METABOLITE TRANSPORT PATHWAYS OF *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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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

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ABSTRACT

Susan Beveridge – Metabolite Transport Pathways of *Plasmodium falciparum*

Metabolite transport pathways of the malaria parasite, *Plasmodium falciparum*, are an important area for study in order to further the understanding of the parasite's biology. Identification and characterisation of the transporters involved in these pathways may also provide potential novel drug targets or drug delivery mechanisms. This is especially valuable as chemotherapy remains one of the main management strategies in the fight against malaria and the usefulness of the current range of antimalarial drugs is seriously threatened by the emergence and spread of resistance.

In this thesis the *Xenopus laevis* oocyte heterologous expression system was used to functionally characterise a gene-specific cDNA library of 48 putative membrane proteins and the previously annotated putative amino acid transporter PFF1430c for the uptake of several amino acids. This screening failed to identify any definite amino acid transport by the cDNA library or PFF1430c, however this could have been due to the fact that uptake of a relatively narrow range of amino acids was tested and these were used at concentrations lower than found physiologically. Inherent issues with the *X. laevis* expression system may also have been an issue, including the expression of endogenous transporters for the substrates being investigated.

The uptake of methionine by intact infected RBCs and free *P. falciparum* parasites was also characterised. No increase in uptake by infected RBCs compared to uninfected RBCs was found, however this is an unexpected

result compared to other nutrients and further experiments need to be carried out before any conclusion can be made. Methionine was found to be transported across the parasite plasma membrane via one or more saturable systems showing partial similarity to the vertebrate systems L-methionine influx into the free parasites was inhibited most effectively by cysteine and to a lesser degree by lysine and histidine.

The whole cell membrane transport of substrates related to folate and methionine metabolism was studied using a pharmacological approach. pABA was the only folate substrate to antagonise sulphadoxine and pyrimethamine at physiological concentrations and growth assays with *E. coli ΔpabA/ΔabgT* expressing *P. falciparum* folate transporters PfFT1 or PfFT2 also showed pABA to be the most efficient folate substrate at increasing bacterial growth. These results suggest that the folate precursor pABA may be the relevant natural folate intermediate substrate for the intraerythrocytic malaria parasite. This is an interesting observation as pABA is not significantly utilised or synthesised in humans making pABA salvage a potential target for chemotherapy.

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PUBLICATIONS

1. Salcedo-Sora, J.E. Ochong, E. Beveridge, S. Johnson, D. Nzila, A. Biagini, G.A. Stocks, P.A.. O'Neill, P.M. Krishna, S. Bray, P.G. Ward, S.A. (2011). The Molecular Basis of Folate Salvage in *Plasmodium falciparum*: Characterization of Two Folate Transporters. *J Biol Chem,* 286(52), 44659-44668

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ABBREVIATIONS

5-MTHF	5-methyl tetrahydrofolate
AAAP	Amino acid/ auxin permease
AAT	Amino acid transporter
ABC	ATP -binding cassette
ABT	Archeal/bacterial transporter
ACT	Artemisinin-based combination therapy
ACT	Amino acid/choline transporter
ADC	4-amino-4-deoxychorismate
AEC	Auxin efflux carrier
AGCS	Alanine/glycine:cation symporter
APA	Basic amino acid/polyamine antiporter
APC	Amino acid-polyamine-organocation
ATP	Adenosine triphosphate
BCH	2-aminobicyclo[2.2.1]heptane-2-carboxylic acid

BCCT	Betaine/carnitine/choline transporter
BLAST	Basic Local Alignment Search Tool
C	Chorismate
CAT	Cationic amino acid transporter
CCG	Cycloguanil
cDNA	Complimentary DNA
cRNA	Complimentary RNA
CSP	Circumsporozoite protein
DAACS	Dicarboxylate/amino acid:cation (Na^+ or H^+) symporter
Dcu	C_4-dicarboxylate uptake
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethylpyrocarbonate
DHFS	Dihydrofolate synthase
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase

DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
E_a	Activation energy
EAT	Ethanolamine transporter
EDTA	Ethylenediamine tetraacetic acid
ESS	Glutamate:Na^+ symporter
FGPS	Folylpolyglutamate synthase
GABA	γ-aminobutyric acid
gDNA	Genomic DNA
GGA	Glutamate:γ -aminobutyrate (GABA) antiporter
GTPC	Guanine triphosphate cyclohydrolase
HAAP	Hydroxyl/amino acid permease
HAAT	Hydrophobic amino acid uptake transporter
hENT1	Human equilibrative nucleoside transporter 1

hRFC	Human reduced folate carrier
hPCFT	Human proton coupled folate transporter
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hFNT1	Human facilitative nucleoside transporter 1
HPPK	Hydroxymethyldihydropterin pyrophosphokinase
IC_{50}	50% Inhibitory concentration
IRS	Indoor residual spraying
ITNs	Insecticide treated bed nets
K_i	Inhibition constant
K_m	Michaelis constant
LAT	L-Type amino acid transporter
LCT	Lysosomal cystine transporter
LIVCS	Branched chain amino acid:cation symporter
LIV-E	Branched chain amino acid exporter
LLINs	Long lasting ITNs

LysE	L-Lysine exporter
MC	Mitochondrial carrier
MFS	Major facilitator superfamily
ITNs	Insecticide treated bed nets
MS	Methionine synthase
MSP	Merozoite surface protein
MTHFR	Methylenetetrahydrofolate reductase
NADPH	Nicotinamide adenine dinucleotide phosphate
NEM	N-ethylmaleimide
NPP	New Permeation Pathway(s)
NSS	Neurotransmitter:sodium symporter
PAAT	Polar amino acid uptake transporter
pABA	*para*-aminobenzoic acid
PabaS	pABA synthase
PBN	Probenecid

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PepT	Peptide/opine/nickel uptake transporter
PfATP4	*P. falciparum* Ca^{2+} ATPase 4
PfATP6	*P. falciparum* Ca^{2+} SERCA ATPase 6
PfCRT	*P. falciparum* chloroquine resistance transporter
PfCRT^{CQR}	Chloroquine resistance conferring form of PfCRT
PfFT1	*P. falciparum* folate transporter 1
PfFT2	*P. falciparum* folate transporter 2
PfHT	*P. falciparum* hexose transporter
PfMDR1	*P. falciparum* multidrug resistance protein 1
Pf(E)NT1	*P. falciparum* (equilibrative) nucleoside transporter 1
PfNT2	*P. falciparum* (equilibrative) nucleoside transporter 2
PfNT3	*P. falciparum* (equilibrative) nucleoside transporter 3
PfNT4	*P. falciparum* (equilibrative) nucleoside transporter 4

PfPiT	*P. falciparum* Na^+: phosphate symporter
PfPTPS	*P. falciparum* pyruvoyltetrahydropterin synthase
PLM	Phospholemman
POT	Proton-dependent oligopeptide transporter
PPM	Parasite plasma membrane
PVM	Parasitophorous vacuole membrane
PYR	Pyrimethamine
QAT	Quartenary amine uptake transporter
RBC	Red blood cell
RESA	Ring-infected erythrocyte surface antigen
RNA	Ribonucleic acid
RhtB	Resistance to homoserine/threonine
S-AdoMet	S-adenosylmethionine
SDX	Sulphadoxine
SERCA	Sarco-endoplasmic reticulum

SGP	Spore germinating protein
SIT	Sterile insect technique
SP	SDX/PYR drug combination
SSS	Solute:sodium symporter
TbAT1	*Trypanosoma brucei* aminopurine transporter 1
THF	Tetrahydrofolate
ThrE	Threonine/serine exporter
TMS	Transmembrane α-helical spanner
TRAP	Thrombospondin related anonymous protein
TRAP-T	Tripartite ATP-independent periplasmic transporter
TS	Thymidylate synthase
V_{max}	Maximum velocity
WHO	World Health Organisation
YAT	Yeast amino acid transporter

CHAPTER 1

INTRODUCTION

1.1 Malaria

Malaria is a vector borne disease caused by apicomplexan parasites of the family *Plasmodiidae* and the genus *Plasmodium.* There are over two hundred species of *Plasmodium* described, and four for which humans are natural hosts: *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale* and *Plasmodium malariae* (Garnham, 1966, Abdalla, 2004, Perkins and Austin, 2009). The simian malaria parasite *Plasmodium knowlesi* has also recently been found in humans as a zoonotic infection (Singh *et al.*, 2004). There have been fatal human infections with this parasite, which naturally infects long-tailed macaques and is widespread throughout Southeast Asia (Cox-Singh and Singh, 2008). In 2006 there were an estimated 3.3 billion people at risk of malaria worldwide, with an estimated 247 million cases of malaria and an estimated 881, 000 deaths (WHO, 2008). Cases in Africa accounted for 91 % of the deaths from malaria, with children under five years of ages being the most affected. *P. falciparum* causes the most severe form of disease and is the most widespread species affecting humans, being responsible for 92 % of cases worldwide in 2006 (figure 1.1) (WHO, 2008).

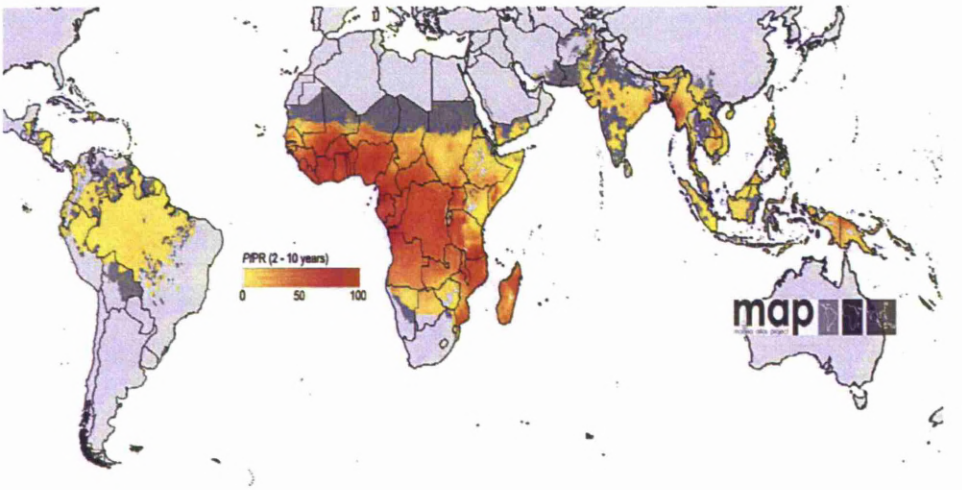

Figure 1.1. The spatial distribution of *P. falciparum* malaria endemicity in 2007 (Hay *et al.*, 2009).

1.1.1 Discovery of the Malaria Parasite

Long before the discovery of the causative agent of malaria evidence of the disease is well documented. Descriptions of symptoms of malaria, such as splenomegaly and intermittent fever, are found in the Ebers Papyrus from Egypt (ca. 1570 B.C.) and also in the Nei Ching from China in 2700 B.C (Sherman, 1998). Further proof of the presence of the malaria parasite in Ancient Egypt comes from the detection of a trophozoite derived *P. falciparum* antigen in skin, lung and muscle samples of mummies from between 3200 and 1085 B.C. (Miller *et al.*, 1994). Malaria is mentioned in Homer's Iliad and the Greek physician Hippocrates described the quartan and tertian fevers of *P. malariae* and *P. vivax* in his Book of Epidemics in the fifth century B.C. (Sherman, 1998). He also discerned a correlation

between malaria cases and marshland areas as well as a seasonal pattern of disease (Bruce-Chwatt, 1989). Despite noting the link between marshlands and malaria Hippocrates did not make the link between the presence of mosquitoes and the disease, instead attributing the cause of malaria to the drinking of stagnant waters. It was not until 1880 that the cause of malaria was correctly discovered. The discovery of the malarial agent, parasites of the genus *Plasmodium*, was made by Alphonse Laveran whilst working at a military hospital in Bône in Algeria (Collins and Jeffery, 2007). On post-mortem examination of blood taken from the spleen of a pernicious malaria case he noted that there was not only free pigment (as had previously been observed), but also observed leukocytes containing ingested particles and pigmented spherical hyaline corpuscles and crescent-shaped bodies (Bruce-Chwatt, 1981). On the 6th of November 1880 he proved that these observed particles were not just degraded erythrocytes by observing exflagellation in blood from a soldier suffering from malaria. From these observations he concluded that he was seeing living organisms in the blood that were the cause of malaria. Examination of blood from marsh fever (malaria) cases in Italy showed the same bodies and further consolidated Laveran's claim that malaria was a parasitic disease. However, despite extensive searching, including examination of water, soil and air, Laveran was unable to discover the parasites outside the patient's body. In the late 1890s Ronald Ross, an army surgeon working in India, carried out a series of experiments to attempt to demonstrate the role of the

mosquito in malaria transmission. In 1887 he made the discovery of foreign bodies in the stomach wall of mosquitoes fed on malarious patients. Further work on avian malaria and the mosquito vector allowed Ross to follow the development of the parasite in the mosquito, and so gave further evidence to the theory of mosquitoes as the vectors of human malaria.

1.1.2 The Life Cycle of the Malaria Parasite

The human malaria parasite has a two host life cycle, the asexual phase of development and replication occurring in the human host and the sexual phase within the anopheline mosquito vector (figure 1.2). When the mosquito takes a blood meal sporozoites in the mosquito's saliva are released into the human host. These sporozoites travel to the liver and invade hepatocytes where they differentiate and undergo asexual replication. Thousands of merozoites develop within each parasitised hepatocyte and are released into the blood stream when the hepatocyte ruptures. Free merozoites then invade red blood cells and undergo either a round of asexual multiplication through trophozoite and schizont stages or else differentiate into sexual forms (micro and macro gametocytes). Once mature the schizonts burst releasing merozoites back into the blood stream and the asexual intraerythrocytic cycle is repeated. Micro and macrogametocytes are ingested by the anopheline mosquito during a blood

meal and develop in the midgut of the mosquito into gametes, with the microgametes undergoing exflagellation. The gametes then fertilise and develop into an ookinete which penetrates the midgut wall and further develops into an oocyst. Sporogenesis occurs within the oocyst and sporozites are released into the salivary glands of the mosquito, which can then be transmitted when the mosquito takes a blood meal.

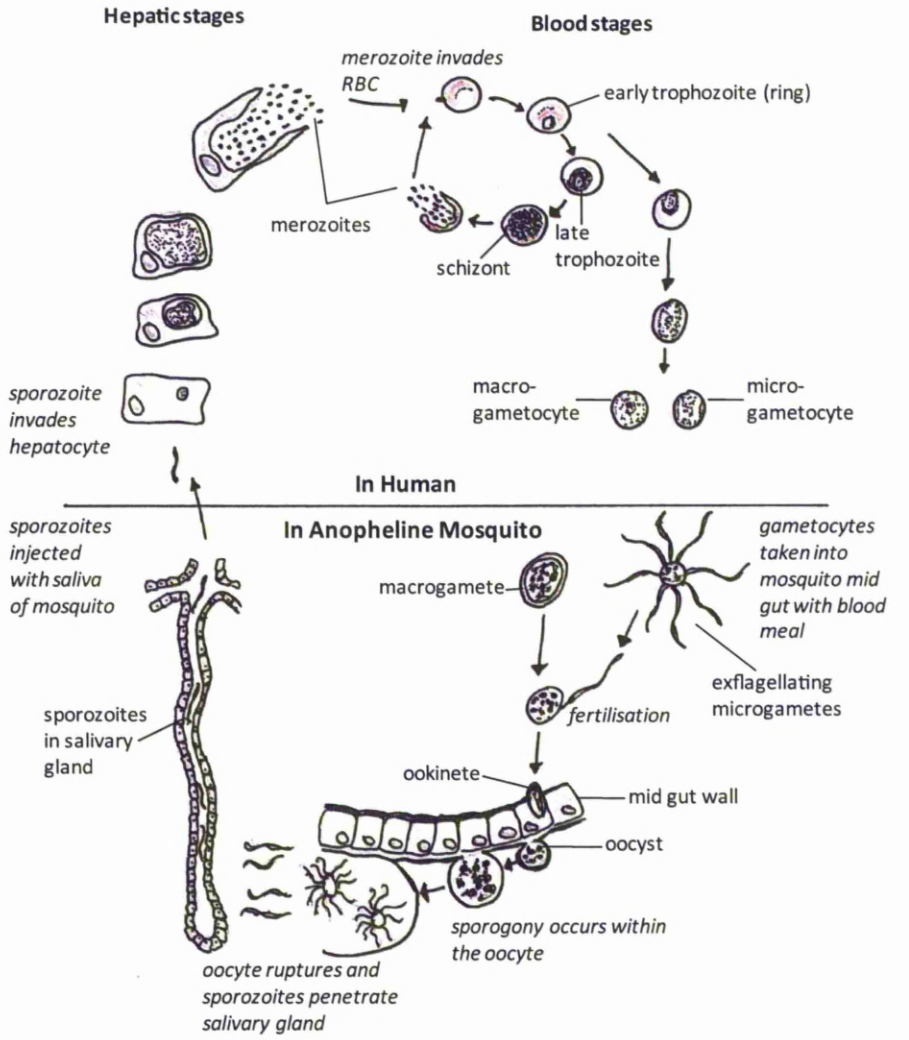

Figure 1.2. The life cycle of the malaria parasite. Modified from Cox (1993).

1.2 Malaria Management Strategies

Different strategies to control malaria have been implemented throughout the ages. Before the discovery of the malaria parasite and its mosquito vector some limited control strategies had already been implemented, for example the draining of marshes and simply the avoidance of malarious regions. Control strategies over the last century have helped reduce the number of people at risk of malaria from 77 % in 1900 to just under 50 % in 2000 (Hay *et al.*, 2004, Enayati and Hemingway, 2010). These control strategies have included vector control and chemotherapy with major advances, and setbacks, occurring in both of these fields over the past hundred years. There is also an ongoing quest to find an effective malaria vaccine (Greenwood and Targett, 2009, Haque and Good, 2009).

1.2.1 Vector Control

Long before Ronald Ross identified the mosquito vector responsible for transmission of malaria it had been noticed that there was a correlation between areas of wetlands and malaria cases. This led to the first malaria control strategy of draining marshlands (Griffitts, 1937). After the identification of mosquitoes as the vector of malaria significant efforts were made to control the insects in an attempt to break the cycle of transmission. Early efforts in this sanitation era included focussing on reducing mosquito breeding sites by drainage or other modifications and

the application of larvicides (Kitron and Spielman, 1989). The discovery of the insecticide properties of dichlorodiphenyltrichloroethane (DDT) by Paul Muller in 1939 and its widespread use in the 1940s and following decades helped to eliminate malaria from previously malarious regions, including Greece and Italy (Enayati and Hemingway, 2010). Despite successes in the developed world, malaria eradication efforts using DDT and subsequent insecticides in combination with chemotherapy were not as successful in the developing world. This was mainly due to operational, political and economic difficulties faced in sustaining long term campaigns of indoor residual spraying (IRS) with insecticides combined with chemotherapeutic interventions (Muturi *et al.*, 2008, Enayati and Hemingway, 2010). Insecticide treated bed nets (ITNs) are another form of vector control that has been developed more recently. These are effective, and although not as powerful as IRS are much simpler and easier to deploy. ITNs have been improved with the introduction of long lasting ITNs (LLINs) that do not require periodic re-treatment with insecticide (Enayati and Hemingway, 2010). Other vector control methods are currently being developed including sterile insect technique (SIT, the release of sterile males to reduce the mosquito population), the release of genetically modified mosquitoes that are refractory to malaria parasite development and the use of entomopathogenic fungi to kill mosquitoes (Kilama, 2009, Mnyone *et al.*, 2010).

1.2.2 Vaccine Development

People living in malaria endemic regions naturally acquire immunity to malaria after repeated infections and this observation has led to the search for a potential malaria vaccine candidate. Immunisation with live irradiated sporozoites via bites from infected irradiated mosquitoes first demonstrated that vaccination against human malaria is possible (Clyde *et al.*, 1973). Since then there has been much research into finding suitable antigens as vaccine candidates. Three stages of parasite development have been targeted for vaccine development so far. The pre-erythrocytic stage vaccines target either sporozoites to block invasion of hepatocytes or the infected hepatocytes, so preventing infection or clinical disease. Erythrocytic stage vaccines inactivate the blood stage merozoites or target infected RBCs to prevent or reduce clinical disease. Transmission-blocking vaccines target the parasite's sexual stages, with the resulting antibodies being ingested by the mosquito along with the parasite and blocking parasite development (Targett and Greenwood, 2008, Greenwood and Targett, 2009, Enayati and Hemingway, 2010). Currently there are still no licensed vaccines on the market despite decades of research spent on vaccine development. However, there are several promising candidates currently going through trials.

Current pre-erythrocytic stage vaccine candidates include whole sporozoites attenuated genetically or by irradiation and sub-unit vaccines

based on the sporozoites circumsporozoite protein (CSP) and thrombospondin related anonymous protein (TRAP) involved in sporozoites motility and invasion of liver cells (Mueller *et al.*, 2005, Targett and Greenwood, 2008). The most promising of the sporozoite sub-unit vaccines so far is RTS, S. This vaccine fuses the tandem repeat tetra peptide (R) and C-terminal T-cell epitope containing (T) regions of the CSP to the hepatitis B surface antigen (S), with the unfused S antigen included. It has been used successfully in trials with the ASO2 adjuvant, which is an oil in water emulsion based adjuvant with the immunostimulants monophosphoryl lipid A and QS-21 added (Targett and Greenwood, 2008).

Merozoite surface proteins have been the most investigated antigens for the development of erythrocytic stage vaccines. A phase 2 clinical trial with a vaccine containing merozoite surface protein 1 (MSP-1), MSP-2 and ring-infected erythrocyte surface antigen (RESA) showed a strain specific reduction in parasite density but no decrease in the prevalence of infection (Genton *et al.*, 2002). This correlates with the theory that erythrocytic stage vaccines would reduce disease but not infection, and mimics naturally acquired immunity (Enayati and Hemingway, 2010).

Transmission-blocking vaccines have focussed on using sexual stage specific surface molecules involved in fertilisation (Pfs 48/45 and Pfs 230

in *P. falciparum*) and two other proteins expressed by zygotes and ookinetes (P25 and P28) as antigens. It has been shown using animal models that these antigens induce effective transmission-blocking immunity and phase I human clinical trials have also shown promising results (Targett and Greenwood, 2008).

1.2.3 Malaria Chemotherapy

With the lack of a licensed vaccine and vector control not effective at eliminating malaria alone, malaria chemotherapy remains the main management strategy in the fight against malaria. Antimalarial drugs have been in use for centuries, with the bark of the cinchona tree being used by the native Quechua people of South America as an effective cure for malaria. The antimalarial property of the bark of the chinchona tree was discovered by Europeans when they travelled to South America and witnessed its use in the early 17^{th} century (Foley and Tilley, 1998). Initially a ground powder of the bark was used to treat malaria but the active form was later discovered and named quinine (Carmargo *et al.*, 2009).

1.2.3.1 The Status of Current Antimalarial Chemotherapy and the Spread of Resistance

Since the discovery of quinine, new antimalarial drugs have been developed and improved. Due to the occurrence and spread of parasites

resistant to antimalarial drugs efforts are ongoing to develop new drugs and prevent the further spread of resistance to existing drugs (Biagini *et al.*, 2003b).

1.2.3.1 Quinoline-Containing Drugs

All the quinoline containing drugs act on the erythrocytic stages of the parasites, with the exception of the 8-aminoquinoline primaquine which also targets hypnozoites of *P. vivax* and *P. ovale* (Foley and Tilley, 1998). A method for producing synthetic quinine was not discovered until 1944 when Woodward and van Doering (Carmargo *et al.*, 2009) discovered a pathway that was, however, too complex (and therefore too expensive) for commercial use (Foley and Tilley, 1998, Carmargo *et al.*, 2009). Because of this delay and difficulty in producing synthetic quinine, research into alternative quinoline-containing antimalarials was carried out and the discovery of 4-aminoquinolines (1), alternative quinoline methanols (2) and bisquinolines (3) achieved (Foley and Tilley, 1998, O'Neill *et al.*, 1998).

(1) 4-Aminoquinolines

The 4-aminoquinoline drugs were discovered during the search for an alternative antimalarial to quinine (Greenwood, 1995). The most widely used drug of this class of antimalarials is chloroquine, which was

discovered by Bayer in 1934 (figure 1.3) (Coatney, 1963). It was initially known as resochin and was thought to be too toxic for human use, until demands for antimalarials during World War II led to its re-evaluation and trials in 1943. These trials showed that is was safe at therapeutic levels and it was renamed chloroquine and brought into use (Foley and Tilley, 1998). Chloroquine has been used extensively as it is cheaply produced, safe and has efficacy against all human malarias, although problems of resistance have since occurred and rendered it ineffective in many areas (Maitland *et al.*, 2004).

Amodiaquine, another 4-aminoquinoline, was discovered by Parke, Davis and Company in the 1950s (figure 1.3) (Greenwood, 1995). It was initially used as a prophylactic drug but reports of neutropenia and hepatitis led to concerns of toxicity and its use was suspended (Jewell *et al.*, 1995, Taylor and White, 2004). The safety of amodiaquine has been thoroughly evaluated since and it was found to be safe and well tolerated (Olliaro and Mussano, 2003).

Both chloroquine and amodiaquine target the erythrocytic forms of the malaria parasite, specifically the stages ingesting and actively degrading haemoglobin (Foley and Tilley, 1998). The site of action of these drugs is the parasite's digestive vacuole and their antimalarial property is due to

haeme-binding activity but there is still no consensus on how the haeme-drug complex is toxic to the parasite (Bray *et al.*, 2005b).

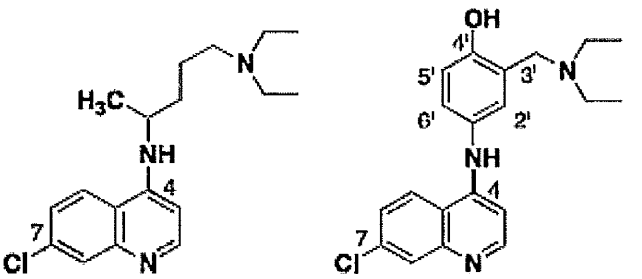

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Figure 1.3. Structures of the 4-aminoquinolines, chloroquine (1) and amodiaquine (2).

Cases of chloroquine resistance were first reported in South America and Southeast Asia in the late 1950s and early 1960s (Eyles *et al.*, 1963, Harinasuta *et al.*, 1965, Foote and Cowman, 1994). Resistance took longer to appear in Africa but by the late 1970s three cases of resistance had been reported in East Africa (Kihamia and Gill, 1982) and resistance is now widespread (Bjorkman and Phillips-Howard, 1990). The major mediator of chloroquine resistance is thought to be point mutations of the *P. falciparum* chloroquine resistance transporter gene (PfCRT), which codes for an integral digestive vacuole membrane protein (Fidock *et al.*, 2000, Bray *et al.*, 2005b, Bray *et al.*, 2005a, Hyde, 2007). In parasites from field samples

a change from lysine to threonine at position 76 is found consistently in chloroquine resistant parasites but this is always accompanied by at least 3 other changes (Hyde, 2007). The levels of chloroquine resistance have been shown to be modulated by mutations and copy number amplifications of the *P. falciparum* multi-drug resistance gene (PfMDR1) (Valderramos and Fidock, 2006, Hyde, 2007). Resistance to amodiaquine may have similar mechanisms to chloroquine resistance due to its similarity in structure and this has been shown to be true in some cases (Ochong *et al.*, 2003, Holmgren *et al.*, 2006). Chloroquine resistance is widespread, however a study in Malawi has shown that there can be a reversal to the wild-type K76 and chloroquine sensitivity after a prolonged break from chloroquine use (Mita *et al.*, 2003).

(2) Quinoline Methanols

Quinine was the first quinoline methanol discovered, and as stated above the first globally administered antimalarial drug (figure 1.4). Quinine is still in use as a treatment for malaria, especially in combination with other drugs including tetracycline (Kremsner and Krishna, 2004). Mefloquine is a synthetic quinoline methanol that was discovered by the Walter Reed Army Institute of Research and marketed in the 1970s (figure 1.4) (Greenwood, 1995). Mefloquine has been, and is still, used as a prophylactic drug alone and for treatment either alone or as the partner

drug in artemisinin-based combination therapy (ACT) (Kremsner and Krishna, 2004).

As with the 4-aminoquinolines the quinoline methanols target the erythroctic stages of the parasite, but the precise mode of action of the quinoline methanols is not fully understood. Unlike the 4-aminoquinolines the quinoline methanols do not cause a build up of undigested haemoglobin, suggesting that they may inhibit the ingestion of the host cell haemoglobin (Famin and Ginsburg, 2002, Bray *et al.*, 2005b).

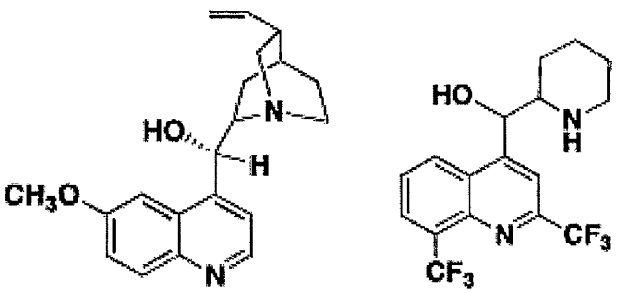

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Figure 1.4. Structures of the quinoline methanols, quinine (1) and mefloquine (2).

Decreased sensitivity to quinine was first reported in Brazil in 1910 (Bjorkman and Phillips-Howard, 1990) but resistance is not as widespread as chloroquine resistance due to it not being used as extensively (Foley and

Tilley, 1998). In areas where resistance is common, for example South East Asia, quinine is often used in combination with tetracycline (Bunnag *et al.*, 1996). Mefloquine resistance has been increasing since its introduction in the 1970s, with resistance occurring within five years of its introduction in an area of Thailand (White, 1994, Foley and Tilley, 1998). Mefloquine is used as a combination therapy where resistance has been noted, being used as an ACT with artesunate as a partner drug (Nosten *et al.*, 2000). Quinine and mefloquine share cross-resistance (O'Neill *et al.*, 1998) and resistance has been shown to be conferred by mutations in Pgh1, an integral membrane protein of the ATP-binding cassette superfamily encoded by PfMDR1 (Reed *et al.*, 2000, Bray *et al.*, 2005b).

(3) Bisquinolines

Piperaquine is a bisquinoline drug that was first synthesised in the 1960s and has been widely used in China since 1978 (figure 1.5) (Chen, 1991, Basco and Ringwald, 2003, Davis *et al.*, 2005). As with the other quinoline-containing drugs piperaquine targets the erythrocytic stages of parasite development.

The exact mode of action of piperaquine is unknown although it does accumulate within the parasite's digestive vacuole suggesting it may have a similar mode of action to that observed for the other quinoline-containing drugs (Warhurst *et al.*, 2007). Resistance has been reported in China but *in*

vitro tests of some African isolates have shown it to be effective there (Basco and Ringwald, 2003). There is some suggestion that piperaquine may be subject to a chloroquine related resistance mechanism, due to its similarity to the 4-aminoquinolines and the fact that it concentrates within the parasite's digestive vacuole, but the exact mechanism has not yet been identified (Warhurst *et al.*, 2007). An ACT partnering piperaquine with dihydroartemisinin is currently under development, although issues of possible antagonism are still being reviewed (Basco and Ringwald, 2003, Fivelman *et al.*, 2007).

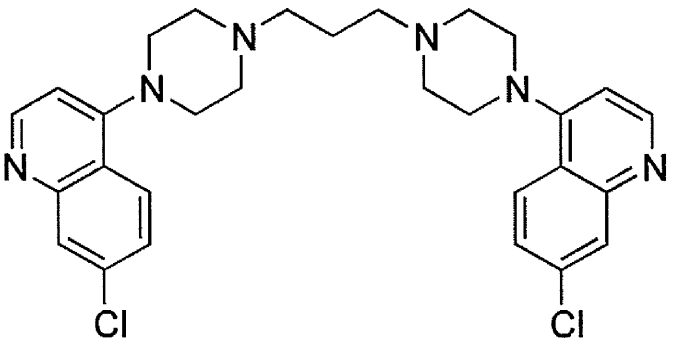


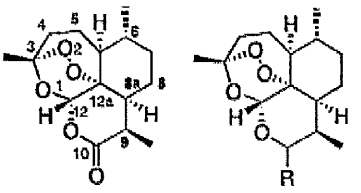
Figure 1.5. Structure of the bisquinoline, piperaquine.

1.2.3.2 Artemisinins

Artemisinin is a sesquiterpene trioxane lactone that was first isolated from the sweet wormwood shrub, *Artemisia annua*, in China in the 1970s (figure 1.6) (O'Neill *et al.*, 2010). Artemisinin itself is poorly soluble in

oil and water which reduces its therapeutic value, so derivatives of artemisinin were prepared to overcome this (Bray *et al.*, 2005b). Artemisinins have been shown to be effective against multi-drug resistant strains of *P. falciparum* (ter Kuile *et al.*, 1993) and are active against both the erythrocytic stages and gametocyte stages of the parasite (Kumar and Zheng, 1990, O'Neill *et al.*, 2010).

There is considerable debate on the precise mode of action and cellular target of artemisinins, although it has been noted that the endoperoxide bridge is essential for antimalarial activity (O'Neill *et al.*, 2010). Artemisinin derivatives are recommended to be used along with a partner drug for artemisinin-based combination therapy (ACT), with artemether-lumefantrine (marketed by Novartis as Coartem®) one of the leading combinations (Mueller *et al.*, 2006). Until recently resistance to artemisinin derivatives has not been a concern, however, resistance to the artesunate-mefloquine has now emerged on the Cambodian-Thai border (Alker *et al.*, 2007, O'Neill *et al.*, 2010).



Artemisinin

First Generation
Artemisinin Analogues

Dihydroartemisinin, R = β + α OH
Artemether, R = β-OMe
Arteether, R = β-OEt
Sodium artesunate, R = α-OC(O)$CH_2CH_2CO_2Na$
C-10-deoxyartemisinin, R = H

Figure 1.6. Structures of artemisinin and the first generation artemisinin analogues. Modified from Bray *et al* (2005b).

1.2.3.3 Antifolates

Antimalarial antifolates have been used extensively for treatment and prophylaxis for over 50 years (Nzila, 2006). As antifolates are a major part of this thesis they are discussed in depth in section 1.3.4.

1.3 *P. falciparum* Folate Pathway and Antimalarial Antifolates

The folate compound was discovered in the 1930s during work on anaemias. It was shown by Lucy Wills that a factor in yeast extract (Marmite) cured macrocytic anaemia of pregnancy (Hoffbrand and Weir, 2001). In 1941 a similar factor was isolated from spinach and was named folic acid from the latin *folium* meaning leaf (Mitchell *et al.*, 1941). Folic acid was synthesised in pure crystalline form in 1945 (Angier *et al.*, 1945)

and was shown to consist of a pteridine moiety, *para*-aminobenzoic acid (pABA) and glutamic acid, with this compound also being known as pteroylglutamic acid (figure 1.7) (Nzila, 2006). It was soon noted that folic acid is not a naturally occurring folate, with natural folate differing from folic acid in three ways: (1) reduction of the pteridine rings to di- or tetrahydro forms at positions 7,8 and 5,6, (2) additional glutamate residues forming polyglutamated derivatives and (3) additional single carbon units (methyl, formyl, methylene and methenyl) (Hoffbrand and Weir, 2001, Nzila, 2006). Higher animals, including humans, are unable to synthesise folate *de novo* and rely on dietary sources of pre-formed folate, whereas some microorganisms and plants are able to synthesise folates *de novo*. The *P. falciparum* malaria parasite is capable of both *de novo* biosynthesis and salvage of folate (Wang *et al.*, 2007). This difference in folate pathways between the malaria parasite and the human host is an attractive drug target and antimalarial antifolates have been used for over 50 years (Nzila, 2006).

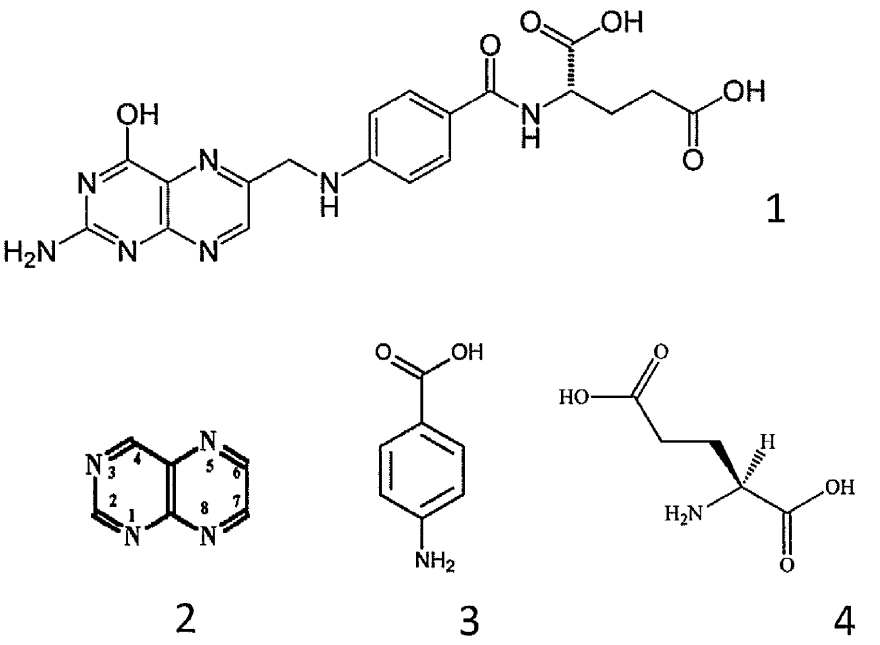


Figure 1.7. Structures of folic acid (1), pteridine (2), pABA (3) and glutamic acid (4).

1.3.1 *De novo* Folate Biosynthesis in *P. falciparum*

Unlike mammalian cells, *P. falciparum* is capable of *de novo* folate biosynthesis. The first evidence of the existence of this pathway in *P. falciparum* came from the susceptibility of the parasites to the antifolate sulphonamide and sulphone drugs which competitively interact with pABA and so inhibit the formation of folate derivitives in the parasite (Ferone, 1977). Further evidence came from the observation that the parasite is able to incorporate the radiolabelled folate precursors guanine

triphosphate (GTP), pABA and glutamic acid into folate end products (Krungkrai *et al.*, 1989b). Folate biosynthesis has been extensively characterised in bacteria and the key genes in the pathway identified (Neidhart, 1996). Biochemical, genomic and molecular studies have also identified key folate pathway enzymes and genes in *P. falciparum* including GTP cyclohydrolase (Krungkrai *et al.*, 1985, Lee *et al.*, 2001), hydroxymethyldihydropterin pyrophosphokinase-dihydropteroate synthase (HPPPK-DHPS) (Triglia and Cowman, 1994), dihydropteroate synthase-folylpolyglutamate synthase (DHPS-FPGS) (Salcedo *et al.*, 2001) and dihydrofolate reductase-thymidylate synthase (DHFR-TS) (Bzik *et al.*, 1987).

Biosynthesis of the pteridine moiety of the folate compounds starts with GTP which is subsequently converted to 7,8-dihydroneopterin triphosphate (DHNTP) (figure 1.8). This is catalysed by GTP cyclohydrolase which removes carbon 8 of the purine ring system forming DHNTP and formate. The next step in the *P. falciparum* pathway differs from that found in the conventional *de novo* folate biosynthesis pathway found in bacteria and plants (Dittrich *et al.*, 2008). In the conventional pathway DHNTP is converted to 6-hydroxymethyl-7,8-dihydropterin (HMDP) via a two step process, first the pyrophosphate group is removed then dihydroneopterin aldolase (DHNA) catalyses the cleavage of the lateral side chain releasing glycoaldehyde and producing HMDP (Bermingham and Derrick, 2002).

No candidate genes for DHNA have been found in *Plasmodium* but an unusual orthologue of 6-pyruvoyltetrahydopterin synthase (PTPS) has been found that could compensate for this (Dittrich *et al.*, 2008). The *P. falciparum* PTPS (PfPTPS) enzyme converts DHNTP to 6-pyruvoyl-5,6,7,8-tetrahydopterin and HMDP. This HMDP is then converted to 6-hydroxymethyl-7, 8-dihydropterin pyrophosphate (DHPPP) in a reaction catalysed by 6-hydroxymethyl-7, 8-dihydropterin pyrophosphokinase (HPPPK) with the transfer of the pyrophosphate moiety from ATP (Bermingham and Derrick, 2002, Hyde, 2005).

The *P. falciparum* parasite is also capable of *de novo* biosynthesis of pABA (figure 1.8). The shikimate pathway is responsible for the production of chorismate, a pABA precursor, and this pathway has been shown to be present in *P. falciparum* (Keeling *et al.*, 1999, Gardner *et al.*, 2002, Triglia and Cowman, 1999b). Chorismate is converted to pABA, via 4-amino-4-deoxychorismate (ADC), a reaction catalysed in *P. falciparum* by PABA synthase (a bipartite protein with domains homologous to *Escherichia coli* PabA and PabB) (Triglia and Cowman, 1999b). Dihydropteroate synthase (DHPS) condenses pABA (either from the *de novo* synthesis mentioned above or salvaged) and DHPPP to dihydropteroate. A glutamate residue is then attached to the carboxyl moiety of pABA in an ATP-dependent reaction catalysed by dihydrofolate synthase (DHFS) converting dihydropteroate to dihydrofolate (DHF). In a

NADPH dependent reaction dihydrofolate reductase (DHFR) reduces DHF to the biologically active tetrahydrofolate (THF). The polyglutamation of synthesised THF or salvaged substituted THF cofactors is an MgATP dependent reaction catalysed by folylpolyglutamate synthase (FPGS) (Nzila *et al.*, 2005a).

