and reduced folates. Although the *in vitro* culture system for *P. falciparum* was rapidly shown to be a robust method for the determination of parasite sensitivity to drugs such as chloroquine, quinine and mefloquine (Desjardins et al. 1979) and subsequently quinidine and amodiaquine, the assay was less reproducible for antifolates. In initial evaluations, some compounds in this class of antifolate drugs failed to kill parasite efficiently. After careful evaluation, Desjardins and co-workers (Desjardins, Canfield et al. 1979) drew attention to the problems presented by the presence of pABA and folic acid (each at 1.0mg/L) in the original RPMI-1640 medium used to support parasite growth and also to the presence of these substrates (in varying concentrations) in human serum. Human serum generally contains pABA at 250-300 µg/L (Tan-ariya et al. 1983) and total folate (TFOL) which

comprises of 5-MeTHF (the predominant circulating form), FA and 5-FoTHF at ~ 36nM. The antagonistic effect of these compounds prompted several investigators to try to standardise the in vitro test system for these drugs. It is now recommended as standard practice to perform *in vitro* antifolate drug tests by eliminating sources of folate in the culture.

We have provided a comprehensive review in **section 1.3.2.4** as evidence on the parasite's ability to salvage folate cofactors. This phenomenon is particularly of concern considering the fundamental role of folate supplementation during pregnancy and the presumptive use of antifolates in the intermittent preventive treatment programme. As we have previously mentioned in **chapter 1,** some studies have shown folate supplementation compromised the efficacy of antifolates and the presence of folate cofactors during *in vitro* tests negates the activities of the antifolates.

Several species of *Plasmodium* were shown to have arrested growth when hosts were fed a milk diet which is deficient in pABA (Jacobs 1964). Here, parasite growth was restored with the addition of pABA. It is these observations together with the informed understanding on the effect of sulfonamides that led to a proposal that in addition to the *de novo* pathway, the parasite utilizes a salvage route to meet its pABA needs. This proposal initially faced challenges as other studies showed the capacity of several lines of *Plasmodium* to grow in undetectable levels of pABA (Milhous et al 1985) and also the presence of fundamental enzymes required for pABA synthesis detected in *P falciparum* extracts (Dieckman and Jung 1986). Support for the need of pABA for parasite growth

was further offered by McConkey and colleagues (McConkey et al 1994). In this experiment directed towards verifying the selection of pABA requiring mutants, several clones were tested with and without pABA. They found out that pABA stimulated growth when added to the pABA deficient medium. One striking observation they made was that a parental strain 3D7 was unaffected by the availability of pABA in the medium. This was an insightful observation as it suggested an inter-strain difference exists in the utilization of pABA.

In line with this unique strain characteristic to respond to pABA (McConkey 1994), Milhous and colleagues (Milhous et al1985) also reveal a similar observation with folic acid. The group identified two groups of strains; an isolated strain, Sierra Leone I/CDC that did not grow in deficient medium with dialyzed plasma but had normal growth rate restored in culture medium supplemented with normal plasma and another set of three strains that grew well irrespective of deficient or normal medium condition and with dialyzed or nondialyzed plasma.

As already discussed in **chapter 1,** the reversal ability of the intact folic acid against sulfonamides was attributed to the breakdown of the molecule and further utilization of its constituent parts for example *p*-aminobenzoylglutamate. We have also continously pointed out the presence of folic acid, pABA, folate derivatives or folate precursors have been shown to compound antifolate *in vitro* testing. See also (Milhous et al 985). Using folic acid as a template molecule and SDX as the antifolate of choice, Wang and colleagues (Wang et al 1999) rigorously assessed this inter-strain difference starting off

with HB3 and DD2 and later proceeding to transfectants and came up with the phenomenon 'folate effect', see (Hyde J 2005). In this phenomenon, low level concentration of folic acid has a comparatively huge mitigation impact on *in vitro* activity of SDX for the Dd2 strain while in the case of HB3 strain, one requires higher amounts of folic acid concentration to make a similar observation.

The work undertaken in this chapter was performed in response to this evidence that the degree of antagonism produced by folate substrates was isolate dependent. In **chapter 1,** we discussed the various parasite isolates harbouring different genotypes of DHFR and DHPS. As a logical interrogation of the role of 'isolate effect', we have selected a panel of isolates of unique DHFR and DHPS genotypes and also of known antifolate susceptibility patterns.

As reviewed in **chapter 1,** the folate moiety also exists in various forms. In addition to the intact folic acid and the precursor pABA, we have included 5-methylTHF and 5-formylTHF, reduced folate derivatives in order to tease out the impact of the substrate in reversing antifolate activity. The test antifolate compounds under investigation are the less potent drugs PYR and SDX and the more potent ones CCG and DDS. Although this is a fundamentally biochemical study, it forms the framework in the molecular probing of the existing transporters as explored in **chapter 4.**

3.2 Materials and Methods

3.2.1 Parasite clones

Four well established laboratory reference parasite clones (HB3, 3D7, K1 and Dd2) were used in the present study, (see table 2.2).

3.2.2 DHFR and DHPS inhibitors: standard stock and working drug preparations

Chlorcycloguanil hydrochloride, CCG.HCl (molecular weight, 288.17) and DDS (molecular weight, 248) were kindly provided as gifts from Glaxosmithkline, UK. SDX (molecular weight, 310.34) and PYR (molecular weight, 248.7) were purchased from Sigma chemicals, Poole, UK. Stock solutions of each drug were prepared by dissolving the individual drugs in tissue-culture grade absolute dimethylsulfoxide (DMSO). The drug solutions were sterilised by passage through a 0.22μm millipore filter and stored at -20°C until used. On the day of the assay, three-fold serial working dilutions were prepared by diluting the stock solutions with the desired amount of culture media. This final, dilution corresponded to the desired maximum concentrations for testing against the reference isolates. The final concentration of DMSO was always maintained below 0.005% and was shown previously to have no effect on parasite viability in the 48h test.

3.2.3 pABA and 'folate' compounds

pABA and the folate compounds were sourced and prepared as previously described in sections 2.10 and 2.10.1

3.2.4 *In vitro* Parasite culture

Parasites were cultured according to the method of Trager and Jensen (1976) with minor modifications as previously described in **section 2.12**

3.2.5 Inoculum preparation

Stock cultures of red blood cells harbouring mainly ring stage parasites were synchronised with 5% ^{w}/v sorbitol treatment and allowed to develop for one more complete asexual cycle before use in *in vitro* sensitivity studies (drug sensitivity testing was not done on the day of synchronisation). The infected cells were washed three times (2000g, 5 min) in HEPES buffered folate free wash media and then resuspended in specific fresh complete folate-free medium containing dialysed serum (FDM). Media was supplemented with specific folate and folate precursors as required with individual final concentrations of pABA at 7.3uM or FA/5MeTHF/5FoTHF at 2.3uM. When parasitemia exceeded 2%, the culture was diluted with washed fresh RBC (wash medium – folate) and all stidies were performed with a 2% parasitemia and 1% hematocrit.

3.2.6 *In vitro* drug susceptibility assay

The *in vitro* drug sensitivity assays of the selected antifolates were determined according to the method of Desjardins and others (1979) with modifications as described below.

The growth inhibition studies were performed in a 96-well Tissue micro-culture plates [flat bottom, with lid, sterile (Greiner-bio-one)]. The boundary wells were omitted since previous experiments had shown that parasites in these wells do not grow well. The two middle rows of wells constituted control cultures where no drug was added. Fifty μl of the culture suspension (2% parasitemia and 1% hematocrit, -/+ test folate or pABA compound) was dispensed into the 96 well tissue culture plate that had been pre-dosed with 50μl of the test drug dilutions at various concentrations, **figure 3.1**. The plates were gently agitated and placed in a humidified gas-tight chamber and flushed with a 3% CO_2, 4% O_2 and 93% N_2 gas mixture for 3 minutes. The chamber was incubated for 24 hr at 37°C. After 24hr, the plates were removed from the chamber and each well pulsed with 10μl of [^{3}H]-hypoxanthine (0.05mCi), gently agitated and returned to the chamber, flushed with a 3% CO_2, 4% O_2 and 93% N_2 gas mixture for 3 minutes and incubated at 37°C for an additional 18 hrs. The plates were later harvested onto filter paper mats for the measurement of radio-isotope incorporation, **(section 2.11.2)**.
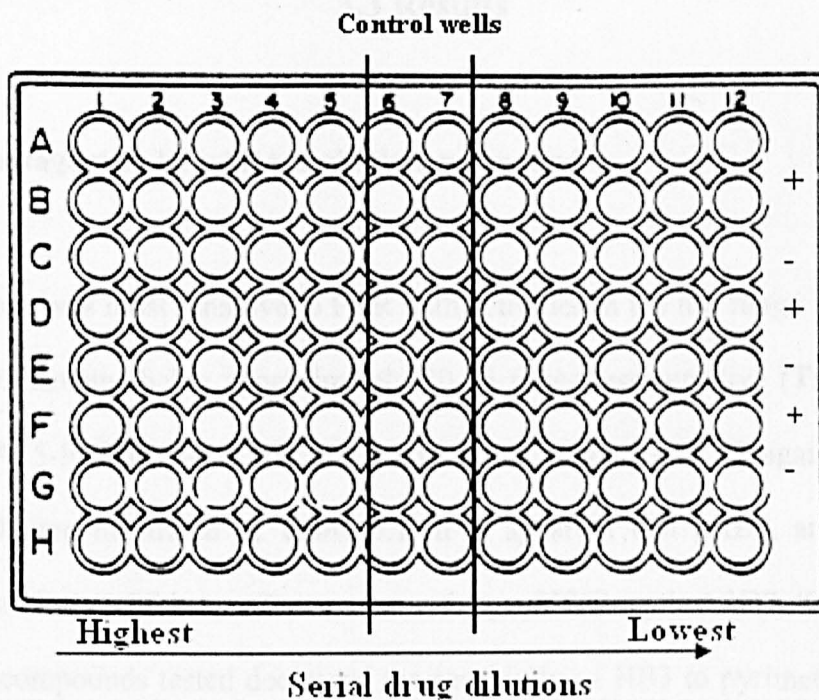
Figure 3.1 Schematic representation of the *in vitro* drug susceptibility plate assay

+ / wells containing folate test substrate
- / control wells without any added folate substrate

3.2.7 Determination of IC$_{50}$

The 50% inhibitory concentration (IC$_{50}$) was determined by the non-linear regression analysis of logarithm of concentrations plotted against the parasite growth inhibition. The best-fitting sigmoid curve was derived by using the Grafit Program; (Erithacus Software, Kent UK)

3.3 Results

3.3.1 PYR antagonism by pABA and folate compounds

The 3D7 clone was most sensitive to PYR with activities in the nM range with the HB3, K1 and DD2 isolates being approximately 20-45 times less sensitive (**Table 3.1**). The effects of FA, 5-FoTHF, 5-MeTHF and pABA on the activity of PYR against HB3, 3D7, K1 and DD2 are illustrated in **Table 3.1**. It is apparent that pABA and the 'folate' substrates had little inhibitory effect on the activity of PYR against 3D7, K1 and DD2. In contrast all compounds tested decreased the sensitivity of HB3 to pyrimethamine 3 to 6 fold (**Table 3.1**).

Test medium	Strain, IC_{50} (μM)			
	HB3	3D7	K1	DD2
Control	1.06 ± 0.65	0.05 ± 0.01	2.23 ± 0.33	2.29 ± 0.47
+ FA	$3.65 \pm 0.35^{b\S}$	$0.10 \pm 0.03^{c\S}$	$3.53 \pm 1.67^{c\P}$	$3.21 \pm 1.52^{d\P}$
+5-FoTHF	$6.57 \pm 0.71^{c¥}$	$0.11 \pm 0.02^{c\S}$	$2.99 \pm 0.39^{c\P}$	$4.19 \pm 2.54^{a\P}$
+5-MeTHF	$3.04 \pm 1.90^{d\S}$	$0.07 \pm 0.01^{c\P}$	$2.70 \pm 0.71^{d\P}$	$2.35 \pm 0.56^{d\P}$
+pABA	$5.61 \pm 0.68^{b¥}$	$0.09 \pm 0.01^{c\P}$	$2.94 \pm 0.38^{a\P}$	$4.02 \pm 1.87^{a\P}$

Table 3.1 Effect of exogenous folic acid, reduced folate derivatives and pABA on the *in vitro* inhibitory activity of PYR against 3D7, K1, DD2 and HB3
P values represented as superscripts; a=0.1, b=0.05, c<0.05, d>0.1
Values are means ± standard deviations of 3-7 experiments
Fold increase; ¶ : negligible, § : 2 to 4.9, ¥ : 5 to 10

3.3.2 SDX antagonism by pABA and folate compounds

The impact of FA, 5-FoTHF, 5-MeTHF and pABA on the *in vitro* inhibitory activities of SDX on the growth of HB3, 3D7, K1 and DD2 are summarised in **Table 3.2**. The results indicate SDX activity was antagonised markedy by FA, 5-FoTHF and pABA in all parasites tested. The effect of 5-MeTHF was less pronounced and was absent in the 3D7 parasite clone. Qualitatively there was no difference in the response of the susceptible HB3 clone compared to the resistant clones 3D7, K1 and Dd2.

	Strain, IC_{50} (μM)			
Test medium	**HB3**	**3D7**	**K1**	**DD2**
Control	175.09 ± 71.61	426.13 ± 85.46	494.59 ± 47.88	517.45 ± 206.43
+ FA	$1272.91 \pm 246.99^{a¥}$	$1572.06 \pm 413.66^{b§}$	$2231.40 \pm 67.96^{b§}$	$1491.77 \pm 240.35^{c§}$
+5-FoTHF	$1221.99 \pm 106.41^{nd¥}$	$1336.65 \pm 220.05^{b§}$	$2256.09 \pm 90.39^{c§}$	$1498.43 \pm 100.60^{nd§}$
+5-MeTHF	$451.17 \pm 13.80^{a§}$	$472.25 \pm 42.11^{nd¶}$	$1116.48 \pm 100.31^{a§}$	$927.86 \pm 145.86^{nd¶}$
+pABA	$1476.92 \pm 35.89^{nd¥}$	$1952.33 \pm 189.98^{b§}$	$2675.69 \pm 533.95^{b¥}$	$1786.09 \pm 438.47^{a§}$

Table 3.2 Effect of exogenous folic acid, reduced folate derivatives and pABA on the *in vitro* inhibitory activity of SDX against 3D7, K1, DD2 and HB3
P values represented as superscripts; a=0.1, b=0.05, c<0.05, d>0.1 and nd=not determined
Values are means ± standard deviations of 2-6experiments
Fold increase; ¶ : negligible, **§** : 2 to 4.9, **¥** : 5 to 10

3.3.3 DDS antagonism by pABA and folate compounds

Table 3.3 shows the effects of pABA and the folate substrates on *in vitro* DDS activity against all the four reference clones studied. Although a sulfa-drug and targeting the same site as SDX, the results show that DDS inhibits all the clones at comparatively lower concentrations than SDX. The results indicate DDS activity was antagonised markedly by FA, 5-FoTHF and pABA in all parasite clones tested. 5-MeTHF had little effect on DDS activity in any of the parasite clone investigated. It was less pronounced and was absent in the 3D7 parasite clone.

| Test medium | Strain, IC_{50} (μM) | | | |
	HB3	3D7	K1	DD2
Control	26.53 ±2.46	38.27 ±0.63	51.22 ±2.62	65.25 ±6.07
+ FA	213.01 ±65.67$^{c¥}$	333.59 ±275.66$^{a¥}$	685.08 ±239.86$^{c♀}$	498.38 ±318.01$^{c¥}$
+5-FoTHF	272.7 ±29.50$^{c♀}$	283.82 ±17.86$^{a¥}$	336.24 ±5.12$^{nd¥}$	416.58 ±251.21$^{c¥}$
+5-MeTHF	40.15 ±9.05^{c¶}	50.70 ±4.18^{a¶}	64.42 ±36.42^{d¶}	185.25 ±17.48^{nd§}
+pABA	544.88 ±188.17$^{e♀}$	524.70 ±309.72$^{a♀}$	740.61 ±144.02$^{nd♀}$	495.51 ±195.77$^{c¥}$

Table 3.3 Effect of exogenous folic acid, reduced folate derivatives and pABA on the *in vitro* inhibitory activity of DDS against 3D7, K1, DD2 and HB3
P values represented as superscripts; a=0.1, b=0.05, c<0.05, d>0.1, e<0.01 and nd=not determined
Values are means ± standard deviations of 3-5 experiments
Fold increase; ¶ : negligible, **§** : 2 to 4.9, **¥** : 5 to 10, **♀** : greater than 10

3.3.4 CCG antagonism by pABA and folate compounds

Unlike the other antifolates discussed, CCG is less well studied and there are suggestions that the actions of the triazine antimalarials are less influenced by folate substrates (Yeo, Seymour et al. 1997). With CCG, we only looked at one PYR-resistant clone HB3 and one PYR-sensitive clone 3D7. Both of these isolates are CCG-sensitive. **Table 3.4** shows the mean IC_{50} of CCG against HB3 and 3D7 under the range of media conditions studied. In comparison to its counterpart PYR, it is clear CCG is more potent than PYR and is consistently more potent against the PYR resistant clone. In comparison to the other three antifolates investigated FA, 5-FoTHF, 5-MeTHF and pABA had no significant influence on drug sensitivity.

	Strain, IC_{50} (nM)	
Test medium	HB3	3D7
Control	107.19 ±13.90	4.36 ±0.24
+ FA	215.28 ±149.64[¶]	6.52 ±1.85[¶]
+5-FoTHF	310.91 ±7.77[§]	4.95 ±1.12[¶]
+5-MeTHF	119.92 ±33.86[¶]	4.64 ±0.47[¶]
+pABA	277.13 ±241.46[§]	5.79 ±2.35[¶]

Table 3.4 Effect of exogenous folic acid, reduced folate derivatives and pABA on the *in vitro* inhibitory activity of CCG against 3D7 and HB3
P values not determined. The control experiments were done twice.
Values are means ± standard deviations of 2-3 experiments
Fold increase; ¶ : negligible, **§** : 2 to 4.9,

3.4 Discussion

In this chapter, the *in vitro* relationship between *P. falciparum* growth inhibition and the pharmacological activities of antifolates under different media conditions has been explored. It is accepted that interference with *P. falciparum* folic acid metabolism is a very effective way to kill malaria parasites as exemplified by the use of drug combinations such as PYR-SDX. There have been concerns that the activity for these combinations could be compromised by exogenous pABA and other folate substrates in serum. The results presented here are clearly of importance in relation to antifolate testing and provide illuminating information on the influence of the media composition on the activity of antifolates. It is evident that IC_{50} of all the antifolates tested in all the clones were consistently lower in the control media compared to the modified and supplemented media.

It was not totally surprising to find decreasing levels of antifolate activity in the presence of pABA or other folate substrates and this data is in agreement with previous studies (Watkins et al. 1985; Tan-ariya et al. 1987) and supports the suggestion that *P. falciparum* exhibits a salvage mechanism for folate molecules.

FA, 5-FoTHF and pABA demonstrated a more marked antagonism on the activity of the anti-DHPS compounds compared to anti-DHFR inhibitors. The fact that pABA reduced the level of anti-DHPS activity is in keeping with the conclusion that pABA is a competitive inhibitor at the DHPS locus. The effects of FA and 5-FoTHF presumably

reflect antagonism at other sites, possibly DHFR, which in the absence of a specific DHFR inhibitor counteracts any inhibition further upstream in the pathway i.e. DHPS although there is a remote possibility that degradation products such as para-aminobenzolglutamate (pABG) or the aldehyde derivative could impact on the pathway. However, antagonism studies comparing FA and its degradation products mentioned above reveal that FA is more superior at antagonising the compounds than the degradation products and the effect has been directly linked to the intact molecule and not the degradation products (Milhous et al. 1985; Watkins, Sixsmith et al. 1985).

FA has been reported to be a competitive inhibitor of dihydrofolate for reduction to tetrahydrofolate by DHFR in other organisms but *P. falciparum* DHFR-TS substrate substrate specificity is very narrow (catalyses only a very limited number of substrates so far it is dihydrofolate only) and no enzymes capable of catalysing the reduction of FA have been identified. Suggestions have been put forward that *P. falciparum* DHFR could perform this task (Hyde 2005). The mechanism of inhibition by 5-FoTHF is unknown. Whatever the detailed mechanism of 5FoTHF antagonism, it seems to be effective against both DHFR and DHPS inhibitors as clearly shown in HB3.

Of particular interest in this study is the surprising finding that 5-MeTHF does not antagonise the activities of antifolates as the counterpart cofactors (except marginally against DDS in HB3) yet it is the predominant folate species present in humans and would be the most likely substrate of critical concern in antifolate chemotherapy. This

observation has been made by others while the current thesis was being written (Nduati et al. 2008).

The IC_{50} results reported here differ from those reported elsewhere, (Milhous, Weatherly et al. 1985; Watkins, Sixsmith et al. 1985; Tan-ariya, Brockelman et al. 1987; Wang, Read et al. 1997; Nduati, Diriye et al. 2008) and depicts the challenges facing *in vitro* antifolate drug susceptibility. The poor reproducibility of the antifolate *in vitro* assay is based on the conditions of the test system and includes; test medium, serum source (human; dialysed vs undialysed or albumax), batch of human erythrocytes and duration of incubation (24 hr vs 48 hr) and lability of folates. Wang and colleagues developed an *in vitro* assay with a near elimination of folate sources and this partly involved the substitution of serum for albumax. An evaluation of this method did not yield consistent results (Ndounga et al. 2001) and adds to the complexities of antifolate drug testing to give reproducible data.

