

**An investigation of novel host-directed antimalarial
therapeutics through genetic and pharmacological
targeting of haem biosynthetic enzymes**

By

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Submitted in fulfilment of the
requirement for the Degree of
Doctor of Philosophy



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January 2012

Summary

Malaria is a lethal disease caused by the *Plasmodium* parasite. The current arsenal of antimalarial therapies targets the parasite, thereby selecting for mutant, resistant parasites. New antimalarials are desperately needed and a potential clue for a new therapeutic strategy has been provided by so-called “natural genetic antimalarials”. Host genetic changes to red cell genes have offered millennia of stable protection to individuals living in endemic regions. By imitating natural resistance, this thesis proposes novel host-directed antimalarial pharmacologic therapies through the targeting of erythrocyte molecules required by the parasite for growth and survival. Work in this thesis investigated several enzymes from the haem biosynthetic pathway as potential targets for a host-directed therapy (HDT).

Here, multiple experimental approaches were used to investigate and validate d-aminolevulinate dehydratase (ALAD), ferrochelatase (FECH) and uroporphyrinogen-III synthase (UROS) as targets for a novel host-directed antimalarial therapy. Firstly, it was demonstrated that host ALAD, FECH and UROS were localised in *Plasmodium* during intraerythrocytic growth. Moreover, the host enzymes were demonstrated to be required for normal parasite development as *Plasmodium* growth *in vitro* was impaired in UROS and FECH deficient red cells. This was shown using genetic models of human and mouse haem synthetic enzyme deficiency.

Finally, the HDT strategy was validated with several inhibitors of ALAD and FECH demonstrating *in vitro* and *in vivo* anti-plasmodial activity. Host ALAD was specifically inhibited with succinylacetone (SA), a non-competitive irreversible ALAD inhibitor, demonstrating parasite growth inhibition in a *P. falciparum* *in vitro* assay with an IC₅₀ of 2.5 µM. The antimalarial activity of SA was also demonstrated *in vivo* with SA treated mice demonstrating a significant reduction in *P. chabaudi* infection and increased survival compared to untreated controls.

The competitive FECH inhibitor N-methylprotoporphyrin (NMPP) demonstrated anti-plasmodial activity *in vitro* with an IC₅₀ of 25 nM, a figure

comparable with many current antimalarials today. Griseofulvin, a second FECH inhibitor, is an antifungal agent, approved for use for over 50 years with an anti-FECH side effect, mediated through NMPP. Griseofulvin inhibited *P. falciparum* growth in an *in vitro* growth inhibition assay, with an IC₅₀ between 10 and 50 μ M on both chloroquine resistant and susceptible parasites. As griseofulvin is FDA and TGA-approved for human use, work in this thesis investigated parasite growth capacity in red cells from individuals taking pharmacologic doses of griseofulvin. It was demonstrated that griseofulvin concentrates in red cells and that parasites were unable to grow in red cells collected from human volunteers eight-hours after taking a clinically relevant dose of griseofulvin. Together, this data suggests that griseofulvin may be a useful antimalarial drug with a novel mode of action, potentially avoiding parasite resistance.

Overall, work in this thesis has demonstrated that the parasite requires several host haem enzymes for growth and has provided proof-of-principle that targeting these enzymes as a HDT is a potentially effective antimalarial strategy. As griseofulvin is FDA and TGA approved for human use, it is quite possible that griseofulvin may be an “off the shelf” next generation antimalarial. The ultimate outcome from this work is a new generation of antimalarial therapies that may target the host and not the parasite, potentially limiting the development of drug resistance.

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Statement of ethical conduct

The research associated with this thesis abides by the International and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University



Clare Smith

5/1/2012

Publications

The following publications resulted from this thesis and allied work during my time as a PhD student at the Menzies Research Institute, University of Tasmania;

Clare Smith, Brendan McMorran, Gaétan Burgio, Noel Davies, Rob Gasperini, Ingrid Winship, Jean-Charles Deybach, Laurent Gouya, Hervé Puy, Peter David, Odile Mercereau-Puijalon and Simon Foote (2012) Genetic and pharmacological targeting of host ferrochelatase prevents the intra-erythrocytic growth of malaria parasites (*Submitted*).

Clare Smith, Brendan McMorran and Simon Foote. The host red cell aminolevulinate dehydratase is an antimalarial target (*manuscript in preparation*).

Clare Smith, Ingrid Winship, Brendan McMorran and Simon Foote. Host uroporphyrinogen-III synthase is scavenged and required for intraerythrocytic growth of the malaria parasite (*manuscript in preparation*).

Rhea Longley, Clare Smith, Anny Fortin, Joanne Berghout, Brendan McMorran, Gaétan Burgio, Simon Foote, Philippe Gros (2011) Host resistance to malaria: using mouse models to explore the host response. *Mammalian Genome* 22: 32-42

Abstracts

The following abstracts for presentation at conferences have resulted from work in this thesis

Smith, C.M., Winship, I., Puy, H., David P., Mercereau-Puijalon, O., Burgio, G., McMorran, B.M and Foote S.J. Host ferrochelatase is required by the malarial parasite and is a potential target for a novel host-directed therapy. Australian Society for Parasitology Annual Conference. Launceston, Tasmania. Oral presentation (2012)

Smith, C.M., McMorran, B.M., Gasperini, R., Burgio, G., Winship, I., Puy, H., Mercereau-Puijalon, O., and Foote S.J. Host ferrochelatase is required by the malarial parasite and is a potential target for a novel host-directed therapy. Molecular Approaches to Malaria Conference. Lorne, Victoria. Poster (2012)

Smith, C.M., McMorran, B.M., Burgio, G. and Foote S.J. Targeting host ferrochelatase as a novel antimalarial therapy. 32nd Annual Lorne Genome Conference. Lorne, Victoria. Poster (2011)

Smith, C.M., McMorran, B.M., and Foote S.J. Towards novel host-directed antimalarial treatments. 1st annual Lorne Infection and Immunity Conference. Lorne, Victoria. Poster (2011)

Smith, C.M., McMorran, B.M., and Foote S.J. Towards novel antimalarial treatments: a host-directed therapy and the haem biosynthetic pathway. XIIth International Congress of Parasitology (ICOPA). Melbourne, Victoria. Oral Presentation (2010)

Smith, C.M., McMorran, B.M., and Foote S.J. Towards novel antimalarial treatments: a host-directed therapy and the haem biosynthetic pathway. 31st annual Lorne Genome Conference. Lorne, Victoria. Poster (2010)

Smith, C.M., McMorran, B.M., and Foote S.J. Utilising host resistance mechanisms for a novel therapeutic strategy against malaria. Host Genetic Control of Infectious Diseases Conference. The Pasteur Institute, Paris, France. Poster (2009)

Smith, C.M., McMorran, B.M., and Foote S.J. Towards novel antimalarial treatments: a host directed therapy and the haem biosynthetic pathway. Gordon Research Conference for Malaria. Magdalen College, Oxford, U.K. Poster (2009)

Smith, C.M., McMorran, B.M., and Foote S.J. Towards novel antimalarial treatments: a host directed therapy and the haem biosynthetic pathway. ASP and ARC/NHMRC Research Network for Parasitology Annual Conference. Sydney, New South Wales. Poster and short oral presentation (2009)

Smith, C.M., McMorran, B.M., and Foote S.J. A novel antimalarial therapy. Sharing Excellence in Research Conference. Hobart, Tasmania. Oral presentation (2008)

Smith, C.M., McMorran, B.M., and Foote S.J. A novel antimalarial therapy. National AusBiotech Conference. Melbourne, Victoria. Poster (2008)

Acknowledgements

I have been privileged to work in the malaria genetics group and I would like to thank my two fantastic mentors, Simon Foote and Brendan McMorran, for their guidance, discussions and endless encouragement over the past few years. Simon was the enthusiastic visionary and Brendan provided a healthy dose of realism. Both of which were essential for the success of this project and very much appreciated.

Thanks also to past and present lab members, in particular Laura Wieczorski, Elinor Hortle, Rhea Longley, Meredith Roberts-Thomson, Gaetan Burgio, Preethi Guru, Claire Howells, Andy Greth, Patrick Lelliot, Mariana Brizuela, Karen Drysdale and Lucy Whittock.

This project would not have been made possible without the enthusiastic volunteers who took part in the study, in particular Richard Bradbury, Ros Herbert, Briony Patterson, John Burgess and Ray Playford. Thanks to Ceri Flowers and Laura Keith for venipuncture skills and support, Noel Davies (CSL) for running my HPLC samples, Rob Gasperini for patiently teaching me to drive the phase-contrast scope and the animal facility staff at Menzies.

Many thanks to Ingrid Winship for travelling the country to collect blood samples from people with porphyria. It may have been a serendipitous event that led to our collaboration but this project could not have been completed without you or the porphyria patients and families involved.

Thank you to Odile Mercereau-Puijalon for hosting my exchange at the Pasteur Institute and to Hervé Puy and associates for access to porphyria blood samples. Thanks also go to Lina, Antony, Robyn, Serge, Michilen, Innes, Olivier and Peter for sharing my Parisian adventure.

I wish to acknowledge the inspiration and encouragement provided on different occasions by Michelle Keske, Fiona Poke, Robert Menard, Genivieve Milon, Elizabeth Blackburn and Oliver Smithies.

Funding from GlaxoSmithKline-Australia, OzEMalaR, The Australian Society of Parasitology, the Lindau Committee and the Australian Academy of Science for the Bede Morris Travelling Fellowship is greatly appreciated.

Finally, this adventure could not have been completed with the smile that it has been, without the support of Scott Hansen. Thanks for the perpetual encouragement and hot meals to keep me going. Thanks also to friends and family, particularly Ros, Phil, Chris, Laurie, Jeanette, Soph and Beck W, for understanding that there are just some things I have to do.

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Abbreviations

AGRF	Australian Genome Research Facility
ALAD	d-aminolevulinate dehydratase
AMA1	apical membrane antigen 1
B-Gal	B-galactosidase
CCM	Complete Culture Media
CR1	complement receptor 1
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	Enhanced Chemiluminescence
FECH	ferrochelatase
g	gram
G6PD	glucose 6 phosphate dehydrogenase
GST	glutathione-S-transferase
h	hour
Hb	haemoglobin
HDT	host directed therapy
HIV	human immunodeficiency virus
HLA	Human Leukocyte Antigen
HMBS	hydroxymethylbilane synthase
ICAM-1	intracellular adhesion molecule-1
i.p.	intraperitoneal
IPTG	isopropyl-thio-galactopyranoside
iRBC	infected red blood cell
KAHRP	knob-associated histidine-rich protein
kb	kilobase
LB	Luria broth
min	minute

ng	nanogram
nM	nanomole
NMPP	N-methylprotoporphyrin
OD ₆₀₀	Optical density at 600 nm
ON	overnight
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PK	pyruvate kinase
PV	parasitophorous vacuole
RBC	red blood cell
RIFIN	repetitive interspersed family
RON	rhoptry neck protein
rpm	revolutions per minute
SA	Succinylacetone
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Sec	second
SOD	superoxide dismutase
ug	microgram
ul	microliter
uM	micromole
UPLC-MS	ultra performance liquid chromatography-mass spectrometry
UROS	uroporphyrinogen-III synthase
VTs	vascular transport signal
WHO	World Health Organisation
WT	wild type

Chapter 1

Introduction

Summary

The current arsenal of antimalarial treatments is losing the battle with parasite resistance. A new therapeutic paradigm that avoids parasite resistance is desperately needed. A clue for a resistance-proof antimalarial strategy has been provided by the numerous red cell mutations known to protect the host from severe malaria. Can we mimic these so-called natural “genetic antimalarials” as a new host directed therapeutic strategy? Work in this thesis investigates this question by targeting red cell enzymes required by the parasite in a strategy coined host directed therapy. Host directed therapy may be a resistance-proof therapeutic strategy as the target is outside of the cellular milieu and genetic control of the parasite.

Malaria is a lethal disease with no easy solution

Malaria is a disease caused by the protozoan parasite *Plasmodium*, which infects over 200 million people worldwide every year. Of these infections, the latest World Health Organisation (WHO) malaria report estimates that that over seven hundred thousand people die from the disease annually, mostly young children and pregnant women (WHO, 2011). Over 40% of the world population lives in at-risk areas. This includes nearly 100 countries with the highest prevalence in sub-Saharan Africa (Bremner et al., 2006). Malaria also has a profound economic impact, having a severe effect of the gross domestic product in countries that are already burdened with poverty (Sachs and Malaney, 2002).

Despite the number of currently available antimalarials, the outlook for eliminating malaria remains daunting. The WHO set the goal of eliminating malaria in 1946; a task that has dismally failed and studies have suggested

that, if unchecked, the number of malaria cases may double in the next 20 years (Sachs and Malaney, 2002). The failure of the eradication of malaria is due in part, to a variety of socioeconomic factors in at-risk communities but is also exacerbated by the increasing parasite resistance towards all antimalarials (Gallup and Sachs, 2001). An additional concern is the limited number of novel antimalarial compounds making their way through the long and arduous pipeline of drug discovery and clinical trials. If we are to ever make inroads into not only treating but also overcoming this lethal disease, there needs to be a concerted effort in identifying compounds with novel modes of action that may avoid parasite resistance common to the current generation of antimalarials.

Plasmodial species and lifecycle

There are a variety of *Plasmodium* species that infect vertebrates including rodents, birds, reptiles and primates. Five species of *Plasmodium* infect humans; *falciparum*, *vivax*, *malariae*, *ovale* (which is now characterized as two separate species *P. ovale curtisi* and *P. ovale wallikeri*) (Liu et al., 2010, Sutherland et al., 2010). Also included is *knowlesi*, a strain infecting long-tailed macaques but field data has shown naturally acquired human infections in Malaysian Borneo (Cox-Singh et al., 2008, Singh et al., 2004, Sutherland et al., 2010). All result in serious and sometimes life-threatening symptoms, but *P. falciparum* is the most lethal, accounting for the majority of deaths (Snow et al., 2005).

The lifecycle of all *Plasmodium* species include stages of sexual and asexual reproduction in the female *Anopheles* mosquito and the human host respectively (Figure 1.1).

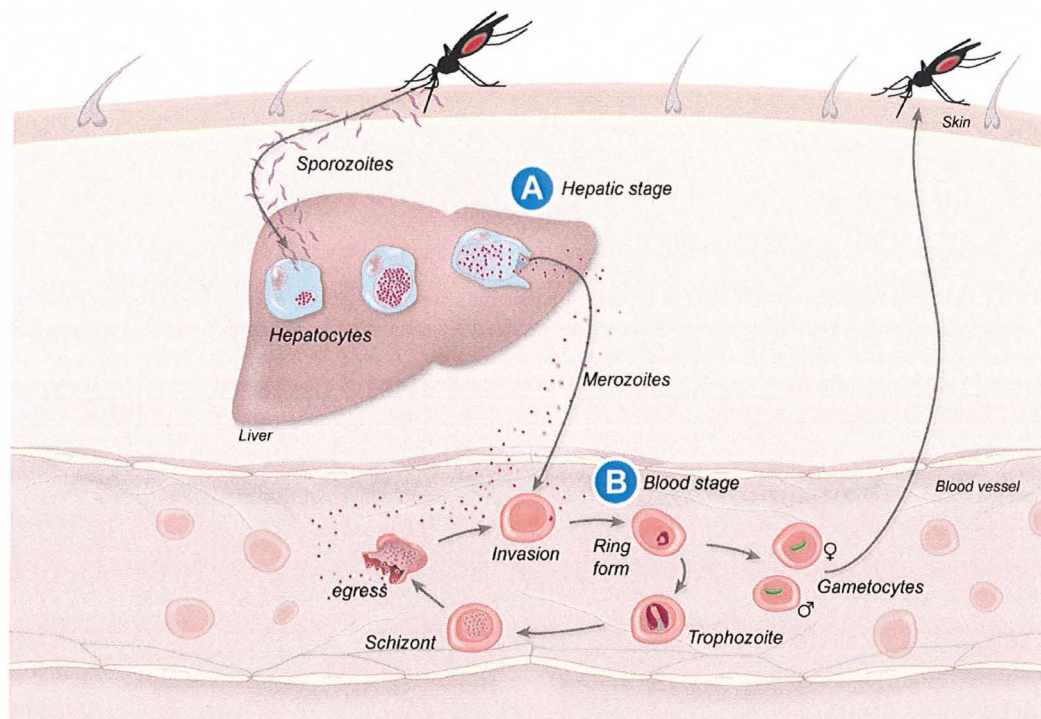


Figure 1.1 Lifecycle of *Plasmodium*. The lifecycle of *Plasmodium* in the human host is initiated upon the bite of a female *Anopheles* mosquito, releasing sporozoites that make their way to the liver. Infection in the human host is made up of the asymptomatic hepatic stage (A) and the clinical blood stage (B) before some merozoites differentiate into gametocytes that get taken back up by the *Anopheles* mosquito. Within the mosquito, the gametes combine, undergo meiosis and subsequently differentiate into new and genetically unique sporozoites capable of infecting other people, thereby continuing the lifecycle of the parasite.

Illustration drawn by Andy Greth, Menzies Research Institute, 2010

Sporozoites residing in the salivary glands of the female mosquito are injected during feeding into subcutaneous tissue of the host (Lasonder et al., 2008). The majority of sporozoites travel to the liver but work in rodent models of malaria demonstrated that a proportion of sporozoites remain in the skin and have the capability to form infective merozoites (Gueirard et al., 2010). Sporozoites that follow the normal infection progression enter the circulation and make their way to the liver where they migrate through several cells before ultimately invading a hepatocyte, initiating the clinically silent stage of infection (Mota et al., 2002, Mota et al., 2001). Sporozoites subsequently undergo asexual schizogony within a parasitophorous vacuole to form schizonts that can release up to several thousand merozoites into the host's circulation (Graewe et al., 2011). Evidence suggests the parasite modifies the host hepatocyte during its development to avoid detection by host immune cells. A specific death and detachment process is induced by the parasites allowing movement towards the liver sinusoids and into circulation (Sturm et al., 2006). The parasite reaches the circulation still enclosed in a vacuole of host origin, named the merozoite, thereby escaping detection from the host immune system, until the merozoites are released and invade the host red cells (Graewe et al., 2011).

Merozoites invade red cells where they undergo successive, synchronised rounds of intracellular asexual reproduction and merozoite release, causing the characteristic periodic fevers that are a classic symptom of the disease (Hill, 2006). Merozoite invasion is a multi-step process including initial recognition, attachment and the formation of a tight or moving junction between the apical end of the merozoite and the red cell membrane (Richard et al., 2010). A parasite actin-myosin motor powers a series of events that include the movement of the moving junction from the apical to the posterior end of the parasite. This process is facilitated through several parasite-derived proteins including rhoptry neck protein (RON) 2 and the apical membrane antigen 1 (AMA1), which form a complex (Tonkin et al., 2011). A parasitophorous vacuole (PV) is formed as the parasites traverse into the host red cell cytoplasm (Aikawa et al., 1978).

Once inside a red cell, the parasite develops through several stages, namely a ring stage, a larger trophozoite stage and a schizont stage. Schizont rupture releases 30-40 merozoites that infect other red cells (Millholland et al., 2011). The parasite undergoes a complete cycle of invasion, replication and release every twenty-four, forty-eight or seventy-two hours depending on the *Plasmodium* species.

Some merozoites differentiate into male or female gametocytes (precursors of gametes), initiating the sexual stage of the *Plasmodium* lifecycle (Hawking et al., 1971). The trigger of parasite differentiation into gametocytes (gametocytogenesis) is unknown but several *Plasmodium*-encoded genes are essential (Gardiner et al., 2005). Gametocytogenesis also involves a specific pattern of gene regulation and expression (Pace et al., 2006). Environmental factors such as stress, pH and temperature may also play a role in gametocytogenesis (Carter and Miller, 1979). The mosquito takes up the gametocyte-infected red cells during a blood meal. Fertilization and subsequent differentiation of the zygote into an ookinete occurs in the midgut lumen of the mosquito (Boisson et al., 2011). The ookinete is motile and crosses the midgut, differentiating into an oocyst that can release up to several thousand sporozoites. The sporozoites make their way to the salivary gland of the mosquito, ready for the next blood meal (Ghosh et al., 2011).

Parasite-erythrocyte interactions

The mature red cell is devoid of organelles and trafficking pathways; hence the parasite must remodel the red cell to create a habitable environment (Gormley et al., 1992). The parasitophorous vacuole (PV) forms an intermediate between the host and parasite and facilitates the exportation of parasite proteins.

The induction of transport pathways and ion channels from the parasitophorous vacuole to the surface of the red cell allows *Plasmodium* to maintain osmotic integrity of the red cell and obtain vital nutrients, ions and amino acids that the red cell cannot supply (Cobbold et al., 2011, Kirk and

Horner, 1995, Kirk et al., 1994, Martin and Kirk, 2007, Saliba and Kirk, 2001). One of the main pathways described is the new permeation pathway (NPP), which is induced around 16 hours after invasion and allows the infected red cell (iRBC) increased permeability to nutrients and inorganic ions (Kirk and Horner, 1995, Martin and Kirk, 2007, Saliba et al., 1998b, Staines et al., 2000).

Additional features of the intraerythrocytic parasite include the formation of a tubulovesicular network, which acts as an intermediary between the parasitophorous vacuole membrane and the outside of the red cell (Lauer et al., 1997). Maurer's clefts are membranous structures formed as part of the network and play an important role in sorting parasite proteins prior to presentation on the surface of the red cell (Aikawa et al., 1986, Hanssen et al., 2008b). The exportation of several parasite proteins is essential for membrane rigidity and parasite virulence, including knob-associated histidine-rich protein (KHARP) and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) (Maier et al., 2008). PfEMP1 is exported to the Maurer's clefts before being targeted to knob-like structures, which form under the red cell membrane (Kriek et al., 2003). Several parasite proteins are essential for this process including the ring exported protein-1 (REX1) (Dixon et al., 2011, Hanssen et al., 2008a), skeleton-binding protein 1 (SBP1) (Cooke et al., 2006) and the membrane-associated histidine-rich protein1 (MAHRP1) (Spycher et al., 2008). These structures and parasite receptors decrease red cell deformability and allow cytoadherence of the iRBC to host receptors on the endothelium, preventing splenic clearance (Wickham et al., 2001). The sequestration of infected cells to the endothelium can occlude blood vessels to the brain, contributing to the pathogenesis of severe malaria (Glenister et al., 2008). Another pathogenesis mechanism associated with severe malaria is the adherence of iRBCs to uninfected red cells, in a process called rosetting (Mercereau-Puijalon et al., 2008). This process is thought to be mediated by PfEMP1 and the host red cell receptor complement receptor 1 (CR1) (Rowe et al., 1997, Fairhurst et al., 2005, Chen et al., 1998).

The display of the parasite proteins on the surface of the iRBC means that they are exposed to the host's immune system and can be targeted for

immune destruction (Vigan-Womas et al., 2011). The parasite relies on a complicated system of antigenic variation to evade the immune system (Barfod et al., 2011). Parasite proteins (such as *PfEMP1*) encoded by the *var* gene family are able to switch and avoid immune detection (Janes et al., 2011, Mok et al., 2008). Other parasite variant surface antigens implicated in either altering the properties of the red cell for cytoadherence or antigenic variation include the STEVOR family of transmembrane proteins (Przyborski et al., 2005, Sanyal et al., 2011, Niang et al., 2009) and RIFIN (repetitive interspersed family) (Joannin et al., 2011, Wang et al., 2009, Haeggstrom et al., 2004).

A number of recent discoveries have shed light on how exported parasite effector proteins traverse the PV. A conserved secretory signal called a PEXEL motif or a vascular transport signal (VTS) was identified in the sequence of proteins that make it to the surface of the red cell (Marti et al., 2004, Hiller et al., 2004). The parasite machinery allowing proteins with this motif to be exported beyond the PV was subsequently identified, termed a translocon. The authors identified the ATP-driven *Plasmodium* translocon of exported proteins (PTEX) and found a range of proteins making up the PTEX machinery (de Koning-Ward et al., 2009). Furthermore, plasmepsin V, the parasite endoplasmic reticulum aspartic protease, cleaves proteins with a PEXEL motif thus allowing their exportation (Boddey et al., 2010, Russo et al., 2010). The PTEX is required for the remodeling process of the host red cell, thus enabling growth and survival of the intraerythrocytic parasite (Boddey et al., 2009).

Clinical outcome to malaria

Malaria infections can range from asymptomatic through to lethal. Pathogenic consequences of infection by the malarial parasite include periodic fever, anaemia and severe malaria, which include cerebral malaria, severe anaemia and metabolic acidosis (Menendez et al., 2000). Fever is caused by a pro-inflammatory cytokine response, which corresponds to the

rupturing of erythrocytic schizonts (Magill, 1998). Anaemia is thought to be due to a combination of increased hemolysis of infected red cells (iRBCs), increased clearance of uninfected red cells and an impaired ability of the infected host to produce an adequate erythropoietic response (Lamikanra et al., 2007, Kai and Roberts, 2008). The development of severe malaria is a multi-system disorder, which is thought to result from a combination of host inflammatory factors such as adherence and sequestration in the vasculature (Mackintosh et al., 2004). The adherence of iRBCs is mediated by the binding of *PfEMP1* to endothelial cell surface receptors such as CD36, thrombospondin and intracellular adhesion molecule 1 (ICAM-1) (Baruch et al., 1996). Adherence of iRBCs to the endothelial cells of various organs such as the heart, liver and brain of the host often causes microvascular occlusions, therefore preventing tissue perfusion (MacPherson et al., 1985).

Host immunity can also influence the clinical outcome to malaria. The host immune response to malaria is complex and varies depending on the age and genetic makeup of the host, species of *Plasmodium*, level of endemicity, the particular stage of infection, and the antigenic makeup of the parasite that produces the immunity (Lopez et al., 2010, Stevenson and Riley, 2004). Individuals who are repeatedly infected and continuously exposed to the parasite over their lifetime develop an acquired immunity, which protects against the clinical symptoms of the disease (Bull et al., 1998). Protection against the symptomatic stage of disease is thought to be due to a variety of innate and adaptive immune responses over the lifetime of the host, which can reduce the levels of circulating parasites but may also result in a persistent infection by low levels of parasites, and therefore continued transmittance of the disease to other individuals (Doolan et al., 2009, Smith et al., 2002). Some of the mechanisms thought to be involved in host immunity to malaria include the production of antibodies that block the invasion of sporozoites into liver cells and invasion of merozoites into circulating red cells (Mueller et al., 2007, Purcell et al., 2008). It is also thought that interferon- γ plays a role with CD8⁺ T cells to inhibit parasite development in hepatocytes, as well as acting with CD4⁺ T cells to activate

macrophages, which are able to phagocytose free merozoites and iRBCs (Hirunpetcharat et al., 1999, Todryk et al., 2008, Langhorne et al., 2008). Platelets have also been implicated as an important immune-mediated defense, with platelets preferentially binding to iRBC over uninfected red cells and directly killing the parasite (McMorran et al., 2009). Other evidence suggests that platelets contribute to the development of cerebral malaria and may actually worsen the clinical outcome to severe disease (Martins et al., 2009).

Evidence for a genetic basis to the immune response to malaria has come from several epidemiological studies in West Africa, showing inter-ethnic differences in antibody titers against several malarial antigens. Despite similar infection levels, the Fulani showed lower parasite rates and fewer malarial attacks than their neighbors. This was hypothesized to be due to a more efficient immune response, as evidenced by a higher antibody titer (Verra et al., 2009). The immune response genetic variants will be discussed later in this literature review, along with red cell variants that confer resistance to severe malaria.

The antimalarial story dates back to ancient times

The history of antimalarials dates back many thousands of years where ancient cultures recognised the medicinal properties of herbs and other natural products. One of the earliest known antimalarials, quinine, was originally isolated from the bark of the cinchona tree and used by the Aztecs. It was introduced into European medicine in the 1630s and quinine was identified as the active ingredient in the cinchona bark in 1820. The synthesis of this compound became a priority in the 1940s when supplies to the Pacific were cut by the onset of war (Ball, 2008). Quinine was reportedly fully synthesized in 1944 by Woodward and Doering, a fact debated over many years and only now has been validated (Smith and Williams, 2008). Quinine was the frontline treatment for severe disease until the synthesis of chloroquine, which was deployed in the 1950s. Other antimalarials include

the sulpha and pyrimethamine drugs, used in combination in an effort to halt the signs of resistance.

The most recently released antimalarial, artemisinin and related compounds, are another example of a natural product whose medicinal properties have been recognised for several thousand years. Artemisinin is derived from the extracts of sweet wormwood (*Artemisia annua* L) and derivatives include dihydroartemisinin, artmetheter and artesunate, which were first prepared in a collaborative effort by Chinese scientists in the 1970s (Krishna et al., 2008).

Current antimalarials are losing the battle with parasite resistance

Drugs in the current antimalarial arsenal inhibit a range of enzymatic and biologic processes specific to the parasite. Despite the range of compounds available in the fight against malaria, a cause for concern is that only one new synthetic antimalarial has been released in the last 30 years (mefloquine). The limited time needed for the parasite to develop resistance to antimalarials in the field is cause for concern. For example, chloroquine was synthesized in 1934 and was an important antimalarial used in the malaria eradication campaign in the 1950s but resistance was observed in Thailand by 1962. The emergence of resistance to chloroquine rapidly extended and by 1988 resistance had spread to essentially all of sub-Saharan Africa where chloroquine is no longer effective (Hyde, 2007). It is a similar story with the remaining antimalarials including the latest addition to the market, artemisinin, showing delayed parasite clearance and increasing resistance throughout Asia (Dondorp et al., 2011).

One problem with these therapies is that they target the parasite, which has a rapid lifecycle in the human host and is under intense selective pressure. Additionally, gametocytes undergo meiosis in the mosquito vector, giving rise to genetic variation. These factors lead to the development of drug resistant parasites that can rapidly become the dominant strain within a geographical area (Vangapandu et al., 2007). There are several ways that resistance to antimalarials can occur. Firstly, mutations to transporters alter the activity

and/or expression of parasite transporters, preventing drug accumulation or increased transportation out of the infected cell (Chinappi et al., 2010, Martin et al., 2009). Alternatively, mutations in the target enzyme can reduce the drug affinity for the target molecule (Cowman et al., 1988, Foote et al., 1989). Several examples of this are illustrated below.

Chloroquine mechanism of action and resistance models

The internalisation of host cytosol is initiated at the mid-ring stage of intraerythrocytic growth and the imported host haemoglobin (Hb) is concentrated into acidic peripheral structures that form a digestive vacuole (Abu Bakar et al., 2010). Here, the imported haemoglobin is degraded and polymerized to the inert malarial pigment hemozoin (Slater and Cerami, 1992). Chloroquine accumulates in the digestive vacuole and prevents the formation of hemozoin, allowing the buildup of toxic byproducts that damage the parasite (Homewood et al., 1972, Sullivan et al., 1996). Chloroquine resistance arises by mutations in parasite genes that encode drug transporters, in particular the *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) (Vangapandu et al., 2007). The *PfCRT* is an integral membrane protein of the digestive vacuole and a variety of mutations have been shown to alter chloroquine transport (Saliba et al., 1998a). Compared to the wild-type protein, mutant *PfCRT* readily transports chloroquine, thus allowing chloroquine to be readily taken away from the site of action in the food vacuole (Martin et al., 2009).

Another gene identified is the *P. falciparum* multiple drug resistance gene 1 (*PfMDR1*), which is a parasite homologue of the mammalian multi-drug resistance (*MDR*) gene in tumour cells (Foote et al., 1990, Foote et al., 1989). Mutations in the *PfMDR1* gene confer chloroquine resistance through decreased accumulation of the drug (Reed et al., 2000). Additionally amplification of *PfMDR1* copy number is thought to contribute to both chloroquine and mefloquine resistance (Wilson et al., 1989, Cowman et al., 1994).

Antifolate mechanism of action and resistance

Folate is required for the pyrimidine, purine and amino acid biosynthetic pathways. Humans obtain folic acid from dietary sources, while the parasite cannot utilise exogenous folic acid, and instead employs a unique *de novo* synthetic pathway (Vangapandu et al., 2007). The sulphonamides and pyrimethamine are often used in combination, each targeting specific enzymes in the folate biosynthetic pathway in the parasite. The sulphonamides target dihydropteroate synthetase (Zhang and Meshnick, 1991), whereas pyrimethamine and cycloguanil target dihydrofolate reductase (Gregson and Plowe, 2005). The genetic basis of resistance to antifolates is largely due to single point mutations in the genes of the target enzymes dihydrofolate reductase and dihydropteroate synthetase (Cowman et al., 1988).

Artemisinin mechanism of action and resistance

The artemisinins play an important role in current antimalarial strategies, with artemisinin combination therapy the first-line treatment worldwide (Dondorp et al., 2009). The mechanism of action is not completely understood with haemoglobin, iron and calcium ATPase processes being implicated as the targets of artemisinin (Coghi et al., 2009, Haynes et al., 2011, Krishna et al., 2008, Krishna et al., 2010). Recent evidence demonstrates that artemisinin inhibits the uptake of host haemoglobin with subsequent parasite growth inhibition (Klonis et al., 2011). Artemisinin has a short half-life but is routinely coupled with other longer-lasting antimalarial compounds (Eastman and Fidock, 2009). However, artemisinin resistance has emerged on the Thai-Cambodian border, with demonstrated *in vivo* delayed parasite clearance (Dondorp et al., 2009), meaning that the future use of this drug may be limited. Work *in vitro* recently demonstrated several mutations in *PfMDR1* were associated with parasite resistance in field isolates from the Thai-Myanmar border (Veiga et al., 2011).

Novel antimalarial targets

With the current antimalarial drugs suffering from parasite resistance, there is a need for new compounds that are effective. One of these routes is the identification of new parasite targets, helped by the recent advances in parasite biology and the completion of the *P. falciparum* genome. While any new antimalarial compound needs to show efficacy against the parasite, bioavailability and limited side effects; another important trait is the cheap production cost. As such, the commercial opportunities of a new antimalarial are limited. Nevertheless, a range of parasite biochemical pathways are being viewed for potential targets, including parasite proteases, membrane biosynthesis, shikimate pathway, isoprenoid biosynthesis, purine metabolism, pyrimidine metabolism, cyclin-dependant proteases and the redox and mitochondrial systems (Grimberg and Mehlotra, 2011). Several large-scale drug screens have also revealed a large range of potential new candidate drugs (Gamo et al., 2010, Guiguemde et al., 2010).

A problem with any newly discovered antimalarial therapeutic candidates is that there is still the potential for parasite resistance to develop, as they are still parasite-directed. Another point to consider is that the above targets are still at the beginning of the drug development pipeline, with many millions of dollars needing to be spent and on clinical testing to evaluate their safety as antimalarials. Is there value in pushing these parasite-directed targets through the long and expensive road of clinical trials, only to see the parasite rapidly develop resistance once deployed in the field? From the multitude of evidence of the failure of the current antimalarial strategy presented above, there is a clear demand for the development of antimalarials for which the parasite cannot develop resistance. This necessitates that not only are new targets needed but also that a new therapeutic strategy is required.