1.3.2 Folate Salvage by *P. falciparum*

As well as *de novo* folate biosynthesis the malaria parasite is also capable of salvaging folate precursors, pre-formed folate and C_1 substituted folate derivatives. Evidence of a need for pABA salvage by *Plasmodium* was first indicated in the 1960s when it was shown that *P. berghei* parasites had reduced growth when the mouse hosts were fed a pABA-deficient diet, with parasite growth returned to normal with the addition of pABA (Jacobs, 1964). This is further supported by the fact that induced folate deficiency in rhesus monkeys conferred protection against disease when injected with *P. cynomolgy* (Das *et al.*, 1992). Experiments *in vitro* have also shown that *P. falciparum* is able to take up exogenous radiolabelled folic acid, folinic acid and pABA and convert it to polyglutamated folate end products (Krungkrai *et al.*, 1989b, Wang *et al.*, 2004). The transport of exogenous folate by *P. falciparum* has now been characterised and shown to be a specific energy-dependent, saturable process, inhibited by the

classical anion transport inhibitors probenecid and furosemide (Wang *et al.*, 2007).

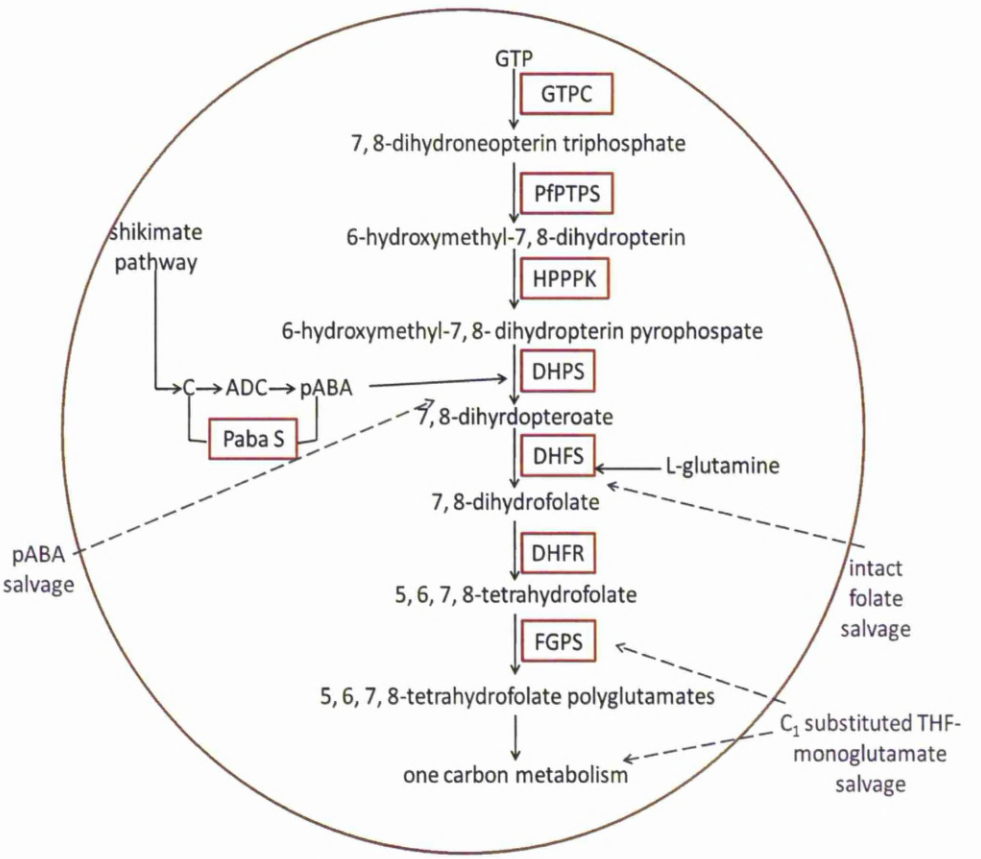


Figure 1.8. *P. falciparum de novo* folate biosynthesis pathway and possible salvage routes. Enzymes are boxed in red and possible salvage is shown with broken arrows. Abbreviations: GTP, guanine triphosphate; GTPC, guanine triphospate cyclohydrolase I; PfPTPS, *P. falciparum* pyruvoyltetrahydropterin synthase; HPPPK, hydroxymethyldihydropterin pyrophosphokinase; DHPS, dihydropteroate synthase; DHFS, dihydrofolate synthase; DHFR, dihydrofolate reductase; FPGS, folylpolyglutamate synthase; C, chorismate; ADC, 4-amino-4-deoxychorismate; pABA, *para*-aminobenzoic acid; Paba S, PABA synthase. Modified from Hyde (2005), Nzila (2005a), Ochong (2008) and Dittrich *et al* (Dittrich *et al.*, 2008).

1.3.3 Role of Folates in *P.falciparum*

The biologically active THF derivatives are essential cofactors for one carbon metabolism. In *P. falciparum* they are essential for major biosynthetic pathways including the pyrimidine deoxythymidine-5'-monophosphate (dTMP) via the thymidylate cycle and the synthesis of methionine. The folate-dependent reactions in *P. falciparum* are summarised in figure 1.9. In mammalian cells THF derivatives are also essential in purine synthesis but the enzymes involved in this pathway are not found in *Plasmodium* and so it has been assumed that the parasite relies on purine salvage (Reyes *et al.*, 1982).

1.3.3.1 Thymidylate Cycle

5, 10-methylene tetrahydrofolate is the single carbon donor involved in converting 2'-deoxyuridine-5'-monophosphate (dUMP) to dTMP (Nzila *et al.*, 2005a). This reaction is catalysed by thymidylate synthase (TS) and the reduction of the methylene to a methyl group is accompanied with the oxidation of THF to DHF. This resulting DHF is recycled back into the folate pathway and reduced by DHFR to the biologically active THF (Nzila *et al.*, 2005a) .

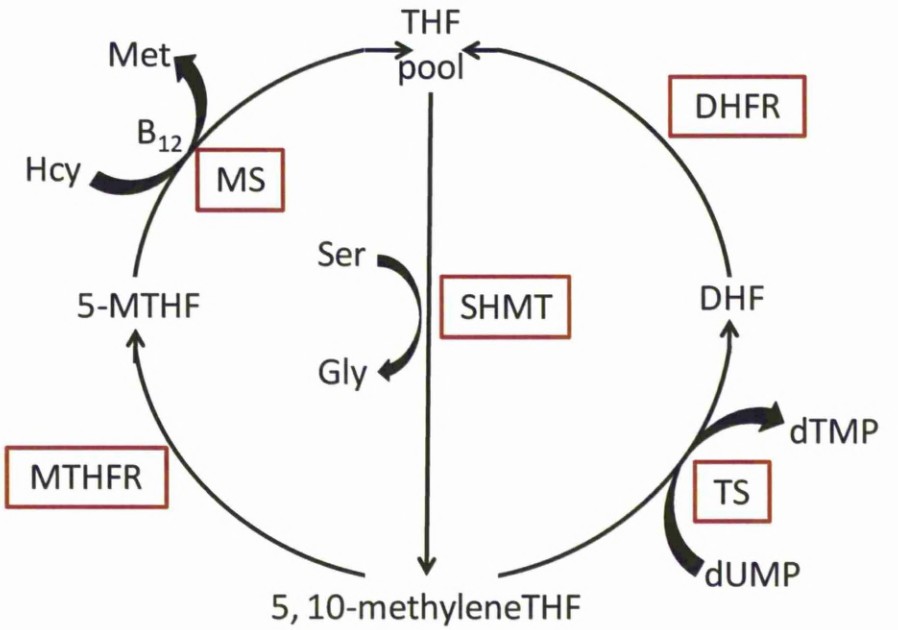


Figure 1.9. Folate-dependent reactions in *P. falciparum.* Enzymes are boxed in red. Abbreviations: THF, tetrahydrofolate; SHMT, serine hydroxymethyl transferase; TS, thymidylate synthase; DHF, dihydrofolate; DHFR, dihydrofolate reductase; MTHFR, methylenetetrahydrofolate reductase; 5-MTHF, 5-methyltetrahydrofolate; MS, methionine synthase; B_{12}, methylcobalamin. Modified from Krungkrai *et al* (1990).

1.3.3.2 Methionine Cycle

The folate pathway provides the one-carbon donor to methionine biosynthesis. 5-methyltetrahydrofolate (5-MTHF) is the methyl donor in the biosynthesis of methionine from homocysteine. This 5-MTHF is derived from 5, 10-methylenetetrahydrofolate in a reaction catalysed by methylenetetrahydrofolate reductase (MTHFR) (Nzila *et al.*, 2005b). The cobalamin-dependent methionine synthase catalyses the conversion

of homocysteine to methionine and results in the demethylation of 5-MTHF to THF. Both MTHFR and methionine synthase have been found in *P. falciparum* showing that the methionine can be synthesised by the parasite (Krungkrai *et al.*, 1989a, Asawamahasakda and Yuthavong, 1993). Methionine is required for protein synthesis and for the synthesis of S-adenosylmethionine (S-AdoMet) the methyl group donor for ubiquitous methylation of nucleic acids, proteins and lipids. Methionine is converted to (S-AdoMet) in an ATP-dependent reaction catalysed by methionine adenosyltransferase. The resulting S-AdoMet is an allosteric inhibitor of MTHFR providing feedback inhibition which is important as the MTHFR catalysed conversion of 5, 10-methyleneTHF to 5-MTHF is irreversible (Bailey, 2010).

1.3.4 Antimalarial Antifolates

Only two enzymes of the folate pathway have been used as pharmalogical targets for malaria chemotherapy so far. These are DHPS and DHFR, with the sulpha drugs targeting DHPS and pyrimethamine, proguanil and cholorproguanil targeting DHFR (Nzila, 2006).

1.3.4.1 Anti-DHFR Antimalarial Antifolates

1.3.4.1.1 Proguanil

The first reported antifolate agent with antimalarial properties was proguanil. It was discovered during a research programme led by Imperial Chemical Industries (ICI) with the first report of its antimalarial properties in 1945 showing that it was more active than quinine on avian malarias (Nzila, 2006). Proguanil itself is a prodrug with the anti-DHFR activity coming from the cycloguanil (CCG) metabolite (figure 1.9). Proguanil has been used as prophylaxis and treatment, with a prophylactic combination of proguanil and atovaquone now being marketed as Malarone® (Kain, 2003).

1.3.4.1.2 Pyrimethamine

The antifolate property of pyrimethamine (PYR) was recognised as other drugs of the same class showed a structural similarity to proguanil (figure 1.9) (Falco *et al.*, 1951). It was first introduced for routine human use in 1952 and is one of the most widely used antimalarial antifolates so far, either used alone or in combination with sulphadoxine (SDX) (Hyde, 1990, Nzila, 2006).

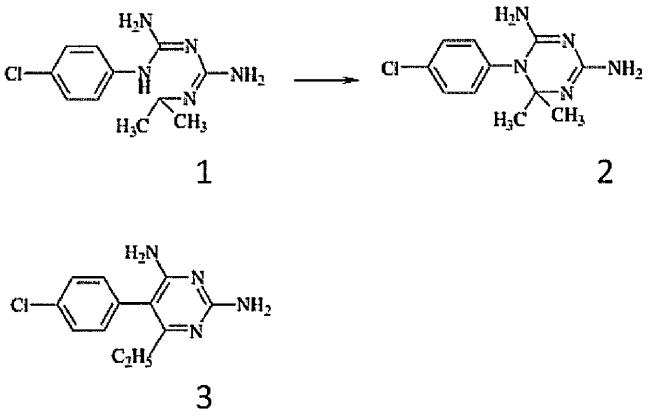


Figure 1.10. Structures of DHFR inhibitors proguanil (1), cycloguanil (2) and pyrimethamine (3).

1.3.4.1.3 Mode of Action of Anti-DHFR Antimalarial Antifolates

PYR and the active metabolite of proguanil, CCG, act on the folate pathway by competitively inhibiting DHFR enzyme (Hyde, 1990). It has been shown that PYR and CCG bind to the *Plasmodium* DHFR more strongly than the human DHFR allowing the drugs to selectively target the parasites (Ferone *et al.*, 1969, Sirawaraporn *et al.*, 1993). This disruption of the folate pathway prevents further folate-dependent reactions (as described in section 1.3.3) and so prevents DNA synthesis, methylation of proteins and nucleic acids and other essential pathways. Anti-DHFR drugs have the most marked effect against the late trophozoite and schizont stages of parasite development (Schellenberg and Coatney, 1961, McCormick *et al.*, 1971, Janse *et al.*, 1986, Banyal and Inselburg, 1986).

This correlates with the fact that these drugs target the folate pathway as this stage of development is where DNA replication reaches a maximum (Hyde, 1990).

1.3.4.2 Anti-DHPS Antimalarial Antifolates

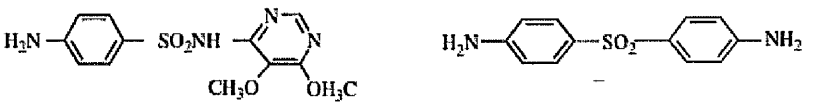
1.3.4.2.1 Sulphadoxine

The bactericidal and antimalarial properties of the sulphonamides was discovered in the 1930s when sulphanilimide, a metabolite of prontosil dye, was observed to have an inhibitory effect (Díaz de León, 1937). Sulphadoxine (SDX) is often used in combination with PYR and the addition of the anti-DHFR synergizes the effect of SDX. This combination is commonly known as SP and marketed as Fansidar®.

1.3.4.2.2 Dapsone

Dapsone is a sulphone drug that was initially synthesised in 1908 in a search for molecules to produce azo dyes. Its microbial properties were noted in the 1930s and in the 1940s it was found to have antimalarial properties (Nzila, 2006). However, dapsone was not initially considered for antimalarial use due to worries about toxicity and the fact that there were other antimalarials already available (Nzila, 2006). Due to increasing worries over resistance to other available antimalarial drugs interest in

dapsone has been renewed, with a combination of the DHFR inhibitor chlorproguanil and dapsone being marketed as Lapdap® (Watkins *et al.*, 1997, Winstanley *et al.*, 1997). As with anti-DHFR and anti-DHPS combination SP, the Lapdap® combination is also synergistic. Dapsone is also used to treat leprosy (Britton and Lockwood, 2004) and pneumocystic pneumonia (Watson, 2002) and it is the most widely used anti-DHPS agent in human chemotherapy (Nzila, 2006).



1 2

Figure 1.11. Structures of the DHPS inhibitors sulphadoxine (1) and dapsone (2).

1.3.4.2.3 Mode of Action of Anti-DHPS Antimalarial Antifolates

SDX and dapsone act on the late erythrocytic stages of the parasite, with the mode of action being inhibition of DHPS (Sibley *et al.*, 2001, Nzila, 2006). These anti-DHPS drugs are structural analogues of pABA and interfere with the folate pathway by blocking pABA incorporation into dihydropteroate (Hyde, 2005). This mode of action has been confirmed by the fact that the addition of an exogenous supply of pABA can reverse the inhibitory effect of the anti-DHPS drugs (Wang *et al.*, 1997b).

1.3.4.3 Mechanisms of Resistance to Antimalarial Antifolates

The combination of SDX and PYR (SP) has been used for almost 20 years as a first line treatment but drug resistance quickly emerged and is now drastically reducing the effectiveness of these drugs (Sibley *et al.*, 2001). The understanding of mechanisms behind the rapid emergence of resistance to antimalarial antifolates is important as it can allow informed choices on how to overcome resistance or develop novel antimalarial antifolates. The mechanisms behind resistance to anti-DHFR and anti-DHPS drugs in *P. falciparum* have been shown to be mainly due to point mutations in the *dhfr* and *dhps* genes (Peterson *et al.*, 1988, Sibley *et al.*, 2001). PYR resistance first occurs with a mutation of serine to asparagine at codon 108 of the *dhfr* gene, which increases resistance approximately 100-fold (Peterson *et al.*, 1988, Sibley *et al.*, 2001). Resistance increases with further mutations from asparagine to isoleucine at codon 51, cysteine to arginine at codon 59 and isoleucine to leucine at codon 164 (Peterson *et al.*, 1988, Sibley *et al.*, 2001, Nzila, 2006). Point mutations in the *dhps* domain of the *dhps-hpppk* gene have been shown to confer resistance to SDX (Nzila, 2006). Combinations of point mutations from serine to alanine or phenylalanine at codon 436, alanine to glycine at codon 437, lysine to glutamate at codon 540, alanine to glycine at codon 581 and alanine to serine or threonine at codon 613 have been shown to play a role in the development of resistance to anti-DHPS compounds (Triglia and Cowman, 1999a, Sibley *et al.*, 2001, Nzila, 2006).

1.4 Membrane Transport Pathways of *P. falciparum*

The *P. falciparum* parasite has to cope with several different extracellular environments during its life cycle. The asexual stage in the RBC of the host is the phase that causes the clinical symptoms observed in malaria patients. This intracellular location allows the parasite to evade the host immune system but also means that it has to deal with surviving within a relatively inert cell with limited transport abilities. The parasite can obtain some nutrients from the digestion of haemoglobin but must rely on a supply of certain nutrients from the external milieu of the RBC (Divo *et al.*, 1985). Unlike other eukaryotic cells the host RBC also has an unusually low Na^+ concentration and high K^+ concentration which poses further problems for the parasite (Kirk, 2001, Kirk, 2004, Biagini *et al.*, 2005). In order to survive within this intracellular environment the parasite must have evolved membrane transport mechanisms to meet its nutritional requirements, to maintain its chemical composition and to allow elimination of waste metabolites from the parasite and the host cell (Kirk, 2001).

The intraerthrocytic stages of the *Plasmodium* life cycle have three membranes separating them from the external environment (blood): the host RBC plasma membrane, the parasitophorous vacuole membrane

(PVM) and the parasite plasma membrane (PPM). The parasite also contains several membrane bound intracellular organelles, including the nucleus, food vacuole, mitochondrion, apicoplast, Golgi apparatus and endoplasmic reticulum (Kirk, 2001, Staines *et al.*, 2010). Therefore, the parasite must use a range of transport pathways to distribute solutes around the intracellular environment, as well as into and out of the cell. The transport proteins that the parasite uses to achieve this can be split into two groups: channels and transporters. Channels are integral membrane proteins that form hydrophilic pores spanning the lipid bilayer and provide a pathway for diffusion (Wolfersberger, 1994). They may be regulated by a range of physiological signals (for example, voltage and ligand gated channels) and allow passage of the appropriately sized and charged solutes down their electrochemical gradient (Kirk, 2004). Transporters (also known as carriers) are also integral membrane proteins but do not provide an open diffusion pathway, instead they transport solutes across a membrane by binding and undergoing a conformational change. The three main classes of transporters are facilitative transporters (uniporters), primary active transporters (pumps) and secondary active transporters (symporters and antiporters) (Staines *et al.*, 2010). Facilitative transporters are the simplest of the three as they facilitate the transport of substrates down their electrochemical gradient. The *P. falciparum* hexose transporter (PfHT) and the human RBC glucose transporter (GLUT1) are examples of facilitative transporters (see figure 1.12). Primary active transporters use

energy derived from the hydrolysis of phosphodiester bonds in molecules like ATP and pyrophosphate in order to drive the active transport of solutes. In the RBC membrane there are several examples of primary active transporters (the plasma membrane Ca^{2+} pump and the Na^{+}/K^{+} ATPase) and in the PPM there is a V-type H^{+} pump (figure 1.12). Secondary active transporters use energy derived from electrochemical gradients of solutes such as Na^{+} and H^{+} and couples the transport of one solute to the transport of another, either in the same direction (symporters) or in the opposite direction (antiporters) (Kirk, 2004, Staines *et al.*, 2010). Examples of secondary active transport include the H^{+} coupled lactate and pantothenate symporters in the PPM and the H^{+} coupled monocarboxylate transporter (MCT1) and the HCO_3^{-}/Cl^{-} exchanger (band 3) in the RBC plasma membrane (figure 1.12) (Staines *et al.*, 2010).

Initial annotation of the *P. falciparum* genome in 2002 identified only 54 putative transport proteins and no obvious homologues of sodium, chloride or potassium ion channels (Gardner *et al.*, 2002). A further analysis of the *P. falciparum* genome by Martin *et al* (2005) revealed a total of 109 putative transport proteins, taking the number of putative transport genes to 2.1% of all genes encoded for. This is a relatively low percentage compared to other eukaryotic genomes, for example 4.2% of the total proteins encoded by the *Saccharomyces cerevisae* genome are transport proteins and in *Homo sapiens* it is 3.4% (Martin *et al.*, 2005).

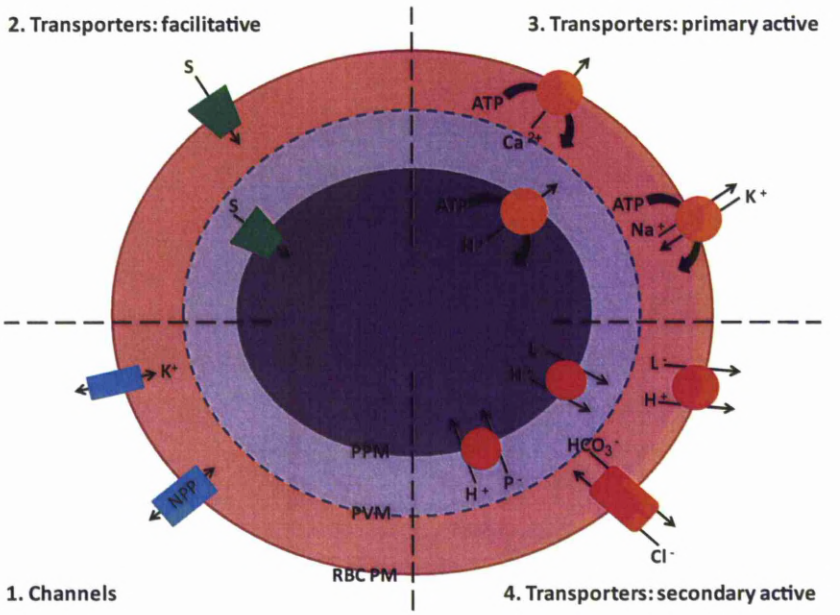


Figure 1.12. Schematic illustration of the classes of transport proteins and examples of parasite and host transport processes in *Plasmodium* infected RBCs. The three membranes shown are the RBC plasma membrane (RBC PM), the parasitophorous vacuole membrane (PVM), and the parasite plasma membrane (PPM). 1. Channels: examples are the parasite-induced new permeation pathways (NPP) and the Gardos channel (Ca^{2+} activated K^+ channel) in the RBC PM. 2. Facilitative transporters: examples are the hexose transporters PfHT (in the PPM) and GLUT1 (in the RBC PM). 3. Primary active transporters: examples are a V-type H^+ pump in the PPM, and the Na^+/K^+ ATPase and the plasma membrane Ca^{2+} pump in the RBC PM. 4. Secondary active transport: examples are the H^+ coupled pantothenate (P^-) and lactate (L^-) symporters in the PPM, and the HCO_3^-/Cl^- exchanger (Band 3) and the H^+-coupled monocarboxylate symporter (MCT1, which is responsible for the efflux of lactate) in the RBC PM. Modified from Staines *et al* (2010).

Infection by the *Plasmodium* parasite causes changes to some of the host RBCs endogenous transport systems (Ginsburg and Stein, 1987, Sherman, 1988, Cabantchik, 1989, Cabantchik, 1990, Gero and Kirk, 1994, Elford *et al.*, 1995). For example, up to a two fold increase in activity of the host cells endogenous Na^+/K^+ ATPase has been observed in *P. falciparum*

parasitised RBCs (Kirk *et al.*, 1991, Staines *et al.*, 2001). As the parasite develops within the host RBC the host cell membrane becomes more permeable to certain ions and small molecules (Kirk, 2004). An increased rate of transport has been demonstrated for certain solutes including monosaccharide sugars (Ginsburg *et al.*, 1985, Kirk *et al.*, 1994, Kirk *et al.*, 1996), peptides (Atamna and Ginsburg, 1997), nucleosides (Gero and Wood, 1991, Upston and Gero, 1995), small ammonium compounds (Staines *et al.*, 2000), monovalent inorganic ions (Cranmer *et al.*, 1995, Kirk *et al.*, 1994), various monocarboxylates (Cranmer *et al.*, 1995) and amino acids (Elford *et al.*, 1985, Ginsburg *et al.*, 1985, Kirk *et al.*, 1994). Some of the increase in uptake of low molecular weight solutes across the host RBC membrane has been attributed to the parasite-induced new permeation pathways (NPP) (Kirk, 2004). The contribution of the NPP to the uptake of particular nutrients depends on the relative rates of uptake by endogenous transporters compared to uptake via the NPP (Kirk and Saliba, 2007). For example, although glucose is a substrate of the NPP the uptake of glucose across the RBC membrane in *Plasmodium* infected RBCs is mainly via an endogenous high capacity equilibrative hexose transporter (GLUT1), with negligible uptake *via* the NPP (Kirk *et al.*, 1996, Kirk and Saliba, 2007). The opposite is true for the essential nutrient pantothenate which relies exclusively on uptake across the RBC membrane via the NPP as the RBC lacks any endogenous transporter of pantothenate (Saliba *et al.*, 1998). It is still unknown if the NPP consist of single or multiple channels

or if they are parasite or host derived (Kirk *et al.*, 1994, Alkhalil *et al.*, 2004, Decherf *et al.*, 2004, Biagini *et al.*, 2005, Staines *et al.*, 2006). Experiments using inhibitors and measuring uptake of various substrates have shown the NPP to be anion-selective although they do also allow transport of cationic and electroneutral solutes at a lower rate (Kirk *et al.*, 1994, Staines *et al.*, 2005). Induction of the NPP appears to take place 10 to 20 hours after invasion (Kirk, 2001, Staines *et al.*, 2004).

Electrophysiological studies of the PVM have indicated the presence of a high conductance channel that is permeable to a range of anions and cations and is open for more than 98 % of the time (Desai *et al.*, 1993, Desai and Rosenberg, 1997). Another study that involved the use of streptolysin O to permeabilise the RBC membrane, but not the PVM, showed that a normally membrane impermeant biotin derivative gained access to parasitophorous vacuole proteins (Nyalwidhe *et al.*, 2002). These findings suggest that the PVM allows free passage of low molecular weight nutrients, ions and metabolic wastes (Kirk, 2004).

The PPM is not freely permeable and maintains substantial ion gradients, with the movement of solutes across the PPM mediated via a range of transporters and channels (Kirk and Saliba, 2007). Transport across the PPM has been investigated for many substrates including nucleosides (Upston and Gero, 1995, Gero and Hall, 1997, Downie *et al.*, 2006,

Downie *et al.*, 2008, Quashie *et al.*, 2008, Quashie *et al.*, 2010), glucose (Goodyer *et al.*, 1997, Kirk *et al.*, 1996, Krishna *et al.*, 2000, Woodrow *et al.*, 2000, Landfear, 2010), choline (Biagini *et al.*, 2004, Lehane *et al.*, 2004), pantothenic acid (Saliba and Kirk, 2001), inorganic phosphate (Saliba *et al.*, 2006), lactate and pyruvate (Kanaani and Ginsburg, 1991, Elliott *et al.*, 2001), ATP/ADP (Kanaani and Ginsburg, 1989, Choi and Mikkelsen, 1990), and isoleucine (Martin and Kirk, 2007). The *Xenopus laevis* oocyte heterologous expression system has been a valuable tool in the expression and characterisation of *Plasmodium* encoded transporters. It has been used to characterise a nucleoside transporter designated either PfNT1 (Carter *et al.*, 2000) or PfENT1 (Parker *et al.*, 2000), a hexose transporter designated PfHT (Krishna and Woodrow, 1999, Woodrow *et al.*, 1999, Woodrow *et al.*, 2000) a Ca^{2+} P-type ATPase designated PfATP4 (Krishna *et al.*, 2001b), and a Na^{+}:phosphate symporter designated PfPiT (Saliba *et al.*, 2006). These functionally characterised transporters are described in more detail in section 1.4.1.

Few transporters responsible for the movement of solutes into and out of the membrane-bound organelles of the intraerythrocytic have been characterised. Several transporters have been identified on the digestive vacuole membrane including a thapsigargin (and cyclopiazonic acid)-sensitive Ca^{2+} pump (Biagini *et al.*, 2003a), at least two discrete H^{+} pumps (a H^{+} -pyrophosphatase and a H^{+} -ATPase (Kirk and Saliba, 2007), and

two integral membrane proteins PfCRT1 (Fidock *et al.*, 2000) and Pgh-1 (Reed *et al.*, 2000). Very few transporters involved in the movement of metabolites and nutrients have been identified in the membranes of other organelles. Using the presence of signal peptides to indicate the targeting of proteins to an organelle has allowed putative organelle associated transport proteins to be identified. Several putative apicoplast transporters have been identified in this way including ABC transporters, P -type ATPases, a putative amino acid transporter, and other putative transport proteins with unknown functions (Martin *et al.*, 2005, Kirk and Saliba, 2007). Signal peptides have also been used to identify nine transporters with putative mitochondrial targeting (Kirk and Saliba, 2007).

As well as transport pathways mediating the movement of nutrients into, out of and around the *Plasmodium* infected RBC the parasite also uses endocytosis to ingest the RBC cytosol (Kirk and Saliba, 2007). To do this the parasite uses the cytostome, a specialised mouth-like structure at the surface of the parasite. Double-membrane transport vesicles are formed from the cytostome with the inner membrane formed from the PVM and the outer from the PPM. These then move to the parasite's digestive vacuole where they fuse and release their contents. Haemoglobin is the major component of the RBC cytoplasm and is broken down in the digestive vacuole and/or the transport vesicle (Hempelmann *et al.*, 2003) by a range of proteases (McKerrow *et al.*, 1993, Coombs *et al.*, 2001, Kirk

and Saliba, 2007). Peptides and amino acids resulting from the digestion of haemoglobin are though to be exported from the digestive vacuole (Kolakovich *et al.*, 1997) with haemozoin crystallised from the residual haem groups in the digestive vacuole (Kirk and Saliba, 2007)

1.4.1 Currently Identified *P. falciparum* Membrane Transporters

1.4.1.1 *P. falciparum* Hexose Transporter (PfHT)

The expression of a facilitative glucose transporter on the PPM was suggested from studies of glucose uptake in parasitised RBCs (Kirk *et al.*, 1996, Landfear, 2010). The presence of a glucose transporter encoded by the parasite was subsequently confirmed using the *X. laevis* oocyte heterologous expression system. *X. laevis* oocytes injected with mRNA from intraerythrocytic stages of *P. falciparum* showed a significant increase in uptake (7 fold) of radiolabelled 2-deoxy-D-glucose compared to control oocytes injected with water (Penny *et al.*, 1998). Comparative searches of a pre-launched version of the *P. falciparum* genome allowed the identification of the *P. falciparum* hexose transporter (PfHT) using the sequence of known glucose transporters, with PfHT sharing 29.8 % amino acid sequence identity with GLUT1 and 20-27 % amino acid sequence identity to other glucose transporters in eukaryotes and prokaryotes (Woodrow *et al.*, 1999). This transporter is a member of the major

facilitator superfamily (MFS). When expressed in *X. laevis* oocytes PfHT induced saturable, stereospecific and Na^+-independent uptake of glucose (K_m = 0.48 mM) and 2-deoxyglucose (K_m = 1.31 mM) (Woodrow *et al.*, 1999). PfHT showed a broad specificity for hexoses with uptake of both substrates inhibited by fructose, mannose, galactose, 6-deoxyglucose and 3-*O*-methylglucose (Woodrow *et al.*, 1999, Landfear, 2010). Cytochalasin B, a classical inhibitor of mammalian GLUTs, also inhibited glucose uptake by PfHT (Woodrow *et al.*, 1999). PfHT mRNA expression was shown to be developmentally regulated using tandem competitive polymerase chain reaction (TC-PCR) with peak mRNA levels at the small ring stage and lowest in gametocytes (Woodrow *et al.*, 1999). This pattern of expression is consistent with the differing levels of glucose utilisation by the maturing intraerythrocytic parasite (Krishna *et al.*, 2001a). PfHT was shown to be localised to the PPM using confocal immunofluorescence microscopy (Woodrow *et al.*, 1999).

Fructose has been shown to be an alternative to glucose as an energy source for the intraerythrocytic parasite and PfHT expressed in *X. laevis* oocytes is capable of transporting fructose as well as glucose (Woodrow *et al.*, 2000). The substrate selectivity of PfHT was investigated in *X. laevis* oocytes with the mutation of the Q169 residue, located within the predicted amphipathic transmembrane helix 5, to an asparagine residue (Q169N). This resulted in transport of glucose similar to that observed for native

PfHT but eliminated fructose transport, indicating that this single residue in helix 5 is involved in determining the substrate specificity of PfHT (Woodrow *et al.*, 2000, Landfear, 2010). Genetic studies have shown PfHT to be essential for parasite growth as parasites were not viable when PfHT was knocked out unless the gene was complemented by an episomal construct (Slavic *et al.*, 2010).

1.4.1.2 *P. falciparum* Nucleoside Transporters

Malaria parasites lack the enzymes required for synthesise of purines but do have the enzymes necessary for de novo synthesis of pyrimidines (Gero and O'Sullivan, 1990, de Koning *et al.*, 2005, Downie *et al.*, 2006). Therefore, the intraerythrocytic parasite must rely on salvaging purines from the external milieu (Divo *et al.*, 1985). Purines have been shown to enter the parasitised RBC mainly via the human equilibrative nucleoside transporter (hENT1) and the human facilitative nucleobase transporter (hFNT1), both of which are endogenous transporters on the host RBC (Quashie *et al.*, 2010).

As with the *P. falciparum* hexose transporter evidence for a purine transporter came from the use of the *X. laevis* oocyte heterologous expression system. *X. laevis* oocytes injected with mRNA from intraerythrocytic stages of *P. falciparum* showed increased transport of

adenosine and hypoxanthine compared to water-injected control oocytes (Penny *et al.*, 1998). Four members of the equilibrative nucleoside transporter (ENT) family have been found in the *P. falciparum* genome (Martin *et al.*, 2005). One of these genes, designated PfNT1 (Carter *et al.*, 2000) or PfENT1 (Parker *et al.*, 2000), was cloned and characterised using the *X. laevis* expression system by two groups at around the same time giving very different results. Expression of PfNT1 in *X. laevis* oocyte by Carter *et al.* (2000) showed high affinity transport of adenosine (K_m = 13 μM), which was inhibited by dipyridamole, but unable to transport nucleobases. This is in contrast to the findings by Parker *et al.* (2000) who reported PfENT1 to have much a lower affinity for adenosine (K_m = 320 μM), which was not inhibited by dipyridamole, and was able to transport nucleobases. Despite these differing results it was clear that PfNT1 is indeed a nucleoside transporter but that further studies were required to reconcile the results from these two studies (de Koning *et al.*, 2005, Downie *et al.*, 2006). Experiments using quantitative PCR showed that PfNT1 is expressed throughout the intraerythrocytic stages of the parasite's lifecycle and immunoelectron microscopy has shown it to be localised to the PPM (Rager *et al.*, 2001). Further characterisation of PfNT1 using the *X. laevis* oocyte expression system showed low affinity transport of adenosine (K_m = 1.86 ± 0.28 mM) and thymidine (K_m = 1.33 ± 0.17 mM) (Downie *et al.*, 2006) and also low affinity transport of adenine (K_m = 0.82 mM) (Downie *et al.*, 2008). Transport measurements by Downie *et al.*

(2006, 2008) with *P. falciparum* trophozoites isolated by saponin treatment showed similar kinetic characteristics for adenosine, thymidine and adenine transport and similar substrate specificity characteristics as observed for PfNT1 in *X. laevis* oocytes above. These results led to the conclusion that PfNT1 is a high-capacity, low-affinity transporter that acts as a major route for the transport of nucleosides and nucleobases across the PPM (Downie *et al.*, 2006, Downie *et al.*, 2008, Kirk *et al.*, 2009). A separate study by Quashie *et al.* (2008) on the purine uptake by *P. falciparum* trophozoites isolated by saponin treatment gave different results to those described above. In this study four purine transport activities were identified in intraerythrocytic parasites with parasite expression of (1) a high-affinity hypoxanthine transporter with a secondary capacity for purine nucleosides (PfNT1), (2) a separate high-affinity transporter for adenine, (3) a low-affinity transporter for adenosine and (4) a low-affinity, high-capacity adenine transporter (Quashie *et al.*, 2008). These results show a similarity with purine salvage reported in other *Plasmodium* species and show purine uptake in *P. falciparum* to be closer to purine transport activity in other protozoa than the results from Downie *et al.* (2008, 2006) suggest (de Koning *et al.*, 2005). A subsequent attempt to reconcile these differing results has been made by Kirk *et al.* (2009) who suggested that Quashie *et al.* (2008) may not have taken enough account of the effect of metabolism.

The other three members of the ENT family encoded by *P. falciparum* (PfNT2, PfNT3 and PfNT4) have not yet been characterised. However, PfNT2 has been shown to be localised to the endoplasmic reticulum using confocal and immunoflourescence microscopy (Downie *et al.*, 2010). PfNT2 is therefore the first intracellular purine permease to be found in apicomplexan parasites.

1.4.1.3 *P. falciparum* Ca^{2+} -ATPase (PfATP4)

The *P. falciparum* genome encodes six P-type ATPases (PfATP1-4, PfATP6 and PfATP7). Immunoflourescence microscopy has shown that the *P. falciparum* Ca^{2+} -ATPase PfATP4 is localised to the PPM of asexual stage parasites (Dyer *et al.*, 1996). Analysis of the hydropathy profile of PfATP4 showed that it does not resemble that of SERCA (sarco/endoplasmic reticulum)-type ATPases or PMCA (plasma membrane)-type ATPases (Krishna *et al.*, 2001b). Characterisation of PfATP4 using the *X. laevis* oocyte expression system showed it had activity that is resistant to inhibition by ouabain (a Na^+/K^+ ATPase inhibitor) and thapsigargin (a selective SERCA inhibitor) but is inhibited by vanadate (a pentacoordinate phosphate analogue) and cyclopiazonic acid (a metabolite from *Aspergillus penicillum* that inhibits SERCA and PMR (yeast Golgi)-type pumps but not PMCA) (Krishna *et al.*, 2001b). The same study also showed that the Ca^{2+} dependent activity of PfATP4 is

stimulated by a broader range of $[CA^{2+}]_{free}$ than the rabbit SERCA1a or the avian SERCA1 pump. Analysis of the expression of PfATP4 throughout the parasite's life cycle was carried out using quantitative PCR and showed that expression varied throughout the life cycle, with a five fold increased expression in meronts compared to ring stages (Krishna *et al.*, 2001b). These results have led to the conclusion that PfATP4 defines a novel subclass of Ca^{2+} -ATPases that are unique to apicomplexan parasites (Krishna *et al.*, 2001b).

1.4.2 *P. falciparum* Membrane Transport Pathways as Drug Targets

With the emergence and spread of resistance seriously threatening the usefulness of the currently available antimalarials it is vital to further understand the biology of the parasite in order to find new novel drug targets and to understand the mechanisms of resistance. Membrane transport pathways are a potential way of delivering drugs into the parasite and the transport proteins are potential targets themselves (Kirk, 2004, Staines *et al.*, 2010, Biagini *et al.*, 2005). The principle behind transporters as drug targets is to disrupt the uptake of one or more essential nutrients or to block the efflux of harmful metabolic waste products. Membrane transporters as a drug delivery strategy allow a route of entry for drugs that target essential cellular mechanisms, and it has been observed that carrier

mediated uptake of drugs may be more common than traditionally thought (Dobson and Kell, 2008). Drugs targeting human membrane transporters are used to treat several diseases including congestive heart failure, blood pressure disorders, diabetes, and depression (Kirk, 2004), with 13 % of human protein drug targets for FDA approved oral drugs being transporters (Overington *et al.*, 2006). However, there are currently very few drugs for treating infectious diseases that are known to inhibit membrane transport processes (Kirk, 2004). The *Trypanosoma brucei* P2/TbAT1 aminopurine transporter is one of the best known examples of a protozoan transporter responsible for the delivery of a drug into a parasite (de Koning *et al.*, 2005, Staines *et al.*, 2010). This transporter is responsible for the transport of adenosine and adenine but also carries melaminophenyl arsenicals including melarsoprol (Carter and Fairlamb, 1993, Barrett and Fairlamb, 1999, Stewart *et al.*, 2005), and trypanocidal diamidines including diminazene and pentamidine (Barrett *et al.*, 1995, Carter *et al.*, 1995, De Koning *et al.*, 2004), which are treatments for human African trypanosomiasis (Lanteri *et al.*, 2006, Staines *et al.*, 2010). The *P. falciparum* Ca^{2+} SERCA pump PfATP6 is a proposed site of action of the antimalarial artemisinins, however there is no consensus over the exact mode of action of these drugs (Eckstein-Ludwig *et al.*, 2003, O'Neill *et al.*, 2010).

For a transport protein to be a suitable potential target it must be essential for the parasite's survival, have no human orthologues that might be detrimentally targeted and it must be druggable. Druggability can be predicted if a target is a member of a family containing a clinically validated drug target or based on structural information (Staines *et al.*, 2010). Genetic and chemical validations are used in the selection of new drug targets. Chemical validation involves the identification of a compound that inhibits the targeted protein specifically (without affecting any host orthologues) and ideally works both *in vitro* and *in vivo* (Cowman and Crabb, 2007). Genetic validation will show if loss of gene function results in a non-viable parasites, indicating essentiality (Cowman and Crabb, 2007).

Currently only a few *P. falciparum* transporters have been validated as potential drug targets. The only potential target to be validated both chemically and genetically is the *P. falciparum* hexose transporter, PfHT (Slavic *et al.*, 2011). 3-*O* derivatives of glucose were shown to inhibit uptake of glucose by PfHT (when expressed in *X. laevis* oocytes), with the glucose derivative 3-*O*-((undec-10-en)-1-yl)-D-glucose (known as compound 3361) inhibiting glucose uptake by PfHT with a K_i of ~50 μM but poorly inhibiting glucose uptake by human GLUT1 (K_i ~3 mM) (Joet *et al.*, 2003, Joet and Krishna, 2004, Landfear, 2010). Compound 3361 has also been shown to kill parasites in culture and in an *in vivo* animal model

(*P. berghei)* (Joet *et al.*, 2003, Staines *et al.*, 2010). PfHT has been shown to be essential for parasite growth as loss of function, by knocking out the *pfht* gene, resulted in non-viable parasites with viability restored if the gene was episomally complemented (Slavic *et al.*, 2010).