These studies also further substantiate the superiority of CCG and DDS over PYR and SDX respectively. CCG and DDS possess potentially useful activity against PYR- and SDX-resistant clones. The data lend support to the further development of these molecules as alternative antifolates.

Findings from this study were quite illuminating. Although the role of the isolate and substrate were noted in some cases, in the case of CCG, there was no reversal despite the changes in substrate for the two strains HB3 and 3D7. This could suggest that the impact

of folate substrates can be linked to the specific nature of the drug itself as some drugs are not markedly affected with the folate or pABA component in the medium.

In conclusion, this work has partly refined the optimum conditions of antifolate sensitivity measurements by depleting the levels of exogenous folates. Although the work specifically addressed antagonism effect, the findings may also have ramifications relating to transport of the substrates across the parasite membrane, metabolism of the compounds and the mechanism of antagonism. The biochemical transport of folate has recently been characterized in both infected RBCs and free parasites (Wang et al. 2007). Taking this new information together with the potential of the folate cofactors to negate antifolate activity we have demonstrated here, it is logical to assume the parasite expresses folate transporters. Based on these findings, we extended this work to explore the existence and molecular characterization of folate transporters in the parasite **(chapter 4).**

CHAPTER 4

Molecular Cloning and Functional Analysis of putative

Plasmodium falciparum folate transporters in *Xenopus* oocytes

4.1 Introduction

The data presented in **chapter 3** demonstrated that the activity of antifolate antimalarials in all the parasite strains tested could be mitigated by both oxidised and reduced folate substrates and pABA regardless of the DHFR/DHPS genetic status. These data strengthen the argument for the ability of the parasites to salvage the folates. Taken together with the evidence for a salvage pathway presented in **chapter 1,** this new information argues against the long held assumption of reliance on *de novo* folate synthesis and point to a robust adaptive salvage mechanism.

We know malaria parasites spend a great deal of their lifespan within the host's RBC which is rich in nutrients but to obtain vital membrane impermeable substances such as the charged folate species, the parasites must evolve appropriate transport networks. Despite the large volume of published work on the acquisition of nutrients by the parasite and on the role of folates in parasite survival, nothing is known about the molecular identity of the malaria parasite's folate transport system. Despite this lack of information it has been shown that the pharmacological inhibition of folate influx by probenecid

potentiates the activity of antifolates (Nzila *et al.* 2003) and that a variety of extracellular and intracellular factors can regulate folate uptake (Wang *et al.* 2007). Understanding the molecular identity and functional properties of folate transporters is considered a priority. This knowledge will help clarify the folate uptake process including identification of critical substrate binding sites and translocation pathways, confirmation of substrate preference and characterisation of inter-strain variation in folate utilisation. It is also hoped that this information will provide excellent opportunities for the design of inhibitors that can specifically target these transporters as demonstrated in human studies (Matherly et al. 2007), in order to enhance antimalarial activity of traditional antifolates such as the DHFR inhibitors.

As discussed in **chapter 1**, our understanding of folate transport in malaria has been largely derived from work carried out on mammalian cells, plants and other protozoa such as *Leishmania*. Following the deciphering of the *Plasmodium* genome, it is now possible to search for genes that might encode for putative folate transport proteins. Three putative genes; MAL8P1.13, PF11_0172 and PF10_0215 have been identified using bioinformatic approaches and these have been preliminarily classified as belonging to the small folate biopterin transporter (FBT) subfamily (Martin et al. 2005). FBT transporters comprise a small subgroup of the Major Facilitator Superfamily (MFS) and members of this FBT subgroup are found in protozoa, plants and cyanobacteria (Saier et al. 1999). In *Leishmania,* it is known that folate transport is regulated in a growth stage dependent fashion (Richard et al. 2004). Martin and colleagues (Martin *et al.* 2005) studied the expression profiles of the three putative malaria folate transporter genes in the

erythrocytic phase of the parasite and found their regulation to be highly dependent on the growth stage of the parasite. All three genes were found to be maximally expressed in the late trophozoite and early schizont stages. This observation appears to be consistent with the fundamental role of folates in DNA synthesis, which occurs mainly in these late stages as described in **chapter 1**.

We used these published data and blast searches to identify, compare, clone and sequence these genes in 5 well established clones of *Plasmodium falciparum* **(see table 2.2).**

In addition to the sequencing analysis, our interest in the genes focused on functional characterization and assessing their role in the mediation of folate transport. Such functional expression would clearly provide the confirmatory data needed to support the role in folate transport. It is difficult to interrogate the role of these genes in the multi-compartment intact parasite system because of the complexities of a transport system with multiple substrates and multiple transport routes. Even transfection and knock-out experiments might not be very informative. To begin to address this challenge we have adopted the well-characterized oocyte of *Xenopus laevis* (originating from South Africa) as a model system to investigate the role of these genes in folate transport. The model has a number of important and attractive features; normally they produce faithful and efficient translation of exogenous mRNA, display correct orientation of the encoded protein and the oocytes themselves are large and easy to handle. The use of this model for heterologous expression of foreign genetic material was pioneered by Gurdon and colleagues (Gurdon et al. 1971) and the model has been widely embraced as an

experimental tool for use with malaria genes following studies by Penny et al 1998. To date several *P. falciparum* transport activities have been extensively studied using this model including the hexose-transporter, the chloroquine resistance-transporter, nucleoside-transporters and the $Ca2^+$-dependent ATPase- transport systems (Eckstein-Ludwig et al. 2003; Nessler et al. 2004; Downie et al. 2006).

A critical concern over the use of this model is the potential for expression of endogenous transporters for the substrate under investigation. Although, oocytes harbour elaborate biosynthetic machinery and are generally independent of nutrient uptake from their environment, they have been shown to express a small number of endogenous membrane transport systems (Sigel et al. 2005). We acknowledge that one study has reported the existence of an endogenous carrier-mediated uptake system for folate in the oocyte model (Lo et al. 1991). Despite this report, a large body of work also exists that shows the extensive use of this system to study a number of folate transporters (Said et al. 1996; Subramanian et al. 2001; Umapathy et al. 2007). Notably one of the studies is from the same group that first characterised the endogenous folate transporter. Based on all the data we decided that the oocyte was a suitable model for the study of these putative *P. falciparum* folate transporters.

In this chapter, we describe the sequence analysis of MAL8P1.13 and PF11_0172 FBT genes in five well established clones **(see table 2.2).** We failed to successfully amplify the PF10_0215. As previsouly stated, xenopus oocyte is a notoriously difficult expression system and together with time constraint, we only give the first demonstration of the

xenopus heterologous functional expression of one of the putative folate genes, establishing its functional identity as a candidate folate carrier.

Antifolate resistance in *P. falciparum* is currently a matter of grave concern **(section 1.7)** and one of the emerging strategies to overcome antifolate resistance is through the application of salvage blockers that target the transport of folates across the membrane. Blocking folate salvage makes all parasite lines more susceptible to antifolates and using this approach it may even be possible to overcome some forms of antifolate resistance, **(see section 1.8 (c)** and Nzila et al. 2003). A clarification of the molecular identity and function of the putative folate transporters clearly offers an opportunity for a better understanding on the molecular mechanism of folate transport inhibition and provides a platform for the testing and development of novel salvage-blocking antifolate improvers.

4.2 Materials and methods

4.2.1 Chemicals

Radiolabeled reagents; Folinic acid [3, 5, 7, 9-^{3}H]-(6S)-Leucovorin Diammonium salt, (250µCi, 30Ci/mmol) and [3, 5, 7-^{3}H] Methotrexate, sodium salt (250µCi, 12.2Ci/mmol, 9.25MBq) were purchased from Moravek Biochemicals (Brea, CA, USA). Folic acid, [3, 5, 7, 9-^{3}H] sodium salt] (250µCi, 9.25MBq, 24.0Ci/mmol, 888GBq/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, USA). Unlabeled folate substrates were sourced and prepared as described in **sections 2.10 and 2.10.1.**

4.2.2 Strains:

See table 2.2

4.2.3 Bioinformatics

Three genes; MAL8P1.13, PF11_0172 and PF10_0215 have been predicted as members of the FBT family (Martin *et al.* 2005). Table 4.1 lists representative protein members of this family (Saier *et al.* 1999). MAL8P1.13 and PF11_0172 have been claimed to possess significant sequence similarity (Martin *et al.* 2005). Attempts to amplify the PF10_0215 gene were unsuccessful and hence we focused our attention on MAL8P1.13 and PF11_0172. The PlasmoDB (http://plasmodb.org/plasmo/) has full-length sequence entries for the two genes. However, their functional features have not been investigated and their predicted role is inferred from homology with the well characterised FBT members. Our own search through Blast analysis identified a number of homologs in plants, protozoa and cyanobacteria that have a reasonable degree of identity and confirmed that MAL8P1.13 and PF11_0172 are indeed putative folate-like transporters. These putative genes were cloned and their identity confirmed by sequencing.

Table 4.1 Sequenced Members of the Folate-Biopterin Transporter (FBT) Family (TC ≠ 2.71)

Abbreviation	Name or Database Description	Organism	Size (No. residues)	Database and Accession No.
BT1 Ldo	BT1 biopterin/folate (not methotrexate) transporter	*Leishmania donovani*	627	gb|L38571
FT1 Ldo	FT1 folate/methotrexate (not biopterin) transporter	*Leishmania donovani*	704	No Acc. #
Orf Lin	Integral membrane protein	*Leishmania infantum*	627	gb|L25643
BT1 Lme	Biopterin transporter	*Leishmania mexicana*	631	gb|AF078929
FT1 Tbr	FT1 (ESAG10) folate/biopterin transporter	*Trypanosoma brucei*	686 (*597)	pir|S33475
Orf1 Ath	Similar to Synechocystis integral membrane protein	*Arabidopsis thaliana*	431	gb|AC002376
Orf2 Ath	Functionally uncharacterized Orf	*Arabidopsis thaliana*	408	gb|AC002332
Orf3 Ath	Putative integral membrane protein	*Arabidopsis thaliana*	429	gb|AC006223
Orf Sco	Functionally uncharacterized Orf	*Synechococcus PCC7942*	453	gb|AF055873
Orf Scy	Integral membrane protein	*Synechocystis PCC6803*	494	gb|D64002

Adapted from Saier, M. H., Jr., J. T. Beatty, et al. (1999) " J Mol Microbiol Biotechnol 1(2): 257-79

4.2.4 PCR and pCR®II-TOPO® cloning.

The sequence information available in the PlasmoDB enabled us to design primers suitable for amplification of the entire coding regions of MAL8P1.13 and PF11_0172. Total RNA isolation and cDNA synthesis were carried out as previously described, **sections 2.14 and 2.15** respectively. PCR was performed using the resultant cDNA according to the conditions described in **section 2.16**. For the full length synthesis of MAL8P1.13 and PF11_0172 products, the primer combination PF7-F / PF7-R and PF9-F / PF9-R were used respectively **(see table 2.1 for primer sequences)**. Because of our interest to extend the work to translation studies in the *Xenopus*, we designed the upstream primers (PF7-F and PF9-F) for generating the PCR template to contain the Kozak consensus sequence (CCACCATG) before the initiator ATG for efficient translation. Each reaction yielded a fragment of the expected size (~ 1518 base pairs and 1368 base pairs for the MAL8P1.13 and PF11_0172 respectively) which were then cloned into the dual promoter pCR®II-TOPO® vector **(see sections 2.23 and 2.24)** and sequenced on both strands in their entirety using the SP6 and T7 promoter sequences **(see**

section 2.29). Sequencing was done for five independent clones for each of the five strains, deriving a consensus sequence that was shared by one of the five clones for each strain. Homology to the known MAL8P1.13 and PF11_0172 in PlasmoDB was confirmed by sequence alignment analysis (BLAST at the National Center for Biotechnology Information). Homology of the sequences to each other and to the existing sequence in PlasmoDB was determined by aligning multiple MAL8P1.13 and PF11_0172 sequences using the Lasergene program suite (DNASTAR, Madison).

4.2.5 Subcloning pCR®II-TOPO® inserts into the KSM pBluescript vector

Although the introduction of the Kozak consensus sequence has been reported to improve translation (Kozak 1987), we initially had difficulties to achieve meaningful functional assay with cRNA transcribed from the insert containing the Kozak sequence only. With the aim of increasing the Xenopus oocyte expression, the MAL8P1.13 and PF11_0172 inserts (from the 3D7 strain only) were released from the pCR®II-TOPO® by the sequential digestion with Xho-I and Spe-I (restriction sites in the vector) and then sub-cloned into the dephosphorylated (see section 2.21) KSM vector at the Xho-I / Spe-I site (internal sites in the 5'- and 3'-untranslated regions of the KSM vector). The KSM vector (kindly provided by Dr Bill Joiner, University of Pennsylvania, USA) is a derivative of the pBluescript in which the insertion site is flanked by the 5'- and 3'-untranslated regions of the Xenopus β-globin gene of the cloning sites to enhance the expression of the heterologous cRNAs in the oocytes.

4.2.6 Linearization of the MAL8P1.13 and PF11_0172 + KSM vector

The plasmid MAL8P1.13+KSM and PF11_0172+KSM were linearized with *XbaI* (New England Biolabs, UK) according to the manufacturer's instructions. The linearized plasmid was extracted with phenol/chloroform, precipitated with ammonium acetate/ethanol, re-suspended in DEPC-H_2O and quantitated using the Nanodrop at A_{260} nm. The integrity of the plasmid was assessed by the agarose gel electrophoresis.

4.2.7 Heterologous cRNA synthesis

Because of the difficulties of delivering cDNA into the nucleus of the oocyte during microinjection, we adopted the use of cRNA for protein expression since it can easily be delivered into the cytoplasm of the oocyte. At least 1μg of the linearized plasmid MAL8P1.13+KSM or PF11_0172+KSM was used as template for *in vitro* transcription of MAL8P1.13 or PF11_0172 capped complementary RNA (cRNA) from the T3 promoter using the Ambion T3 mMessage mMachine kit (Ambion, Huntingdon, UK) according to the manufacturer's instructions. The *in vitro* transcription products were treated with 1 μl Turbo DNase (RNase-free) for 15min at 37°C followed with phenol/chloroform extraction, Li-Cl and ethanol precipitation, re-suspended in DEPC-H_2O and quantitated using the Nanodrop at A_{260} nm. The cRNA quality was verified by agarose gel electrophoresis under denaturing conditions. cRNA was stored at -80°C as 10μl aliquots of 1μg/ul.

4.2.8 Amphibian husbandry

Healthy sexually mature female *Xenopus laevis* frogs were procured in batches of 20 from Xenopus Express (Haute-Loire, France). On arrival, they were inspected for red leg infection (an infectious disease affecting the underneath of the thighs) and for any damage. The frogs were housed at 18°C aquarium within The School of Biological Sciences, University of Liverpool in specially designed Premier tanks [118cmX50cmX50cm, Schwarz, Germany] containing 2/3rd level of non-chlorinated water. Feeding and a 1/3rd water change was performed three times a week with the tank cleaned once per week. During water change, waste food particles and any debris were siphoned from the tank.

4.2.9.1 Preparation and selection of oocytes

A selected frog from the tank was euthanised by immersion in pre-chilled water containing 5g/L of 3-Aminobenzoic acid ethyl ester (methane sulphate, MS222, A5040, Sigma, Poole, UK) for 45 min. After death, the frog was rinsed in running tap water, a surgical abdominal cavity incision made and the ovarial lobes removed. The oocyte sacs were cut open and the separated oocytes treated with collagenase (1mg/ml, 2 hr, 60rpm shaking, room temperature, Sigma, Poole, UK) in Modified Barths Solution (MBS, containing in mM: 88 NaCl, 1 KCl, 2.4 NaHCO$_3$, 0.82 MgSO$_4$.7H$_2$O, 15 HEPES adjusted to pH 7.6 with NaOH) minus Ca2$^+$ to ensure complete enzymatic defolliculation. After thorough washing, initially with MBS and then Complete Barths solution (CBS), MBS

containing Ca2$^+$; 0.3mM CaNO$_3$.6H$_2$O and supplemented with 1ml of 10mg/mL penicillin-streptomycin mix. The oocytes were kept in CBS and stored at 18°C for two hours. Healthy undamaged oocytes stage V-VI oocytes (Dumont 1972) showing even colouration and well defined borders of the poles with no residues of follicular tissues were selected under Stereomicroscopy (Leica microsystems, China) and left to recover in CBS for 24 hr at 18°C before microinjection.

4.2.9.2 Microinjection and incubation of oocytes

Glass capillaries [Thinw W/FIL, 1.0mm 4 IN, World Precision Instruments, (WPI), Sarasota, FL, USA] for oocyte microinjection were pulled using a puller (Pul-1, WPI), fixed onto the microinjector (WPI) and the tip manually nicked under the stereomicroscope. cRNA or water control was then loaded into the capillary under suction. For expression studies, oocytes were injected with 50 nl (containing 50ng) of the cRNA. Oocytes injected with similar volume of water were used as controls. Injected oocytes were separated and maintained in CBS at 18°C for 3-5 days with daily changes of the medium and removal of damaged or dead oocytes. Differences in the days of incubation resulted from the unpredictability in the quality of eggs that would vary from frog donor to donor. At the end of the incubation, oocytes were used for uptake studies.

4.2.10 Radioactive Tracer Flux assays

Flux studies were performed at room temperature in Ringers buffer (containing in mM; 120 NaCl, 2 KCl, 1.8 $CaCl_2$ and 5 HEPES) as the transport medium. Batches of 10 oocytes were removed from CBS, gently rinsed 4 times in Ringers and then preincubated in the same buffer for 5 min before initiation of uptake assays. Oocytes were transferred to 500 μl Ringers buffer, pH7.4 supplemented with 100 nM radiolabelled compound minus/plus unlabeled compound and incubated either for 1 hr or 2 hr depending on the assay. Uptake was terminated by gently immersing the oocytes in ice-cold Ringers followed by four successive washes with the same buffer. The oocytes were carefully transferred individually to scintillation vials and digested in 200μl of 1M NaOH. 3 ml of scintillation cocktail was added and radioactivity counted using the Liquid Scintillation Analyser [2250 CA, Tris-Carb®, Packard, Pangboune, UK]. Figure 4.1 provides a schematic illustration of the *Xenopus* oocyte functional study from infected RBCs to radioactive tracer studies.

4.2.11 Data Analysis

Data for oocyte flux studies are presented as mean ± SE of 8-10 oocytes and are expressed as femtomoles per oocyte per hour / 2 hr. *P* values were calculated using the Student's t-test.