Vaccine prospects

The prospect of a vaccine to protect against malaria is based on evidence that individuals repeatedly exposed to *Plasmodium* develop an acquired immunity

(Hommel, 1981). However, immunity occurs over the lifetime of an individual and is incomplete (Cohen, 1977). There are several potential strategies for vaccine targeting, including exoerythrocytic (liver stage) or intraerythrocytic. Out of over 47 new vaccine candidates, only one (The RTS,S vaccine) has reached phase III clinical trials (Langhorne et al., 2008). The RTS,S vaccine targets the circumsporozoite protein and recent preliminary results from a phase III clinical trial demonstrate between 35-50 percent efficacy (Agnandji et al., 2011). Important factors that must be considered include the low level of efficacy, length of protection and potential cost of the vaccine (White, 2011, Duncan and Hill, 2011). Overall, much is unknown about the actual mechanisms of natural immunity to malaria and future work in this area may provide novel insights into eliciting a sustained immune response.

Vector control

With widespread antimalarial resistance and no vaccine on the market, currently the most effective means of controlling malaria is by way of insecticide treated bed nets. The efficacy of bed-nets is dependent on several factors including mosquito sensitivity to insecticides, high malaria incidence and rate of community participation (Gu and Novak, 2009). A recent concern is the emergence of mosquito resistance to the insecticides used to treat bed nets (Fane et al., 2011, Gamo et al., 2010). As mosquito resistance spreads, this important control method is at risk of becoming ineffective, just as antimalarials have done. With a fully protective vaccine unlikely in addition to increasing mosquito resistance to insecticides and widespread parasite resistance to drugs, a novel strategy is required to lessen the impact of this disease.

Natural host resistance

Malaria has coexisted with humans for many millennia, a recent study suggesting that the human and *Plasmodium* genomes have been evolving

together as *Homo sapiens* migrated out of Africa nearly 80,000 years ago (Tanabe et al., 2010). Given the high mortality rate it causes, malaria has had a profound impact on the human genome. A variety of mutations that protect against malarial infection and the symptoms of the disease have arisen in human populations living in malaria-endemic regions of the world (Weatherall et al., 2002). These include many polymorphisms affecting the red cell. Considering that the parasite spends a large proportion of its lifecycle in a red cell, it is not surprising that an ongoing evolutionary battle has been fought between the host and parasite genomes for thousands of years, the battleground being the red cell; the outcome ultimately deciding the result of infection for the human host and the continuing cycle of the parasite.

Haldane (1949) first recognized the importance of host genetics in malaria resistance when he postulated that the high rates of thalassemia in Mediterranean populations were due to a heterozygous advantage afforded to carriers (Haldane, 1949). According to Haldane's malaria hypothesis, the heterozygous resistance to malaria balances the homozygote hematological disadvantage. Since this initial observation, numerous other mutations that have an impact on the host resistance to malaria have been identified. These mutations affect red cell proteins, factors involved in the sequestration of parasites or the host immune response. These have often been initially suggested through coincidence of malarial endemicity (Hill et al., 1987). Hemoglobin S (HbS) is one of the best-characterised mutations influencing infection but studies suggest there are many genes yet not identified, each resulting in small population effects (Mackinnon et al., 2005). New tools including genome-wide association studies may help identify other traits and increase our understanding of protective immunity (Verra et al., 2009). With the current drug regime failing due to profound parasite resistance, can we instead use what we know about host resistance to infection to discover novel antimalarials that avoid the problem of parasite resistance?

The host immune response variants

There are several variants of immune response genes that confer resistance to malarial infection. The major histocompatibility complex (referred to as the human leukocyte antigen; HLA) and is comprised of many polymorphic genes, and subdivided into 3 main groups (Class I, II and III) (Verra et al., 2009). Several studies have previously shown that carriers of certain HLA class I and II alleles have different susceptibility to malaria (Hill et al., 1991, Hill et al., 1992). Furthermore, carriers of the Class I HLA antigen HLA-Bw53 were protected against severe malaria, an antigen that frequently occurs in sub-Saharan Africa (Hill et al., 1991, Hill et al., 1992, Gilbert et al., 1998).

Complement receptor 1 (CR1) is a red cell surface protein responsible for complement-regulatory processes and for the removal of immune complexes (Stoute, 2011, Reinagel et al., 1997). CR1 has been implicated in the process of rosetting by interacting with *PfEMP1* (Rowe et al., 1997). Polymorphic variants of CR1 confer reduced expression of CR1 on the red cell surface, a feature hypothesised to reduce the ability to rosette and therefore reduce severe malaria complications. One study by Thathy et al (2005) confirmed that low CR1 phenotype protects against cerebral malaria but not against malaria-associated anaemia (Thathy et al., 2005). These results have been contradicted by several studies showing no association between the low-expression of CR1 and reduced severe malaria (Nagayasu et al., 2001, Zimmerman et al., 2003, Sinha et al., 2009). Recent evidence has demonstrated that CR1 is a host red cell receptor required for *P. falciparum* invasion (Tham et al., 2011, Tham et al., 2010, Awandare et al., 2011).

Nitric oxide synthase 2 (NOS2) is an enzyme that produces nitric oxide, a free radical important in several immune-mediated responses (Lopez et al., 2010). It has been suggested that polymorphisms in the NOS2 promoter may increase nitric oxide production and provide the host with some protection against severe malaria (Perkins et al., 1999). The effect of NOS2 polymorphisms on severe malaria is not fully supported; with one study finding that increased nitric oxide production had a detrimental effect on disease (Kremsner et al., 1996). Levesque and colleagues also recently found

no association between polymorphisms in the NOS2 promoter and malarial severity in a cohort of Tanzanian children (Levesque et al., 2010).

Tumor necrosis factor- α (TNF- α) is a cytokine produced by monocytes, macrophages and T- and B-cells and is important for the presentation of pro-inflammatory events (Wilson et al., 1997). TNF- α has been implicated in severe malaria, including cerebral malaria, severe anaemia and lactic acidosis (Odeh, 2001). High levels of TNF- α were also shown to disrupt erythropoiesis and red cell proliferation (Tchinda et al., 2007). Several studies have identified TNF- α polymorphisms that are independently associated with cerebral malaria in infant populations in Africa (Knight et al., 1999, McGuire et al., 1994).

Haemoglobinopathies

The haemoglobinopathies are a group of inherited disorders affecting haemoglobin structure and production. They are amongst the most common monogenic diseases worldwide (Weatherall et al., 2002). This group includes haemoglobin variants and the thalassemias, which have high population frequencies correlating with the historic incidence of malaria (Hill et al., 1988). Haemoglobinopathies themselves are a worldwide health burden, with a recent minimum estimate suggesting in excess of 300,000 children each year born with a haemoglobin disorder (Weatherall, 2011).

There are hundreds of known low-prevalence Hb variants but only 3 structural variants are observed at polymorphic frequency, being Haemoglobin C (HbC), Haemoglobin E (HbE) and Haemoglobin S (HbS). The sickle cell trait (HbS) has one of the highest frequencies of these variants with carriers reaching 15-20% in some areas of sub-Saharan Africa (Weatherall et al., 2002, Hardison et al., 2002). HbS is caused by a point mutation in the haemoglobin β -chain leading to an altered red cell shape. The most common resulting amino acid change is the substitution of valine for glutamic acid in the β -globin subunit ($\beta 6\text{Glu}\rightarrow\text{Val}$) (Wood and Granger, 2007). Upon deoxygenation, this change induces polymer formation leading to the classic “sickle” shape (Lopez et al., 2010). Findings from studies suggest that while

the homozygous HbSS suffer a severe hemolytic syndrome, the heterozygous HbAS are healthy and protected from severe malaria (Williams et al., 2005a). Case control studies demonstrated heterozygous carriers were at least 70 percent protected against severe malaria (Allison, 1954, Flint et al., 1986).

HbC is another protective variant, but unlike HbS where selection for the heterozygous state has occurred, the protective advantage of HbC is greatest in homozygotes. This was demonstrated in a large study by Modiano and colleagues (2001) who showed a 90 percent protection rate of clinical malaria in homozygotes and 30 percent in the heterozygote carriers (Modiano et al., 2001). Prior to this report, there were contrasting results in the protective capability of this variant (Guinet et al., 1997, Mockenhaupt et al., 2004a, Rihet et al., 2004). Unlike the severe condition of the HbS variant, individuals homozygous for HbC are characterized by mild haematological symptoms (Agarwal et al., 2000). The mechanism for the protective effect of both HbS and HbC is not fully elucidated with several hypotheses postulated. Parasite entry into the red cell may be impaired by the altered exterior membrane structure of the red cell and additionally, low oxygen conditions and the altered red cell structure may impair subsequent parasite growth (Fairhurst et al., 2005, Cooke et al., 2001, Williams, 2006). Additionally, HbC heterozygotes and homozygotes have been shown to display an abnormal *PfEMP1*, resulting in reduced cytoadhesion and impaired rosetting *in vitro* (Cholera et al., 2008). This mechanism would hinder the pathogenesis of disease and may prevent the onset of severe disease exacerbated by due to the sequestration of parasites in the microvasculature.

HbE is the third haemoglobin variant thought to afford host protection against malaria. It is most widely distributed in South East Asia, with Laos, Cambodia and Thailand recording the highest carrier frequency of 55 percent (Flatz, 1967). Homozygotes have less efficient production of haemoglobin, resulting in red cells with a lower haemoglobin concentration and are microcytic (low mean corpuscular volume) (Bunyaratvej et al., 1992a). It has also been observed *in vitro* that HbE red cells show reduced plasticity and deformability (Bunyaratvej et al., 1992b). Clinical presentation of

homozygotes is similar to β -thalassemia, while heterozygous carriers are asymptomatic. A case-control study in Thailand suggested that the HbE variant affords protection only against severe malaria, due to reduced invasion efficiency at high parasitaemias (Hutagalung et al., 1999, Chotivanich et al., 2002).

Thalassemias are caused by mutations in the promoters of either the α or β globin genes, causing α -thalassemia or β -thalassemia respectively (Williams, 2006). A mutation in either one of the globin genes results in the reduced expression and formation of abnormal haemoglobin molecules (Mockenhaupt et al., 2004b), of which there are over 180 known mutations (Lopez et al., 2010). The population frequencies of both of these conditions are unexpectedly high in countries where malaria is (or previously was) endemic, including the southwest Pacific, sub Saharan Africa and the Mediterranean (Flint et al., 1986). Individuals homozygous for α^+ thalassemia suffer from anaemia with hyperchromic red cells while heterozygotes are clinically normal (Fowkes et al., 2008). α^0 thalassemia occurs when both linked alpha globin genes are lost and is lethal in the homozygous state (Hill et al., 1987). Evidence for a protective role comes from α^+ thalassemia but the mechanism of protection is unclear, with no evidence of reduced rate of invasion or parasite growth (Flint et al., 1986). An immune-mediated protection has instead been postulated, with α^+ thalassemic iRBCs binding more antibody from immune plasma compared to control iRBCs (Luzzi et al., 1991, Williams et al., 2002). Alternatively, the increased red cell number and decreased red cell size seen in homozygotes may offset anemia during severe disease (Fowkes et al., 2008). The protective effect of α^+ thalassaemia has only repeatedly been observed in severe malarial anemia, while the observed effect on mild malaria has not been consistent between studies (Williams et al., 2005b, Mockenhaupt et al., 2004b, Wambua et al., 2006, Oppenheimer et al., 1987, Allen et al., 1997). The reproducibility of these studies may be affected by the finding of a negative epistatic effect between HbAS and α^+ thalassaemia. When these two conditions are inherited together, the protective effect of either trait is lost (Williams et al., 2005a).

β -thalassemia is usually caused by mutations affecting the promoter region of HbB, which causes a premature termination or a change in the correct splicing (Hill et al., 1988). This leads to a decreased synthesis of β -globin and a subsequent excess of α -globin, which promotes oxidative membrane destruction and mechanical damage to precursors (Weatherall, 2000). Individuals heterozygous for the mutation display mild anemia and a lower erythropoiesis due to red cell morphological changes, while homozygous individuals display severe anemia and regular blood transfusions are required (Weatherall, 1997, Galanello and Origa, 2010). A variety of resistance mechanisms for malarial protection have been suggested: that fetal haemoglobin is sustained for longer in newborn carriers (Pasvol et al., 1976); a higher phagocytosis rate in β -thalassaemic iRBCs (Ayi et al., 2004); and that antibodies bind at a higher rate to β -thalassaemic red cells compared to normal red cells (Smith et al., 2002).

Glucose-6-phosphate dehydrogenase deficiency

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is an X-linked disease, which affects over 400 million people worldwide and is mainly found in tropical regions with the highest frequencies observed in Africa, Asia, Papua New Guinea and the Mediterranean (Ruwende et al., 1995). Glucose-6-phosphate dehydrogenase catalyses the first step of the pentose phosphate pathway, providing NADPH, which is required to protect red cells from oxidative damage (Clarke et al., 2001, Frank, 2005). A G6PD deficient red cell is more susceptible to oxidative stress, as NADPH is not being produced in sufficient quantity to fully protect the cell. Although most affected individuals are asymptomatic, the clinical manifestations can include episodes of red cell hemolysis, which is associated with exposure to certain drugs (such as primaquine), chemicals (such as naphthalene) or other redox compounds (Piomelli, 1981). In the literature, there is conflicting *in vitro* evidence of the protective mechanism of G6PD deficiency, with invasion thought to be normal but the growth of parasites in G6PD deficient red cells being impaired and a higher rate of phagocytosis (Roth et al., 1983, Cappadoro et al., 1998). Upon *P. falciparum* invasion, there is a higher level of oxidative stress in

G6PD deficient red cells, leading to the accumulation of toxic oxidised substances in the host red cell that impairs parasite development (Wajcman and Galacteros, 2004, Beutler, 1996). It is also hypothesized that due to parasitic infection, the extra oxidative stress put on the host red cells can also lead to the premature lysis of infected G6PD deficient red cells (Ruwende and Hill, 1998). A third proposal is that parasitaemia is limited in G6PD deficient red cells due to the earlier onset of phagocytosis (Gallo et al., 2009).

Pyruvate kinase deficiency

Pyruvate kinase (PK) is an enzyme in the glycolysis pathway, a pathway required in the red cell to maintain cellular energy (Chan and Sim, 2005). A deficiency in this enzyme causes hereditary non-spherocytic hemolytic anemia, which has a worldwide distribution pattern (Wang et al., 2001). PK deficiency was initially suggested to confer host resistance to malaria from mouse studies. Min Oo and colleagues (2003) showed that mice homozygous for a PK deficiency were resistant to *P. chabaudi*, a rodent model of infection (Min-Oo et al., 2003). This prompted suggestions that a similar protective effect might be seen in human populations and has been confirmed by *in vitro* studies with PK deficient human blood (Durand and Coetzer, 2008). Further work by Ayi and colleagues (2008) suggested that blood from homozygous patients was resistant to *P. falciparum* invasion (Ayi et al., 2008). This supported previous work demonstrating PK deficient red cells have altered membrane rigidity impairing parasite invasion (Min-Oo et al., 2003). Furthermore Ayi and colleagues (2008) suggested that infected red cells at the ring stage were preferentially cleared by macrophages (Ayi et al., 2008).

Duffy antigen

The Duffy antigen (also known as DARC; Duffy antigen receptor for chemokines) is normally expressed on the surface of red cells and functions as a chemokine receptor (Mallinson et al., 1995). The receptor is also used by *P. vivax* to obtain entry into the host red cell (Singh et al., 2006). Certain populations, especially those in West Africa, do not express the Duffy antigen on their red cells due to a mutation in the promoter preventing erythrocytic expression (Tournamille et al., 1995). People who are Duffy negative are

resistant to infection by *P. vivax* (Miller et al., 1976), which probably explains the absence of *P. vivax* in West Africa today. A recent study in Madagascar identified Duffy negative individuals infected with *P. vivax* (Menard et al., 2010). This is the first report of *Plasmodium* adaptation to host genetic variants but may be influenced by the unique Madagascan population that contains many diverse ethnic groups, as well as the high rate of co-infection with *P. falciparum*.

Ovalocytosis

Ovalocytosis is a disease caused by a 27 base-pair deletion in the gene encoding band 3, a red cell membrane protein (Liu et al., 1990, Jarolim et al., 1991). The band 3 variant is endemic throughout Malaysia, Papua New Guinea and the Philippines, sometimes referred to as Melanesian or south-east Asian ovalocytosis (Liu et al., 1990). It is a homozygous embryonic lethal disease, while heterozygotes are asymptomatic but have red cells with a distinctive elliptical shape (Amato and Booth, 1977). Carriers are susceptible to infection by the parasite but the mutation appears to offer strong protection against the development of cerebral malaria (Allen et al., 1999). Individuals with ovalocytosis may be protected from cerebral malaria due to an altered interaction between the infected red cell and the vascular endothelium (Genton et al., 1995).

Sequestration variants

The sequestration of infected blood cells is also a major cause of malarial pathogenesis. Several parasite antigens (*PfEMP1*) are known to bind and sequester to a number of host endothelial surface receptors such as ICAM-1, CD36 and CD31 (Baruch et al., 1996, Janes et al., 2011). Polymorphisms of these host receptors is thought to impact on severity of malarial infection, with several studies showing associations between decreased expression of host receptors and protection from malaria (Kun et al., 1999, Bellamy et al., 1998, Amodu et al., 2005).

Red cell polymorphisms are still effective as “natural antimalarials”

Some of these red cell polymorphisms, such as sickle cell, were discovered in the 1840s but are thought to have been present in human populations for thousands of years (Lell et al., 1999). Thus, although the parasite has had ample time to change and overcome these protective mechanisms, this has not happened (excluding the possible recent exception of Duffy-negative individuals being infected with *P. vivax* on Madagascar (Menard et al., 2010)) and contrasts with the resistance that has rapidly developed to parasite-directed chemotherapy. Consequently populations with high frequencies of these red cell polymorphisms are able to avoid many of the serious complications associated with malarial infection. Therefore the host red cell variations can be regarded as “resistance-proof natural antimalarials” (Foote, 2004).

Host-directed therapy, a novel antimalarial strategy based on genetic resistance to malaria

Evidence from the above described host genetics implies that red cell variants are an effective means of controlling malarial infection. Unlike current antimalarials that are parasite-directed, targeting host proteins could be a way of escaping parasite resistance that has hampered the current range of antimalarials. This thesis focuses on investigating and validating a novel host-directed therapy (HDT), targeting host proteins in the mature red cell that the parasite relies on for survival. A HDT is based on the evidence that red cell polymorphisms have a detrimental effect on invasion, growth or egress of the parasite.

HDT: a way forward for infectious disease treatment?

A host-directed therapy is a new and emerging field for treating not only malaria but also other infectious diseases caused by bacteria, viruses and other parasites. The role of the host in pathogenic infection has long been

investigated; in the late 1870s Louis Pasteur recognized the importance of the host as a “culture vessel” (Brown et al., 2008). Furthermore, Garber (1960) described the host’s role as a growth medium, alluding that this may be exploited for therapeutic advantage (Garber, 1960). As our knowledge of host-pathogen interactions increases, identifying novel host factors required by pathogenic organisms has become a feasible approach to novel treatment avenues. Several examples below demonstrate the benefit of targeting host molecules needed by the pathogen for growth.

Human immunodeficiency virus (HIV) is an example of a virus where therapies that previously targeted the causative agent have begun to fail. There has been a rapid emergence of resistance to available therapies due to the high viral mutation rate (Pinggen et al., 2011). HIV drugs have classically targeted the viral replication machinery and proteases used for viral production in the host cell (Lewin et al., 2011). Along with poxvirus, HIV uses host chemokine receptors to enter the host cell, namely CCR5 and CXCR4 (Oppermann, 2004, Lalani et al., 1999). One of the most recent drugs Maraviroc (Trade names Selzentry/Celsentri, FDA approved in 2007) makes use of this phenomenon and targets CCR5 on the host cell membrane. CCR5 inhibition prevents the interaction with the viral protein gp120, necessary for viral entry (Ferain et al., 2011). A downside to this therapy is Maraviroc is only effective against strains that use the CCR5 receptor for host cell entry (Lederman et al., 2006). Other host chemokine receptor inhibitors are being developed and are at various stages through pre-clinical and clinical trials.

Large-scale screens using short interfering RNA (siRNA) libraries have been used to great effect to identify other host factors needed by viruses. One such study identified over 250 host factors required by HIV-1 (Brass et al., 2008), the challenge being to identify inhibitors of these novel targets. This technique has also been used to identify several novel host factors needed in bacterial infections. *Chlamydia caviae* infection in mammalian cells was reduced when the host Tim-tom multiprotein complex was knocked down. This complex is used by the bacterium to recognize and import nuclear-encoded proteins into the mitochondria (Derre et al., 2007). Additionally,

several host kinases have been identified that support the growth of *Salmonella* and *Mycobacterium* (Kuijl et al., 2007). These host kinases could provide novel targets for future drug targeting.

Celgosivir is another host-directed drug currently in phase II clinical trials, this time for Hepatitis C infection. Celgosivir inhibits host endoplasmic reticulum glycosidase, an enzyme important for the folding of the virus envelope proteins. Inhibition of this enzyme results in damaged viral assembly and release (Durantel et al., 2007).

The host nutrient status has previously been investigated as a potential line of therapeutic targeting. Host cholesterol is needed by certain pathogens to establish infection (Riethmuller et al., 2006, Lafont and van der Goot, 2005). Drugs that reduce cholesterol such as statins have shown therapeutic promise against HIV infections by reducing viral load (del Real et al., 2004). Other pathogens thought to rely on host cholesterol include *Leishmania spp.* and *Staphylococcus aureus*, using cholesterol for binding or activity of virulence factors (Pucadyil and Chattopadhyay, 2007).

Another host factor influenced by nutrition is iron, which is thought to play an important role in the onset of infection as many pathogens utilize host iron for growth (Weinberg, 1974). Animal studies have shown iron chelator treatment decreases the growth of *M. tuberculosis* (Gordeuk et al., 1996, Gangaidzo et al., 2001). Iron chelator therapy has also shown some success in human malarial infection (Mabeza et al., 1999).

Finally, along with the increase in knowledge of host-pathogen interactions, the technological advances in target selection from genomic and proteomic techniques may lead the way for the rapid identification of further host targets. The examples described in this thesis demonstrate that host-directed therapies are emerging as a novel avenue for therapeutic intervention in the fight against pathogenic infection. Next, this review focuses on the literature surrounding host factors required for malarial infection.

Potential host-directed antimalarial targets

Numerous host factors have been identified that *Plasmodium* either scavenges or requires to invade red cells, replicate or egress. As previously discussed, therapeutic targeting of these factors could be an important new class of antimalarials. Several examples are illustrated below.

Host proteins necessary for parasite invasion

Parasite invasion of the red cell is vital for the intraerythrocytic infection to begin. Several host proteins are required for this process, including the Duffy antigen receptor for *P. vivax* invasion, previously described in this review. Murphy and colleagues used an erythrocyte guanine nucleotide regulatory protein Gs antagonist to show that this protein was necessary for parasite invasion into red cell ghosts (Murphy et al., 2006). Additionally, Bei and colleagues used shRNA to knock down gene expression of glycophorin A in red cells isolated from haemopoietic stem cells and subsequently terminally differentiated into mature red cells *in vitro* (Bei et al., 2010). This knock-down showed that glycophorin A was required for strain-specific invasion. Most recently basigin (the Ok blood group antigen) was shown to be essential for *P. falciparum* invasion, through both knock-down and inhibition of the host protein (Crosnier et al., 2011). If novel drugs can target host proteins that the parasite requires for invasion, the ensuing parasitaemia levels may be decreased and severe malaria may be prevented.

Host proteins in parasite egress

An important step in the intraerythrocytic lifecycle of the parasite is when multiple new merozoites must escape the encompassing parasitophorous vacuole and red cell to enter into the blood stream. The process of parasitophorous vacuole and red cell rupture is referred to as egress. Without efficient egress, new merozoites are unable to continue the asexual lifecycle of the parasite. Calpain-1 has been demonstrated as an essential host factor for parasite egress, as *Plasmodium* was unable to egress from calpain-1 immunodepleted red cells, instead remaining arrested in the schizont stage (Chandramohanadas et al., 2009).

Host protein kinases

Host kinases have been a recent area of interest, with several studies investigating their efficacy as novel drug targets (Doerig et al., 2009). One study used the power of a large-scale RNAi screen of the 727 known kinase-related genes to identify five host kinases influencing the liver stage of *P. berghei* infection. When the expression of any of these five host kinases was knocked down, the level of infection was severely reduced (Prudencio et al., 2008). Recently host protein kinases have been implicated in malarial infection with the finding that host red cell protein kinases PAK-1 and MEK-1 are upregulated in iRBCs and may be used in the parasite's protein kinase-mediated signaling pathway (Sicard et al., 2011).

Red cell eryptosis

Another suggestion for new therapeutic targeting is to increase the rate of suicide death of iRBCs (also called eryptosis). Upon parasite invasion a cascade of events is induced that leads to high levels of oxidative stress on that cell, eventually causing it to undergo eryptosis (Foller et al., 2008). These cells display phosphatidylserine residues on the surface that act as flags for macrophages to engulf and destroy the dying cell. Foller and colleagues (2009) suggest using this strategy to therapeutically induce eryptosis in an effort to decrease parasitaemia and have a positive impact of the clinical course of disease (Foller et al., 2009). This idea builds on the observation that red cells with a haemoglobinopathy or that are deficient in G6PD are prone to early eryptosis once infected (Lang et al., 2002, Ayi et al., 2004).

Host nutrients

Another avenue for potential host targets is that of the nutrients and cofactors the parasite relies on scavenging from the host. The importation of host nutrients by the parasite is well established, initially from studies revealing that malarial parasites endocytose and digest host haemoglobin (Sherman, 1977). The parasite makes use of the abundance of host haemoglobin (Hb) in the red cell, digesting it for amino acids prior to storage in the digestive vacuole as hemozoin (Esposito et al., 2008). Channels such as the new permeation pathway (NPP) are induced in the red cell membrane

12-15 h after invasion, allowing infected red cells to more readily take up anions (Kirk et al., 1994), sugars (Ginsburg et al., 1985), purines (Upston and Gero, 1995), organic cations (Staines et al., 2000) and pantothenate (the precursor of coenzyme A) (Saliba et al., 1998b) from the host serum. Methionine (an amino acid missing from adult haemoglobin) and isoleucine also enter the iRBC through the NPP (Cobbold et al., 2011, Martin and Kirk, 2007) and are required for parasite growth. It has also been suggested that *P. falciparum* scavenges fatty acids from the host red cell that are used in numerous parasite processes including phospholipid and triacylglycerol synthesis (Vial et al., 1982).

Host enzymes

One of the first studies to identify the acquisition of host red cell enzymes by the parasite was by Fairfield and Meshnick (1983) who found that the parasite adopts host superoxide dismutase (Fairfield et al., 1983). This enzyme is vital to the oxidant defense system of cells, particularly important for the parasite in the lysis prone red cell (Fairfield et al., 1986). SOD activity in isolated *P. berghei* parasites was identified as being of host origin using enzyme activity assays, isoelectric focusing and polyacrylamide gel electrophoresis examining host and parasite enzymes. They surmised that the parasite lacked its own SOD therefore requiring the import of the host enzyme as part of its antioxidant defense (Fairfield et al., 1983). Another more recent study has shown that the human redox protein peroxiredoxin-2 is scavenged and colocalised to the parasite cytosol and Maurer's clefts. The hijacked host protein was also found to have a functional role in the redox system of the parasite (Koncarevic et al., 2009). Enzymes of the haem biosynthetic pathway have also been of considerable interest as it has been suggested several host haem enzymes are scavenged by the parasite and used to fulfill its own haem synthetic requirements, namely aminolevulinate dehydratase (ALAD) and ferrochelatase (FECH) (Bonday et al., 2000, Bonday et al., 1997, Varadharajan et al., 2004). These enzymes are of interest to this thesis and are further discussed below.

The examples above display the wide-variety of potential novel host targets but it barely scrapes the surface of the many host proteins or transporters that may be utilized for a host-directed antimalarial therapeutic strategy. Proteomic studies have identified over 751 proteins in mature red cells, many of which may be scavenged or required by the parasite (Alvarez-Llamas et al., 2009, Goodman et al., 2007, Kakhniashvili et al., 2004, Pasini et al., 2006, Zhang et al., 2008). Using the powerful and vast tools of the “-omics” generation, high throughput chemical screening libraries and traditional genetic techniques, the time is ripe to pursue a host-directed therapy as the antimalarial therapeutic strategy of the future.

Identification of host targets using a bioinformatics approach

Work done in my honours year examined potential host targets using microarray analysis on red cell precursors and bioinformatics methods to investigate parasite and host pathways. Firstly, host proteins present in the mature red cell were identified using microarray data from precursor red cells (unpublished data) as well as the red blood cell proteome database (Pasini et al., 2006). Parasite genome and pathway data was then analysed to investigate pathways that were vital to the parasite and corresponding protein homologs that may be absent in the parasite. It was hypothesised that the parasite may scavenge the host version to fulfill its own biochemical pathways. Several potential targets were identified including Uroporphyrinogen-III synthase (UROS), an enzyme in the haem biosynthetic pathway (Smith, 2007). The parasite has a complete repertoire of genes coding for all of the enzymes in this pathway, with the exception of UROS (Gardner et al., 2002). As the parasite lacks a gene coding for UROS, it was hypothesised that the host version may be imported by the parasite to supplement its UROS requirements for haem synthesis. Additionally, previous data suggests that other haem biosynthetic enzymes, ALAD and FECH, may be scavenged by the parasite (Bonday et al., 2000, Bonday et al., 1997, Varadharajan et al., 2004). Considering the numerous potential targets in this pathway, enzymes in the haem biosynthetic pathway have

subsequently been the focus of my current investigation into host-directed therapeutics.

Haem synthesis in red cells

Haem is required by all organisms as a prosthetic group or cofactor for a variety of proteins including haemoglobin (Hb), myoglobin, cytochrome C, cytochrome p450, catalase, peroxidase and B12 (Heinemann et al., 2008, Layer et al., 2010). Haem synthesis occurs in high levels in liver and progenitor red cells (Ponka, 1999). The haem biosynthetic pathway is comprised of eight enzymes (Figure 1.2) and in red cells, this pathway occurs in the cytosol and mitochondria (Sassa, 1990). The terminal enzyme, ferrochelatase, is mitochondrially located, and hence the mitochondria are the ultimate site of haem formation (Wu et al., 2001, Chen et al., 2010).

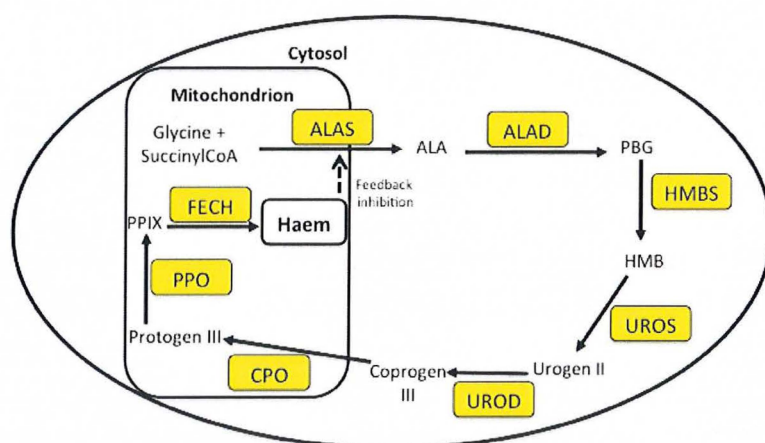


Figure 1.2 The mammalian haem biosynthetic pathway. In black are substrates/products and in yellow boxes are the haem synthetic enzymes. ALAS, aminolevulinate synthase; ALA, aminolevulinate; ALAD, aminolevulinate dehydratase; PBG, porphobilinogen; HMBS, hydroxymethylbilane synthase; HMB, hydroxymethylbilane; UROS, uroporphyrinogen III synthase; Urogen III, uroporphyrinogen III; UROD, uroporphyrinogen decarboxylase; Coprogen III, coproporphyrinogen III; CPO, coproporphyrinogen oxidase; Protophyrin III, protoporphyrinogen III; PPO, protoporphyrinogen oxidase; PPIX, protoporphyrin IX; FECH, ferrochelatase

As progenitor red cells undergo maturation, haem supplies the prosthetic group for Hb assembly (Thorell, 1947). At the reticulocyte stage of red cell development, organelles and cell components are eliminated from the cell via exosomes (Carayon et al., 2011). This process completes the final transition into a mature circulating red cell. The final mature red cell form is devoid of a nucleus and organelles and therefore unable to transcribe and translate new proteins. The mature red cell is therefore also unable to synthesise haem. The red cell cytosol is comprised mostly of haemoglobin (98%) but a variety of proteomic studies have revealed the presence of over 700 other proteins (Alvarez-Llamas et al., 2009, Goodman et al., 2007, Kakhniashvili et al., 2004, Pasini et al., 2006, Zhang et al., 2008). The three haem synthetic enzymes, ALAD, FECH and UROS, currently under investigation in this thesis were identified in one of these studies (Pasini et al., 2006). A range of technical difficulties, including sample preparation and the detection limit, may have precluded their identification in the other studies. A question therefore remains if the haem biosynthetic enzymes are present in mature red cells, which are no longer undergoing haem synthesis.

***Plasmodium* haem synthesis**

The parasite also has an active haem biosynthetic pathway that is compartmentalised between the mitochondria, apicoplast and cytosol (Figure 1.3) (van Dooren et al., 2006, Sato et al., 2004). *Plasmodium* haem synthesis begins in the mitochondrion with the first enzyme of the pathway, aminolevulinate synthase (ALAS) localised to this organelle (Varadharajan et al., 2002). The second enzyme, ALAD is targeted to the apicoplast (Dhanasekaran et al., 2004, Sato and Wilson, 2002). Transporters have not been identified that take the substrate aminolevulinic acid from the mitochondrion, across the cytosol and into the apicoplast, a process that must traverse several membranes. The next enzyme, hydroxymethylbilane synthase (HMBS) is also targeted to the apicoplast (Sato et al., 2004).