1.4.3 *P. falciparum* Membrane Transport Pathways as Antimalarial Resistance Mediation Targets

Membrane transport is also of importance regarding antimalarial resistance as transporters can be involved in mediating drug resistance. There are three main ways that transporters can mediate drug resistance: (1) by reducing the effective concentration of the drug at its target with the provision of an efflux pathway, (2) by mutation of the transporter that reduces drug affinity and effectiveness if the transporter is the target, and (3) by mutation of the transporter that reduces drug levels at the target if the transporter is a delivery route (Staines *et al.*, 2010).

The *P. falciparum* chloroquine resistance transporter (PfCRT), a member of the drug/metabolite transporter (DMT) superfamily, is an example of the first mechanism and is a key determinant of resistance to chloroquine (Bray *et al.*, 2005a). In resistant parasites with mutated PfCRT the transporter reduces the concentration of chloroquine in the food vacuole. This resistance is reversible with the calcium channel blocker verapamil.

Attempts to reverse resistance to chloroquine with verapamil or other agents (including antipsychotic drugs, histamine receptor antagonists, and antidepressant agents) has been attempted but problems with side effects and toxicity at the needed concentrations has prevented further trials (van Schalkwyk *et al.*, 2001, Henry *et al.*, 2006, Egan and Kaschula, 2007, Masseno *et al.*, 2009).

1.4.4 Amino Acid Transport

Amino acids form the building blocks of proteins and are important intermediates in metabolism. They are necessary for a variety of cellular processes including nitrogen metabolism, energy generation, cell wall synthesis and intercellular communication (Saier, 2000a). Although the digestion of haemoglobin releases amino acids it has been shown that only up to 16 % of the amino acids released by haemoglobin digestion are used by the parasite (Krugliak *et al.*, 2002). Haemoglobin is also unable to supply the parasite with all amino acids as it contains no isoleucine and only low levels of methionine (Sherman, 1977). From *in vitro* growth experiments it has been shown that the parasite requires an exogenous supply of isoleucine, methionine, cysteine, glutamic acid, glutamine, proline and tyrosine to maintain normal growth (Divo *et al.*, 1985). It has also been shown that the parasite is capable of taking up and incorporating into protein all 20 naturally occurring amino acids (Sherman, 1977, Elford

et al., 1985, Kirk *et al.*, 1994, Divo *et al.*, 1985). This evidence suggests that the parasite must have developed pathways to import amino acids, and transport can be assumed to be essential for parasite survival in the cases of isoleucine, methionine, cysteine, glutamic acid, glutamine, proline and tyrosine. Inhibition of the uptake of these amino acids could therefore be a good target for potential new antimalarial drugs.

In adult human RBCs there are a number of different amino acid transport systems with varying substrate specificities present at the plasma membrane (Barker and Ellory, 1990, Kirk, 2001). Apart from glutamate, all amino acids are transported across the RBC plasma membrane via one or more of these transporters (Elford *et al.*, 1985). As glutamate is an essential amino acid for normal growth of the parasite the parasite must have a mechanism of transporting it through the parasitised RBC membrane. It has been shown that the parasite-induced NPP provide a major route of entry for most amino acids, including glutamate, but not the cationic amino acids arginine and lysine (Elford *et al.*, 1985, Lauer *et al.*, 1997). The NPP show an apparent preference for hydrophobic neutral amino acids over similar sized hydrophilic neutral amino acids (Kirk *et al.*, 1994). Amino acid efflux from the infected RBC is also required to remove the unused amino acids released by haemoglobin digestion and this may be achieved via the NPP (Kirk, 2001, Staines *et al.*, 2005). Without this expulsion of excess amino acids there would be substantial increase in the

osmotic content of the infected RBC which would eventually lead to haemolysis. The relative contributions of the endogenous and parasite-induced transport pathways for the uptake of amino acids into infected RBCs are not yet known for all amino acids. The transport of isoleucine into trophozoite stage *P. falciparum* parasitised RBCs has been characterised and shown to be 5 fold higher than that observed in uninfected RBCs, with this transport attributable to parasite-induced NPP and not an increase in endogenous transport (Martin and Kirk, 2007). In a recent study, published at the end of this thesis' work, it was shown that methionine is taken up by trophozoite stage *P. falciparum* parasitised RBCs at a rate 15 fold higher than uninfected RBCs (Cobbold *et al.*, 2011). As with isoleucine this increase in transport was attributed to the NPP. These two studies also characterised the transport of isoleucine and methionine across the parasite plasma membrane. Isoleucine was shown to be taken up into the parasite via a high capacity saturable ATP, Na^+ and H^+ independent system (Martin and Kirk, 2007). Methionine was shown also shown to be taken up by the parasite via a saturable ATP, Na^+ and H^+ independent system that is also temperature dependent (Cobbold *et al.*, 2011).

1.4.5 Amino Acid Transporter Gene Families

Amino acid transport systems were initially characterised phenotypically with transport assigned to different systems according to characteristics such as ion dependence, substrate specificity and stereospecificity (Christensen, 1985, Barker and Ellory, 1990). Expression cloning and gene sequencing technology have given more information on transport proteins and the genes responsible. Gene sequencing has allowed transporters to be grouped into families based on the degree of similarity observed for their amino acid sequence. There are currently approximately thirty four transporter gene families that are recognised as transporters of amino acids and their conjugates. These include one family of channel proteins, twenty eight families of secondary active transporters and five families of primary active transporters (Saier, 2000b, Saier, 2000a, Saier *et al.*, 2009). The characteristics of these families are summarised in table 1.1.

TC no.	Family	Substrate	Size Range (residues)	No. of TMS	Distribution	No. of Members	Example
1. A. 27	Phospholemman (PLM) family	Cl^- (anion selective), taurine, lactate, glutamate, isethionate, gluconate	70 – 100	$1_{(m)}$	E (An)	>10	Phospholemman; Cl^- conductance inducer protein, Mat-8
2.A.1.6	Metabolite:H^+ symporter (MHS) family of the MFS Superfamily	Various small molecules	400-600	6, 12 or 14	B, A, E	>1000	Proline permease, PutP of *Escherichia coli*
2.A.3	The Amino Acid-Polyamine-Organocation (APC) Superfamily (includes 10 families that transport amino acids: AAT, APA, CAT, ACT, EAT, ABT, GGA, LAT, SGP and YAT)	Amino acids, polyamines, choline	440-630	10, 12 or 14	B, A, E	>1000	Lysine permease, LysP of *E. coli*
2.A.13	C4-dicarboxylate uptake (Dcu) family	C_4-dicarboxylates	440	12	Gram negative B	5	Dicarboxylate uptake porter A, DcuA of *E. coli*
2.A.15	Betaine/Carnitine/Choline transporter (BCCT) family	Glycine, betaine, carnitine, choline	480-680	12	B, A, E	10	Carnitine transporter, CaiT of *E.coli*

TC no.	Family	Substrate	Size Range (residues)	No. of TMS	Distribution	No. of Members	Example
2.A.17	Proton-dependent oligopeptide transporter (POT) family	Peptides, nitrates and amino acids	450-600	12	B, E	>30	Dipeptide transporter, DtpT of *Lactococcus lactis*
2.A.18	Amino acid/auxin permease (AAAP) family	Amino acids and their derivatives	400-710	11	E (An, Pl, Y, F)	>30	Amino acid auxin:H^+ symporter, AAP3 of *Arabidopsis thaliana*
2.A.21	Solute:sodium symporter (SSS) family	Sugars, amino acid, vitamins, nucleobases, inositols, iodine, urea	400-700	15	B, A, E	>30	Pantothenate:Na^+ symporter, PanF of *E. coli*
2.A.22	Neurotransmitter:sodium symporter (NSS) family	Neurotransmitters, amino acids, osmolytes, taurine, creatine	600-700	12	B, A, E (An)	>50	Glycine: Na^+ symporter, GLYT1 of *Rattus norvegicus*
2.A.23	Dicarboxylate/amino acid:cation (Na^+ or H^+) symporter (DAACS) family	C_4-dicarboxylates; acidic and neutral amino acids	420-580	10 to 12	B, A, E	>20	Glutamate/aspartate permease, GltP of *E. coli*
2.A.25	Alanine or glycine:cation symporter (AGCS) family	Alanine, glycine	440-540	8 to 12	B	10	Alanine/glycine: Na^+ symporter, DagA of *Alteromonas haloplanktis*

TC no.	Family	Substrate	Size Range (residues)	No. of TMS	Distribution	No. of Members	Example
2.A.26	Branched-chain amino acid:cation symporter (LIVCS) family	Branched-chain amino acid	~440	12	B	10	Branched chain amino acid transporter, Brab of *Pseudomonas aeruginosa*
2.A.27	Glutamate:Na^+ symporter (ESS) family	Glutamate	400	12	B	5	Glutamate:Na^+ symporter, GltS of *E. coli*
2.A.29	Mitochondrial carrier (MC) family	ATP/ADP/AMP, P_i, organic anions, H^+, carnitine/acyl carnitine, basic amino acids, FAD	300	6	E	>100	Mitochondrial basic amino acid carrier, BAAC of *Neurospora crassa*
2.A.42	Hydroxyl/aromatic amino acid permease (HAAAP) family	Hydroxy and aromatic amino acids	400-450	11	B	>20	Tyrosine permease, TyrP of *E. coli* and serine permease, SdaC of *E. coli*
2.A.56	The Tripartite ATP-independent Periplasmic Transporter (TRAP-T) Family	C_4-dicarboxylates, acidic amino acids, sugars (?)	~1000 (3 components)	12+4	B, A	>20	Dicarboxylate transporter, DctPQM of *Rhodobacter capsulatus*

TC no.	Family	Substrate	Size Range (residues)	No. of TMS	Distribution	No. of Members	Example
2.A.75	L-Lysine exporter (LysE) family	Basic amino acids	190-240	5	B	10	Lysine/arginine exporter, LysE of *Corynebacterium glutamicum*
2.A.76	Resistance to homoserine/threonine (RhtB) family	Neutral amino acids and their derivatives	190-230	5	B	10	Neutral amino acid exporter, RhtB of *E. coli*
2.A.78	The Branched Chain Amino Acid Exporter (LIV-E) Family	Leucine, Isoleucine, Valine	~250 + ~110	7+4	A, B, E (Pr)	>50	Branched chain amino acids efflux pump, AzlC and AzlD of *Bacillus subtilis*
2.A.79	The Threonine/Serine Exporter (ThrE) Family	Threonine, serine	450-600	10	A, B, E	>50	Threonine and serine efflux, ThrE of *Corynebacterium glutamicum*
3.A.1	The ATP-binding cassette (ABC) superfamily (includes 5 familes that transport amino acids: PAAT, HAAT, PepT, QAT and TauT)	All sorts of inorganic and organic molecules of small, intermediate, and large sizes, from simple ions to macromolecules	1,000 –2,000 (multidomain; usually multisubunit)	10 or 12, variable	B, A, E	>1000	Maltose permease, MalEFGK of *E. coli* and multidrug resistance protein, MDR of *Homo sapiens*

Table 1.1. Amino acid transporter gene families. Abbreviations: B, bacteria; A, archea; E, eukaryote; An, animal; Pl, plant; Y, yeast; F, fungi; TMS, transmembrane α-helical spanners. Modified from Saier (2000a), Busch and Saier (2002) and Saier *et al.* (2009).

1.4.5.1 Families of Channel Proteins Capable of Transporting Amino Acids

The only family of channel proteins known to transport amino acids is the phospholemman (PLM) family (TC 1.A.27). They include mammalian phospholemmans, are found in heart muscle and other body tissues and are a major substrate for phosphorylation by protein kinase A and C (Kirk and Strange, 1998, Saier, 2000a). Expression in *Xenopus* oocytes induces a hyperpolarisation-activated chloride current (Moorman *et al.*, 1992). The chloride-conductance inducer protein, Mat-8, is a PLM like protein that is expressed in human breast tumours (Morrison *et al.*, 1995). PLM channels are anion selective and permeable to chloride, taurine, glutamate, lactate, isethionate and gluconate (Kirk and Strange, 1998).

1.4.5.2 Families of Secondary Active Transporters Capable of Transporting Amino Acids

The majority of amino acid transporters are secondary active transporters, with twenty eight families having members that transport amino acids. These secondary active transporters function by proton motive force driven uptake or efflux, sodium-ion motive force driven uptake, by uniport or by solute-solute exchange (Saier, 2000a). Eight of the currently recognised secondary active transporters capable of transporting amino acids are found only in prokaryotes, two are found only in eukaryotes and

the remaining nine are ubiquitous (Saier, 2000a, Saier, 2000b). The amino acid transporter gene families that fall into the category of secondary active transporters are discussed below.

1.4.5.2.1 The amino acid-polyamine-organocation (APC) superfamily

Ten families in the amino acid-polyamine-organocation (APC) superfamily (TC 2.A.3) include transporters that have been shown to transport amino acids. These are: the amino acid transporter (AAT) family (TC 2.A.3.1); the basic amino acid/polyamine antiporter (APA) family (TC 2.A.3.2); the cationic amino acid transporter (CAT) family (TC 2.A.3.3); the amino acid/choline transporter (ACT) family (TC 2.A.3.4); the ethanolamine transporter (EAT) family (TC 2.A.3.5); the archeal/bacterial transporter (ABT) family (TC 2.A.3.6); the glutamate:γ-aminobutyrate (GABA) antiporter (GGA) family (TC 2.A.3.7); the L-Type amino acid transporter (LAT) family (TC 2.A.3.8); the spore germination protein (SGP) family (TC 2.A.3.9); and the yeast amino acid transporter (YAT) family (TC 2.A.3.10). The APC superfamily includes members that function as solute:cation symporters and solute:solute antiporters (Jack *et al.*, 2000, Saier, 2000a). There is a large size range between members with the larger proteins generally of eukaryotic origin and the smaller proteins generally of prokaryotic origin. The *E. coli*

lysine permease, LysP (TC 2.A.3.1.2), is a lysine:H^+ symporter in the APC superfamily (Steffes *et al.*, 1992, Ellis *et al.*, 1995).

1.4.5.2.2 The major facilitator superfamily (MFS)

The metabolite:H+ symporter (MHS) family (TC 2.A.1.6) in the major facilitator superfamily (MFS, TC 2.A.1) includes transporters that have been shown to transport amino acids (Saier *et al.*, 1999a, Saier, 2000a). The *E. coli* (proline/glycine-betaine):(H^+/Na^+) symporter, ProP (TC 2.A.1.6.4), is a member of the MHS family which transports proline and other osmoprotectants (Keates *et al.*, 2010).

1.4.5.2.3 The C_4-dicarboxylate uptake (Dcu) family

Members of the C_4-dicarboxylate uptake (Dcu) family (TC 2.A.13) have been sequenced from Gram negative bacteria (Engel *et al.*, 1994, Golby *et al.*, 1998, Saier, 2000a, Saier *et al.*, 1999b). The C_4-dicarboxylate uptake transporters DcuA (TC 2.A.13.1.1) and DcuB (TC 2.A.13.1.2) from *E.coli* are the best characterised transporters of the Dcu family. These proteins are antiporters for any two of aspartate, malate, fumarate and succinate.

1.4.5.2.4 The betaine/carnitine/choline transporter (BCCT) family

The betaine/carnitine/choline transporter (BCCT) family (TC 2.A.15) contains members from Gram negative and Gram positive bacteria (Saier *et al.*, 1999b, Saier, 2000a). All members transport molecules with a quarternary ammonium group and are energised by proton motive force driven proton symport. One member of the BCCT family is the EctP is an ectosine/proline/glycine/betaine:Na^+ symporter from *Corynebacterium glutamicum* (Peter *et al.*, 1998).

1.4.5.2.5 The proton-dependent oligopeptide transporter (POT) family

Transport proteins in the proton-dependent oligopeptide transporter (POT) family (TC 2.A.17) are mainly peptide transporters but some members are known to transport nitrates and amino acids (Saier *et al.*, 1999b). Members of this family are proton symporters but there is variable substrate:H^+ stoichiometry between members (Saier, 2000a). The BnNRT1;2 transporter from the plant *Brassica napus* belongs to the POT family and exhibits histidine and nitrate elicited currents when expressed in *Xenopus* oocytes (Zhou *et al.*, 1998).

1.4.5.2.6 The amino acid/auxin permease (AAAP) family

Transporters of the amino acid/auxin permease (AAAP) family (TC 2.A.18) have been shown to transport auxin, single amino acids or multiple amino acids, with some having broad substrate specificities for naturally occurring amino acids (Saier, 2000a). Six amino acid permeases in *Arabidopsis thaliana* (AAP1-6) belong to the AAAP family (Young *et al.*, 1999, Fischer *et al.*, 2002). The *Arabidopsis thaliana* general amino acid permease 1, AAP1 (TC 2.A.18.2.1) transports most neutral and acidic amino acids but not basic amino acids or aspartate (Hsu *et al.*, 1993). AAP3 and AAP5 do transport basic amino acids but AAP6 is the only one to transport aspartate (Fischer *et al.*, 2002).

1.4.5.2.7 The solute:sodium symporter (SSS) family

There is broad substrate specificity between members of the solute:sodium symporter (SSS) family (TC 2.A.21) but they all catalyse solute transport via Na^+ symport (Saier, 2000a). An example of a member of the SSS family that transports amino acids is the *E.coli* proline:Na^+ symporter, PutP (Nakao *et al.*, 1987, Jung *et al.*, 1998).

1.4.5.2.8 The neurotransmitter:sodium symporter (NSS) family

Members of the neurotransmitter:sodium symporter (NSS) family (TC 2.A.22) use solute: Na^+ symport to catalyse uptake of neurotransmitters,

amino acids, osmolytes, taurine and creatine (Saier, 2000a). Two members of the NSS family in the brown rat, *Rattus norvegicus*, are the proline:Na^+ symporter, NTPR (Fremeau *et al.*, 1992), and the glycine:Na^+ symporter, GLYT1 (Guastella *et al.*, 1992).

1.4.5.2.9 The dicarboxylate/amino acid:cation (H^+ or Na^+) symporter (DAACS) family

The dicarboxylate/amino acid:cation (H^+ or Na^+) symporter (DAACS) family (TC 2.A.23) contains members that catalyse H^+ or Na^+ symport with (1) small, semipolar, neutral amino acids (alanine, cysteine, serine and threonine), (2) both neutral and acidic amino acids, (3) most zwitterionic or dibasic amino acids (Saier, 2000a). The glutamate/aspartate:H^+ symporter, GltP, of *E. coli* is a member of the DAAC family (Wallace *et al.*, 1990, Tolner *et al.*, 1992).

1.4.5.2.10 The alanine/glycine:cation symporter (AGCS) family

Members of the alanine/glycine:cation symporter (AGCS) family (TC 2.A.25) are found in Gram positive and negative bacteria and transport alanine and/or glycine in symport with Na^+ or H^+ (Saier, 2000a). The alanine (or glycine):Na^+ symporter of the marine bacterium *Alteromonas haloplanktis*, DagA, is a member of the AGCS family (MacLeod and MacLeod, 1992).

1.4.5.2.11 The branched chain amino acid:cation symporter (LIVCS) family

The branched chain amino acid:cation symporter (LIVCS) family (TC 2.A.26) includes members that transport the branched chain aliphatic amino acids leucine, isoleucine and valine via Na^+ or H^+ symport (Saier, 2000a). The branched chain amino acid transporter:Na^+ symporter, BraB, of *Pseudomonas aeruginosa* is a member of this family (Hoshino *et al.*, 1990).

1.4.5.2.12 The glutamate:Na^+ symporter (ESS) family

The glutamate:Na^+ symporter (ESS) family (TC 2.A.27) contains members found in bacteria that transport glutamate via Na^+ symport (Saier, 2000b). One example from this family is the *E. coli* glutamate:Na^+ symporter, GltS, which transports L- and D-glutamate, α-methylglutamate and homocyteate (Dobrowolski *et al.*, 2007).

1.4.5.2.13 The mitochondrial carrier (MC) family

Members of the mitochondrial carrier (MC) family (TC 2.A.29) transport are only found in eukaryotes (Saier, 2000a). They are mostly found in mitochondria and many members are solute:solute antiporters. The

mitochondrial basic amino acid transporter (BAAC) of *Neurospora crassa* is a member of the MC family (Liu and Dunlap, 1996).

1.4.5.2.14 The hydroxy/aromatic amino acid permease (HAAAP) family

The hydroxy/aromatic amino acid permease (HAAAP) family (TC 2.A.42) are found in bacteria and includes aromatic and hydroxy amino acid:H^+ symporters (Saier, 2000a). The tyrosine-specific permease, TyrP, the high-affinity tryptophan-specific permease, Mtr, and the low affinity tryptophan permease, TnaB, of *E. coli* are examples of this family that transport aromatic amino acids (Wookey and Pittard, 1988, Sarsero *et al.*, 1991, Sarsero and Pittard, 1995). The serine permease, SdaC, and the threonine permease, TdcC, of *E. coli* are members of the HAAAP family that transport hydroxy amino acids (Goss *et al.*, 1988, Shao *et al.*, 1994).

1.4.5.2.15 The tripartite ATP-independent periplasmic transporter (TRAP-T) family

Members of the tripartite ATP-independent periplasmic (TRAP-T) family (TC 2.A.56) are generally heterotrimeric with three non-homologous, dissimilar subunits (Saier, 2000a). The *Rhodobacter capsulatus* DctMQP system is one of the best characterised members of this family, with all

three proteins necessary for dicarboxylate uptake implying that the subunits function together (Saier, 2000a). DctM is a 12 TMS protein, DctQ is a 4 TMS integral membrane protein and DctP is a periplasmic integral membrane protein (Forward *et al.*, 1997, Rabus *et al.*, 1999).

1.4.5.2.16 The L-lysine exporter (LysE) family

The L-lysine exporter (LysE) family (TC 2.A.75) are found in bacteria and two members have been functionally characterised (Saier, 2000a). The two characterised members are the lysine/arginine exporter, LysE, of *Corynebacterium glutamicum* (Vrljic *et al.*, 1996, Vrljic *et al.*, 1999) and the arginine exporter protein, ArgO, of *E. coli* (Nandineni and Gowrishankar, 2004). LysE provides the only route for L-lysine excretion from *C. glutamicum* and catalyses unidirectional efflux of L-lysine and other basic amino acids (Saier, 2000a).

1.4.5.2.17 The resistance to homoserine/threonine (RhtB) family

Members of the resistance to homoserine/threonine (RhtB) family (TC 2.A.76) are efflux pumps that may catalyse the efflux of threonine, homoserine and homoserine lactones (Zakataeva *et al.*, 1999, Saier, 2000a). One characterised member id this family is the homoserine/homoserine lactone/β-hydroxynorvaline efflux permease,

RhtB, of *E. coli* which is likely to be a substrate:H^+ antiporter (Zakataeva *et al.*, 1999).

1.4.5.2.18 The branched chain amino acid exporter (LIV-E) family

The branched chain amino acid exporter (LIV-E) family (TC 2.A.78) are pairs of integral membrane proteins. The two-component permease, BrnFE, of *C. glutamicum* is a member of this family and has been shown to catalyse the efflux of methionine, isoleucine, leucine and valine (Kennerknecht *et al.*, 2002, Trotschel *et al.*, 2005). Another member is the branched chain amino acid efflux pump, AzlCD, of *Bacillis subtilis* (Belitsky *et al.*, 1997). This is another two-component permease (AzlC and AzlD) and is an efflux pump for branched chain amino acids.

1.4.5.2.19 The threonine/serine exporter (ThrE) family

One member of the threonine/serine exporter (ThrE) family (TC 2.A.79) has been functionally characterised to date. The threonine/serine exporter, ThrE, of *C. glutamicum*, catalyses the proton motive force driven efflux of threonine and serine (Simic *et al.*, 2002, Eggeling and Sahm, 2003).

1.4.5.3 Families of Primary Active Transporters Capable of Transporting Amino Acids

The ATP-binding cassette (ABC) superfamily (TC 3.A.1) contains five families containing members that have been shown to transport amino acids (Saier, 2000a, Saier, 2000b, Saier *et al.*, 2009). These are: the polar amino acid uptake transporter (PAAT) family (TC 3.A.1.3); the hydrophobic amino acid uptake transporter (HAAT) family (TC 3.A.1.4); the peptide/opine/nickel uptake transporter (PepT) family (TC 3.A.1.5); the quarternary amine transporter (QAT) family (TC 3.A.1.12): and the taurine uptake transporter (TauT) family (TC 3.A.1.17). Proteins in the ABC superfamily bind ATP and use the energy to drive transport of a range of molecules (Dean *et al.*, 2001).

The ABC superfamily includes members of both prokaryotic and eukaryotic origins and includes both uptake and efflux transporters (Saier, 2000b). The families with members known to transport amino acids are found in prokaryotes and are uptake permeases (Saier, 2000a). Two amino acid transporters in the ABC superfamily have been found in the filamentous cyanobacteria *Anabaena sp*: the high-affinity basic amino acid uptake transporter, Bgt, belongs to the PAAT family (Pernil *et al.*, 2008), and the neutral amino acid permease, N-I, belongs to the HAAT family (Picossi *et al.*, 2005).

1.4.5.4 Amino Acid Transporter Genes in *P. falciparum*

No *P. falciparum* amino acid transporters have yet been identified at the molecular level and to date only six amino acid transporters have been putatively annotated in the *P. falciparum* genome (Martin *et al.*, 2005). Of the six putative transporters currently annotated three show similarity to members of the amino acid/auxin permease (AAAP) family (PFF1430c, PFL0420w and PFL1515c) and the other three show similarity to members of the neurotransmitter:Na^+ symporter (NSS) family (PFB0435c, PFE0775c and PF11_0334) (Martin *et al.*, 2005). Despite the putative function of these genes being known no functional characterisation has yet been carried out.

1.5 Aims of Thesis

The focus of the thesis is on transport and salvage in the *Plasmodium falciparum* malaria parasites. This work has been constructed on the following themes of interest:

1. A broad ranging study to identify and characterise potential *P. falciparum* amino acids transporters, with the functional characterisation of a gene-specific cDNA library of putative membrane transporters using the *Xenopus laevis* oocyte heterologous expression system carried out. The putatively annotated amino acid transporter PFF1430c has also been screened using this system to characterise its transport properties.
2. A targeted approach to characterise the properties of methionine uptake by *P. falciparum* infected RBCs and the free parasites.
3. A biochemical approach to understand the role of transport-mediated salvage of folate pathway substrates by determining their effects on antifolate drugs *in vitro* in the presence and absence of a transport inhibitor, and also a targeted investigation of the substrate specificity of two likely *P. falciparum* folate transporters (PfFT1 and PfFT2).

CHAPTER 2

Materials and Methods

2.1 Culturing of *P. falciparum* parasites *in vitro*

Cultivation of *P. falciparum* was carried out following a modified version of the method described by Trager and Jensen (Trager and Jensen, 1976). *P. falciparum* parasites were kept in O positive human RBCs (2.8 % haematocrit; 1 ml washed erythrocytes in 35 ml culture) and culture medium, under an atmosphere of 3 % CO_2, 4 % O_2 and 93 % N_2 in sterile 160 ml culture flasks (Nunc^{TM} Rosklide, Denmark). The culture medium was replaced daily and the parasitaemia adjusted if required by the addition of washed O positive human RBCs. The cultures were maintained at 37 °C and all culture work was carried out under aseptic conditions in a laminar flow hood (class II biological safety cabinet, Nuaire). Cultures were examined using thin blood smears and the parasitaemia and growth stage assessed before the cultures were used for assays.

The strain of *P. falciparum* used throughout this work was 3D7, a strain sensitive to chloroquine and pyrimethamine, and moderately resistant to

sulphadoxine originating from an airport malaria case at Schiphol Airport, The Netherlands.

2.1.1 Preparation of Red Blood Cells

Type O positive human blood from the National Blood Service blood bank at Speke, Liverpool was washed with wash medium containing either RPMI-1640 (Sigma, Poole, UK) supplemented with 0.02 mg/ml gentamycin (Sigma, Poole, UK) for the experiments requiring normal medium (with folate) or folate and pABA-free RPMI-1640 (HyClone, ThermoScientific, UK) supplemented with 0.02 mg/ml gentamycin for the experiments requiring parasites to be cultured in the absence of folate and pABA. Wash steps were repeated three times when using wash medium with folate and pABA present and four times more when using folate and pABA-free wash medium. Washed blood was stored at 4 °C and used for no longer than 6 days.

2.1.2 Preparation of Culture Media

Culture medium used for parasites grown in the presence of folate and pABA contained RPMI-1640 supplemented with 25 mM HEPES pH 7.4, 0.25 % $^{w}/_{v}$ Albumax II (GIBCO, Invitrogen), 36 μM hypoxanthine (Sigma, Poole, UK) and 0.02 mg/ml gentamycin. This culture medium was used for parasite cultures from which total RNA or genomic DNA was extracted

and for the experiments described in chapter 4. Parasites used for experiments in the absence of folate and pABA were grown in folate and pABA-free but otherwise standard RPMI-1640 (HyClone, ThermoScientific, UK) supplemented with 25 mM HEPES pH 7.4, 0.25 % Albumax II, 36 μM hypoxanthine and 0.02 mg/ml gentamycin. Parasites were cultured in this folate and pABA-free medium for the experiments described in chapter 5. HEPES was used as buffer to prevent the acidification of the culture during parasite growth. A stock solution of 1 M HEPES was prepared in distilled water with the pH adjusted to 7.4 with 5 M NaOH. This was filter sterilised and stored at 4 °C. Albumax II was used in place of the traditional serum as this is folate and pABA-free and a 5 % $^{w}/_{v}$ stock solution in distilled water was made. This was filter sterilised and stored at 4 °C for no longer than 30 days.

2.1.3 Examination of Parasites and Calculation of Parasitaemia Using Giemsa Stained Slides

Parasite cultures were examined daily using giemsa stained thin blood smears. The thin blood smear was made from a drop of dense culture suspension (taken from the bottom of the flask) then fixed with methanol for 5 seconds and stained with 10 % giemsa for 10 minutes. The slide was then examined to check for the parasite stage using a light microscope with a 100 x oil immersion objective and the parasitaemia calculated (Zeiss,

Germany). Parasitaemia was calculated as the percentage of parasitised red blood cells out of the total number of red blood cells (with at least 500 cells in total counted).

2.1.4 Synchronisation of Parasite Cultures

In order to have synchronous cultures of parasites, cultures were synchronised using sterile 5 % D-sorbitol (Sigma, Poole, UK) in distilled water, following a modification of the procedure described by Lambros and Vanderberg (Lambros and Vanderberg, 1979). D-sorbitol removes trophozoites and schizont stages from the culture by selective osmotic lysis leaving a culture with mainly ring stage parasites. Synchronisation was carried out on mixed cultures or cultures with mainly ring stage parasites present. The cultures were centrifuged at 805 x g for 5 minutes and the supernatant removed. Five pellet volumes of 5 % D-sorbitol was added to the pellet, mixed thoroughly and kept for 20 minutes at room temperature. The suspension was centrifuged as before and the pellet washed once with an excess of the appropriate culture medium. The pellet was then resuspended in culture medium and transferred to a fresh culture flask, gassed and kept at 37 °C.

2.1.5 Gassing of Parasite Cultures

The parasite cultures were kept under an atmosphere of 3 % CO_2, 4 % O_2 and 93 % N_2. This was achieved by using a pipette fitted with a 0.22 μm filter (Millipore) to pass the gas into the culture flask. The flasks were flushed with gas for 1 minute then the lid was quickly and tightly replaced and the flasks returned to the 37 °C incubator.

2.1.6 Cryopreservation of Parasites

Parasites were cryopreserved for future use from ring stage cultures above 5 % parasitaemia. Cultures were centrifuged at 805 x g for 5 minutes and the supernatant removed. One pellet volume of sterile cryopreservation solution (28 % glycerol; 72 % of 4.2 % $^{w}/v$ D-sorbitol in 0.9 % $^{w}/v$ NaCl) was added to the pellet and gently mixed. This suspension was then divided into 1 ml aliquots in sterile cryogenic vials (non-pyrogenic polypropylene, Corning Incorporated, Corning, USA). Vials were then stored under liquid nitrogen (Union Carbide, UK) in K series cryostorage tanks (JENCONS, Scientific LTD).

2.1.7 Retrieval of Cryopreserved Parasites

Vials of cryopreserved parasites were taken from the liquid nitrogen storage and allowed to thaw in the 37 °C incubator. Once thawed the

contents were transferred to a 15 ml centrifuge tube (Greinerbio-one, Germany) and centrifuged at 805 x g for 5 minutes. The supernatant was removed and the pellet resuspended in an equal volume of sterile 3.5 % $^{w}/v$ NaCl and centrifuged as before. The supernatant was removed and the pellet washed once with culture medium (with folate and pABA). The resulting pellet was resuspended with 15 ml culture medium (with folate and pABA) and 0.5 ml washed red blood cells, then transferred to a 40 ml sterile culture flask (Nunc^{TM} Rosklide, Denmark). This was gassed for 30 seconds and then transferred to the 37 °C incubator.

2.2 Total RNA Extraction from *P. falciparum* Cultures

RNA was isolated from mixed and trophozoite stage *P. falciparum* infected erythrocytes with approximately 10 % parasitaemia. The culture was centrifuged at 805 x g for 5 minutes and the supernatant removed. The pellet was transferred to a 1.5 ml microcentrifuge tube and 1/10th pellet volume of 5 % $^{w}/v$ saponin in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, at pH 7.4) was added to the pellet and mixed by inversion for under 1 minute until a bright red supernatant appeared. This was centrifuged immediately at 17000 x g for 1 minute in a bench top microcentrifuge (Heraeus Freco 17, Thermo

Scientific) and the supernatant discarded. One ml of Trizol (Invitrogen) was added and the pellet was resuspended thoroughly by pipetting. This suspension was left for 5 minutes at room temperature then 200 µl of chloroform was added, mixed by vortexing and then centrifuged at 17000 x g for 15 minutes at 4 °C. The upper aqueous phase was carefully removed, taking care not to contaminate it with the DNA containing interphase, and transferred into a fresh 1.5 ml microcentrifuge tube. Five hundred µl of high quality isopropanol was added and left at room temperature for 10 minutes. The sample was then centrifuged at 17000 x g for 10 minutes at 4 °C. The supernatant was discarded and the pellet washed by adding 750 µl of 70 % ethanol and centrifuging at 17000 x g for 5 minutes at 4 °C. The pellet was dried then resuspended in diethylpyrocarbonate (DEPC) treated water, the volume depending on the size of the pellet (small pellets resuspended in 20 µl and larger pellets in 50 µl). The concentration was determined using a NanoDrop Nd-1000 UV/Vis 1 µl Spectrophotometer (Labtech International, UK) at 260 nm and samples with an A260:A280 of over 1.7 deemed to be of suitable purity. The RNA was then aliquoted and stored at -80 °C. All water, solutions, microcentrifuge tubes and pipette tips used for RNA extraction were RNase-free and DNase-free.

2.3 cDNA Synthesis from Total *P. falciparum* RNA

cDNA was produced from isolated *P. falciparum* RNA by reverse transcription. Contaminating genomic DNA was degraded before cDNA synthesis by incubating total RNA with 1µl Turbo DNase (Ambion, UK) and incubated at 37 °C for 30 minutes to degrade any contaminating DNA. The sample was then heat inactivated at 65 °C for 20 minutes. The ThermoScript™ RT-PCR System (Invitrogen) was used for cDNA synthesis, with 2 µg of isolated RNA, 2 µl random hexamers, 2 µl dNTPs and 5.074 µl DEPC treated water incubated at 65 °C for 5 minutes. This mix was then added to 4 µl 5 X buffer, 1 µl 0.1 M DTT, 1 µl DEPC treated water, 1 µl RNaseOUT, and 1 µl ThermoScript RT (reverse transcriptase) and incubated overnight at 37 °C. The reaction was terminated by heating to 85 °C for 5 minutes. The RNA template was removed by adding 2 U RNase H and incubating at 37 °C for 20 minutes. The cDNA was stored in aliquots at -20 °C (for use within one month) or -80 °C for longer term storage.

2.4 Genomic DNA Extraction from *P. falciparum* Cultures

Genomic DNA was isolated from mixed and trophozoite stage *P. falciparum* infected erythrocytes with approximately 10% parasitaemia. The culture was centrifuged at 805 x g for 5 minutes and the supernatant removed. Five pellet volumes of ice-cold 0.05 % $^{w}/v$ saponin in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, at pH 7.4) was added to the pellet, mixed and left on ice for 10 minutes then centrifuged at 2000 x g for 5 minutes. The supernatant was discarded and the pellet washed twice with PBS. After the second wash the pellet was resuspended in $1/100^{th}$ of the original culture volume of TE buffer (10 mM Tris-Cl, pH 7.5 and 1 mM EDTA) and transferred to a 1.5 ml microcentrifuge tube. Proteinase K and SDS were added to respective final concentrations of 20 μg/ml and 0.5 % and mixed slowly by inversion. This mix was then incubated at 56 – 65 °C for 2 hours. An equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) to the current mix was added and mixed thoroughly by inversion until a mixture of organic and aqueous phases occurred. This was then centrifuged at 17000 x g at 4 °C for 5 minutes and the resulting upper aqueous phase transferred into a fresh 1.5 ml microcentrifuge tube. This was followed by two more phenol/chloroform extractions carried out in the same way. One tenth volume of 3 M sodium acetate and 2.5 volumes of 99 % ethanol were then

added to the final aqueous phase and chilled at -20 °C overnight. After chilling the mix was centrifuged at 17000 x g for 10 minutes and the supernatant discarded. The pellet was air dried then resuspended in DEPC treated water, the volume depending on the size of the pellet (small pellets resuspended in 20 µl and larger pellets in 50 µl). The concentration was read using a NanoDrop Nd-1000 UV/Vis 1 µl Spectrophotometer (Labtech International, UK) at 260 nm and samples with an A260:A280 of over 1.8 deemed to be of suitable purity. The DNA was then aliquoted and stored at -80°C. All water, solutions, microcentrifuge tubes and pipette tips used for genomic DNA extraction were DNase and RNase-free.

2.5 Agarose Gel Electrophoresis

Agarose gel electrophoresis was used for the separation and identification of DNA fragments from restriction digests and PCR amplifications. Agarose gels were prepared with 1 % agarose in TBE buffer (89 mM Tris, 89 mM Boric acid, 2 mM EDTA pH 8) and TBE was used as the running buffer. Before loading, samples were mixed with loading buffer II (Ambion) and the TrackIt™ 1Kb plus DNA ladder (Invitrogen) was used as a molecular weight marker. Electrophoresis was performed at 90 V for 1 hour and the gel incubated in a solution of 0.5 µg/ml ethidium bromide for 20 minutes. The bands were analysed for the expected DNA fragment size

using Gene Genius Bio-imaging Gel Documentation System (Syngene, Synoptics, Ltd. Cambridge, U.K.).

CHAPTER 3

Xenopus laevis Oocyte Screening of Putative *P. falciparum* Amino Acid Transporters

3.1 Introduction

Malaria parasites spend a large part of their lifespan within the host's RBC and so they must evolve appropriate transport networks to acquire vital nutrients that are not present, or that are in limited supply, within these cells (see section 1.4 for more details on *Plasmodium* membrane transport). It is important to identify and characterise these transport pathways and the transport proteins responsible to further understand the biology of the malaria parasite and as they may be potential antimalarial chemotherapeutic targets. Transporters can be targeted either as way of delivering drugs into the parasite or by targeting the transport proteins directly as described in section 1.4.2 (Biagini *et al.*, 2005, Kirk, 2004). Evidence has shown that the intraerythrocytic malaria parasite cannot meet its demand for amino acids by the digestion of haemoglobin alone (as discussed in section 1.4.4). The parasite also requires an exogenous supply of isoleucine, methionine, cysteine, glutamic acid, glutamine, proline and tyrosine for normal growth *in vitro*, suggesting that the parasite must have

transport pathways to import them (Divo *et al.*, 1985). The presence of amino acid transport pathways in the parasite is also supported by the fact that the parasite is able to take up and incorporate all 20 naturally occurring amino acids into protein (Sherman, 1977, Elford *et al.*, 1985, Kirk *et al.*, 1994, Divo *et al.*, 1985).

To date only six amino acid transporters have been putatively annotated in the *P. falciparum* genome (Martin *et al.*, 2005). Three of the six transporters currently annotated as amino acid transporters show similarity to members of the amino acid/auxin permease (AAAP) family (PFF1430c, PFL0420w and PFL1515c) and the other three show similarity to members of the neurotransmitter:Na+ symporter (NSS) family (PFB0435c, PFE0775c and PF11_0334) (Martin *et al.*, 2005). The characteristics of these two families of amino acid transporter genes, as well as other families of amino acid transporter genes, are discussed in section 1.4.5. Despite the putative function being known for these six proteins no functional characterisation has previously been carried out and so this is addressed in this chapter.

As there is a possibility that there are more amino acid transporters than the six described above a gene-specific cDNA library was constructed. This was constructed by Dr Enrique Salcedo Sora before the start of the work described in this thesis. Genes with three or more transmembrane domains

and lacking signal peptides were selected, suggesting that they may code for plasma membrane bound proteins. One hundred and fifteen genes were identified in this way and forty eight cloned (by Invitrogen) for functional characterisation. These forty eight genes were not chosen specifically out of the one hundred and fifteen but because the remaining sixty seven genes were unable to be cloned.