Infected RBCs ≥10% parasitemia

↓

Total RNA isolation

↓

cDNA synthesis

↓

PCR: F' primers + Kozak consesus

↓

pCR®II-TOPO® cloning

↓

KSM sub-cloning (5'-3' Xenous β-globin)

↓

Linearization

↓

cRNA ⟶

3 – 5 days
Incubation

cRNA

1. translation
2. modification
3. sorting
4. membrane insertion

↓

Radioactive tracer fluxes
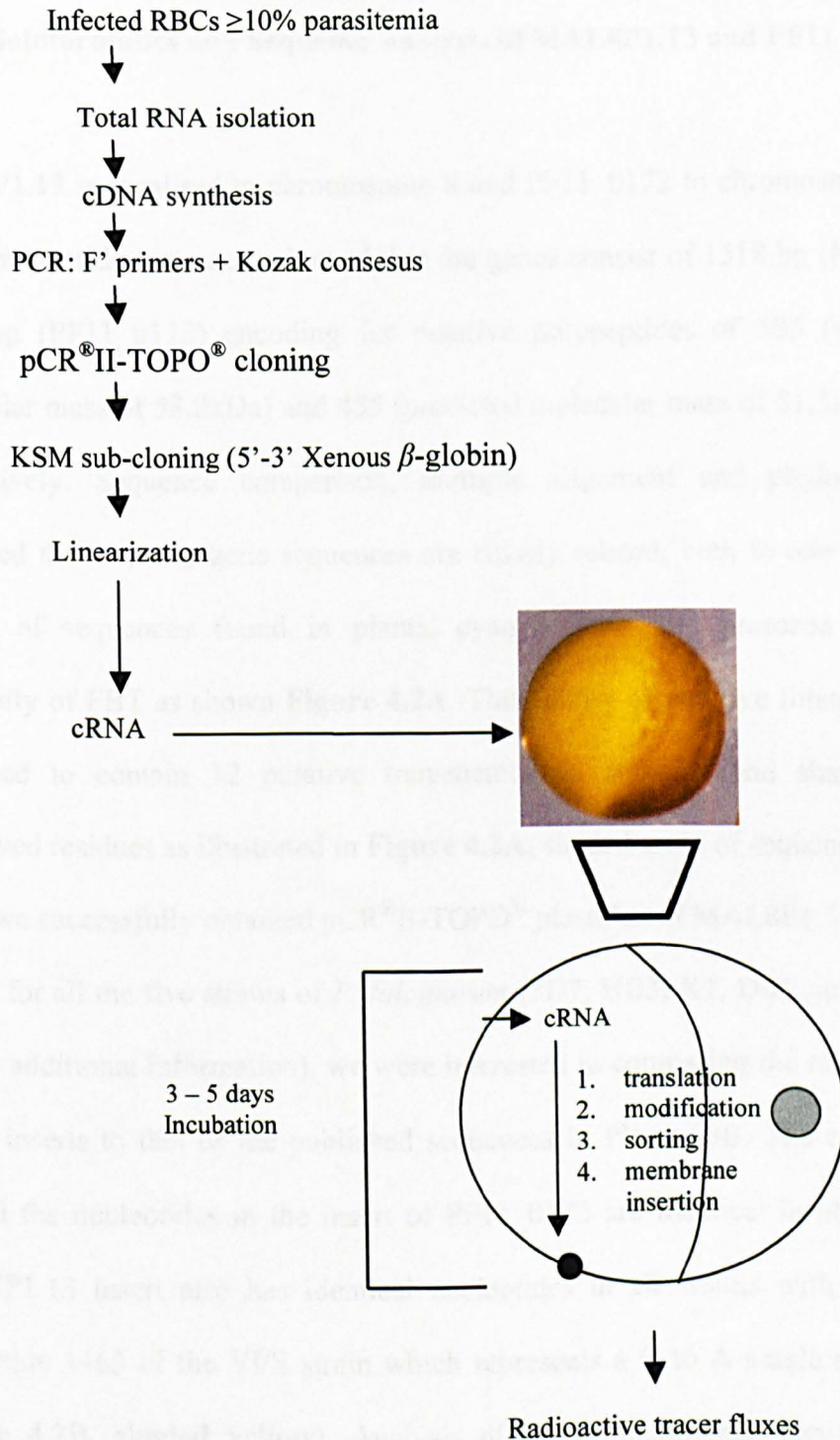
Figure 4.1 Schematic illustration of the *Xenopus* laevis oocyte functional study

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4.3 Results

4.3.1 Bioinformatics and Sequence analysis of MAL8P1.13 and PF11_0172

MAL8P1.13 is localised to chromosome 8 and PF11_0172 to chromosome 11. Analysis of the nucleotide sequences showed that the genes consist of 1518 bp (MAL8P1.13) and 1368 bp (PF11_0172) encoding for putative polypeptides of 505 (with a predicted molecular mass of 58.0kDa) and 455 (predicted molecular mass of 51.3kDa) amino acids respectively. Sequence comparison, multiple alignment and phylogenetic analysis indicated that the two gene sequences are closely related, both to one another and to a cluster of sequences found in plants, cyanobacteria and protozoa belonging to a subfamily of FBT as shown **Figure 4.2A**. This family of putative folate trasnporters are predicted to contain 12 putative transmembrane domains and share a number of conserved residues as illustrated in **Figure 4.2A**, shaded areas of sequence.

Since we successfully obtained pCR®II-TOPO® plasmids of MAL8P1.13 and PF11_0172 inserts for all the five strains of *P. falciparum* (3D7, HB3, K1, DD2 and VI/S. **See table 2.2** for additional information), we were interested in comparing the nucleotide sequence of the inserts to that of the published sequences in PlasmoDB. The comparison shows that all the nucleotides in the insert of PF11_0172 are identical in all the strains. The MAL8P1.13 insert also has identical nucleotides in all strains with the exception of nucleotide 1463 of the VI/S strain which represents a C to A single nucleotide change **(figure 4.2B, shaded yellow)**. Analysis of the chromatogram chart at this position

(figure 4.2B, vertical line shown) clearly indicates the quality of the output was good and not an artefact.

Of the two cloned sequences, the *Xenopus* functional expression and analysis data is presented for MAL8P1.13.

```
                    TM1                                    TM2
              οοοοοοοοοοοοοοοοοο                     οοοοοοοοοοοοοοοοοο
Synechocys(I)  ....APSWELLAILSIYFVC VL.GLSRLAVSFFL ELGLS AAMGALIGLGAA WIL PVLGLMS TVPLF Y RRS   104
At2g32040(I)   KFYGVELSPDNVAVAMVYFVC VL.GLARLAVSFYL DLHLD AETAVITGLSSL WLV PLYGFIS SVPLF Y RRS   195
At1g04570(II)  RFECVGGKGISVLCGLGYWVC SR.CFPWLALNFHMVHSLALQ STLCLVCYSCSL MVA PLYGVLS VLYIGSG R VP   156
MAL8P1.13(II)  NHENEYMIFNKISSSLIAMLC VE.VLCNLSIYLL NYHLH ASLSIVMCFIKI WSI LVWAVIS NYPIF Y RKY   122
PF11.0172(II)  EKTKKGFDFTCIVVYLVGLSD LT.HLASLAIYYLF YFRLT YQVSLILMYFYI FIL PVIALIT SFSIF M KP    109
Osativa(III)   LGRELHWSFVAGVVATYGASC LGGGVMRVASDYYW VCRVQ SAACVYCGVTSI WMV PLWGLLT VLPIA C YR RP   132
Ldonovani(IV)  AVKGYGLKFIVALGASNLLCK VADCILYGQTYAMMI RYGIDVARYCRLSSISTMGWSI AFTAMLC GFAFL CYTKRW  135
Rsolanace(VI)  ..........WLTLFIYLATFAG.YLLDIQTSYLL NCLHATATQISIFRLVTGI VYIAFAFGLAR HWNPL CL DRG    90


                   TM3                           TM4                              TM5
           οοοοοοοοοοοοοοοοοο          οοοοοοοοοοοοοοοοοο              οοοοοοοοοο
Synechocys(I)  YLWLSGLMGSAGWLLFAAWVSS..GTCAGLVLLFTSLS.VAIGDVIVDSL VW RACRESLACVGSLC LTWGAAAV GII   181
At2g32040(I)   YLVLSGLLGAFSWSLMAGFVDS..KYSAAFCILLGSLS.VAFSDVVVDSM VV RARGESQSVSGSLC LCWGSSAF GIV   272
At1g04570(II)  YIAIGVFLCVLAWGSMGIFQGAREVLPSLVACVLLSNLGASITEVAKDAL LA YGLRYRING...LCS YALMASAA GVL   233
MAL8P1.13(II)  YLLLIGSFLCILSLICLGLITHN..NLFITILLLFIYFFGSSLCNVIGEAC VV SNFNCSINSSARNV LFFAFRKLSFAI   200
PF11.0172(II)  YLFLFSLFCSLNFLALAFINLS..VICASLILFFISLC ASFCTTVAEAL VV SSMGQTYSQGTNKVIEFIASKAI CSLS   186
Osativa(III)   YFVIAGFMGVVAMLVLSLHSKI..HVLFALLALMAGSASVAIADVTIDAC LA NS.IVHPHLAADMISLNGFCASV GLI   209
Ldonovani(IV)  YMFISCVGGGAFALIYGLLPAKEASANVACAFIFLSSWGKANVDILSCGHYSRLMRCNPKPGP.ALVS WMWFCIMI SLI   214
Rsolanace(VI)  FFLIFAPTTAAALIWMAFSGFS...YLGLLVGMLLAMLSSRFVAAAYQGLIALVGCERMSG..RLSALWNVVSAVPVVA   165


               TM5                     TM6                                     TM7
           οοοοοοο           οοοοοοοοοοοοοοοοοο                          οοοοοοο
Synechocys(I)  TAYA CA LEWFSTRTV AITAIFFLLTVGAAFLIS....VSTAEEEEK..........PQPKACIKLVWQAVRQKTILL   248
At2g32040(I)   SSYF CS VESYGVRFV GVTALLFLITSAVAVLVN CRVVRPASGQKENITLLSFGFLCTSKCNMICLWGAIKQPNVFL   352
At1g04570(II)  GNLLC CY LLTTFPKIS LVFSALLSLQLVVSLSSK ESFGLPRIAETSS......VLESVKKCISNLKEAIQADEISQ   306
MAL8P1.13(II)  MSYL CY LLLISKHI LIGSFLFICVFTSGFFII....KRNYTKSS.......IKDCIKCIYSIIKLSYLKN         264
PF11.0172(II)  VAYF CYFLEKMFREYI IATSIFFLIISLSCLFLK....KEYSTNFN..........IFNCLSCLIKFINTPIFLG    250
Osativa(III)   GFSI CF VHAIGSQGALGMLAIFSALVILAGMMIKFVHM FNFPYELAHMK..........FVEASRTMMATLKCPEVWR   279
Ldonovani(IV)  ATVM CP ACAGKPQISIFVSAALCAITCVFYLFRS IALFILEETRKERERLIAMLCANILADTLGLLVACVVVSVIAK   294
Rsolanace(VI)  GAFA CYISDHLAPCGA FLVAAVTVLIALLGLWKPISVFGQLYEKPCARG..........TDFIGNVRRLVTHRAIYP   234


                   TM7                             TM8                          TM9
           οοοοοοοοοο                      οοοοοοοοοο                   οοοοοοοοοο
Synechocys(I)  PTLFIFFWCA.TF SAESAF YFT EG EPKFL RVR LVTSVAGLIGVGL QRFL TLPF VIMGWS VISSLLG      323
At2g32040(I)   PTLFIFLWCA.TF HSDSAM YFT KG TPEFL RVK LVTSIASLIGVGL NGFL TVPL KIFLVT IFGTGLG      427
At1g04570(II)  PLIWAVVSIAMV LLSGSVF CYC C VNLDPSVI MSK VIGQLMLLCLTVV DRYL TLPM FLIHII QLLYGLSI     382
MAL8P1.13(II)  FIIFIFIMMS.TF SCXNTLF FYI E NSPNLL KVA MFQSLASFISIIS MLFFTKIDI KLLLYS IIITPFC      339
PF11.0172(II)  PFLYIFVYMS.FG DYDDAPF FFC KG RPSFM TLR LTYGIASLIGIII RVFL NCSLF KTLIIT LVSFPIY     325
Osativa(III)   PCVYMYMSLALSVDIQEXM YWY DFNAG S SEBLI FIF AVGSVGSLIGVIL QNIL DHSF SVLCLSQLLLSLSG   358
Ldonovani(IV)  ANVFAYLDKAVSIRVSGPLNAFYL TYNLFN TYTFYNTVAGVINTIVGMITVTLFNFLFAKHGY LTFIVT IMQVLAA   374
Rsolanace(VI)  AVLICFLWNF.AF GSATPLCFYL A HASDSVYSYYN.GIFAAAFIPTFLL GLLC KVSLCKLLWWG LVAVP.Q     308


                   TM9                      TM10                              TM11
           οοοοοο                    οοοοοοοοοοοοοοοοοο               οοοοοοοοοοοοοοοοοο
Synechocys(I)  LTTL LITHANRAM DDHWFSLGLSIIL TVTGCIAFM VL VT AARL CF CI ATLFALLM VMNLAGVLSFEVCSLLT 402
At2g32040(I)   MTCV LVSGFNRCL SDEWFAIGLSLIL TVLACASFM VL VT AARL CF CM ATLFATLM ISNGGSVLGGLM AGLT 506
At1g04570(II)  LLDY LVKCINLGF SNEVYVLCFSSLA EILACFKIL FA VRLASM CG CO GSVTSFLA TLCLSCIVSAFLCVGLA  461
MAL8P1.13(II)  LLPLVVIKKVNYFLF PNTLFFIILTVLI EFIAEFCTM IL VL CSRLIF PC EFF STIYSLLL SNNFASIISSFLSSLLT 418
PF11.0172(II)  ISPI LTEHINKFL SNELFVLSGGFLI EAITEICLI LFI TANIC PCL ASVFATIL VKNLGSLTKKGTSSFIT  404
Osativa(III)   MLDL LVLRLNIKL PDYYFAVILEGVS KMINRIKWM LL VT SSKLC PCI GTFYALLM IDNVGLLSGSWA GLIL 437
Ldonovani(IV)  LFDI MVKRWNLYI PDHAMYIWGLAVVGEIVYMLGFM CI VL LSRLC RCS SVVYALMAGFARLGRTTAASLCAILL 454
Rsolanace(VI)  MIPLAFIHSAN.........LALVLAAPI.GLM33VATAAYFD AMRS FCLCGTIMMLVDGVLALSARAGDLL SWIY   378


                    TM12
           οοοοοοοοοοοοοοοοοο
Synechocys(I)  HWLGVTETCFDN ALLVIITNLST... L PFLG L ....AGDPQVKDKTEKEDNPDDPGDRLVLFFAEVF   468
At2g32040(I)   QAFGITRDSFG L STLIILCNLSS... L PLLG L ....RDSP...DTLTKED.........ADVE        556
At1g04570(II)  NLIGITSSNYS L SSGILCSLAA... A CFMH V ...MSEPVIEKEGKRGISKRSRFNRRVGRVVDKE       527
MAL8P1.13(II)  YSLNITSTNFT N PYMIIICCLTN... II IFFLYIL ....NHSQKFNLCHSNSHTQKYYSYPSTCYISSQK    484
PF11.0172(II)  YLMKIDSYNFD N SLYILTCGFFL... FS TLVP L ....NEEHIEKLKNKETSKG...............   455
Osativa(III)   HVLNITRTEFK N WAAILINAMR... L ALLF V RSEQNSNLLPADLLFECLAACYQMDNVELTSLTVD       507
Ldonovani(IV)  EYDDGSRCNYDN PLLLFLCSMCTFLLAI SIIL FKARICDDIDIDGKVVRCAVDKQVAAAPLSSSCSDAV     527
Rsolanace(VI)  NSSPTHGFTYCVIATTVVYALILL... I PTPKT IATR..DGEPNPEAEVRNEFREAEPT..........   435
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Figure 4.2A Predicted amino acid sequences and transmembrane domains of MAL8P1.13 and PF11_0172. The sequences of the putative transporters available in PlasmoDB were compared with representative FBT proteins from cyanobacteria (*Synechocys*), plants (*Arabidopsis*; At1g04570, At2g32040, Rice; *O. sativa*), protozoa (*L. donovani*) and bacteria (*R solanace*). Conserved amino acid residues are shaded.

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               1370      1380      1390      1400      1410      1420      1430      1440
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V1S-PF7_T7P    tatcctaccaaaccattcccaaaaaaaaaaatttacaacattcaaactcacatactcaaaaatattattcatacccatcca
Other_strains_Pf tatcctaccaaaccattcccaaaaaaaaaaatttacaacattcaaactcacatactcaaaaatattattcatacccatcca

               1450      1460      1470      1480      1490      1500      1510      1520
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
V1S-PF7_T7P    ccgattatatatAatctcaaaaatcggattcatctgaaataaccaaattttctgcagacatgcaaattgatgacataacc
Other_strains_Pf ccgattatatatCatctcaaaaatcggattcatctgaaataaccaaattttctgcagacatgcaaattgatgacataacc

               1530
               ....|....|....|.
V1S-PF7_T7P    ttggaataaggttacc
Other_strains_Pf ttggaataaggttacc
```

ock Columns: 1 2 3 4 Rows: 1 2 3 4 5 6 ☐ Show confidence   Save settings   ☑ Compact   Close | Help >>

Figure 4.2B Nucleotide sequence comparison of MAL8P1.13 insert against the consensus sequence of four strains (3D7, HB3, DD2 and K1). Top nucleotide sequence/chromatogram is for VI/S and the lower one for the four strains. Nucleotide change at position 1463 is highlighted in yellow and the corresponding chromatogram with a blue vertical line.

4.3.2 Functional characterization of the putative MAL8P1.13

4.3.2.1 Comparison of the endogenous folate uptake system to MAL8P1.13 cRNA for folic acid, methotrexate and folinic acid at 2 hours.

The putative folate transporter (MAL8P1.13) has not previously been functionally characterized. To correlate the report from Martin and colleagues (Martin *et al.* 2005) and our above results on the predicted functionality of the gene, we microinjected *Xenopus* oocytes with *in vitro* transcribed cRNA from the cloned plasmid insert of MAL8P1.13 and assayed for the uptake of radiolabelled methotrexate (MTX), folic acid (FA) and folinic acid (5-FoTHF). As explained in the introduction Lo and co-workers (Lo *et al.* 1991) have previously reported the existence of an endogenous folate uptake system in oocytes. They demonstrated uptake of radiolabelled FA in both collagenase treated and untreated oocytes over a period of 2 hours. Due to concerns that a similar phenomenon could interfere with our heterologous expression results, we evaluated the functional expression of the transporter by comparing the uptake of radiolabelled FA between cRNA-injected oocytes and water-injected oocytes at 2 hr. Bearing in mind that most folate carrier systems display substrate specificity, we expanded the substrates to include the antifolate (MTX) and the reduced folate derivative (5-FoTHF). **Figure 4.3** shows that the uptake of all three substrates was significantly higher in cRNA injected oocytes compared to water injected oocytes. The uptake of FA was ~3.9 fold- (31.23 ± 7.598 vs 7.854 ± 0.7982), MTX ~2.6 fold- (18.98 ± 3.569 vs 7.121 ± 2.655) and 5-FoTHF ~11.2 fold-higher (81.95 ± 7.034 vs 7.270 ± 0.4939) in cRNA injected oocytes compared to

water injected oocytes. These data show that the cloned *P. falciparum* MAL8P1.13 is indeed capable of mediating the transport of oxidised and reduced folate species and antifolates.
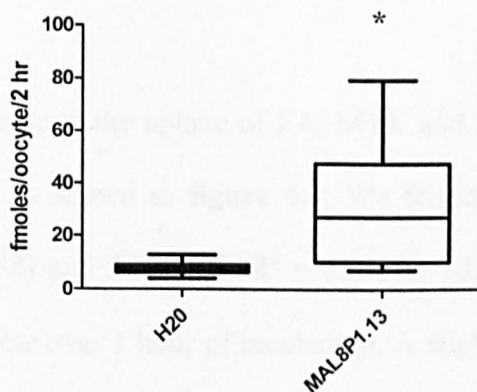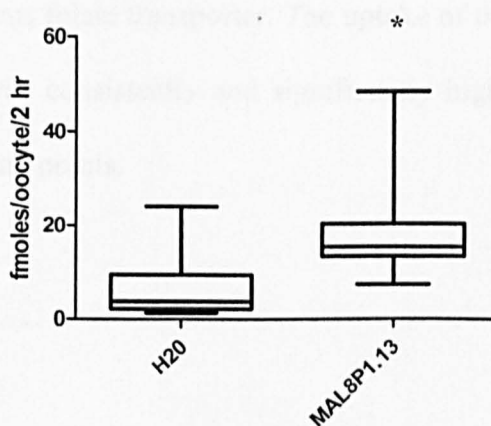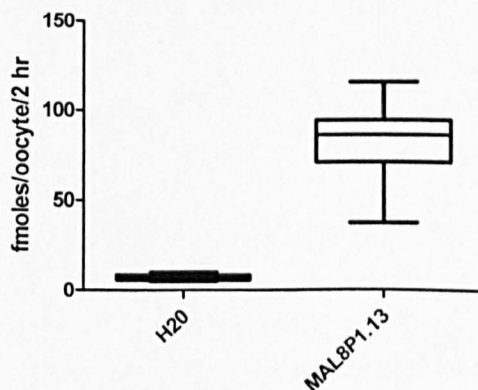
Figure 4.3 Uptake of radiolabelled folic acid, methotrexate and folinic acid each at 100nM in water injected and MAL8P1.13 cRNA injected oocytes.
* Significance for all substrates in cRNA injected compared to uptake in water-injected oocytes (folic acid, p<0.0067, methotrexate, p<0.0157 and folinic acid p<0.0001)

4.3.2.2 Time-dependent uptake of folic acid, methorexate and folinic acid.

In this experiment, we examined the uptake of FA, MTX and 5-FoTHF as a function of time (20, 40 and 60 min) as shown in **figure 4.4**. We found the uptake of FA ($R^2 = 0.9998$), MTX ($R^2 = 0.9588$) and 5-FoTHF ($R^2 = 0.96$) by MAL8P1.13 cRNA-injected oocytes to be linear with time over 1 hour of incubation. A slight rise in the uptake of the compounds over time was also observed in water-injected oocytes. This observation could be due to the endogenous folate transporter. The uptake of the labelled compounds in cRNA injected oocytes was consistently and significantly higher than in the water-injected oocytes for all the time points.