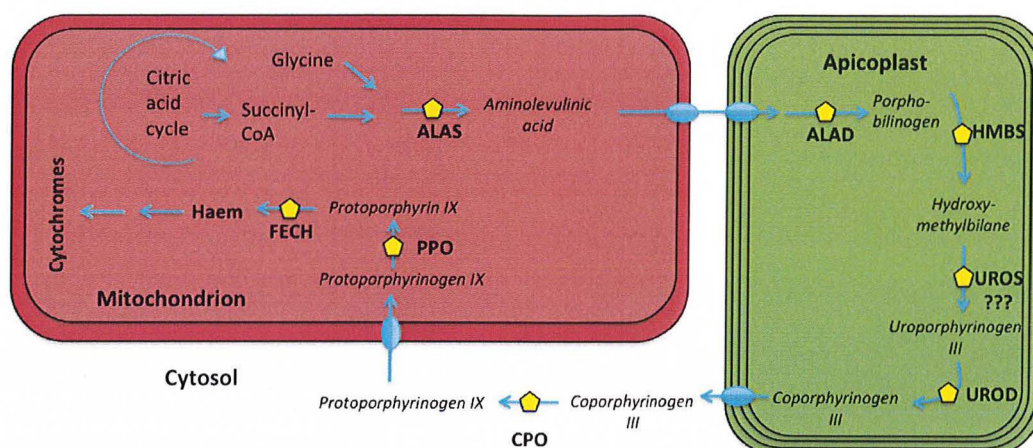


Figure 1.3 The hybrid haem biosynthetic pathway of *Plasmodium*. In bold black next to yellow boxes are the haem biosynthetic enzymes, which are compartmentalized between the cytosol, mitochondrion and apicoplast in *Plasmodium*. ALAS, aminolevulinic synthase; ALAD, aminolevulinic dehydratase; HMBS, hydroxymethylbilane synthase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen decarboxylase; CPO, coporphyrinogen oxidase; PPO, protoporphyrinogen oxidase; FECH, ferrochelatase

Hypothetically the following enzyme uroporphyrinogen-III synthase (UROS) would also be targeted to the apicoplast, after HMBS but there is question over the presence of UROS, with no parasite-encoded genes having been annotated as UROS (Gardner et al., 2002). An orthologue of UROS has been identified in the genome of *Toxoplasma gondii* and is localised to the apicoplast (Ralph et al., 2004). The following enzyme in the pathway, uroporphyrinogen III decarboxylase (UROD) has been shown using bioinformatics to contain an apicoplast leader sequence, which was confirmed by immunohistochemistry studies (Nagaraj et al., 2009a). Interestingly, the pathway seems to continue into the cytosol with the next enzyme coporphyrinogen III oxidase (CPO) being identified in this location using western blotting, immunohistochemistry and enzyme assay techniques (Nagaraj et al., 2010b). The next haem synthetic pathway enzyme,

protophyrinogen IX oxidase (PPO), was cloned, expressed and localised to the mitochondrion using immunohistochemistry techniques (Nagaraj et al., 2010a). These findings indicate the presence of further transporters involved in the shuttling of intermediates between the apicoplast, cytosol and mitochondria. The final enzyme in the pathway, ferrochelatase (FECH) has been surrounded by controversy regarding its location. Initially a study by Varadharajan and colleagues (2004) demonstrated an apicoplast location (Varadharajan et al., 2004) but evidence by Ralph and colleagues (2004) suggested a mitochondrial location (Ralph et al., 2004). Using the *Plasmodium*-trained mitochondrial transit peptide predictor tool PlasMit, FECH was shown to contain a short N-terminal extension that may function as a mitochondrial transit peptide. Recent work by Bonday and colleagues confirmed the mitochondrial location of FECH using immunohistochemical techniques (Nagaraj et al., 2009b). Their prior mistake on its location was suggested to be due to an alternate promoter sequence used in their initial cloning experiments. A mitochondrial location of FECH makes sense as FECH is mitochondrially located in all other organisms and is therefore the ultimate site of haem production. It is unknown how the parasite coordinates this hybrid pathway, including the shuttling of enzymes or intermediates between the mitochondria, cytosol and apicoplast.

A role for host haem enzymes in the *Plasmodium* haem synthetic pathway

Interestingly, even though the parasite contains genes encoding ALAD and FECH, there is evidence that the parasite scavenges the host versions of these enzymes. Dhanasekaran and colleagues showed over 90% of the total parasite ALAD activity is derived from a host-encoded enzyme (Dhanasekaran et al., 2004). Similarly, over 80% of total parasite FECH activity is that of host origin (Varadharajan et al., 2004). The role of host haem enzymes in malarial infection has been unclear, considering the parasite contains genes encoding active ALAD and FECH enzymes (Sato and Wilson, 2002, Sato and Wilson, 2003). It has also been suggested that the

parasite contains a specific transporter for co-opting the host enzymes, as opposed to passive consumption when Hb is imported and proteolysed in the digestive vacuole (Bonday et al., 2000). It is unsure if the host enzymes are utilised in the cytosol or transported into the organelles of the parasite to supplement haem synthesis. Overall, the importation of the host haem enzymes ALAD and FECH has not been independently investigated. Furthermore, it is not known if the parasite requires the host enzymes for normal growth. These questions will be investigated in this study.

A potential role for host UROS in *Plasmodium* haem synthesis?

The absence of a *Plasmodium*-encoded UROS remains a question in parasite biology. UROS plays a vital role in the haem synthetic pathway, responsible for the inversion and closing of the linear hydroxymethylbilane into the uroporphyrinogen III porphyrin ring (Tsai et al., 1988). Without UROS, the substrate is unstable and non-enzymatically converts into the physiologically unusable uroporphyrinogen I isomer (Freeseemann et al., 1998). With a parasite-encoded UROS yet to be identified, Nagaraj and colleagues (2008) provided evidence that the preceding haem biosynthetic enzyme, hydroxymethylbilane synthase (HMBS), acts as a dual function enzyme (Nagaraj et al., 2008). *P. falciparum* HMBS (*Pf*HMBS) was cloned in a bacterial expression system and found to have limited UROS activity (Nagaraj et al., 2008). However, it was noted that purified red cell UROS was catalytically more efficient than the *Pf*HMBS activity. Considering the previously discussed evidence that the parasite scavenges other host haem synthetic enzymes and that the host enzyme is more efficient than the dual-function *Pf*HMBS, it is possible that host UROS is also scavenged by the parasite. This is the case in other parasites such as *Trypanosoma* and *Leishmania*, which rely on scavenging haem enzymes due to the partial loss of their own pathways (Toh et al., 2010). This thesis proposes that *Plasmodium* does not require its own version of UROS with the availability of the host enzyme, which is scavenged like other haem enzymes.

Host directed therapy: a resistance proof therapeutic strategy?

If the parasite relies on UROS, ALAD and FECH for essential metabolic functions, inhibitors of these host enzymes may be useful antimalarial compounds. Drugs against these host haem synthetic enzymes may be an important new class of antimalarial. This therapeutic strategy has been coined a “host directed therapy” (HDT). A HDT could be a potentially resistance-proof antimalarial strategy, as compounds targeting host enzymes would be acting outside the cellular milieu and genetic control of the parasite.

An irreversible inhibitor of one of these targets could last the life of the mature red cell, about 120 days, thus creating a unique therapeutic window that may be useful in prophylactic as well as chemotherapeutic treatment. The red cell is the target cell of a HDT but potential side effects may be limited considering that other cells that rely on haem synthesis (such as hepatocytes) can easily overcome inhibition due to their transcriptional ability.

Overall, current parasite-directed antimalarials are losing the battle with resistance. Host genetics reveals that changes to the red cell disrupt parasite invasion or growth and may give us clues to a novel therapeutic strategy to overcome parasite resistance. Work in this thesis investigates the haem synthetic enzymes ALAD, FECH and UROS as targets for a novel host-directed antimalarial therapy.

Thesis aims

All of the current chemotherapeutic antimalarial research is focused on identifying processes and targets unique to the parasite. The failure of this strategy is evidenced by both the field and *in vitro* data, which tells a story of parasite resistance to all current antimalarials. This thesis instead investigates a novel host-directed therapy, targeting host enzymes required by the parasite for growth and survival. The host targets are outside of both the cellular milieu and genetic control of the parasite, making it difficult for the parasite to develop resistance.

Plasmodium requires haem for growth and several studies suggest that the parasite scavenges two host haem synthetic enzymes, ALAD and FECH. UROS, a third enzyme from this pathway is seemingly absent in the parasite. It is therefore hypothesised that *Plasmodium* also utilises the host enzyme to provide UROS activity for haem synthesis during intraerythrocytic growth.

This thesis focused on investigating these three enzymes as potential targets for a host directed therapy using a variety of techniques including western blot and immunofluorescence; *in vitro* and *ex vivo Plasmodium falciparum* growth inhibition assays with specific inhibitors; knock-out mouse models; rodent studies using *Plasmodium chabaudi* and human studies with porphyria patient blood (haem enzyme deficiencies).

Aim 1. To investigate the Plasmodium location of host haem enzymes.

Previous evidence suggests host ALAD and FECH are scavenged by the parasite but this has not been independently investigated. The potential importation of host UROS has not previously been investigated. Here, western blot and immunofluorescence techniques were employed to verify whether the host proteins are located in *Plasmodium* during intraerythrocytic growth.

An additional aim of this chapter was to further investigate the potential UROS activity provided by *PfHMBS*. Here, cloning and protein purification techniques were employed to investigate if *PfHMBS* can fulfill the enzymatic function of UROS.

Aim 2. Assess Plasmodium growth capacity in haem enzyme deficient red blood cells

The requirement of host haem enzymes for parasite growth was investigated in this chapter through assessment of *Plasmodium* growth kinetics in human and mouse models of haem enzyme deficiency. The porphryias are a group of diseases caused by mutation(s) in the genes encoding haem enzymes, resulting in loss of its activity below a critical threshold. For this study, blood samples were obtained from patients with FECH (Erythropoietic protoporphyria) and UROS (Congenital Erythropoietic porphyria “Gunther’s disease) deficiencies. *P. falciparum* growth was quantified in red cells deficient in UROS and FECH using an *in vitro* parasite culture system. Additionally, mice deficient in FECH (*Fech*^{m1Pas}) were infected with the murine malaria parasite, *P. chabaudi*, to investigate the requirement of FECH during an *in vivo* malaria infection.

Aim 3. Target host haem enzymes with chemical inhibitors as a novel antimalarial therapy

The host haem synthetic enzymes ALAD and FECH were targeted with chemical inhibitors to determine their potential anti-plasmodial activity. Inhibitors were tested in an *in vitro* *P. falciparum* growth inhibition assay. Additionally, *in vivo* antimalarial effects were investigated in a murine model of malaria, *P. chabaudi*. One inhibitor that demonstrated *in vitro* effects is also FDA-approved for human use. *In vitro* *Plasmodium* growth was then examined in red cells collected from individuals taking pharmacologic doses of this compound.

Chapter 2

Materials and methods

2.1 Ethics

Ethics approval for animal experiments was received from the University of Tasmania Animal Ethics Committee (A0001049). Ethics approval for the griseofulvin treatment and human blood studies was received from the Human Research Ethics Committee Networks of Tasmania (H0011444) and Melbourne Health (2011.013).

This work was supported in part by a GlaxoSmithKline Australia Postgraduate support grant. This grant was competitively obtained for work in this thesis involving validation of a high-throughput UROS assay for identifying inhibitors. Synthesis of UROS inhibitors by collaborators in the UTAS School of Chemistry was ultimately unsuccessful. The postgraduate support grant obtained for the UROS work was completely unrelated and independent of other work in this thesis, which identified griseofulvin as a ferrochelatase inhibitor. Griseofulvin is made by several companies (one of which is GlaxoSmithKline) but is a drug that is off-patent and is therefore not a conflict of interest.

2.2 Cloning, of human UROS and HMBS and *Plasmodium falciparum*

HMBS

2.2.1 PCR of cDNA and gel electrophoresis of PCR products

Human *Uros* and *Hmbs* genes were amplified from cDNA derived from human PC-1 cells (A gift from Alison West). *P. falciparum Hmbs* was amplified from purified *P. falciparum* DNA (Table 2.1 for primers; all primers from Geneworks, South Australia, Australia). PCR products were analysed for correct product size using agarose gel electrophoresis, followed by purification using the Promega PCR clean-up kit (Promega, Wisconsin, USA).

2.2.2 Ligation of PCR products into pGEM-T Easy Vector system

PCR fragments were ligated into the pGEM-T easy vector system (Promega) overnight at 4 °C, as per manufacturers instructions. A standard reaction contained 5 µl of rapid ligation buffer, 1 µl pGEM-T easy vector (50 ng), 1 µl T4 DNA ligase (3 Weiss units/µl) and 1-3 µl PCR product (to give a molar ratio of PCR product: vector of 3:1). The reaction volume was increased to 10 µl with dH₂O. A positive control was control insert DNA, instead of PCR product; background negative control was water instead of PCR product added to the reaction.

2.2.3 Transformation using the pGEM-T easy vector ligation reactions

2 µl of each ligation reaction was added to 50 µl of competent *E. coli* JM109 cells (Promega) before being gently resuspended and placed on ice for 20 min. The cells were heat shocked for 45 sec in a water bath at 42 °C before being returned to ice for 2 min. 950 µl room temp LB medium was added to the tubes containing cells transformed with ligation reactions. Cells were incubated at 37 °C for 1.5 h with shaking at 150 rpm. 100 µl of each transformation culture was plated onto duplicate LB plates with ampicillin (1 µg/ml), IPTG (0.1 M) and X-GAL (50 mg/ml). Plates were incubated ON at 37 °C.

2.2.4 Colony selection and analysis for insert by PCR

Upon growth of colonies, at least 8 white colonies (containing the insert) were picked with a pipette tip, dipped into 10 µl dH₂O before being added to 2 ml LB both with ampicillin (1 µg/ml). Media with tips were incubated ON at 37 °C with shaking and added 1:1 to 80 % glycerol before being placed at -80 °C, as a backup of selected colonies. The water inoculated with a selected colony was subjected to PCR using the T7 and SP6 primers (Table 2.1) to determine if the correct sized insert was present. The PCR reaction consisted of 10 µl GoTaq Green (Promega), 1 µl T7 (10 µM), 1 µl SP6 (10 µM), 8 µl colony inoculated dH₂O. PCR conditions consisted of 95 °C denaturation 5 min; 95 °C 30 sec, 52 °C 30 sec, 72 °C 30 sec (35 cycles); 72 °C 10 min. PCR products were analysed using 1 % agarose gel electrophoresis.

2.2.5 Restriction enzyme digest on colonies with correct sized insert

Colonies displaying the correct sized insert as determined by PCR analysis, were grown overnight in LB broth before being purified using a plasmid mini prep kit (Promega). Plasmid DNA was quantified on a spectrophotometer (NanoDrop Technologies, Delaware, USA). To ensure the correct insert was in the purified plasmid DNA, a restriction enzyme digest was performed using *EcoRI* (New England Biolabs, Massachusetts, USA). Reactions consisted of 100 ng purified plasmid, 1 µl *EcoRI*, 2 µl 10x buffer and dH₂O up to 10 µl. An uncut plasmid control was run for each reaction, consisting of a reaction without *EcoRI*. Restriction enzyme digests were incubated at 37 °C for 4 h prior to analysis using 1 % gel agarose electrophoresis.

2.2.6 Sequencing of recombinant plasmids

Purified plasmids were sent to the Australian Genome Research Facility (AGRF; St Lucia, Qld) for sequencing using the SP6 and T7 primers. Reactions contained 150 ng purified plasmid, 2 µL of primer (3.3 µM) and brought up to 10 µL with dH₂O. Sequences were analysed using VectorNTI to check for changes against the reference sequences (NCBI).

2.2.7 Subcloning amplified and cloned genes in to pGEX expression vector.

Clones with correct sequences and start sites were then digested from the pGEM-T easy vector with *EcoRI* restriction enzyme (New England Biolabs). Restriction digest reactions consisted of 500 ng purified plasmid, 1 µl *EcoRI* (12 units), 2 µl 10x buffer and dH₂O up to 20 µl. Products were analysed on agarose gel electrophoresis for correct size insert, which was then cut from the gel and purified using a PCR and gel clean-up kit (Promega). The purified fragment was then quantified on the nanodrop spectrophotometer (NanoDrop Technologies, Delaware, USA) before being ligated into the pGEX-6-P-3 expression vector (GE Healthcare, Wisconsin, USA).

The pGEX plasmid was prepared for the subcloning by digestion with *EcoRI* (New England Biolabs) for 4 h at 37 °C. Reactions contained 10 µl pGEX DNA (500 ng), 10 µl 10X buffer, 1 µl *EcoRI* (12 u) and dH₂O up to total volume of 50 µl. Digestion progress was monitored by agarose gel electrophoresis. The

linearized pGEX vector was dephosphorylated using RAPID alkaline phosphatase (Roche, New South Wales). Reaction contained 10 µl linearized pGEX DNA, 10x phosphatase buffer, 1 µl alkaline phosphatase and 7 µL dH₂O. Reaction was incubated for 30 min at 37 °C, followed by phosphatase inactivation for 2 min at 75 °C.

Gel-purified insert sequences were then ligated into the linearized and dephosphorylated pGEX vector. The ligation reaction contained 2 µl linearized dephosphorylated pGEX DNA, 5 µl gel purified insert DNA, 2 µl 10x T4 ligase buffer, 1 µl T4 DNA ligase, dH₂O up to 20 µl. Reactions were incubated for 2 h at 10 °C and terminated at 65 °C for 10 min.

Purified recombinant plasmids were then transformed into competent *E.coli* BL21 cells (GE Healthcare) for expression of the fusion protein. BL21 cells were made competent using MgCl₂ (Sigma; Missouri, USA). Briefly, a single BL21 colony was isolated from an LB plate and incubated at 37 °C until and optical density (600 nm wavelength; A₆₀₀) of 0.4-0.5 was reached. Cells were sedimented at 2500 x g for 15 min at 4 °C, then gently resuspended in 1/10 volume of ice-cold transformation and storage solution (1.0 g tryptone, 0.5 g yeast extract, 0.5 g NaCl, 10 g polyethylene glycol, 5 ml dimethylsulfoxide, 5 ml MgCl₂ (1 M) (All from Sigma) and dH₂O up to 100 ml, pH 6.5) and placed on ice.

For transformation, 20 µl of the ligation reaction or 1 ng uncut vector (positive control) or 20 µl dH₂O (negative control) was added to 1 ml freshly prepared competent BL21 cells. Cells were incubated at 42 °C for 2 min before being returned to ice. 100 µl of transformed cells were added to 900 µl warmed LB broth and incubated for 1 h at 37 °C with shaking (250 rpm). 100 µl of diluted transformed cells from the samples were plated on LB/amp plates at 37 °C ON. Up to 50 colonies were selected and subjected to PCR analyses using the pGEX primers (Table 2.1) to determine if the recombinant plasmid was present. Several colonies were sequenced (as above) to check for correct insert and orientation. Colonies with correct recombinant plasmids were grown ON in LB broth with ampicillin. 500 µl of ON stock was

added to 500 µl of 100% glycerol and placed at -80 for future induction of fusion protein.

Table 2.1 Oligonucleotide primer sequences.

Oligonucleotide name	Oligonucleotide sequence
HsUROS -F	ATGAAGGTTCTTTTACTGAAGGATGC
HsUROS-R	CAGGCTTGAGGCAGGAGTCTGAC
HsHMBS -F	ATGTCTGGTAACGGCAATGCGGCTG
HsHMBS - R	CCCTGCAGTTCAGTCTCCCGGGGTAA
PfHMBS-F	GGGCTGGCAAGCCACGTTTGGTG
PfHMBS-R	CCGGGAGCTGCATGTGTCAGAGG
SP6	GATTTAGGTGACACTATAG
T7	TAATACGACTCACTATAGGG
pGEX-F	GGGCTGGCAAGCCACGTTTGGTG
pGEX-R	CCGGGAGCTGCATGTGTCAGAGG

F= forward primer, R= reverse primer; all primers from Geneworks

2.3 Expression of recombinant proteins

2.3.1 Screening pGEX recombinants for fusion protein expression

2 ml warmed LB broth with ampicillin were inoculated from the frozen culture stocks and grown ON at 37 °C. For comparison, a control tube was inoculated with bacteria transformed with parental pGEX plasmid. ON cultures were diluted 1:100 into fresh LB and grown to an A₆₀₀ of 0.6-0.8 with vigorous agitation at 37 °C. Fusion protein expression was induced with 2 µl of 100mM IPTG to give a final concentration of 1 mM. Incubation was continued for additional 1-2 h. Cultures were centrifuged in a microcentrifuge for five min at 500 x g and the supernatant was discarded. Each pellet was resuspended in 200 µl of ice-cold PBS. 10 µl of each cell

suspension was transferred into separate tube for SDS-PAGE analysis. 2 µl of lysozyme solution (Sigma) (at 10 mg/mL) was added to remaining cells and were then vortexed and incubated at room temperature for 5 min. Cells were then lysed by repeated cycle of freeze/thaw in dry-ice/warm water. The lysates was centrifuged in a microcentrifuge for 10 min to remove insoluble material. 10 µl of insoluble material was reserved for SDS-PAGE analysis. The supernatant (soluble material) was transferred to fresh tubes, with 10 µl being reserved for SDS-PAGE analysis.

2.3.2 SDS-PAGE analysis of fusion protein expression

The 10 µl samples previously saved from each induced recombinant pGEX colony were added to 2 µl of 6x SDS-PAGE loading buffer (0.35 M Tris-HCL, 10.28 % SDS, 36 % glycerol, 0.6 M dithiothreitol, 0.012 % bromophenol blue). Samples were mixed and heated at 95 °C for 5 min before being placed on ice. Samples were loaded onto 12.5 % SDS-polyacrylamide gel and electrophoresed at 200 volts for 1 h. Gels were stained with BioSafe Coomassie stain (Biorad, California, USA) for 1 h, followed by 1 h of rinsing with dH₂O to visualise fusion protein induction.

2.3.3 Optimisation of fusion protein expression

Colonies that did not express any fusion protein as indicated by SDS-PAGE analysis, were subjected to varying fusion protein induction conditions. These included varying IPTG amount (0.1-10 mM), length of induction (1-18 h), A₆₀₀ level (0.5-2), aeration level and induction temperature (18 to 37 °C). Each attempted induction was analysed by SDS-PAGE analysis (as above).

2.3.4 Preparation of large-scale recombinant protein from bacterial lysates

When the optimal induction condition for each fusion protein was identified, preparation of large-scale recombinant protein lysates for purification was attempted. A single colony of BL21 cells containing recombinant plasmid was used to inoculate 12 ml of LB medium with ampicillin. Inoculated cultures were grown at previously optimised conditions with the addition of IPTG to induce expression the fusion proteins. Induced cultures were treated as above to lyse cells and separate the soluble and insoluble fractions.

2.3.5 GST tagged protein purification

The soluble protein was subjected to purification using the GST Spintrap purification columns, according to standard protocol (GE healthcare), with samples collected throughout the purification process for analysis by SDS-PAGE. Briefly, resin was resuspended in column by vortexing briefly prior to use. Columns were centrifuged at 735 x g in a microcentrifuge and resulting buffer from each column was discarded. Up to 600 µl of supernatant from culture lysate was added to each column, before being mixed gently at room temperature for 10 min to ensure optimal binding of GST proteins to the glutathione sepharose matrix. Columns were placed in a clean Eppendorf tube and spun in a microcentrifuge for 1 min at 735 x g to collect flow through. Columns were placed into clean Eppendorf tubes and 600 µl of PBS was added and spun as above to wash the matrix. This was repeated twice with 10 µl of each wash collected for SDS-PAGE analysis. 100 µl of glutathione elution buffer was added to each column and incubated at room temperature for 10 min. Columns were placed into clean Eppendorf tubes before being spun as above for 1 min at 735 x g to collect eluate. This was repeated to collect the second eluate with samples of both elutions collected for SDS-PAGE analysis.

2.3.6 Cleavage of GST tag by PreScission protease

On-column cleavage of the fusion protein was conducted when the fusion protein was bound to the glutathione sepharose column (as above). Upon binding of the GST-fusion protein to the matrix, the column was washed with 1 ml of cleavage buffer at 4 °C. 40 µl of PreScission protease (80 units) (GE healthcare) was then added to 960 µl cleavage buffer (50 mM Tris-HCL, 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol) and added to the fusion protein-bound column, followed by incubation at 4 °C for 4 h. Eluate was collected by centrifugation at 735 x g for 5 min. The eluate contained the protein of interest, while the GST portion of the fusion protein and the PreScission protease remain bound to the glutathione sepharose matrix. The eluates were subjected to SDS-PAGE analysis and immunoblotting to confirm cleavage of the GST moiety.

Samples were immunoblotted and visualised (as described in section 2.6); antibodies used were anti-human UROS (Santa Cruz; sc-100637), anti human HMBS (Sigma; HPA006114), anti-glutathione-S-Transferase (GST) (Sigma; G7781)(all at 1:500 dilutions) and goat anti-mouse HRP conjugated antibody (Sigma) (at 1:1000 dilution).

2.4 Coupled enzyme assay to measure UROS activity

The coupled enzyme assay to measure UROS activity was performed as described by Omata and colleagues (Omata et al., 2004). Briefly, HMBS generates the hydroxymethylbilane substrate for UROS from porphobilinogen and the ratio of uroporphyrinogen-I and III are measured by ultra performance liquid chromatography-mass spectrometry (UPLC-MS). Initially, the units of purified recombinant HMBS were defined by optical quantification of the amount of uroporphyrin produced, as described by Omata and colleagues. The standard assay mixture for the coupled-enzyme assay contained 0.2 M sodium phosphate buffer (pH 8.0), 0.6 mM EDTA, 100 units HMBS, 0.5 mM porphobilinogen and UROS (equivalent to 5 ng of purified enzyme per assay) in a total volume of 0.75 ml. The reaction mixture without porphobilinogen was pre-incubated at 37 °C for 5 min prior to the addition of pre-warmed porphobilinogen to initiate the reaction. After 3 min, 0.4 ml of 0.5% I₂ in 1% KI was added to terminate the reaction and oxidise the uroporphyrinogens to uroporphyrins. This also acted to convert the remaining HMBS to non-porphyrin products. Excess I₂ was quenched with 0.1 ml of 1% sodium disulfite and proteins were precipitated with 1.75 ml of 7.1% TCA. Assay samples were analysed by UPLC-MS to separate the uroporphyrins I and III on a reversed-phased column attached to a waters 600E HPLC system. Elution was carried out with a 1 mL/min isocratic flow of 1 M ammonium acetate containing 0.27 mM EDTA and 12.5% acetonitrile. The eluate was monitored with a Waters 996 photodiode array detector and the Soret band maximum at 400 nM was extracted. One unit of activity was defined as the amount of enzyme that catalyses the formation of 1 µM uroporphyrin-III per hour under the reaction conditions used.

2.5 *P. falciparum* culture

P. falciparum strain 3D7, K1 and W2 mef (a gift from R. Anders and L. Tilley, La Trobe University, Victoria) were maintained at between 0.5 and 10% parasitaemia in purified AB+ human erythrocytes in a 1 % O₂/5 % CO₂ atmosphere according to the method of Trager and Jensen (Trager and Jensen, 1976). The cell culture medium (CCM) comprised of RPMI 1640 supplemented with 1X Glutamax, 0.2 % Albumax (all from Gibco, New York, USA), 4 % pooled AB+ human serum (Invitrogen, New York, USA), 10 mM D-glucose, 25 µg/ml gentamycin, 6 mM HEPES and 0.2 mM hypoxanthine (all from Sigma).

2.6 Immunoblotting

Late-stage parasitised cells (trophozoites and schizonts) were harvested from *P. falciparum* cultures (at approximately ~10% parasitaemia) using Percoll density gradient centrifugation (Rivadeneira et al., 1983); 80-90% of the harvested cells were infected. To isolate the parasites from their host red cells, the harvested parasites were treated with 10 volumes of saponin (0.15 % w/v) for 10 minutes on ice. Following centrifugation, the resulting supernatant (containing the hemoglobin, red cell cytosol components and red cell plasma membranes) was removed and the parasite pellet was washed with RPMI and then resuspended in PBS containing protease inhibitors (Complete® protease cocktail from Roche). The parasites were then subjected to sonication (3 x 30 sec) and protein quantified using a Bradford protein assay (ThermoScientific, Illinois, United States). Purified parasites and equivalent numbers of uninfected red blood cells were lysed in equal volume of 2 x SDS loading buffer (containing 0.5 % β-mercaptoethanol), heated at 95 °C for 5 min, and subjected to SDS-PAGE, 12 % gel from Biorad (Laemmli, 1970), and then transferred to nitrocellulose membrane. Membranes, blocked in 1 % blocking reagent (Roche) overnight, were then blotted with antibodies (in 0.5 % blocking reagent); mouse monoclonal anti-human FECH (Santa Cruz, sc-271434), mouse monoclonal anti-human ALAD (Santa Cruz; sc-271585), mouse monoclonal anti-human UROS (Santa Cruz;

sc-100637), mouse monoclonal anti-human hexokinase (Santa Cruz; 46695), all diluted at 1:100, followed by goat anti-mouse conjugated peroxidase (1:1000 dilution Sigma). Blotted membranes were visualised using enhanced chemiluminescence (ECL) reagents (Thermoscientific) using a Chemi-smart 5000 (Vilber Lourmat, Marne-la-Vallee, France).

2.7 Immunofluorescence staining

Thin blood smears prepared from *P. falciparum* cultures were air dried, fixed for 30 sec in 100 % methanol, and then fixed for 20 min in 1 % paraformaldehyde in PBS. Slides were washed in PBS and blocked with 1 % BSA/0.1% Triton X100 in PBS (PBT). Slides were then incubated in the following primary antibodies (overnight at 4 °C, diluted in PBT); mouse monoclonal anti-human FECH (Santa Cruz, sc-271434) or mouse monoclonal anti-human ALAD (Santa Cruz; sc-271585) or mouse monoclonal anti-human UROS (Santa Cruz; sc-100637) (all 1:100 dilution) and rabbit anti-*PfEXP2* (1:1000; a gift from P. Gilson, Burnet Institute, Victoria). After washing, slides were incubated in donkey anti-mouse Alexa Fluor® 488 (1:1000) and goat anti-rabbit Alexa Fluor® 594 (1:1000; both from Invitrogen) for 90 min at room temperature in the dark. Slides were counter-stained with DAPI (1:5000), washed and mounted in Slowfade Gold (Invitrogen). Slides were viewed on a Nikon inverted microscope at 60x magnification with a water immersion lens. Images were obtained using an Evolve camera and NIS-Elements software.

2.8 Collection and preparation of purified human red blood cells

Blood from individuals with porphyria and from individuals treated with griseofulvin was collected by venipuncture into 5 ml sodium citrate tubes. Blood samples collected at the Menzies Research Institute Tasmania were used immediately. Blood samples collected from EPP patients at the Royal Melbourne Hospital (by Prof Ingrid Winship) were shipped immediately to the Menzies Research Institute Tasmania at 4 °C prior to further processing.

XLDP patient blood samples were collected at Centre Français des Porphyries, Hôpital Louis Mourier, Colombes (France) by Hervé Puy prior to immediate shipment to the Pasteur Institute, Paris. Blood was then centrifuged at 170 x g for 13 minutes and the plasma, platelet and white cell fractions removed. Blood was washed two times in RPMI and stored at 4 °C, and then washed further prior to use.

2.9 *P. falciparum* growth inhibition assay

For succinylacetone (SA) growth inhibition assays, two experimental approaches were taken. Firstly, synchronized trophozoite-stage *P. falciparum* (3D7) were incubated continuously with the inhibitor for 48 h. The second approach involved preincubating uninfected red cells with SA for 2 h, prior to repeated washing with RPMI to remove excess unbound SA. Aminolevulinic acid (2 mM) was then added to pre-treated red cells to non-enzymatically convert any unbound SA to a pyrrole (Ebert et al., 1979). Synchronised trophozoite-stage *P. falciparum* (3D7) parasites were then added to the pre-treated red cells (at a final percentage between 0.5-1 %) and cultured for a further 48 h prior to analysis.

For N-methylprotoporphyrin (NMPP) *in vitro* growth inhibition assays, synchronized ring-stage *P. falciparum* (3D7) parasites were grown in the presence of increasing concentrations of NMPP (Frontier Scientific, Utah, USA) for 48 h prior to analysis of growth and subsequent comparison with an untreated control.

For the growth assays involving *in vitro* griseofulvin treatment, uninfected red cells were incubated with griseofulvin for 3 days prior to the addition of synchronized ring-stage *P. falciparum* (3D7 and K1) parasites, and then cultured for an additional 48 h prior to analysis. The medium and griseofulvin was replaced every 24 h during the preincubation and growth assay periods.

For *ex vivo* growth assays using the porphyric blood and blood from the griseofulvin-treated individuals, *P. falciparum* (3D7) parasites grown in normal untreated blood were harvested by Percoll density gradient centrifugation or MACS column (Ribaut et al., 2008, Rivadeneira et al., 1983) to collect trophozoite and schizont-stage parasites. All assays were conducted by infecting the red cells with 0.01 x volume of purified trophozoite- and schizont- stage infected cells at a final percentage parasitaemia of approximately 0.5 %. Parasites were grown in treated/porphyric blood and untreated/normal blood for up to 72 h, with samples collected for analysis at varying time-points.

For all growth inhibition experiments, thin-blood smears were obtained after incubation of the parasites for up to 72 h, stained with 10% Giemsa stain and the percentage of infected parasites (% parasitaemia) was determined by counting cells under a light microscope. At least 1000 cells were counted on each slide. In some experiments parasite growth was also quantified by flow cytometric analysis using YOYO-1 dye (Invitrogen) (Li et al., 2007). Percentage parasite growth (parasitemia divided by the untreated or normal blood control parasitemia x 100) and percentage growth inhibition (the calculated % parasite growth of each sample subtracted from 100) were determined by comparison of growth in cultures treated without drug, or in blood from normal, untreated individuals. Each drug-treated sample, porphyria blood sample or control sample was completed in triplicate. Each entire experiment was independently repeated at least twice.

2.10 Experimental *P. chabaudi* infection

There are several murine models of *Plasmodium* that replicate different aspects of *P. falciparum* infection. *P. chabaudi* was used in this study as it is most similar to *P. falciparum* blood stage of infection, with the exception that cerebral malaria does not occur. Upon intravenous injection with *P. chabaudi*, a 24-hour cycle of red cell invasion, growth and rupture occurs, resulting in increased proportions of infected red cells (% parasitaemia) over the ensuing

days. Upon peak parasitaemia, mice will either survive (with a resulting decrease in circulating infected red cells) or succumb to infection. Survival is also dependent on the sex and genetic background of the mice (Stevenson et al., 1982, Stevenson et al., 1990).