The functional characterisation of these forty eight genes and the six putatively annotated genes was important in order to provide the confirmatory data needed to support their role in amino acid transport. The *Xenopus laevis* oocyte expression system was chosen as a heterologous expression system to investigate the roles of these genes in amino acid transport. This is a well characterised system and was chosen as it would have been difficult to ascertain the role of individual genes by investigating transport in the intact parasite due to the complex nature of different transport routes within the parasite. The *X. laevis* oocyte expression system has been used extensively in the past for expression of membrane transporters and has several important and attractive features: they normally produce faithful and efficient translation of exogenous RNA; display the correct orientation of the encoded protein; and the oocytes themselves are large and easily handled. This model has been used for the expression of foreign genetic material since the 1970s, with initial work on the model carried out by Gurdon and colleagues (Gurdon

et al., 1971). To date it has also been used to study *P. falciparum* transport activity with the hexose transporter, the chloroquine resistance transporter, nucleoside transporters and the Ca^{2+} dependent ATPase transport systems being studied (Joet *et al.*, 2003, Martin *et al.*, 2009, Downie *et al.*, 2007, Downie *et al.*, 2006, Krishna *et al.*, 2001b). It has also been used previously for the characterisation of amino acid transporters (Closs *et al.*, 1997). The AT-rich nature of the *P. falciparum* genome can cause problems with expression of *P. falciparum* proteins in heterologous systems but it does not seem to cause as much of a problem in *X. laevis* oocytes as it does in other systems (Krishna *et al.*, 2001a, Kirk *et al.*, 2005). There are a number of potential drawbacks to the *X. laevis* oocyte expression system including: the misfolding or degradation of the protein; inefficient translation or degradation of the mRNA; the protein not being targeted to the oocyte plasma membrane; the protein may be part of a heteromeric complex and not function alone (for example members of the TRAP-T family and ABC superfamily); endogenous *X. laevis* proteins may interact with expressed foreign proteins and so affect their functional properties; and the protein may not be active as a transporter because of problems in posttranslational modifications (Wagner et al., 2000b, Kirk et al., 2005).

Yeast is another potential eukaryotic system for expressing *Plasmodium* proteins. However, the AT bias of the *P. falciparum* genome can cause

problems with expression in yeast (Kirk *et al.*, 2005). Codon optimisation can be used to reduce the AT content and therefore improve expression in yeast, but as the cDNA library was already under construction without codon optimisation this would have introduced a further step if yeast was to be used as the expression system. Manipulation of gene expression in the parasite itself is also a potential method of investigating the function of transport proteins. Knock-out experiments can establish gene function but may be problematic if the transporter is essential for parasite survival. If there is a known substrate and it can be transported via another uptake route then increased concentrations of the substrate can be added in order to allow parasite survival, or if there is no alternative uptake route then relevant downstream metabolites can be provided instead as long as they can be taken up by the parasite (Kirk *et al.*, 2005). Mutation of transporters can also be used to investigate specific amino acid substitutions. For the number of genes being investigated genetic manipulation would have been too time consuming for the time-frame of this thesis. This is, however, a worthwhile approach as it would allow validation of the gene function and the essentiality of the genes to be investigated.

After a number of rounds of PCR, only one of the six putatively annotated genes was successfully amplified (PFF1430c, the putative amino acid transporter of the AAAP family). This chapter describes the

screening and characterisation of this gene as well as the forty eight strong gene-specific cDNA library. The uptake of the substrates methionine, isoleucine, leucine, glutamic acid, taurine, pantothenic acid and choline by proteins encoded by the cDNA library or PFF1430c was measured. Methionine, isoleucine, glutamic acid, and the vitamin pantothenic acid were used as these have been shown to be essential for parasite growth *in vitro*. Leucine was used as it has been shown to be a substrate for the *P. falciparum* isoleucine transporter (Martin and Kirk, 2007) so it would be interesting to investigate if any gene was responsible for transport of both leucine and isoleucine.

3.2 Materials and Methods

3.2.1 Chemicals

Radiolabelled reagents: [methyl-^{3}H] choline chloride, 81Ci/mmol (Amersham); [3', 5', 7, 9-^{3}H] folic acid (American Radiolabeled Chemicals, Inc.); L-[2, 3, 4-^{3}H] glutamic acid, 50Ci/mmol (American Radiolabeled Chemicals, Inc.); L-[4, 5-^{3}H] isoleucine, 60Ci/mmol (American Radiolabeled Chemicals, Inc.); L-[4, 5-^{3}H] leucine, 166Ci/mmol (GE Healthcare, UK); L-[methyl-^{3}H] methionine, 83Ci/mmol (GE Healthcare, UK); D-[2, 3-^{3}H] pantothenic acid, 50Ci/mmol (American Radiolabeled Chemicals, Inc.); [2-^{3}H] taurine, 20Ci/mmol (American Radiolabeled Chemicals, Inc.); and L-[2,3,4-^{3}H] glutamine, 44Ci/mmol (American Radiolabeled Chemicals, Inc.).

3.2.2 Bioinformatics

Potential integral membrane proteins were identified by bioinformatic methods. This identification was carried out by Dr Enrique Salcedo Sora before the start of this project. The HMMTOP transmembrane topology prediction server (http://www.enzim.hu/hmmtop/index.html) (Tusnady and Simon, 2001) and the TMHMM server (http://www.cbs.dtu.dk/services/TMHMM/) were used to select genes with more than 3 transmembrane domains. Only non-organellar proteins,

identified as lacking signal peptides, were chosen, suggesting that they may code for plasma membrane bound proteins. One hundred and fifteen genes were identified in this way.

As well as this screening, six putative *P. falciparum* amino acid transporters have been previously identified through homology (Martin *et al.*, 2005). These are PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c and PF11_0334. PFF1430c, PFL0420w and PFL1515c are similar to members of the amino acid/auxin permease (AAAP) family and PFB0435c, PFE0775c and PF11_0334 are similar to members of the neurotransmitter:Na+ symporter (NSS) family (Martin *et al.*, 2005).

3.2.3 Gene Specific cDNA Library

A gene-specific cDNA library of 48 clones was produced by Invitrogen from the 115 genes identified as potential integral membrane proteins above (table 3.1). These 48 genes were not chosen specifically out of the 115 identified but because the remaining 67 genes were unable to be cloned. Genes were cloned from cDNA (generated from total 3D7 *P. falciparum* RNA) into the pDEST47 vector and propagated in *Escherichia coli*. The genes were provided as glycerol stocks and stored at -80 °C.

Group	Gene Name	Internally Assigned Number	Number of Trans-membrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
1	PF14_0593	16	3	Conserved Plasmodium protein, unknown function	Multi-pass transmembrane protein (*Cryptosporidium hominis*) E-value = 3e-10
1	MAL13P1.210	41	11	Dolichyl-phosphate-mannose-glygolipidalpha-mannosyltransferase, putative	Dolichyl-phosphate-mannose-glycolipid alpha-mannosyltransferase involved in GPI anchor biosynthesis (*Cryptosporidium parvum* Iowa II) E-value = 4e-26
1	PFE0340c	43	6	Rhomboid protease ROM4	Rhomboid-like protease 4 (*Toxoplasma gondii* GT1), E-value = 0
1	PF14_0054	45	4	Conserved protein, unknown function	Circumsporozoite protein (*Plasmodium vivax*), E-value = 2e-112
1	PF11_0384	49	6	Cleft lip and palate associated transmembrane protein-related	Transmembrane CLPTM1 family protein (*Babesia bovis*), E-value = 1e-155
1	MAL13P1.126	51	8	DHHC-type zinc finger protein, putative	Palmitoyltransferase TIP1 (*Cucumis melo*), E-value = 9e-15
1	PF08_0032	52	3	DnaJ protein, putative	DnaJ domain-containing protein (*Cryptosporidium muris RN66*), E-value = 2e-46
1	PF11_0310	54	12	Transporter, putative	Major facilitator superfamily domain-containing protein (*Toxoplasma gondii*), E-value = 2e-61

Group	Gene Name	Internally Assigned Number	Number of Transme-mbrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
2	PF08_0097	59	7	Conserved protein, unknown function	Erythrocyte membrane-associated antigen (*Plasmodium yoelii yoelii* 17XNL), E-value = 9e-133
2	PFE1130w	62	10	Conserved protein, unknown function	Apicomplexan conserved protein with 9 transmembrane domain (*Cryptosporidium parvum* Iowa II), E-value = 1e-92
2	PFE0785c	65	10	Metabolite/drug transporter, putative	GDP-fucose transporter (*Ascaris suum*), E-value = 9e-08
2	PF07_0064	66	9	Drug/metabolite exporter, drug/metabolite transporter	Transporter, EamA (*Bacillus cereus* AH603), E-value = 2e-13
2	PF10_0295	68	5	Conserved Plasmodium protein, unknown function	Multitransmembrane protein with signal peptide and GMGPP repeat at C-terminus, related (*Neospora caninum* Liverpool), E-value = 4e-40
2	PF10_0223	69	5	Conserved Plasmodium membrane protein, unknown function	Biofilm-associated protein (*Staphylococcus simulans*), E-value = 1.5
2	MAL13P1.329	71	6	Conserved Plasmodium membrane protein, unknown function	SNARE associated golgi family protein (*Fischerella sp*. JSC-11), E-value = 1e-23
2	PF14_0258	72	10	Conserved Plasmodium membrane protein, unknown function	No known proteins (with E-values of under 10)

Group	Gene Name	Internally Assigned Number	Number of Transme-mbrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
3	PF14_0253	73	7	Conserved Plasmodium membrane protein, unknown function	Nuclear import and export protein Msn5 (*Aspergillus fumigatus* Af293), E-value = 0.062
3	PFD1110w	74	6	PfM6Tα, a glideosome associated protein with multiple membrane spans (Rayavara K. *et al.*, 2009)	Cytoachrome c oxidase subunit III with 6 transmembrane domains (*Cryptosporidium parvum* Iowa II), E-value = 4e-10
3	MAL7P1.64	75	8	Serpentine receptor, putative	No known proteins (with E-values of under 10)
3	PFB0845w	77	5	Conserved Plasmodium membrane protein, unknown function	Insulin-degrading enzyme (*Cryptosporidium hominis* TU502), E-value = 6e-24
3	PF14_0453	78	3	Conserved Plasmodium protein, unknown function	Phospholipid/glycerol acyltransferase (*Cordyceps militaris* CM01), E-value = 2e-06
3	PF14_0279	80	3	Conserved Plasmodium protein, unknown function	WSC domain-containing protein (*Dictyostelium fasciculatum*), E-value = 0.050
3	MAL13P1.130	84	6	PfM6Tγ, glideosome associated protein with multiple membrane spans (Rayavara K. *et al*., 2009)	Cytoachrome c oxidase subunit III with 6 transmembrane domains (*Cryptosporidium parvum* Iowa II), E-value = 0.010
3	PFL0775w	86	5	Conserved Plasmodium protein, unknown function	No known proteins (with E-values of under 10)

Group	Gene Name	Internally Assigned Number	Number of Transme-mbrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
4	PF14_0065	90	6	Conserved Plasmodium protein, unknown function	Cytochrome c oxidase subunit III (*Cryptosporidium parvum* Iowa II), E-value = 2e-40
4	PFE1490c	91	4	RING zinc finger protein, putative	Zinc finger, C3HC4 type domain-containing protein (*Cryptosporidium muris* RN66), E-value = 4e-16
4	PF10_0070	92	6	Conserved Plasmodium protein, unknown function	AGAP008757-PA (*Anopheles gambiae* str. PEST), E-value = 1e-09
4	PFI0800c	93	4	Conserved Plasmodium protein, unknown function	3-hydroxy-3-methylglutaryl-Coenzyme A reductase (*Scophthalmus maximus*), E-value = 9.9
4	PF10_0276	95	4	Zinc finger, C3HC4 type, putative	Zinc finger (C3HC4 RING finger) protein (*Toxoplasma gondii* VEG), E-value = 3e-91
4	PFL1140w	96	5	Putative $Fe2^{+}$:H^{+} antiporter (Martin, Ginsburg and Kirk, unpublished)	Vacuolar iron family transporter (*Micromonas sp.* RCC299), E-value = 3e-60
4	PF10_0317	98	5	DER1-like protein, putative	Der1-like family protein (*Babesia bovis*), E-value = 6e-74
4	PF13_0124	99	4	SFT2-like protein, putative	SFT2-like domain-containing protein (*Toxoplasma gondii* ME49), E-value = 6e-37

Group	Gene Name	Internally Assigned Number	Number of Transme-mbrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
5	MAL7P1.34	102	4	Conserved Plasmodium protein, unknown function	Extracellular metalloprotease (*Simonsiella muelleri* ATCC 29453), E-value = 1.9
5	PFL1825w	105	5	Conserved Plasmodium membrane protein, unknown function	ALI0B08899p (*Yarrowia lipolytica*), E-value = 0.002
5	PFI0150c	106	3	Retrieval receptor for endoplasmic reticulum membrane proteins, putative	RER1 protein (*Cryptosporidium muris* RN66), E-value = 9e-55
5	MAL13P1.94	109	4	Conserved Plasmodium membrane protein, unknown function	Chitin synthase 1 (*Candida tropicalis* MYA-3404), E-value = 1.9
5	PF11_0394	110	4	Conserved Plasmodium membrane protein, unknown function	ABC transporter related protein (*Pseudoxanthomonas spadix* BD-a59), E-value = 0.52
5	PFL2035c	111	3	Conserved Plasmodium membrane protein, unknown function	Bumetanide-sensitive Na-K-Cl cotransport protein, putative (*Aedes aegypti*), E-value = 2.6
5	PF10_0112	115	4	Conserved Plasmodium membrane protein, unknown function	Response regulator receiver (*Planococcus donghaensis* MPA1U2), E-value = 7.4
5	PFE0725c	107	4	Conserved Plasmodium membrane protein, unknown function	No known proteins (with E-values of under 10)

Group	Gene Name	Internally Assigned Number	Number of Transme-mbrane Domains	Current Annotation (in PlasmoDB)	Similar known proteins
6	PFA0205w	40	5	Conserved Plasmodium membrane protein, unknown function	S24 sporozoite-expressed protein (*Plasmodium yoelii*), E-value = 1e-150
6	PFC0530w	56	12	Transporter, putative	Transporter, major facilitator family domain containing protein (*Toxoplasma gondii*), E-value = 4e-11
6	PFI0720w	62	11	Transporter, putative	Major facilitator superfamily domain containing protein (*Toxoplasmsa gondii* ME49), E-value = 2e-47
6	MAL13P1.117	82	4	DHHC-type zinc finger protein, putative	Zinc finger family protein (*Arabidopsis lyrata* subsp. *Lyrata*), E-value = 5e-38
6	PF11_0217	97	4	Conserved Plasmodium membrane protein, unknown function	Zinc finger DHHC domain-containing protein (*Toxoplasma gondii* ME49), E-value = 1e-62
6	PF10_0023	94	3	Plasmodium exported protein (hyp16), unknown function	Plasmodium exported protein (hyp2) (*Plasmodium falciparum* 3D7), E-value = 1e-07
6	PFA0670c	101	3	Plasmodium exported protein (hyp8), unknown function	Voltage-dependent calcium channel (*Schistosoma mansoni*), E-value = 4.4
6	PFB0910w	103	3	Plasmodium exported protein, unknown function	Liver stage antigen 3 (*Plasmodium falciparum*), E-value = 3.7

Table 3.1. Table of gene-specific cDNA library. Similar known proteins identified using blastp (Altschul *et al.*, 1997, Altschul *et al.*, 2005).

3.2.3.1 Plasmid Preparation from cDNA Library Glycerol Stocks

Overnight cultures were set up as follows; 50 ml LB medium, 100 μg/ml Ampicillin, and 20μl of transformed *E. coli* glycerol stock (from gene-specific DNA library). Either cultures from individual clones or groups of 8 clones together were set up (see table 1 for group assignments). In the case of group 1 the overnight culture also included human reduced folate carrier 1 (hRFC1_pKSM, provided by Dr Enrique Salcedo Sora). Flasks were incubated in a shaking incubator at 37 °C overnight. Twenty five OD_{600} units of culture were centrifuged, in 15 ml or 50 ml tubes as required, at 4000 rpm for 10 minutes. The supernatant was discarded and the plasmid extracted and purified using the commercially available QIAprep spin miniprep kit (QIAGEN) following the kit protocol. The concentration of DNA was determined using a NanoDrop Nd-1000 UV/Vis 1μl Spectrophotometer (Labtech International, UK) at 260 nm (with the purity measured as the ratio of absorbance at 260 and 280 nm, $A_{260/280}$).

3.2.3.2 Linearisation of Plasmid DNA

DNA was linearised by adding 20 units of XbaI (New England Biolabs) restriction enzyme to 45 μl plasmid DNA, 1 X end concentration buffer 2 and 1 X end concentration BSA, and incubating at 37 °C for 1 hour. After 1 hour a further 1 μl restriction enzyme was added and incubated at 37 °C for

another hour. The mix was heat inactivated at 65 °C for 20 minutes, then treated with 8 µl 5% SDS, 8 µl 2.5 mg/ml Proteinase K and 11.5 µl DEPC treated water and incubated at 50 °C for 30 minutes. The DNA was cleaned by phenol/chloroform extraction as follows: 120 µl DEPC treated water was added to the linearisation mix to make up to 200 µl total volume then 200 µl phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) was added and mixed by inversion. This was then centrifuged at 17000 x g for 1 minute in a bench top microcentrifuge to separate the aqueous and organic phase. The aqueous phase was taken into a fresh 1.5 ml microcentrifuge tube and the phenol/chloroform extraction was repeated. Commercially available Wizard SV Gel and PCR Clean-up System (Promega) was used to purify the linearised DNA following the kit protocol. The DNA was eluted in DEPC treated water and the concentration was determined using a NanoDrop Nd-1000 UV/Vis 1µl Spectrophotometer (Labtech International, UK) at 260 nm (with the purity measured as the ratio of absorbance at 260 and 280 nm, $A_{260/280}$). DNA was analysed on 1 % agarose gel (as detailed in section 2.4) and stored at -20 °C.

3.2.3.3 Heterologous cRNA Synthesis

For *X. laevis* oocyte expression it is simpler to use capped complimentary RNA (cRNA) than cDNA as it is much easier to inject the cRNA into the cytoplasm of the oocyte than to inject cDNA into the nucleus. cRNA was

transcribed from the linearised cDNA (produced as described in section 3.2.3.2) using the commercially available mMessage mMachine High Yield RNA Transcription Kit T7 (Ambion) following the manufacturer's instructions. Briefly, this included 1 μg linearised DNA, 1 X NTP/CAP, 1 X reaction mix, 2 μl enzyme mix and nuclease-free water to a total volume of 20 μl, incubated at 37 °C for 6 hours. In order to remove the template DNA from the *in vitro* transcription products 1 μl of TURBO DNase was added to the reaction mix and incubated at 37 °C for 15 minutes. RNA was recovered by lithium chloride precipitation by adding 30 μl nuclease-free water and 30 μl lithium chloride precipitation solution (mMessage mMachine High Yield RNA Transcription Kit T7, Ambion) and incubating overnight at -20 °C. After incubation the precipitation mix was centrifuged at 17000 x g at 4 °C for 10 minutes, washed once with 70 % $^{v}/_{v}$ ethanol and the pellet dried in the laminar flow hood. The pellet was resuspended in 15 μl nuclease-free water and concentration was read using a NanoDrop Nd-1000 UV/Vis 1μl Spectrophotometer (Labtech International, UK) at 260 nm (with the purity measured as the ratio of absorbance at 260 and 280 nm, $A_{260/280}$). The cRNA was analysed on 1 % agarose gel as described in section 2.4 with the samples prepared up to 10 μl with nuclease-free water with 2 μl formaldehyde (to denature the cRNA) and 3 μl gel loading buffer II also added. The cRNA concentration was adjusted to 1 μg/μl, split into 5 μl aliquots in 1.5 ml nuclease free microcentrifuge tubes and stored at -80 °C.

3.2.4 Cloning of PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c, PF11_0334

3.2.4.1. PCR Amplification of PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c, PF11_0334

Amplification of PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c, and PF11_0334 was attempted using the PCR method. Primers were designed based on the published sequences of these genes and are shown in table 3.2. An initial amplification reaction was set up with the end concentration of the following reagents: 2 X amplification buffer, 2 X PCR_x enhancer solution, 2.3 mM $MgSO_4$, 0.2 mM dNTPs mix, 1 unit Platinum® *Pfx* DNA Polymerase (Invitrogen), 2 μM of the forward and reverse primer (Invitrogen), 4 μl template 3D7 cDNA (produced following the protocol described in section 2.3) and DEPC-H_2O to a total volume of 21.4 μl. PCR amplifications were all carried out using the Biometra-T-Personal-Thermal-Cycler (Anachem, Luton, UK) and the initial amplification attempt used the following cycling conditions: initial denaturation (94 °C for 4 minutes), 40 cycles of denaturation (94 °C for 15 seconds), annealing (53 °C for 30 seconds) and extension (72 °C for 2 minutes) and a final extension (72 °C for 10 minutes). Primers for PfCRT (provided by Dr Enrique Salcedo Sora) were used as a positive control for the PCR reactions. The size and integrity of the PCR products was visualised on a 1 % $^{w}/v$ agarose gel as described in section 2.5.

Primer Name	Sequence (5' to 3')
PFF1430c Forward Primer	ATATAAGCTTATGAATAAAAAGTATGG
PFF1430c Reverse Primer	ATATGAATTCTTATAAAATTAAATTTAAGG
PFL0420w Forward Primer	ATATAAGCTTATGAGTATATGCATATC
PFL0420w Reverse Primer	ATATGAATTCTTAATATATAAAATCATAAAGG
PFL1515c Forward Primer	ATATAAGCTTATGAATCTAAAATGG
PFL1515c Reverse Primer	ATATGAATTCTTAACCAAATAATAAATCAG
PFB0435c Forward Primer	ATATAAGCTTATGCTACAAAAGTTAAG
PFB0435c Reverse Primer	ATATGAATTCTCATATTTTACTTTTAGTAG
PFE0775c Forward Primer	ATATAAGCTTATGTCGAGGGAAACATC
PFE0775c Reverse Primer	ATATGAATTCCTACATATTTATAAATTCAC
PF11_0334 Forward Primer	ATATAAGCTTATGGGAACACCTG
PF11_0334 Reverse Primer	ATATGAATTCTTAATAGGAAATATGAGC

Table 3.2. Primer combinations used to amplify PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c and PF11_0334.

Optimisation of the PCR was carried out as the initial PCR did not successfully amplify any of the six genes of interest. Firstly, several attempts at PCR using the same mix of reagents, with new batches of cDNA from freshly extracted RNA, and only increasing the extension time to 5 minutes were tried but were also unsuccessful. A gradient PCR was used to attempt to amplify PFF1430c using 2 different reaction mixes, again with a new batch of cDNA from freshly extracted RNA. Mix one included 1 X amplification buffer, 1.5 mM $MgSO_4$, 0.2 mM dNTPs mix, 1

unit Platinum® *Pfx* DNA Polymerase (Invitrogen), 1.6 μM of the forward and reverse primer, 1 μl template 3D7 cDNA and DEPC-H_2O to a total volume of 25 μl. Mix two was the same but also included 1 X PCR_x enhancer solution. The following cycling conditions were used for the gradient PCR: initial denaturation (94 °C for 2 minutes), 40 cycles of denaturation (94 °C for 15 seconds), annealing (75 °C, 73.3 °C, 70.3 °C, 65.8 °C, 59.5 °C, 55 °C, 51.9 °C, or 50 °C for 30 seconds) and extension (72 °C for 2 minutes) and a final extension (72 °C for 10 minutes).

As the gradient PCR did not successfully amplify PFF1430c amplification of PFL0420w was attempted using genomic DNA as a template, as this gene has no introns. Three different reaction mixes were used with three different DNA polymerases. The first mix used Platinum® *Pfx* DNA Polymerase and consisted of the following reagents: 2 X amplification buffer, 2 X PCR_x enhancer solution, 2.5 mM $MgSO_4$, 0.2 mM dNTPs mix, 1 unit Platinum® *Pfx* DNA Polymerase (Invitrogen), 2 μM of the forward and reverse primer, 2 μl template 3D7 genomic DNA (produced as described in section 2.4) and DEPC-H_2O to a total volume of 20 μl. Mix two used Platinum® taq DNA Polymerase and consisted of the following reagents: 1 X amplification buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs mix, 1 unit Platinum® taq DNA Polymerase (Invitrogen), 0.2 μM of the forward and reverse primer, 2 μl template 3D7 genomic DNA and DEPC-H_2O to a total volume of 20 μl. Mix three used Platinum® taq DNA Polymerase

High Fidelity and consisted of the following reagents: 1 X amplification buffer, 2 mM $MgSO_4$, 0.2 mM dNTPs mix, 1 unit Platinum® taq DNA Polymerase High Fidelity (Invitrogen), 0.2 μM of the forward and reverse primer, 2 μl template 3D7 genomic DNA and DEPC-H_2O to a total volume of 20 μl. The following PCR cycling conditions were used for all mixes: initial denaturation (94 °C for 2 minutes), 40 cycles of denaturation (94 °C for 15 seconds), annealing (55 °C for 30 seconds) and extension (68 °C for 2 minutes) and a final extension (68 °C for 10 minutes). PFL0420w was found to be successfully amplified using mix two with Platinum® taq DNA Polymerase and so this could be used for further cloning work.

Touchdown PCR was used in a final attempt to amplify PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c and PF11_0334 from cDNA. An amplification reaction was set up with the end concentration of the following reagents: 1 X amplification buffer, 2.3 mM $MgSO_4$, 0.25 mM dNTPs mix, 2.5 units Platinum® taq DNA Polymerase High Fidelity (Invitrogen), 2 μM of the forward and reverse primer (Invitrogen), 2 μl template 3D7 cDNA and DEPC-H_2O to a total volume of 21 μl. The following cycling conditions were used for this touchdown PCR: initial denaturation (94 °C for 2 minutes), 40 cycles of denaturation (94 °C for 15 seconds), annealing (63 °C for 30 seconds, dropping by 0.5 °C for each cycle) and extension (68 °C for 4 minutes) and a final extension (68 °C for

10 minutes). PFF1430c was successfully amplified using this method and was used for cloning.

3.2.4.2 Deoxyadenosine (dA)-Tailing

As the PFF1430c was amplified using a proofreading enzyme the fragment lacked 3' A-overhangs that are necessary for cloning and so these were recreated by dA-tailing. The reaction mixture contained the following ingredients at the final concentration; 1X Taq buffer, 2.5mM $MgCl_2$, 0.1mM dATP, 2.5 units Taq DNA polymerase (Promega) and PCR product. The reaction was incubated at 68 °C for 1 hour. The product was then purified using the commercially available Wizard SV Gel and PCR Clean-Up System (Promega) and eluted with 20 µl DEPC-H_2O.

3.2.4.3 TOPO® Cloning

The ligation reaction was carried out using the commercially available TOPO® TA cloning kit (Invitrogen). A molar ratio of 3:1 (insert DNA : vector DNA) was used. For ligation of PFF1430c and the pCR II TOPO® vector the reaction was set up as follows: 4 µl dA-tailed PCR product, 1 µl pCR II TOPO® vector and 1 µl salt solution. The ligation reaction mix of PFL0420w and the pCR II TOPO® vector was set up as follows; 6 µl PCR product, 1 µl pCR II TOPO® vector and 1 µl salt solution. Both reactions

were incubated for 30 minutes at room temperature then used directly for transformation.

3.2.4.4 Transformation Into One Shot® TOP10 competent E. coli cells

The ligation mix was transferred directly into a vial of One Shot® TOP10 competent cells and incubated on ice for 30 minutes followed by a 45 second heat-shock at 42°C. The vial was immediately transferred to ice and 250 µl of room temperature SOC medium was added then transferred to a shaking incubator for 1 hour at 37 °C. Forty µl 40 mg/ml X-gal and was added to the mix and spread out onto pre-warmed 100 µg/ml ampicillin treated LB agar plates. The plates were incubated at 37 °C overnight.

3.2.4.5 Plasmid Preparation and Clone Selection

Individual white colonies from LB agar plates were picked and cultured overnight in 5 ml LB medium, containing 100 µg/ml ampicillin, at 37 °C and shaking at 200 rpm. The plasmids were prepared from this culture using the commercially available QIAGEN QIAprep miniprep kit following the kit protocol. A restriction digest was performed on the eluted DNA to check for the presence of the insert. The restriction digest reactions were set up as follows: 1 µg plasmid DNA, 1 X EcoRI buffer (Promega), 1 X BSA, 10 units of EcoRI restriction enzyme (Promega) with DEPC treated water to a total volume of 20 µl. The reaction was incubated

for one hour at 37 °C after which the size and integrity of the digested DNA was checked on a 1 % ^{w}/v agarose gel electrophoresis (as detailed in section 2.5). This confirmed the presence of ligated PFF1430c_pCR®II-TOPO® but provided no confirmation of successful ligation of PFL0420w_ pCR®II-TOPO®.

The orientation of the PFF1430c insert in the pCR®II-TOPO® vector was determined using another restriction digest. This digest was set up as for the previous digest but using EcoRV restriction enzyme (New England Biolabs) and buffer 3 (New England Biolabs) and the presence of the correctly orientated insert was determined by 1 % ^{w}/v agarose gel electrophoresis.

3.2.4.6 Subcloning PFF1430c from pCR®II-TOPO® into the KSM pBluescript vector

The pKSM vector (originally provided by Dr Bill Joiner, University of Pennsylvania, USA) is a derivative of pBluescript in which the insertion site is flanked by the 5'- and 3'-untranslated regions of the *Xenopus* β-globin gene to enhance the expression of heterologous cRNAs in the oocytes. Using DNA from colonies showing the PFF1430c insert in the correct orientation, PFF1430c was released from the PCR II TOPO® vector using the Xho1 and BamH1 restriction enzymes (New England Biolabs).

This restriction digest reaction was set up as follows: 18 µg plasmid DNA, 1 X buffer 3 (New England Biolabs), 1 X BSA, 200 units of XhoI restriction enzyme (New England Biolabs), 200 units BamHI restriction enzyme (New England Biolabs) and DEPC treated water to a total volume of 100 µl. The reaction was incubated for four hours at 37 °C, after which the DNA was cleaned to remove enzyme using the commercially available QIAamp PCR Purification kit (QIAGEN) following the kit protocol and eluted from the column with 30 µl DEPC treated water. The eluted DNA was then checked for the presence of the correctly sized excised insert by 1 % ^{w}/v agarose gel electrophoresis as described in section 2.5. The pKSM vector was digested from hRFC1_pKSM (provided by Dr Enrique Salcedo Sora) using the same restriction enzymes and reaction mix as for PFF1430c_pCR®II-TOPO®. The digested DNA was cleaned using the commercially available QIAamp PCR Purification kit (QIAGEN) following the kit protocol and eluted from the column with 30 µl DEPC treated water. This was also checked for the presence of the correctly sized cut vector by 1 % ^{w}/v agarose gel electrophoresis. The excised PFF1430c fragment and cut pKSM vector were excised from agarose gel and extracted using the commercially available Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions and eluted from the membrane with 30 µl DEPC treated water.

Ligations of PFF1430c into pKSM were carried out at molar ratios of 2:1 or 4:1 (insert DNA : vector DNA) and the reaction set up as follows: 200 ng or 400 ng PFF1430c DNA and 200 ng pKSM DNA with T4 DNA ligase buffer at 1 X final concentration and 3 units of T4 DNA ligase. The reaction was incubated for 2 hours at room temperature for the 2:1 molar ratio reaction or overnight at 4 °C for the 4:1 molar ratio reaction then used directly for transformation. The ligase mix was added directly to a vial of One Shot® TOP10 competent cells and the transformation carried out as described in section 3.2.4.4, with the exception of no addition of X-gal when plating out. No colonies were observed after the initial ligation and transformation attempt so the same procedure was repeated with a 2:1 molar ratio and ligation for 2 hours at room temperature, but this time using new T4 ligase, T4 ligase buffer and One Shot® TOP10 competent cells. This time one colony was observed and plasmids were prepared from this colony as described in section 3.2.4.5. Digestion with EcoRI as described in section 3.2.4.5 was carried out but no correctly sized insert for PFF1430c was observed when observed on 1 % agarose gel.

A different approach was then taken to attempt to successfully subclone PFF1430c into pKSM. Both PFF1430c_pCR®II-TOPO® and hRFC_pKSM were digested using XhoI and SpeI (restriction sites in pCR®II-TOPO® vector and internal sites in the 5'- and 3'-untranslated regions of the pKSM vector). The digest reaction was set up as follows: 25 µg plasmid DNA, 1

X buffer 2 (New England Biolabs), 1 X BSA, 200 units of XhoI restriction enzyme (New England Biolabs), 200 units SpeI restriction enzyme (New England Biolabs) and DEPC treated water to a total volume of 100 µl. The reaction was incubated at 37 °C for 4 hours and heat inactivated at 65 °C for 20 minutes. A different procedure for cleaning up the digested DNA was also followed. The digested PFF1430c_pCR®II-TOPO® and hRFC_pKSM were treated with proteinase k (250 µg/ml end concentration) and SDS (1 % end concentration) at 50 °C for 30 minutes. This was followed by a phenol/chloroform extraction with the proteinase K treated digest mixes made up to 200 µl with DEPC treated water and 200 µl phenol/chloroform/isoamyl alcohol (25 : 24 : 1, v/v/v) added and mixed by inversion. The mixes were then centrifuged at 17000 x g for 1 minute and the aqueous phases transferred to new 1.5 ml microcentrifuge tubes. The phenol/chloroform extraction was repeated once more and the DNA recovered using an overnight ethanol precipitation at -20 °C with the following reagents used: $1/10^{th}$ volume 3 M sodium acetate (pH 5.2) and 4 volumes of ice-cold absolute ethanol. After incubation the mixes were centrifuged at 17000 x g at 4 °C for 10 minutes, washed once with 70 % $^{v}/v$ ethanol and the pellet dried in the laminar flow hood. The dried pellets were resuspended in 30µl DEPC treated water and loaded onto a 1 % agarose gel, made and run as described in section 2.5. The excised PFF1430c fragment and cut pKSM vector were observed to be the correct size and were excised from agarose gel and extracted using the

commercially available Wizard SV Gel and PCR Clean-Up System (Promega) following the manufacturer's instructions and eluted from the membrane with 15 µl DEPC treated water.

Ligations of PFF1430c into pKSM using insert and vector from the XhoI and SpeI digests of PFF1430c_pCR®II-TOPO® and hRFC1_pKSM were carried out at molar rations of 3:1 and 6:1 (insert DNA : vector DNA) and the reaction set up as follows: 300 ng or 600ng PFF1430c DNA and 200 ng pKSM DNA with T4 DNA ligase buffer at 1 X final concentration and 3 units of T4 DNA ligase. The reaction was incubated for 2 hours at room temperature for then used directly for transformation as described for the previous ligation and transformation attempt. Four colonies were obtained (1 from the 3:1 molar ratio and 3 from the 6:1 molar ratio) and plasmids prepared from these as described in section 3.2.4.5. Digestion of the DNA from the colonies with XhoI and SpeI (1 µg DNA, 1 X buffer 2, 1 X BSA, 20 units of XhoI and SpeI made up to 20 µl with DEPC treated water) was carried out at 37 °C for 1 hour. The reaction was then heat inactivated at 65 °C for 20 minutes and analysed on a 1 % agarose gel. No band corresponding to the size of PFF1430c was observed so further work on these colonies was discontinued.

A further step to try and achieve successful ligation and transformation was attempted by treating the digested vector with Antarctic phophatase to

prevent self ligation. After a fresh digestion of PFF1430c_pCR®II-TOPO® and hRFC_pKSM using XhoI and SpeI, carried out in same way as previously described in this section, the digested hRFC1_pKSM was treated with Antarctic phosphatase. This would help to prevent self ligation of the vector by dephosphorylation of the 5'-cohesive ends. The heat inactivated digestion reaction was incubated with 25 units of Antarctic phosphatase (New England Biolabs) and 1 X Antarctic phosphatase buffer (New England Biolabs) at 37 °C for 15 minutes then heat inactivated at 65 °C for 5 minutes. Further clean-up of the digested PFF1430c_pCR®II-TOPO® and Antarctic phosphatise treated hRFC_pKSM was carried out using proteinase K treatment, phenol/chloroform extraction and ethanol precipitation as described for the previous digest. The commercially available Wizard SV Gel and PCR Clean-Up System (Promega) was used (following the kit protocol) to excise and extract the PFF1430c fragment and cut pKSM vector from a 1 % agarose gel. A sample of the pKSM DNA eluted from the spin column of this kit was also run on a 1 % agarose gel and showed as a clear compact band at the expected size (3.2 kb).

Ligations of PFF1430c into pKSM using the vector treated with Antarctic phosphatase were carried out in the same way as the previous ligation and transformation attempt (with 3:1 and 6:1 molar rations of insert DNA : vector DNA). However, this was also unsuccessful with no colonies observed after overnight culture of the transformations on LB agar plates.

After these unsuccessful rounds of ligation and transformation attempts it was decided to use a synthesised gene for further work on PFF1430c as several other researchers in the group had also had difficulty subcloning successfully into the pKSM vector and the codon-optimised synthesised genes were ordered together.

3.2.4.7 Synthesis of Optimised PFF1430c

After multiple unsuccessful attempts at subcloning PFF1430c into the KSM pBluescript vector a codon-optimised PFF1430c flanked by the 5'- and 3'-untranslated regions of the *Xenopus* β-globin gene was designed and synthesised (synthesised by GenScript Corp., Piscataway, NJ).

3.2.4.8 Plasmid Preparation, Linearisation and cRNA Transcription of Optimised PFF1430c

Carried out as described for the gene-specific DNA library in sections 3.2.3.1 to 3.2.3.3, with the exception of using the mMessage mMachine High Yield RNA Transcription Kit Sp6 (Ambion) instead of the T7 kit.

3.2.5 *X. Laevis Oocyte* Expression System

3.2.5.1 Animal Husbandry

Healthy wild laboratory conditioned sexually mature female *X. laevis* frogs were obtained in batches of 20 from Xenopus Express (Haute-Loire, France). The frogs were kept in specially designed Premier tanks (measuring 118 cm x 50 cm x 50 cm) filled 2/3rd full with non-chlorinated water. The tanks were housed in an aquarium within the School of Biological Sciences at the University of Liverpool which was kept at a constant 18 °C. Feeding with pellet food specifically for amphibians was carried out 3 times a week along with a water change with any uneaten food and other debris removed by siphoning. The tank was thoroughly cleaned once a week. The frogs were examined frequently for any sign of disease or damage, with special attention for any sign of red leg (an infectious disease affecting the underneath of the thighs).

3.2.5.2 Preparation and Selection of Oocytes

For each experiment one frog was selected and euthanised by submersion in 1 litre euthanising dose of ice-cold ethyl 3-aminobenzoic acid ethyl ester methanesulfonate salt (MS222, Sigma), 5g/L in distilled water, pH 7.4 for at least 40 minutes. The euthanised frog was washed thoroughly to remove the anaesthetic solution, and placed on a clean tray lined with absorbent tissue for the surgical removal of oocytes. The forceps and scissors used

for the removal of the ovary sacs were sterilised with 70 % ethanol. Using scissors, a lateral cut through the outer layer of skin across the lower abdomen was made with 2 flaps created by making an upwards cut at the midline. The skin was parted to expose the internal body wall which was cut in the same way as the outer skin, taking care not to sever the artery running along the midline under the body wall. Ovary sacs were then removed from the abdomen of the frog using forceps and the sacs opened up to allow separation of the oocytes.

Two methods for isolating individual oocytes were used. The first method isolated single denuded oocytes from the ovaries enzymatically with collagenase treatment (1 mg/ml, Sigma, Poole, UK) in Barths solution minus calcium (containing 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4.7H_2O$, 15 mM HEPES adjusted to pH 7.6 with NaOH) for 2 hours, shaking at 60rpm on a flat bed shaker, at room temperature. This was followed by thorough washing with Barths solution minus calcium and finally with Barths solution with calcium (as Barths minus calcium with 0.3mM $CaNO_3.6H_2O$ added). Oocytes were then kept at 18 °C for 1 to 2 hours to allow the oocytes to rest. Healthy looking (with animal and vegetal poles sharply contrasting), morphologically intact stage V-VI oocytes were selected and kept in petri dishes filled with Barths solution with calcium supplemented with penicillin and streptomycin at 18

°C overnight to allow another selection of the healthiest looking oocytes before injection.

The second method used a platinum loop to pull individual oocytes from the ovary sacs, with healthy oocytes selected and kept overnight as for the previous method. When using this method injected oocytes were treated with collagenase (as above) for 20 minutes, shaking at 60 rpm on a flat bed shaker, at room temperature after injection and incubation, and prior to the uptake assays being carried out.

3.2.5.3 Microinjection of Oocytes

Needles for injection of cRNA into oocytes were made from R-series borosilicate glass capillaries (World Precision Instruments) using the PUL-1 Micropipette Puller (World Precision Instruments). Briefly, this involved heating the centre point of the capillary until it reached melting point then using the Micropipette Puller to pull the two ends apart, and so creating two needles with long fine points. The needle was then loaded onto the KITE micromanipulator (World Precision Instruments) and the end trimmed off to allow sample to be loaded as the Micropipette Puller creates needles with sealed ends. A stereomicroscope was used to visualise the needle cutting and injection process due to small size of the needle and oocytes being injected. A PV830 Pneumatic PicoPump (World Precision

Instruments) and DA7C vacuum pump (Charles Austen Pumps) were linked to the KITE micromanipulator to provide regulated positive and negative pressure to allow uptake of sample and injection of a specified volume of cRNA or water. The appropriate volume of cRNA (or DEPC treated water as negative control) for the experiment being carried out was loaded into the needle by suction from a droplet placed on film on the stage of the stereomicroscope. The drop size was checked and the size adjusted to approximately 50 nl (therefore approximately 50 ng 1 ng/nl cRNA per drop).

One final selection of the healthiest oocytes from the isolation on the previous day was carried out before injection in order to improve the chances of oocyte survival and expression of the injected cRNA. Not all injected oocytes survive the 4 day incubation period so approximately double the number of oocytes required for the planned uptake experiment were injected (as 10 oocytes were required per condition or time point approximately 20 were injected for each). The selected oocytes were then aligned onto a groove on a plastic rack and injected with approximately 50 nl (~50 ng) of cRNA or approximately 50 nl of DEPC treated water for the negative control.