Folic acid

$y = 0.2659x + 14.425$
$R^2 = 0.9998$

$y = 0.0636x + 2.8002$
$R^2 = 0.8443$

uptake fmols/oocyte

time (min)

Methotrexate

$y = 0.2161x + 4.1076$
$R^2 = 0.9588$

$y = 0.0606x + 2.9885$
$R^2 = 0.98$

uptake fmols/oocyte

Time (min)

Folinic acid

$y = 0.6667x + 36.683$
$R^2 = 0.96$

$y = 0.0155x + 3.9306$
$R^2 = 0.5224$

uptake fmols/oocyte

time (min)
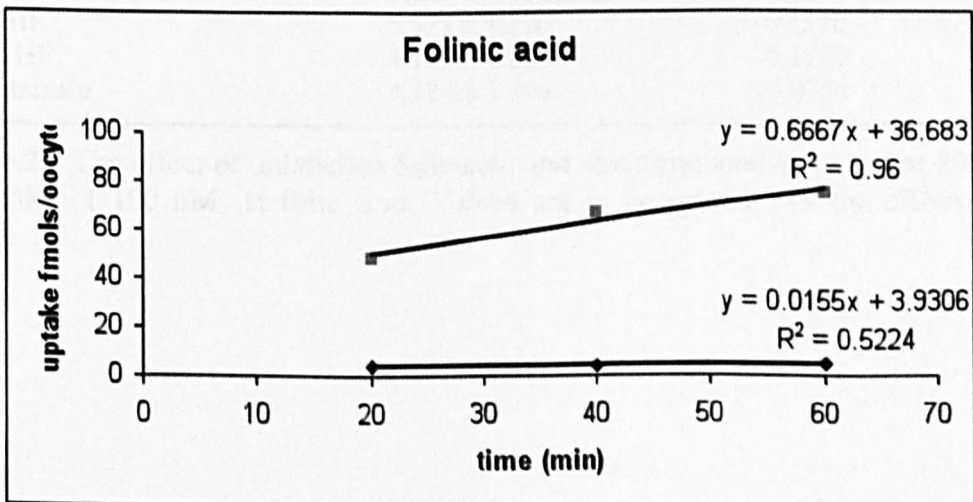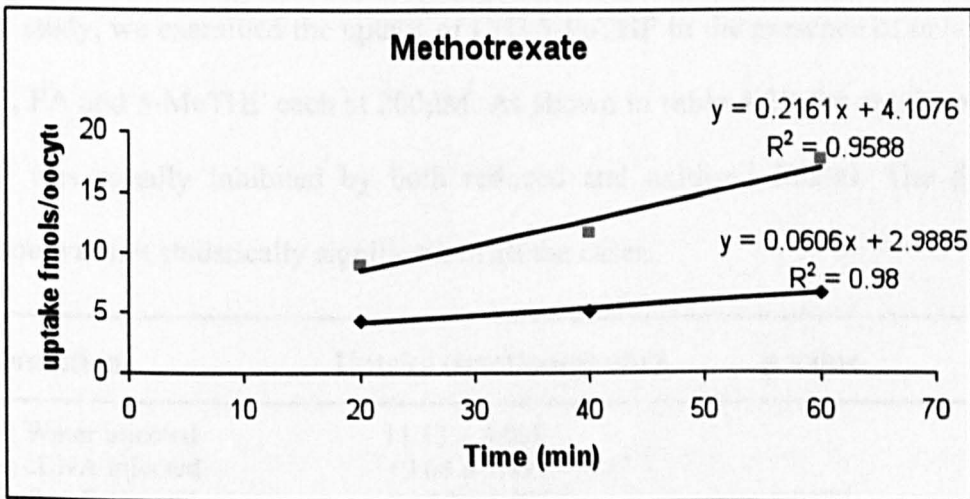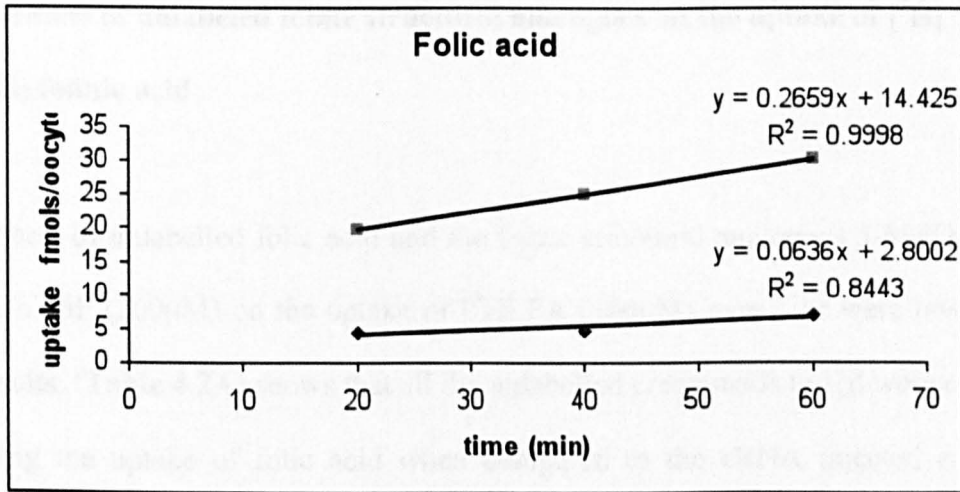
Figure 4.4 Time-dependent uptake of folic acid, Methotrexate and folinic acid each at 100nM by water (■) and MAL8P1.13-injected oocytes (■)

4.3.2.3 Effect of unlabeled folate structural analogues on the uptake of [^{3}H] folic acid and [^{3}H] folinic acid

The effects of unlabelled folic acid and the folate structural analogues 5-MeTHF, MTX and 5-FoTHF (200µM) on the uptake of [^{3}H] FA (100nM) over 1 hr were investigated. The results, **(Table 4.2A)** shows that all the unlabelled compounds tested were capable of inhibiting the uptake of folic acid when compared to the cRNA injected control. In another study, we examined the uptake of [^{3}H] 5-FoTHF in the presence of unlabelled 5-FoTHF, FA and 5-MeTHF each at 200µM. As shown in **table 4.2B** the uptake of [^{3}H] 5-FoTHF was equally inhibited by both reduced and oxidised folates. The degree of inhibition was not statistically significant in all the cases.

Test Condition	Uptake (fmol/oocyte/hr)	p value
Control; Water injected	11.13 ± 4.098	
Control; cRNA injected	19.08 ± 7.411	
+ Unlabelled Folic acid	3.957 ± 0.8415	0.0577
+5-FoTHF .	5.573 ± 1.770	0.1310
+5-MeTHF	4.132 ± 1.584	0.1190
+Methotrexate	4.784 ± 1.596	0.0756

Table 4.2A The effect of unlabelled folic acid and structural analogues (all at 200µM) on the uptake of 100 nM ^{3}H folic acid. Values are in comparison to the cRNA injected control.

Test Condition	Uptake (fmol/oocyte/hr)	p value
Control; Water injected	15.70 ± 6.037	
Control; cRNA injected	29.85 ± 10.18	
+ Unlabelled Folinic acid	14.85 ± 4.893	0.2005
+Folic acid	14.54 ± 5.316	0.1989
+5-MeTHF	12.72 ± 5.795	0.1608

Table 4.2B The effect of unlabelled folinic acid and structural analogues (200µM) on the uptake of 100 nM ^{3}H folinic acid. Values are in comparison to the cRNA injected control.

The Xenopus oocyte model is a notoriously difficult system and the expression of the foreign genetic material can vary tremendously depending on the quality of the batch of oocytes and the incubation period. We observed a great deal of variability in the quality of oocytes. Some studies have reported uptake of radiolabelled substrates to range from a simple 2- to 100-folds. This is certainly the case with our study where for the same substrate and same incubation time, variations were noted in the level of uptake. Our attempts to functionally characterise the MAL8P1.13-mediated FA, MTX and 5-FoTHF transport activity in more detail were unsuccessful and this was partly related to the oocytes quality and the technical reasons as mentioned in **sections 4.2.5.and 4.2.7.** Although we failed to establish a precise K_m value for FA, MTX and 5-FoTHF in the cRNA injected oocytes, the K_m for the endogenous folate carrier was reported to be ~ 42 nM for FA and uptake was also observed to increase when the concentrations were raised up to 270nM. In this work, we used 100 nM concentration of radiolabelled FA and the other substrates. Our cRNA injected oocytes demonstrated a significantly higher uptake than the water-injected oocytes in all the functional assays carried out. This proves the enhanced uptake of the substrates is attributable to the foreign transporter in the cRNA

injected oocytes and that with comparison to FA, the transporter has a better affinity than the endogenous transport system.

4.4 Discussion

Our objective was to establish the molecular identity and functional role of the reported *P. falciparum* putative folate transporter genes. As mentioned in **chapter 1** and backed up by the results in **chapter 3** there is clear evidence of the parasite's ability to salvage folates. A number of studies have provided compelling evidence on the existence of the folate salvage system in *Plasmodium* that can influence antifolate drug activity against malaria **(see section 1.3.2.4).** Of critical concern is the impact of folate supplementation during pregnancy since antifolates play a key role in the intermittent preventive treatment in this population (Ouma *et al.* 2006). Therefore, there is an urgent need to dissect the salvage system further and fully understand the underlying mechanisms.

The folate salvage phenomenon in *Plasmodium* has now been studied in some detail at the biochemical level (Wang *et al.* 2007) but the molecular components of the transport mechanism remained unknown. This is in stark contrast to the wealth of molecular information available on folate transporters in other organisms that are capable of salvaging folate from the environment such as mammalian cells (folate receptors and reduced folate carrier), (Matherly et al. 2003) and other protozoa such as *Leishmania* (biopterin and folate transporter), (Richard *et al.* 2004). A good starting point in the effort to identify relevant transporters is to interrogate the available genome-wide information.

Thus far, Martin and colleagues (Martin *et al.* 2005) identified three genes; MAL8P1.13, PF11_0172 and PF10_0215. The functional role of these genes was unknown but sequence analysis indicates that MAL8P1.13 and PF11_0172 share homology to members of the FBT sub-family present in plants, cyanobateria and protozoa (Saier *et al.* 1999).

Based on the identification of the genes, our attention turned to the functional examination of their activities. Our initial approach was to isolate cDNA from a range of parasites and generate the appropriate PCR products. We performed a preliminary analysis on the sequence homology of MAL8P1.13 and PF11_0172 against other homologous gene members of the FBT family. Since the amino acid sequence underlies the function of the protein, there is usually a higher degree of amino acid sequence conservation when compared to the nucleotide sequence (Nguyen et al. 1997). As shown in **figure 4.2A**, the two genes share a number of conserved residues with members of the FBT family across the 12 transmembrane domains in addition to similarities with one another. Such conserved residues are known to play a significant role in the function of the protein and their conservation would imply similarity in function.

Cloning of the PCR products into the pCR®II-TOPO® vector was straightforward and we capitalised on this to clone the PCR products of the two genes from five strains (3D7, HB3, DD2, K1 and VI/S, **see table 2.2**) for subsequent sequence analysis. Unlike amino acid sequence, nucleotide sequences tend to accumulate silent changes over time (Nguyen *et al.* 1997) and we suspected because of the strains' unique geography, DHFR /

DHPS genotypes and folate effect characteristics (Wang *et al.* 1997), there may have been intra-strain differences in sequence that have evolved with the functions of MAL8P1.13 and PF11_0172. In particular it would be interesting to test the hypothesis the intra-strain variability in folate utilisation was a function of differences in gene structure (Wang *et al.* 1997) as the underlying molecular mechanism remain unknown (Wang et al. 2004). Studies in *Leishmania* have shown that mutations on the high affinity folate transporter impair the function of the protein and induce an alternative pathway for folate uptake (Kundig et al. 1999). From the sequencing data, the nucleotide sequence of PF11_0172 was the same for all strains but a single change of C to A was noted for the MAL8P1.13 gene of VI/S against the other four strains as shown in **figure 4.2B**. Further studies will be required to investigate the impact of this mutation on the function of the protein once it's fully characterised.

To initiate the functional characterization, we successfully cloned the MAL8P1.13+pCR®II-TOPO® derived insert into the Xenopus 5'-3' β-globin vector and micro-injected the oocytes with the cRNA synthesised from this plasmid. Although PCR-TOPO cloning was straight-forward, sub-cloning the fragments into the *Xenopus* expression vector was a challenging undertaking. However, *Xenopus* oocytes are generally able to process foreign *Plasmodium* RNA and synthesize the corresponding proteins in the correct orientation with the eventual targeting to the plasma membrane (Penny et al. 1998). This property has made the model a useful tool for molecular characterization.

The suspected functional role of MAL8P1.13 was confirmed by the demonstration of enhanced uptake of FA, 5-FoTHF and MTX in cRNA injected oocytes compared to water-injected oocytes. Although an endogenous folate carrier system has been identified in the *Xenopus* oocyte (Lo *et al.* 1991), the results clearly show the level of uptake of the cRNA injected oocytes was consistently and significantly higher than the water-injected oocytes. Folate transporters have been shown to exhibit substrate specificity, it is interesting to observe that MAL8P1.13 cRNA injected oocytes were capable of transporting both the oxidised, FA and reduced, 5-FoTHF folates and the antifolate, MTX. The affinity for 5-FoTHF was demonstrably higher compared to the other substrates, FA and MTX.

Although we noted significant changes in the levels of uptake between cRNA-injected and water injected oocytes, we observed a marginal rise in the uptake of the substrates as a function of time in the water-injected oocytes. These results offer support to the suspected existence of low levels of endogenous folate transport activity. However, the impact of the endogenous system apparently appears to be negligible when the heterologously expressed folate transporter is fully functional as found here and this could be the underlying reason why the system has been extensively applied for folate transport studies as previously mentioned.

We did not assess the possibility of substrate metabolism as a contributing factor to the enhanced levels of uptake. Neither did we investigate the breakdown products although the compounds are known to be labile. Some studies have reported the identification of

the parent 5-MeTHF and FA as the intact metabolic form incorporated into the *Xenopus* oocyte (Lo *et al.* 1991; Said *et al.* 1996). Substrate uptake was time dependent. In addition, uptake of 5-FoTHF acid and FA could be inhibited by their unlabeled structural analogues. Based on these findings, we drew a firm conclusion that the observed transport phenomena are directly linked to the expression of the MAL8P1.13 protein and not the endogenous activity of the model. This work represents the first definitive evidence on the expression and functional role of MAL8P1.13 in the transport of folate.

It is notable that the diversity in folate substrate uptake and the inhibition by folate structural analogues are characteristic behaviours of the recently reported study of folates transport in intact malaria parasites (Wang *et al.* 2007). However, some puzzling features were noted with regard to pH and the predicted mechanism of H^+ symporters for these putative folate transporters (Martin *et al.* 2005). The work reported here was performed at pH 7.4 yet the optimal pH range reported earlier in the biochemical study for folate uptake is 6.5-7.0. However, these workers do not rule out folate transport that is independent of the proton gradient (Wang *et al.* 2007). Characterization of other folate transporters using the *Xenopus* oocyte model has shown differences between the optimal pH for folate transport in the Xenopus oocyte system compared to the native cell (Said *et al.* 1996). One classical example is the intestinal folate carrier. The transporter is known to operate in a tightly regulated pH range with optimal performance at pH 5.5 yet the expression of the transporter in the *Xenopus* oocyte reveals a greater uptake at pH 7.5 (Nguyen *et al.* 1997). It is still not clear what the molecular basis for this discrepancy is

but speculation points towards cell-specific post-translational modification mechanisms (Kumar et al. 1998).

Because of the challenges associated with the xenopus system such as batch to batch variation in the quality of the oocytes, different days of the flux assays, and operational technicality, we did not comprehensively assess the kinetic parameters for example the precise Km and Vmax of this transporter and so we don't know as yet how it compares to other folate transporters that have been functionally characterised. Alternative approaches to explore include the use of derived folate transporter null mutants of other protists or use of yeast to functionally express these *Plasmodium* folate transporters.

We do not know the localization of MAL8P1.13 in the infected erythrocyte but from comparison with recent characterization of folate transport in naked parasites we speculate that it may be located on the parasite plasma membrane. It is also possible that the transporter is exported to the host cell membrane. Malaria parasites are known to induce extensive modifications in the host's erythrocyte membrane. However, folate species although charged are thought to traverse the parasite membrane via endogenous carriers or possibly via the new permeability pathway (Ginsburg et al. 2005). Regardless of its sub-cellular localization, this study provides the first proof that a specific parasite gene (MAL8P1.13) encodes for a protein that functions as a folate transporter. We hypothesise the potential of folate species to abrogate the *in vitro* activities of antifolates as the data in **chapter 3** show could be tied in part to the utilization of this transport system. Therefore, this work is a significant advance in our understanding of folate

transport. Additional studies are required to fully characterise the gene as well as the second one, PF11_0172.

CHAPTER 5

Transcriptional analysis of the putative folate transporter genes

5.1 Introduction

As discussed in **chapter 1**, malaria parasites possess well characterized genes required for the *de novo* biosynthesis of folates. Although the genes are known to be operational, the results in **chapter 4** demonstrated the parasites have also acquired the necessary genetic machinery required for the salvage of folate substrates. This is especially significant considering the abundance of extracellular substrates available to the parasite during the erythrocytic phase of the life cycle. We have also demonstrated that the parasite expresses MAL8P1.13 and PF11_0172 and that these novel genes (preliminary data for PF11_0172,) are essential in facilitating the scavenging of folate molecules from the host. The expression of these gene functions by the parasite is not a surprising phenomenon as parasites are known to induce novel transport systems in order to accomplish pathogenecity and improve survival within their intracellular environment. Of relevance to the focus of this thesis is the view that in addition to the well accepted mechanisms of antifolate resistance based on point mutations in *dhfr* and *dhps* in the parasite, resistance to SDX has been reported to be influenced by an additional factor

linked to the parasite's ability to utilise folic acid (Wang *et al.* 1997). It is our hypothesis that this "additional factor" is linked with the functional expression of folate transporters.

Several lines of evidence indicate that the ability to salvage folate substrates varies from strain to strain. Using HB3 and Dd2 as model strains, Wang et al (1997) showed low levels of extracellular folate to have a profound effect on the antimalarial activity of SDX in Dd2 while HB3 required much higher amounts of folate to effect a similar inhibitory activity. McConkey et al (McConkey et al. 1994) have also reported several lines of *P. falciparum* that grow at normal rates in cultivation without detectable levels of PABA while others require supplemental PABA to grow. The PABA-requiring clones were shown to take up PABA normally. Another group, Milhous et al (Milhous *et al.* 1985) have described an isolate that was incapable of sustained growth in deficient folate medium and dialysed serum. We have observed a similar case with the Vietnamese strain, VI/S and this has been reported elsewhere (Nzila et al. 2005). Under low levels of folate, strains of this nature exhibit decreased growth and viability and increased apoptotic susceptibility. Growth in these isolates is restored only with the transfer to culture in folate supplemented culture medium. In another recent study on characterisation of folate transport in *P. falciparum*, Wang et al (Wang *et al.* 2007) have further shown there was a more rapid uptake of folate into the K1 strain compared to the sensitive FCB strain. An observation they suggest could be linked to the increased efficiency of folate salvage.

As stressed already the differential ability of parasites to salvage folates has been known for some time. However, at present, the exact mechanism underlying this difference is largely unknown and could be explained by postulating the differential expression of the putative folate transporters we have discovered. Other suggested speculations include; differences in the efficiency of reducing the intact folic acid to dihydrofolate or differences in the ability to break-down the intact folic acid and re-use the metabolites, see Wang et al 1997.

A number of biochemical and genetic strategies have been applied in order to tease out the relevant mechanisms to explain folate salvage and how it contributes to the inter-strain variability to survive under depleted folate conditions or respond to low levels of exogenous folate during antifolate drug inhibition. Based on a clear understanding of the *de novo* biosynthesis pathway and the molecular mechanism of antifolate resistance, the main studies addressing this issue have focused on the potential role of *dhps* and *dhfr-ts* (Wang *et al.* 1997; Nirmalan *et al.* 2002; Wang *et al.* 2004; Hyde 2005).

Following the observation of the difference between HB3 and Dd2 in folate utilization, Wang et al (1997) used the progeny of 'Wellems' genetic cross between the two strains (Wellems et al. 1990). They established that certain progeny responded in an analogous fashion to Dd2 while others were unresponsive and in the same way as HB3 for the same low levels of added folate. Analysis of the progeny in relation to the derived *dhps* genotype of the parental strains (HB3-wild type and Dd2-triple mutant) revealed the phenotype did not correlate to the *dhps* genotype while a good correlation was observed

with the *dhfr* genotype (**see table 2.2** for HB3 and Dd2 *dhfr* genotype characteristics). However, an extension of this observation to well characterised laboratory reference strains (See Wang et al 1997) failed to show any evidence for an association between the *dhfr* genotype and the folate utilization phenotype. A follow up study (Nirmalan et al. 2002) on the transcriptional analysis of these folate genes identified two copies of a unique tandemly repeated 256bp sequence in the 5' UTR of the Dd2 *dhfr-ts* while only one was found in HB3. A metabolic analysis of a link between this unique sequence and folate utilization in well characterised laboratory strains again did not correlate (Wang *et al.* 2004).

As mentioned in **chapter 4,** folate transport is a comparatively well studied topic in mammalian cells and *Leishmania.* Studies of these cells have shown examination of individual mRNA expression levels of candidate transporter molecules provide insight into the mechanisms involved in the transport-mediated process of folates. There is existing evidence that transcriptional down-regulation of the folate transporter genes result in reduced uptake of folate substrates and the antifolate drug MTX (Richard et al. 2002; Liu et al. 2006).

As comprensively reviewed in **chapter 1,** monoglutamate folate based compounds are high molecular weight divalent membrane impermeable species. We went further to demonstrate in **chapter 3** and references therein that these substrates mitigate the activities of known antifolates. We also showed substrate response to be highly strain specific. In order for the substrates to exert their effect, the compounds must traverse the

parasite's membrane. The genes discussed in **chapter 4** are so far the only known putative transporters existing in *Plasmodium falciparum.* To try and understand the mechanisms behind the inter-strain variability in folate utilization, we used Real-Time PCR to analyse MAL8P1.13 and PF11_0172 gene expression profiles in reference isolates of known folate utilization status and extended the work to a limited number of newer isolates originating from South-East Asia and Africa. Differences in the expression levels of the putative transporter candidate genes amongst strains may in part reflect the differences in the level of the proteins which play a crucial role in the uptake of the folate substrates. This is based on the premise that mRNA and protein levels are correlated to some degree. We accept that at times this correlation does not hold true as protein expression may be subject to differential stability, inefficient translation or may result in a mere non-functional activity. But in the absence of commercially available antibodies and insufficient funding for custom synthesis, we were forced to assume that the abundance of putative transporter proteins is proportional to the levels of expression of the corresponding mRNA.