2.10.1 *P. chabaudi* infection of *Fech*^{m1Pas} mice

Fech^{m1Pas} mice, originally produced from an ENU-mutagenesis screen at the Pasteur Institute (Paris, France) were kindly provided by X. Montagutelli. Mice were genotyped as described by Abitbol (Abitbol et al., 2005). Female and male *Fech*^{+/+}, *Fech*^{+/-} and *Fech*^{-/-} mice on an isogenic (C57BL/6) or mixed (C57BL/6 and BALB/c background were infected with the rodent malarial species *Plasmodium chabaudi adami* DS at 7-12 weeks of age. Female mice were routinely infected intravenously with 5 x 10⁵ iRBC/ml and male mice were infected with 2.5 x 10⁵ iRBC/ml. Blood stage parasitaemia (% infected red blood cells) for each mouse during infection was determined by counting cells on Giemsa-stained (10 % Giemsa solution) thin blood smears taken from tail bleeds of mice during the course of infection. Percentage parasitaemia was calculated by counting at least 500 cells per slide.

2.10.2 *P. chabaudi* infection of SA-treated mice

Male C57BL/6 mice between 7-12 weeks of age received an established dose of SA (in saline) the day prior to infection, the day of infection and everyday for eight days. Control mice received saline at the same time-points. All mice were infected with 2.5 x 10⁵ iRBC/ml RBC. Blood stage parasitaemia (% infected red blood cells) for each mouse during infection was determined by counting cells on Giemsa-stained thin blood smears (as above).

2.11 ALAD assay of peripheral mouse blood

Blood samples were obtained from experimental mice and assayed for peripheral blood ALAD activity according to the method of Wigfield and Farant (Wigfield and Farant, 1981). ALAD activity was expressed as μ M porphobilinogen produced per h at 37 °C, relative to the untreated control sample.

2.12 Haematological analysis of SA-treated mice

Blood samples were obtained by mandibular bleeding from experimental mice (~250 uL collected) and complete blood analysis was performed using an Advia 2120 haematology system (Bayer Healthcare, New York, USA) using the CBC/DIFF/RETIC and C57BL/6 mouse settings.

2.13 UPLC-MS analysis of griseofulvin levels in blood

Quantification of griseofulvin in blood from griseofulvin treated volunteers or griseofulvin pre-incubated red cells was analysed by Ultra-Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS) using a Waters Acquity H-series UPLC coupled to a Waters Xevo triple quadrupole mass spectrometer as described previously (Mensch et al., 2007).

2.14 Statistical Analysis

P values were calculated in Microsoft Excel software using two-tailed t-tests assuming equal variance. $P < 0.05$ was determined as significant.

2.15 General recipes

LB broth

12.5 g LB broth (Astral), with 500 ml distilled H₂O (dH₂O), autoclaved

LB agar plates with ampicillin

12.5g LB, 7.5g agar into 500mL dH₂O

Autoclave then add 1uL ampicillin (1 µg/ml) per 100mL broth

SDS PAGE electrophoresis buffer (10 X)

30 g Tris base, 144 g glycine, 10 g SDS up to 1 L dH₂O, pH 8.3

50mL of 10x stock was diluted with 450 mL dH₂O for each electrophoresis run.

Western transfer buffer

25mM Tris, 182 mM glycine up to 1L dH₂O, pH 8.3

Chapter 3

An investigation of the localisation of host haem enzymes during intraerythrocytic growth of the malarial parasite

Introduction

Host enzymes and proteins have been increasingly shown as important factors for intraerythrocytic malarial infection. Recently, several host factors have been implicated as essential requirements for parasite invasion, growth or egress. Further characterisation of essential host factors required for intraerythrocytic infection could pave the way for novel antimalarial therapeutic targets.

It has been reported that two host haem synthetic enzymes aminolevulinate dehydratase (ALAD) and ferrochelatase (FECH) are scavenged by *Plasmodium* during intraerythrocytic infection (Bonday et al., 2000, Bonday et al., 1997, Varadharajan et al., 2004). However, these findings have been questioned because the presence of these enzymes in the mature red cell is unproven. It is also unclear why the parasite would need to scavenge host haem synthetic enzymes considering the parasite genome encodes a functional ALAD and FECH (Sato et al., 2004, Sato and Wilson, 2003, Sato and Wilson, 2002, Ralph et al., 2004).

ALAD and FECH are abundant in progenitor red cells, which have a highly active haem synthetic pathway. Haem synthetic enzymes are distributed between the red cell cytosol and mitochondria. ALAD is a cytosolic enzyme, while FECH is located in the mitochondria where it inserts iron in protoporphyrin IX to form haem (Sellers et al., 2001). The mitochondria are therefore the ultimate site of mammalian haem synthesis. The current paradigm suggests that haem synthetic enzymes are expelled from the

developing red cell along with the mitochondria and other organelles. Recent proteomic studies have provided conflicting evidence, some confirmed ALAD and FECH were present in the mature red cell (Pasini et al., 2006), while others did not identify either enzyme (Alvarez-Llamas et al., 2009, Goodman et al., 2007, Kakhniashvili et al., 2004, Zhang et al., 2008). Therefore, the current study aimed to verify if ALAD and FECH are present in the mature red cell.

If the mature red cell does contain residual ALAD and FECH, the parasite may indeed scavenge the host enzymes, as previously suggested (Bonday et al., 2000, Bonday et al., 1997, Varadharajan et al., 2004). The parasite localisation of ALAD and FECH during intraerythrocytic infection remains to be independently confirmed and was therefore investigated in this chapter.

A third host enzyme from the haem biosynthetic pathway, uroporphyrinogen-III synthase (UROS) is also of interest, considering there is no gene for *Uros* in the *P. falciparum* genome. The absence of a parasite UROS is an anomaly because UROS is an essential enzyme in the haem biosynthetic pathway and endogenous haem synthesis is essential for all cellular forms of life. Therefore, if UROS is present in the mature red cell, this enzyme may also be scavenged by the parasite to fulfill its UROS requirements for haem synthesis. A further aim of this chapter was to therefore investigate if host UROS is localised in the parasite during intraerythrocytic growth.

An alternative explanation for the source of parasite UROS is that the enzyme in the haem synthesis pathway prior to UROS, hydroxymethylbilane synthase (HMBS; also known as porphobilinogen deaminase) may possess a dual HMBS and UROS function. Nagaraj and colleagues demonstrated parasite encoded HMBS (*PfHMBS*) contains UROS activity, although the activity was very low compared to the human version of the enzyme (Nagaraj et al., 2008). A final aim of this chapter was therefore to further investigate the role of *PfHMBS* as a potential source of *Plasmodium* UROS.

Results

The goals of this work were to initially investigate whether the host enzymes ALAD, FECH and UROS were present in the mature red cell and secondly if the host enzymes were localised in the parasite during intraerythrocytic infection. Two different approaches were used that took advantage of the availability of monoclonal antibodies specific to each of the human enzymes. These antibodies were used in Western blotting to probe protein extracts purified from cultures of saponin-lysed *P. falciparum* and uninfected red cells; and in immuno-staining of fixed *P. falciparum*-infected red blood cells.

Analysis of the parasite-specific localisation of red cell ALAD

Western blot analysis of uninfected human red cells was undertaken with a monoclonal anti-ALAD antibody against the amino acids 1-300 mapping at the N-terminus of ALAD of human origin. Results demonstrated the presence of ALAD in mature red cells (Figure 3.1 A). Furthermore, the same ALAD antibody was used to probe protein extracts purified from *P. falciparum* cultures, demonstrating a band at the same predicted size for that of the human ALAD (~37-39kDa) (Figure 3.1 A). It is unlikely that the band detected in the parasite fraction is anything other than host red cell ALAD. Firstly, host ALAD is not a membrane-associated protein; therefore it is unlikely the enzyme was adhering to the red cell membrane or parasitophorous vacuole membrane (PVM) of the saponin-lysed parasites. Secondly, the *P. falciparum* version of ALAD has a predicted size of 53kDa, distinct from the host protein at ~37-39kDa. Finally, the human and *Plasmodium* ALAD share only 31% amino acid sequence identity therefore it is unlikely that the host antibody cross-reacts with the parasite ALAD.

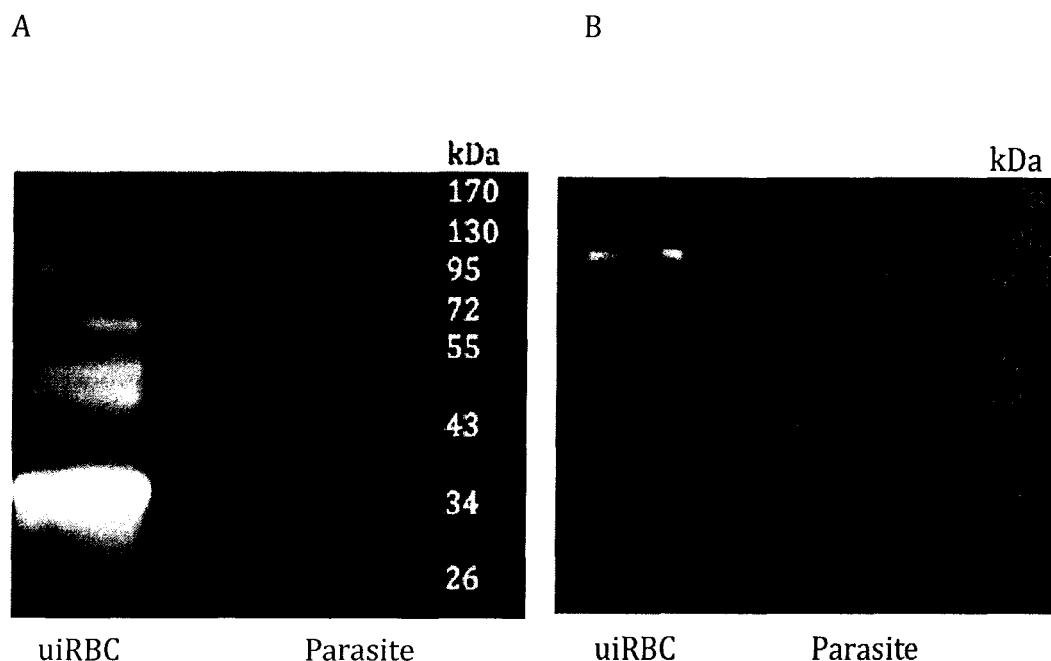


Figure 3.1 Host aminolevulinate dehydratase (ALAD) is present in uninfected red cells (uiRBC) and purified parasites

(A and B) Western blot analyses of protein samples of uninfected red cells and purified saponin-lysed *P. falciparum* parasites (parasites). Each lane was loaded with protein prepared from equal numbers of cells. Proteins were separated on denaturing SDS polyacrylamide gels, transferred to nitrocellulose membranes and immuno-blotted with anti-human ALAD antibody (A) and anti-human hexokinase antibody (B).

(A) A band corresponding to the predicted size of human ALAD (37-39 kDa) is present in both the red cell and parasite samples.

(B) A band corresponding to the predicted size of human hexokinase (130 kDa) is present in the red cell sample but not in the parasite sample, indicating that the parasite sample is free of red cell cytosol.

Another possibility that may explain the presence of red cell ALAD in the parasite fraction was contamination with red cell cytosol. To rule this out, the presence of an additional and unrelated red cell cytosolic enzyme hexokinase was investigated as a control to provide evidence against the possibility of host red cell contamination. The same blots from the results above were analysed with an anti-hexokinase antibody. A band at the expected 120 kDa was observed in the uninfected red cell fraction but not the purified parasite fraction, indicating the purified parasite fraction is free of red cell contaminants (Figure 3.1 B). This data indicates the presence of host ALAD in the parasite fraction is unlikely a result of host red cell cytosolic contamination in the parasite lysate. However, it must be noted that the intensity of the host hexokinase band in the red cell fraction is very weak relative to that of the ALAD band in the same fraction. It is therefore possible that host hexokinase is present in the parasite fraction but is not abundant enough to be detected, given the weak signal in the red cell fraction.

To further investigate the parasite localisation of host ALAD, a second approach involved immunostaining analysis of fixed preparations of cells from *P. falciparum* cultures. The same antibody as above, directed against human ALAD was used in conjunction with fluorescently labelled secondary antibodies and epi-fluorescence microscopy. An anti-EXP2 antibody, directed against the parasitophorous vacuole membrane (PVM), was used to delineate the boundary of the intracellular parasite and the DNA-specific dye DAPI was used to mark the parasite nucleus.

Examples of uninfected red cells, ring, trophozoite and schizont iRBCs stained with these markers are shown in Figure 3.2. The host ALAD was evenly distributed in the uninfected red cells, consistent with a cytosolic location. This staining was distinct from that of the isotype control. The host ALAD staining was unchanged when an early ring-stage parasite (based on the absence of hemozoin pigment) was present. There was some concentration of host ALAD observed around the PVM marker at the late-ring and trophozoite stage, consistent with the growth stages of the parasite.

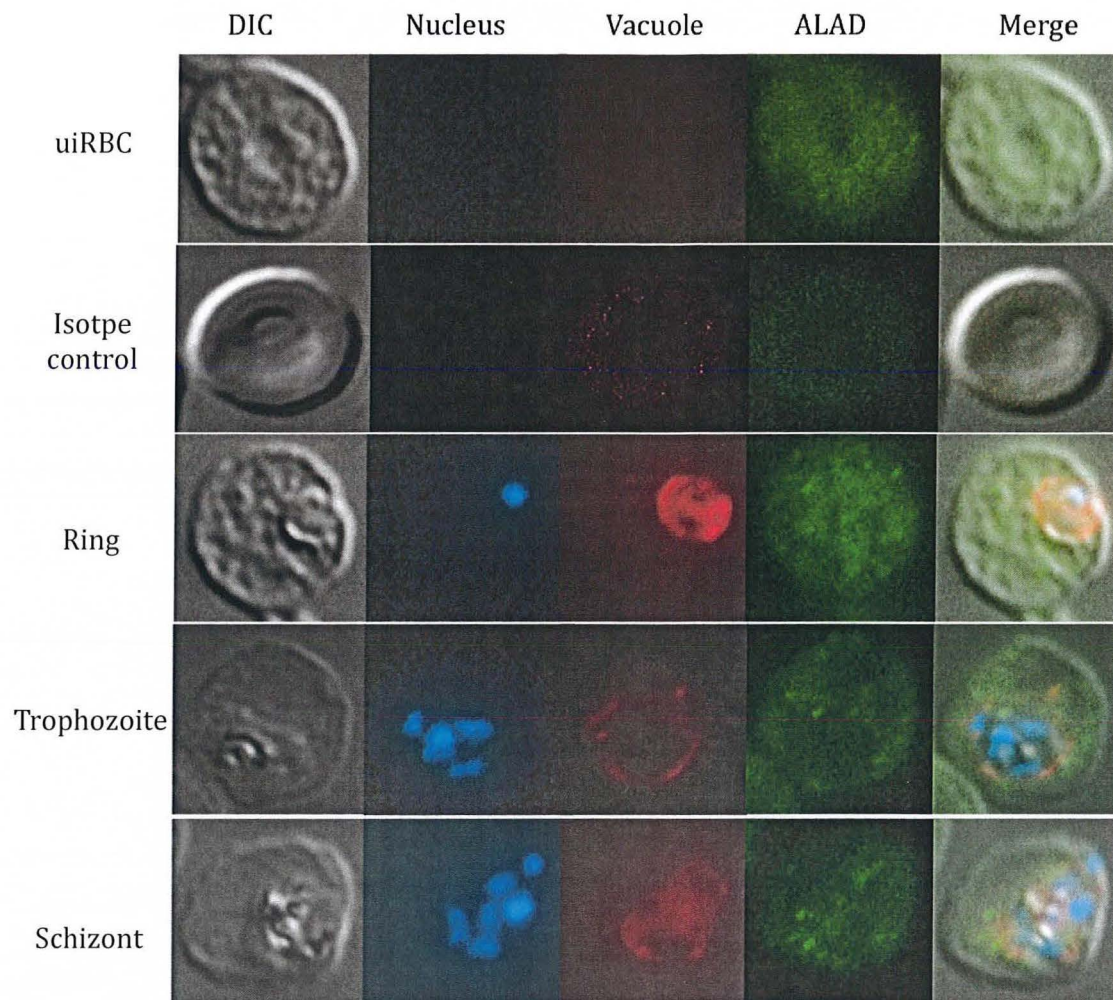


Figure 3.2. Host aminolevulinate dehydratase (ALAD) is present in uninfected RBCs (uiRBC) and localised in the parasite during intraerythrocytic growth

Representative images showing *P. falciparum* infected red blood cells immunostained with anti-human ALAD and anti-EXP2 antibodies, and stained with the DNA-specific dye DAPI (to detect parasite nuclei). Anti-EXP2 was used to detect the parasitophorous vacuole membrane (PVM). The images overlaid (merge) to show colocalisation (anti-ALAD in green, anti-EXP2 in red and DAPI in blue). Anti-ALAD staining is evident in uninfected red cells (uiRBC), this staining was distinct as compared to background levels of signal detected by isotype control antibodies. Different developmental stages of the parasite are depicted in early ring-stage, mature trophozoite stage, and schizont stage. ALAD staining is unchanged in ring-stage iRBCs but starts to accumulate around the PVM at the trophozoite stage of infection. ALAD is almost entirely within the PVM by the schizont stage.

By the late-stage of development (schizont stage), the host stain was almost entirely within the PVM. Additional varying punctate ALAD staining was also observed within the parasite at this stage, possibly due to a specific sub-cellular location or compartmentalisation of the host enzyme. These observations indicate that the host ALAD is progressively accumulated within the PV of the parasite, and is consistent with the uptake of this host enzyme during the growth of the parasite. This observation is consistent with previous published findings (Bonday et al., 2000, Bonday et al., 1997, Dhanasekaran et al., 2004).

Analysis of the parasite-specific localisation of host red cell FECH enzyme.

Western blot analysis of uninfected human red cells was undertaken with a monoclonal anti-FECH antibody raised against amino acids 124-243 mapping at the C-terminus of FECH of human origin. Results demonstrated the presence of FECH in mature red cells (Figure 3.3). This result indicates that there is still some residual FECH present in the mature red cell, even though FECH is a mitochondrial enzyme. Furthermore, the same FECH antibody was used to probe the same protein extracts purified from *P. falciparum* cultures (as used above) and previously shown to be free of red cell cytosol (Figure 3.1 B). Results demonstrated a band at the same predicted size for that of the human FECH (~40-43 kDa) (Figure 3.3). While the parasite FECH is estimated at the same size as the human FECH, there is only 28% sequence identity between the amino acid sequences and therefore likely that the band detected in the parasite fraction is host red cell FECH.

Fixed preparations of cells from *P. falciparum* cultures were then used for immunostaining analysis to further investigate the parasite localisation of host FECH (As for ALAD). The same antibody as above, directed against human FECH, was used in conjunction with fluorescently labelled secondary antibodies, anti-EXP2, DAPI and epi-fluorescence microscopy. Examples of uninfected red cells, ring, trophozoite and schizont iRBCs stained with these markers are shown in Figure 3.4. The host FECH was evenly distributed in the uninfected red cells, which is interesting considering the mitochondrial location of this enzyme in developing red cells. This staining was distinct

from that of the isotype control. The host FECH staining was mostly unchanged in early ring-stage parasites but some concentration of host FECH was observed (Figure 3.4; ring). By the late ring and trophozoite stage of infection, the host FECH staining was observed around the PVM and at the late-stage of development (schizont stage), the host stain was almost entirely within the PVM. Additionally, the host signal appeared comparatively weaker in the schizont stage, possibly due to metabolism or breakdown of the host enzyme inside the parasite. These results are similar to that observed for ALAD, in that the host FECH is progressively accumulating around or within the PVM during growth stages of the parasite.

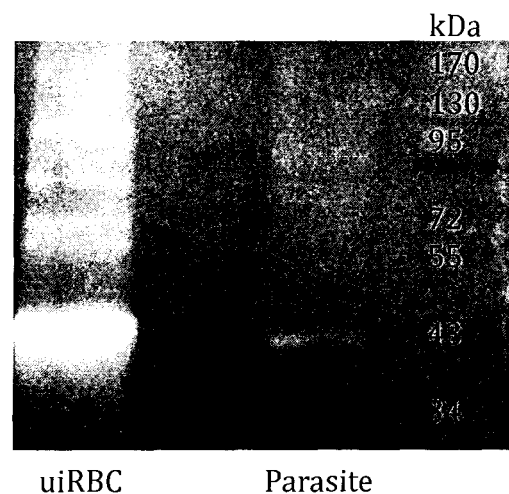


Figure 3.3 Host ferrochelatase (FECH) is present in uninfected RBCs (uiRBC) and purified parasites

Western blot analyses of protein samples prepared from uiRBC and purified saponin-lysed *P. falciparum* parasites (prepared from equal numbers of cells). Proteins were separated on denaturing SDS polyacrylamide gels, transferred to nitrocellulose membranes and immuno-blotted with anti-human FECH antibody. A band corresponding to the predicted size of human FECH (40-43 kDa) is present in both the red cell and parasite samples.

The same parasite preparation was used, as previously shown to be free of red cell cytosol (Figure 1B)

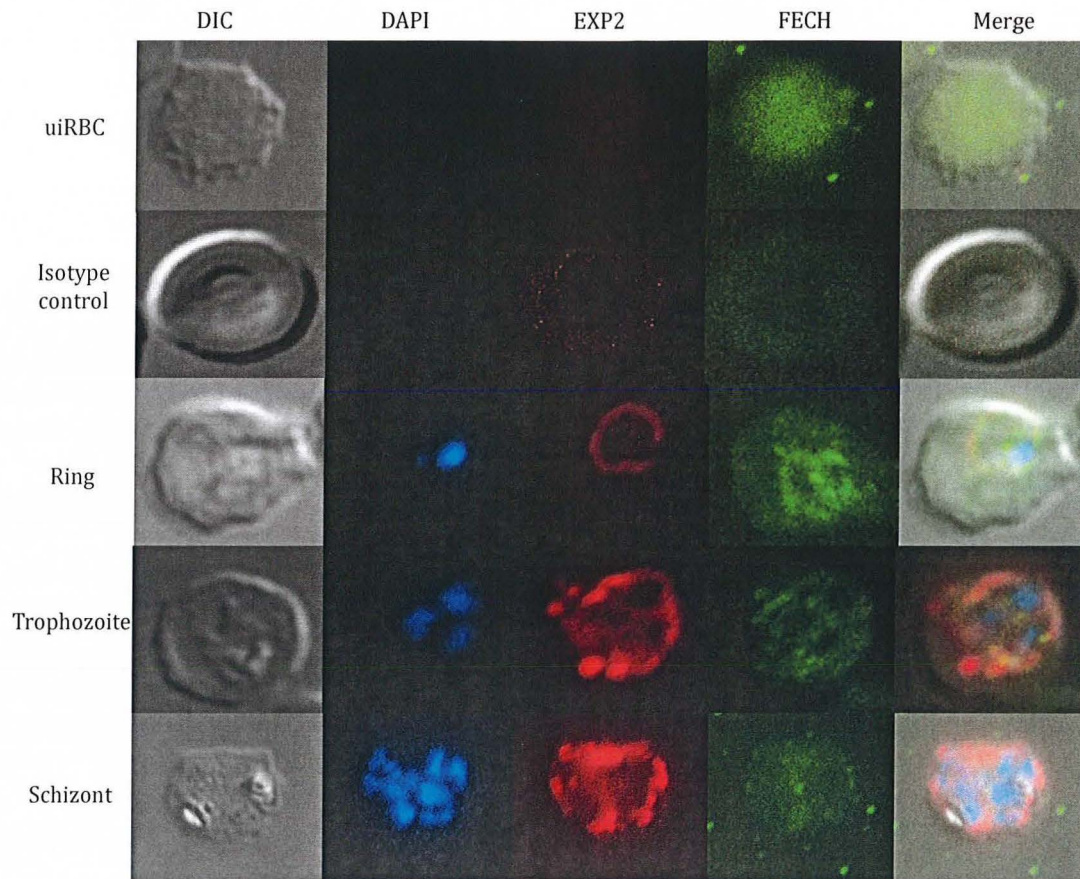


Figure 3.4. Host ferrochelatase (FECH) is present in uninfected RBCs (uiRBC) and localised in the parasite during intraerythrocytic growth

Representative images showing *P. falciparum* infected red blood cells immunostained with anti-human FECH and anti-EXP2 antibodies, and stained with the DNA-specific dye DAPI (to detect parasite nuclei). Anti-EXP2 was used to detect the parasitophorous vacuole membrane. The images overlaid (merge) to show colocalisation (anti-FECH in green, anti-EXP2 in red and DAPI in blue). Disperse anti-FECH staining is evident in uninfected red cells (uiRBC), this staining was distinct as compared to background levels of signal detected by isotype control antibodies (Isotype control); Different developmental stages of the parasite are depicted in early ring-stage, mature trophozoite stage (Troph), and schizont stage. In ring-stage iRBCs the anti-FECH staining is distinct from that of uninfected red cells and this staining appears to be colocalising around the PVM by the trophozoite stage. By the schizont stage of infection, the host staining is entirely within the PVM.

Analysis of the parasite-specific localisation of host red cell UROS enzyme.

Western blot analysis of uninfected human red cells was undertaken with a monoclonal anti-UROS antibody raised against recombinant UROS of human origin. Results demonstrated that host UROS is present in mature red cells (Figure 3.5); this was not unexpected considering the cytosolic location of this enzyme in progenitor red cells. Additionally, the same UROS antibody was used to probe the same protein extracts purified from *P. falciparum* cultures (as used above) and previously shown to be free of red cell cytosol (Figure 3.1 B). Results demonstrated a band at the same predicted size for that of the human UROS (~26 kDa) (Figure 3.5). The parasite does not contain its own UROS; hence the band detected in the parasite fraction is of host origin.

To further investigate the parasite localisation of host UROS, immunostaining analysis of fixed preparations of cells from *P. falciparum* cultures was also utilised (As for ALAD and FECH). The same antibody as above, directed against human UROS, was used in conjunction with fluorescently labelled secondary antibodies, anti-EXP2, DAPI and epi-fluorescence microscopy. Examples of uninfected red cells, ring, trophozoite and schizont iRBCs stained with these markers are shown in Figure 3.6. The host UROS was evenly distributed in the uninfected red cells, consistent with its cytosolic location and the staining was distinct from the isotype control antibody. By the late ring and trophozoite stage of infection, the host UROS staining was concentrated around the PVM. At the schizont stage of infection, the host UROS was entirely within the PVM. The staining was not dispersed throughout the parasite, indicating there may be further compartmentalisation of the host enzyme within the parasite. These results are similar to that observed for ALAD and FECH, in that the host UROS is progressively accumulating within the PVM during growth stages of the parasite. Together, the Western blotting and immunofluorescence results demonstrate that host UROS is localised in the parasite during growth. Considering the parasite does not contain its own UROS, the host enzyme may play a functional role in the parasite.

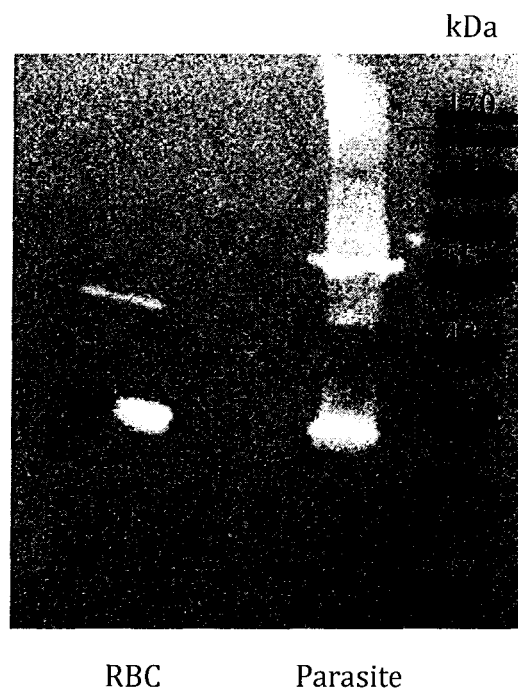


Figure 3.5. Host Uroporphyrinogen-III synthase (UROS) is present in uninfected RBCs (uiRBC) and purified parasites

Western blot analyses of protein samples prepared from uiRBC and purified saponin-lysed *P. falciparum* parasites (prepared from equal numbers of cells). Proteins were separated on denaturing SDS polyacrylamide gels, transferred to nitrocellulose membranes and immuno-blotted with anti-human UROS antibody. A band corresponding to the predicted size of human UROS (29 kDa) is present in both the red cell and parasite samples.

The same parasite preparation was used, as previously shown to be free of red cell cytosol (Figure 1B).

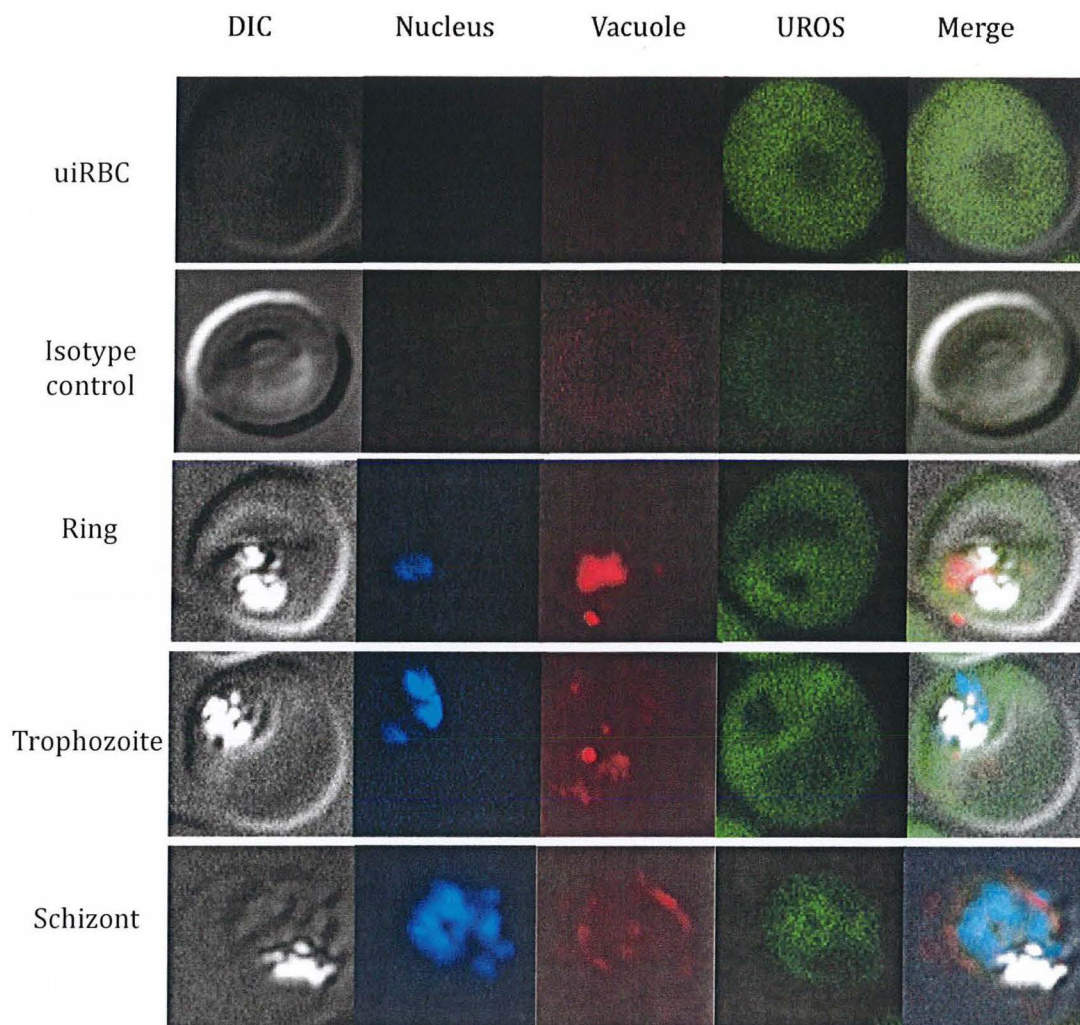


Figure 3.6. Host Uroporphyrinogen-III synthase (UROS) is present in uninfected red cells and localised in the parasite during growth

Representative images showing *P. falciparum* infected red blood cells immunostained with anti-human UROS and anti-EXP2 antibodies, and stained with the DNA-specific dye DAPI (to detect parasite nuclei). The images overlaid (merge) to show colocalisation (anti-UROS in green, anti-EXP2 in red and DAPI in blue). Disperse anti-UROS staining is evident in uninfected red cells (uiRBC), this staining was distinct as compared to background levels of signal detected by isotype control antibodies. Different developmental stages of the parasite are depicted in early ring-stage, mature trophozoite stage, and schizont stage. The UROS stain starts to concentrate and colocalise with the parasite vacuole at the trophozoite stage and by schizont stage, the host stain is entirely within the parasite vacuole and around the nucleus.

Can PfHMBS fulfill the enzymatic function of UROS?

Results so far presented in this chapter indicate host UROS is localised in the parasite during intraerythrocytic growth and may act as the parasite's UROS source for haem synthesis. An alternative hypothesis suggests that *Plasmodium*-encoded HMBS (*PfHMBS*) has dual-functionality and possesses UROS activity (Nagaraj et al., 2008). Work here further investigated this reported finding, and aimed to assess the contribution of host UROS and *PfHMBS* towards overall UROS activity in the parasite. To do this, purified recombinant forms of both *PfHMBS* and human HMBS and UROS were produced to test and compare levels of UROS activity.

Cloning and expression of PfHMBS

PfHMBS was successfully amplified and cloned into the pGEX-3-P expression vector (Figure 3.7 A and B), via a sub-cloning using the pGEM-T easy plasmid. Clones with no sequence errors (as determined by sequencing; figure 3.7 C) were transformed into BL21 *E. coli* cells, a strain designed to maximize expression of full-length proteins. The *PfHMBS* fusion protein could not be induced in BL21 *E. coli* cells under a variety of trial induction conditions including varying IPTG amounts, temperature of induction and length of induction (Table 3.1). Several *PfHMBS*-pGEX clones were trialed under these conditions. Overall, the expression of *PfHMBS* as a fusion protein in this chapter was unsuccessful; hence the large-scale induction and purification of this protein was unable to be carried out.