After injection the injected oocytes were transferred to petri dishes containing Barths solution with calcium supplemented with penicillin and

streptomycin. Each cRNA (or group cRNA) injected oocytes and water-injected negative control oocytes were kept in separate dishes but the same number of oocytes were kept in each and the same batch of Barths solution with calcium (supplemented with penicillin and streptomycin) was used for all oocytes. Injected oocytes were incubated for 4 days to allow expression of the injected cRNA. Throughout this incubation period the oocytes were kept at a constant 18 °C in an incubator kept in the cold room, as this allowed a more accurate maintenance of temperature. This temperature was used as it has been found to be the optimal temperature for maintenance of *X. laevis* oocytes (Sigel and Minier, 2005). This is because frogs are cold blooded animals so oocytes will die at higher temperatures. The Barths solution with calcium supplemented with penicillin and streptomycin was changed daily, and the same batch was used for all oocytes. Any dead or dying oocytes were removed to prevent contamination of the solution. At the end of the incubation period the oocytes were used for uptake studies.

3.2.5.4 Radiolabelled Transport Assays

Uptake assays were performed at room temperature (21 °C) and carried out in Ringers buffer pH 7.4 (106 mM NaCl, 24 mM $NaHCO_3$, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1 mM Na_2HPO_4, 25 mM HEPES). Room temperature was used as all literature examined appeared to use around 21 °C and this was

the standard procedure used within the group (Penny *et al.*, 1998, Carter *et al.*, 2000, Parker *et al.*, 2000, Woodrow *et al.*, 2000, Joet *et al.*, 2003, Downie *et al.*, 2006, Downie *et al.*, 2008). Using this temperature, rather than 37 °C, will affect the V_{max} but should not affect the K_m of transport. The consequences of using this temperature are discussed in the critical assessment section of this chapter (section 3.4.1).

Assays were carried out using a final specific activity of 2 μCi/ml and the concentrations of substrates as follows: choline chloride 30.47 nM, glutamic acid 40 nM, isoleucine 33.34 nM, leucine 12.05 nM, methionine 24.1 nM, pantothenic acid 40 nM, taurine 100 nM and folic acid 30nM), plus or minus unlabelled substrate. For each cRNA and substrate 10 oocytes were incubated with the radiolabelled amino acid for 45 minutes. For the time course experiments 10 oocytes were used for each time point. After incubation oocytes were washed in 4 consecutive baths of ice-cold Barths solution with calcium and placed in individual 1.5 ml microcentrifuge tubes. 1ml of scintillation fluid (Ultima Gold, Corning) was added to each tube, and the uptake measured using liquid scintillation counting (β-counter, Wallac). Figure 3.1 shows a diagram of the *X. laevis* oocyte expression system and the preparation of the cRNA.

The initial group screening consisted of one round of experiments, with ten replicates of each data point measurement within each experiment. This

screening was broken down into several parts. Groups 1 to 3 were investigated first, due to the glycerol stocks of the individual genes in these groups arriving first, with the uptake of isoleucine, methionine, glutamic acid and folic acid measured on the same day, using oocytes from the same frog that were injected on the same day and kept under identical conditions. Uptake of taurine, pantothenic acid, choline chloride and leucine was measured in a subsequent independent experiment, using oocytes from the same frog that were injected on the same day and kept under identical conditions. Groups 4 to 6 were investigated in the same way as groups 1 to 3.

The screening of the individual genes in group 1 consisted of three to six independent experiments for each substrate tested, with ten replicates of each data point measurement within each experiment. In each experiment oocytes from one frog were used. All subsequent experiments were carried out once, with ten replicates of each data point measurement within each experiment, using oocytes from one frog per experiment.

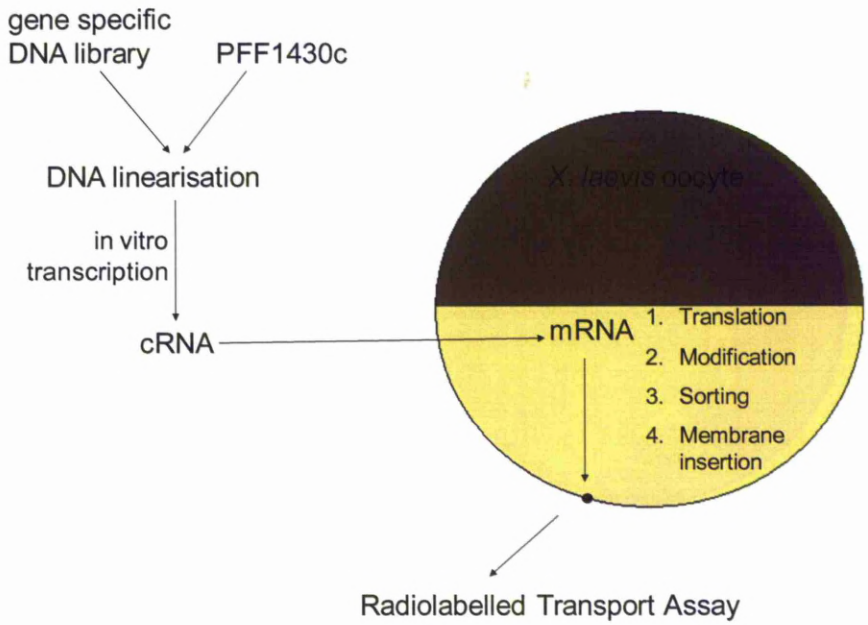


Figure 3.1 Schematic illustration of the *X. laevis* oocyte expression system.

3.2.6 Statistical Analysis of Results

Non-parametric tests were used as the distribution of values within each group could not be determined to be Gaussian. Therefore the Mann-Whitney U test was used to establish whether there was a significant difference between the groups. Bonferroni correction of p values was carried out where multiple comparisons were made to reduce the chances of type I errors occurring. Significance was counted with p values of <0.05.

3.3 Results

3.3.1 Functional characterisation of the gene-specific cDNA Library

3.3.1.1 Screening of Grouped Gene Specific cDNA Library

The gene-specific DNA library of 48 genes was divided into 6 groups of 8 genes for initial screening using the *X. laevis* oocyte expression system (see table 3.1 for group allocations) with the human reduced folate carrier, hRFC1, added to group 1 as a positive control. Water-injected control oocytes, treated in the same way as cRNA injected oocytes, were included in the experiment to allow any endogenous transport by the oocytes to be accounted for. The uptake of methionine, isoleucine, leucine, glutamic acid, taurine, pantothenic acid and choline chloride for each cRNA group and water-injected control oocytes was measured after 45 minutes. These substrates were chosen due to the need for supplementation with them for parasite growth *in vitro* and so increasing the likelihood that the parasite will transport these substrates (Divo *et al.*, 1985, Geary *et al.*, 1985). The uptake of folic acid by oocytes injected with group 1 cRNA (with hRFC1 present) and water-injected control oocytes was also measured after 45 minutes. Uptake of a substrate was counted when there was significantly increased uptake by the group cRNA injected oocytes compared to the water-injected controls or else if a high number of positive oocytes (those

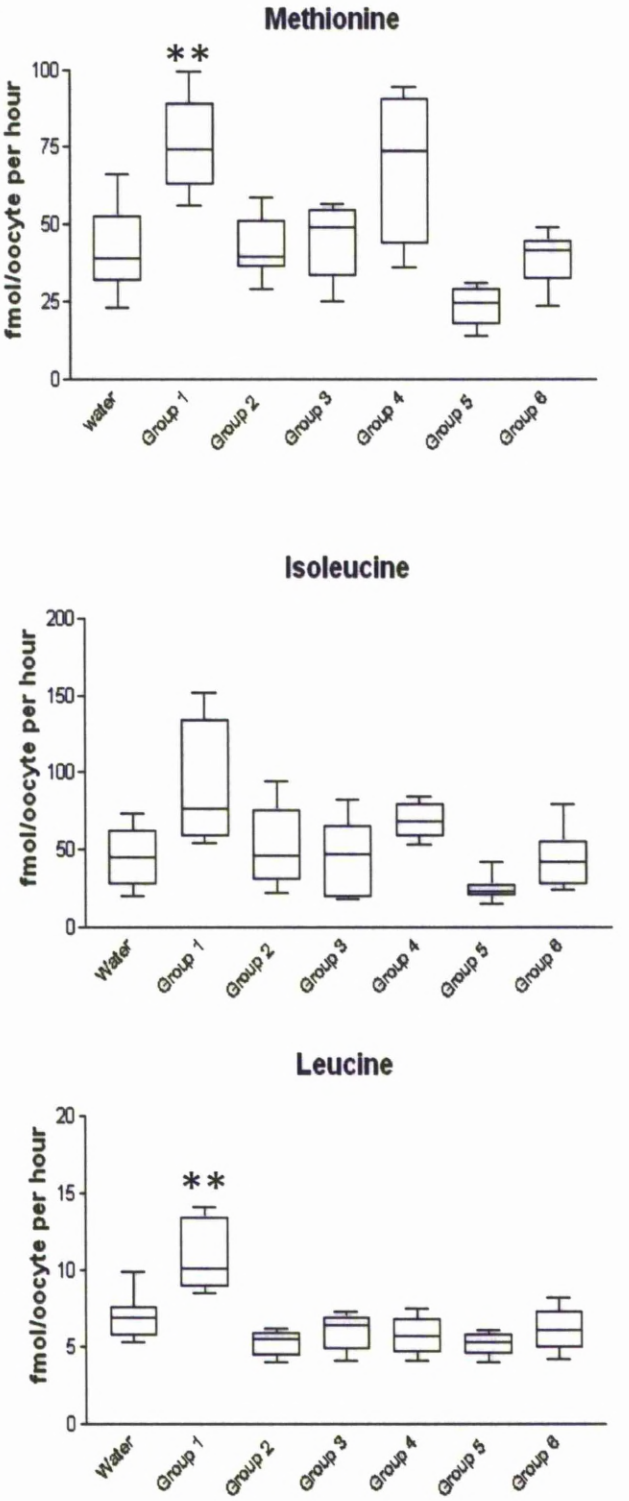
oocytes in cRNA injected groups taking up obviously more than the mean water-injected control value) were observed even if significance between group and water control was lacking. This method was used as the variability between the 10 oocytes within a group was sometimes high making it difficult to prove any statistical difference between the cRNA injected oocytes and the water control oocytes.

The uptake of methionine by oocytes injected with group 1 cRNA was significantly higher than the water-injected oocytes, with a p value of 0.001 (figure 3.2). Group 1 cRNA injected oocytes also appeared to take up more isoleucine than the water-injected controls, however this was not significant. Leucine was also taken up by group 1 cRNA injected oocytes more than the water-injected controls ($p = 0.004$). Group 4 cRNA injected oocytes showed a higher uptake of glutamic acid than the water-injected oocytes ($p = 0.03$). Group 4 cRNA injected oocytes also appeared to take up more methionine than the water-injected controls, however this was not significant. Group 1 and group 4 cRNA injected oocytes showed higher uptake of taurine than the water-injected oocytes, with respective p values of 0.008 and 0.0072. Pantothenic acid appeared to be taken up more by group 4 and group 6 cRNA injected oocytes than the water-injected control oocytes, with p values of 0.0005 and 0.001. No groups showed significantly more uptake of choline chloride than the water-injected controls oocytes. Folic acid was taken up by group 1 cRNA injected

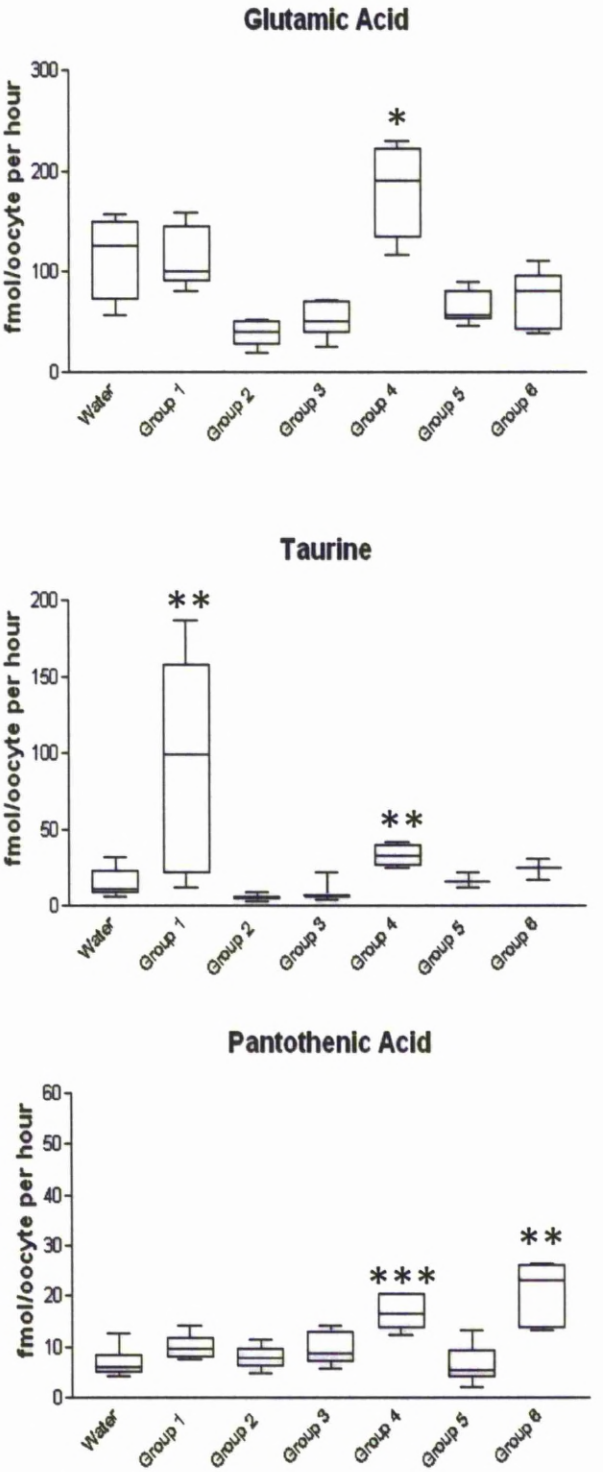
oocytes significantly more than by the water-injected control oocytes with a p value of 0.0003, suggesting that the injected cRNA was successfully expressed on the oocyte plasma membrane.

From these results it was decided to further investigate individual genes in groups 1, 4 and 6 as these showed the most uptake beyond that of the water-injected control oocytes.

A.


Methionine
fmol/oocyte per hour
100
75
50
25
0
**
water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Isoleucine
fmol/oocyte per hour
200
150
100
50
0
Water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Leucine
fmol/oocyte per hour
20
15
10
5
0
**
Water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6

B.


Glutamic Acid
fmol/oocyte per hour
300
200
100
0
*
Water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Taurine
fmol/oocyte per hour
200
150
100
50
0
**
**
Water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6
Pantothenic Acid
fmol/oocyte per hour
60
50
40
30
20
10
0
***
**
Water
Group 1
Group 2
Group 3
Group 4
Group 5
Group 6

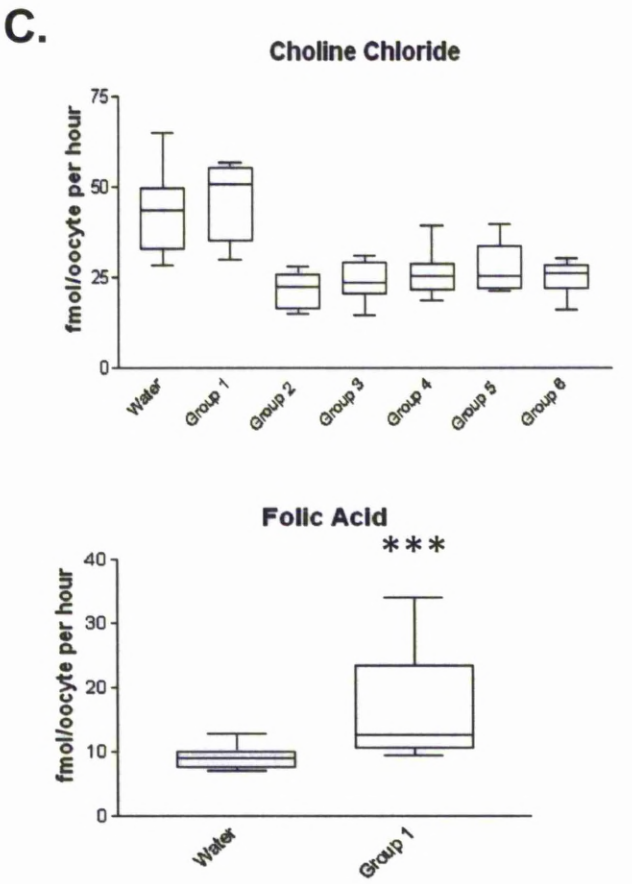


Figure 3.2. Uptake of radiolabelled methionine, isoleucine and leucine (A), glutamic acid, taurine and pantothenic acid (B), and choline chloride and folic acid (C) in water-injected and group 1 to 6 cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point (from the same frog for each data point). Significance values between water-injected and group cRNA injected oocytes were calculated using the Mann-Whitney U test and are Bonferroni corrected (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

3.3.1.2 Screening of Individual Genes from Group 1

The initial screening of grouped cRNA showed group 1 to take up methionine, isoleucine and taurine above the level of water-injected control oocytes (figure 3.2), so the individual genes in this group were further investigated using the *X. laevis* oocyte expression system. The 8 genes from the cDNA library in group 1 (table 3.1) were individually linearised and cRNA was synthesised *in vitro* from the linearised cDNA. In order to characterise these genes *X. laevis* oocytes were microinjected with the cRNA from individual genes and assayed for the uptake of radiolabelled methionine, isoleucine and taurine. There was no obvious increase in uptake of isoleucine or taurine by any of the oocytes injected with individual gene cRNA compared to water-injected oocytes (figure 3.3). Although there was no significant increase in uptake of methionine compared to water-injected oocytes there was an increased number of positive oocytes for those injected with gene 41 (MAL13P1.210) and gene 49 (PF11_0384) cRNA (figure 3.3).

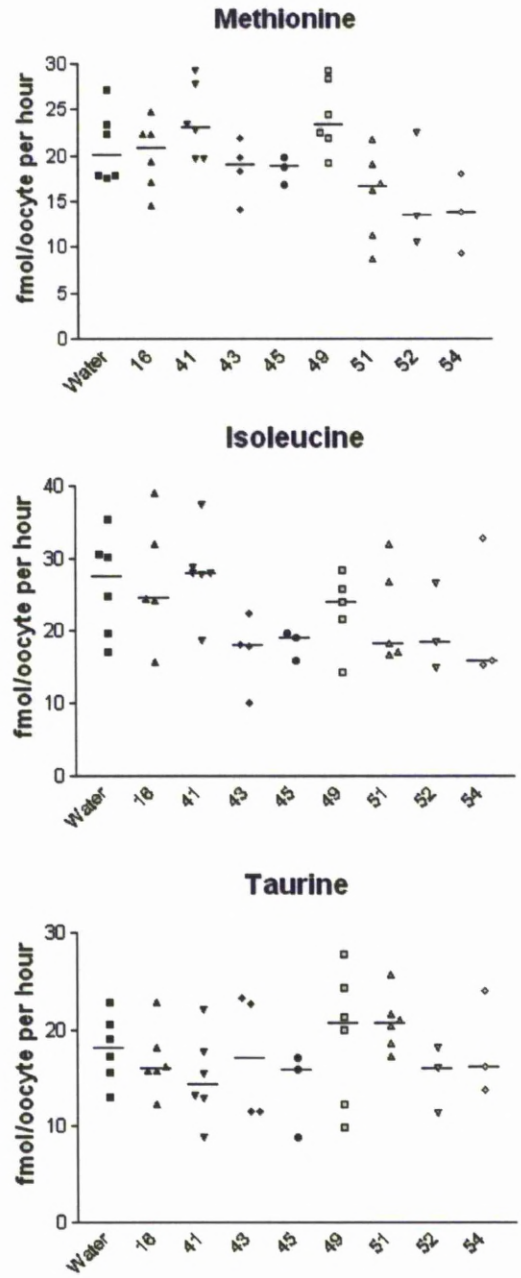


Figure 3.3. Uptake of radiolabelled methionine, isoleucine, and taurine in water and group 1 individual cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data points shown are the averages from 3 to 6 independent experiments (with each independent experiment carried out using oocytes from one frog), each with 10 oocytes per data point, and the median line shown.

In order to further investigate the potential methionine transport by genes 41 (MAL13P1.210) and 49 (PF11_0384) the effect of 0.2 mM unlabelled methionine on the uptake of [^{3}H] methionine over 45 minutes was examined. The results (figure 3.4) showed no significant inhibitory effect by the unlabelled methionine, with no significant decrease in the uptake of methionine with the addition of unlabelled methionine observed for either 41 (MAL13P1.210) or 49 (PF11_0384). Neither gene showed an increase in uptake of methionine compared to the water-injected oocytes. These results suggest that there may be no specific uptake of methionine by either gene. However, as only one concentration of unlabelled methionine was used it is not possible to conclude that there is no specific uptake as the concentration used may have been too low to measure any inhibition. The results presented in the following chapter showed that methionine uptake by free *P falciparum* trophozoites has a K_m value of 1.13 ± 0.16 mM and that there was no significant inhibition of methionine uptake with the addition of 2 mM of unlabelled methionine. Therefore, the concentration of unlabelled methionine added here was likely to be too low (far below the K_m value and, therefore, the K_i value) to measure inhibition of transport by a *P. falciparum* encoded protein. As gene 49 (PF11_0384) showed very little uptake compared to the water-injected oocytes it was concluded that it was unlikely to be a transporter of methionine, and so work on this gene was stopped. However, as accumulation of methionine by gene 49 injected oocytes was significantly lower than the water-injected controls ($p = 0.001$)

there is a possibility that this gene is involved in the efflux of methionine from the oocyte, and in doing so cancels out the oocytes endogenous methionine uptake transporters. This is interesting as *Plasmodium* parasites must release large quantities of the amino acids generated from haemoglobin digestion, although in the case of methionine only small quantities are found in haemoglobin. However, six previous experiments had shown accumulation of methionine by gene 49 injected oocytes to be slightly, but not significantly, higher than the control so it was decided not to further investigate possible methionine efflux by gene 49.

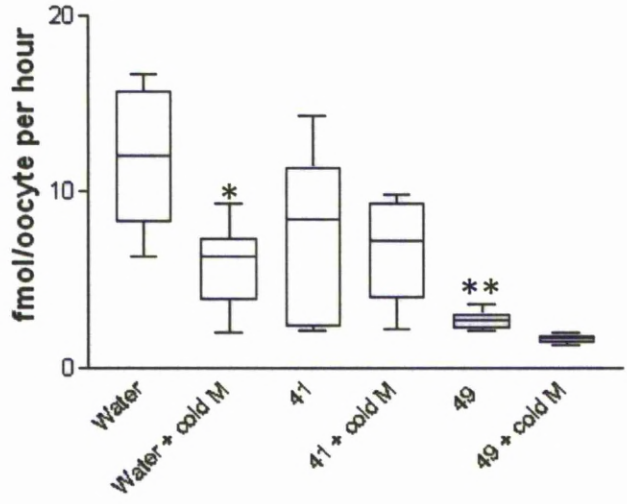


Figure 3.4. Uptake of radiolabelled methionine with and without 0.2 mM unlabelled methionine in water and gene 41 and 49 cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point (from the same frog). Significance values shown are between water-injected and other results. P values were calculated using the Mann-Whitney U test and are Bonferroni corrected (* = $p < 0.05$, ** = $p < 0.01$).

There was a decrease in the uptake of methionine by water-injected oocytes when unlabelled methionine was added ($p = 0.025$) (figure 3.4). One potential reason for the inhibition of [^{3}H] methionine uptake by unlabelled methionine in the water-injected control oocytes is that there is an endogenous low-capacity high-affinity transporter which has reached

saturation at the concentrations used. As this endogenous transport could have been masking any methionine transport by gene 41 (MAL13P_1.210) a time course experiment was carried out to find out if there was a time point where the transport of methionine by gene 41 (MAL13P_1.210) was significantly higher than uptake by the water-injected oocytes. Uptake of methionine by gene 41 (MAL13P1.210) cRNA and water-injected oocytes was measured at 10, 20, 30, 45 and 60 minutes (figure 3.5). There was no significant increase in uptake of methionine by gene 41 (MAL13P1.210) cRNA injected oocytes compared to water-injected oocytes at any time point, and so work on this gene was discontinued.

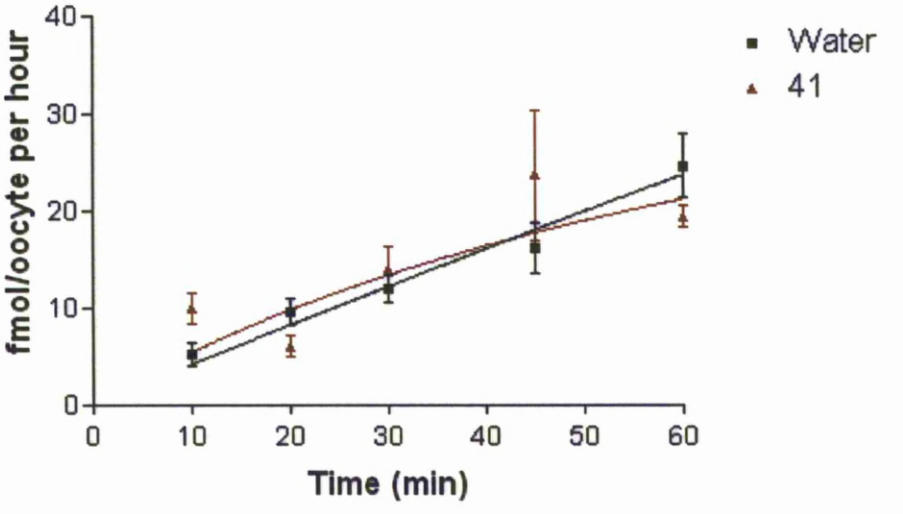


Figure 3.5. Time course uptake of radiolabelled methionine in water-injected and gene 41 cRNA injected oocytes. Data shown is the median ± standard error from 10 individual oocytes for each time point (from the same frog).

3.3.1.3 Screening of Individual Genes from Group 4

The results from the initial screening of grouped cRNA showed group 4 to take up glutamic acid, taurine and pantothenic acid above the level of water-injected control oocytes (figure 3.2), so the individual genes in this group were further investigated using the *X. laevis* oocyte expression system. The genes in group 4 (table 3.1) were individually linearised and cRNA synthesised *in vitro*. In order to characterise these genes *X. laevis* oocytes were microinjected with the cRNA from individual genes and assayed for the uptake of radiolabelled glutamic acid, taurine and pantothenic acid. The uptake of taurine and pantothenic acid was higher in gene 98 (PF10_0317) cRNA injected oocytes than in water-injected oocytes, with p values of 0.0021 and 0.0007 respectively (figure 3.6). Gene 95 (PF10_0276) cRNA injected oocytes showed a lower accumulation of glutamic acid than water-injected oocytes. This is interesting as *Plasmodium* parasites must release large quantities of the amino acids generated from haemoglobin digestion, therefore, amino acid efflux transporters are physiologically important. Gene 93 (PFI0800c) cRNA injected oocytes showed more uptake of glutamic acid than water-injected oocytes, $p = 0.004$ (figure 3.6). However, the uptake of glutamic acid by gene 93 (PFI0800c) and the efflux of glutamic acid by gene 95 (PF10_0276) were not further investigated due to time constraints.

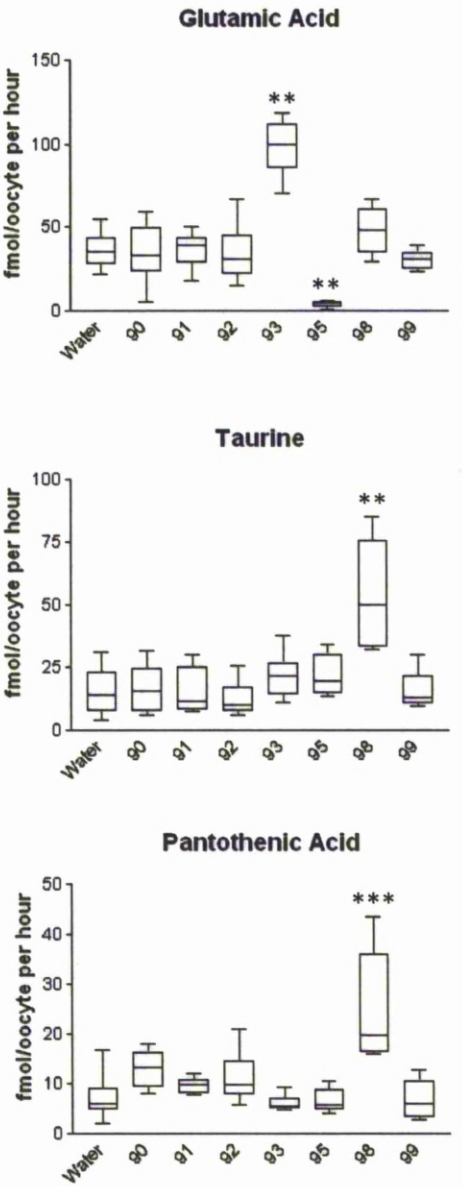


Figure 3.6. Uptake of radiolabelled glutamic acid, taurine and pantothenic acid in water-injected and individual group 4 cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point (from the same frog). Significance values between water-injected and cRNA injected oocytes were calculated using the Mann-Whitney U test and are Bonferroni corrected (** = $p < 0.01$, *** = $p < 0.001$).

The uptake of taurine and pantothenic acid by gene 98 (PF10_0317) was further investigated by examining the effect of the addition of 0.2 mM unlabelled taurine and pantothenic acid on [^{3}H] taurine and [^{3}H] pantothenic acid over 45 minutes. The effect of both unlabelled taurine and pantothenic acid on [^{3}H] taurine and [^{3}H] pantothenic acid was investigated in order to see if these substrates are transported via the same route; as inhibition of the radiolabelled substrate would be inhibited by the addition of either of the unlabelled substrates if they do use the same route of uptake. Unlike the previous experiment these results showed no increase in uptake of [^{3}H] taurine or [^{3}H] pantothenic acid by gene 98 (PF10_0317) cRNA injected oocytes compared to water-injected controls (figure 3.7). There does appear to be specific uptake of pantothenic acid by water-injected oocytes as the addition of 0.2 mM unlabelled pantothenic acid significantly reduced the uptake of [^{3}H] pantothenic acid (figure 3.7). One potential reason for the inhibition of [^{3}H] pantothenic acid uptake by unlabelled pantothenic acid in the water-injected control oocytes is that there is an endogenous low-capacity high-affinity transporter which has reached saturation at the concentrations used. The results also showed no significant inhibitory effect on taurine uptake by unlabelled taurine for either gene 98 cRNA injected oocytes or water-injected oocytes. However, as only one concentration of unlabelled taurine was used it is not possible to conclude that there is no specific uptake as the concentration used may have been too low to measure any inhibition. Unlabelled taurine at 200 μM

is likely to be well below the predicted K_m and K_i (the inhibition constant, the inhibitor concentration required to produce half maximal inhibition) values of taurine transport. This would mean that competitive inhibition of taurine transport would not be observed with a decrease in accumulation of [^{3}H] taurine, even if specific transport was occurring. In this case the competitive inhibitor is the same as the substrate being investigated, so the K_i value is likely to be similar to the K_m value (Van Winkle, 1999).

Due to the fact that there appeared to be no specific uptake of either substrate by gene 98 (PF10_0317) it was decided not to carry out any further studies into this gene. This decision was taken as the two results (figures 3.6 and 3.7) did not show consistent uptake of pantothenic acid or taurine by gene 98 (PF10_0317) and also due to time constraints. However, further experiments are necessary before it is possible to be confident that this gene does not encode a transporter for these substrates, especially as there was no proof of heterologous expression in these experiments.

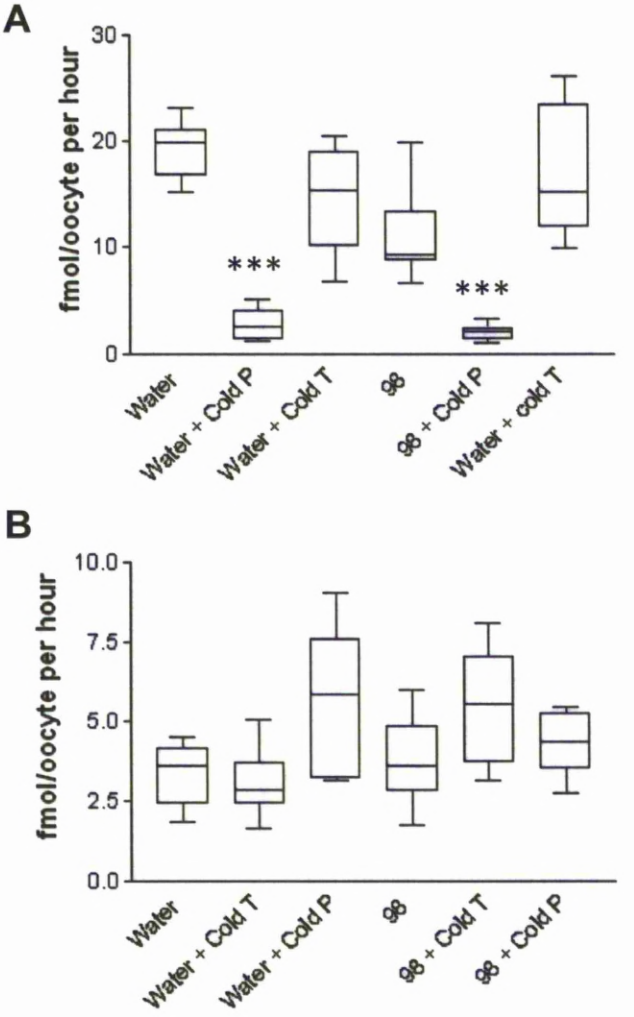


Figure 3.7. Uptake of radiolabelled pantothenic acid (A) and taurine (B) plus competition with 0.2mM unlabelled pantothenic acid (Cold P) and taurine (Cold T) in water-injected and gene 98 cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point (from the same frog). Significance values between water-injected and cRNA injected oocytes were calculated using the Mann-Whitney U test and are Bonferroni corrected (** = $p < 0.01$, *** = $p < 0.001$).

3.3.1.4 Screening of Individual Genes from Group 6

The results from the initial screening of grouped cRNA showed oocytes injected with group 6 cRNA to take up pantothenic acid above the level of water-injected control oocytes (figure 3.2), so the individual genes in this group were further investigated using the *X. laevis* oocyte expression system. The genes in group 4 (table 3.1) were individually linearised and cRNA synthesised *in vitro* from the linearised cDNA. In order to characterise these genes for pantothenic acid transport *X. laevis* oocytes were microinjected with the cRNA from individual genes and assayed for the uptake of radiolabelled pantothenic acid for 45 minutes. No uptake was observed for any of the genes and due to time constraints no investigation into the uptake of taurine was carried out (figure 3.8).

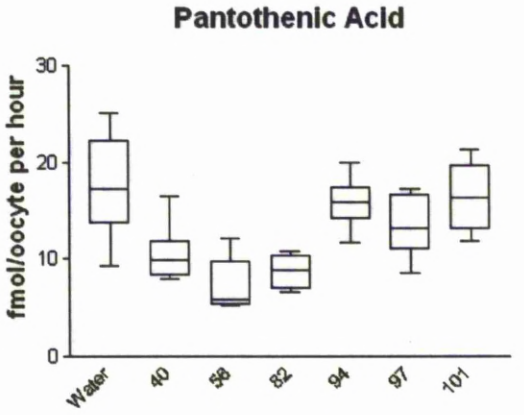


Figure 3.8. Uptake of radiolabelled pantothenic acid in water-injected and group 6 individual cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point (from the same frog).

3.3.2 Cloning of PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c, PF11_0334

The initial PCR of PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c and PFF_0334 from cDNA was carried out using Platinum® *Pfx* DNA Polymerase (Invitrogen) and using the cycling conditions given in section 3.2.4.1. cDNA synthesised from RNA isolated from mixed stage cultures was used as gene expression has been shown to vary slightly between these genes (Martin *et al.*, 2005). This initial amplification attempt did not show any bands of the correct size for the six genes of interest but did a band was observed for the PfCRT control (figure 3.9).

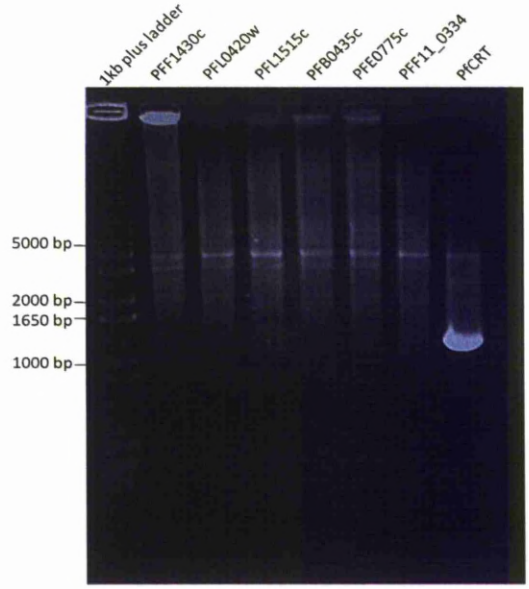


Figure 3.9. Agarose gel visualisation of PCR products from the initial PCR reaction.

In order to achieve amplification several methods were attempted to optimise the PCR reaction. The extension time was increased in order to improve amplifaction and gradient PCR (with and without PCR_x enhancer) was carried out but both of these were unsuccessful in amplifying any of the six genes of interest. As PFL0420w does not contain any exons an attempt at amplifying it using genomic DNA with three different polymerases was carried out. This resulted in a band of the correct size after amplification with Platinum® taq DNA Polymerase (Invitrogen) when visualised by agarose gel electrophoresis (figure 3.10).

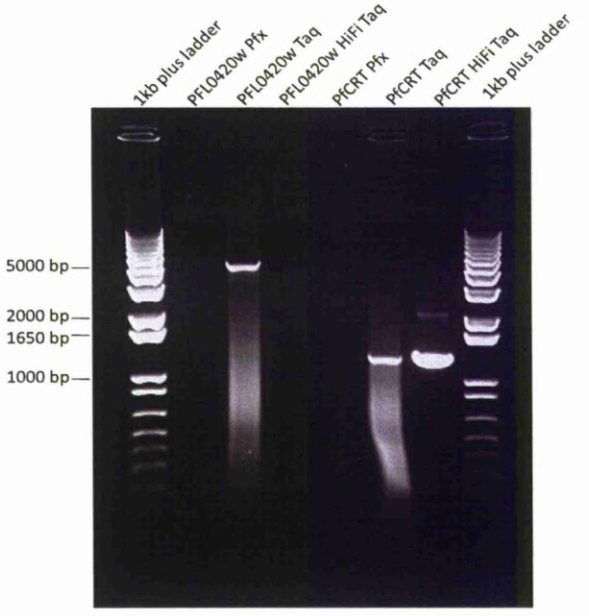


Figure 3.10. Agarose gel visualisation of PCR products from amplification of PFL0420w or PfCRT with Platinum® *Pfx* DNA Polymerase, Platinum® taq DNA Polymerase or Platinum® taq DNA Polymerase High Fidelity.

Touchdown PCR was used in a final attempt to amplify PFF1430c, PFL0420w, PFL1515c, PFB0435c, PFE0775c and PF11_0334 from cDNA. This was successful for PFF1430c with a band of the correct size visualised by agarose gel electrophoresis (figure 3.11).

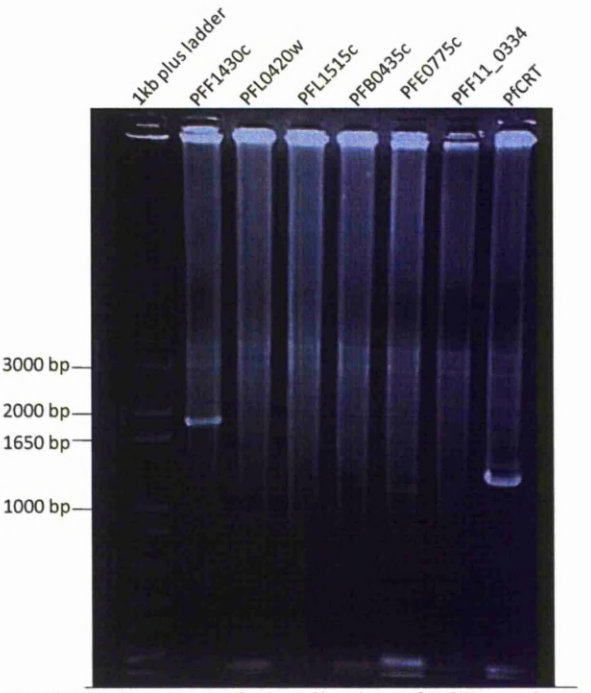


Figure 3.11. Agarose gel visualisation of PCR products from touchdown PCR.

PFF1430c was amplified using a proofreading enzyme so the resulting PCR product lacked 3' A-overhangs necessary for cloning so these were recreated by dA-tailing of the PCR product. The resulting dA-tailed PFF1430c and the PFL0420w amplified from genomic DNA were ligated

into the pCR II TOPO® vector and transformed into One Shot® TOP10 competent cells. Overnight cultures from individual white colonies on LB agar plates were made and the plasmids prepared from these cultures. A restriction digest with EcoRI was used to check for the presence of insert and agarose gel electrophoresis of the digest product showed that PFF1430c_pCR®II-TOPO® had been successfully ligated but that PFL0420w_ pCR®II-TOPO® had not been. Another restriction digest, this time using EcoRV, was used to determine the orientation of the PFF1430c insert in the pCR®II-TOPO® vector. Visualisation by agarose gel electrophoresis showed that the orientation was correct and so this was used for subcloning into the pKSM *Xenopus* optimised vector.

Five rounds of subcloning were attempted with varying molar ratios, different ligation conditions and using a different combination of restriction enzymes to digest the pKSM vector and PFF1430c_pCR®II-TOPO®. As none of these resulted in successful ligation it was decided to use a synthesised gene for further work on PFF1430c, and a codon-optimised PFF1430c flanked by the 5'- and 3'-untranslated regions of the *Xenopus* β-globin gene was synthesised (GenScript Corp., Piscataway, NJ). Several other people in the group also had difficulty subcloning successfully into the pKSM vector and the codon-optimised synthesised genes were ordered together.