5.2 Materials and Methods

5.2.1 Strains

Reference clones (**see table 2.2** for folate effect status and *dhfr* genotype) and culture adapted isolates from Thailand (South-East Asia) and Kenya (Africa) available in our laboratory were used in the study.

5.2.2 Cell culture

Cells were cultured as described in **section 2.12**.

5.2.3 RT-PCR for mRNA expression of MAL81P1.13 and PF11_0172

Total RNA isolation and cDNA synthesis were carried out as previously described in sections **2.14 and 2.15** respectively. The primer and probe sequences are shown in **Table 5.1**. **EF1-alpha** reference gene; EF1_ALPHA-OPT5F / EF1_ALPHA-OPT5R and EF1_ALPHA-OPT5M1-PROBE, **MAL8P1.13**; MAL81P1.13-F/ MAL81P1.13-R and MAL81P1.13-PROBE and **PF11_0172**; PF11_0172-F / PF11_0172-R and PF11_0172-PROBE. The primers and probes were sourced from Applied Biosystems', Cheshire, UK.

All reactions were carried out in duplicate in a total volume of 25 µl using the PTC-200 Peltier Thermal Cycler with Chromo 4 Continuous Fluorescence Detector (Bio-Rad). Each reaction mixture contained 1 X Absolute QPCR mix (ABgene), 250nM of the probe and 900nM of both the forward and the reverse primers. The programme consisted of an initial activation at 95°C for 10 min followed by 45 cycles of denaturation at 92°C for 14 s, annealing/extension at 60°C for 60s, followed by a fluorescence measurement.

5.2.4 Real-Time PCR quantitation of MAL81P1.13 and PF11_0172 copy number

Genomic DNA was isolated as previously described in **section 2.13**, normalised to 20ng/μl and the copy number determined by Real-time PCR using the PTC-200 Peltier Thermal Cycler with Chromo 4 Continuous Fluorescence Detector (Bio-Rad). The primer and probe sequences are shown in **Table 5.1** while that of EF1-alpha used for this copy number assay is available in **Table 6.1**. The primers and probe sequences were sourced from Applied Biosystems', Cheshire, UK. The reaction was carried out essentially as described in **section 5.2.3** except the genomic DNA template (40ng) was used in this case.

Oligo name	Sequence	Dye
EF1_ALPHA-OPT5F	GGC AGA AAG AGA AAG AGG TAT TAC CA	-
EF1_ALPHA-OPT5R	CCT GGT GCA TCA ATG ACA GTA AAG A	-
EF1_ALPHA-OPT5M1	CTT ATG GAA ATT TGA AAC CCC	FAM
MAL81P1.13-F	GAG AAG CTC AGG TGG TAG AAT CTA ATA G	
MAL81P1.13-R	ACA TGA TAG CAA AAC TTA ATT TCC TAA AAG CAA	
MAL81P1.13-PROBE	TCT GCT AGG AAT GTT TCT CTT TT	FAM
PF11_0172-F	AGC GTT TTT AAA TTT ATC AGT TAT ACA AGC AAG TT	
PF11_0172-R	ACT AAA GCT TCT GCC ACA GTT GT	
PF11_0172-PROBE	CAA AAG GAT GCA CAT AAT GAT	FAM

Table 5.1 Primer and probe sequences utilised for mRNA expression study and copy number analysis.

5.2.5 Growth experiment

Unlike the reference clones, the growth characteristics of the Thailand and Kenyan isolates in relation to folate media conditions were unknown. In order to assess this, we performed growth experiment assays for 3 days in culture media conditions without folic acid or with folic acid at 2.3μM. Cells were maintained as described in **section 2.12** and on the day of the assay, the cultures were washed in wash media minus folic acid and the inoculum prepared according to **section 3.2.5** with minor modifications. Cultures mainly at the ring stage were diluted with washed uninfected erythrocytes (**see section 2.1**) to make up a culture suspension of 1% parasitemia and 1% hematocrit. 200 μl aliquots of the culture suspension were plated out in duplicate wells of a 96-well microculture plate. The plate was incubated at 37°C in a gas-tight box and flushed with gas as described in **section 2.8**. After 24, 48 and 72 hr, the culture media was removed and replaced with fresh medium minus folic acid or containing the indicated concentration of folic acid. Thin blood films were prepared as described in **section 2.3** at the specified times. Assessment of parasite growth was evaluated using ^{3}H-Hypoxanthine incorporation. The growth assays were prepared as above and labelling was performed as described in **section 2.11.1** at the following times; 24 hr test labelled from the start of the assay, the 48 hr test labelled after 24 hr and the 72 hr test labelled after 48 hr. The labelled plates were harvested and counted as **sections 2.11.2 and 2.11.3** respectively.

5.3 Results

5.3.1 mRNA gene expression profiles of MAL81P1.13 and PF11_0172

The reference clones were continuously cultured in the standard medium containing 2.3μM folic acid. As previously mentioned, isolates are either poor (HB3 and VI/S) or efficient (K1, 3D7 and Dd2) folate utilizers in relation to the effect of low levels folates on SDX inhibition or survival. We assessed the gene expression profiles in all the five clones under the stated folic acid conditions. Examination of the expression profiles of the two genes, MAL8P1.13 and PF11_0172, **(figure 5.1)** indicated there was differential expression pattern between the clones. The three isolates in which efficient folate salvage has been reported exhibited significantly lower levels of expression of both genes compared to HB3 and VI/S where expression was higher. The p values for VI/S and HB3 in both genes are significant when compared to 3D7, Dd2 and K1 (**see figure 5.1** legend for precise p values).

Following the observation of the differential pattern of mRNA levels, we assessed the changes in the copy number of the genes by performing Real-Time PCR as previously described in section 2.5. We rounded off the copy number values to the nearest integer as shown in figure 5.2 and compared them with the alterations in the mRNA expression levels reported above. The results showed there was a correlation, as between the copy number and the changes in the mRNA expression levels.

MAL8P1.13

PF11_0172

Figure 5.1 mRNA expression pattern for MAL8P1.13 and PF11_0172 in 3D7, HB3, Dd2, VI/S and K1.
PF11_0172: VI/S compared to 3D7/ Dd2 p value < 0.0001, VI/S to K1 p value = 0.0001
HB3 to 3D7 p value = 0.0001, HB3 to Dd2 p value < 0.0001, HB3 to K1 p value = 0.0019
MAL8P1.13: VI/S compared to 3D7/ Dd2 p value < 0.0001, VI/S to K1 p value = 0.0001
HB3 to 3D7/Dd2 p value < 0.0001, HB3 to K1 p value = 0.0478

5.3.2 Determination of MAL81P1.13 and PF11_0172 genes copy number

Following the observation of the differential expression of mRNA levels, we assessed the

changes in the copy number of the genes by performing Real-Time PCR as previously

described in **section 5.2.4**. We rounded off the copy number values to the nearest integer

as shown in **figure 5.2** and compared them with the differences in the mRNA expression

levels reported above. The results showed there was no clear association between the

copy number and the changes in the mRNA levels of the two genes.
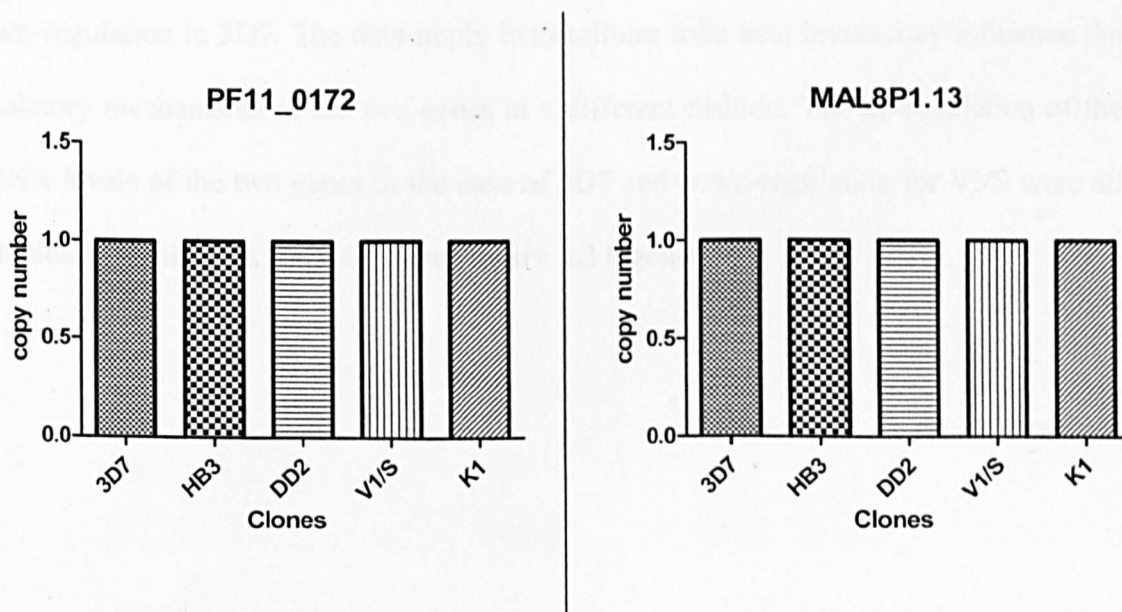
Figure 5.2 MAL81P1.13 and PF11_0172 genes copy number in 3D7, HB3, DD2, VI/S
and K1.

5.3.3 Effect of exogenous folic acid on the mRNA expression level of MAL8P1.13

and PF11_0172 in 3D7 AND VI/S

Since we have previously shown in **chapter 4** that the two genes are linked to folate transport, the differential expression profiles results above led us to investigate whether or not the genes are influenced by extracellular folate concentration. To establish whether the observed differences in the expression pattern were associated with folates recognition, we performed the assays using 3D7 and VI/S clones grown overnight in culture medium with or without folic acid. The results show folate deficiency up-regulated the mRNA expressions of both genes in 3D7. However, in VI/S there was down-regulation of the genes **(figure 5.3)**. Under supplemented folic acid conditions, the reverse was the case with up-regulation of both genes observed in the case of VI/S and down-regulation in 3D7. The data imply extracellular folic acid levels may influence the regulatory mechanisms of the two genes in a different fashion. The up-regulation of the mRNA levels of the two genes in the case of 3D7 and down-regulation for VI/S were all statistically significant, $p < 0.001$, **(see figure 5.3 legend)**.
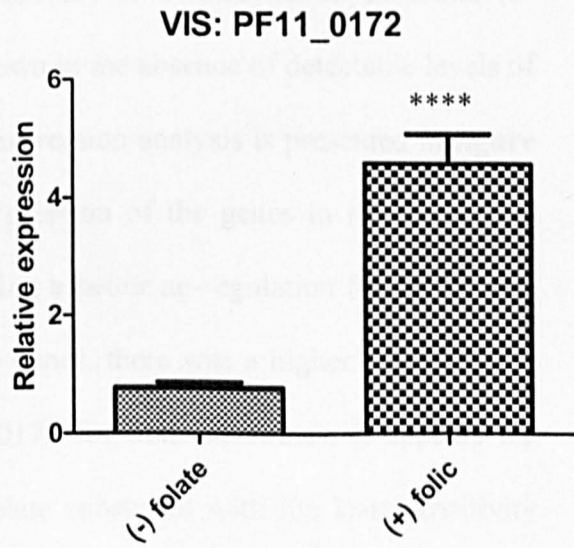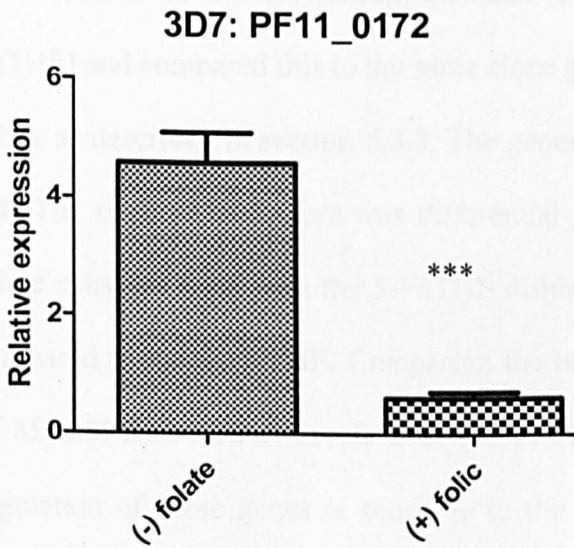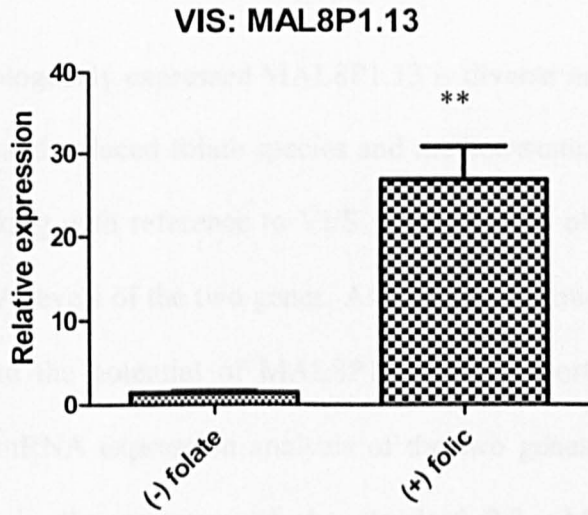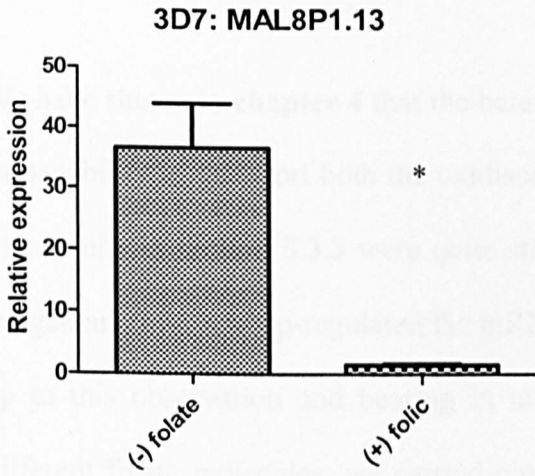
Figure 5.3 Effect of folate levels in culture medium on the mRNA expression of MAL8P1.13 and PF11_0172 in 3D7 AND VI/S
P values; * = 0.0013, ** = 0.0003, *** < 0.0001 and **** < 0.0001

5.3.4 Effect of exogenous 5-Methyltetrahydrofolate and 5-formyltetrahydrofolate on the mRNA expression level of MAL8P1.13 and PF11_0172 in VI/S

We have shown in **chapter 4** that the heterologously expressed MAL8P1.13 is diverse in its capability to transport both the oxidised and reduced folate species and methotrexate. The results in **section 5.3.3** were quite striking with reference to VI/S. The presence of exogenous folic acid up-regulated the mRNA levels of the two genes. As a logical follow up to this observation and bearing in mind the potential of MAL8P1.13 to transport different folate molecules, we carried out mRNA expression analysis of the two genes using the VI/S clone cultured overnight in the presence of the standard 2.3 μM concentration of 5-Methyltetrahydrofolate (5-MeTHF) or 5-formyltetrahydrofolate (5-FoTHF) and compared this to the same clone grown in the absence of detectable levels of folate as described in **section 5.3.3.** The genes expression analysis is presented in **figure 5.4.** The results show there was differential expression of the genes in relation to the folate substrate used with the 5-FoTHF displaying a better up-regulation for both genes compared to the 5-MeTHF. Comparing the two genes, there was a higher up-regulation of MAL8P1.13 mRNA levels than the PF11_0172 for both substrates. It appears the regulation of these genes is sensitive to the folate substrates with the least sensitivity observed for 5-MeTHF especially for PF11_0172. The p value for MAL8P1.13 for both substrates was statistically significant, p = 0.0043 and 0.0190 for 5-MeTHF and 5-FoTHF respectively, **(see figure 5.4 legend)**

We have focused on the effect of folic acid as a folinate substrate that has been extensively studied and also as the only folate metabolite present in standard media. To determine the potential effects of folic acid substrates, folate dependent parasite invasion of isolates were grown in folate depleted media and supplemented with folate and also compared to fresh media that contained the standard folate metabolites (figure 5.5). We observed no significant effects of folate metabolites, with parasites however, at 72 hours, growth was suppressed when folic acid was removed.

Figure 5.4 Effect of 5-MethylTetrahydrofolate (5-MeTHF) and 5-formyltetrahydrofolate (5-FoTHF) on the mRNA expression levels of MAL8P1.13 and PF11_0172 in VI/S
P values; * = 0.0043, ** = 0.3667, *** = 0.0776 and **** = 0.0190

5.3.5 Effects of folic acid on growth of South-East Asian and African isolates

We have focused on the effect of folic acid as the folate substrate that has been extensively studied and also as the main folate molecule present in standard medium. To determine its potential effects on growth and survival of the S. E. Asian and African isolates, the isolates were grown in culture media as described in **section 2.12**, washed and then resuspended in fresh medium with and without folic acid. The data presented in figure 5.5 demonstrate a minimal effect of 24 or 48hr growth with or without folate. However, at 72 hr growth was suppressed when folic acid was removed.
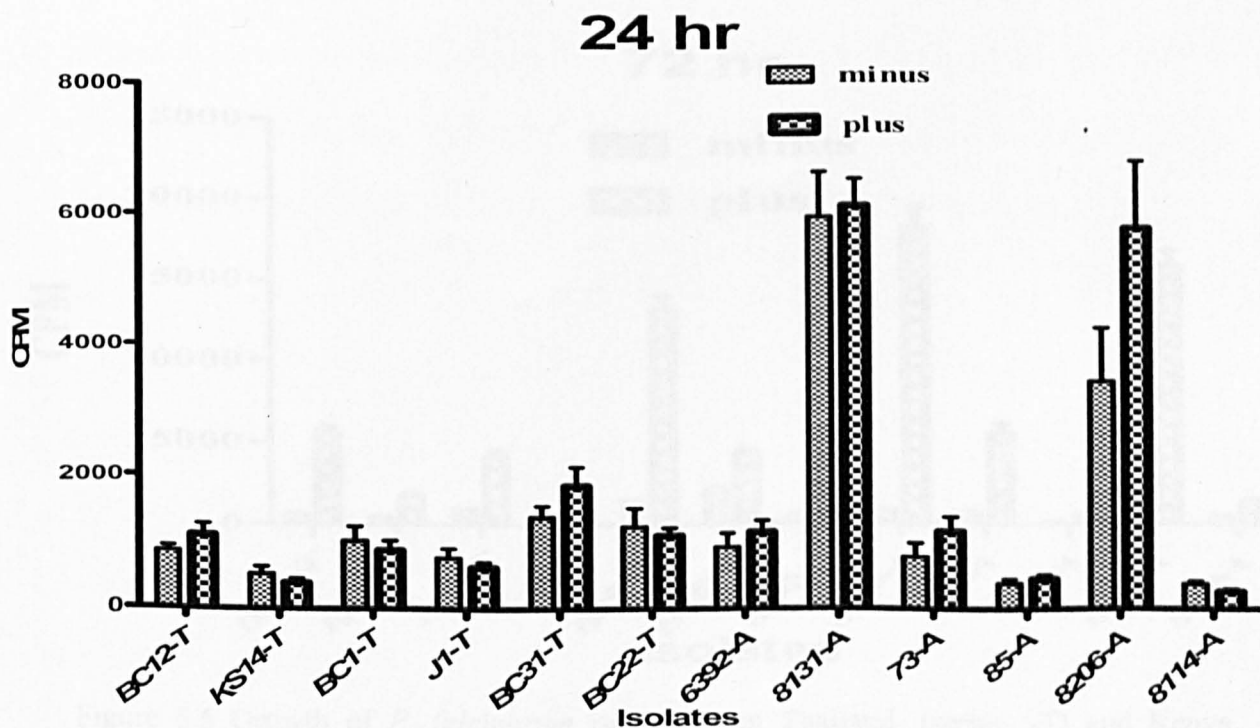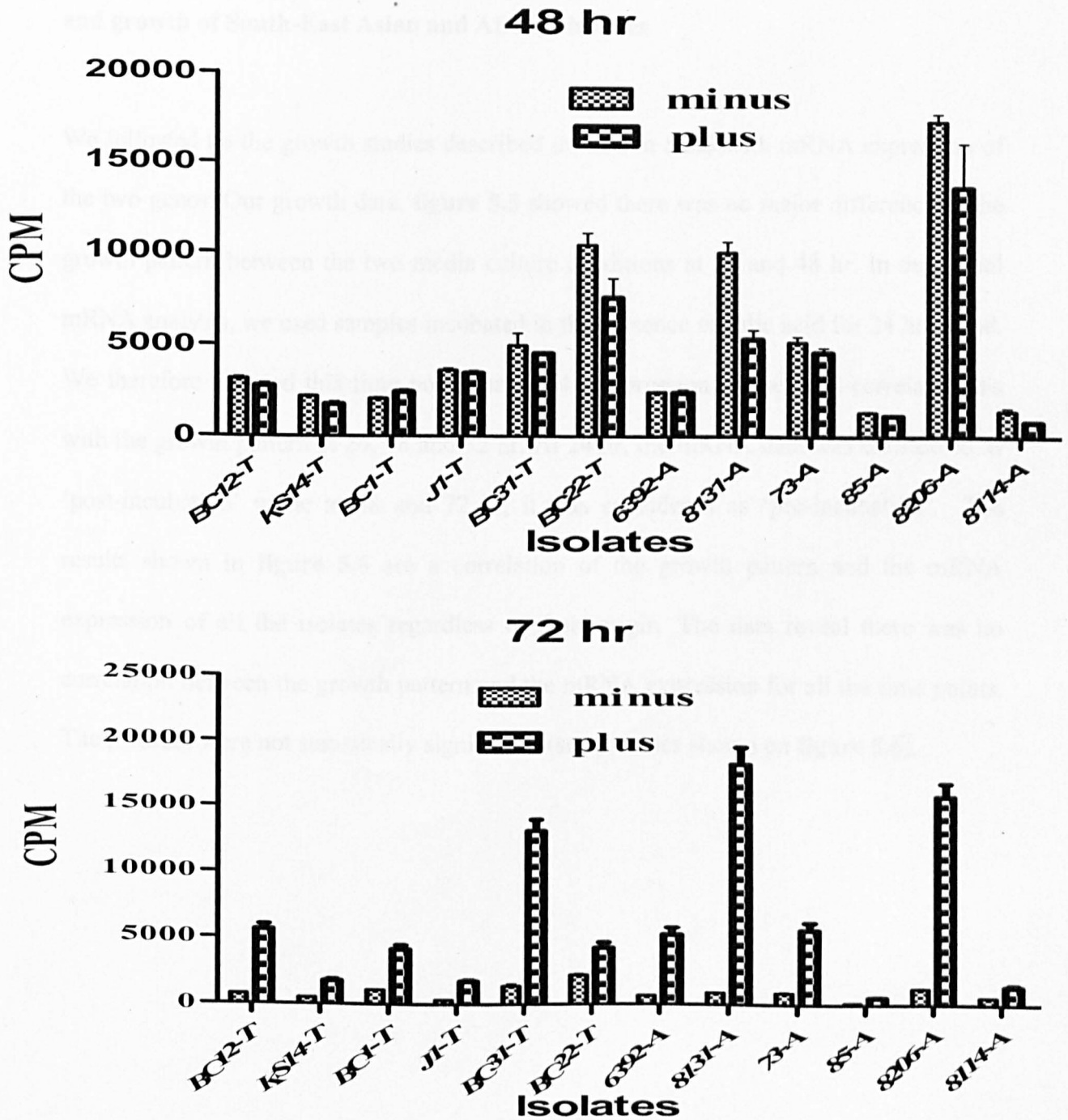
Figure 5.5 Growth of *P. falciparum* isolates from Thailand, (series, -T) and Kenya, (series, -A) at 24, 48 and 72 hr interval in minus/plus folic acid culture medium containing 0.0045mCi of ^{3}H-Hypoxanthine