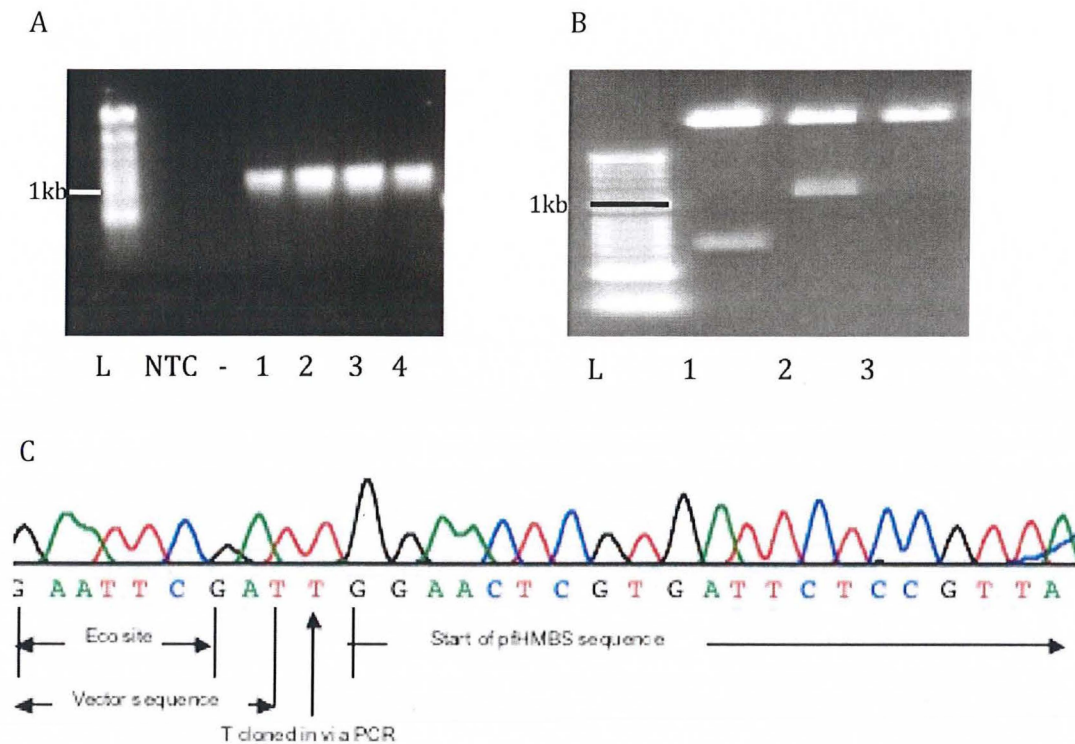


Figure 3.7 Amplification, cloning and sequencing of *PfHMBS*

(A) Amplification of the N-terminal truncated *PfHMBS* from purified parasite DNA using gradient PCR (Temperature range 55 to 65°C). Lane 1-4 show product 1.2 kb as compared to the no template control (NTC).

(B) Restriction enzyme digest (using *EcoRI* restriction enzyme) of purified DNA from selected pGEX fusion protein clones transformed with positive control DNA (1), *PfHMBS* (2) or pGEX vector alone (3). The positive control demonstrates a product that is cut from the vector at the expected 700bp (1). The purified *PfHMBS* colony in (2) has a product at the expected 1.1kb as compared to the pGEX vector alone (3).

(C) Selected segment from sequencing of the *PfHMBS* clone (from purified DNA of the correct clone above), demonstrating the start site of the *PfHMBS*-pGEX sequence, an additional T cloned in via PCR from the original sub-cloning into pGEM-T easy, the pGEX vector sequence and one of the *EcoRI* restriction enzyme sites. The *PfHMBS* sequence showed no base pair changes from the reference sequence (accession # XP_001350505, NCBI).

Table 3.1 Summary of induction conditions trialed for *Pf*HMBS fusion protein expression.

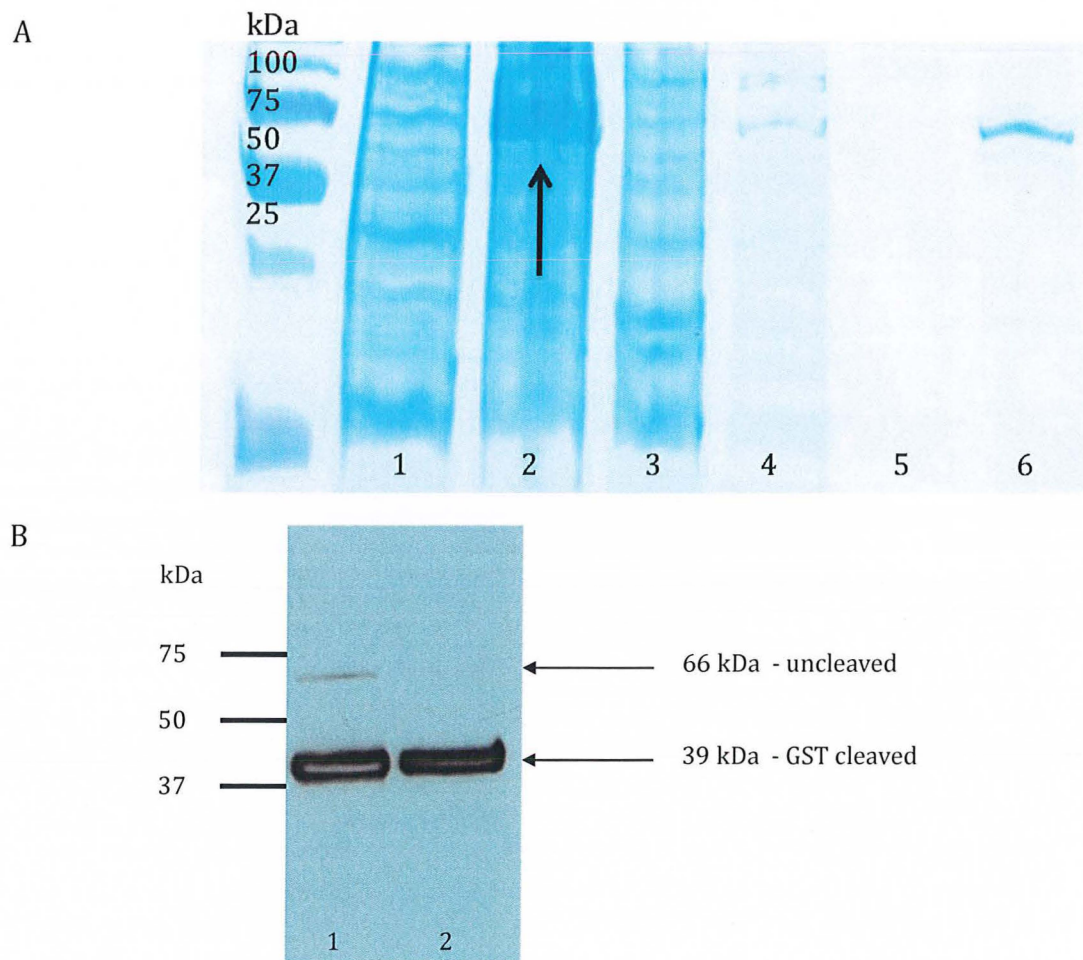
<i>Trial #</i>	<i>IPTG concentration</i>	<i>Temperature (°C)</i>	<i>Length of induction</i>	<i>Induction of fusion protein</i>
1	0.1 mM	37	1 h	No
2	0.1 mM	37	2 h	No
3	0.1 mM	37	Overnight	No
4	0.1 mM	20	1 h	No
5	0.1 mM	20	2 h	No
6	0.1 mM	20	Overnight	No
7	1 mM	37	1 h	No
8	1 mM	37	2 h	No
9	1 mM	37	Overnight	No
10	1 mM	20	1 h	No
11	1 mM	20	2 h	No
12	1 mM	20	Overnight	No
13	10 mM	37	1 h	No
14	10 mM	37	2 h	No
15	10 mM	37	Overnight	No
16	10 mM	20	1 h	No
17	10 mM	20	2 h	No
18	10 mM	20	Overnight	No

Cloning, expression and purification of human HMBS and UROS as enzyme sources for the coupled-enzyme assay

While the attempted expression of *Pf*HMBS occurred, an assay system to test for UROS activity was concurrently developed. The hydroxymethylbilane substrate of UROS is highly unstable; as such the assay is a coupled-enzyme system requiring both UROS and HMBS sources, as based on the described method (Omata et al., 2004). Successful cloning of the full-length human UROS and HMBS into the expression vector pGEX was conducted as for *Pf*HMBS. Several clones for each protein were sequenced prior to transformation into BL21 *E.coli* cells for induction of fusion protein expression.

Proteins of the correct size for HMBS were induced with 1 mM IPTG at 37 °C for 1 hour with the subsequent large-scale expression and purification completed successfully (Figure 3.8 A and B). Proteins for the correct size for UROS were induced with 1 mM IPTG at 37 °C for 1 hour but the majority of protein was insoluble (Figure 3.9 A). Conditions were altered to try and increase the soluble yield without any success. The final purified UROS protein could not be detected on an SDS-PAGE gel using Coomassie stain but was present at the correct size upon western blot analysis, indicating there was some soluble protein able to be expressed and purified (Figure 3.9 A and B).

The expressed and purified HMBS and UROS were initially tested in the couple-enzyme assay (as described in section 2.4) with no detectable product. Subsequently, the GST-tag was cleaved from each of the purified proteins to be trialed for activity in the assay. Due to the failure of *Pf*HMBS to be expressed, subsequent development of the assay was put on hold and ultimately was not completed. Future work will aim to continue development of the coupled-enzyme assay using the UROS and HMBS sources purified in this study.



GST cleaved = HMBS without GST

Figure 3.8 Expression and purification of Human HMBS-pGEX fusion protein

(A) Coomassie stained SDS-PAGE gel of protein lysate from a selected HMBS clone that was uninduced (1); induced with 1mM IPTG for 1 hour at 37°C, with an arrow showing the induced protein band (2); insoluble HMBS protein from the induced clone (3); insoluble HMBS protein from bacterial lysate (4); soluble HMBS protein that was subsequently purified on a glutathione sepharose column; flow through of unbound protein from the column purification (5) and purified HMBS-pGEX protein at the estimated 66kDa (6).

(B) Western blotting of purified HMBS using an anti-HMBS antibody after cleavage of the GST tag. The first elution after on column purification and cleavage demonstrates both uncleaved (66kDa) and cleaved (39kDa) HMBS (1), while the second elution (2) only contains pure HMBS with no residual GST tag.

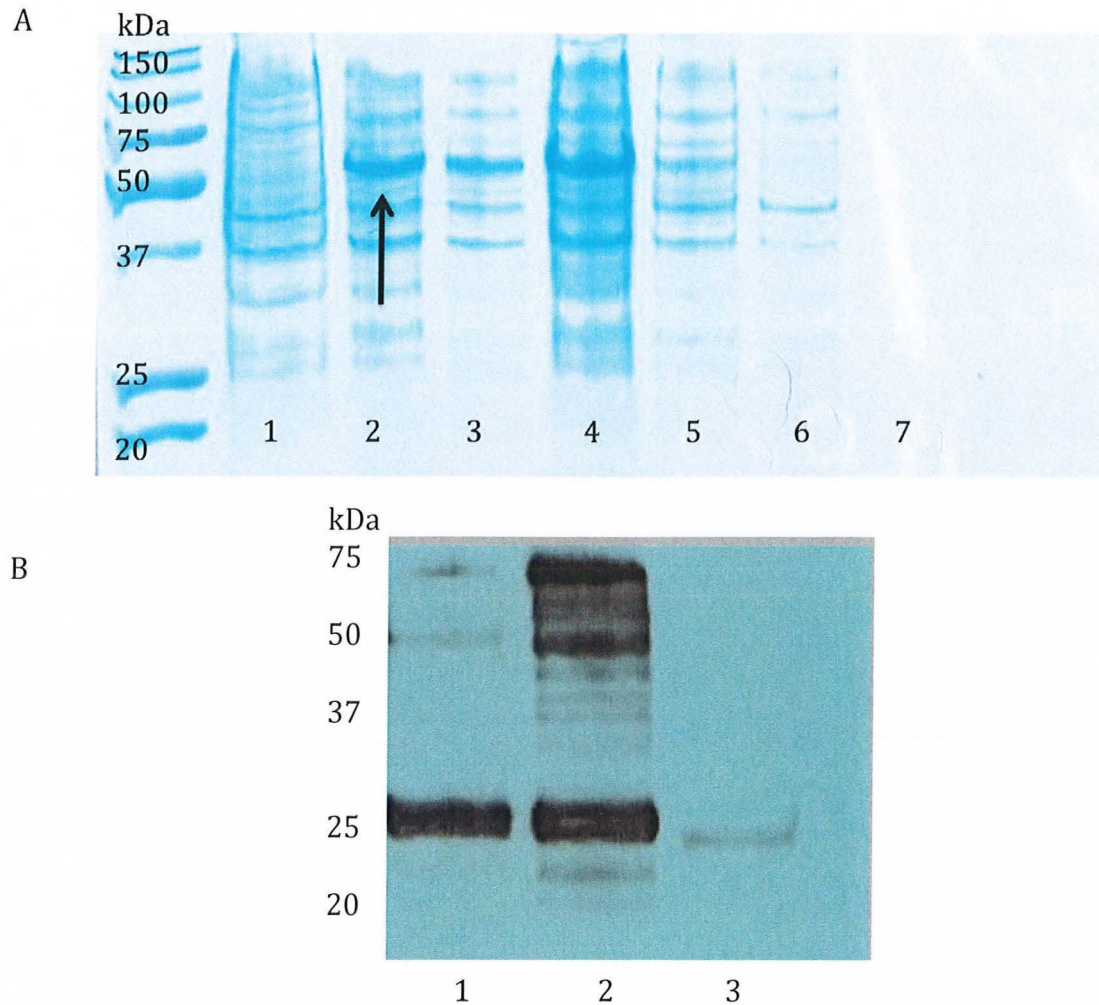


Figure 3.9 Expression and purification of Human UROS-pGEX fusion protein

(A) Coomassie stained SDS-PAGE gel of lysate from an uninduced UROS-pGEX clone (1); induced with 1mM IPTG for 1 hour at 37°C, with an arrow showing the induced fusion protein band at 55 kDa (2); soluble UROS protein (3); insoluble UROS protein from the induced clone (4); flow-through eluate of unbound protein from the column purification (5) and a washing step (6). There was no visible band in the final elution product (7).

(B) Western blotting of purified UROS with an anti-UROS antibody after cleavage of the GST tag. The first and second elutions off the column (1 and 2) show a band at the expected size of 26kDa but both have some residual uncleaved UROS-pGEX protein, as compared to RBC lysate run in (3).

Discussion

This chapter investigated the intraerythrocytic parasite localisation of several host red cell enzymes from the haem biosynthetic pathway, namely ALAD, FECH and UROS. Host ALAD and FECH were previously demonstrated to be localised in the parasite (Dhanasekaran et al., 2004, Varadharajan et al., 2004) and results in this chapter confirmed this. The *P. falciparum* genome does not contain a gene for *UROS*, therefore red cell UROS was hypothesised to be scavenged by the parasite. Results from this chapter demonstrated that host UROS is present in the mature red cell and is also located in *Plasmodium* during the growth-stages of parasite development.

Haem synthetic enzymes are present in the mature red cell

Results in this thesis demonstrated the host haem enzymes ALAD, FECH and UROS are present in the mature red cell. It was important to establish this, considering the mature red cell does not require an active biosynthetic pathway. If these enzymes were not present in the mature red cell, they obviously would not be available to be scavenged by the parasite as this chapter proposed. Results from Western blotting and immunofluorescence studies using antibodies against the host ALAD and UROS demonstrated that the enzymes were present throughout the uninfected red cell. These results were consistent with the cytosolic location of ALAD and UROS in progenitor red cells. FECH was also demonstrated to be present in the mature red cell, which was unexpected considering that it is located on the inner and outer membrane of the mitochondria in progenitor red cells (Chen et al., 2010). A possible explanation for the presence of this mitochondrial enzyme is that some FECH remains when the mitochondria are degraded in exosomes prior to removal from the red cell; or alternatively, after FECH is post-translationally cleaved in the cytosol, not all of the enzyme may be translocated into the mitochondria for enzymatic function. As such, there may be some residual FECH remaining in the cytosol but this remains an unanswered question.

Host enzymes are localised in the parasite during growth

Both western blotting and immunofluorescence techniques were employed in this chapter to investigate the localisation of the host haem enzymes ALAD, FECH and UROS during intraerythrocytic *P. falciparum* infection. Western blotting was conducted using monoclonal antibodies against the human proteins. All of these anti-host enzyme antibodies detected the human enzyme in both uninfected red cells and the purified parasite protein. These results indicate that the host enzymes are firstly present in the mature red cell and secondly, that they are localised inside the parasite. Parasites for Western blotting experiments were prepared by Percoll gradient density purification (enabling the concentration of late-stage trophozoites and schizont parasites), followed by saponin treatment. Saponin is a detergent that lyses the red cell membrane and the parasitophorous vacuole membrane but not the parasite membrane (Hsiao et al., 1991). Serial washing steps then removed red cell cytosolic contaminants and parasitophorous vacuole (PV) contents. The removal of red cell contaminants from the parasite protein was confirmed with the use of an anti-hexokinase antibody, a red cell cytosolic enzyme. Therefore the host enzymes must be located inside the parasite plasma membrane, although the amount of each host protein was lower in the parasite preparation compared to the uninfected red cell preparation. Considering that equal cell numbers were loaded for SDS-PAGE analysis, the reduced host protein in the parasite may be due to the parasite metabolising the imported host protein. Alternatively, the percoll purification of parasites for Western blotting may have been yielded more trophozoites, which have not yet imported the host proteins (as evidenced by immunofluorescence studies below).

Immunofluorescent analysis of thin blood smears of *P. falciparum* cultures, using the same human monoclonal antibodies, confirmed the western blotting results. ALAD, FECH and UROS staining were the same in uRBCs and early ring-stage parasites but were concentrated around the PVM marker (EXP2) by late-ring and trophozoite stage of infection. By the schizont stage, the enzymes were mostly absent from the remaining red cell compartment

and instead entirely surrounded or were within the PVM marker. The host staining was not evenly distributed throughout the parasite, indicative of further sub-cellular compartmentalisation of the host enzymes. Further work utilising other parasite markers (particularly organelle and digestive vacuole markers) may provide further insight. An additional observation was that each of the host enzymes had dissipated by the late-schizont stage, possibly due to the host enzyme being metabolised by the parasite. Further analysis and quantification of this observation is required.

One question arising from the western blotting and immunofluorescence work is if the host antibodies are also cross-reacting with the parasite enzymes. In the case of ALAD this is unlikely as the parasite and host ALAD amino acid sequence only share 31% sequence identity. Additionally, parasite protein versions have a larger molecular weight than the host version (53 kDa for the parasite ALAD, 37 kDa for the host ALAD). In contrast, the human and parasite FECH proteins both have the same predicted molecular weight although cross-reaction between the parasite and human protein is unlikely as the amino acid sequence between the host and parasite FECH only share 26% sequence identity. In the case of UROS, the host anti-UROS antibody showed a protein band in the parasite fraction at the correct predicted size for the human protein (~29kDa), indicating the host protein is present in the parasite. Additionally, the monoclonal antibody against the human protein is unlikely to be cross-reacting with a plasmodial UROS, as there have been no genes coding for UROS annotated in the *Plasmodium* genome. Protein alignment tools have also not revealed any parasite proteins homologous to that of the human UROS.

A role for host haem enzymes in parasite intraerythrocytic development

ALAD and FECH were previously demonstrated to be scavenged by the parasite (Bonday et al., 1997, Dhanasekaran et al., 2004, Varadharajan et al., 2004). It was also previously demonstrated that the host enzymes account for most of the total ALAD and FECH enzyme activity in the parasite (Dhanasekaran et al., 2004, Varadharajan et al., 2004). This finding has been received with considerable skepticism considering the parasite contains

genes encoding functional ALAD and FECH (Sato and Wilson, 2003, Sato and Wilson, 2002, van Dooren et al., 2006). Results presented here confirm that host ALAD, FECH and UROS are localised in the parasite during growth, with the host enzyme entirely within the parasite by the schizont stage of infection. Host UROS was also demonstrated to be localised within the parasite during intraerythrocytic growth, which has not been previously reported. Further questions remain regarding potential transport mechanisms and functional necessity of these host haem synthetic enzymes.

The uptake of host haem synthetic enzymes may be a passive process with the endocytosis of haemoglobin and other red cell contents or through active transporters. One report by Bonday and colleagues identified a 65-kDa protein on the surface of *P. berghei* membrane that specifically bound host ALAD. They further identified that a truncated fragment of host ALAD competed with native red cell ALAD for binding to the parasite membrane, with parasite haem synthesis and subsequent growth impaired after the uptake of the truncated ALAD (Bonday et al., 2000). This work demonstrates the existence of transporters allowing the importation of host haem synthetic enzymes and also showed that the uptake of host ALAD is important for parasite haem synthesis. Equivalent transporters in *P. falciparum* have not been described and the specificity of the described transporter warrants further investigation. Future work could utilise live-cell imaging and further parasite organelle markers in conjunction with phase-contrast or confocal imaging to further investigate the uptake and final parasite colocalisation of these host haem synthetic enzymes.

Regardless of how the host haem synthetic enzymes are transported into the parasite, a further question remains whether the host enzymes play a functional role in the parasite's own biosynthetic pathways. During intraerythrocytic growth the parasite has a high haem requirement, as haem is required for protein synthesis and as a cofactor for cytochrome c oxidase and cytochrome P450 (Surolia and Padmanaban, 1992). As these three host enzymes are localised in the parasite during growth, they may also play an essential role in supplementing the haem biosynthetic requirements of the

parasite during intraerythrocytic development. This remains to be confirmed; especially considering the parasite contains genes encoding active versions of FECH and ALAD (Sato et al., 2004, Sato and Wilson, 2003). Work in the next chapter aims to establish if these host enzymes are required by the parasite for normal intraerythrocytic growth.

Investigation of the UROS activity of PfHMBS

The absence of a gene coding for UROS in the *P. falciparum* genome has been a continuing question in parasite biology (Gardner et al., 2002, van Dooren et al., 2006). UROS is an essential enzyme in the haem pathway responsible for the cyclisation and inversion of the linear hydroxymethylbilane into the porphyrin ring, forming the basis of the haem molecule (Tsai et al., 1988). Work in this chapter has presented novel evidence that the host UROS is localised in the parasite during the late stages of intraerythrocytic infection. If *Plasmodium* can simply co-opt the host enzyme to maintain its metabolic haem requirements, it may in part explain the apparent absence of a parasite-encoded UROS.

Additional evidence has demonstrated that the parasite encoded HMBS (*PfHMBS*) is a dual function enzyme that also possesses UROS enzymatic activity. Nagaraj and colleagues expressed a N-terminus truncated *PfHMBS* and found it possessed a low level of UROS activity (Nagaraj et al., 2008). The *PfHMBS* protein was found to have extra residues that may be responsible for UROS activity. They also found that the UROS activity of *PfHMBS* was much lower than that of the host red cell enzyme. It may be possible that the parasite *PfHMBS* can yield enough UROS activity to keep minimal haem synthesis occurring while the parasite is not in a host red cell where it can freely scavenge the more efficient host enzyme.

Work in this chapter attempted to further investigate the previous report that *PfHMBS* contained UROS activity. A coupled enzyme assay was being developed in conjunction with *PfHMBS* expression, in order to measure UROS activity. The assay development included cloning, expressing and purifying human UROS and HMBS, which was successful. *PfHMBS* was successfully

cloned into the expression vector but ultimately this protein was unable to be expressed; therefore the potential UROS activity could not be examined. Further work is needed to optimize induction of the fusion protein to verify the dual-enzyme activity of this enzyme and continue testing the coupled enzyme assay with purified UROS and HMBS cleaved from the GST-tag to allow activity. The contribution of the *Pf*HMBS and host UROS for overall parasite UROS activity and ultimately haem synthesis may then be examined.

Summary

Overall this work verifies the previous reports that host ALAD and FECH are scavenged by the parasite (Bonday et al., 2000, Bonday et al., 1997, Varadharajan et al., 2004). The observations here also suggest that the import of these enzymes is dependent on the developmental stage of the parasite, with greater amounts present at later stages of parasite development. An additional finding of this chapter was that host UROS is also localised in the parasite during intraerythrocytic growth. This has not previously been demonstrated. If *Plasmodium* can scavenge the host enzyme, this may go some way toward explaining the absence of a parasite-encoded UROS. Work in the next chapter aims to establish if these host enzymes are required by the parasite for normal intraerythrocytic growth.

Chapter 4.

Host haem enzyme deficiencies and *Plasmodium* growth

Introduction

Results in the previous chapter of this thesis support published data that the red cell haem enzymes ferrochelatase (FECH) and aminolevulinate dehydratase (ALAD) are localised in the parasite during intraerythrocytic growth. This was also demonstrated for additional host haem synthetic enzyme uroporphyrinogen-III synthase (UROS). These three host haem biosynthetic enzymes may be scavenged by *Plasmodium* as an essential growth requirement for parasite metabolism. This chapter proposes that parasite growth is impaired in red cells deficient in any of these haem enzymes. The requirement of host haem enzymes for sustenance of intraerythrocytic malarial infection was investigated in this chapter by examining parasite growth in red cells deficient in these enzymes, using both human and mouse models of haem enzyme deficiency.

Porphyria is a rare disease caused by severe deficiencies in any one of the eight haem biosynthetic enzymes due to genetic or environmental factors (Puy et al., 2010). Each enzyme deficiency results in the accumulation of toxic porphyrin precursors, manifesting in different presentations of porphyria (Figure 4.1). The porphyrias can be grouped into hepatic or erythropoietic, depending on the location of substrate porphyrin accumulation (Kauppinen, 2005).

This investigation focused on deficiencies involving the three haem enzymes that were previously determined to be localised in the intraerythrocytic parasite; ALAD, FECH and UROS. While all three deficiencies are rare, samples were available from UROS and FECH deficient patients (For overview of these porphyrias, see table 4.1). ALAD deficiency is particularly rare (with about six reported cases worldwide) and samples were not available for this study.

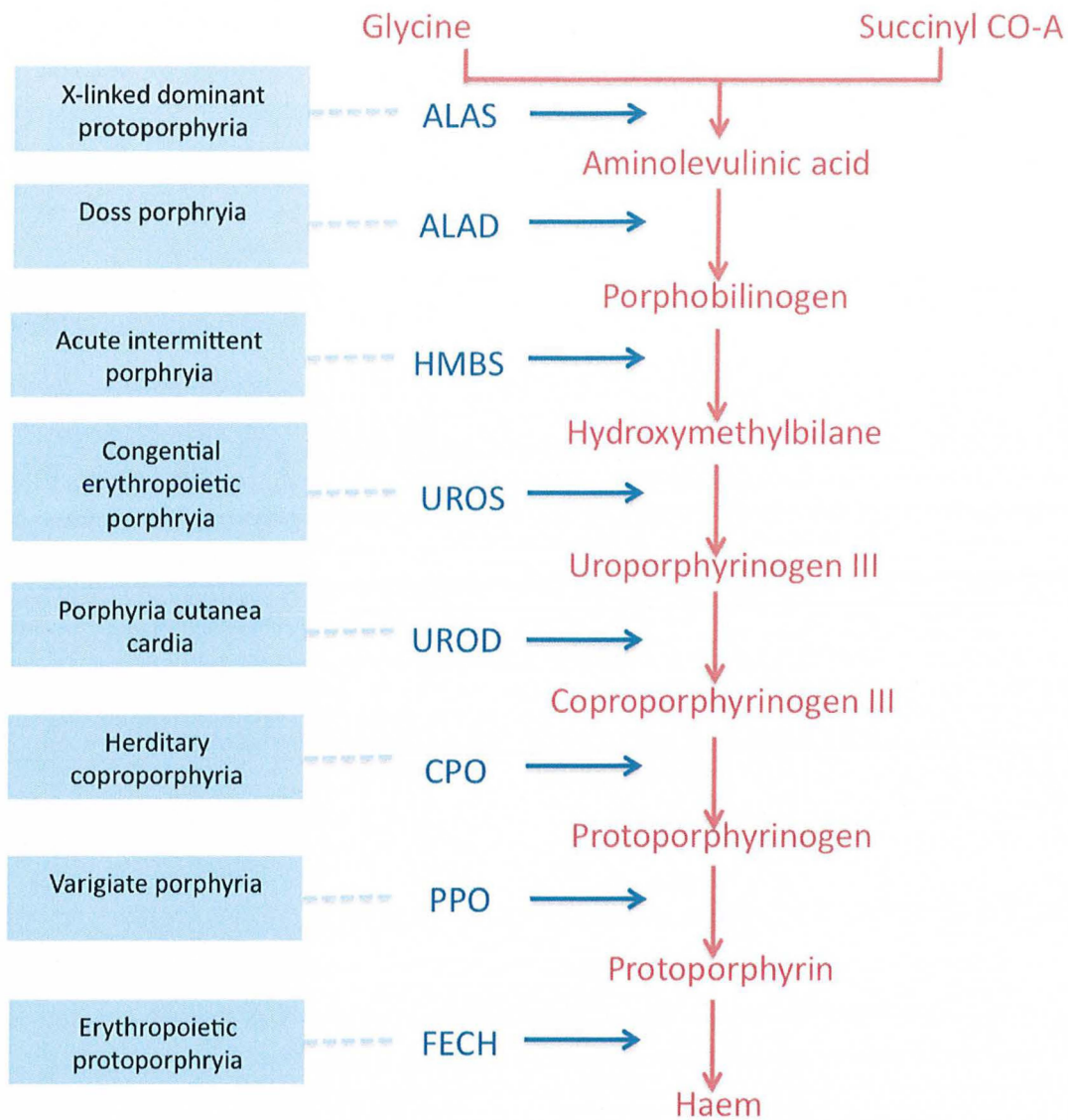


Figure 4.1. The haem biosynthetic pathway with corresponding enzymes and porphyria. In red are substrates, blue is haem synthetic enzymes and blue boxes the corresponding clinical porphyria syndrome manifesting as the deficiency or absence of each of the enzymes. ALAS, aminolevulinate synthase; ALAD, aminolevulinate dehydratase; HMBS, hydroxymethylbilane synthase; UROS, uroporphyrinogen III synthase; UROD, uroporphyrinogen decarboxylase; CPO, coproporphyrinogen oxidase; PPO, protoporphyrinogen oxidase; FECH, ferrochelatase

**Table 4.1 Characteristics of each of the haem enzymes deficiencies used
in this study**

Enzyme deficiency	Porphyria	Mode of inheritance	Prevalence	Symptoms
UROS	Congenital erythropoietic porphyria (CEP), also called Gunther's disease	Autosomal recessive	1:1,000,000	Anemia, Hepatosplenomegaly, Erythrodontia, Cutaneous photosensitivity
FECH	Erythropoietic protoporphyria (EPP)	Predominantly autosomal dominant (requires mutant allele co-inherited with common hypermorphic allele); Also rarer autosomal recessive cases	1:75,000 (The Netherlands) 1:200,000 (Wales)	Cutaneous photosensitivity, Liver disease in severe cases

In addition to the human EPP patient blood samples obtained for this investigation, the availability of a FECH deficient mouse provided an ideal model in which to further examine the role of host FECH in an *in vivo* malarial infection. The *Fech*^{m1Pas} mice, generated from an ENU-mutagenesis screen, were kindly provided by X. Montagutelli (The Pasteur Institute, Paris, France). *Fech*^{m1Pas} have an A to T substitution at nucleotide 293, which leads to a methionine to lysine substitution at position 98 (mutation M98K) in the murine *Fech* homolog (Boulechfar et al., 1993). The mutation results in 5% residual FECH activity in homozygotes and 45-65% FECH activity in heterozygotes (Tutois et al., 1991). The mice used in this study were maintained on an isogenic (C57BL/6) or mixed (C57BL/6 and BALB/c) background.

Overall this chapter sought to determine whether haem enzyme deficient red cells were able to support malaria parasite growth. *P. falciparum* growth was quantified in red cells deficient in UROS and FECH using an *in vitro* parasite culture system. *Fech*^{m1Pas} mice were infected with the murine malaria parasite, *P. chabaudi*, to investigate the requirement of FECH during an *in vivo* malaria infection.

Results

Reduced Plasmodium growth in UROS-deficient human red cells

Congenital erythropoietic porphyria (CEP; also known as Gunther's disease) is a rare condition, with only ~150 cases reported worldwide to date (Wiederholt et al., 2006). One patient with confirmed CEP (Homozygous for a mutation in *Uros*), based in Australia, was recruited into the study (See table 4.2; CEP 001). Both CEP 001 and non-porphyria control blood were infected with synchronized late stage (trophozoite and schizont) *P. falciparum* (3D7 strain) and grown for a further 72 hours. Overall average parasite growth was significantly impaired in CEP 001 blood at each time point, after the addition of parasites, relative to the normal blood control (Figure 4.2 A). At T=24, parasites in UROS deficient blood had grown to only 45 % of the control blood levels. A reduction in new ring-stage parasites in the UROS deficient cells was also observed at this time-point, indicating that parasite invasion of new red cells may be impaired (Figure 4.2B). The number of new rings at the second round of reinvasion (at T=72) was also lower in CEP blood than the control, confirming that overall reinvasion was impaired in CEP blood (Figure 4.2B). Additionally, parasites grown in CEP blood did not have the same proportion of ring to trophozoite stage parasites as the control at T=48 and 72, indicating overall growth was also impaired in CEP blood (Figure 4.2B).

Table 4.2 Overview of porphyria patient samples obtained for this study.

Patient #	Porphyria	Specific enzyme activity*	Porphyrins in RBC nmol/L RBC (Normal ref:<1.8nmol/L)
CEP 001	CEP	Unknown	Not available
EPP 001	EPP	Unknown	37.6
EPP 002	EPP	Unknown	84.8
EPP 003	EPP	Unknown	43.9
EPP 004	EPP	Unknown	17.9
XLDPP 001	XLDPP	ALAS2: +200%*, FECH: 4.1*	86.3
XLDPP 002	XLDPP	ALAS2: +200%*, FECH: 3.7*	51.2
XLDPP 003	XLDPP	ALAS2: +300%*, FECH: 3.9*	25.6

- : - ALAS2 mutants were expressed as % of wild-type activity using prokaryotic expression vector as previously described (Cotter et al., 1994); N: 100%

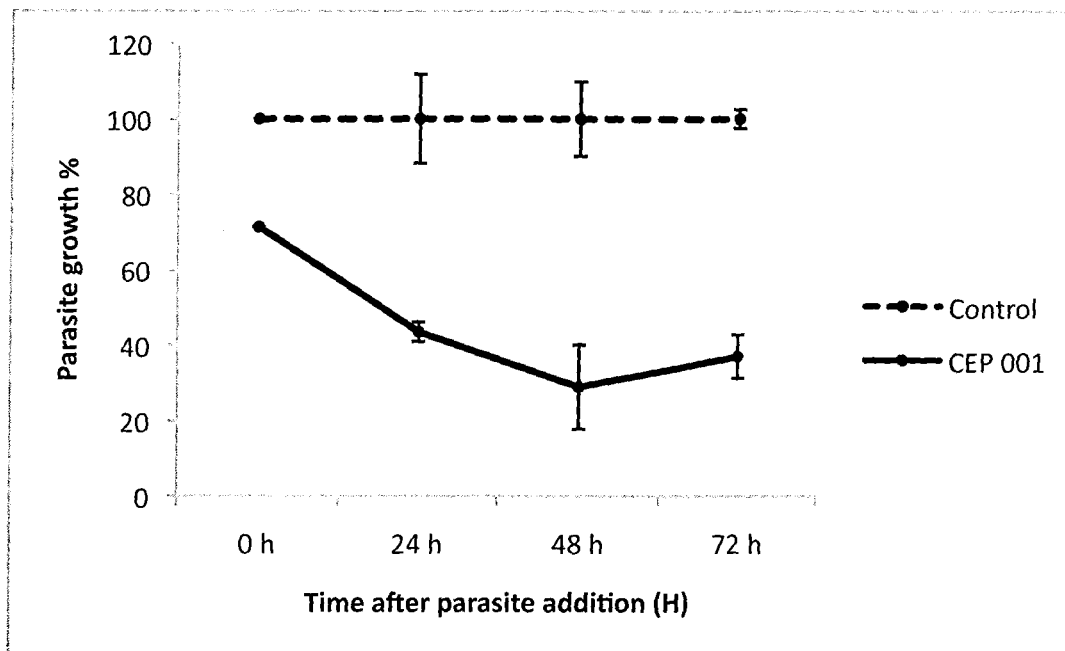


Figure 4.2 *P. falciparum* growth in UROS deficient blood.