3.3.3 Functional characterisation of PFF1430c

PFF1430c has been designated a putative amino acid transporter but has not yet been functionally characterised (Martin *et al.*, 2005). Subcloning of this gene into the optimal vector for *X. laevis* expression, KSM pBluescript, was unsuccessful. To address this problem a codon-optimised PFF1430c flanked by the 5'- and 3'-untranslated regions of the *Xenopus* β-globin gene was synthesised and functionally characterised (GenScript, USA). In order to characterise this gene *X. laevis* oocytes were microinjected with *in vitro* transcribed cRNA from the cloned plasmid insert of PFF1430c and assayed for the uptake of radiolabelled methionine, isoleucine, leucine, glutamic acid, taurine and pantothenic acid. PFF1430c cRNA injected oocytes showed no increase in uptake of methionine, isoleucine, leucine, glutamic acid, taurine and pantothenic acid compared to water-injected oocytes (figure 3.12).

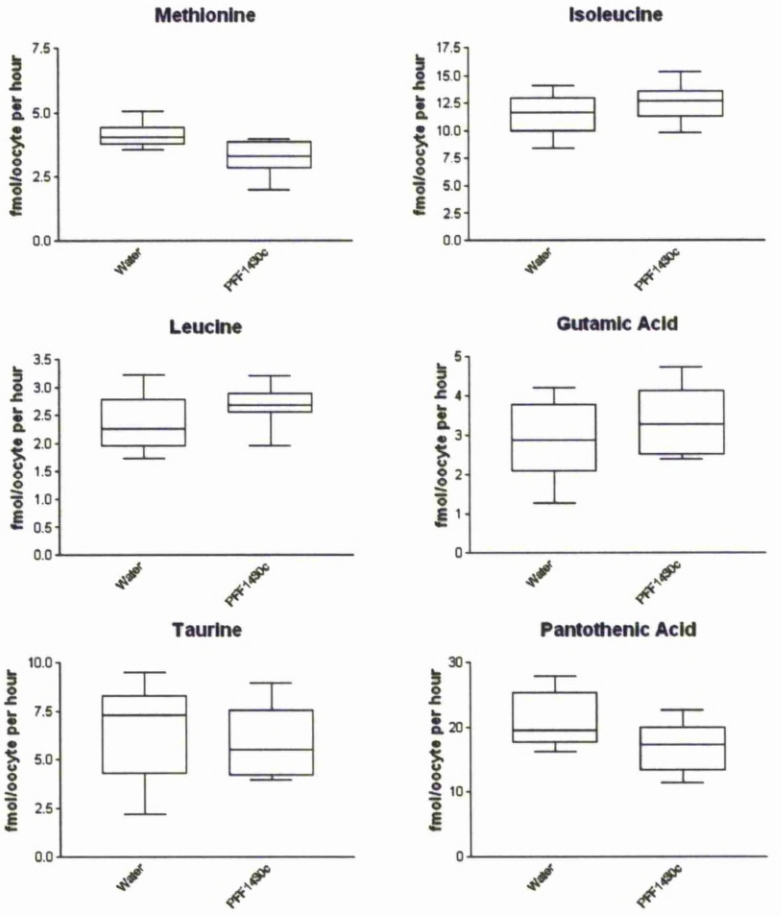


Figure 3.12 Uptake of radiolabelled methionine, isoleucine, leucine, glutamic acid, taurine, pantothenic acid and choline chloride in water-injected and PFF1430c cRNA injected oocytes. Uptake was measured over 45 minutes in Ringers pH 7.4. Data shown is the central median line, with the box representing the central 50 % of data and the whiskers showing the data range, from 10 individual oocytes per data point, (from the same frog).

As PFF1430c shows some homology to system N amino acid transporters the uptake of glutamine was assayed, as this is the classical substrate for system N (Barker and Ellory, 1990). A time course experiment was used in order to be able to identify if there was any time dependent uptake and also

so that the time point where the greatest potential difference between PFF1430c cRNA injected oocytes and water-injected oocytes could be identified. There was no increase in uptake of glutamine by PFF1430c cRNA injected oocytes compared to the water-injected oocytes over all time points (figure 3.13).

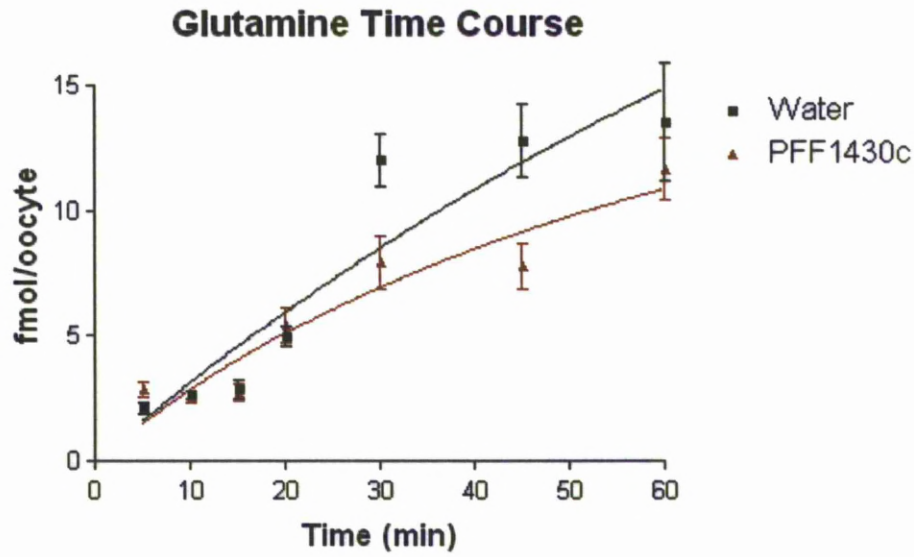


Figure 3.13. Time course uptake of radiolabelled glutamine in water-injected and gene PFF1430c cRNA injected oocytes. Data shown is the median ± standard error from 10 individual oocytes per time point (from the same frog).

The lack of uptake of any of the amino acids tested with PFF1430c is surprising as this gene shows homology to the AAAP family of amino acid transporters and similarity to system N amino acid transporters.

3.4 Discussion

Amino acid transport is important to the malaria parasite as it has been shown *in vitro* that the presence of certain amino acids is required for growth (Divo *et al.*, 1985). Currently there are only six genes annotated as putative amino acid transporters in the *P. falciparum* genome, and none of these have yet been functionally characterised (Martin *et al.*, 2005). As it is possible that there are more, as yet undiscovered, *P. falciparum* amino acid transporters a gene-specific DNA library of forty eight genes was created. Genes were chosen based on having more than three trans-membrane domains and lacking signal peptides. In this chapter the gene-specific DNA library and PFF1430c (one of the putatively annotated amino acid transporters) were functionally characterised for amino acid transport using the *X. laevis* oocyte expression system. The original intention was also to characterise the other five putatively annotated amino acid transporters (PFL0420w, PFL1515c, PFB0435c, PFE0775c and PF11_0334). However, attempts to amplify these genes were not successful and so no further work was carried out on them.

The *X. laevis* oocyte expression system was chosen over other heterologous expression systems as it is a well characterised eukaryotic system, normally produces faithful and efficient translation of exogenous RNA and it has previously been used successfully to study several *P.*

falciparum transporter proteins (Joet *et al.*, 2003, Martin *et al.*, 2009, Downie *et al.*, 2007, Downie *et al.*, 2006, Krishna *et al.*, 2001b). Initial screening of the gene-specific DNA library was carried out with the genes collected into groups of 8. This allowed more rapid identification of any potential transporters and saved time. Genes in groups that showed evidence of transport were then studied individually. From the initial grouped screening there was evidence of transport of methionine, isoleucine, taurine, glutamic acid and pantothenic acid. However, when the individual genes were studied no genes responsible for this transport were definitively identified. Characterisation of the PFF1430c gene showed no evidence of transport of glutamic acid, glutamine, isoleucine, leucine, methionine, taurine, pantothenic acid or choline chloride.

The lack of any conclusive evidence of amino acid transport by the genes in the gene-specific cDNA library or the putative amino acid transporter PFF1430c could be due to several factors. One could be that simply none of the genes in the cDNA library or PFF1430c are transporters of the amino acids tested; however there are too many other factors to conclude that this is the case. The fact that very low substrate concentrations were used erroneously may not have allowed detectable uptake by scintillation counting even if any of the proteins expressed are amino acid transporters. This problem is discussed further in the critical assessment section below. Only one round of expression was carried out for the group screening, and

along with the multiple comparisons carried out this could have resulted in false positive results. Multiple comparisons increase the familywise error rate and, therefore, increase the chance of type I errors occurring. This would mean that it is possible that in some cases the null hypothesis could be erroneously rejected (i.e. the uptake of substrate by cDNA injected oocytes was falsely identified as being significantly different to uptake by water-injected control oocytes). The Bonferroni correction was used throughout this chapter where multiple comparisons were made in order to correct for this error.

Another potential reason for transport being observed in the group stage but not from individual gene expression is that transport activity could have been dependent on the presence of co-factors or heterodimers within the groups. An example of this is the heteromeric system L transporter unit which is composed of a heavy (4F2hc) and light chain (LAT1) heterodimer (Wagner et al., 2000a, Broer et al., 1998, Ritchie and Taylor, 2001). Amino acid transporters in the TRAP-T family and ABC superfamily are also composed of multiple subunits (Saier, 2000a).

There are limitations within the *X. laevis* oocyte expression system including variation in oocyte quality between different frogs, which could affect the reproducibility of results, and the potential for endogenous transporters to mask any transport activity of the inserted protein (Taylor *et*

al., 1996). The characteristics of endogenous *X. laevis* oocyte transport of folic acid, leucine glutamic acid and glutamine are shown in table 3.3 as these are the only substrates used in this study for which detailed characterisation has been carried out. Leucine, glutamic acid and glutamine are all transported via a Na^+ dependent system (Belle *et al.*, 1976, Taylor *et al.*, 1989, Steffgen *et al.*, 1991, Marciani *et al.*, 1998). The transport of glutamine appears to be via the Na^+ dependent system $B^{0,+}$ and this is the only well documented amino acid transporter definitely found in *X. laevis* oocytes (Taylor *et al.*, 1989, Van Winkle, 1993). In *X. laevis* oocytes system $B^{0,+}$ appears to transport a broad range of zwitterionic and cationic amino acids (Van Winkle, 1993). Glutamine transport has been shown to be inhibited by BCH and a range of amino acids including alanine (table 3.2) (Taylor *et al.*, 1989). The addition of these to the glutamine transport assays might have allowed reduction of the endogenous transport background. This was not used in the experiments carried out in this chapter but would have been a very useful addition.

Substrate	K_m in *X. laevis* oocytes	Characteristics
Folic Acid	42 ± 7 nM (Lo *et al.*, 1991)	Na^+ independent, highly pH dependent (severely inhibited below pH 6.5) Inhibited by anion transport inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) and 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SIDS) (Lo et al., 1991)
Leucine	57 ± 21 µM (Marciani *et al.*, 1998)	Na^+ dependent Partially inhibited by ouabain, an inhibitor of membrane ATP-ases (Belle *et al.*, 1976)
Glutamic Acid	Two saturable components, one low affinity (Km 9 mM) and one high affinity (Km 0.35 µM) (Steffgen *et al*, . 1991)	Na^+ dependent (Steffgen et al,. 1991)
Glutamine	0.12 ± 0.02 mM (Taylor *et al.*, 1989)	Na^+ dependent Inhibited by L-alanine, D-alanine, L-asparagine and L-arginine (~60 % inhibition at 1 mM); L-histidine, L-valine and L-glycine (25-40 % inhibition at 1 mM); L-serine, L-lysine, L-phenylalanine and L-glutamate (40-55 % inhibition at 10 mM); BCH (50 % inhibition at 10 mM) (Taylor et al., 1989)

Table 3.3. Substrates used in this study for which the transport characteristics in *X. laevis* oocytes are known.

3.4.1 Critical Assessment

To address the failure of the cloning efforts, one step RT-PCR, using gene-specific primers, could have been attempted. The reason for performing reverse transcription using random hexamers was to increase versatility as the resulting cDNA could then be used for multiple PCRs. Carrying out

two step RT-PCR also allowed optimisation of the two steps without compromising one for the other. Using random hexamers to prime the reverse transcription could, however, have resulted in the generation of a very small amount of cDNA of the genes of interest which would have made amplification of these genes by PCR difficult. This potential problem could have been remedied by using gene-specific primers.

Subcloning into the pKSM *X. laevis* vector has been found to be problematic by various other researchers in our group. A possible way around these problems would have been to use an alternative *X. laevis* expression vector as used by other groups, for example the pBSTA plasmid (Very and Gaymard, 1995) and the pXBG plasmid (Preston *et al.*, 1992, Francis *et al.*, 2000). A codon-optimised synthesised PFF1430c, flanked by the 5'- and 3'-untranslated regions of the *X. laevis* β-globin gene, was created instead of trying a different vector in order to save time.

Ideally, codon optimisation would have been carried out for all genes rather than just for PFF1430c. The reason it was not carried out for the cDNA library was an issue of cost as the cDNA library was already in the process of being created when this project began. For PFF1430c creating a codon-optimised synthesised gene was deemed to be worth the expense as it is annotated as a potential amino acid transporter (Martin *et al.*, 2005) and was created during the course of this project. *Xenopus* 3' and 5' β-

globin UTR flanking sequences were added to the codon-optimised synthesised PFF1430c as it was simple to do so at the time (no subcloning would be required) and previous studies have shown that they increase expression (Woodrow *et al.*, 2000). Ideally these flanking sequences would have been incorporated into the genes in the cDNA library but the time it would have taken to subclone all 48 genes into the pKSM vector with the *Xenopus* flanking sequences would have made it very difficult to carry out in the time frame of this thesis. This is a weakness in the methodology used as Woodrow *et al.*, (2000) noted that when *Xenopus* β-globin UTR flanking sequences were added to the *P. falciparum* hexose transporter (PfHT) ORF uptake of glucose was increased compared to PfHT ORF alone.

The temperature used for the radiolabelled uptake assays carried out in this chapter was 21 °C. This temperature was chosen instead of 37 °C for practical reasons, as it was much simpler to use room temperature (which was measured to be consistently 21 °C). Temperature is an important factor affecting transporters and enzymes, however it usually only affects the V_{max} but not the K_m of transport. The K_m gives important information on the affinity of the transporter for the substrate, with a relatively high K_m signifying low affinity and a relatively low K_m signifying high affinity. The V_{max} gives less information on the nature of the transport, but is useful if the transport is to be modelled. Temperature changes can affect the lipid

environment in which transporters reside, as the lipid bilayers that make up cell membranes undergo phase transitions with changes in temperature. Therefore, this can have an indirect effect on membrane transporters (Carruthers and Melchoir, 1988). In order to comprehensively characterise a transporter, reaction parameters need to be investigated at a range of temperatures. This can yield information on the way the transporter operates at different temperatures which can give insights into the nature of the transporter. Results gathered from experiments carried out over a range of temperatures can be plotted on an Arrhenius plot. This is a plot of the logarithmic value of the rate constant, K, versus the inverse temperature, 1/T, which results in a negatively sloped line that is equal to the negative activation energy divided by the gas constant, R. The activation energy (E_a), the minimum energy required before a reaction can occur, can then be calculated and this can give information on the nature of the transporter being investigated. Channels typically have E_a values of 4 to 8 kcal.mol^{-1} (Hille, 1978, Maurel *et al.*, 1994, Chraibi and Horisberger, 2002) and carriers typically have E_a values of 9 to 40 kcal.mol^{-1} (Loo *et al.*, 1999, Zhang and Kaback, 2000, Derbyshire *et al.*, 2008, Martin *et al.*, 2008). Transport by a carrier typically results in a high E_a as the carrier protein must undergo a conformational change to translocate the substrate across the membrane. The lower E_a for transport via a channel is due to the fact that channel proteins do not undergo as extensive conformational changes as carrier proteins and are less temperature dependent. This technique has

been utilised to analyse whether the chloroquine resistance conferring form of PfCRT ($PfCRT^{CQR}$) behaves like a channel or carrier when expressed in *X. laevis* oocytes (Summers and Martin, 2010). $PfCRT^{CQR}$ was shown to have an E_a of 41.3 $kcal.mol^{-1}$ for transport of chloroquine, suggesting that a substantial conformational change occurs during the translocation of chloroquine and supporting the theory that PfCRT is a carrier. If any definite uptake had been observed by the genes in the cDNA library, or PFF1430c, then further characterisation on the temperature dependence of transport would have been extremely useful for determining the nature of transport.

A major problem with the experimental work described in this chapter is that very low concentrations of substrates were used erroneously in the uptake experiments. This error was not realised in time to repeat the experiments in this chapter but the following work in this thesis does make use of physiological concentrations of substrates. The substrate concentrations used, compared to the concentrations found in human plasma are shown in table 3.4, and in all cases except folic acid the concentrations used in this study are much lower. This may have resulted in uptake that was insufficient to be detected by scintillation counting and so makes it difficult to draw any conclusions. Radiolabelled uptake assays should have been carried out using concentrations of substrates close to or just below the expected K_m values. The use of only one, relatively low,

concentration of unlabelled substrate (0.2 mM) in the inhibition experiments may also have been too low to measure inhibition of transport by a *P. falciparum* encoded protein, as this concentration may be well below the K_m and K_i values for the transport activity under investigation. This limitation would result in a lack of inhibition being noted: the low concentrations used would mean no competitive effect between the radiolabelled and unlabelled substrates would be observed by a decrease in accumulation of the radiolabelled substrate. If any *P. falciparum* encoded protein investigated does transport the substrates tested, inhibition by unlabelled substrate would only be observed when the substrate concentration becomes high enough to allow competition between the radiolabelled and unlabelled substrate to be observed. This would correlate with the results obtained in the next chapter where it was observed that inhibition of radiolabelled methionine uptake by the free *P. falciparum* parasite was only achieved when 10 mM unlabelled methionine was added. This should have been further investigated by repeating the uptake experiments with higher concentrations of unlabelled substrates as this would have allowed saturation to be reached. The inhibition of radiolabelled methionine uptake by unlabelled methionine in the water-injected control oocytes could be explained by the presence of an endogenous low-capacity high-affinity transporter which has reached saturation at the concentrations used.

Substrate	Concentration Used In This Study	Physiological Range in Human Plasma	K_m in Plasmodium or other organism
Folic Acid	30 nM	0.5 nM	*X. Laevis* oocytes - 42 ± 7 nM (Lo *et al.*, 1991)
Methionine	24.1 nM	16 - 30 µM	*P. falciparum* trophozoites (free) - 1.132 ± 0.16 mM (from this thesis, chapter 4) 1.6 ± 0.3 mM (Cobbold *et al.*, 2010)
Isoleucine	33.34 nM	42 - 100 µM	Human RBCs - 10 ± 1 mM *P. falciparum* trophozoites (free) - 0.93 ± 0.26 µM (Martin and Kirk, 2006)
Leucine	12.05 nM	66 - 170 µM	Human RBCs - 12.9 mM (Young *et al.*, 1980)
Glutamic Acid	40 nM	18 - 98 µM	*Leishmania amazonensis* - 0.59 ± 0.04 mM (Paes *et al.*, 2008)
Glutamine	45.46 nM	390 - 650 µM	Human RBCs - 2.5 ± 3.5 µM (Ellory *et al.*, 1983)
Taurine	100 nM	45 - 130 µM	*Anguilla japonica* (eel) RBCs - 22.6 ± 2.3 mM (Fincham *et al.*, 1987)
Pantothenic Acid	40 nM	1.57 - 2.66 µM	*P. falciparum trophozoites* (free) - 22.6 ± 2.3 mM (Saliba and Kirk, 2001)
Choline Chloride	30.47 nM	10 - 20 µM	*P. falciparum* trophozoites (free) - 25 ± 3.5 µM (Biagini *et al.*, 2004)

Table 3.4. Substrates concentrations used in this study, their concentrations in human plasma and the *K*m of transport in *Plasmodium* or other cells.

An issue in the experimental design of the group screening experiments is that they were broken down into sections. This was done by first investigating groups 1 to 3 then groups 4 to 6, and splitting the uptake experiments into two (with one experiment for isoleucine, methionine, glutamic acid and folic acid, and another for taurine, pantothenic acid,

choline chloride and leucine). The reasons why groups 1 to 6 were investigated first were because the glycerol stocks of the individual genes in these groups arrived first and it was decided that it was simpler to start with a more manageable number of groups of cRNA. However, this means that variability between oocytes from different frogs and any slight changes in the assay conditions could have confounded the results. Carrying out the group screening all in one experiment would, however, have been very difficult due to the number of oocytes to be injected and the large number of oocytes to process during the transport assays. A better option would have been to wait until the glycerol stocks for all the individual genes in the cDNA library had arrived before starting the screening. This would have allowed all the groups to be investigated for a substrate at the same time, minimising any confounding factors.

The positive control used in the group screening stage was the human reduced folate carrier 1 (hRFC1). This did show significantly increased uptake of folic acid compared to water-injected controls and so provided proof that the expression system was working. However, the same problems are found with this result as the other results in the group screening stage; specifically that this experiment was only carried out once so there is a potential for statistically spurious results to be generated. Inclusion of a positive control in subsequent experiments would have also been extremely useful and should have been included. Ideally a known

amino acid transporter would have been included as a positive control but hRFC1 was available within our group at the time so it was used instead. Using an amino acid transporter as a positive control would have provided an opportunity for the problem with using very low substrate concentrations to be noticed early enough to have time to correct the error. There are low physiological concentrations of folic acid in human plasma (0.5 nM) and as the concentration of [^{3}H] folic acid used in this experiment was 30 nM it did not show up the issue with substrate concentrations.

In this study the expression of proteins resulting from the injection of cRNA was not checked. To have assurance that the proteins were being expressed in the *X. laevis* oocytes the expression should have been checked using specific antibodies and visualised with Western blotting or fluorescence microscopy, or by RT-PCR on oocytes after injection and incubation. This was planned to be carried out if reproducible transport was observed for any gene, but if it had been carried out at the start it would have identified any problems with the expression. Using one of these techniques to test expression of just one or two proteins would have given some proof that the expression system was working and should have been carried out.

Further repeats of most of the experiments in this chapter are required to confirm the results found, as only the screening of the individual genes in

group 1 was repeated more than once. This is a major flaw as it is difficult to draw conclusions based on the reliability of individual experiments. However, any repeats of the radiolabelled transport assays would only be useful if they took into account the problem of using too low substrate concentrations and incorporated robust controls. Further work to investigate the uptake of taurine and pantothenic acid by gene 98 (PF10_0137), the uptake of glutamic acid by gene 93 (PPFI0800c), and the efflux of glutamic acid by gene 95 (PF10_0276) is also required. Gene 98 (PF10_0137) significantly increased uptake of taurine and pantothenic acid in one experiment (figure 3.6) but no increase in uptake was observed in the following experiment (figure 3.7). As these results did not show consistent uptake of these substrates by oocytes injected with the cRNA of this gene it was decided not to continue working on this gene due to time constraints. The lack of further work on gene 93 (PFFI0800c) and gene 95 (PF10_0276) was also due to time constraints. However, a more critical approach to the *X. laevis* oocyte work should have been taken: correct substrate concentrations used, a positive control included and the expression of the heterologous proteins checked. This would have given more credibility to the results obtained and allowed more reliable identification of any transporters for the substrates tested.

The intraerythrocytic *P. falciparum* parasite digests up to 65 % of the host cell's haemoglobin, but only up to 16 % of the amino acids released from

this degradation are utilised by the parasite (Krugliak et al., 2002). This means that the parasite must mediate the disposal of waste amino acids generated by haemoglobin digestion. In this study the potential for some of the genes in the gene-specific cDNA library to encode amino acid efflux transporters was not investigated. These should have been further studied as amino acid efflux transporters are physiologically important and could be potential drug targets.

A problem with the gene-specific cDNA library used in this study is that only twenty of the forty eight genes have six or more trans-membrane domains. Only three amino acid transporter gene families, out of seventeen found in eukaryotes, have fewer than ten trans-membrane domains (Saier, 2000b). These are the MHS, LysE and PLM families. The characteristics of these and other amino acid transporter gene families are discussed in section 1.4.5. This indicates that the work carried out on the cDNA library would have been better focused to the twenty genes with six or more trans-membrane domains.

CHAPTER 4

Methionine Transport in the *P. falciparum* Parasite

4.1 Introduction

The use of the *X. laevis* oocyte expression system in the previous chapter did not lead to the functional characterisation of any *P. falciparum* amino acid transporters. A more targeted approach was, therefore, taken to study methionine transport by the *P. falciparum* parasite. Methionine is required for protein synthesis as well as for the ubiquitous methylation of proteins, nucleic acids and lipids. In the malaria parasite there is a functioning methionine synthase, allowing homocysteine to be converted to methionine with the addition of a methyl group from the folate product 5-MTHF (Krungkrai *et al.*, 1989a, Asawamahasakda and Yuthavong, 1993). Through this process the methionine cycle is linked to the folate pathway, as the demethylation of 5-MTHF produces THF, which is recycled back through the folate one-carbon metabolism enzymes (see section 1.3.3 for more details). Despite the presence of a functioning methionine synthase an exogenous source of methionine has been shown to be necessary for parasite growth, as discussed in section 1.4.4 (Divo *et al.*, 1985). The characterisation of the transporter(s) responsible for the uptake of

exogenous methionine may provide useful information for the identification of new drug targets, as inhibition of methionine influx could potentially starve the parasite of an essential amino acid.

The membrane transport of methionine into human RBCs is known to be facilitated by the Na^+ independent system L (Winter and Christensen, 1964, Rosenberg, 1982, Barker and Ellory, 1990, Tunnicliff, 1994). Until very recently the transport of only one amino acid, isoleucine, had been characterised in *P. falciparum* parasites (Martin and Kirk, 2007). In this chapter the methionine transport in *P. falciparum* parasites has been characterised. The objectives of the work carried out in this chapter were to discern any changes in methionine transport by RBCs when infected with *P. falciparum* parasites, to characterise the kinetics of methionine transport by free *P. falciparum* parasites and to further characterise this transport using transport inhibitors and inhibition with other amino acids.

4.2 Materials and Methods

4.2.1 Parasite Cultivation

The *P. falciparum* clone 3D7, originating from an airport malaria case at Schiphol, the Netherlands, was used for all experiments described in this chapter. Cultivation was carried out following the modified method of Trager and Jensen as described in section 2.1 (Trager and Jensen, 1976).

4.2.2 Transport Assays In *P. falciparum* Parasitised Red Blood Cells

4.2.2.1 Magnetic Separation of Trophozoite Stage *P. falciparum* Infected Red Blood Cells

Cultures of trophozoite stage *P. falciparum* infected RBCs at approximately 5 % parasitaemia or above were used. Trophozoites were concentrated to 40 % parasitaemia using a VarioMACS magnetic separation unit (Miltenyi Biotec, Germany). The parasite cultures were centrifuged at 805 x g for 5 minutes, the supernatant discarded and the pellet resuspended in solution 1 (2 % BSA and 20 mM glucose in PBS). The resuspended pellet was then passed through the VarioMACS column attached to the magnetic separation unit and washed thoroughly with solution 1. The bound trophozoites were eluted with solution 1 and washed

3 times with Ringers buffer pH 7.4 (106 mM NaCl, 24M m $NaHCO_3$, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1 mM Na_2HPO_4, 0.8 mM $MgCl_2$, 25 mM HEPES and 10 mM glucose). The cell density was then estimated using an Improved Neubauer haemocytometer.

4.2.2.2 Methionine Uptake in *P. falciparum* Infected Red Blood Cells and Uninfected Red Blood Cells

Trophozoite stage *P. falciparum* infected RBCs at 40 % parasitaemia (from magnetic separation) or uninfected RBCs, that had been maintained in culture under the same conditions as the infected RBCs, were suspended is Ringers buffer pH 7.4 (as previously described) at a concentration of approximately 1 x 10^8 cells/ml. An appropriate volume of radiolabelled substrate solution was made with Ringers buffer pH 7.4 (as above) supplemented with 34 μM unlabelled methionine and L-[methyl-^{3}H] Methionine (GE Healthcare, UK) at a specific activity of 20 μCi/ml. The prepared cell suspensions and substrate solution were kept at 21 °C and the uptakes carried out at this temperature. The uptake was started with the mixing of an equal volume of the cell suspension with the radiolabelled substrate solution (giving a final concentration of 17 μM unlabelled methionine, the approximate physiological concentration in adult human plasma, and a final activity of [^{3}H] methionine of 10 μCi/ml). For time courses, at predetermined intervals 200 μl aliquots of the mixed cell and

substrate suspension were transferred to 1.5 ml microcentrifuge tubes containing 300 µl Dow Corning oil with 1ml ice-cold Ringers buffer pH 7.4 (as previously described) supplemented with 1.7 mM unlabelled methionine layered on top (in order to stop the transport immediately). The tubes were then immediately centrifuged at 17000 x g for 1 minute to pass the cells through the oil layer. The supernatant layer above the oil was removed by aspiration, the walls of the tube washed 3 times under flowing water and the oil layer removed by aspiration. The cell pellet was dissolved by adding 100 µl boiling distilled water and mixing. This was transferred to a 6 ml scintillation vial and 5 ml scintillation fluid (Optima Gold, Corning) added. The radioactivity was measured using β-scintillation counter (Packard). All points in each uptake were carried out in triplicate.

As 40 % parasitaemia was used for the uptake experiments using *P. falciparum* infected RBCs the uptake was adjusted to 100 % parasitaemia. This was carried out by removing the uptake by the uninfected cells (based on data from time courses using uninfected cells) then adjusting to 100 % parasitaemia.

Each experiment was carried out three times, with three replicates of each data point measurement within each experiment.

4.2.3 Transport Assays in Free *P. falciparum* Parasites

4.2.3.1 Free *P. falciparum* Trophozoites Isolated by Saponin Lysis

P. falciparum trophozoites were isolated from the host RBC by saponin lysis. Treatment with saponin permeabilises the host RBC and parasitophorous vacuole membranes, leaving the parasite plasma membrane intact (Saliba and Kirk, 1999).

Cultures of trophozoite stage *P. falciparum* infected RBCs at above 5 % parasitaemia were used for free parasite generation by saponin lysis. Cultures were centrifuged at 805 x g for 5 minutes and the infected RBC pellet washed once with Ringers buffer pH 7.4 (as previously described). Five pellet volumes of 0.15 % saponin in PBS was added to the pellet and gently mixed until red blood cell lysis was observed. This was then centrifuged at 2000 x g, 4 °C for 5 minutes, the supernatant carefully removed and the pellet washed and resuspended four times in Ringers buffer pH 7.4 (as previously described) to remove all traces of saponin. The cell density was then estimated using an Improved Neubauer haemocytometer and the success of the saponin lysis checked by wet smear. Briefly, a drop of the washed, saponin treated, suspension was placed on a microscope slide, a cover slip placed over the drop and examined using a light microscope with a 100 x oil immersion objective. For the experiments where methylation and/or protein synthesis inhibitors

were used 20 mM cycloleucine and/or 40 μM cycloheximide were added to the cell suspensions at this stage and incubated for 30 minutes at 21 °C.

4.2.3.2 Methionine Uptake in Free *P. falciparum* Parasites

All of the [^{3}H] methionine uptake experiments in free *P. falciparum* parasites were carried out at 21 °C. For time course assays the uptake was started with the mixing of an equal volume of the cell suspension with the radiolabelled substrate solution (20 μCi/ml [^{3}H] methionine and 34 μM unlabelled methionine in Ringers buffer pH 7.4, as previously described). At predetermined intervals 200 μl aliquots of the mixed cell and substrate suspension were transferred to 1.5 ml microcentrifuge tubes containing 1 ml ice-cold Ringers buffer pH 7.4 (as previously described) supplemented with 1.7 mM unlabelled methionine (in order to stop the transport immediately). The tubes were then immediately centrifuged at 17000 x g, 4 °C for 1 minute to sediment the cells. The supernatant was removed by aspiration and the walls of the tube washed with 1ml ice-cold Ringers buffer pH 7.4 (as previously described) supplemented with 1.7 mM unlabelled methionine. The cell pellet was dissolved by adding 100 μl boiling distilled water and mixing. This was transferred to a 6 ml scintillation vial and 5 ml scintillation fluid (Optima Gold, Corning) added.

For single time point experiments where uptake over 30 seconds was measured the uptake was started by the addition of 100 µl cell suspension to 100 µl radiolabelled substrate solution in individual 1.5 ml microcentrifuge tubes. After 30 seconds the uptake was terminated by the addition of 1 ml ice-cold Ringers buffer pH 7.4 (as previously described) supplemented with 1.7 mM unlabelled methionine (in order to stop the transport immediately). The tubes were then immediately centrifuged at 17000 x g for 1 minute to sediment the cells. The cell pellets were then treated the same way as in the time course experiments. The radioactivity was measured using β-scintillation counter (Packard).

In the experiments where transport inhibitors were used these were added to the substrate solution prior to mixing with the cell suspension to start the uptake (so double the required end concentration added). The system L inhibitor 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) (Christensen *et al.*, 1969) was used at end concentrations of 2 mM and 20 mM and the system y+ inhibitor N-ethylmaleimide (NEM) (Soriano-Garcia *et al.*, 1998) was used at end concentrations of 0.1 mM and 1 mM. NEM is, however, a generally unspecific inhibitor as it is reactive towards all sulfhydryl groups.

For the experiments investigating inhibition by other amino acids the uptakes were carried out over 30 seconds as described above, but with the

addition of 0.4 mM, 4 mM or 20 mM appropriate unlabelled amino acid to the radiolabelled substrate solution (giving end concentrations of 0.2 mM, 2 mM or 10 mM when mixed with the cell suspension to start the uptake). An electronic metronome was used for uptakes with time points of 30 seconds or less to improve accuracy of the timing. All points in each uptake were carried out in triplicate.

Each experiment was carried out three times, with the exception of the experiment investigating inhibition by 10 mM of other amino acids where only one experiment was carried out. Each individual experiment consisted of three replicates of each data point measurement.

4.2.4 Statistical Analysis of Results

Non-parametric tests were used as the distribution of values within each group could not be determined to be Gaussian. The Mann-Whitney U test was used to establish whether there was a significant difference between the groups. Bonferroni correction of p values was carried out where multiple comparisons were made to reduce the chances of type I errors occurring. Significance was counted with p values <0.05.

4.3 Results

4.3.1 Uptake of Methionine by *P. falciparum* Parasitised Human Red Blood Cells

Time courses of the uptake of methionine into trophozoite stage *P. falciparum* infected and uninfected RBCs were carried out at 21 °C in the presence of the approximately physiological concentration of methionine (17 μM) and the absence of all other amino acids (figure 4.1). This temperature was used as it was simpler to carry out for an initial study. The influx of methionine into *P. falciparum* infected RBCs was not higher than that observed for uninfected RBCs. The initial rate of influx was calculated from the linear phase of uptake (inset, figure 4.1), which was shown to be significantly linear with r^2 values of 0.65 for infected RBCs and 0.78 for uninfected RBCs (calculated using Graphpad Prism). No significant difference was observed between the initial rate of influx of methionine into uninfected RBCs and that of infected RBCs, with respective rates of 991 ± 208 fmol/10^6 cells/minute and 608 ± 301 fmol/10^6 cells/minute (mean from three independent experiments on different days, each in triplicate, ± standard error, with significance values calculated using the Mann-Whitney U test). Due to the lack of parasite-induced change in methionine influx in RBCs it was decided to focus further mainly on methionine transport in free *P. falciparum* parasites.

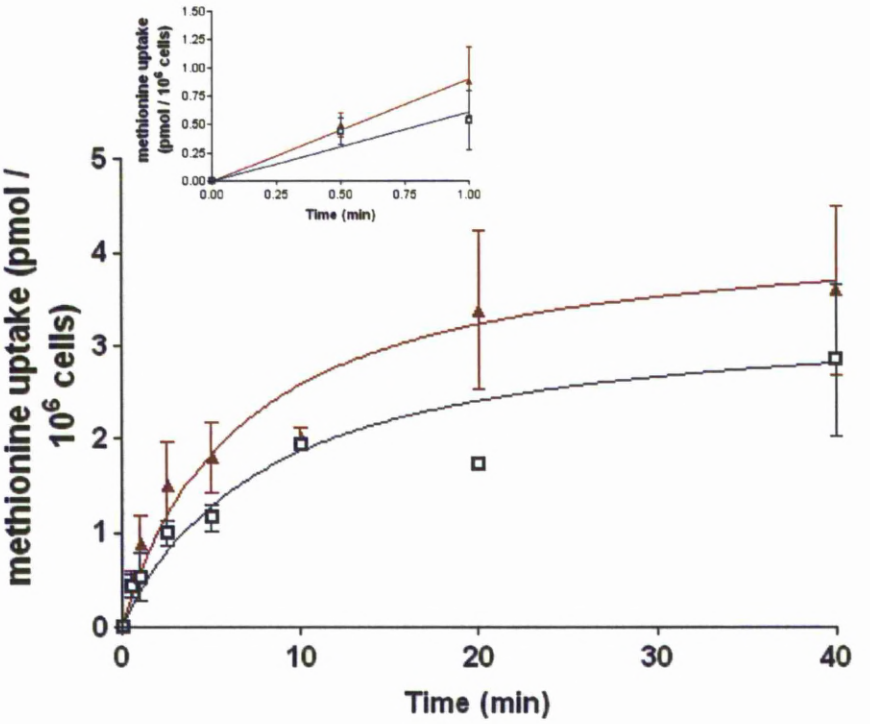


Figure 4.1. Uptake of methionine by *P. falciparum* infected and uninfected RBCs at 21 °C. Measured in Ringers pH 7.4 in the presence of 17 μM methionine and the absence of other amino acids. Empty blue squares denote uptake by *P. falciparum* infected RBCs and solid red triangles denote uptake into uninfected RBCs. The inset graph shows the initial linear element of uptake. Values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error.

4.3.2 Uptake of Methionine by free *P. falciparum* parasites

The transport of methionine by free *P. falciparum* parasites was measured using trophozoite stage parasites isolated from the host RBCs using the saponin lysis technique, which frees the parasite from the RBC and parasitophorous vacuole membrane. Time courses of methionine uptake were carried out at 21 °C in the presence of the approximately

physiological concentration of methionine (17 μM) and the absence of all other amino acids. To make sure only the true transport of methionine was being measured, and not the knock on effect of further methionine metabolism, the effects of a methionine adenosyltransferase inhibitor (cycloleucine) and a protein synthesis inhibitor (cycloheximide) were examined. Figure 4.2 shows the time courses for the uptake of methionine into free *P. falciparum* parasites in the absence of inhibitor, or in the presence of 20 mM cycloleucine or 40 μM cycloheximide. The initial rate of influx was calculated from the linear phase of uptake (inset, figure 4.2), which was shown to be significantly linear and with r^2 values of 0.81 for uninhibited parasites, 0.82 for parasites with 20 mM cycloleucine and 0.89 for parasites with 40 μM cycloheximide (calculated using Graphpad Prism). The initial rate of influx of methionine into the free parasites was 248 ± 86 fmol/10^6 cells/minute for the uninhibited parasites, 220 ± 38 fmol/10^6 cells/minute for parasites with 20 mM cycloleucine and 210 ± 32 fmol/10^6 cells/minute for the parasites with 40 μM cycloheximide, with no significant difference between the uninhibited parasites and either inhibitor (mean from three independent experiments on different days, each in triplicate, ± standard error, with significance values calculated using the Mann-Whitney U test). The effect of inhibiting methylation or protein synthesis was only noticeable after 1 minute, but to make sure that only true transport of methionine was measured, and not the effect of

methylation or protein synthesis, both cycloleucine and cycloheximide were included as a precaution in further experiments.

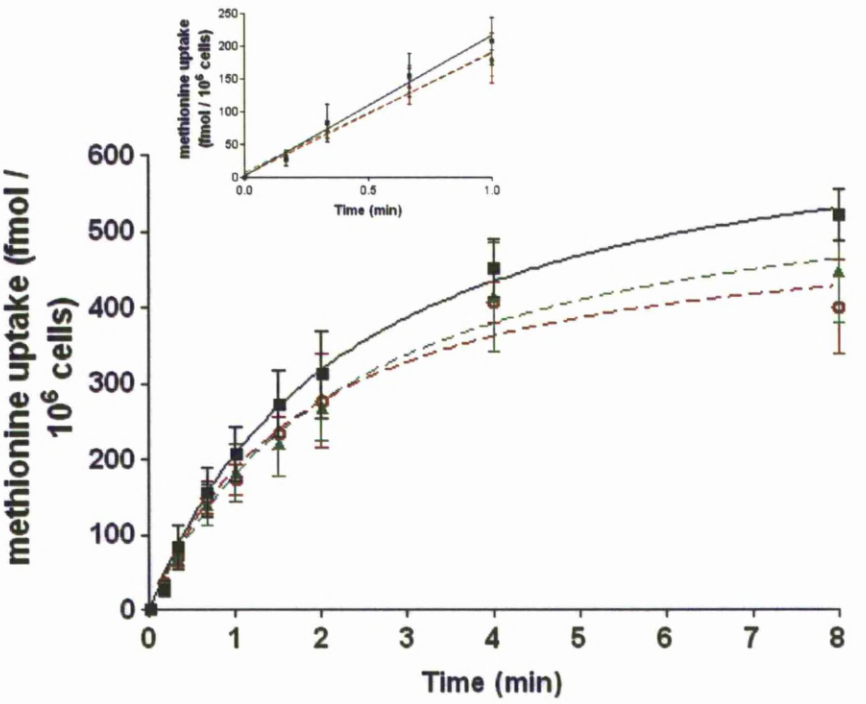


Figure 4.2. Uptake of methionine by free *P. falciparum* trophozoites at 21°C. Measured in Ringers pH 7.4 in the presence of 17 μM methionine and the absence of other amino acids. Time courses shown in the absence of inhibitors (denoted by solid black squares), with methionine adenosyltransferase inhibition (20 mM cycloleucine, denoted by empty red triangles) or protein synthesis inhibition (40 μM cycloheximide, denoted by empty green circles). The inset graph shows the initial linear element of uptake. Values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error.