5.3.6 Relationship between MAL8P1.13 and PF11_0172 mRNA genes expression and growth of South-East Asian and African isolates

We followed up the growth studies described in **section 5.3.5** with mRNA expression of the two genes. Our growth data, **figure 5.5** showed there was no major difference in the growth pattern between the two media culture conditions at 24 and 48 hr. In our initial mRNA analysis, we used samples incubated in the presence of folic acid for 24 hr period. We therefore adopted this time point for mRNA expression studies and correlated this with the growth pattern at 24, 48 and 72 hr. At 24 hr, the mRNA data was considered as 'post-incubation' while at 48 and 72 hr, it was considered as 'pre-incubation'. The results shown in **figure 5.6** are a correlation of the growth pattern and the mRNA expression of all the isolates regardless of their origin. The data reveal there was no correlation between the growth pattern and the mRNA expression for all the time points. The p values were not statistically significant, (see p values shown on **figure 5.6**).
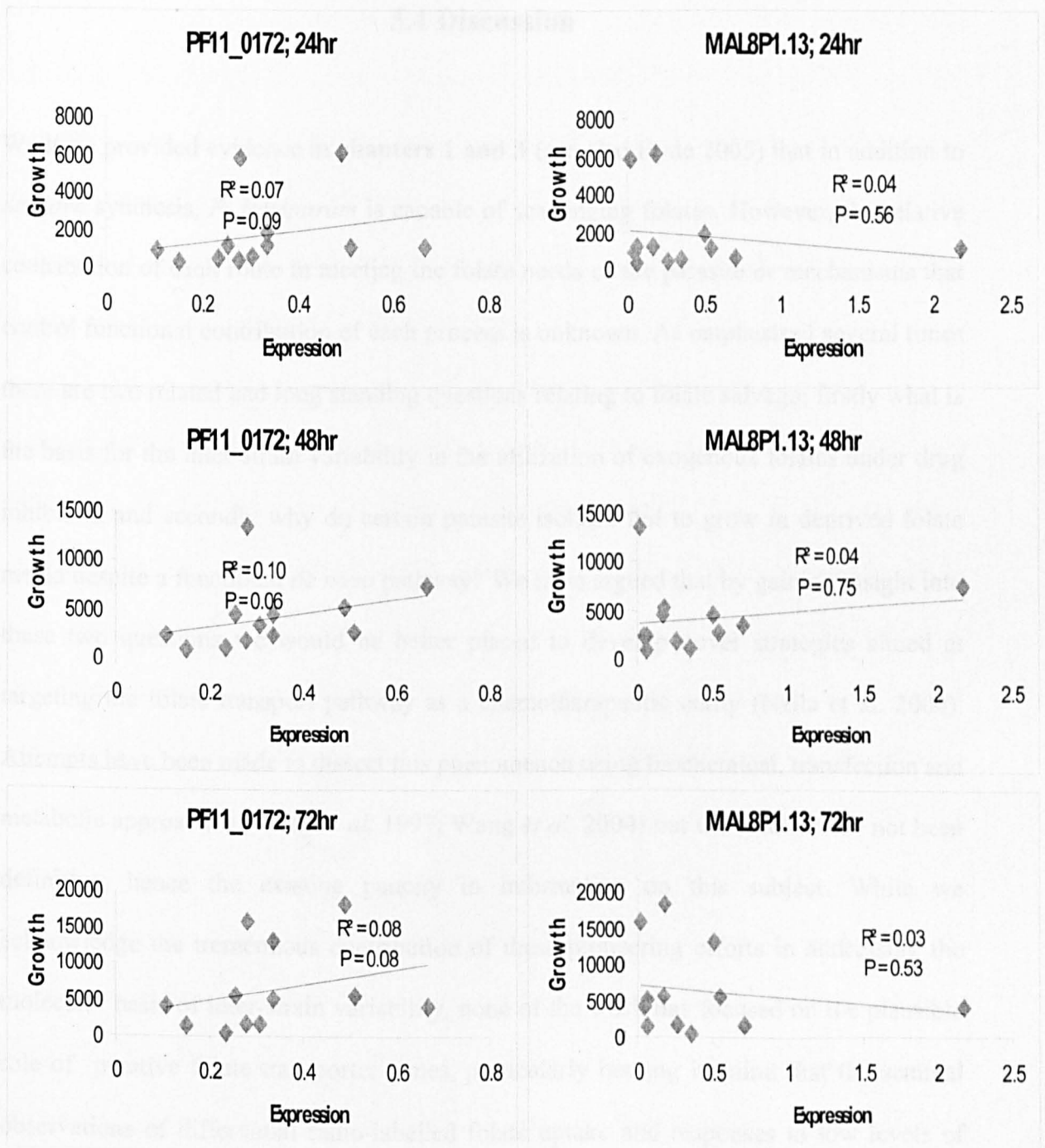
Figure 5.6 Correlation between growth at 24, 48 and 72 hr and the mRNA genes expression of PF11_0172 and MAL8P1.13.

5.4 Discussion

We have provided evidence in **chapters 1 and 3** (see also Hyde 2005) that in addition to *de novo* synthesis, *P. falciparum* is capable of scavenging folates. However, the relative contribution of each route in meeting the folate needs of the parasite or mechanisms that control functional contribution of each process is unknown. As emphasised several times there are two related and long standing questions relating to folate salvage; firstly what is the basis for the inter-strain variability in the utilization of exogenous folates under drug inhibition and secondly why do certain parasite isolates fail to grow in deprived folate media despite a functional *de novo* pathway? We have argued that by gaining insight into these two questions we would be better placed to develop novel strategies aimed at targeting the folate transport pathway as a chemotherapeutic entity (Nzila et al. 2004). Attempts have been made to dissect this phenomenon using biochemical, transfection and metabolic approaches (Wang *et al.* 1997; Wang *et al.* 2004) but the results have not been definitive, hence the existing paucity in information on this subject. While we acknowledge the tremendous contribution of these pioneering efforts in addressing the molecular basis of inter-strain variability, none of the work has focused on the plausible role of putative folate transporter genes, particularly bearing in mind that the seminal observations of differential radio-labelled folate uptake and responses to low levels of folate during antifolate inhibition (Wang *et al.* 1997; Wang *et al.* 2007) have been linked to differences in the capability of the isolates to transport folates.

The identification of the putative folate transporter genes and their corresponding functional link to folate transport as shown in **chapter 4** offers a new dimension to our understanding of the interaction between gene expression and exogenous signals such as folate substrates. With this information, it is now possible to assess the role of the expression of the putative transporter genes, MAL8P1.13 and PF11_0172 and relate them to the inter-strain variability in folate utilization.

Using RT-PCR, we analysed the mRNA expression profiles of MAL8P1.13 and PF11_0172 in five laboratory reference strains **(figure 5.1)** from diverse geographic regions with well characterised DHFR genotype, drug sensitivity profiles and folate effect status **(see table 2.2)**. Our primary objective here was to identify the dynamics in the expression profiles amongst the strains and relate it to the folate effect in a series of parasites, K1, HB3, Dd2 and 3D7 that have previously been extensively studied (Wang *et al.* 1997).

We deliberately included the VI/S strain. Although not extensively studied like its counterpart strains in terms of the strict definition of folate effect as defined by Wang et al (1997), this clone has some remarkable properties related to antifolate resistance and survival in folate-based medium conditions and may thus provide quite illuminating information on the inter-strain variability. Unlike the other strains, VI/S cultured in medium with minimal folate is not viable and exhibits significant growth retardation (Nzila *et al.* 2005), it possesses the 164 mutant allele on the *dhfr* gene and hence it is a true representative isolate of the S. E. Asian region where this mutation is well

established (Ochong et al. 2008). Yeast studies have also shown the DHFR enzyme possessing this change (dhfr quadruple mutant) are enzymatically compromised (Sibley et al. 1997).

It is apparent from the RT-PCR analysis in **figure 5.1** that VI/S and HB3 demonstrate statistically significant higher expression of the MAL8P1.13 and PF11_0172. This observation was quite striking considering these two clones HB3 and VI/S have been linked to a particular unique folate phenotype status as poor response to low levels of folate during SDX inhibition and poor survival in low folate levels respectively. We have ascribed the two genes to functions related to folate transport **(chapter 4)** and therefore the inter-strain differential expression pattern noted might be linked to specific inter-strain variations associated with the functions of folate transport activities. This particular work was done under folate supplemented conditions. We think here that at high levels of extracellular folate concentrations, there is an adaptive up-regulation of the putative folate transporters in these two isolates with a pronounced activity in the VI/S strain. We suspect that this phenomenon probably helps the strain to scavenge for folates from the environment and hence its ability to grow well in folate supplemented conditions compared to under depleted folate levels.

Malaria parasites are known to modulate the levels of certain transporter genes by altering the copy number. For example mefloquine and halofantrine sensitivity have been linked to changes in the copy number of the *Pfmdr-1* gene (Wilson et al. 1993; Price et al. 2004). We assessed whether the changes in the mRNA expression profiles of

MAL8P1.13 and PF11_0172 between the strains was associated with copy number changes, **figure 5.2**. Rounding off the values into integers, our data showed there was no correlation between mRNA expression levels and copy number. This implies the changes in the mRNA expression are under a different mechanism.

Prior to the identification of the putative folate transporters, there were suggestions that under folate-rich medium, folates would enter cells independently of the transporter and that if the transporters were present for the salvage pathway, they would be functionally important under folate-poor conditions or during antifolate drug inhibition (Hyde 2005). Human studies on folate transport indicate that the levels of expression of folate transporter genes are influenced by extracellular folate concentration whereby the expression of the transporter levels increase in low levels folate compared to the folate-rich conditions (Jhaveri et al. 2001). Applying this approach to the mRNA expression analysis in VI/S and 3D7 cells previously treated overnight with or without folic acid shows conflicting results, **figure 5.3**. The up-regulation of the mRNA levels of the two putative folate transporter genes in the case of 3D7 is consistent with the expected compensatory cellular responses to folate deficiency and this phenomenon has been observed with other folate transporters genes in human cells and *Leishmania* (Richard *et al.* 2002; Novakovic *et al.* 2006). The down-regulatory effect of folate deficiency on the two genes in the case of VI/S is a surprising finding and is of great interest because it would suggest a new regulatory mechanism that warrants further investigation. Apparently this VI/S observation was also noted when other folate structural analogues, 5-MeTHF and 5-FoTHF were tested, **figure 5.4** with an exception of the PF11_0172

where the mRNA expression levels was not statistically significant for both 5-MeTHF and 5-FoTHF, p values = 0.3667 and 0.0776 respectively. This is the first study in *Plasmodium* to have reported an association between mRNA expression levels of putative folate transporter genes and extracellular folate signals.

We also extended this work to a limited number of isolates from S. E.-Asia and Africa. We have previously shown that the *dhfr* 164 mutant allele is rampant in South-East Asia (Ochong *et al.* 2008) and hypothesised that isolates originating from here have devised a compensatory mechanism to withstand the selection of the 164 mutant allele which has been associated with a compromised DHFR enzymatic function in the case of VI/S. We started off by establishing the growth pattern of the isolates in medium with or without folic for 3 days and monitored the growth at 24, 48 and 72 hr using ^{3}H-Hypoxanthine incorporation, **figure 5.5**. All our isolates showed equivalent growth in media with and without folic acid at 24 and 48 hr. However, growth was only viable at 72 hr in isolates grown in media with folic acid. Because the growth at 24 hr is not different between the media conditions and we have previously used this time point for the analysis of mRNA expression in the reference strains, we therefore compared these isolates mRNA expression of MAL8P1.13 and PF11_0172 in relation to the parasite growth in medium with folic acid at 24, 48 and 72 hr. The results, **figure 5.6** showed no correlation between the mRNA expression and the growth pattern since all the isolates examined had better growth. It is important that this experiment is repeated with large number of samples and also after 72h folate deprivation.

Using the results of 3D7 and VI/S as a platform, we propose a model to explain the down-regulation of the mRNA levels of the two putative transporter genes. The inability for VI/S to grow in folate-deprived conditions suggests it cannot meet its folate needs through synthesis and therefore has to maximise folate uptake from its environment. In this regard, the apparent low levels of expression in VI/S cultured in a low folate media may be a function of the parasite's susceptibility to apoptosis or parasite death. On the other hand, 3D7 is capable of its own synthesis of folate and therefore we propose that to prevent toxic levels of folate in a folate-rich environment, the strain minimises folate uptake through transrepression of the transporters. Based on knowledge from mammalian systems in the same way it was applied in the DHFR and its cognate mRNA interaction (Zhong and Prathod 2002), there are a number of potential ways in which this could occur. These are summarised in **Figure 5.7**.
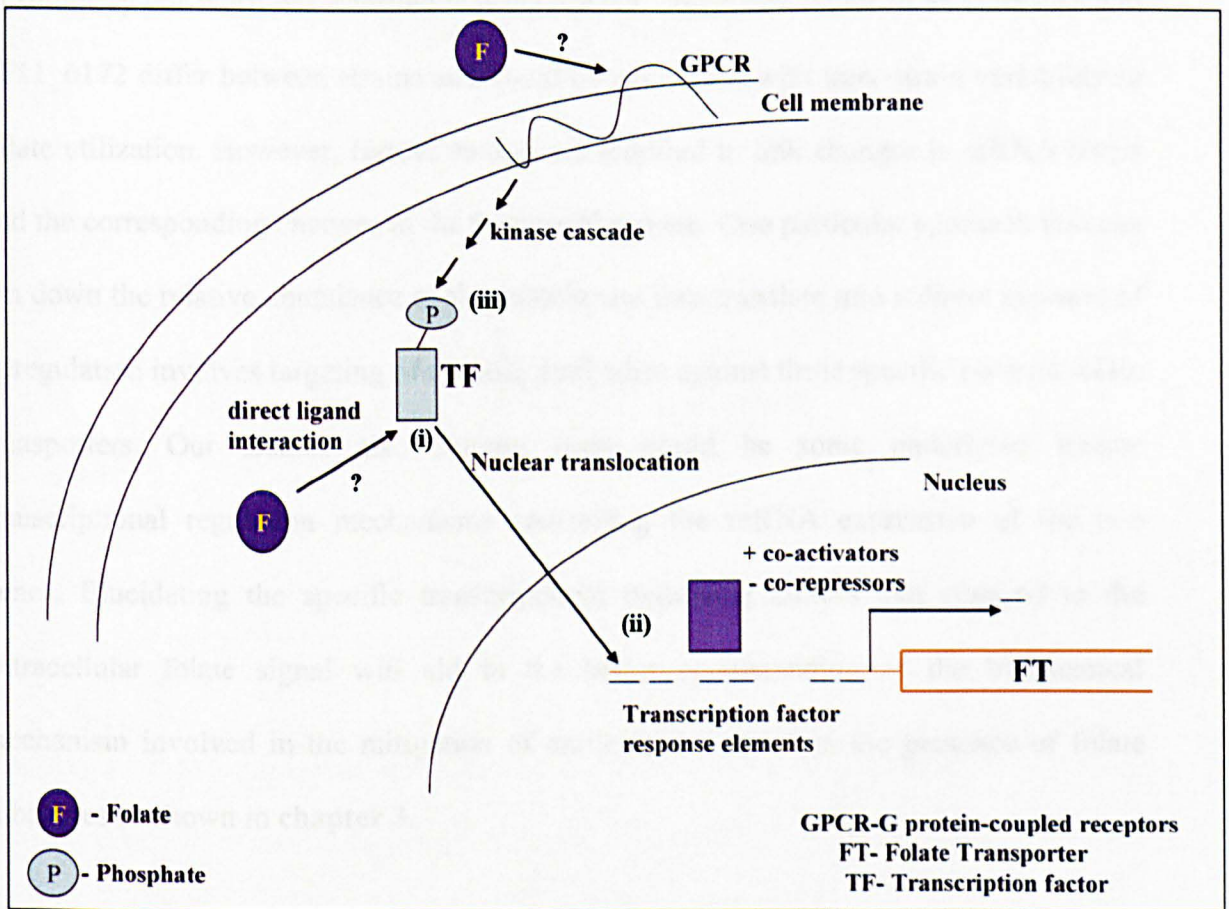
Figure 5.7 Transcriptional regulation of the putative folate transporter genes.

(i)   The transcription factor interacts with the negative response elements (co-repressors)

(ii)   Transcription factor is constitutively active but ligand (folate) binding results in a switch from co-activator to co-repressor usage.

(iii)   Phosphorylation decreases activity of the transcription factor.

Another possible route through which modulation of the transporter expression can occur is by the dynamics of the metabolic flux. As discussed in **chapter 1,** the relative catalytic efficiency of the DHFR enzyme can be compromised due to point mutations. Under these circumstances, the delivery of the substrates at the required target site can be severely hampered and an organism may switch to an alternative route by upregulating it.

In summary our work has revealed that the mRNA expression levels of MAL8P1.13 and PF11_0172 differ between strains and could be associated with inter-strain variability in folate utilization. However, further studies are required to link changes in mRNA levels and the corresponding changes in the functional protein. One particular approach that can pin down the relative abundance of the protein and thus translate into a direct measure of upregulation involves targeting of specific antibodies against these specific parasite folate transporters. Our studies also suggest there could be some underlying unique transcriptional regulation mechanisms controlling the mRNA expression of the two genes. Elucidating the specific transcriptional regulating factors that respond to the extracellular folate signal will aid in the better understanding of the biochemical mechanism involved in the mitigation of antifolate activities in the presence of folate substrates as shown in **chapter 3**.

CHAPTER 6

Validation of a Real-Time PCR method for the detection of the mutant DHFR 164 allele

6.1 Introduction

The focus of this thesis has been on folate transporters as mechanisms of modifying parasite response to antifolate drugs. However, it is well known that mutations in target enzymes are the key resistance mechanisms to antifolates operational in *P. falciparum*.