Parasite growth is impaired when cultured in red cells from an individual with UROS deficiency (congenital erythropoietic porphyria; CEP 001).

Average percent parasite growth was determined at different time points after *in vitro* infection of red blood cells purified from blood samples collected from one individual with CEP, and compared to similarly infected red cells from a non-porphyrin control individual. Parasite growth relative to the normal control was significantly impaired in the UROS-deficient blood at all time-points (T=24 p=0.0093; T=48 p=0.0088; T=72 p=0.0005).

Data represents the mean of triplicate cultures at each time point; at least 900 cells were counted for each replicate. Error bars indicate SEM.

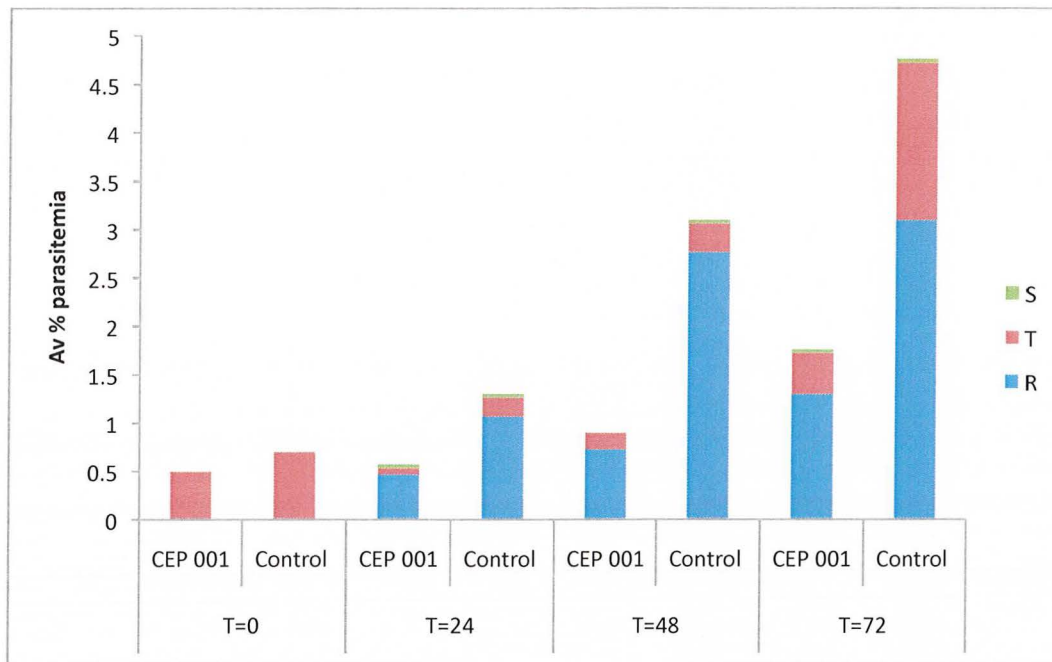


Figure 4.3 Individual growth stages of *P. falciparum* in UROS deficient blood.

Parasite invasion and growth is impaired when cultured in red cells from an individual with UROS deficiency (congenital erythropoietic porphyria; CEP 001).

Average % infected red cells (% parasitaemia) including percent schizonts (S), trophozoites (T) and rings (R) at each time point for UROS deficient and control blood samples. Blood was infected at T=0 with late stage parasites (Trophozoite-schizont). UROS deficient blood has a lower % of rings at T=24 and T=72, indicating invasion is impaired ($p=0.001$ and 0.004). Additionally, a lower proportion of trophozoites at T= 72, was observed in the UROS deficient blood, indicative of impaired growth ($p= 0.004$).

Host FECH deficiencies impair normal parasite growth

Human studies

There are ~20 known EPP cases in Australia. Four patients with laboratory confirmed *FECH* mutations were recruited into this study, all compound heterozygotes (inheriting one deleterious *FECH* mutation and the common low expression allele, IVS3-48 T/C) (Table 4.2). Collaborator Ingrid Winship confirmed the *FECH* mutations in each patient and conducted total RBC porphyrin analysis. The total RBC porphyrin levels were above the normal reference value in all four patients (>1.8 nmol/L; table 4.2), a characteristic of EPP due to decreased FECH with subsequent substrate build-up.

Purified red cells from EPP001-EPP004 and non-porphyria control bloods were infected with late stage (trophozoite and schizont) *P. falciparum* (3D7 strain) and grown for a further 72 hours. Erythrocytes from the four individuals with EPP displayed impaired *P. falciparum* growth *in vitro* compared to normal controls at all time-points (Figure 4.4 A, B, C and D). Further analysis of individual stages of parasite development in each EPP patient sample demonstrated a reduction in new ring-stage parasites compared to each control at T=24 (Figure 4.5 A, B, C and D). This was also observed at T=72, when a second round of reinvasion occurred in the control samples. Together, this data indicates that invasion is impaired in FECH deficient blood. The progression of parasites from the ring stage to trophozoite and schizont stage of development was variable between the four patients but generally parasites grown in the EPP patient blood did not demonstrate the same proportion of late-stage parasites as the control at T=48, indicating growth may also be impaired. Overall, results in the 4 EPP blood samples indicate that *P. falciparum* intraerythrocytic growth is severely compromised in FECH deficient blood.

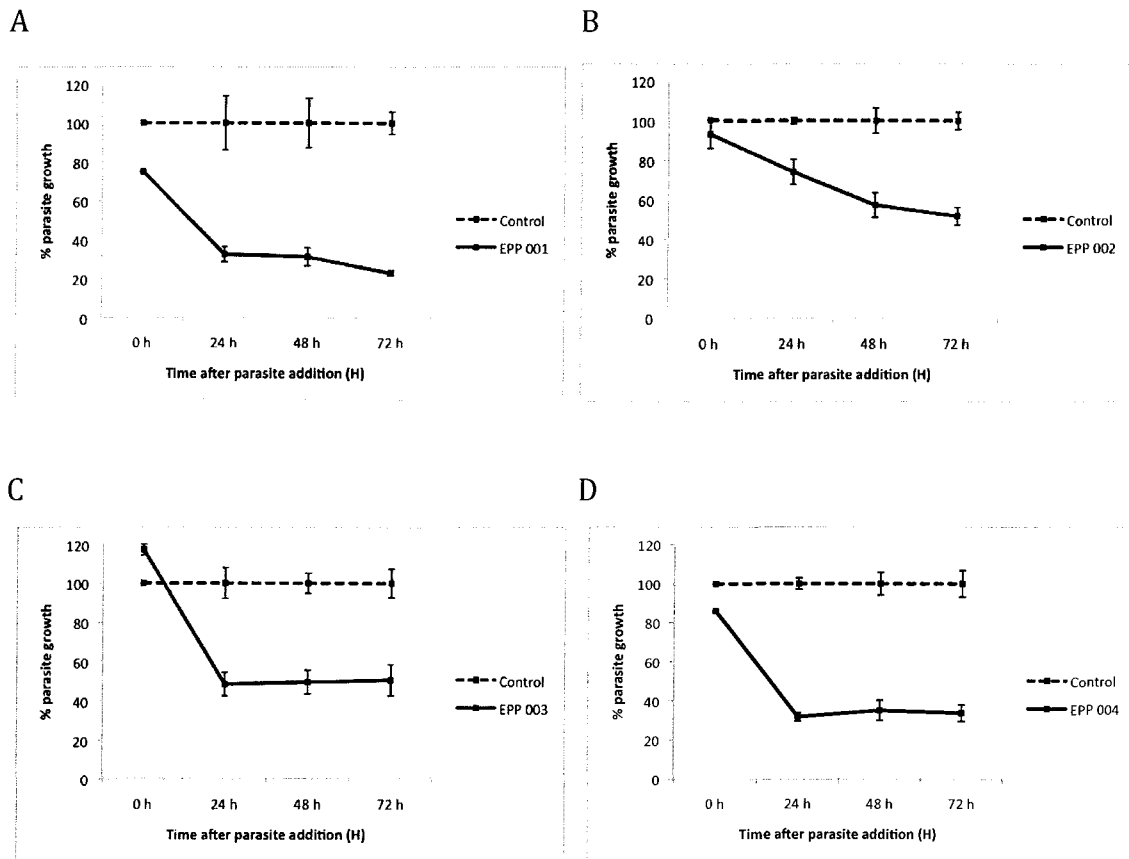


Figure 4.4 *P. falciparum* growth in FECH deficient blood

Parasite growth is impaired when cultured in red cells from individuals with FECH deficiency (Erythropoietic protoporphyria; EPP 001-004).

(A-D) Average percent parasite growth was determined at different time points after *in vitro* infection of red blood cells purified from blood samples collected from 4 individual with EPP, and compared to similarly infected red cells from four non-porphyric control individuals. Parasite growth was significantly impaired in the FECH-deficient blood compared to normal blood control by 72 h after parasite addition in all four EPP blood samples (P-values at T=72: EPP 001 $p=0.00020$; EPP 002 $p=1.58708E-05$, EPP 003 $p=0.0010$; EPP 004 $p=0.0011$).

Data represents the mean of triplicate cultures at each time point (EPP 002 and EPP 003 were both independently repeated twice); at least 900 cells were counted for each replicate. Error bars indicate SEM.

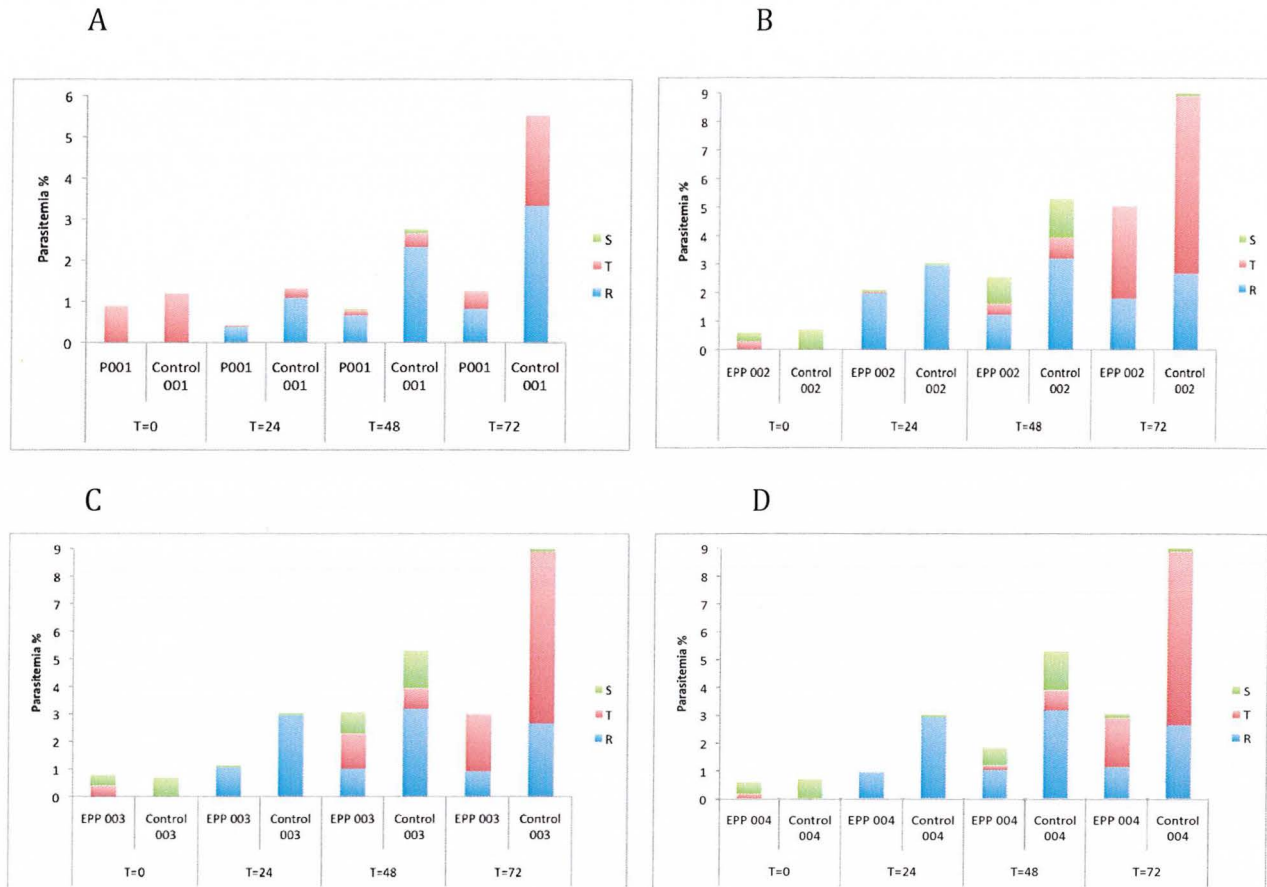


Figure 4.5 Individual growth stages of *P. falciparum* in FECH deficient blood

Parasite invasion is impaired when cultured in red cells from individuals with FECH deficiency (Erythropoietic protoporphyria; EPP 001-004).

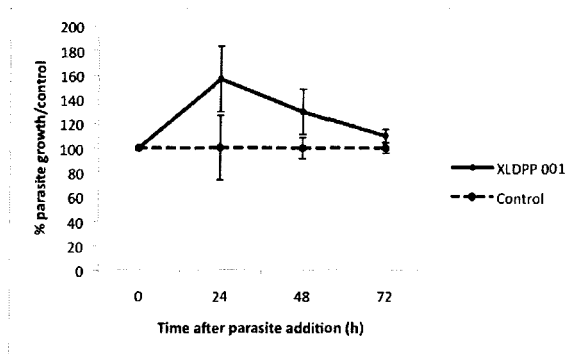
(A-D) Average % infected red cells (% parasitaemia) including percent schizonts (S), trophozoites (T) and rings (R) at each time point for each individual FECH deficient and control blood patient sample. Blood was infected at T=0 with late stage parasites (Trophozoite-schizont). FECH deficient blood had a lower proportion of new rings at T=24 and 72 compared to control blood, indicating invasion is impaired in the EPP red cells (T=24 and T=72 $p < 0.05$ for all EPP individuals).

Data represents the mean of triplicate cultures at each time point, from the same experiment as shown in figure 4.4 (note that results presented in the above graphs are from one of the independent repeats of EPP 002 and EPP 003). At least 900 cells were counted for each replicate.

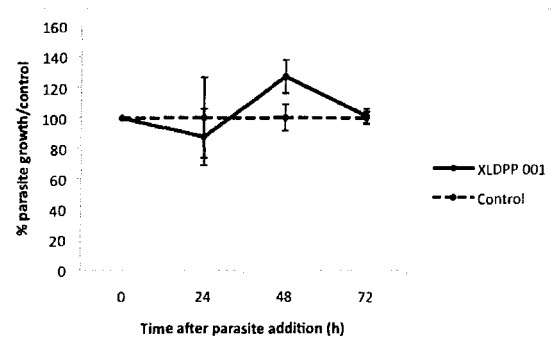
Parasite growth impairment is independent of protoporphyrin

A characteristic of EPP is a significant increase in red cell protoporphyrin additional to the FECH deficiency. To investigate if impaired parasite growth in EPP blood was due to FECH deficiencies or a direct inhibitory effect of protoporphyrin, *P. falciparum* was additionally grown in blood samples from patients with X-linked dominant protoporphyria (XLDPP); a recently recognised porphyria caused by gain of function deletions in aminolevulinate synthase (ALAS2) (Whatley et al., 2008). ALAS2 is the major rate-limiting enzyme in the haem biosynthetic pathway, therefore gain of function deletions in the C-terminus of the ALAS2 protein inhibit its ability to regulate the pathway. This leads to greatly increased substrate presentation to the remainder of the pathway (Whatley et al., 2008). The next rate-limiting enzyme is FECH; therefore XLDPP patients demonstrate a buildup of protoporphyrin (with normal FECH). When grown in the XLDPP red cells, parasites showed the same growth kinetics as non-porphyria control blood (Figure 4.6 A, B and C; Figure 4.7 A, B and C), indicating that the growth inhibition seen in the EPP patient blood was due to host FECH deficiency and not increased protoporphyrin.

A



B



C

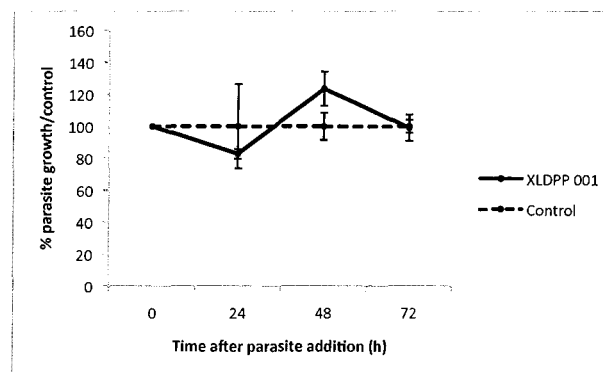


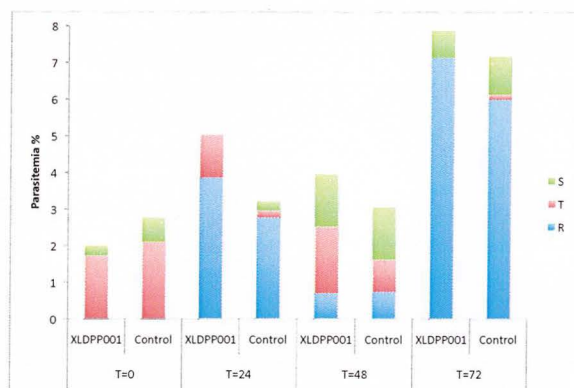
Figure 4.6 Parasite growth is not impaired when cultured in XLDPP patient blood

(A-C) Parasite growth is not significantly impaired when cultured in red cells from individuals with XLDPP (X-linked dominant protoporphyria; XLDPP 001-003).

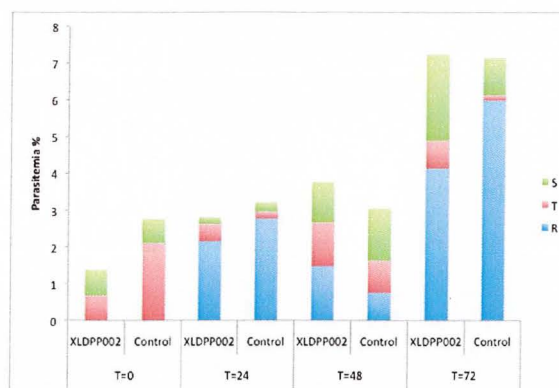
Average percent parasite growth was determined at different time points after *in vitro* infection of red blood cells purified from blood samples collected from three individuals with XLDPP, and compared to similarly infected red cells from a non-porphyric control individual. *P. falciparum* growth was not significantly impaired when cultured in blood from XLDPP individuals compared to a normal blood control.

Data is an average of parasite growth from 3 XLDPP patients compared to 1 non-porphyria control blood and represents the mean of triplicate cultures at each time point; at least 900 cells were counted for each replicate. Error bars indicate SEM.

A



B



C

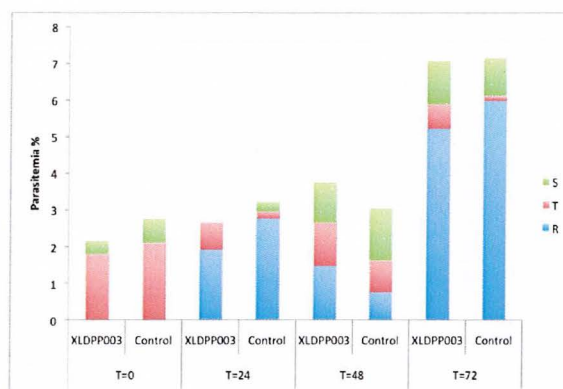


Figure 4.7 Individual stages of *P. falciparum* growth in XLDPP patient blood

(A-C) Average % infected red cells (% parasitaemia) including percent schizonts (S), trophozoites (T) and rings (R) at each time point for each XLDPP patient and the control blood sample. Blood was infected at T=0 with late stage parasites (Trophozoite-schizont). There was no significant difference of parasitaemia or stage of development between XLDPP and control blood samples at any of the time points after parasite addition.

Data represents the mean of triplicate cultures at each time point, from the same experiment as shown in figure 4.6. At least 900 cells were counted for each replicate.

Mouse studies

Mice carrying the *Fech*^{m1Pas} mutation were maintained on two different genetic backgrounds; inbred C57BL/6 and mixed (C57BL/6 and BALB/c). Mice homozygous for the *Fech*^{m1Pas} mutation (*Fech*^{-/-}), as well as heterozygous mice (*Fech*^{+/-}) were infected with *P. chabaudi* and the course of infection compared to similarly infected wild-type littermates (*Fech*^{+/+}). Parasite growth was monitored daily for each mouse over the course of infection. The dose of iRBCs was different for female and male mice, given that male mice are more susceptible to *P. chabaudi* infection (Stevenson et al., 1982, Stevenson et al., 1990).

In the isogenic background (C57BL/6) strain female *Fech*^{-/-} mice had a significant difference in average percent parasitaemia at the peak of infection (day 6) and a significant increase in survival compared to *Fech*^{+/+} and *Fech*^{+/-} mice (Figure 4.8 A and B). Similar to the females, male *Fech*^{-/-} mice also demonstrated a marked resistance to *P. chabaudi* infection, with a significant difference in average percent parasitaemia at the peak of infection (Day 8) and significantly increased survival compared to *Fech*^{+/+} and *Fech*^{+/-} (Figure 4.9 A and B).

In the mixed background strain, female *Fech*^{-/-} mice had a significant difference in average percent parasitaemia at the peak of infection compared to *Fech*^{+/+} mice (Figure 4.10 A). Interestingly mice heterozygous (*Fech*^{+/-}) for the *Fech* mutation (with ~45-65% residual FECH activity) also displayed the same course of infection as the *Fech*^{-/-} mice, with a significant decrease in percent parasitaemia at peak compared to wild-type mice. These results confirm that host FECH is important for malarial infection, regardless of the genetic background of the host. Male *Fech* mice on the mixed background were also challenged with *P. chabaudi* but continuing problems with frozen parasite stocks resulted in failed challenges (i.e. none of the control or mutant mice displayed evident parasitaemia).

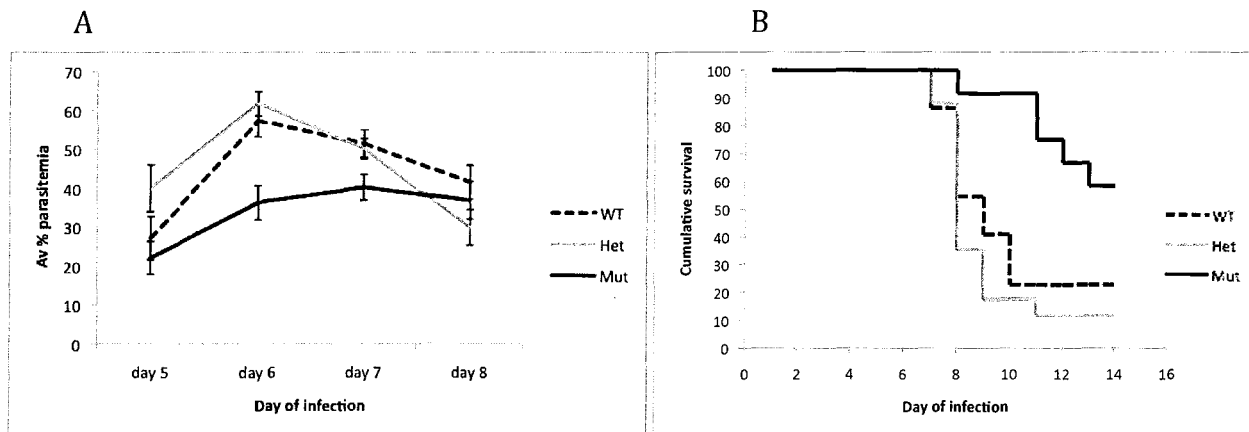


Figure 4.8 *P. chabaudi* infection kinetics and survival in female *Fech^{m1Pas}* mice (Isogenic background).

Homozygous female ferrochelatase deficient mice (C57BL/6 background) display a significantly lower infection and increased survival compared to mice with normal FECH levels when infected with a murine model of malaria, *P. chabaudi*.

(A) Parasitaemia (% of iRBC) of *P. chabaudi* infected female mice. Data represent the mean percentage of infected red cells in mice on the indicated day following infection with *P. chabaudi* (5×10^5 iRBC). The strains of mice used (all on a C57BL/6 genetic background) were: *Fech^{+/+}* (WT, n=22), *Fech^{+/-}* (Het, n=17) and *Fech^{-/-}* (Mut, n=12). There was a significant difference between *Fech^{-/-}* mice and both the *Fech^{+/+}* and *Fech^{+/-}* strains at the day of peak infection (female, day 6, $p < 0.003$). Error bars indicate SEM.

(B) Survival of *P. chabaudi* infected female mice from the same experiment as (A). Mice were infected with *P. chabaudi* (as above) and monitored for survival over the time period indicated. There was a significant difference in survival between *Fech^{-/-}* mice and both the *Fech^{+/+}* and *Fech^{+/-}* strains (female, $p < 0.01$ using Mantel-cox Log-rank test of survival).

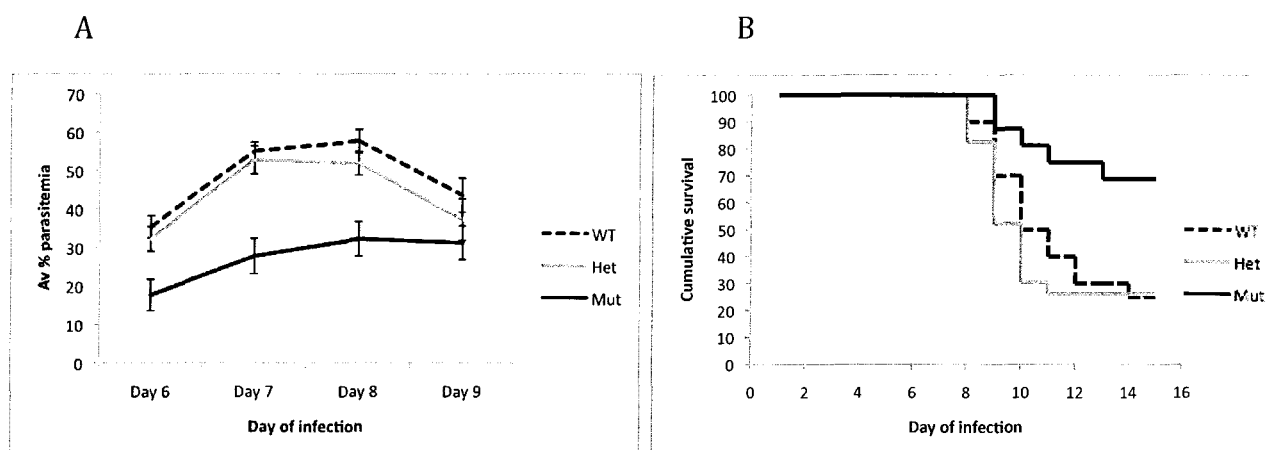


Figure 4.9 *P. chabaudi* infection kinetics and survival in male *Fech*^{m1Pas} mice (Isogenic background).

Homozygous male ferrochelatase deficient mice (C57BL/6 background) display a significantly lower infection and increased survival compared to mice with normal FECH levels when infected with a murine model of malaria, *P. chabaudi*.

(A) Parasitaemia (% of iRBC) of *P. chabaudi* infected male mice. Data represent the mean percentage of infected red cells in mice on the indicated day following infection with *P. chabaudi* (Male 2.5×10^5 iRBC). The strains of mice used (all on a C57BL/6 genetic background) were: *Fech*^{+/+} (WT, n= 20), *Fech*^{+/-} (Het, n= 23) and *Fech*^{-/-} (Mut, n= 16). There was a significant difference between *Fech*^{-/-} mice and both the *Fech*^{+/+} and *Fech*^{+/-} strains at the day of peak infection (male, day 8, $p < 0.002$). Error bars indicate SEM.

(B) Survival of *P. chabaudi* infected male mice from the same experiment as (A). Mice were infected with *P. chabaudi* (as above) and monitored for survival over the time period indicated. There was a significant difference in survival between *Fech*^{-/-} mice and both the *Fech*^{+/+} and *Fech*^{+/-} strains (male $p < 0.01$ using Mantel-cox Log-rank test of survival).

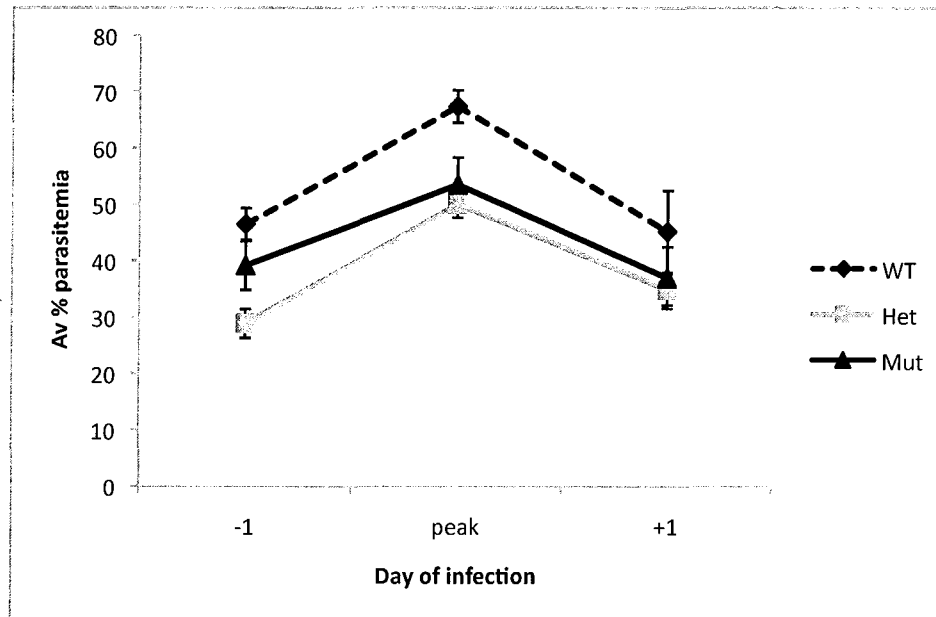


Figure 4.10 *P. chabaudi* infection kinetics in female *Fech^{m1Pas}* mice (mixed background)

Mixed background *Fech^{-/-}* and *Fech^{+/-}* mice (C57BL/6 / BALB/c) display a significantly lower infection compared to *Fech^{+/+}* mice when infected with a murine model of malaria, *P. chabaudi*.

Parasitaemia (% of iRBC) of *P. chabaudi* infected female mice. Data represent the mean percentage of infected red cells in mice normalised for the peak of infection from four independent challenges with *P. chabaudi* (5×10^5 iRBC). The strains of mice used (on a mixed C57BL/6 and BALB/c genetic background) were: *Fech^{+/+}* (WT, n=16), *Fech^{+/-}* (Het, n=14) and *Fech^{-/-}* (Mut, n=14). The *Fech^{-/-}* and *Fech^{+/-}* mice both displayed a significantly lower % parasitaemia at the peak of infection, compared to *Fech^{+/+}* ($p < 0.01$). Error bars indicate SEM.

Discussion

The previous chapter of this thesis investigated the intraerythrocytic localisation of the host haem synthetic enzymes ALAD, FECH and UROS but it is unknown whether these host enzymes are required for normal parasite growth. This chapter aimed to investigate the growth of parasites in haem synthetic enzyme deficient red cells, using genetic models of UROS and FECH deficiency. UROS and FECH deficient human red cells were infected *in vitro* with *P. falciparum*, while FECH deficient mice were infected with an *in vivo* murine model of malaria, *P. chabaudi*. Results in this chapter demonstrate dramatically reduced parasite growth in the UROS and FECH deficient models, indicating that the host enzymes are important for normal intraerythrocytic infection and may play a functional role in the parasite.

Host FECH and UROS deficiencies inhibited parasite growth

Haem enzyme deficiencies manifest as porphyrias in susceptible humans and mice. UROS deficiencies result in congenital erythropoietic porphyria (CEP) while severe FECH deficiencies lead to erythropoietic porphyria (EPP). This study utilised available blood samples from human patients with CEP and EPP, as well as a mouse model of FECH deficiency to investigate the importance of these host enzymes in malarial infection. Complete absence of enzyme activity is not compatible with life; therefore although these red cells have enzyme deficiencies, they contain some residual enzyme activity. Human red cells were infected with late stage *Plasmodium falciparum* parasites (trophozoites and schizonts) *in vitro*. Parasites in the enzyme-deficient red cells displayed overall lower growth relative to normal blood controls. Parasite growth was also impaired in the FECH deficient mice, with mice homozygous for the *Fech* mutation (and 5% residual FECH activity) displaying a significantly lower course of infection and increased survival compared to the wild-type mice (with normal FECH levels).

The developmental stages of intraerythrocytic *P. falciparum* growth were analysed in the human haem synthetic enzyme deficient red cells. Results demonstrate that parasite invasion was impaired in both UROS and FECH deficient red cells, as evidenced by the lower percentage of new rings 24 and 72 hours after parasite addition. It is difficult to conclude if invasion *per se* is

impaired or if this is due to impaired progression of late-stage parasites (trophs to schizonts), subsequently resulting in reduced ring-stage parasites. To further dissect the mechanism of impairment, whether invasion or growth, additional future studies could add purified merozoites to the haem enzyme deficient red cells in a specific invasion assay, recently described (Riglar et al., 2011). Additionally, TUNEL-staining could be used to investigate whether parasites are undergoing apoptosis in the UROS or FECH deficient red cells.