4.3.2.2 The Kinetics of Methionine Transport

The kinetics of methionine transport in free trophozoite stage *P. falciparum* parasites was investigated by measuring the uptake of [^{3}H] methionine at 21 °C over 30 seconds. A range of methionine concentrations were used, from 120.5 nM to 10.6 mM. Figure 4.3 shows the concentration dependence of the uptake of methionine and shows that methionine uptake was saturable and conforming to Michaelis-Menten kinetics. The K_m (Michaelis constant, the substrate concentration that gives the half maximal rate of uptake) of methionine uptake was determined to be 1.13 ± 0.16 mM with a V_{max} (maximum rate of uptake) of 3200 ± 120 pmol/10^6 cells/hour.

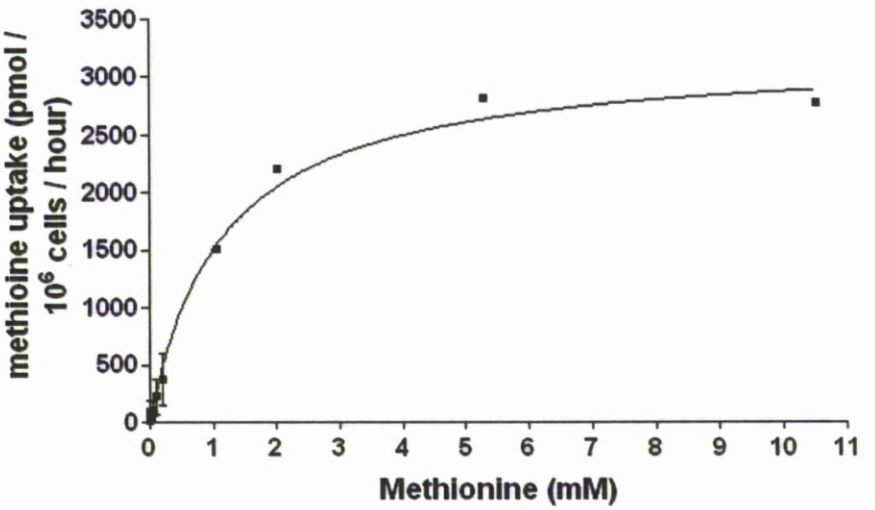


Figure 4.3. The uptake of increasing concentrations of methionine by free trophozoite stage *P. falciparum* parasites at 21 °C. Uptake was measured over 30 seconds in Ringers pH 7.4 with a range of concentrations of methionine (120.5 nM to 10.6 mM) and methionine adenosyltransferase and protein synthesis inhibitors added (20 mM cycloleucine and 40 μM cycloheximide). Values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error. The curve was drawn using the equation: methionine uptake = V_{max} [met]/(K_m + [met]), with K_m = 1.13 ± 0.16 mM and V_{max} = 3200 ± 120 pmol/10^6 cells/hour.

4.3.2.3 The Effect of Transport Inhibition

In order to investigate the nature of the methionine transport over the *P. falciparum* plasma membrane uptake experiments were carried out with the addition of inhibitors of known amino acid transport systems. 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) is an inhibitor of system L amino acid transport, a system that is responsible for transport of neutral amino acids in mammalian cells (Christensen *et al.*, 1969). The effect of BCH on the uptake of [^{3}H] methionine over 30 seconds at 21 °C is shown in figure 4.4. No significant decrease in uptake was observed with the addition of 2 mM and 20 mM BCH using the Mann-Whitney U test, but a drop in uptake without a dose response can be observed from figure 4.4.

N-ethylmaleimide (NEM) is an inhibitor of system y+ amino acid transport (Soriano-Garcia *et al.*, 1998) but will also bind to, and inactivate, any integral plasma membrane protein with a free sulfhydryl group. The addition of even just 0.1 mM NEM reduces the uptake to background levels (figure 4.5). The incomplete inhibition by BCH and lack of dose response suggests that there may be more than one transporter responsible for the uptake of methionine. BCH is a more specific inhibitor than NEM and could, therefore, be targeting a particular transport route. If more than one methionine transporter is present and BCH is only targeting one of these then even increasing the concentration of BCH will not reduce the

transport occurring via alternate transporters. NEM is generally an unspecific inhibitor and so may target more than one transport route.

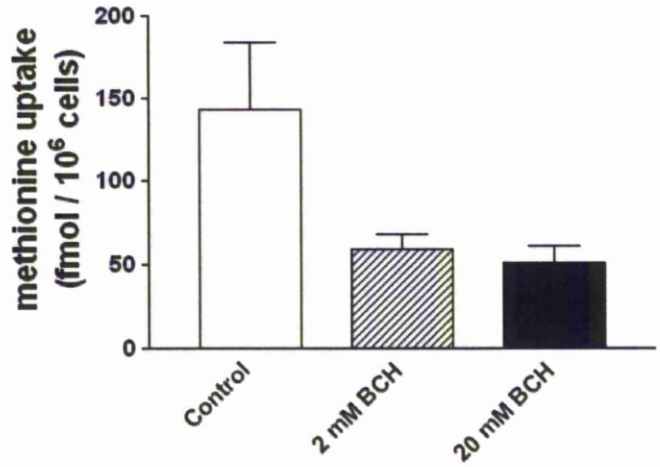


Figure 4.4. Methionine uptake by free trophozoite stage parasites with the addition of 2 mM or 20 mM 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH), at 21 °C over 30 seconds. Measured in Ringers pH 7.4 in the presence of 17 μM methionine and the absence of other amino acids. Methionine adenosyltransferase and protein synthesis inhibitors were also added (20 mM cycloleucine and 40 μM cycloheximide). Values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error.

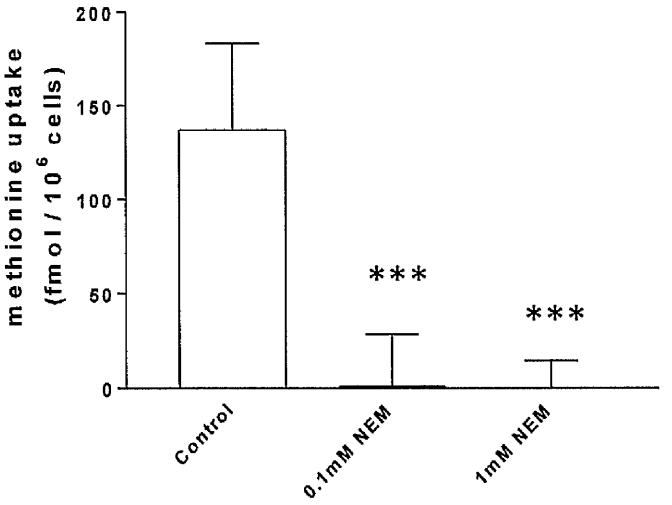


Figure 4.5. Methionine uptake by free trophozoite stage parasites with the addition of 0.1 mM or 1 mM N-ethylmaleimide (NEM), at 21 °C over 30 seconds. Measured in Ringers pH 7.4 in the presence of 17 μM methionine and the absence of other amino acids. Methionine adenosyltransferase and protein synthesis inhibitors were also added (20 mM cycloleucine and 40 μM cycloheximide). Values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error. Significance values between control and parasites treated with NEM were calculated using the Mann-Whitney U test, are Bonferroni corrected, and shown as *** representing a p value < 0.001.

4.3.2.4 Inhibition by other Amino Acids

The ability of 0.2 mM, 2 mM or 10 mM unlabelled naturally occurring amino acids and taurine (only 0.2 mM and 2 mM unlabelled glutamate, tryptophan and arginine were used) to inhibit the influx of [^{3}H] methionine was investigated (figure 4.6). Cysteine was the only amino acid to inhibit the influx of [^{3}H] methionine at all the concentrations used with the influx of [^{3}H] methionine completely reduced when 10 mM unlabelled cysteine

added. No other unlabelled amino acids appeared to reduce [^{3}H] methionine influx when 0.2 mM or 2 mM was added. Unlabelled lysine and histidine caused non significant inhibition of [^{3}H] methionine influx when 10 mM was used. The addition of unlabelled methionine did not inhibit [^{3}H] methionine influx when 0.2 mM or 2 mM was added, only showing inhibition when 10 mM was added, although this was not significant. No significant inhibition of [^{3}H] methionine influx was observed by D-methionine but further experiments would have to be carried out in order to make any conclusions on the stereospecificity of methionine transport. The inhibition observed here cannot be assumed to be competitive as further experiments would need to be carried out to allow the nature of inhibition to be analysed.

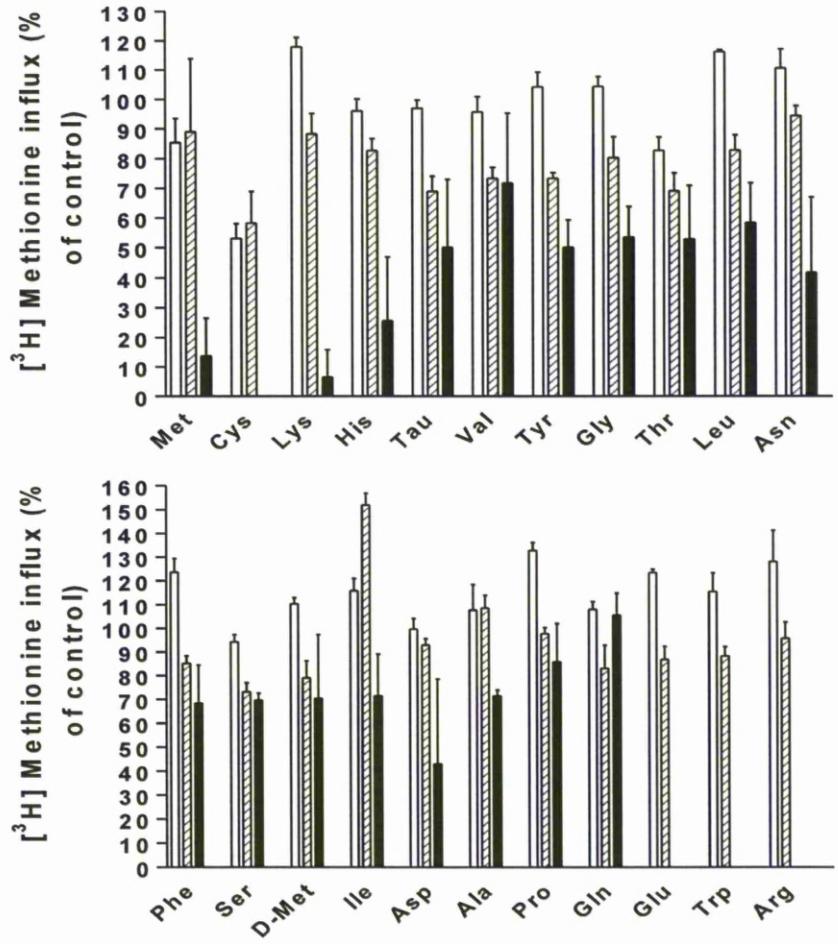


Figure 4.6. Inhibition of [^{3}H] methionine influx into free trophozoite stage *P. falciparum* parasites by unlabelled amino acids. Influx is shown as a percentage of the influx measured in the absence of unlabelled amino acids (control). Measurements were carried out at 21 °C over 30 seconds in Ringers pH 7.4 in the presence of methionine adenosyltransferase and protein synthesis inhibitors (20 mM cycloleucine and 40 μM cycloheximide). The influx of [^{3}H] methionine was measured in combination with 0.2 mM, 2 mM or 10 mM unlabelled amino acids (with the exception of glutamate, tryptophan and arginine where only 0.2 mM and 2 mM were used). White bars show 0.2 mM unlabelled amino acid, dashed bars show 2 mM unlabelled amino acid and black bars show 10 mM unlabelled amino acid. For 0.2 mM and 2 mM amino acids values are represented as mean from three independent experiments carried out on different days, with each data point in triplicate, ± standard error. For 10 mM values are the mean from one experiment, with each data point in triplicate, ± standard error.

4.4 Discussion

In this chapter the methionine transport in intact *P. falciparum* parasitised RBCs and free *P. falciparum* parasites has been investigated. In adult human RBCs methionine is taken up via the saturable system L (Barker and Ellory, 1990). Previous studies have shown that RBCs infected with the malaria parasite, *P. falciparum*, to have an increased permeability to amino acids (Elford *et al.*, 1985), and the increase in uptake of isoleucine by parasitised RBCs has been well characterised (Martin and Kirk, 2007). In the study carried out here, RBCs infected with the 3D7 strain of *P. falciparum* did not show significantly increased uptake of methionine compared to uninfected RBCs. A characterisation of the transport of methionine by *P. falciparum* strain FAF6 infected RBCs and isolated parasites has recently been published by Cobbold *et al* (2011). This study found methionine to be taken up by RBCs infected with *P. falciparum* parasites 15 fold faster than uninfected RBCs. This increase in transport was attributed to the parasite-induced NPP, with transport rates decreasing to that of uninfected RBCs with the addition of an anion transport inhibitor, furosemide, which inhibits the NPP. There are a number of differences between the study described in this chapter and that carried out by Cobbold *et al* (2011). For example: different strains of *P. falciparum* were used in each study; different temperatures were used for the influx assays (21°C was used in this study and 37°C was used by Cobbold *et al*); and the

reaction medium in the study by Cobbold *et al* was supplemented with the full range of amino acids at approximately physiological concentrations, whereas the reaction medium used in this study was not supplemented with any amino acids other than the physiological concentration of methionine. However, the lack of increase in methionine uptake by *P. falciparum* infected RBCs observed in this study is highly unusual compared to other nutrients. Isoleucine has been shown to be taken up 5 fold faster in parasitised RBCs compared to uninfected RBCs (Martin and Kirk, 2007), glutamine uptake has been shown to be increased up to 100 fold (Elford *et al.*, 1985) and glucose uptake has been shown to be increased 40 to 100 fold (Woodrow *et al.*, 2000, Kirk, 2001). In order to conclude that there is definitely no increase in methionine uptake by parasitised RBCs more experiments would have to be carried out and a control included of a substrate known to be transported at a greater rate in parasitised RBCs.

Methionine uptake in various vertebrate cells and other organisms has previously been characterised. As well as the ubiquitous Na^+-independent system L, the sodium dependent system A has been characterised and found to be responsible for methionine transport in many vertebrate cell types, except for RBCs and reticulocytes (Barker and Ellory, 1990). Systems B^{o+} and b^{o+} are Na^+-dependent systems shown to transport methionine in blastocytes. All of these systems are also responsible for the transport of a wide range of neutral amino acids as well as methionine

(Barker and Ellory, 1990). In the yeast *Saccharomyces cerevisae* three distinct methionine transport systems have been found, with one high affinity, high specificity methionine permease, named Mup1, with a K_m of 12 μM (Isnard *et al.*, 1996). In the procyclic and blood stream forms of the kinetoplastid parasite *Trypanosoma brucei brucei* methionine is taken up by a relatively high affinity carrier, with a K_m of 30 μM (Hasne and Barrett, 2000). Methionine transport in *T. brucei* procyclic forms was shown to be weakly inhibited by leucine, phenylalanine, tryptophan and D,L-homocysteine, with blood stream forms also inhibited by D-methionine, isoleucine and glycine.

Transport of methionine into the free *P. falciparum* parasite was shown to be a saturable process, with a K_m of 1.13 ± 0.16 mM. This is similar to that found in the study by Cobbold *et al* (2011) who found a K_m of 1.6 ± 0.3 mM. Compared to the methionine transport characterised in other systems, for example the Mup1 methionine permease in *S. cerevisae* and *T. brucei* methionine transport, the transport into free *P. falciparum* trophozoites is relatively low affinity. Methionine transport into free *P. falciparum* trophozoites was inhibited approximately 50 % by BCH, a specific system L transport inhibitor (Christensen *et al.*, 1969). There is a lack of dose response with BCH inhibition which suggests that there may be more than one transporter responsible for the uptake of methionine and so transport via the BCH inhibited pathway may be via a system L like transport

system. NEM, an inhibitor of system y+ (Soriano-Garcia *et al.*, 1998), completely inhibited transport of methionine into the free parasites. However, as NEM binds covalently and without specificity to accessible sulfhydryl residues in proteins it is difficult to know if this is only inhibiting transport via a system like the vertebrate system y+ or another pathway (Janick *et al.*, 1977).

From influx assays carried out with the addition of unlabelled amino acids it was observed that methionine influx into the free parasites was inhibited most effectively by cysteine. Transport also appeared to be inhibited by lysine and histidine but only when 10 mM unlabelled amino acid was used. This pattern of inhibition is different to that found by Cobbold *et al* (2011), who found inhibition by a wide range of neutral amino acids, including cysteine, and no significant competition by either acidic or basic amino acids. The inhibition of methionine uptake by lysine is interesting when combined with the inhibition observed with the system y+ inhibitor NEM as lysine is one of the main substrates of this system. Although system y+ is predominantly a cationic amino acid transporter it has been shown to transport neutral amino acids when Na^+ is present, as it was in the reaction medium used in transport assays in this chapter. However, no definite conclusions about the contribution of a system y+ like transport system can be made based on the results from NEM inhibition as NEM is a very unspecific inhibitor.

In conclusion, the results from this chapter show that methionine is taken up by the *P. falciparum* parasite via one or more saturable systems, with one component showing similarity to the vertebrate systems L.

4.4.1 Critical Assessment

The "free" *P. falciparum* parasites referred to in this work were isolated from infected human RBCs using saponin lysis. This is a relatively simple technique where saponin, a plant-derived detergent, interacts with cholesterol in the cell membrane and disrupts the integrity of these membranes (Ansorge *et al.*, 1996). The technique has been widely used since its introduction by Christophers and Fulton (1939). Treatment of malaria-parasitised RBCs with saponin does not completely remove the RBC membrane surrounding the intracellular parasite, instead it forms pores in the RBC membrane allowing it to be permeable to solutes as large as soluble proteins (e.g. haemoglobin) (Kirk, 2001). Treatment with saponin also appears to permeabilise the PVM without damaging the PPM (Ansorge *et al.*, 1997). Studies have shown that the PPM can maintain a large, inwardly negative, membrane potential (Allen and Kirk, 2004) and generate and maintain ionic gradients (Saliba and Kirk, 1999) after treatment with saponin for permeabilisation purposes. These attributes allowed saponin lysis to be deemed suitable for use in the experiments described in this chapter, despite the fact that the parasites still reside in

the, permeabilised, ghosts of the RBC membrane. It is possible to culture *Plasmodium* parasites axenically, but the yields are low with only approximately 1 % of harvested merozoites developing into trophozoites (Trager *et al.*, 1992, Trager *et al.*, 1996). This means that for experiments using isolated ("free") parasites it is still necessary to use parasites isolated from host RBCs in order to obtain large enough yields. As well as saponin there are other methods that can be used to isolate parasites from infected RBCs. These include: digitonin, another plant-derived detergent that also permeabilises the RBC membrane (Desai *et al.*, 1993); Sendai virus, which induces fusion of erythrocytes and leaves the parasite intact within freely permeable erythrocyte ghosts (Kanaani and Ginsburg, 1988, Kanaani and Ginsburg, 1989); complement-mediated immune lysis (Trager *et al.*, 1990); streptolysin O, a bacterial protein that permeabilises the RBC membrane but not the PVM (Ansorge *et al.*, 1996, Ansorge *et al.*, 1997); isoosmotic lysis with sorbitol (Hoppe *et al.*, 1992); and nitrogen cavitation followed by free flow electrophoresis (Heidrich *et al.*, 1986). However, saponin lysis still remains the most widely used method for obtaining isolated *Plasmodium* parasites from the host RBC. Its ease of use, permeabilisation of both RBC membrane and PVM whilst leaving the PPM intact, and the fact that the technique was already established within the group, led to the decision to use saponin lysis as the method of obtaining "free" parasites in this chapter.

The methionine uptake experiments were carried out at 21 °C as initial experiments at this temperature showed uptake to be rapid. Increasing the temperature to 37 °C would have increased the initial rate of uptake and made it more difficult to accurately carry out the short time courses required, so 21 °C was continued to be used. There is also precedent for transport assays to be carried out at non-physiological temperatures (Biagini *et al.*, 2004, Downie *et al.*, 2006, Martin and Kirk, 2007). Using 21 °C instead of 37 °C will affect the V_{max} but should not affect the K_m of transport. As discussed in section 3.4.1, the K_m of transport gives important information on the affinity of the transporter for the substrate while the V_{max} gives less information on the nature of the transport, but is useful if the transport is to be modelled. In order to comprehensively characterise the transport of methionine by infected and uninfected RBCs and free parasites, reaction parameters need to be investigated at a range of temperatures. This allows the E_a to be determined (from an Arrhenius plot, as described in section 3.4.1) which can give further information on whether transport is channel or carrier mediated. Cobbold *et al* (2011) showed methionine uptake by free *P. falciparum* parasites to be temperature dependent, however only two temperatures were used to investigate this (4 °C and 22 °C) and the E_a was not determined.

The transport assays were terminated with the addition of ice-cold buffer containing 1.7 mM unlabelled methionine. This should have been much

higher considering that the K_m of methionine transport in free parasites was 1.13 ± 0.16 mM and the addition of 2 mM unlabelled methionine does not reduce methionine uptake. It was initially assumed that using 100 fold increased concentration of unlabelled methionine from the physiological concentration (17 μM) would be sufficient for the stop solution. A time course experiment carried out on ice, using ice-cold 1.7 mM methionine stop solution, showed only background uptake (figure 4.7). This experiment was only carried out once (in triplicate) so cannot be completely relied on, but it may negate this problem slightly as the temperature reduction and physical dilution of the substrate could be assumed to be sufficient for inhibiting further transport of [^{3}H] methionine.

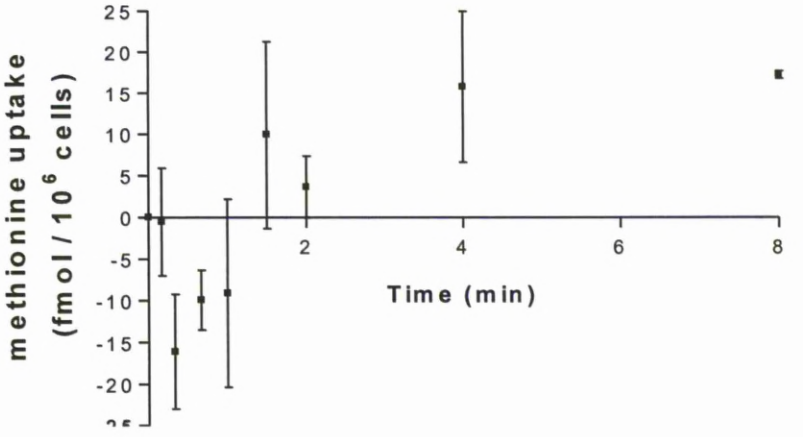


Figure 4.7. Uptake of methionine by free *P. falciparum* trophozoites on ice. Measured in Ringers pH 7.4 in the presence of 17 μM methionine and the absence of other amino acids. Values are represented as mean from one experiment, with each data point in triplicate, ± standard error.

Conclusions on the nature of the inhibition of methionine uptake by cysteine, lysine, histidine, taurine, BCH and NEM cannot be drawn from the current data. The inhibition by other amino acids is not necessarily competitive. In order to find out if the inhibition is competitive more comprehensive experiments would have to have been carried out, using several concentrations of methionine and a wider range of concentrations of potentially inhibiting amino acids. The results from these would have allowed further analysis of the nature of the inhibition using a Dixon plot. The Dixon plot is a graph of the reciprocal of the initial velocity, 1/V, against the concentration of inhibitor which produces a set of intersecting lines representing substrate concentration. A competitive inhibitor graphed using a Dixon plot would have lines converging above the x axis whereas a non-competitive inhibitor would have lines converging on the x axis. Competitive inhibition would also cause an increase in the *K*m with the addition of inhibitor, but no change in the V_{max}. Non-competitive inhibition would cause a decrease in the V_{max} with the addition of inhibitor, but no change in the K_m.

A control of a substrate known to be transported at a greater rate in parasitised RBCs and with known transport kinetics in free parasites should have been included in order to give the results more reliability. This would have been especially useful with the experiments comparing uptake

of methionine by *P. falciparum* infected and uninfected RBCs as these, surprisingly, showed no increased uptake by parasitised RBCs.

CHAPTER 5

Antagonism of the Natural Folate Precursor para-Aminobenzoic Acid on the Action of Anticancer and Antimalarial Antifolates

5.1 Introduction

After investigating methionine transport in the *P. falciparum* infected RBC and free trophozoites (chapter 4) it was decided to investigate the transport-mediated salvage of folate pathway substrates. The folate pathway provides the one-carbon donor to methionine biosynthesis with 5-methyltetrahydrofolate (5-MTHF) the methyl donor in the biosynthesis of methionine from homocysteine (see section 1.3 for the folate pathway and methionine cycle). Antifolates have been effective antimalarial drugs with DHPS and DHFR inhibitors still in use. There is marked synergy of SDX (anti-DHPS) with PYR (anti-DHFR) and this combination (marketed as Fansidar®) is at present used as an intermittent preventive treatment in infants (IPTi) in some areas of Africa (Conteh *et al.*, 2010), and in the treatment of uncomplicated malaria in combination with artesunate and amodiaquine (WHO, 2010). Antimalarial antifolates have been well studied and their modes of

action and mechanisms of resistance have been described in section 1.3.4. One of the most effective and best studied antifolate is the anticancer drug methotrexate (MTX). Although it is a potent inhibitor of DHFR it has not been deployed as an antimicrobial besides being shown to be effective against *P. falciparum in vitro* (Kiara *et al.*, 2009, Nzila *et al.*, 2010, Dar *et al.*, 2008, Huennekens, 1994).

A need for salvage of the folate precursor pABA by *Plasmodium* was first indicated in the 1960s when it was shown that *Plasmodium berghei* parasites had reduced growth when the mouse hosts were fed a pABA-deficient diet, with parasite growth returned to normal with the addition of pABA (Jacobs, 1964). *P. falciparum* parasites have been shown to be capable of salvaging exogenous folate precursors, pre-formed folate and C_1 substituted folate derivatives, as reviewed in section 1.3.2 (Krungkrai *et al.*, 1989b, Wang *et al.*, 2004). However, there is also pharmacological and genetic evidence of *de novo* pABA synthesis via the shikimate pathway in *P. falciparum* (McConkey *et al.*, 1994, Roberts *et al.*, 1998). Unlike the malaria parasite, humans are unable to synthesise pABA *de novo* and also do not significantly utilise it. This makes pABA metabolism a useful potential chemotherapeutic target.

Early *in vitro* methods used to investigate antimalarial drug activity in the 1970s proved difficult to use for the antifolate class of drugs even when they worked well for other major classes of antimalarials (Desjardins *et al.*, 1979, Wang *et al.*, 1997b). It was noticed by Desjardins and colleagues that the RPMI-1640 used to culture the parasites *in vitro* had concentrations of pABA (1.0 mg/L) and folic acid (1.0 mg/L) in excess of the concentrations in the human host plasma. It was concluded that these substrates must have an effect on the action of the antifolate drugs (Desjardins *et al.*, 1979). Subsequent *in vitro* studies have confirmed that salvage of folate substrates from the medium reduces the sensitivity of the parasite to antifolate drugs (Watkins *et al.*, 1985, Tan-ariya *et al.*, 1987, Wang *et al.*, 1999, Wang *et al.*, 1997b). Work by Wang *et al* (1997b) showed that strictly controlled low levels of folate and pABA allowed reliable results to be obtained from *in vitro* drug assays. The antagonism of antifolate drugs by folate salvage has been assumed to be due to the salvaged folate substrates bypassing steps in the *de novo* synthesis pathway (Hyde, 2005).

In this chapter the effects of the salvage of folate substrates and products found in human plasma will be investigated on the antimalarials SDX and PYR, and the anticancer drug MTX. In order to investigate the effect of transport-mediated salvage of folate pathway substrates probenecid (PBN) was used as a membrane transport inhibitor. PBN inhibits organic anion

transport and has previously been shown to chemosensitise *P. falciparum* to antifolates and other antimalarial agents (Sirotnak *et al.*, 2000, Nzila *et al.*, 2003, Masseno *et al.*, 2009). The possible effects of PBN on folate metabolism in *P. falciparum* are shown in figure 5.1.

As previous studies have shown folate salvage to antagonise antimalarial antifolates all experiments carried out in this chapter utilised custom made folate and pABA-free RPMI-1640 and albumax II (which is folate and pABA-free) for the culture medium for *P. falciparum* instead of standard RPMI-1640 and serum respectively. This has been used in previous studies although it has generally been supplemented with low concentrations of folic acid for continuous culture (Wang *et al.*, 1997b). Investigations into the effect of the folate pathway substrates pABA, pABAG1, 5-MTHF and folic acid on antifolate drugs were performed using physiological concentrations. In humans the blood concentration of folate pathway substrates is as follows: pABA concentration is variable with a mean around 150 nM (Schapira *et al.*, 1986); pABA monoglutamate is around 160 nM (Lin *et al.*, 2004); 5-MTHF (the predominant circulating folate product) is around 25nM (Duthie *et al.*, 2010); and folic acid is around 0.5 nM (McDowell *et al.*, 2008). In previous work by Wang *et al* (1999) the effect of folic acid and pABA on SDX was examined, however, in that study the lowest concentration of folic acid used was 11 nM, and unlike the

work carried out in this chapter the effect of transport-mediated salvage was not investigated.

To check if there was any correlation between antagonism of antifolates by folate substrates and folate salvage by specific parasite transporters the substrate specificity of folate and pABA salvage was investigated using *E. coli* expression of two putative *P. falciparum* folate transporters, MAL8P1.13 (designated PfFT1) and PF11_0172 (designated PfFT2). These two likely folate transporters belong to the high affinity BT1 folate transporter group (TC 2.A.71) and were identified from the *P. falciparum* genomic database by Martin *et al* (2005). This group falls within the major facilitator superfamily (MFS) and members are found in protozoa, plants and cyanobacteria (Saier, 2000b, Saier *et al.*, 1999c). Work by Martin *et al* (2005) showed that the two genes are maximally expressed in the late trophozoite and early schizont stages, which is consistent with the role of folates in DNA synthesis as these are the stages when DNA synthesis is at its highest (Hyde, 1990). A double gene knock out has been developed in *E. coli*, of a pABA synthesis enzyme (*pabA*) and the pABA and monoglutamated pABA transporter (*abgT*), to express and study folate transporters (Eudes *et al.*, 2010) and this has been used to investigate the substrate specificity of PfFT1 and PfFT2 using growth rescue experiments.

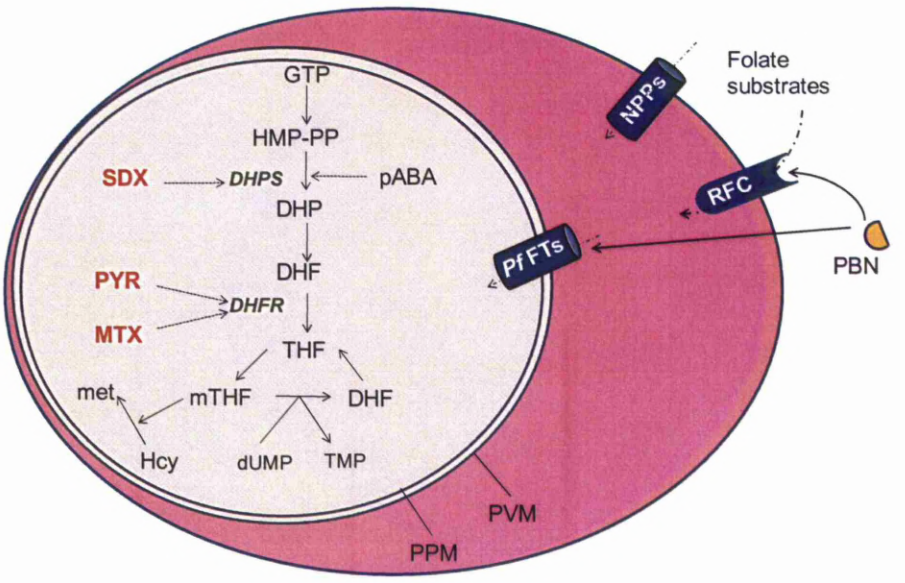


Figure 5.1. The possible effect of PBN on folate metabolism in *P. falciparum* parasites. RBCs salvage exogenous folate derivatives via mammalian RFC. Parasite-induced NPP have also been shown to be a possible route of folates into the infected RBC (Wang *et al.*, 2007). The parasites may take up these via their own folate transporters. PBN inhibits organic anion transporters and so may decrease the uptake of folate derivatives into the RBC and/or also inhibit the uptake of folate derivatives within the parasite, therefore decreasing the levels of folate derivatives within the parasite. Abbreviations: SDX, sulphadoxine, PYR, pyrimethamine; MTX, methotrexate; GTP, guanine triphosphate; HMP-PP, amino-hydroxy-methyl-dihydro-pteridine-pyrophosphate; DHPS, dihydropteroate synthase; pABA, para-aminobenzoic acid; DHP, 7,8-dihydroteroate; DHF, 7,8-dihydrofolate; DHFR, dihydrofolate reductase; THF, 5,6,7,8-tetrahydrofolate; mTHF, methylene-tetrahydrofolate; DHF, dihydrofolate; dUMP, deoxyuridine monophosphate; TMP, thymidine monophosphate; Hcy, homocysteine; met, methionine; PVM, parasitophorous vacuole membrane; PPM, parasite plasma membrane; NPP, new permeation pathway(s); RFC, reduced folate carrier; *Pf*FTs, *P. falciparum* folate transporters; PBN, probenecid. Modified from Nzila *et al* (2004).

5. 2 Materials and Methods

5.2.1 Parasite Cultivation

Cultivation of parasites was carried out following the modified method of Trager and Jensen described in section 2.1 (Trager and Jensen, 1976). Culture medium made using folate and pABA-free but otherwise standard RPMI-1640 was used (HyClone, ThermoScientific, UK) for all parasite cultivation and experiments as described in section 2.1. The 3D7 strain of *P. falciparum*, originating from an airport malaria case at Schiphol, the Netherlands, was used for all *in vitro* sensitivity assays described in this chapter. Although the 3D7 strain has partial resistance to SDX, through a DHPS mutation at position 437 from alanine to glycine, it was used for the antagonism experiments as the SDX sensitive strain HB3 could not be grown acceptably in folate and pABA-free medium (Wang *et al.*, 1997a). Attempts to culture the parasite strains VI/S and K1 in folate and pABA-free medium also resulted in poor growth, making them unsuitable for use in the antagonism experiments. The 3D7 strain grew acceptably in folate and pABA-free medium after approximately 5 to 7 asexual cycles, with growth observed to be only slightly slower than parasites maintained in standard folate and pABA complete medium otherwise under the same conditions. After successful adaption to the folate and pABA-free medium the 3D7 parasites appeared morphologically healthy. Using a

strain that could be cultured in folate and pABA-free medium made it possible to examine substrate effects at their physiological levels.

5.2.2 Stocks of Inhibitors

Stocks of SDX (Sigma, UK) and PYR (Wellcome, UK) were made in DMSO. The working stocks of MTX and PBN (Sigma, UK) were made in 0.1 N sodium hydroxide and filtered through a 0.2 μm Millipore filter to ensure sterility. PBN was used at 150 μM in the experiments described in this chapter as this concentration had previously been shown to have no antimalarial effect (Nzila *et al.*, 2003).

5.2.3 Stocks of Folate Pathway Substrates

Stocks of the folate substrates pABA, pABA monoglutamate (pABAG1), folic acid and folinic acid (5-formylTHF) were made in 0.1 N sodium hydroxide and filtered through a 0.2 μm Millipore filter to ensure sterility. The stock of 5-MTHF was made in 0.5 M potassium phosphate, pH 7.0, supplemented with 40 mM 2-mercaptoethanol, and filtered through a 0.2 μm Millipore filter to ensure sterility.

5.2.4 Parasite Inocula Preparation

Parasites used for *in vitro* sensitivity assays were synchronised at ring stage, using 5 % $^{w}/_{v}$ sorbitol (see section 2.1.4), one full asexual cycle before use. Inocula for use in the assays were diluted in culture medium to a final parasitaemia of 2 % (using uninfected RBCs washed 4 times in folate and pABA-free RPMI-1640 wash medium) and a haematocrit of 2 %, giving an end haematocrit in the assay of 1 % after 50 % dilution to give the final assay volume.

5.2.5 *In Vitro* Sensitivity Assay

Eight serial dilutions of SDX, PYR and MTX were made in folate and pABA-free culture medium with and without the folate pathway substrates being investigated. Four-fold serial dilutions of SDX and PYR were made, with respective starting concentrations of 8 mM and 2 nM. Two fold serial dilutions of MTX were made with a starting concentration of 2 μM. The end concentrations of folate substrate pathway substrates were used at physiological levels as follows: 150 nM pABA, 160 nM pABAG, 25 nM 5-MTHF and 0.5 nM folic acid. In the plates being used to investigate the effect of probenecid, dilutions of inhibitors were made with 300 μM probenecid (giving 150 μM end well concentration) in addition to the folate pathway substrates also being investigated.

The assays were performed in flat-bottomed sterile 96-well tissue culture plates (Nunc). First, 50 µl of each drug dilution was added to 3 wells, with concentration increasing from left to right in the 96-well plate. Each plate was used for two folate pathway substrates and one inhibitor, with rows B to D used for one substrate and E to G for another. In each plate column 1 was used for the negative growth control with 100 µM chloroquine present in the folate and pABA-free culture medium. Columns 6 and 11 were used as the positive growth control with 50 µl of the medium alone. Fifty µl of the prepared inocula was then added to each well taking the total volume in the well to 100 µl. The outside wells in rows A and H and column 12 were not used as it is known that growth is poorer in these, instead 100 µl of sterile distilled water was added to all wells in these rows and columns to prevent evaporation in the neighbouring wells (figure 5.2). The plates were placed in a gas-tight, humidified incubation chamber and gassed for 3 minutes with the same gas mixture used for parasite growth (3 % CO_2, 4 % O_2 and 93 % N_2), then incubated at 37 °C for 48 hours (one full asexual cycle).

Each experiment was carried out four to six times, with three replicates of each data point measurement within each experiment.

5.2.6 Determination of IC_{50}

Plates were removed from the chambers after 48 hours and 100 µl of lysis buffer combined with the fluorescent dye sybr green (0.2 µl sybr green / 1 ml lysis buffer). The lysis buffer contained Tris (20 mM, pH 7.5); EDTA (5 mM); Saponin (0.008 % (w/v); Triton X-100 (0.08 % v/v) (Bennett *et al.*, 2004). Plates were then incubated in the dark for 1 hour and subsequently read with a Varioskan fluorescence reader, at 485 nm excitation and 535 nm emission using SkanIt software. The mean values of the 3 repeats of each data point were calculated using Microsoft Excel. The relative growth of the parasites was then calculated by subtracting the mean background value (the mean of the negative growth control wells) and calculating the resulting number as a percentage of the maximum growth (the mean of the positive growth control wells). The best-fitting sigmoid curve and the 50% inhibitory concentration (IC_{50}) were calculated using the Grafit Data and Analysis Graphics Program (Erithacus Software, UK). The experiments were conducted at least 4 times.

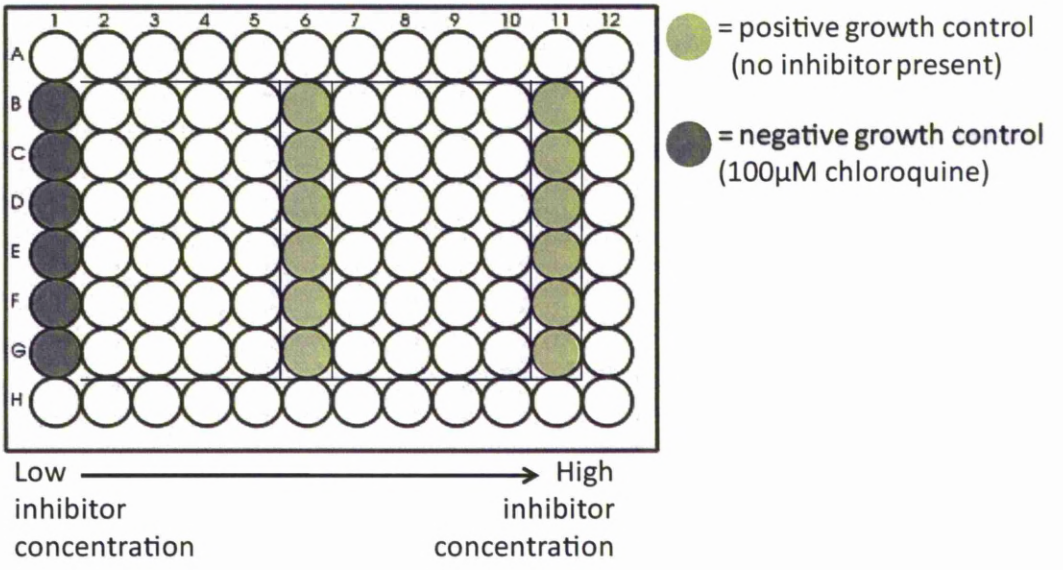


Figure 5.2. Diagram of the layout of the 96-well plate used in the *in vitro* sensitivity assay.

5.2.7 *E. coli* expression of PfFT1 and PfFT2 and growth assays

The *E. coli* strain BN1163 with knockouts of the *pabA* and *abgT* genes (Klaus *et al.*, 2005, Eudes *et al.*, 2010) was used to study the uptake of folate substrates by the likely *P. falciparum* folate transporters MAL8P1.13 (designated PfFT1) and PF11_0172 (designated PfFT2). The PfFT1 and PfFT2 genes were synthesised with codon usage optimised for *E. coli* expression (GenScript Corp., Piscataway, NJ) with the first 49 and 35 amino acids of *Pf*FT1 and *Pf*FT2 respectively replaced by codons 1-37 of the *Synechocystis sp.* PCC6803 gene slr0642 (Klaus *et al.*, 2005).

These were cloned into the pLOI707HE plasmid between *Not*I and *Sac*I sites (Arfinan *et al.*, 1992). A Shine-Dalgarno sequence also preceeded the PfFT1 and PfFT2 genes in order to improve prokaryotic expression. Glycerol stocks of the *E. coli* BN1163 strain harbouring the PfFT1, PfFT2, a S*ynechocystis* folate transporter (Slr0642), the human proton coupled folate transporter (hPCFT) or the human reduced folate carrier (hRFC) pLOI707HE recombinant constructs were kindly provided by Dr Enrique Salcedo-Sora.