We have discussed the enzyme kinetics of DHFR in **chapter 1** and the observation that mutant forms especially the quadruple one, have a comparatively reduced catalytic efficiency would lend support to the possibility of compensatory mutations. Following molecular characterization of the folate transporter, **chapter 4** and subsequent mRNA expression analysis of laboratory strains, **chapter 5**, our special interest moved to the verification of the observed mRNA differences in lab strains to field samples of South East Asia and Africa, also part of **chapter 5**. A well known significant difference in the two sites is the dhfr 164L.

As pointed out in **chapter 1**, the antifolate combination PYR-SDX was deployed in response to chloroquine resistance. However, the greatest handicap to its use has been the rapid evolution of drug-resistant parasites. It is now an accepted fact that PYR-SDX resistance is mediated by the sequential acquisition of point mutations in the genes *Pfdhfr* and *Pfdhps* (Hyde 1990; Plowe et al. 1998). The nature, relevance and contribution of each point mutation have been discussed extensively in **section 1.7.1.** Of critical concern to this work, is the fourth point mutation in *Pfdhfr*, I164L. This mutation is found extensively in South-East Asia and South America but there is debate over its existence in Africa (Masimirembwa *et al.* 1999; Biswas *et al.* 2000; Vasconcelos *et al.* 2000; Krudsood *et al.* 2005; Nzila *et al.* 2005). The presence of the I164L quadruple mutant confers high-level resistance to PYR and this mutation would severely compromise the continued use of PYR-SDX in Africa as in any clinical setting. This mutation would also have compromised the use of Lapdap®, a combination of chlorproguanil (CPG) and dapsone (DDS) (Wilairatana et al. 1997), and CPG-DDS-artesunate in Africa although development and use of these compounds have now been halted due to toxicity in children with glucose-6-phosphate dehydrogenase deficiency.

Many studies have looked for the presence of the I164L mutant allele in Africa; Using conventional polymerase chain reaction (PCR), most studies have not detected this mutation (Plowe et al. 1995; Parzy et al. 1997; Plowe et al. 1997; Nzila et al. 2000; Mutabingwa et al. 2001; Anderson et al. 2003; Ochong et al. 2003; Bates et al. 2004; Tahar et al. 2006; Djaman et al. 2007; Schonfeld et al. 2007) but it has been reported at low prevalences in 5 different African countries.

Of particular interest is the study by Alker et al (Alker et al. 2005) who used real-time PCR with fluorescent probes specific for the mutation and described a 4.7% prevalence in parasites collected from HIV positive pregnant women in Malawi between 2001 and 2003 (it is notable that Malawi was the first African country to switch from chloroquine to PYR-SDX in 1993). A finding that they validated more recently using a heteroduplex tracking assay (Juliano et al. 2008). However, on biological grounds, if the quadruple mutant alleles were present in reasonable proportions, it is hard to imagine that they would not have been selected to high levels by this time due to extensive reliance on PYR-SDX as first-line treatment in this country for over a decade. This is the highest prevalence of the 164 mutant reported to date and it is a high priority for Public Health reasons that this finding be further evaluated.

The aims of this study are to validate the sensitivity, specificity and reproducibility of the assay reported by Alker et al (Alker *et al.* 2005) and to confirm or refute the presence of the mutant 164 allele in parasites collected from the same location in Malawi. We also tested the hypothesis that sustained antifolate use would have resulted in an increased prevalence in the I164L mutant over a sustained period. Finally, we wanted to determine if treatment failure after treatment with PYR-SDX results in the selection of this mutation. The prevalence of the I164L allele was also investigated in clinical isolates from Zambia, a neighbouring country with a shorter history of PYR-SDX deployment, and in clinical isolates from the Thailand-Myanmar border in South-East Asia, an area

known to have a high prevalence of the I164L mutation (Biswas *et al.* 2000; Khim *et al.* 2005).

6.2 Materials and Methods

6.2.1 Samples

6.2.1.1 Reference strains:

Reference isolates (**see table 2.2**). The genotype of each isolate was determined by PCR-Restriction fragment length polymorphism (RFLP) and real time PCR. Phenotype was characterised by the IC_{50} to PYR as previously described in (Mberu et al. 2000) and in **chapter 3**. A further confirmation was performed by DNA sequencing the region of *Pfdhfr* encompassing the I164L allele.

6.2.1.2 Field isolates:

Field isolates were obtained from studies conducted in Malawi between 2003-2005 (n=210), Zambia in 2005 (n=55) and the Thailand-Myanmar border in 2005 (n=50). The Malawian samples were from children aged less than 5 years presenting with uncomplicated malaria. The clinical details of the Malawian study, which includes *Pfdhfr* 164 genotyping using a less sensitive methodology (PCR and allele-specific restriction analysis) are published elsewhere (Bell et al. 2008). However, we need to point out that

the pre-treatment prevalence of *Pfdhfr* triple mutant parasites was 96%, compared to a prevalence of 80% in the parasites described by Alker et al. The Zambian samples were collected from adults with uncomplicated malaria before treatment and the clinical details of the study have been published previously (Mulenga et al. 2006; Van Geertruyden et al. 2006). The Thai samples were from adults with falciparum malaria, before treatment.

Whole blood from the patients in these studies was spotted onto Whatman 3MM filter paper, air dried at room temperature and stored in individual plastic bags with desiccant. In addition, for some children in the Malawi study, venous blood was collected in EDTA tubes and stored at −80°C. All the studies contributing samples to this work were conducted under clinical protocols approved by the corresponding institutional review boards.

6.2.2 Isolation and Extraction of total DNA

Total genomic DNA (host and parasite) was extracted from either EDTA treated whole blood or from blood spotted filter papers with the QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions.

6.2.3 Whole genome amplification

A high degree of variability in parasitemia and a low recovery rate of parasite genomic DNA from filter paper was observed. Therefore, when total parasite DNA concentration

was below 10000 copies per µl whole genome amplification by improved primer extension PCR (IPEP) was conducted. This was performed as previously described (Dietmaier et al. 1999) in order to increase the quantity of DNA and maximise the number of experiments that could be conducted on each sample.

6.2.4 Real Time PCR based discrimination of *Pfdhfr* I164L alleles by the method of Alker

In order to assess the sensitivity of the assay, real-time PCR was conducted using the methodology of Alker et al (Alker *et al.* 2005) using a PTC-200 Peltier Thermal Cycler with Chromo 4 Continuous Fluorescence Detector (Bio-Rad). This was conducted on reference strains as well as samples obtained from Malawi which had not undergone whole genome amplification. Primer and probe sequences are identical to those published previously (Alker *et al.* 2005), **see also Table 6.1** and were sourced from Applied Biosystems', Cheshire, UK.

6.2.5 Normalisation of *Plasmodium* DNA by quantification of *EF1-α*

Owing to the high degree of variability in parasite DNA content, it was necessary to normalise the samples according to parasite DNA copy number. This was achieved by quantification of elongation factor 1 alpha (*EF1-α*) as a marker for parasite genome copy number. Primers and fluorescent probes specific for the *EF1-α* gene were designed so as to avoid introns (**Table 6.1**).

In order to construct a standard curve, DNA from five reference strains (K1, Dd2, HB3, 3D7 and Vl/S) were normalized to 20ng/µl in a 25 µl final reaction volume with 1 X Absolute QPCR Mix (ABgene, Surrey, UK), 0.9 µM of *EF1-α* forward and reverse primers and 0.25 µM of *EF1-α* probe. The samples were amplified in a PTC-200 Peltier Thermal Cycler with Chromo 4 Continuous Fluorescence Detector with an initial activation at 95°C for 10 min followed by 45 cycles of denaturation at 92°C for 14 s and annealing/extension at 60°C for 60s.

Amplicons were checked for the presence of non-specific products by electrophoresis on a 1% agarose gel. Each amplicon was gel purified using the Promega Gel wizard prep kit according to the manufacturer's instructions. The copy number of amplicons was then quantified as described previously (Owen et al. 2005) and diluted to 1, 10, 100, 1000, 10000 and 100000 copies per µl. *EF1-α* was then quantified alongside clinical samples as described above. At the end of amplification, the cycle threshold (Ct) was determined for each sample and standard. The data were plotted and the equation of linear regression used to determine the number of copies of parasite DNA in the clinical samples. Parasite DNA was then normalised to 100, 250, 500, 1000 and 10,000 copies per µl for validation of the assay.

Oligo name	Sequence	Dye
EF1-α forward primer	AAA ACA CCA TGG TAT AAA GGA AGA ACC TT	-
EF1-α reverse primer	AGT GGA ATT CTT AAT GGC TTG TCG TAA	-
EF1-α probe	TTT GGT GGT TGC ATA GTA TC	FAM
Pfdhfr forward primer	ATC ATT AAC AAA GTT GAA GAT CTA ATA GTT TTA C	-
Pfdhfr reverse primer	TCG CTA ACA GAA ATA ATT TGA TAC TCA T	-
Pfdhfr wild-type probe	ATG TTT TAT TAT AGG AGG TTC CGT T	FAM
Pfdhfr mutant probe	ATG TTT TAT TTT AGG AGG TTC CGT T	VIC

Table 6.1 All primer and probe sequences utilised in this study.

6.2. 6 Validation of real time PCR based discrimination of *Pfdhfr* I164L alleles

For quantification of the *Pfdhfr* I164L, mutant (V1/S) and wild-type (K1) genomes were combined in fixed ratios of 100:0, 99:1, 95:5, 90:10, 75:25, 50:50, 25:75 and 0:100. These standards were then amplified and the Ct values for the mutant probe divided by the corresponding Ct values for the wild-type probe. These values were then plotted against log transformed % values as a standard curve.

All reactions were carried out in duplicate in a total volume of 25 μl. Each reaction mixture contained 1 X Absolute QPCR mix (ABgene), 200nM of each probe, 288nM of the forward primer and 490nM of the reverse primer (table 1). The programme consisted

of an initial activation at 95°C for 10 min followed by 45 cycles of denaturation at 92°C for 14 s and annealing/extension at 60°C for 60s.

Initial experiments were conducted with 100, 250, 500, 1000 and 10000 copies per µl of parasite DNA in order to determine the optimum concentration. Intra and inter-run precision of the analyses was calculated as [100 – ((Standard deviation / mean) x 100)]% and the intra and inter-run precision was calculated as [(Calculated log-copy number)/(nominal log-copy number added)] x 100%. Determinations were performed using the same amplicon stock solutions. Intra-run precision and accuracy were assessed on 6 replicates of standards containing 10% and 75% mutant genome. Similarly, inter-run precision and accuracy were assessed on 6 separate runs of standards containing 10% and 75% mutant genome. The limit of detection was defined as the percentage at which the mutant allele could reliably be differentiated from the wild-type allele and the limit of quantification was defined as having both intra- and inter-run accuracy and precision between 90 and 110%.

For quantification of the percentage of an individuals total parasite population containing the mutant allele, normalised DNA were amplified as described above. A standard curve was co-amplified on each plate and both standards and samples were assessed at least in duplicate.

6.3 Results

6.3.1 Limitations of the original methodology

When transferring the methodology of Alker et al (2005) to our laboratory, a number of problems were encountered. Firstly, we observed large inter-sample variability in amplification **(Figure 6.1A)**. Secondly, the wild-type probe was found to bind non-specifically to the mutant sequence **(Figure 6.1B)** and vice versa **(Figure 6.1C)**. This observation could lead to isolates being interpreted as mixed alleles, particularly in high transmission areas such as Africa where most infections are polyclonal. The combination of inter-sample variability and sub-optimal probe specificity led us to conclude that samples with a high parasite DNA concentration may be classified as mutant, even if they were actually wild-type. A strategy was therefore developed to normalise the starting parasite DNA concentrations (irrespective of total DNA) and subsequently validate a method for detection of the *Pfdhfr* I164L mutant by capitalising on higher specificity of mutant probe for mutant sequence and wild-type probe for wild-type sequence. In order to achieve this, an optimised standard curve was included within every run.
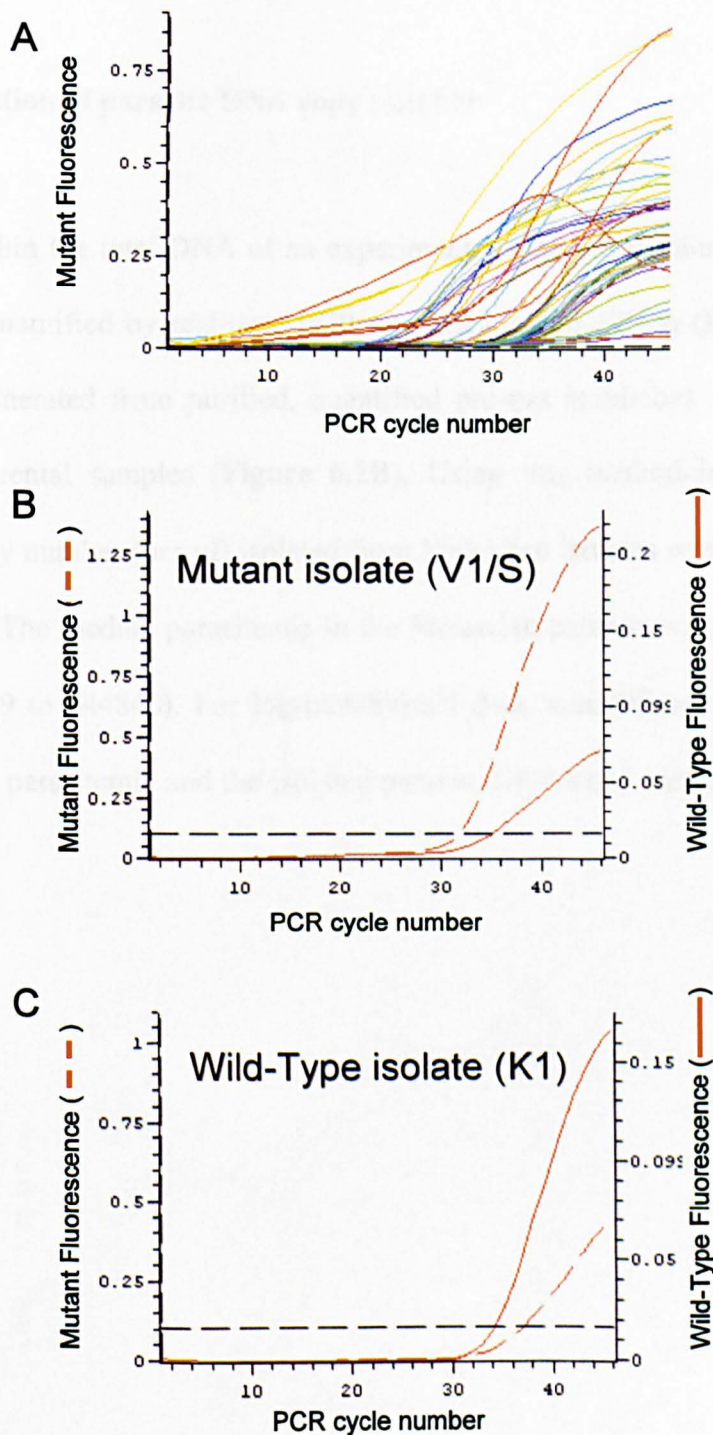
Figure 6.1 Problems experienced with real-time PCR prior to validation. (A) Real-time PCR trace for the mutant allele with Malawian isolates illustrating the high degree of inter-sample variability. (B) Real-time PCR trace for wild-type and mutant alleles in an isolate known to be entirely mutant for this mutation (V1S), illustrating lack of specificity of the wild-type probe. (C) Real-time PCR trace for wild-type and mutant alleles in an isolate known to be entirely wild-type for this mutation (K1), illustrating lack of specificity of the mutant probe.

6.3.2 Standardisation of parasite DNA copy number

Parasite DNA within the total DNA of an experimental sample (containing parasite and host DNA) was quantified by real-time PCR amplification of *EF1-α* (**Figure 6.2A**). A standard curve generated from purified, quantified pre-run amplicons was constructed alongside experimental samples (**Figure 6.2B**). Using this methodology the median parasite DNA copy number (per μl) isolated from Malawian isolates was 27776 (range = 4 to 948664062). The median parasitemia in the Malawian patients was 66585 parasites per μl (range = 39 to 644840). For log-transformed data, a significant correlation was observed between parasitemia and the isolated parasite DNA copy number (R^2 = 0.13; p < 0.0001).
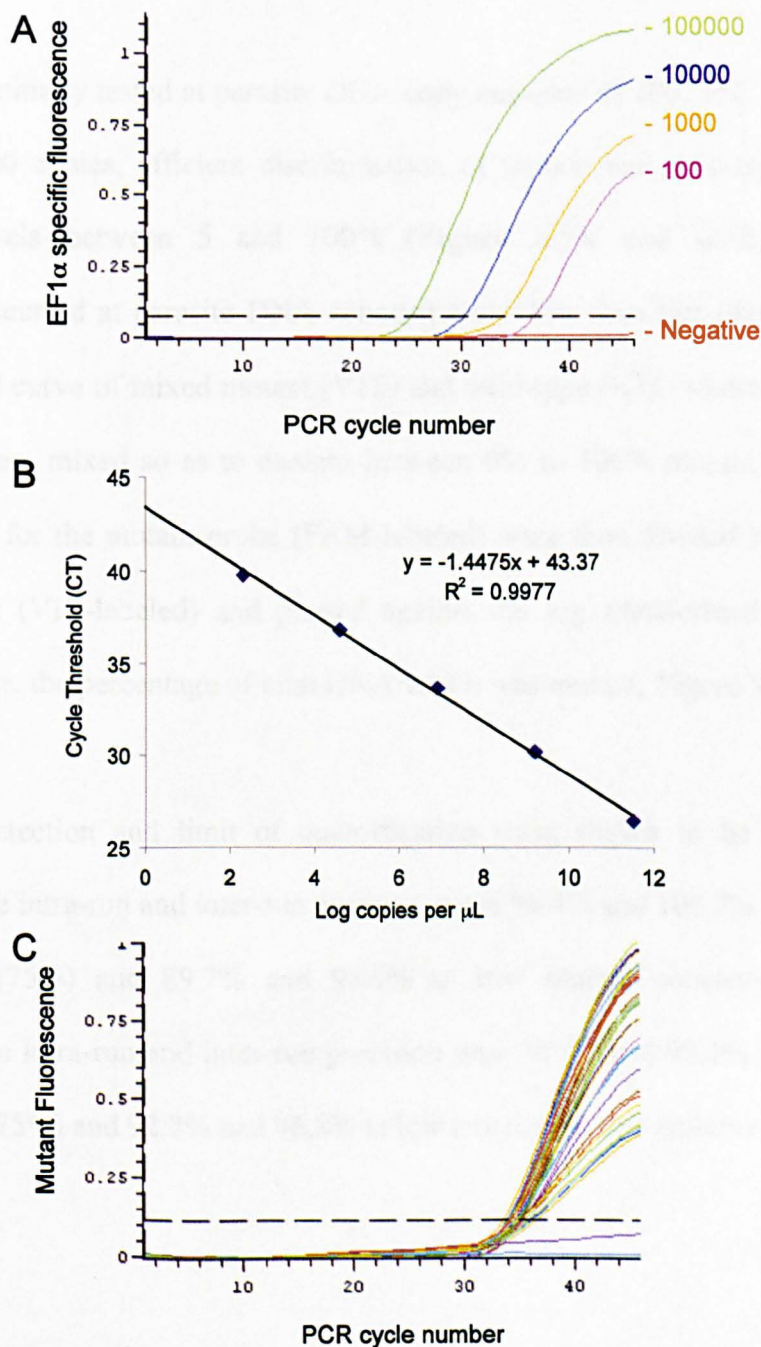
Figure 6.2 Normalisation of parasite DNA in order to resolve inter-sample variability. (A) Representative real-time PCR trace using specific *EF1-α* directed primers and probes. For template, *EF1-α* amplicons were gel extracted, quantified and diluted appropriately prior to the real-time PCR assay, as described under materials and methods. (B) Representative standard curve for quantification of *EF1-α*. Samples of DNA from clinical isolates underwent quantification of *EF1-α* and Ct values were read from this curve in order to normalise for copy number of parasite genome. (C) Real-time PCR trace for the mutant allele within Malawian isolates following normalisation to 10000 copies of *EF1-α* per reaction. Comparison with figure 1A illustrates effective normalisation of parasite DNA.

6.3.3 Validation of real-time PCR genotyping methodology

The assay was initially tested at parasite DNA copy numbers of 100, 250, 500, 1000 and 10000. At 10000 copies, efficient discrimination of mutant and wild-type alleles was possible at levels between 5 and 100% (**Figure 6.3A and 6.3B**). Insufficient amplification occurred at parasite DNA concentrations less than this (data not shown). For the standard curve of mixed mutant (V1S) and wild-type (K1) (where wild-type and mutant DNA were mixed so as to contain between 0% to 100% mutant DNA), the Ct values obtained for the mutant probe (FAM-labeled) were then divided by those of the wild-type probe (VIC-labeled) and plotted against the log transformed mutant DNA concentration (i.e. the percentage of total DNA which was mutant; Figure 5.3.3C).

The limit of detection and limit of quantification were shown to be 5% and 10% respectively. The intra-run and inter-run accuracy were 98.4% and 106.7% at high mutant concentrations (75%) and 89.7% and 96.9% at low mutant concentrations (10%), respectively. The intra-run and inter-run precision was 92.1% and 95.1% at high mutant concentrations (75%) and 92.2% and 96.8% at low mutant concentrations (10%).