Parasite growth inhibition in FECH-deficient red cells is independent of protoporphyrin

Results from this chapter demonstrate that the parasite growth impairment in EPP red cells is due to FECH deficiency, and not due to protoporphyrin substrate levels. It was important to make this distinction, to rule out the possibility that protoporphyrin itself was not directly inhibiting growth of the parasite, considering that EPP patient red cells contain high protoporphyrin (Table 4.2). Parasites grown in red cells from patients with XLDPP, with normal FECH but increased RBC protoporphyrin, showed the same rates of growth compared to normal control blood. This indicates that protoporphyrin does not affect parasite growth and confirms that the parasite impairment demonstrated in EPP patient cells is due to FECH deficiency.

Host resistance to *P. chabaudi* infection is irrespective of genetic background

The importance of host FECH was also demonstrated in FECH deficient mice infected with *P. chabaudi*. *Fech*^{-/-} mice (with ~5% residual FECH activity) displayed a lower peak of infection and increased survival compared to *Fech*^{+/+} mice (with normal FECH activity). The protective effect of the FECH deficiency was observed irrespective of the genetic background of the mouse. Since this factor is known to strongly influence the host response and resistance to infection (Stevenson et al., 1982, Stevenson et al., 1990), FECH deficiency must play an important role in the infection. The protective effect was also observed in both female and male mice. The lower peak parasitaemias can be explained by reduced rates of parasite growth and/or invasion, consistent with the parasite growth and invasion differences in the *P. falciparum*-infected EPP human cells. This therefore suggests that like its human-host-specific counterpart, *P. chabaudi*

also utilizes host red cell FECH for its own growth requirements. Interestingly, heterozygous mice on the mixed background exhibited the same course of infection as the resistant mutant mice, possibly due to the wide range of FECH activity in the heterozygous mice (~45-65% activity)(Tutois et al., 1991), or alternatively due to the effect of a modifier gene co-segregating with the mutant *Fech* allele in mixed background mice. Together these results demonstrate the importance of red cell FECH for normal malarial infection, regardless of the genetic background of the host.

Contribution of parasite vs. host enzyme to overall parasite growth

Results presented in this chapter demonstrate overall impaired parasite growth in FECH or UROS deficient host cells; however, parasite growth was not completely abolished in these red cells. This may be due to the small amount of residual host enzyme activity, as complete absence of these enzymes is not compatible with life. Alternatively, the parasite-encoded enzymes may provide sufficient enzyme activity to sustain limited infection. It has been demonstrated that *P. falciparum* encodes an active FECH that is localized to the mitochondria (Sato and Wilson, 2003, Nagaraj et al., 2009b) but additional studies found that the imported host version of FECH accounts for up to 80% of the parasite's FECH activity (Varadharajan et al., 2004). Work in this chapter provides additional evidence that the majority of FECH activity in the parasite is provided by the host enzyme, considering that parasites have impaired growth in FECH deficient red cells and FECH deficient mice.

Unlike the case with FECH, the parasite does not contain a gene coding for UROS but previously published work has demonstrated that parasite encoded HMBS (*PfHMBS*) contains UROS activity, potentially alleviating the need for a separate functional UROS (Nagaraj et al., 2008). However, the host enzyme activity was 25-30 fold higher than that of the dual function *PfHMBS* (Nagaraj et al., 2008). Considering the low UROS activity of the *PfHMBS* compared to that of the red cell enzyme, results in this chapter demonstrate that the parasite enzyme is insufficient to sustain growth. This indicates the parasite has an absolute requirement for host UROS for normal intraerythrocytic growth and replication. Overall, for both UROS and FECH it cannot be definitely concluded that the host

version of these enzymes are exclusively used by the parasite but results in this chapter indicate that it is likely they comprise a major source of the parasite's haem synthetic capacity.

In order to further investigate the contribution of host or parasite encoded FECH for parasite haem synthesis; it would be useful to create a *FECH* and *HMBS* knockout line of *P. falciparum*. If *FECH* or *HMBS* deficient parasites were viable with normal growth kinetics, this would provide further evidence of the host enzyme as the major contributor to haem synthesis in the parasite. This work could also be extended to testing the knockout parasites in UROS and FECH deficient blood to more closely examine the contribution of host or parasite enzymes for overall haem synthesis in the parasite.

Potential for haem enzyme deficiencies to protect the host against malarial infection

Work in this chapter has shown that host UROS and FECH are required for intraerthrocytic growth, raising the possibility that people with these haem enzyme deficiencies may exhibit natural protection against blood stage malarial infection. While porphyrias are rare, the global distribution of asymptomatic carriers of certain porphyria mutations may have wider implications in identifying new malaria protective red cell variants. In compound heterozygous EPP patients, the IVS3-48T/C common allele is co-inherited with a deleterious *Fech* mutation, acting in concert to decrease FECH activity below a critical threshold and therefore causing the porphyria phenotype (Gouya et al., 1996, Gouya et al., 1999, Gouya et al., 2004). There is evidence that carriers of the IVS3-48T/C common allele (without a deleterious *Fech* allele) also have reduced FECH activity without any porphyric symptoms (Gouya et al., 2006). The IVS3-48 T/C common allele is carried at a high rate in Japanese (54%), Chinese (43%), Mexican (44%) and South-East Asian (39%) populations (Hapmap data). It was also hypothesized that this allele was positively selected for in the Southeast Asian population (Gouya et al., 2006). These are areas where malaria has historically been present. It would be of interest to investigate *Plasmodium* growth kinetics in blood samples from asymptomatic carriers of the IVS3-48 T/C

common allele, as results from this chapter indicate that FECH deficiencies may also impair parasite growth.

Future work

A limiting factor in the investigation of parasite growth in haem enzyme deficient blood was the availability of small numbers of UROS and FECH deficient patients. Future work will continue investigating the role of host FECH in several other EPP patients in Australia and Europe, in collaboration with Ingrid Winship (Melbourne Royal hospital) and Hervé Puy (Centre Français des Porphyrries, Hôpital Louis Mourier, France). As mentioned above, further study will also involve an investigation of the IVS3-48 T/C common allele and potential contribution towards host protection against malaria.

In the case of UROS deficiency, there is one known CEP patient in Australia. This patient participated in the current study. Consent has been obtained from the offspring to also contribute blood samples to this study in the future. As UROS is inherited in an autosomal recessive fashion, the offspring are presumably heterozygous for the UROS mutation. The potential dose-response of residual enzyme activity and inhibition of parasite growth could be examined by correlating UROS activity and parasite growth in red cells from CEP001 and offspring.

Additionally, considering the rarity of human patients with severe haem enzyme deficiencies, mouse models may provide a useful tool for investigating the role of other haem enzymes in an *in vivo* malarial infection. Additional to the *Fech^{m1Pas}* mouse used in the present study, there are mouse models for both CEP and Doss porphyria (ALAD deficiency). Investigation of parasite growth in these haem enzyme deficient mice may provide further insight into the importance of host haem enzymes for parasite growth.

Summary

Overall, this chapter presents novel findings that genetic deficiency of the host haem enzymes UROS and FECH render the red cells resistant to *Plasmodium* growth. Data in the previous chapter supports the hypothesis that host enzymes are scavenged and data in this chapter confirms that the host enzymes play a

functional role in the intraerythrocytic parasite. This chapter has provided genetic validation that targeting host enzymes, required by the parasite for growth, may be a feasible host-directed therapeutic strategy. The next chapter in this thesis investigates the effects of pharmacologically inhibiting these host enzymes as a novel host-directed antimalarial therapy.

Chapter 5.

Pharmacological targeting of host haem enzymes as a novel antimalarial therapy

Introduction

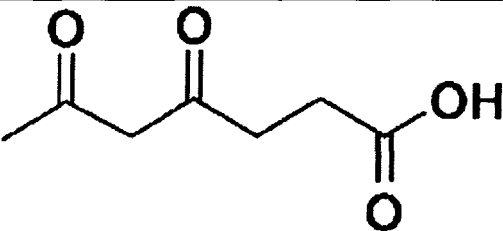
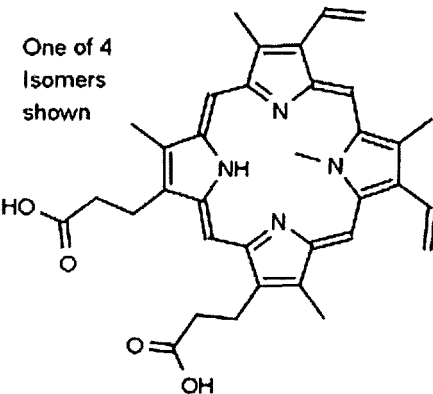
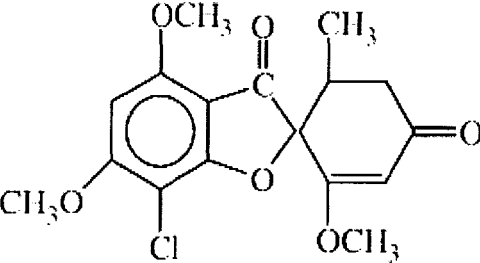
Work in this thesis has demonstrated that the host haem enzymes ALAD, UROS and FECH are localised in *Plasmodium* parasites during intraerythrocytic growth. Host UROS and FECH were then established as essential factors for parasite development, as parasite growth is impaired in UROS and FECH deficient red cells. The aim of this chapter was to determine if specific pharmacological inhibitors of these host enzymes can prevent parasite growth, and if they can thus act as novel host-directed antimalarial therapies.

Here, chemical inhibitors of the haem enzymes aminolevulinate dehydratase (ALAD) and ferrochelatase (FECH) were tested for antimalarial activity. UROS inhibitors were not commercially available and were therefore unable to be tested.

Several inhibitors were used in this section, including succinylacetone (SA), N-methylprotoporphyrin (NMPP) and griseofulvin (Table 5.1). SA is a highly active non-competitive irreversible inhibitor of ALAD ($K_i \sim 0.03 \mu\text{mol/l}$) (Ebert et al., 1979, Sassa and Kappas, 1982, Tschudy et al., 1981, Berger et al., 1983). Evidence suggests that SA is a suicide substrate inhibitor, binding covalently to a conserved active site lysine in ALAD, (Erskine et al., 2001). NMPP is a potent FECH inhibitor ($K_i \sim 10 \text{ nM}$) (Shi and Ferreira, 2006, Cochran and Schultz, 1990). NMPP is a transition-state analogue of protoporphyrin IX, the porphyrin substrate for FECH; NMPP competes with the substrate for FECH binding (Shi and Ferreira, 2006). Griseofulvin is a FECH inhibitor, a pro-drug that reacts with the haem moiety of cytochrome P450 or haemoglobin to form the alkylated porphyrin

NMPP (Bellingham et al., 1995). Studies in this chapter investigated the potential anti-plasmodial activity of these three compounds.

Table 5.1 Structures of inhibitors tested for anti-plasmodial activity

Inhibitor	Structure
Succinylacetone (SA)	
N-methylprotoporphyrin (NMPP)	<p>One of 4 Isomers shown</p> 
Griseofulvin	 <p>GRISEOFULVIN</p>

Results

Inhibition of *P. falciparum* growth by succinylacetone

The ALAD inhibitory capacity of succinylacetone (SA) was verified by measurement of ALAD activity in uninfected human red cells incubated with SA. ALAD activity decreased with increasing concentrations of SA, indicating SA is an effective ALAD inhibitor (Figure 5.1).

The ability of SA to inhibit the growth of *Plasmodium* was tested in a direct *P. falciparum* growth inhibition assay, where parasites were cultured continuously in the presence of SA for 48 hr. There was a dose-dependant impairment of parasite growth, with an IC_{50} of 12.5 μ M (Figure 5.2 A). These results demonstrated that parasite growth is impeded by SA, which presumably acts through the inhibition of red cell ALAD. However, because *Plasmodium* also produces its own version of ALAD, SA could be targeting either host or parasite enzymes. The continuous culture assay used in the above experiments could therefore not distinguish if the antimalarial effect was due to inhibition of the host or parasite ALAD.

SA is a non-competitive inhibitor that irreversibly binds to ALAD (Bourque et al., 2010). This pharmacological feature of SA allowed direct targeting of the host enzyme. Uninfected red cells were pre-incubated with SA for two hours and then repeatedly washed to remove SA. Any remaining unbound SA was non-enzymatically converted to a pyrrole by the addition of 2 mM of aminolevulinate (Ebert et al., 1979) prior to the addition of *P. falciparum* (3D7). When parasites were added to red cells preincubated with increasing concentrations of SA, again, a dose-dependant inhibition of parasite growth was demonstrated after 48 hours, with an IC_{50} of 2.5 μ M (Figure 5.2 B). Closer examination of the individual growth stages at each time-point indicated invasion was affected, with less ring-stage parasites in SA-treated cultures compared to the untreated control (Figure 5.3). The parasite culture is seeded in untreated red cells. These parasites therefore are able to produce normal viable merozoites. The observed reduction in new ring-stage parasites 24 hours later is therefore due to impaired parasite invasion in the SA-pretreated red cells. There was also a dose-dependant

decrease in the proportion of parasites progressing from ring to trophozoite and schizont stage in the SA pre-treated red cells, indicating growth is also impaired. These results confirm that the anti-plasmodial activity of SA is due to specific inhibition of host red cell ALAD, and that levels of parasite-derived ALAD are not sufficient to compensate for this inhibition.

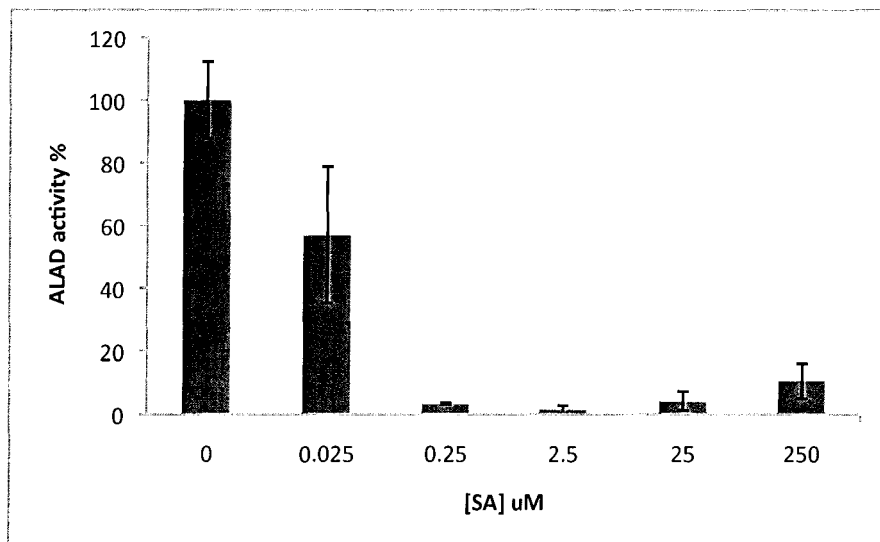


Figure 5.1 ALAD activity in uninfected red cells incubated with succinylacetone (SA)

ALAD activity was measured in uninfected human red cells incubated with increasing concentrations of SA. ALAD activity is expressed as the amount of porphobilinogen (μM) produced per h at 37 °C relative to that of the untreated control activity; each SA concentration was replicated in triplicate; error bars are SEM.

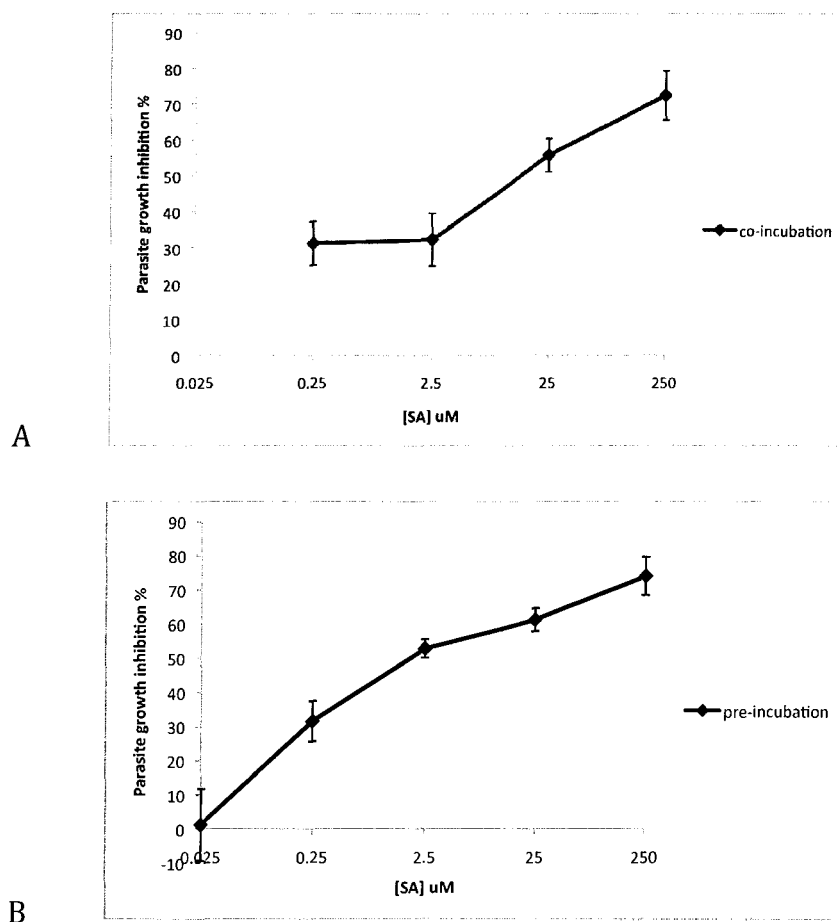


Figure 5.2 Growth inhibition of *P. falciparum* infected human red cells by SA

(A) Parasite growth inhibition (relative to untreated control) with increasing concentrations of SA. Assays were conducted using trophozoite-stage *P. falciparum* 3D7 parasites and growth was determined after 48 h incubation. Data represent the mean of two independent assays (each concentration assayed in triplicate). Error bars indicate SEM.

(B) Parasite growth inhibition (relative to untreated control) in red cells pretreated with SA. Red cells were pretreated in medium with SA for 2 h, prior to multiple washing steps to remove excess SA and the addition of 2 mM aminolevulinic acid (to non-enzymatically convert any unbound SA to a pyrrole) before infection with *P. falciparum* (3D7) parasites. Cells were infected with trophozoite-stage infected cells and parasite growth was determined after a further 48 h incubation. Data represent the mean of four independent assays (each concentration assayed in triplicate). Error bars indicate SEM.

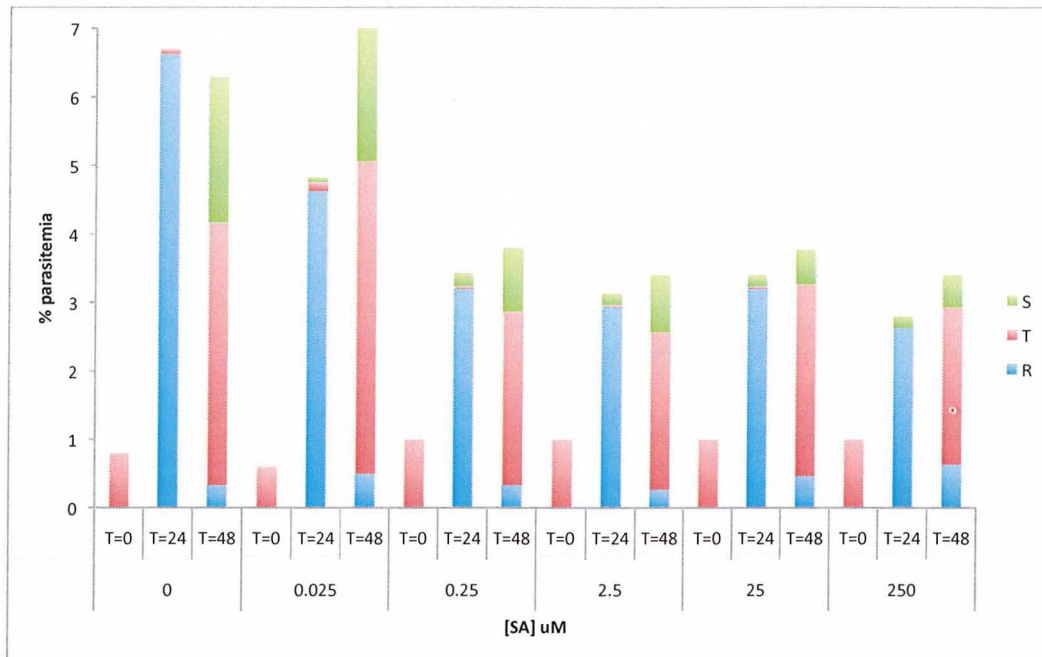


Figure 5.3 Individual growth stages of *P. falciparum* after incubation in red cells pretreated with SA

Average percent parasitaemia (of one of the repeat experiments from above) including percent schizonts (S), trophozoites (T) and rings (R) at each time point after specific targeting of host ALAD with SA. Blood was infected at T=0 with trophozoite-stage parasites. There is a dose-dependent decline in the percentage of new ring-stage parasites at T=24 ($p < 0.05$ for cultures pretreated with 0.25 μM SA and above). Subsequently, there is also a lower proportion of ring-stage parasite progressing through to trophozoite and schizont stage parasites at T=48 in cultures pre-treated with 0.25 μM SA and above ($p < 0.05$).

Red cell ALAD inhibition after SA administration in mice.

The SA dose required to completely inhibit red cell ALAD in mice was determined by measuring ALAD enzyme activity. ALAD enzyme assays were conducted on red cells taken from mice 24 hours after receiving increasing amounts of SA delivered by intraperitoneal injection (i.p.). Results showed a dose of 160 mg/kg SA was required for near complete inhibition of red cell ALAD in C57BL/6 mice (Figure 5.4 A). This once-off dose inhibited red cell ALAD for 24 hours, with ALAD levels back to control levels by four days (Figure 5.4 B). Red cell ALAD was fully inhibited in mice that received 160 mg/kg SA each day for seven days, with no significant differences in haematological cells due to the SA treatment compared to that of untreated mice (Table 5.2). Therefore to achieve sustained ALAD inhibition in the mouse, it was concluded that a daily dose of 160 mg/kg was required.

Effect of administration of SA in a murine model of malaria

The antimalarial capacity of SA was then investigated in mice infected with a murine-specific species of malaria parasite, *P. chabaudi*. Based on the above-demonstrated pharmacokinetic data, male C57BL/6 mice were given daily 160mg/kg i.p. injections of SA (or saline treatment for controls) the day prior to and everyday for eight days during a *P. chabaudi* infection. The untreated control mice demonstrated a gradual increase in percentage of iRBCs (% parasitaemia), peaking at around 50% parasitaemia (Figure 5.5 A). At this point the adaptive immune system responds to plasmodial infection, killing parasites and resulting in a decline in infected red cells. C57BL/6 mice are known to be relatively resistant to *P. chabaudi* infection, and in these experiments, around 50% of saline-treated control mice survived infection (Figure 5.5 B). SA-treated mice had a lower overall % parasitaemia throughout infection, with a significant 48 percent decrease in average parasitaemia than control mice at the peak of infection (Figure 5.5 A). Additionally, SA-treated mice were significantly more likely to survive infection than saline-treated controls (Figure 5.5 B). These results demonstrate that targeting ALAD with the specific inhibitor SA prevents severe *in vivo* malarial infection.

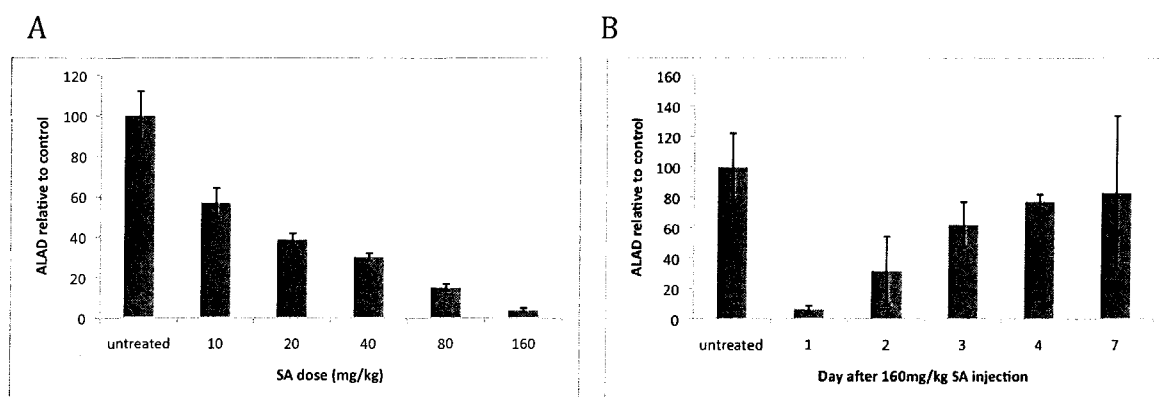


Figure 5.4. ALAD activity in red cells after treatment of mice with SA

(A) ALAD activity in peripheral mouse blood 24 h after receiving varying doses of SA. ALAD activity was measured as the amount of porphobilinogen (μM) produced in one h at 37°C with data representing the average ALAD activity relative to control mice. Three mice per dose were injected i.p with SA; error bars are SEM.

(B) Recovery of ALAD activity after a single 160 mg/kg i.p injection of SA (3 mice per time point). ALAD levels had returned to control ALAD activity by 4 days. Data represents the average ALAD activity (Porphobilinogen (μM) produced in one h at 37°C) relative to control mice; three mice per time-point were injected i.p with 160 mg/kg SA; error bars are SEM.

Table 5.2 Whole blood analysis of ALAD activity and haematological parameters of mice during SA treatment. Average ALAD activity (μM porphobilinogen produced, relative to the untreated control mice) was significantly impaired throughout the daily SA treatment of 160 mg/kg for seven days. Average haematological counts ($\pm\text{SD}$) showed no significant differences to that of untreated mice over the treatment period.

	<i>Untreated</i>	<i>Day 1</i>	<i>Day 5</i>	<i>Day 7</i>
ALAD activity (relative to control)	100	5.4 ± 6.1 (negligible)	1.44 ± 4.9 (negligible)	10.49 ± 4.95 (negligible)
Red blood cells ($\times 10^{12}$ cells/L)	9.71 ± 0.04	9.01 ± 0.23	9.86 ± 0.23	9.41 ± 0.48
Haemoglobin(g/L)	145.5 ± 2.12	137 ± 1.15	144 ± 5.66	140.5 ± 6.36
Haematocrit(L/L)	0.49 ± 0.01	0.46 ± 0.01	0.49 ± 0.01	0.47 ± 0.03
Mean corpuscular volume (fL)	50.3 ± 0.99	51.35 ± 0.06	49.7 ± 0.42	49.55 ± 0.35
Mean corpuscular haemoglobin (pg)	15 ± 0.14	15.25 ± 0.29	14.4 ± 0.57	14.95 ± 0.07
Mean corpuscular haemoglobin concentration (g/L)	298 ± 2.83	296.3 ± 6.35	294 ± 8.49	300.5 ± 3.54
Corpuscular haemoglobin concentration mean (g/L)	283 ± 2.83	280.5 ± 1.73	283 ± 5.66	280.5 ± 0.71
Corpuscular haemoglobin content (pg)	14.2 ± 0.14	14.4 ± 0.12	14 ± 0.14	13.9 ± 0.14
Platelets ($\times 10^9$ cells/L)	1194 ± 28.9	1022 ± 123.5	1232 ± 158.3	1391 ± 265.1
Percentage reticulocytes (%)	4 ± 2	4 ± 0.01	4 ± 0.5	3 ± 1

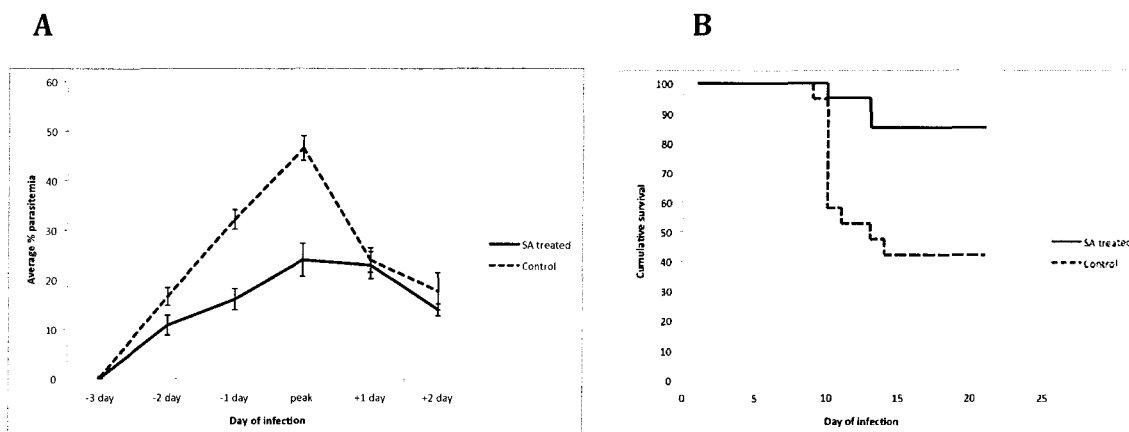


Figure 5.5 *P. chabaudi* infection kinetics and survival in SA-treated mice

(A) Parasitaemia of *P. chabaudi* infected male mice receiving once-daily SA treatment mice for 7 days compared to saline-treated controls. Data represents the mean percentage of infected red cells in mice infected with *P. chabaudi* (1×10^5 iRBC/mL) and normalised for the peak of infection. There was a significant difference between SA-treated and untreated control mice at the day prior to peak ($P = 2.62084 \times 10^{-6}$) and the peak ($P = 5.60601 \times 10^{-6}$) of infection; $n = 19$ SA-treated mice; $n = 18$ untreated controls; Results are the average of two independent experiments; Error bars indicate SEM.

(B) Survival of *P. chabaudi* infected male mice from the same experiment as (A). Mice were infected with *P. chabaudi* (as above) and monitored for survival over the time period indicated. There was a significant difference in survival between SA-treated and untreated control mice ($P = 0.005$ using Mantel-cox Log-rank test of survival).

In vitro analysis of P. falciparum growth after NMPP treatment

N-methylprotoporphyrin (NMPP) is an analogue of protoporphyrin IX and is a competitive FECH inhibitor. The anti-plasmodial activity of NMPP was investigated in a direct *P. falciparum* growth inhibition assay, where ring-stage *P. falciparum* (3D7) parasites were cultured continuously in the presence of NMPP for 48 hours. There was a dose-dependent impairment of parasite growth, with an IC_{50} of around 25 nM (Figure 5.6), a figure comparable with many current antimalarials. Further investigation of the individual growth stages demonstrated a dose-dependent decrease in the proportion of rings (at T=0) progressing through to trophozoites and schizonts at T=24 (Figure 5.7), indicating parasite growth impairment. There was also a lower percentage of new ring-stage parasites in the NMPP-treated cultures at T=48, but it could not be determined if invasion was being impaired or if NMPP was killing the seed parasites prior to invasion. As NMPP is a competitive inhibitor of FECH, the specific targeting of host FECH by preincubation could not be investigated. It is therefore possible that NMPP is targeting both host and parasite FECH. However, NMPP demonstrated potent anti-plasmodial activity, for which the parasite FECH could not compensate for.

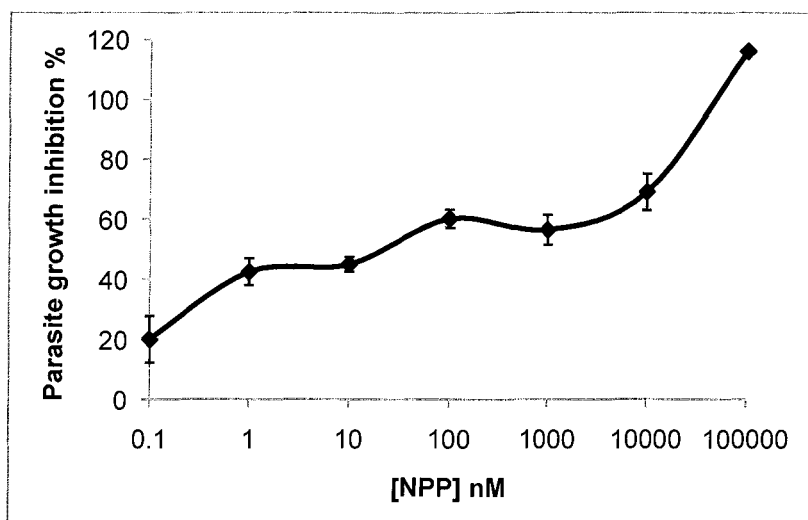


Figure 5.6 Anti-FECH mediated growth inhibition of *P. falciparum* infected human red cells by N-methylprotoporphyrin (NMPP).

Parasite growth inhibition (relative to untreated control), with increasing concentrations of NMPP. Assays were conducted using *P. falciparum* (3D7) parasites and growth determined after 48 hours incubation.

Data represent the mean of two independent assays (each concentration assayed in triplicate). Error bars indicate SEM.

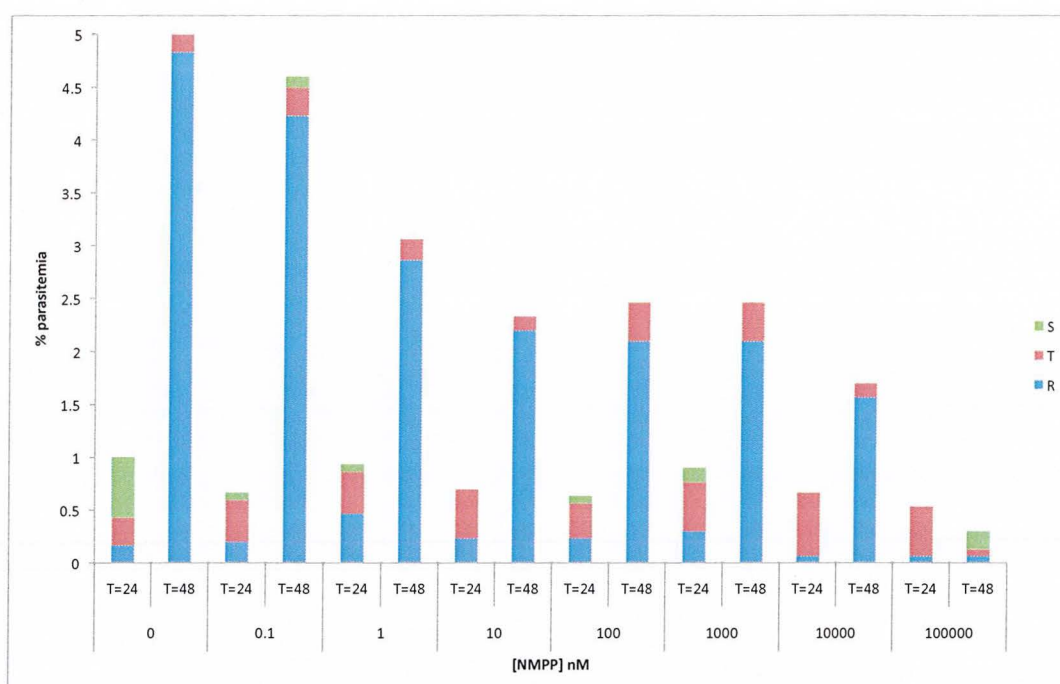


Figure 5.7 Individual growth stages of *P. falciparum* after NMPP treatment

Average percent infected red cells (percent parasitaemia) including percent schizonts (S), trophozoites (T) and rings (R) at each time point after growth of *P. falciparum* in the presence of NMPP. Blood was infected at T=0 with ring-stage parasites (at ~1% parasitaemia).