For the growth assay *E. coli* BN1163 harbouring the pLOI707HE recombinant constructs and *E. coli* BN1163 with the pLOI707HE plasmid alone were cultured in 96-microwell plates in minimal medium (M9 salts, 0.1 mM $CaCl_2$, 0.5 mM $MgSO_4$, 0.4 % glucose, tetracycline 10 μg/mL, kanamycin 50 μg/mL, chloramphenicol 20 μg/mL). The wells were supplemented with folate substrates across a range of concentrations. Four wells were used for each concentration with each recombinant construct. Two fold dilutions of each substrate were made starting at 20 nM for pABA, 10 μM for pABAG1, and 20 μM for both folic acid and folinic acid. The plates were incubated overnight at 37 °C and the wells thoroughly mixed before the culture absorbance at OD_{600} was measured with a Varioskan spectrophotometer using SkanIt software.

5.2.8 Statistical Analysis of Results

Non-parametric tests were used as the distribution of values within each group was shown to be non Gaussian (using the Kolmogorov-Smirnov test, Graphpad InStat). The Mann-Whitney U test was used to establish whether there was a significant difference between the data. Bonferroni correction of p values was carried out where multiple comparisons were made to reduce the chances of type I errors occurring. Significance was counted with p values of <0.05.

5.3 Results

5.3.1 Sulphadoxine Antagonism by Folate Substrates

The effects of the folate pathway substrates and the organic anion transport inhibitor probenecid on the activity of SDX are summarised in table 5.1. The activity of SDX against *P. falciparum* 3D7 in the absence of folate is in the nM range, with a mean IC_{50} of 27.7 nM. The only folate substrate at physiological levels to antagonise SDX was pABA, increasing the IC_{50} approximately 19 fold ($p = 0.03$). The activity of SDX with a combination of pABA and 5-MTHF was similar to that seen with only the addition of pABA. The IC_{50}s of SDX with 5-MTHF and SDX with folic acid are similar to that of SDX alone. The effect of the addition of pABA on SDX inhibition is presented in figure 5.3 as a right shift of the dose response curve.

Unexpectedly, PBN showed a summatory effect with SDX inhibition, with a further 17 fold decrease to the original IC_{50} value of SDX ($p = 0.045$, table 5.1) and a shift of the dose response curve to the left (figure 5.3). PBN also reduced the activity of SDX in combination with the folate substrates.

	IC_{50} (nM)
WITHOUT PROBENECID	
SDX	27.7 ± 13.3
SDX + 150nM pABA	572.7 ± 145.2 *
SDX + 25nM 5-MTHF	23.3 ± 15.3
SDX + 0.5nM Folic Acid	33.2 ± 22.2
SDX + 150nM pABA + 25nM 5-MTHF	578.2 ± 103.6 *
WITH PROBENECID	
SDX	1.6 ± 0.6
SDX + 150nM pABA	158 ± 33.7 *
SDX + 25nM 5-MTHF	1.2 ± 0.4
SDX + 0.5nM Folic Acid	1.5 ± 0.3
SDX + 150nM pABA + 25nM 5-MTHF	156.2 ± 22.2 *

Table 5.1. Sulfadoxine IC_{50} values. SDX in folate and pABA-free culture media, with supplementation of pABA, 5-MTHF, folic acid or pABA and 5-MTHF combined. Carried out in the absence and presence of 150μM probenecid. Values are represented as mean from four to six independent experiments on different days, with each data point in triplicate, ± standard deviation. Significance was calculated using the Mann-Whitney U test, Bonferroni corrected, and shown as * representing a p value < 0.05, comparing the supplemented groups with SDX alone or with SDX + PBN.

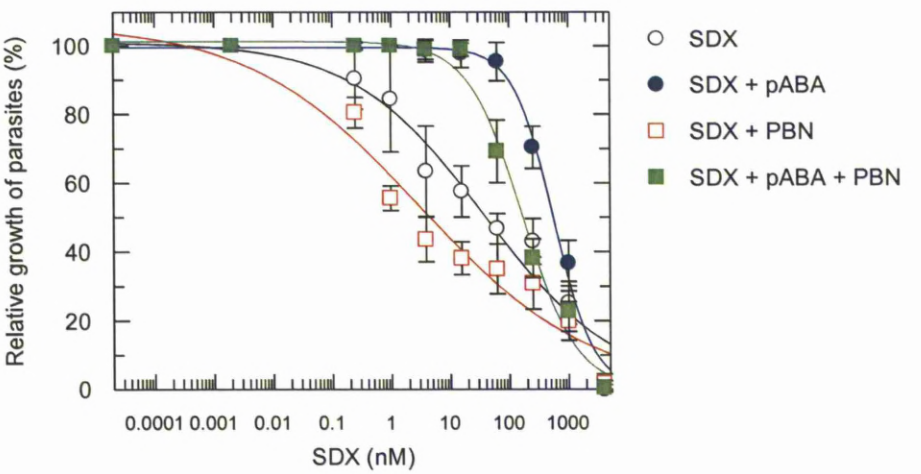


Figure 5.3. Semi-logarithmic dose response graph of SDX concentration plotted against the percentage parasite growth, for SDX alone and in combination with 150nM pABA, both in the absence and presence of 150μM probenecid. Values are represented as mean from four to six independent experiments on different days, with each data point in triplicate, ± standard deviation.

5.3.2 Pyrimethamine Antagonism by Folate Substrates

The effects of the folate substrates and PBN on the activity of PYR are summarised in table 5.2. The activity of PYR against *P. falciparum* 3D7 with the absence of folates and pABA is in the pM range, with a mean IC_{50} of 0.5 pM. The only folate substrate at physiological levels that appeared to antagonise PYR was pABA, increasing the IC_{50} 91 fold. The IC_{50}s of PYR with pABAG1, 5-MTHF or folic acid are similar to that of PYR alone. The activity of PYR with all folate substrates combined was similar to that seen with only the addition of pABA. The addition of pABA causes a shift of the dose response curve to the right (figure 5.4).

There is a slight, but not significant, increase in the inhibitory effect of PYR with the addition of PBN compared to PYR alone. There is no obvious shift of the dose response curve (figure 5.4) and the IC_{50} values are very low so we could not conclude that there was any definite summatory effect.

	IC_{50} (pM)
WITHOUT PROBENECID	
PYR	0.4 ± 0.04
PYR + 150nM pABA	36.6 ± 3.1
PYR + 160nM pABAG1	0.4 ± 0.03
PYR + 25nM 5-MTHF	0.3 ± 0.03
PYR + 0.5nM Folic Acid	0.3 ± 0.05
PYR + 150nM pABA + 25nM 5-MTHF	35.9 ± 2.7 *
WITH PROBENECID	
PYR	0.2 ± 0.04
PYR + 150nM pABA	21.9 ± 2.0 *
PYR + 160nM pABAG1	0.3 ± 0.1
PYR + 25nM 5-MTHF	0.2 ± 0.02
PYR + 0.5nM Folic Acid	0.2 ± 0.01
PYR + 150nM pABA + 25nM 5-MTHF	20.3 ± 0.9 *

Table 5.2. Pyrimethamine IC_{50} values. PYR in folate and pABA-free culture media, with supplementation of pABA, pABAG, 5-MTHF, folic acid or pABA and 5-MTHF combined. Carried out in the absence and presence of 150μM probenecid. Values are represented as mean from four or five independent experiments on different days, with each data point in triplicate, ± standard deviation. Significance was calculated using the Mann-Whitney U test, Bonferroni corrected, and shown as * representing a p value < 0.05, comparing the supplemented groups with PYR alone or with PYR + PBN.

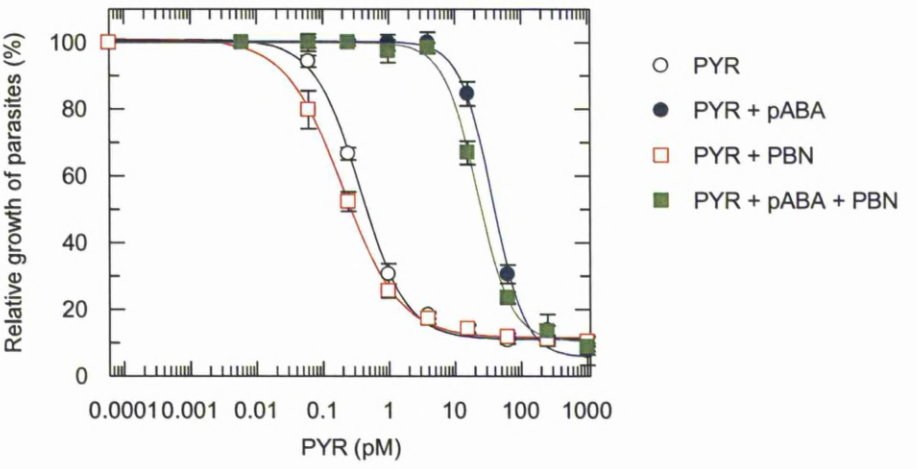


Figure 5.4. Semi-logarithmic dose response graph of PYR concentration plotted against the percentage parasite growth, for PYR alone and in combination with 150nM pABA, both in the absence and presence of 150μM probenecid. Values are represented as mean from four or five independent experiments on different days, with each data point in triplicate, ± standard deviation.

5.3.3 Methotrexate Antagonism by Folate Substrates

The activity of MTX in the absence and presence of folate substrates was in the nM range, with MTX alone having a mean IC_{50} of 92.7 nM (table 5.3). Supplementation with pABAG1, 5-MTHF or folic acid did not appear to cause an obvious change in the IC_{50} value. Supplementation with pABA did not significantly antagonise MTX, although a slight increase in the IC_{50} value was observed. The activity of MTX with the addition of all folate substrates was similar to that seen with the addition of pABA alone. The effect of the addition of pABA on MTX inhibition does not cause a shift in the dose response curve (figure 5.4).

PBN causes a marginal, non significant, reduction of the MTX IC_{50} value but not enough for a summatory effect to be assumed (table 5.3). Probenecid does not cause the dose response curve to obviously shift from that seen with MTX alone or MTX with pABA (figure 5.5).

	IC_{50} (nM)
WITHOUT PROBENECID	
MTX	92.7 ± 6.7
MTX + 150nM pABA	106.5 ± 11.3
MTX + 160nM pABAG1	92.6 ± 7.2
MTX + 25nM 5-MTHF	93.9 ± 7.7
MTX + 0.5nM Folic Acid	94.8 ± 6.1
MTX+ 150nM pABA + 25nM 5-MTHF	121.1 ± 8.9 *
WITH PROBENECID	
MTX	84.7 ± 5
MTX + 150nM pABA	147 ± 14.6 *
MTX + 160nM pABAG1	96.7 ± 3
MTX + 25nM 5-MTHF	97.3 ± 10.1
MTX + 0.5nM Folic Acid	84 ± 5.8
MTX + 150nM pABA + 25nM 5-MTHF	159.5 ± 13 *

Table 5.3. Methotrexate IC_{50} values. MTX in folate and pABA-free culture media, with supplementation of pABA, 5-MTHF, folic acid or pABA and 5-MTHF combined. Carried out in the absence and presence of 150μM probenecid. Values are represented as mean from five to seven independent experiments on different days, with each data point in triplicate, ± standard deviation. Significance was calculated using the Mann-Whitney U test, Bonferroni corrected, and shown as * representing a p value < 0.05, comparing the supplemented groups with MTX alone or with MTX + PBN.

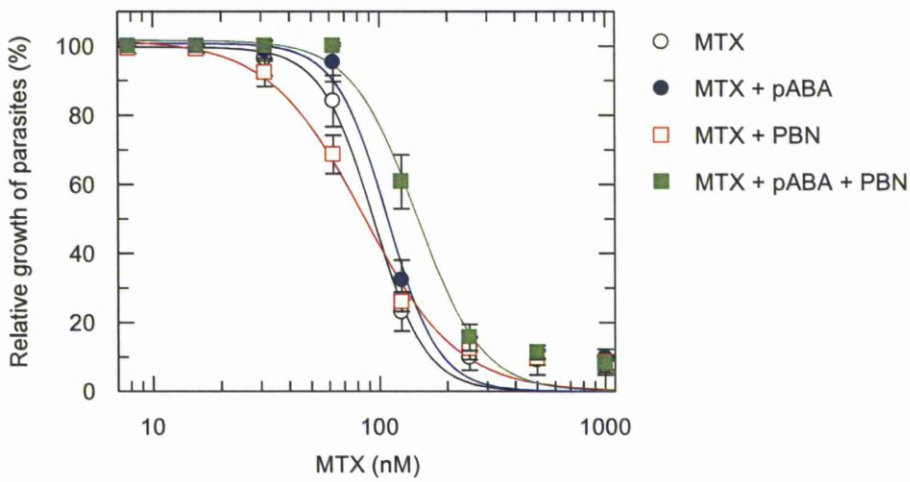
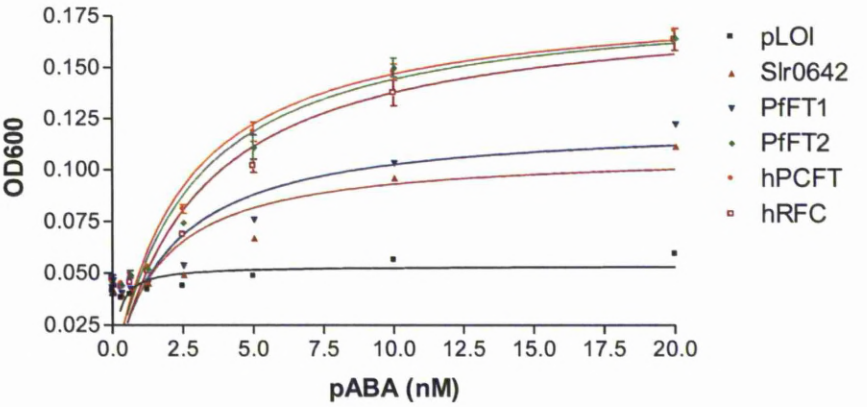


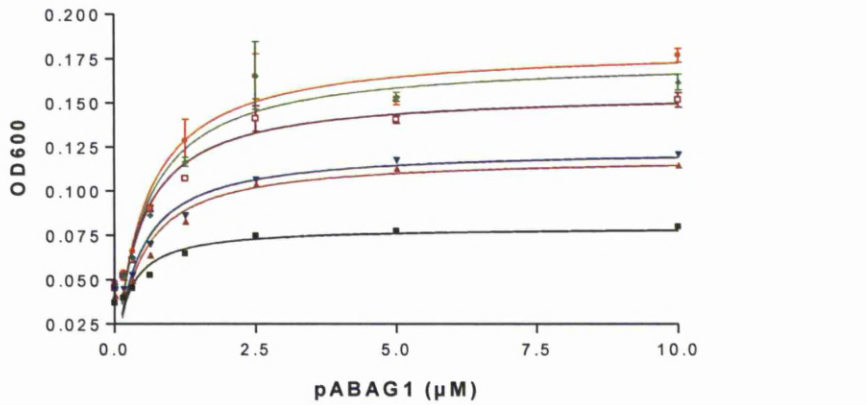
Figure 5.5. Semi-logarithmic dose response graph of MTX concentration plotted against the percentage parasite growth, for MTX alone and in combination with 150nM pABA, both in the absence and presence of 150μM probenecid. Values are represented as mean from four to six independent experiments on different days, with each data point in triplicate, ± standard deviation.

5.3.4 Expression of PfFT1 and PfFT2 in *E. coli* and Growth Rescue Experiments with Folate Substrates

The *E. coli* strain BN1163 with knockouts of the *pabA* and *abgT* genes was used to study the uptake of folate substrates by *P. falciparum* folate transporters MAL8P1.13 (PfFT1) and PF11_0172 (PfFT2). E. co*li* does not transport folates and the ability of this strain to synthesise or salvage pABA is impaired by the knockout of the *pabA* and *abgT* genes, allowing the potential effect of the salvage of folates or pABA by *E. coli* (*ΔpabA/ΔabgT*) expressing PfFT1 or PfFT2 to be observed. As positive controls a *Synechocystis* folate transporter, Slr0642, (Eudes *et al.*, 2010) and two human folate transporters, hPCFT and hRFC, (Subramanian *et al.*, 2008) were also expressed in *E. coli* (*ΔpabA/ΔabgT*). Figure 5.6 shows the growth curves of the *E. coli* (*ΔpabA/ΔabgT*) expressing PfFT1, PfFT2, Slr0642, hPCFT or hRFC along with the plasmid only control (pLOI) in combination with increasing concentrations of pABA, pABAG1, folic acid or folinic acid. Increasing concentrations of all substrates showed an increase in growth in the *E. coli* (*ΔpabA/ΔabgT*) expressing folate transporters with PfFT2 and the 2 human folate transporters showing the highest increase in growth compared to the plasmid only control. Out of all the substrates pABA was the most effective at increasing growth in all the *E. coli* (*ΔpabA/ΔabgT*) expressing folate transporters in comparison to the plasmid only control, with maximum bacterial growth observed at 20 nM.

The monoglutamated form of pABA (pABAG1) was the next most effective at increasing growth in all the *E. coli* (*ΔpabA/ΔabgT*) expressing folate transporters in comparison to the plasmid only control, with maximum bacterial growth observed at 2.5 μM. Folic acid and folinic acid both increased growth to the maximum level at 10 μM for all the *E. coli* (*ΔpabA/ΔabgT*) expressing folate transporters in comparison to the plasmid only control.





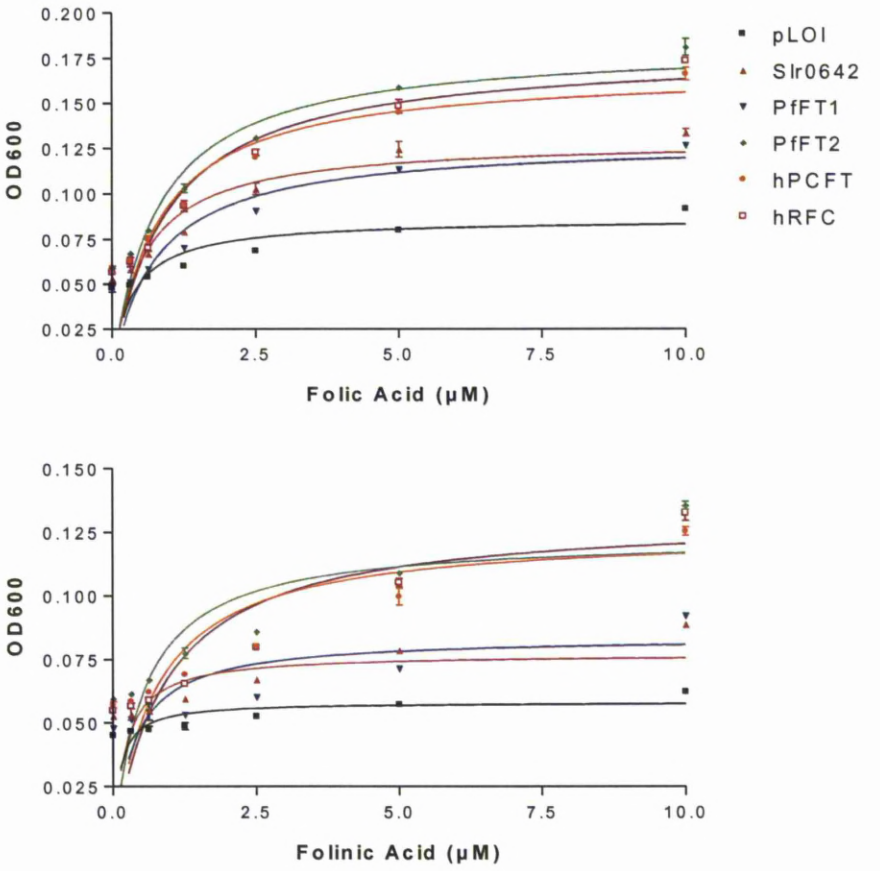


Figure 5.6. Growth response of *E. coli* strain BN1163 (*ΔpabA/ΔabgT*) expressing PfFT1 and PfFT2 to pABA, pABAG1, folic acid or folinic acid. Curves of *E. coli* strain BN1163 (*ΔpabA/ΔabgT*) expressing empty plasmid only (pLOI), *synechocystis* folate transporter (slr0642), *P. falciparum* folate transporters (PfFT1 and PfFT2), human proton coupled folate transporter (hPCFT) or human reduced folate carried (hRFC) in combination with increasing concentrations of pABA, pABAG1, folic acid or folinic acid. OD_{600} on the y-axis represents culture growth based on the absorbance of the broth measured at 600 nM. Values are represented as mean from two experiments, with each data point repeated four times, ± standard error.

5.4 Discussion

In this chapter the antagonistic effect of folate substrates and products on the antifolates SDX, PYR and MTX were investigated. At the physiological levels found in human plasma, pABA was the only folate substrate to antagonise SDX and PYR. pABA did not have a significant antagonistic effect on MTX. MTX is a very potent inhibitor of DHFR which might explain why salvage of folate substrates is unable to rescue the parasites to the same extent as that observed for SDX and PYR (Huennekens, 1994). The antagonistic effect of pABA at physiological concentrations suggests that it may be the relevant natural folate intermediate substrate for *P. falciparum*, at least for the 3D7 strain used in this study. This is further supported by the fact that pABA is the most efficient folate substrate at increasing the growth of *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2. However, there is a possibility that the parasite could utilise other transporters to transport additional folate metabolic intermediates. Indeed, there is one other *P. falciparum* gene (PF10_0215) that has been putatively annotated as a member of the folate-biopterin transporter (FBT) family (Martin et al., 2005). This gene shows significant sequence divergence from other known members of the FBT family, so it possible that it could transport different metabolites. The possibility that the parasite could express other

transporters than PfFT1 and PfFT2, that transport additional folate metabolic intermediates, was not tested in this study.

The lack of any antagonistic effect by 5-MTHF is interesting as this is the main folate product present in human plasma and it has occasionally been assumed to be the folate source for the parasite to salvage (Hyde, 2005). These results are, however, consistent with recent reports showing the lack of rescue by 5-MTHF of parasites under inhibition by antifolates (Ochong, 2008, Nduati *et al.*, 2008). It is possible that the parasite actually has little access to the pre-formed 5-MTHF as this binds electrostatically to deoxy-haemoglobin in the RBC and becomes physically trapped as the molecule picks up oxygen and switches quaternary structure (Wright *et al.*, 1998). This would force the parasite to rely on more available folate substrates, including pABA, and could account for the lack of antagonism observed with 5-MTHF. The effect of 5-MTHF on the growth of *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2 was not tested and so parallels between salvage of 5-MTHF by PfFT1 or PfFT2 and the lack of any antagonistic effect of 5-MTHF on the DHPS and DHFR inhibitors tested cannot be drawn.

The IC_{50} values obtained for SDX and PYR in the pABA and folate free culture medium were 27.7 nM and 0.4 pM respectively, both much lower than values previously reported in literature in the presence of folates and

pABA. Previous work by Wang and colleagues on the *P. falciparum* 3D7 strain gave a SDX IC_{50} value of approximately 48 μM when in the presence of folates and pABA (Wang *et al.*, 1997b). Reynolds and colleagues obtained a PYR IC_{50} value of 5 nM with the *P. falciparum* 3D7 strain (Reynolds *et al.*, 2007). The MTX IC_{50} value obtained in this study was 92.7 nM compared to the IC_{50} value of 49.9 nM previously reported for the *P. falciparum* 3D7 strain in the presence of folates and pABA (Kiara *et al.*, 2009). The dramatic reduction in the SDX and PYR IC_{50} values when the possibility of folate salvage (especially pABA) is withdrawn provides indirect evidence of the need for folate salvage. This requirement makes folate salvage still a relevant target for chemotherapeutic development.

PBN showed an unexpected summatory effect with SDX inhibition indicating that PBN may be restricting the parasite's salvage of one or more metabolite that can help rescue the parasite from the effect of SDX. As the folate cycle is linked to the methionine cycle, with 5-MTHF being the methyl donor for the conversion of homocysteine to methionine, methionine (which was still present in the RPMI-1640 used here) was considered to be a potential metabolite that PBN was inhibiting from being transported into the parasite. Ideally this could have been further investigated but folate and pABA-free RPMI-1640 that was also free of methionine was not available at the time. No obvious summatory effect of

PBN was observed with PYR and MTX to the same extent as that noted with SDX. This difference could be explained by the metabolite(s) whose transport is being reduced by PBN supplement the cell metabolism under SDX (DHPS) inhibition but not under MTX or PYR (DHFR) inhibition.

5.4.1 Critical Assessment

Relying on using only the 3D7 strain of *P.falciparum* was not ideal, but attempts at growing HB3, V1/S and K1 in the folate and pABA-free medium were unsuccessful. The folate effect observed by Wang *et al* (1997) was shown to be variable between strains and to have a genetic basis (Wang *et al.*, 1999). Using several strains with different folate utilisation phenotypes would, therefore, have been useful in drawing conclusions on the effect of physiological concentrations folate pathway substrates on antifolate treatment. The 3D7 strain used here grew acceptably in folate and pABA-free medium with growth observed to be only slightly slower than parasites maintained in standard folate and pABA complete medium. This suggests that it may have adapted to rely on folate biosynthesis making it difficult to draw conclusions from the results about the relevance of folate pathway substrate salvage by *P falciparum* in general. One way around this would have been to use low levels of folic acid in the continuous culture of alternative strains and

only transferring the parasites to folate and pABA-free culture medium one cycle before the *in vitro* sensitivity. This was used successfully by Wang *et al* (1997) but was not attempted during this study. The growth rescue experiments in *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2 showed pABA to be the most efficient folate pathway substrate at rescuing growth. This does help to back up the assumption that pABA may be the relevant natural folate intermediate substrate for *P. falciparum* (at least for the 3D7 strain used in this study). Variation between strains also is expected to be in relation to the extent of pABA uptake rather than the salvage of different folate precursors and products altogether.

The investigation of the substrate preference of PfFT1 and PfFT2 using *E. coli* (*ΔpabA/ΔabgT*) growth rescue experiments was deemed to be the best available method, even though it does not provide direct measurement of folate and pABA transport. Previous work by Dr Enrique Salcedo-Sora using the *X. laevis* oocyte expression system encountered problems with high backgrounds of pABA uptake by control oocytes, making it unsuitable for use (Salcedo-Sora *et al.*, 2011). Using the bacterial system, however, still does not allow direct measurement of folate and pABA transport. This is due to the fact that only very low levels of protein expression and low levels of pABA and folate transport are required to salvage the *E. coli* (*ΔpabA/ΔabgT*) mutants. The concentrations of folate

and pABA required to salvage the *E. coli* (*ΔpabA/ΔabgT*) mutants fall below the analytical sensitivity limits of available methods. This problem has been reported previously for studies on other BT1 homologues by other groups, with an inability to increase expression levels to those suitable for direct kinetic analysis reported (Eudes *et al.*, 2010). The lack of a direct method to measure folate and pABA transport activity by PfFT1 and PfFT2 led to the decision to use an indirect method, and *E. coli* growth rescue was deemed to be the most sensitive indirect method available.

Further repeats of the growth rescue experiments in *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2 were required in order to provide more reliability of the results. These have since been carried out by Dr Enrique Salcedo Sora (Salcedo-Sora *et al.*, 2011) and showed that, as with the results here, the folate precursor pABA was more efficient than pre-formed folate products at rescuing growth.

CHAPTER 6

6.1 General Discussion

It is necessary to study the metabolite transport pathways of the malaria parasite *P. falciparum* in order to further the understanding of the parasite's biology. As well as this crucial reason identifying and characterising the transport pathways may provide potential novel drug targets or drug delivery mechanisms (Biagini *et al.*, 2005, Kirk and Saliba, 2007). This is especially valuable as chemotherapy remains one of the main management strategies in the fight against malaria and the usefulness of the current range of antimalarial drugs is seriously threatened by the emergence and spread of resistance. No *P. falciparum* membrane transporter has been shown unequivocally to be the site of action of any current antimalarials, but the *P. falciparum* Ca^{2+} SERCA pump PfATP6 is a proposed site of action of the antimalarial artemisinins (Eckstein-Ludwig *et al.*, 2003, O'Neill *et al.*, 2010).

The work in this thesis focuses on the transport of amino acids and folate pathway substrates. A broad ranging approach using the *X. laevis* oocyte heterologous expression system to screen a gene-specific cDNA library, of potential membrane proteins, and the putative amino acid transporter PFF1430c for amino acid transport activity was used first. This was

followed by a more targeted approach with the characterisation of methionine transport by *P. falciparum* infected RBCs and the free parasites. Finally, a biochemical approach to investigate the transport-mediated salvage of folate pathway substrates, by determining their effects on antifolate drugs *in vitro* with and without a transport inhibitor, and a targeted investigation of the specificity of two likely *P. falciparum* folate transporters (PfFT1 and PfFT2) was carried out.

The erythrocytic stage of the *P. falciparum* parasite requires an exogenous source of seven amino acids but no transporters facilitating the transport of these across the parasite plasma have not been previously identified at the molecular level (as discussed in section 1.4.5.4). Six putative amino acid transporters have been annotated in the *P. falciparum* genome (Martin *et al.*, 2005) but these have also not yet been functionally characterised. Chapter 3 focuses on the use of the *X. laevis* oocyte expression system to functionally characterise a gene-specific cDNA library of 48 putative membrane proteins and the previously annotated putative amino acid transporter PFF1430c (a member of the AAAP family) for the uptake of several amino acids. No definitive transport of amino acids by the cDNA library or PFF1430c was observed. However, this could have been due to several factors and may not necessarily mean that none of the expressed proteins are amino acid transporters. The main factor being that the erroneous use of very low substrate concentrations may have resulted in

too low levels of transport to be detectible using scintillation counting (this problem has been discussed in section 3.4.1).

The PFF1430c putative amino acid transporter was identified from the *P. falciparum* genome database by Martin *et al* (2005) and shows similarity to members of the AAAP family. This family of transporters are found in eukaryotes and have been shown to transport auxin, single amino acids or multiple amino acids, with some having broad substrate specificities for naturally occurring amino acids (Saier, 2000a). PFF1430c appears to show most sequence similarity with AAAP transporters from other protozoans, yeast and mammals, including the human amino acid transporter A3 (hATA3) which transports neutral and cationic amino acids (Hatanaka *et al.*, 2001). This informed the choice to investigate glutamine as a potential substrate as hATA3 shows structural similarity to members of the glutamine transporter family. Further research into the potential transport activities of this gene are necessary due to the inconclusive nature of the results presented in chapter 3. In contrast to PFF1430c the genes in the cDNA library were not identified through similarity to known amino acid transporters but were simply potential membrane transporters based on having three or more trans-membrane domains. As discussed in section 3.4.1, only twenty genes in the cDNA library contain six or more trans-membrane domains. In eukaryotes only three amino acid transporter gene families have fewer than ten trans-

membrane domains: the phospholemman (PLM) family of channel proteins have one trans-membrane domain; the metabolite:H+ symporter (MHS) family of the MFS superfamily have six, ten or twelve trans-membrane domains; and the mitochondrial carrier (MC) family have six trans-membrane domains. This makes it highly unlikely that the genes containing fewer than six trans-membrane domains are amino acid transporters. The fact that none of the genes in the cDNA library are annotated as putative amino acid transporters makes it possible that they are transporters of other substrates. This could mean that the range of substrates investigated in chapter 3 was too narrow to discern any transport activity by these proteins. This is supported by the fact that several of the genes in the cDNA library show homology to genes in families other than amino acid transporter families.

The *X. laevis* oocyte expression system has previously been used to characterise several *P. falciparum* proteins and amino acid transporters (Closs *et al.*, 1997, Woodrow *et al.*, 2000, Downie *et al.*, 2006). It has many attractive qualities and the reasons why it was chosen for this study are discussed in section 3.1. One concern for the use of the *X. laevis* oocyte system is the potential for an endogenous transport system to mask any transport by the expressed protein. The *X. laevis* oocyte does have endogenous amino acid transport systems, although these have not all been fully characterised as yet. An alternative eukaryotic heterologous

expression system is yeast; however this was deemed to be a less favourable model for functional characterisation of putative *P. falciparum* transport proteins, as yeast appears to have more problems with the AT-rich nature of the *P. falciparum* genome than the *X. laevis* oocyte does (Kirk *et al.*, 2005).

The lack of identification of amino acid transporters using the *X. laevis* oocyte system led to a more targeted approach of characterising of methionine transport in *P. falciparum* parasitised RBCs and free trophozoites (chapter 4). Studying the transport of amino acids by *P. falciparum* parasitised RBCs and free parasites is also important as it allows the characterisation of the transport processes involved in the whole cell. The transport of isoleucine into trophozoite stage *P. falciparum* parasitised RBCs and free trophozoites has been characterised by Martin and Kirk (2007) and the findings of this study have been detailed in section 1.4.4. A study by Cobbold *et al* (2011), published at the end of this thesis' work, showed methionine to be taken up by trophozoite stage *P. falciparum* parasitised RBCs at a rate 15 fold higher than uninfected RBCs. This increased rate of uptake was attributed to the NPP rather than increased endogenous transport. In the study by Cobbold *et al* (2011) methionine was shown to be taken up by the parasite via a saturable ATP, Na^+ and H^+ independent system that is also temperature dependent.

Methionine transport in the trophozoite stage *P. falciparum* parasite has also been carried out in this thesis (chapter 4). In contrast to the findings of Cobbold *et al* (2011), however, no increase in uptake of methionine was seen in parasitised RBCs compared to uninfected RBCs. This is a very unusual result, not only because of the discrepancy with the findings of Cobbold *et al* (2011), but because most other nutrients studied have been shown to be taken up at an increased rate by the parasitised RBC. More extensive experiments, with an appropriate control, would have to be carried out before definitive conclusions can be drawn from the results in chapter 4.

From the use of kinetic transport studies and specific transport inhibitors it was inferred that methionine is taken up by free *P. falciparum* parasites via one or more saturable systems showing partial similarity to the vertebrate system L. Methionine uptake was most effectively inhibited by cysteine and to a lesser degree by lysine and histidine. Despite the fact that methionine and cysteine contain a sulphur group it is unlikely that the transporter responsible requires the presence of a sulphur group for recognition, as the sulphur residue is in different environments in each of these amino acids. The inhibition by lysine and histidine also makes this theory unlikely.

Methionine is required for several important processes within the parasite, including protein synthesis and methylation of protein, nucleic acids and lipids. The malaria parasite has been shown to have a functioning methionine synthase as well as a requirement for an exogenous source of methionine (Divo *et al.*, 1985, Krungkrai *et al.*, 1989a, Asawamahasakda and Yuthavong, 1993). Methionine synthase converts homocysteine to methionine with the addition of a methyl group from the folate product 5-MTHF. This links the methionine cycle to the folate pathway, as the demethylation of 5-MTHF produces THF, which is recycled back through the folate one-carbon metabolism enzymes (see section 1.3.3 for more details).

Chapter 5 focuses on investigating the role of salvage of folate pathway substrates by determining their effects on antifolate drugs *in vitro* and also by investigating the substrate specificity of two likely *P. falciparum* folate transporters (PfFT1 and PfFT2). Physiological concentrations of folate pathways substrates were used in combination with the antifolate drugs SDX, PYR and MTX. There has been work previously carried out on the effect of exogenous folates on the antifolate antimalarials but these studies have generally used concentrations above physiological levels (Watkins *et al.*, 1985, Kinyanjui *et al.*, 1999, Wang *et al.*, 1999, Wang *et al.*, 1997b). Previous studies have also only looked at the dynamics of folate salvage in the presence of antimalarial antifolates whereas the work in chapter 5 also

looks at the effect of transport mediated salvage. The only folate pathway substrate found to antagonise SDX and PYR at physiological concentrations was pABA. The significance of pABA was highlighted again as it was the most efficient folate substrate at rescuing growth of *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2.

The organic anion transport inhibitor PBN has been shown to chemosensitise parasites resistant to the antifolate antimalarials (Nzila *et al.*, 2003, Nzila *et al.*, 2004, Masseno *et al.*, 2009). The uptake of exogenous folate by *P. falciparum* infected RBCs and free parasites has also been shown to be inhibited by PBN (Wang *et al.*, 2007), suggesting that the reversal of resistance is due to PBN blocking the transport of folates into the parasite and preventing the parasite overcoming the effect of the antifolates with salvaged folates. Initial analysis of the results in chapter 5 appeared to support this theory, as a reduction of the antagonism of SDX and PYR by pABA was observed with the addition of PBN. However, the addition of PBN also increases the inhibitory effect of SDX and PYR in the absence of folates so the experiments used in this study are not definitive enough to support the theory that PBN elicits its chemosensitising properties through blocking folate salvage. As the addition of PBN should inhibit organic anion transport, the similar effect of pABA with and without PBN could be due to pABA entering the

intracellular parasite in another way, potentially via passive diffusion. This result is interesting when correlated with the fact that pABA is the most efficient folate substrate at increasing the growth of *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2. The fact that pABA was the only folate pathway substrate to antagonise SDX and PYR, and that it was the most effective at rescuing growth of *E. coli* (*ΔpabA/ΔabgT*) expressing *P. falciparum* folate transporters PfFT1 or PfFT2 suggests that it could be the most influential folate pathway substrate for the malaria parasite during the erythrocytic stages. This is an interesting observation as pABA is not significantly utilised or synthesised in humans making pABA salvage a potential target for chemotherapy. Targeting pABA salvage may improve the antimalarial efficacy of the antifolates SDX and PYR, and this could be achieved by blocking pABA salvage with inhibitors or using pABA-deficient diets.

As discussed previously the current antimalarial armamentarium is under threat due to the emergence and spread of resistance. To overcome this there is a need to find new drug targets or overcome resistance to current drugs. There are no current antimalarial drugs that target either amino acid transporters themselves or utilise them as drug delivery strategies. Despite this, there is precedence for drugs targeting amino acid transporters in other organisms. Drugs targeting members of the NSS family account for 2.7 % of all FDA approved drugs (Overington *et al.*, 2006, Staines *et al.*, 2010),

with many widely known psychiatric drugs, including fluoxetine (Prozac), targeting NSS family members (Bingham and Napier, 2009). Three of the six putatively annotated amino transporters identified in the *P. falciparum* genome (PFB0435c, PFE0775c and PF11_0334) show similarity to members of the NSS family and so are of particular interest for further study (Martin *et al.*, 2005). The antimalarial antifolates are well known and characterised but they are becoming less useful with the spread of resistance. The reversal of resistance to current drugs is a good potential way to extend the usage of antimalarial drugs currently available. Transporters have been shown to be involved in reversing drug resistance (as discussed in section 1.4.3) so transport inhibitors may be good potential reversal agents of resistance to certain drugs. In the case of the antimalarial antifolates the organic anion transport inhibitor PBN has been shown to reverse resistance (Nzila *et al.*, 2003, Nzila *et al.*, 2004, Masseno *et al.*, 2009). Concentrations of PBN capable of chemosensitising parasites are easily and safely achieved *in vivo* in humans and clinical trials combining SP with PBN have been carried out in African children suffering from *P. falciparum* malaria (Sowunmi *et al.*, 2004b, Sowunmi *et al.*, 2004a). The results from these trials showed that PBN significantly increased the efficacy of SP. This information is very interesting as it suggests that inhibiting *Plasmodium* anion transporters may be of clinical importance in the use of antimalarial antifolates (Nzila, 2006). This is therefore a topic of

real clinical translational value that should encourage the research on agents capable of reversing antimalarial resistance.

6.2 Future Work

In order to determine if any of the genes in the cDNA library are actually transporters the *X. laevis* oocyte expression system could be used to look again at these. This work should focus only on the nineteen genes with six or more trans-membrane domains. Using a wider range of substrates would also be useful as no genes in the cDNA library are currently annotated as amino acid transporters. Increasing the substrate concentrations to physiological levels would need to be done as the low substrate concentrations used in chapter 3 may have led to transport being present but undetectable. Any known endogenous transport by the *X. laevis* oocytes would also need to be taken into account. However, as the endogenous transport systems in *Xenopus* oocytes have not been characterised completely (Van Winkle, 1993) further characterisation of these would also be worthwhile future work. Using the *X. laevis* oocyte expression system to characterise PFF1430c more thoroughly and the other five putative amino acid transporters identified in the *P. falciparum* genome is also worthwhile.

There is a need to further investigate the transport of methionine by *P. falciparum* infected RBCs to ascertain whether the observation in chapter 4, that the *P. falciparum* infected RBC does not have an increased rate of uptake of methionine compared to uninfected RBCs, is correct. This result is at odds with that observed for other nutrients and the results from Cobbold *et al* (2011) so further work, including appropriate controls, is needed. More comprehensive characterisation of the transport of methionine is also required, in both *P. falciparum* infected RBCs and free parasites. This should examine energy dependence, ion dependence, the effect of temperature (to allow the E_a to be calculated, which will give more information on the nature of transport) and the profile of inhibition by other amino acids and classical transport inhibitors. Only methionine and isoleucine transport have been characterised in *P. falciparum* infected RBCs and free parasites. These are potentially the most interesting, and relevant, as the parasite cannot rely on degradation of haemoglobin alone for these. However, *in vitro* growth of the parasite has been shown to rely on the presence of a further five amino acids: cysteine, glutamic acid, glutamine, proline and tyrosine. Therefore, characterisation of the transport of these would prove to be useful future work.

Only the 3D7 strain of *P. falciparum* was used in the antagonism experiments in chapter 5. The 3D7 strain has been successfully cultured continuously in folate and pABA-free medium which allowed the effects

of low levels of folates at physiological concentrations to be examined. Other strains were unable to be grown in this medium and so were not used in chapter 5. However, it may be possible to culture other strains by following the protocol described by Wang *et al* (1997b) in which parasites are cultured in medium containing low levels of folic acid then transferred to folate and pABA-free medium one cycle before being used for an *in vitro* drug sensitivity assay. This would be useful future work as it would allow strains with different folate utilisation phenotypes to be used and examine the extent of pABA salvage for these as well as 3D7. It would also be useful to further investigate the observed summatory effect of PBN with SDX by eliminating other potential nutrients from the culture medium that may be being blocked from being salvaged.

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