A

Mutant Dye

Mutant Fluorescence

0.2
0.15
0.1
0.05
0

- 100%
- 75%
- 50%
- 10%
- 0%
- PCR NEG

10   20   30   40

PCR cycle number

B

Wild-Type Dye

Wild-Type Fluorescence

1
0.75
0.5
0.25
0

- 0%
- 10%
- 50%
- 75%
- 100%
- PCR NEG

10   20   30   40

PCR cycle number

C

Mutant CT / Wild-Type CT

1.08
1.06
1.04
1.02
1
0.98
0.96
0.94
0.92
0.9

$y = -0.0635x + 1.2045$
$R^2 = 0.9913$

2   2.5   3   3.5   4   4.5   5

Log percentage mutant
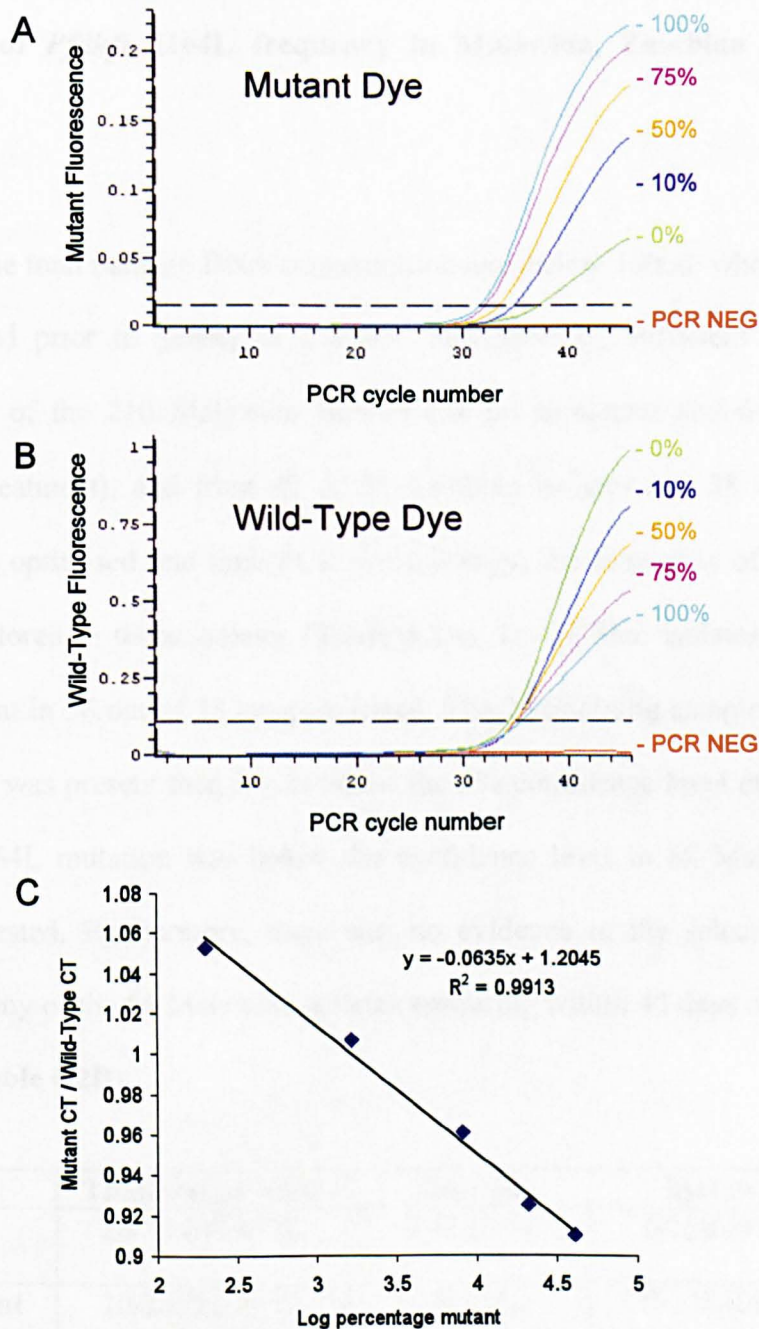
Figure 6.3 Quantification of *Pfdhfr* I164L mutant alleles by real-time PCR. (A) Representative real-time PCR trace for the mutant probe conducted on mixtures of mutant and wild-type alleles. Parasite DNA from a mutant strain (V1S) and a wild type strain (K1) were quantified by *EF1-α*. The DNA for the mutant allele was then diluted appropriately with that of the wild-type allele so as to obtain wild type DNA containing between 0% to 100% mutant DNA. (B) Representative real-time PCR trace for the wild-type probe conducted on mixtures of mutant and wild-type alleles (reciprocal of (A)). (C) Representative standard curve used for quantification of the mutant allele in clinical isolates. The CT values obtained from Figure 3A were divided by those obtained from Figure 3B and plotted against the log of percentage mutant values. See text for limits of detection, limits of quantification, inter and intra run accuracy and precision

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6.3.4 Assessment of *Pfdhfr* I164L frequency in Malawian, Zambian and Thai cohorts

In samples where the total parasite DNA concentration was below 10000, whole genome PCR was conducted prior to genotypic analysis. Subsequently, sufficient DNA was obtained from 158 of the 210 Malawian isolates (94 pre-treatment and 64 recurrent parasitemia after treatment), and from 42 of 55 Zambian isolates and 38 of 50 Thai isolates. Using the optimised real time PCR methodology, the frequency of the I164L mutation was monitored in these isolates (**Table 6.2A**). In the Thai isolates the I164L mutation was present in 36 out of 38 samples tested. The 2 remaining samples indicated that if the mutation was present then it was below the 5% confidence level of the assay. Conversely, the I164L mutation was below the confidence level in all Malawian and Zambian isolates tested. Furthermore, there was no evidence of the selection of this I164L mutation in any of the 64 Malawian isolates appearing within 42 days of treatment with PYR-SDX (**Table 6.2B**).

Genotype	Thailand (n = 38)	Zambia	Malawi
Pure mutant (100%)	26/38 (68.42%)	0/42 (0%)	0/158 (0%)
Partial mutant (5 – 99%)	10/38 (26.32%) (80-97% within patients)	0/42 (0%)	0/158 (0%)
Wild-type (<5%)	2/38 (5.26%)	42/42 (100%)	158/158 (100%)

Table 6.2A Frequency of the *Pfdhfr* I164L alleles in isolates from Thailand, Zambia and Malawi.

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	Ile164 < 5%	Leu164 > 5%
Pre-treatment	94 (100%)	0 (0%)
Post-treatment	64 (100%)	0 (0%)
Matched pre- and post- treatment	57 (100%)	0 (0%)

Table 6.2B Frequency of different *Pfdhfr* I164L alleles in Malawian isolates from patients naive to therapy and from patients having received therapy.

6.4 Discussion

We observed a large variability in the degree of amplification from field isolates. This was partly explained by variability in parasitemia but the correlation coefficient was low (0.13) indicating that other factors contribute to this variability (e.g. DNA extraction efficiency and sample deterioration). Furthermore, we observed only partial selectivity of the mutant probe for the mutant sequence and wild-type probe for the wild-type sequence. These discrepancies will result in high incidence of wild-type sequences being incorrectly identified as partially mutant which would cast doubt on the validity of the real time-PCR based data reported by Alker et al (Alker *et al.* 2005). However, one patient was reported whose entire parasite population contained the mutant allele and this was confirmed by sequencing. Clearly, this cannot be explained by the lack of specificity of the real-time PCR assay. However, binding of minor groove binder probes prohibit the sequencing of the resultant amplicon and the article does not provide an explanation for how this problem was circumvented.

Our approach was both accurate and precise for the detection of the I164L mutation and was able to detect the mutation above a level of 5% of the total parasite population. Furthermore, the assay was able to accurately identify the presence of this mutation in Thai isolates at a frequency comparable to previous reports (Biswas *et al.* 2000; Nair *et al.* 2003).

Appraisal of **figure 6.1A** might suggest that there was a large inter-sample variability in the mutant composition of the samples and that the majority of isolates were positive for the *Pfdhfr* I164L mutation. However, following standardisation **(Figure 6.2C)** the fluorescence associated with the mutant probe is in fact similar in all isolates. The ratio of mutant to wild-type fluorescence for all of these isolates was identical to that observed on amplification of the wild-type reference isolate, K1.

The assay described here allows improved sensitivity over conventional methodologies but owing to the incomplete specificity of the probes it is not possible to quantify the mutation if present at less than 5% of the total parasite population within an individual. However, of the Malawian isolates studied here, 64 were parasitological failures after treatment with PYR-SDX and 57 of these had matched pre-treatment isolates (**Table 6.2B**). Antifolate therapy with PYR-SDX would be expected to select for the I164L mutation if it existed even at low levels in our patient population yet it was not detected in any of the samples tested. The Malawian samples used in this study were collected from young children who self-presented to a health centre situated 10 km away from the hospital where the samples for the Alker study were collected. It is unlikely that the

parasites were under different drug pressures and suggests that the I164L mutation was not pre-existent within the samples tested in this study.

Our samples were collected between 2003 and 2005 whereas the samples in the previous study were collected between 2001 and 2003. Therefore, if it were present one would expect that over this time a significant expansion in the mutant population would have occurred under continued high level PYR-SDX selection as first line treatment in the Malawi. Finally, the previous study utilised isolates from pregnant mothers that were HIV positive (Alker *et al.* 2005). It is possible that the immunosuppression associated with HIV infection or exposure to co-trimoxazole prophylaxis may have influenced the development of the *Pfdhfr* I164L mutation, particularly if a high fitness cost is incurred or that other, as yet unknown selection pressures, were present in this patient group and not in children. It is also important to emphasise that each sample assayed in this paper could have had less than 5% of the parasite population with the mutant allele, and if so, it would not have been detected. Nonetheless, the rarity of this allele in Africa despite over a decade of use of PYR-SDX as frontline antimalarial therapy is an intriguing phenomenon. Particularly given its rapid selection in South East Asia under similar circumstances (Biswas *et al.* 2000; Nair *et al.* 2003) and the fact that it is easily selected for in *in vitro* studies (Paget-McNicol et al. 2001).

Using a fully validated methodology we could not identify I164L mutants within clinical isolates from Malawi and Zambia, even in post treatment failure parasites from Malawi. These data are reassuring as even though CPG-DDS and CPG-DDS-artesunate have now

been withdrawn, PYR-SDX is still used extensively for treatment in Africa and plays a major role in intermittent presumptive therapy programmes in pregnancy. Our results are in agreement with the majority of previous reports and coupled with the need for specialised equipment and cost associated with real-time PCR, there appears to be no urgent need for field application of this method. The mechanisms which underlie the failure of antifolate chemotherapy to select of I164L mutant parasites in Africa compared to S.E Asia is an important phenomenon which clearly requires further investigation.

CHAPTER 7

General Discussion and Conclusion

As already discussed in **chapter 1,** chemotherapy remains a key strategic approach in the battle against malaria. The antimalarial armament we have focused on in this thesis is the folic acid antagonists referred throughout the thesis as antifolates. The antifolates of clinical importance have been extensively discussed in **chapter 1.** These compounds have proved to be of excellent therapeutic value against chloroquine resistant parasites and even today SP remains the leading agent in intermittent preventive treatment in infancy (IPTi) and pregnancy (IPTp), a targeted scheme that is directed at two of the most vulnerable groups at risk of malaria attacks (Greenwood 2007; Vallely et al. 2007). However, as earlier stated, the greatest concern to the clinical use of the class is the development of resistance.

The biochemical rationale for the incorporation of these antifolate molecules in the treatment of malaria is based on their enhanced binding affinity to the Plasmodial DHFR compared to the human DHFR, (PYR and CCG) or the ability to bind to the unique enzyme, DHPS which is absent in humans, (SDX and DDS). These compounds are generally characterized as small, lipid-soluble and lack the distal glutamate moiety of the classical antifolates hence they are assumed to easily traverse biological membranes by simple diffusion or facilitated diffusion. Because of these unique properties, they are

unable to provoke a transport- or a polyglutamation-mediated resistance phenomenon as seen with MTX and consequently the generation of a mutated DHFR/DHPS have been the favoured resistance mechanism **(see section 1.7.1).**

Although widely accepted as the principal resistance mechanism **(see section 1.7.1),** the impact of exogenous folate substrates in modulating the activities of these antifolates has attracted a great deal of interest and is often referred to in the literature as the folate effect of the folate salvage pathway. Based on the argument that folate salvage modifies the activity of antifolates, it was proposed that inhibition of salvage would have a chemosensitising effect. Using probenecid as the inhibitor Nzila et al (2004) provided experimental evidence in support of this argument. It is these observations that have prompted the focus of this thesis towards understanding the transport of folates in *P. falciparum* infected red blood cells.

Chapter 3 of the thesis focused on the potential of extracellular folate cofactors to diminish the activity of PYR, CCG, SDX and DDS in a panel of well characterized sensitive and resistant isolates of *P. falciparum*. The addition of folate substrates was associated with diminished drug activities in all the strains, a result that supports the suggestion that all strains have the capacity to transport folate cofactors to some degree. The only exception was with 5-MeTHF where the effect was not apparent. The influence of the folate cofactors was evident even in isolates that are fully sensitive to these compounds. The inability of 5-MeTHF to abrogate the antifolate activity was quite

striking and physiologically very important since this is the predominant folate substrate in human plasma.

As pointed out above, these drugs are essentially 'unpolyglutamatable' and so the impact of the folate substrates to negate their activities cannot be related to an interruption of the formation of polyglutamatable products. There appears to be a novel mechanism responsible for this effect that could be related to the uptake of the supplied folate, subsequent inter-conversions and eventual targeting of specific folate-based enzymes. Quantification of the precise intracellular folate cofactor pool in relation to the perturbations of the specific extracellular folate substrate and its concentration has not been conclusively determined in malaria parasites.

We know these compounds are competitive inhibitors of their target sites in the folate pathway. Although we did not specifically use the particular enzymatic substrates apart from pABA, a mechanistic understanding akin to resistance of the lipid-soluble antifolates in human cells can be inferred (Stark et al. 2003). The results presented in **chapter 3** suggest malaria parasites accumulate folate cofactors from the external environment which may or may not undergo inter-conversions that in turn suppress or counteract the interaction between the antifolate and its target enzyme resulting in an apparent reduction in susceptibility. This novel mechanism is independent of the acquired resistance through genetic point mutations on the *dhfr* or *dhps* domains.

The data in **chapter 3** also revealed a degree of variation in the ability of the investigated folate cofactors to mitigate the activity of the antifolate, an observation that may have several interpretations. It is possible that this difference reflects the presence of the folate transport system in the malaria parasite which has different affinities for the different folate substrates. A comparative *in vitro* analysis of the DHFR inhibitors (PYR vs CCG) and DHPS inhibitors (SDX vs DDS) also indicated that CCG and DDS were superior inhibitors compared to PYR and SDX respectively. The impact of mutations in the target enzymes on the *in vitro* sensitivity of each antifolate was significantly noted between the wild-type and resistant strains when studied in the control medium. However, the addition of folate substrates of interest to the test medium showed the association between point mutations and the resistance level was highly masked. For example, under this folate supplemented conditions, the resistance level of some less mutated isolates almost matched the level of the highly mutated ones. This raises the possibility that there could be differences in the way the strains utilize the added folate cofactors. The failure of 5-MeTHF to influence antifolate activity and the observed differences in the degree of inhibition amongst the folate substrates warrants further investigations.

Since we know the folate substrates are charged molecules, the general assumption we are making on the impact of these substrates on the activities of the antifolates is that they traverse the membrane and concentrate in the intracellular site. A different approach towards the precise biochemical characterization of the folate substrate effect is through radio-active flux experiments. In this way, the rate of transport of the substrates can be measured, the fate of the transported substrate can be traced and its precise subcellular

location identified. Isolated enzyme assays can also tremendously improve our understanding of the mechanisms of behind the mitigation of antifolate activity.

The hunt for novel molecular mechanisms that underlie the transport of folate substrates suggested the use of the *Xenopus* oocyte as the model of choice for delineating the exact molecular determinant underpinning this biochemical process. This was the basis of **chapter 4.** We focused on two putative folate transporter genes (MAL8P1.13 and PF11_0172) in *P. falciparum* and functionally characterized MAL8P1.13. Sequence analysis established that the two genes were related to the FBT sub-family that are known to participate in the transport of folates in plants, cyanobacteria and other protozoa. The heterologous expression of the cloned MAL8P1.13 in an isolated *Xenopus* oocyte system allowed the direct examination of the role of this transporter in the uptake of MTX, FA and 5-FoTHF. Furthermore we demonstrated that the uptake of the radio-labeled substrates, FA and 5-FoTHF could be inhibited by folate structural analogues. As stressed several times, our interest in the folate transport system stems from the findings regarding the use of probenecid to potentiate the *in vitro* activities of antimalarial antifolates (Nzila *et al.* 2004), a phenomenon that we thought was linked to folate transport and which might have some therapeutic potential (Sweet et al. 2001).

The confirmation of folate transport prompts a series of additional studies which should be conducted if we are to fully exploit this process. With recombinant transporter expressed in suitable model systems it should be possible to probe the recognition and binding sites within the transporter and use this information in rational inhibitor design.

The identification and functional characterization of the putative folate transporter genes in **chapter 4** provided an excellent platform to investigate a long-standing question in folate biochemistry related to two phenotypes; (I) inter-strain variation in response to SDX inhibition in the presence of low levels of added folate. (II) Why do some isolates fail to grow in deprived levels of folate? As we have pointed out before, the majority of published studies have tried to address the first phenotype focussing on the contribution of the *dhfr* or *dhps* genotype or the neighbouring sequences of the *dhfr* domain. In contrast the second phenotype has been less well investigated.

One great concern for the use of the xenopus model is the potential for the induction of an endogenous transport system which might lead to the modulation of the heterlogously expressed protein or competition for the translational machinery. Apart from the functional characterization of the transporters in an intact cell, the expression, isolation and purification of the protein can be adopted to further characterize its functional properties. Other heterologous expression systems might be used to overcome inherent problems with this model.

The work described in **chapter 5** has taken a molecular approach to investigating the described transporters as the mechanism behind these two phenotypes. Evaluation of the mRNA expression pattern of the two genes in reference isolates from diverse geographic backgrounds demonstrated considerable inter-strain variability. This finding raised the possibility that there exist an isolate-specific phenotype that could be linked to this

variation. An extension of this work to look at gene expression under defined folate environments (folate-free/supplemented) suggested that mRNA expression levels of the two genes were sensitive to the extracellular folate concentrations. We have proposed a model to explain these findings based on the involvement of transcription factors (figure 6.7). The fact that these changes in expression occurred in such a short period of time means that this is an adapted response of the parasite, and not a change in phenotype that is mediated by selection. Definitive confirmation of this working model will require comprehensive analysis of the MAL8P1.13 and PF11_0172 gene regulatory regions. For example, cloning the core promoter regions of these genes and evaluating their ability to drive expression of the luciferase reporter would provide a tool for confirming this observation but also would be an excellent system in which to search for the specific parasite transcription factors, chaperones and coregulatory proteins (coactivators and corepressors) that are involved in this phenomenon.

The correlation of mRNA changes and protein expression profiles is not absolute. However, the mRNA regulation analysis provides illuminating insights into what could be happening at the protein level. Despite this, further protein studies are necessary a confirmatory evidence of the mRNA regulation pattern observed.

Although the focus of this thesis was on the biochemical and molecular determinants of folate transport and modulation of resistance, one big question that has puzzled many is the slow selection of the DHFR 164 mutant allele in Africa. As reviewed in **chapter 1,** resistance to anti-DHFR (PYR) normally follows an ordered sequential pattern in the

accumulation of point mutations ultimately leading to the I164L. It has generally been argued that the low level of detection of the rare resistant allele in Africa is associated with the low sensitivity of detection by the traditional PCR tools. In **chapter 6,** we developed a refined Real-Time PCR technique for the detection of the I164L. The report by Alker et al (2005) prompted us to investigate for the presence of this mutant allele in Malawian samples using this technique. In this work, we addressed the potential sources of the problem that could lead to the erroneous detection of the I164L and went further to confirm its absence in a collection of Africa isolates and its existence in a large proportion of Thai isolates. The rarity of the allele in Africa where the triple mutant *dhfr* and double mutant *dhps* are nearly fixed compared to its widespread existence in Asia or South-America could imply the involvement of other compensatory mechanisms. We believe that folate transport may have a role to play in these discrepancies between S.E Asia and Africa. The work described in this thesis provides new areas of investigation in folate biochemistry that might explain some of the previously described parasite phenotypes and which may offer new approaches to anti-folate chemotherapy.

Although a sensitive technique, the Real-Time PCR approach applied in the detection of the DHFR 164 allele has limitations. It is very expensive for large epidemiological studies and may not be appropriate for routine diagnosis in Africa.

In **chapter 1 section 1.9,** we discussed potential challenges facing the future development of antifolates. Currently, PYR/SDX resistance is spreading at an alarming rate. This is of great concern as there are no cheap affordable alternatives. The only

strategy to overcome antifolate resistance is to develop novel and potent antifolates against these mutant forms. This is an expensive process and time consuming. Thus the quickest strategy that can be implemented rapidly is the use of the already existing reversal agents that target folate transport, an approach that has been adopted in cancer chemotherapy.

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