At T=24, there was no significant difference in total parasitaemia between any of the NMPP treatments and control.

At T=48 there was a lower percent parasitaemia in cultures treated with NMPP concentrations of 1 nM and above ($P < 0.01$). There was also a dose-dependent decrease in percentage of new ring-stage parasites in cultures treated with NMPP concentrations of 1 nM and above ($P < 0.05$).

Results are the averages of percent parasitaemia from triplicate cultures in one experiment; error bars are SEM.

In vitro analysis of P. falciparum growth after griseofulvin treatment

A search for additional FECH inhibitors revealed that griseofulvin has anti-FECH activity that is a side effect of its normal antifungal activity, precluding its use by porphyria patients (Holley et al., 1991, Bellingham et al., 1995). Griseofulvin has been used to treat dermatophytoses by oral administration in people for over 50 years (De Carli and Larizza, 1988). The drug concentrates in the keratinous layer of the skin, and targets fungal mitotic spindles (Develoux, 2001). The fungicidal activity of griseofulvin is independent of its FECH inhibitory capacity.

Initial experiments with griseofulvin using the co-incubation growth inhibition assay (As for NMPP) showed no effect on parasite growth at a range of doses. However, reports in the literature suggest that griseofulvin is a pro-drug, binding to the haem moiety of haemoglobin or cytochrome P450 to form the alkylated porphyrin, NMPP (Bellingham et al., 1995). This process may require the accumulation of griseofulvin. Subsequently, uninfected red cells were pre-incubated with griseofulvin for several days prior to parasite addition, with cell media and drug replacement every 24 hours. Using this assay, there was a dose-dependent inhibition of *P. falciparum* 3D7 strain (chloroquine sensitive) at 48 hours (Figure 5.8). Parasite growth inhibition with a similar IC_{50} was also observed in a chloroquine resistant *P. falciparum* strain (K1) (Figure 5.8).

Analysis of griseofulvin in uninfected red cells using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) demonstrated that the drug accumulates in red cells over several days of pre-incubation and constant co-incubation (Figure 5.9). This is consistent with the parasite inhibitory effect only being observed after three days of pre-treatment in red cells.

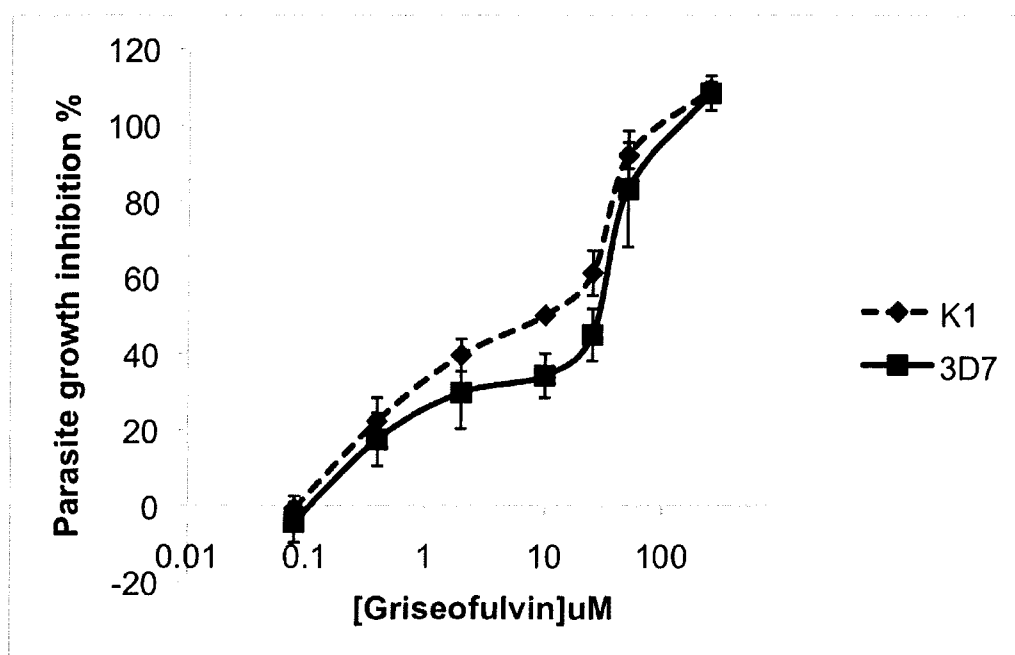


Figure 5.8. Anti-plasmodial activity of griseofulvin

Parasite growth inhibition (relative to untreated control) in cells pretreated with griseofulvin. Red cells were pretreated in medium with griseofulvin for three days with medium replaced every 24 h prior to infection with parasites. The medium and griseofulvin was replaced at the time of parasite addition and every 24 h during subsequent culturing. Parasite growth was determined 48 h after infection. Two strains of *P. falciparum* were tested, 3D7 (chloroquine sensitive), and K1 (chloroquine resistant). Data represent the mean of two independent assays (each concentration assayed in triplicate). Error bars indicate SEM.

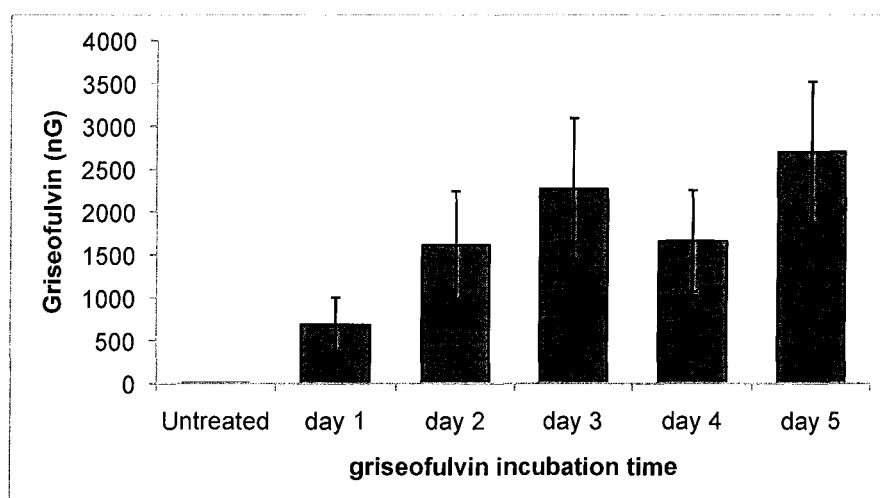


Figure 5.9 Accumulation of griseofulvin in red blood cells

UPLC-MS analysis of griseofulvin in red cells treated with 10 μ M griseofulvin for up to five days, and sampled each 24 h. Griseofulvin-containing medium was replaced each 24 h. Griseofulvin levels were quantified by comparison to a standard curve of known griseofulvin amounts. Each sample contains equal cell numbers; data represent the mean of three independent assays; error bars indicate SEM.

***Ex vivo* analysis of *Plasmodium* growth in red cells from griseofulvin-treated human volunteers**

After demonstrating antimalarial properties *in vitro*, most experimental compounds progress to animal studies of infection for drug safety and tolerability testing. However griseofulvin is FDA and TGA-approved for use in humans to treat fungal skin infections, has very mild or no side effects, and has been used safely in the clinic for over 50 years. Griseofulvin could therefore be administered to human volunteers to further investigate its anti-plasmodial activity.

Two different dose regimens were used. Firstly, volunteers took a clinically standard oral dose of griseofulvin (500 mg/day) for seven days. Blood samples were collected from each patient prior to and at the conclusion of the seven-day treatment period. Following blood collection, both blood samples were washed three-times in RPMI medium to remove white-blood cells, platelets and residual griseofulvin present in the serum. *P. falciparum* (3D7) parasites were seeded into the blood samples and cultured for 24 hours. Parasites grew normally in the blood taken before the griseofulvin treatment, but were unable to grow in the cell collected after the seven-day treatment regimen (Figure 5.10 A). The number of parasites measured in the blood after griseofulvin treatment was less than the original number of seed parasites added. This suggests that the parasites were being killed and as well as growth retarded.

In a second set of experiments, red cells were obtained everyday over the seven-day dosing regime from one volunteer, in order to examine when the parasite growth inhibitory effect occurred. *P. falciparum* (3D7) parasites were again seeded into the blood samples immediately after collection and cultured for 24 hours. A time course showed that the antiplasmodial effect was observed three days after the onset of griseofulvin treatment and parasite growth was fully inhibited by four days of treatment (Figure 5.10 B). UPLC-MS analysis showed a gradual accumulation of red cell griseofulvin, with the level peaking at three to four days, explaining the delay in the onset of parasite inhibition (Figure 5.11).

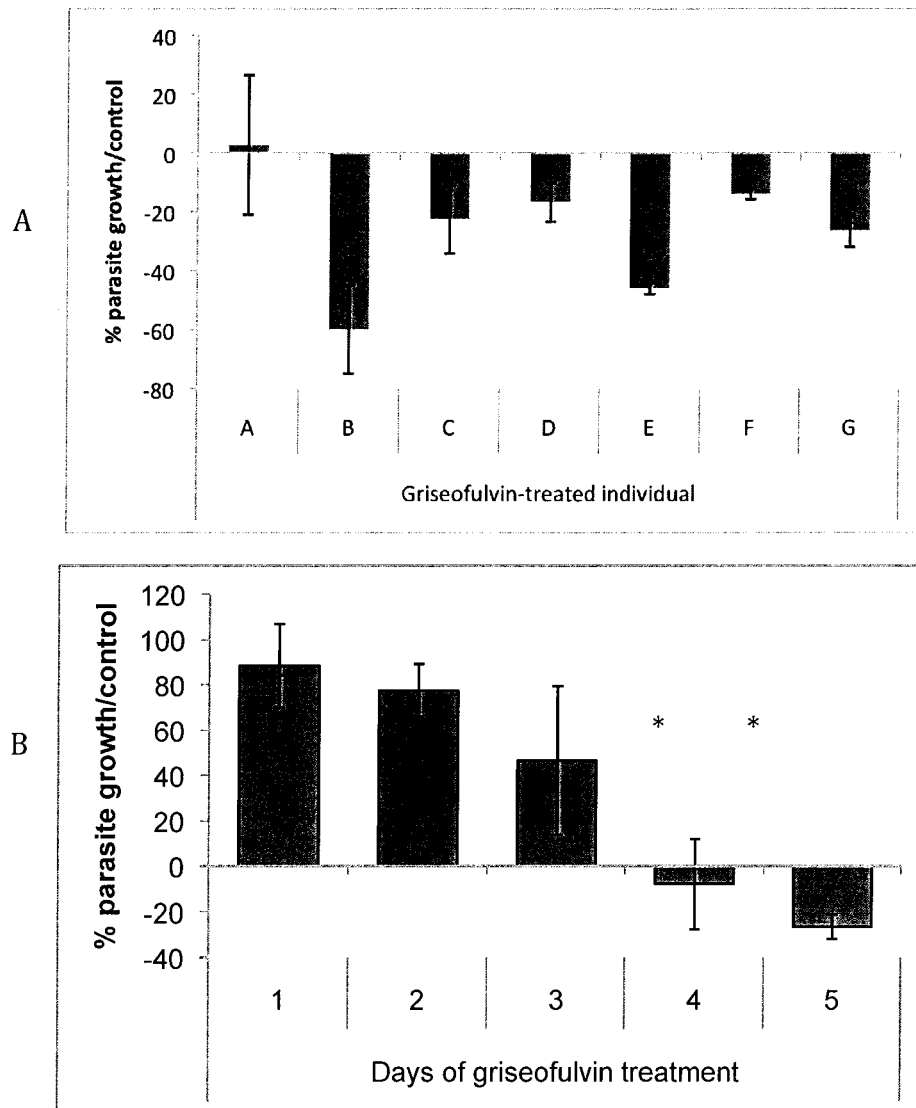


Figure 5.10. Parasite growth in red cells from human volunteers receiving 500 mg/day griseofulvin

(A) Percentage of parasite growth in red cells collected from seven different individuals (A-G) after taking griseofulvin for seven days (500 mg/day). $p < 0.01$ for all seven individuals, comparing parasite growth in cells before and after treatment.

(B) Percentage of parasite growth in red cells collected each day from an individual taking a five-day course of griseofulvin (500 mg/day). * $p < 0.01$ comparing growth in cells before and after treatment.

Mean (\pm SD) initial percentage parasitaemia in all the assays was 1% ($\pm 0.5\%$). Growth was determined (after 24 h) by subtracting the starting parasitaemia, and expressed as a percentage of parasitaemia in cells collected before the griseofulvin treatment (initial levels subtracted). Negative values reflect the reduction in numbers of parasitised cells from initial levels. Data represent the mean of 3 replicate cultures for each griseofulvin treated individual. Error bars indicate SEM.

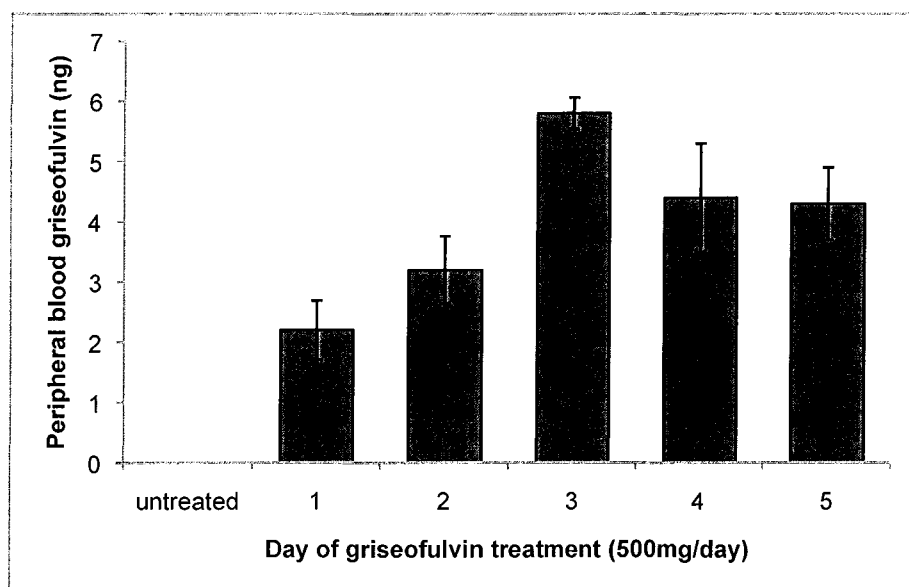


Figure 5.11 Griseofulvin accumulation in red cells from a griseofulvin-treated volunteer

UPLC-MS analysis of griseofulvin in red cells from an individual taking griseofulvin for five days (500 mg/day). Blood samples were collected and analysed at the indicated times, and quantified by comparison to a standard curve of known griseofulvin amounts. Data represent the mean of two independent assays. Error bars indicate SEM.

The previous data demonstrating that several days of treatment was required to see an anti-plasmodial effect, suggested that the accumulation of griseofulvin in the red cell may be the rate-limiting step. A once-off two-gram loading dose of griseofulvin was then administered to test if a higher dose of griseofulvin may result in more rapid accumulation inside the red cell and a therefore more rapid anti-plasmodial effect. Analysis of blood samples taken eight hours post treatment, from three different individuals, consistently demonstrated that they could not support parasite growth (Figure 5.12). Red cells from samples collected from these individuals at later time points (24, 48 and 72 hours) showed a progressively reduced capacity to prevent parasite growth (Figure 5.13 A).

Griseofulvin levels were quantified in samples from one of the volunteers receiving a once-off two-gram dose. UPLC-MS analysis demonstrated a spike in red cell griseofulvin at eight hours with a progressive decline in griseofulvin levels over the subsequent time points (Figure 5.13 B). The griseofulvin levels measured are consistent with the anti-plasmodial effect occurring at eight hours after the two-gram dose. Griseofulvin levels were not detectable in human red cells one-week post griseofulvin treatment. These results show that the two-gram loading dose accumulates more rapidly in red cells, causing the crucial threshold for parasite killing to be reached at an earlier time-point. The growth inhibition effect declines over time, the antiplasmodial activity is present for at least two days. Therefore at clinical doses, administration of griseofulvin may act as an effective therapy against malaria infection.



Figure 5.12 Parasite growth in red cells from human volunteers eight-hours after a once-off two-gram dose of griseofulvin

Percentage of parasite growth in red cells collected from individuals eight-hours after taking a single 2000mg dose of griseofulvin, relative to the untreated control blood form each individual.

Data represent the mean of three replicate cultures in blood from three individuals. Error bars indicate SEM. In all three individuals, $p < 0.01$ comparing growth in cells before and after treatment.

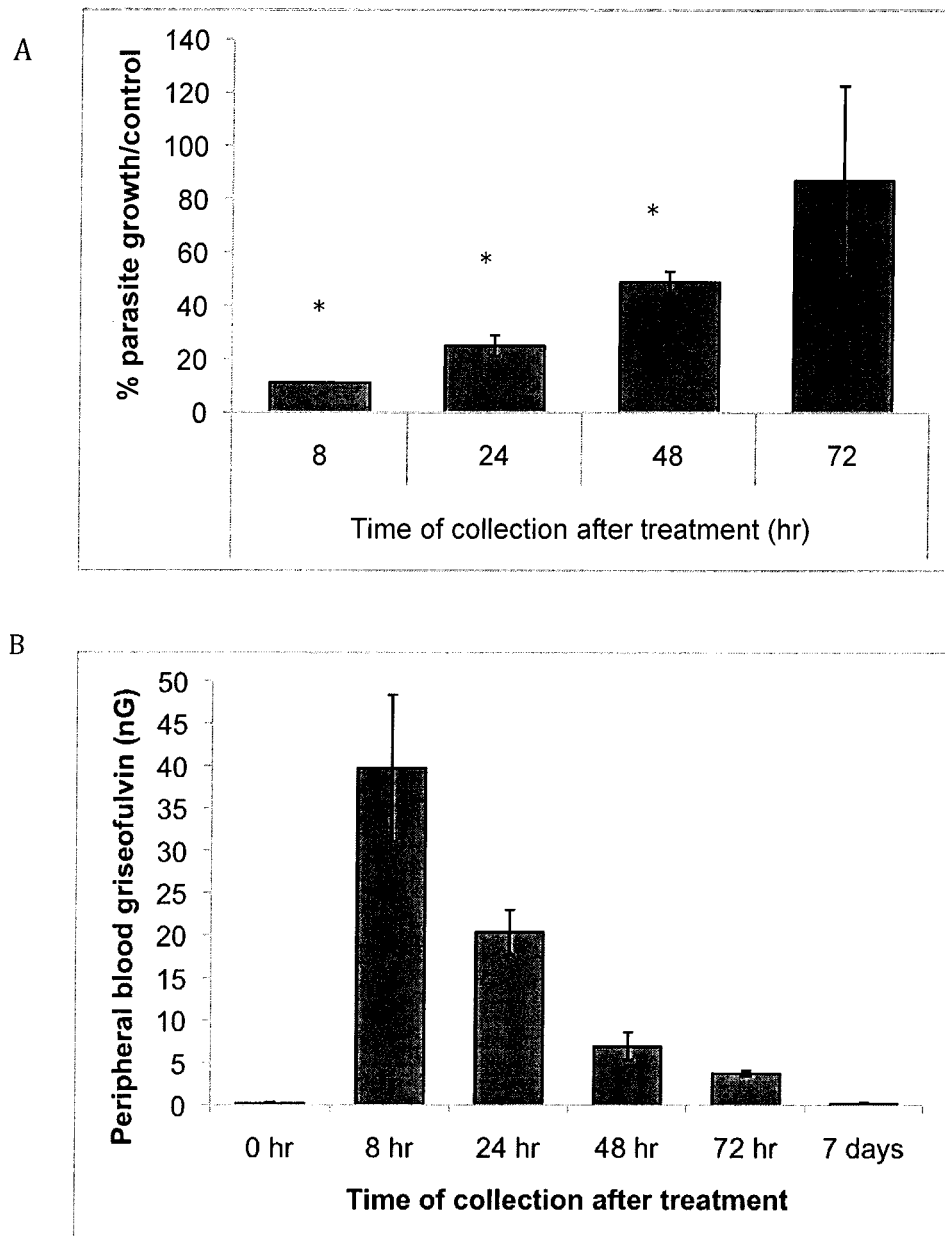


Figure 5.13 Parasite growth and griseofulvin levels in red cells from human volunteers up to 72 h after a once-off two-gram dose of griseofulvin

(A) Percentage of parasite growth in red cells collected from individuals taking griseofulvin at indicated time-points after a single dose (2000 mg) of griseofulvin. Data represent the mean of 3 replicate cultures for three individual volunteers receiving a once-off 2000mg dose of griseofulvin. Error bars indicate SEM. * $p < 0.01$ comparing growth in cells before and after treatment.

(B) UPLC-MS analysis of griseofulvin in red cells from one individual taking a once-off 2000mg dose of griseofulvin. Blood samples were collected and analysed at the indicated times, and quantified by comparison to a standard curve of known griseofulvin amounts. Data represent the mean of three independent assays. Error bars indicate SEM.

Discussion

Studies presented in chapters 3 and 4 of this thesis demonstrate that the host enzymes ALAD, FECH and UROS are localised in the intraerythrocytic parasite and that host FECH and UROS are required by the parasite for normal growth. It was therefore hypothesised that these host haem synthetic enzymes are essential growth factors for the parasite. To test this hypothesis further, and pave the way for the development of novel anti-malarial lead compounds, work in this chapter tested if pharmacological inhibitors of these enzymes could prevent parasite growth. The results showed that SA, a specific and irreversible inhibitor of ALAD, and NMPP, a substrate analog inhibitor of FECH were potent anti-plasmodial agents in a *P. falciparum* *in vitro* growth inhibition assay. The irreversible nature of SA provided a means to directly demonstrate that specifically targeting the red cell ALAD prevented parasite growth. SA was also administered to mice infected with *P. chabaudi*, and significantly improved outcome to infection (survival) and reduced parasite growth. Lastly, griseofulvin demonstrated novel anti-plasmodial properties *in vitro* and in red cells collected from human volunteers taking pharmacologic doses of griseofulvin.

Specific inhibition of host ALAD with SA prevents parasite growth

This chapter has established that the ALAD inhibitor SA blocks *P. falciparum* growth using *in vitro* and *in vivo* models. SA demonstrated antimalarial activity when directly added to *P. falciparum* cultures with an IC₅₀ of 12.5 µM. As the parasite contains a gene encoding ALAD, the anti-plasmodial effect of SA could be due to targeting the host, parasite or both species of enzymes. Subsequent experiments investigated parasite growth after specific inhibition of host ALAD. Uninfected red cells were preincubated with SA for several hours prior to removal of excess unbound SA and addition of parasites. Results demonstrate significant growth impairment 48 hours after the addition of parasites, suggesting that the antimalarial effect of SA is due to the specific inhibition of host ALAD.

Closer analysis of the individual intraerythrocytic parasite stages demonstrated the progression from ring to late-stage parasites was impaired in a dose-dependent manner by SA, indicating impaired growth. Results here also

indicated that invasion was reduced in drug-treated cultures, evidenced by the lower proportion of new rings in drug treated-cultures compared to the untreated control. The reduction in rings is likely to be due to impaired invasion, rather than direct killing of the seed parasites, as the trophozoite-infected red cells used to seed the culture are growing in SA untreated red cells and can therefore be presumed to have normal growth and merozoite formation.

SA has previously demonstrated parasite growth inhibition *in vitro* (Dhanasekaran et al., 2004) the investigations in this chapter have shown the anti-plasmodial effect is due to targeting of the host enzyme, resulting in both impaired invasion and growth. The impaired invasion was a surprising finding and has not been previously been demonstrated but is consistent with findings from the previous chapter where parasite invasion was also impaired in FECH and UROS deficient red cells. An alternative explanation is that the impaired parasite invasion may be due to a direct effect of SA on the host red cell, making the red cell inhospitable for normal parasite development. This may be in addition to the parasite scavenging inhibited ALAD. Regardless of either proposed mechanism, the results indicate that invasion is impaired after SA treatment and any parasites that successfully invade the red cell are further impaired in their intraerythrocytic development.

The anti-plasmodial activity of SA was also tested *in vivo* using *P. chabaudi*, a mouse model of malaria. Results demonstrated the SA-treated mice had a significantly lower course of infection and increased survival compared to the untreated controls. This effect may be due to inhibition of host or parasite ALAD. However the previous pre-incubation experiment specifically targeting host ALAD, demonstrated that the majority of antimalarial activity of SA comes from inhibition of the host ALAD. Considering the similarity of *P. falciparum* and *P. chabaudi*, the inhibition of parasite growth due to host ALAD targeting may also translate into the mouse model.

***In vitro* parasite growth is impaired NMPP**

This chapter demonstrated that NMPP, a specific FECH inhibitor, blocks the intraerythrocytic development of *P. falciparum in vitro*. The drug was directly

incubated with ring-stage parasites and 48 hours later, there were significantly less new ring-stage parasites in drug-treated cultures incubated with NMPP (1 nM and above) compared to the untreated control. This indicates invasion may be impaired, but this may also be a consequence of delayed parasite growth in the first 24 hours of incubation. Overall, NMPP significantly impaired parasite intraerythrocytic development in the growth inhibition assay with an IC_{50} of 25 nM, comparable with several current antimalarials. As NMPP is a competitive inhibitor, unlike SA, specific targeting of the host enzyme was unable to be investigated. Therefore NMPP may be targeting both host and parasite FECH. However, results from the previous chapter demonstrated that host FECH is required for normal *Plasmodium* intraerythrocytic infection, as parasites displayed impaired growth in EPP red cells. The main effect of NMPP-mediated FECH inhibition is therefore likely to be targeting of the human enzyme. Overall, regardless of the species of enzyme inhibition, this work has demonstrated that FECH is a potential antimalarial target and that NMPP possesses potent anti-plasmodial activity *in vitro*. Future work will continue to investigate the anti-plasmodial activity of NMPP *in vivo*, using the *P. chabaudi* murine model of infection, as for SA.

Griseofulvin has both prophylactic and fast-acting treatment properties

Griseofulvin is a pro-drug that forms the FECH inhibitory compound NMPP, independent of its normal antifungal activity. This drug is routinely used to treat human fungal skin infections caused by dermatophytes but is contraindicated in people with porphyria because of its FECH inhibitory side effect. This compound was initially tested in the standard *Plasmodium* co-incubation assay (as performed for NMPP) with no demonstrable effect. Literature suggests that griseofulvin reacts with the haem moiety of cytochrome C or haemoglobin to form NMPP (Bellingham et al., 1995, Cole and Marks, 1984). The concentration and accumulation of griseofulvin may affect the rate of NMPP formation. Therefore in the co-incubation assay, griseofulvin may not have had time to accumulate and form the inhibitory compound NMPP, thus explaining why no growth inhibition was observed.

Subsequently, red cells were pre-incubated with griseofulvin for three days prior to the addition of parasites (to allow for maximal drug accumulation), resulting in marked anti-parasitic effects. Griseofulvin demonstrated an IC_{50} between 10 and 50 μ M, with similar efficacy against chloroquine sensitive and resistant parasites. Furthermore, measurement of griseofulvin in red cells demonstrated that a minimum of three days of griseofulvin pretreatment is needed to achieve maximal loading in red cells. Parasite growth inhibition is therefore dependent on the accumulation of griseofulvin in red cells and may explain why griseofulvin has not been previously identified in large-scale screens for new antimalarial agents from currently FDA-approved drugs.

As griseofulvin is FDA and TGA-approved for human use, its anti-plasmodial properties were further investigated in red cells from human volunteers receiving the drug. Parasites were not able to grow in red cells from human volunteers after seven days of daily griseofulvin (500 mg/day) and the anti-parasitic effect was demonstrable from three days, coinciding with the peak in red cell griseofulvin level. These results indicate griseofulvin may be useful as a prophylactic agent. Griseofulvin is well tolerated, as evidenced by the extended period of time (months or longer) that human patients take griseofulvin to treat fungal skin infection. Therefore, griseofulvin could be taken at least three days prior to potential *Plasmodium* exposure and ongoing preventative treatment could be sustained for long periods of time.

Results from this chapter indicate that griseofulvin requires several days to accumulate in red cells to form the active FECH inhibitor NMPP. Considering the accumulation of griseofulvin may be the rate-limiting step, a higher loading dose of griseofulvin was investigated to see if there was also a more rapid accumulation of the drug and therefore more rapid antimalarial response. Red cells collected from volunteers eight hours after receiving a once-off two-gram oral dose of griseofulvin were resistant to parasite growth, with an observable effect sustained up to 48 hours after the once-off dose. This was explained by further griseofulvin quantification, demonstrating a peak in red cell griseofulvin levels at eight hours, followed by a subsequent decline corresponding to the decrease in anti-parasitic effect. Note that these parasite growth experiments

were performed *in vitro* on purified and washed erythrocytes from griseofulvin-treated human volunteers in the absence of additional exogenous griseofulvin, therefore the growth inhibition is reliant on residual accumulated erythrocytic griseofulvin.

This work demonstrates griseofulvin accumulates in the red cell prior to observable anti-parasitic effects *in vitro*, but attempts to measure NMPP formation in griseofulvin treated red cell were unsuccessful. A UPLC-MS method developed by Noel Davies (Central Science Laboratory, University of Tasmania) detected NMPP in uninfected red cells spiked with known NMPP quantities but was unable to detect NMPP formation in red cells pretreated with griseofulvin. A potential problem is that NMPP is likely bound in a haem complex in the red cells and future work will attempt to proteolyse the complex to measure NMPP.

A host or parasite-targeted therapy?

The aim of this thesis was to investigate targeting host enzymes that the parasite requires as a novel host-directed antimalarial therapy. Previous chapters of this thesis report that host ALAD, FECH and UROS are localised in the parasite during growth and additionally that host UROS and FECH are necessary for intraerythrocytic infection of red cells. Work in this chapter targeted ALAD and FECH with chemical inhibitors. In the case of ALAD, SA inhibition of the host enzyme resulted in parasite growth inhibition but SA treatment in the mouse model may have been targeting host or parasite ALAD. Additionally, FECH inhibition with NMPP and griseofulvin could not differentiate between host and parasite FECH. Therefore, this thesis cannot rule out the possibility that there is some parasite ALAD or FECH contributing to haem synthesis in the parasite and that these drugs may act through targeting both parasite and host enzymes. Additionally, it is theoretically possible for the parasite to develop resistance by increasing the expression of its own gene during blood-stage infection. However, it is likely that these drugs would still target the parasite enzyme as well, ensuring that the parasite would need to accumulate mutations in the parasite genes to overcome this type of therapy. Therefore although resistance is possible, it would likely take considerable time.

UROS was a third host target in the haem biosynthetic pathway that was localised in *Plasmodium* and required for normal parasite growth. As the parasite does not contain a gene coding for UROS, this enzyme is an ideal target for a host-directed therapy as resistance is unlikely. Unfortunately there were no commercially available inhibitors and collaboration with a chemistry team to make compounds also failed to yield any compounds. Recently, a compound called sampangine was identified as a UROS inhibitor (Huang et al., 2011), and future work will investigate its potential anti-plasmodial properties.

Considering the widespread drug resistance to all current antimalarials and the lack of new antimalarial compounds in the drug-discovery pipeline, results in this thesis have demonstrated several novel targets for antimalarial therapeutic intervention. Additionally, as griseofulvin is already FDA-approved for human usage, phase I clinical trials can be bypassed enabling a more rapid and cost-effective route for the introduction of a novel antimalarial agent.

Summary

Overall this chapter investigated pharmacological targeting of the host haem enzymes ALAD and FECH as a novel antimalarial treatment. Results demonstrated that chemical inhibition of host ALAD (with SA) or host FECH (with NMPP or griseofulvin) results in *P. falciparum* growth inhibition *in vitro*. SA also showed *in vivo* antimalarial activity in a murine model of malaria, protecting mice from severe infection. This chapter has also shown that clinical doses of oral griseofulvin render the red cells of a human volunteer resistant to parasite growth. This data indicates that griseofulvin has potential as both a prophylactic and an acute antimalarial treatment. Griseofulvin is already FDA and TGA-approved for human use, paving the way for further investigations of the antimalarial treatment capacity of this compound in humans. An exciting future prospect of this work is a phase II B clinical trial of this compound, in collaboration with James McCarthy at the University of Queensland. This thesis has provided novel evidence that griseofulvin could also be a useful antimalarial drug; with a novel mode of action that potentially avoids parasite resistance.

Chapter 6 - Final discussion

Malarial parasites have developed resistance to all current antimalarial therapies. All of these drugs target parasite enzymes or processes, thereby selecting for mutant, resistant parasites. New antimalarials are desperately needed and a potential clue for a new therapeutic strategy has been provided by so-called “natural genetic antimalarials”. Host genetic changes to red cell genes have offered millennia of stable protection to individuals living in endemic regions. By imitating natural resistance, the work described in this thesis proposed a novel host-directed antimalarial therapy through the targeting of erythrocytic molecules required by *Plasmodium* for growth and survival.

Here, multiple experimental approaches were used to investigate and validate several enzymes from the haem biosynthetic pathway as targets for a novel host-directed antimalarial therapy. Firstly, it was demonstrated that the host haem synthetic enzymes ALAD, FECH and UROS are localised in *Plasmodium* during intraerythrocytic growth. Moreover, parasites showed impaired growth in red cells deficient in UROS and FECH, using both human and mouse genetic models of haem enzyme deficiency. Finally, the HDT strategy was validated with several inhibitors of ALAD and FECH demonstrating *in vitro* and *in vivo* antimalarial activity. One of these inhibitors, griseofulvin is of particular interest considering it is already FDA and TGA approved for human use. The anti-FECH activity of griseofulvin is a side effect additional to its normal antifungal activity. This drug has been used over the last 50 years to treat human skin infections caused by certain species of dermatophytes. Griseofulvin was demonstrated to have novel antimalarial activity *in vitro* (on both chloroquine susceptible and resistant parasites) and additionally in red cells from human volunteers receiving pharmacological doses of griseofulvin. Parasites were unable to grow in red cells collected from human volunteers eight-hours after taking a clinically relevant dose of griseofulvin. Overall, this work has provided proof-of-concept that an

HDT is a feasible approach to antimalarial treatment and also demonstrated griseofulvin has potential clinical benefits as an antimalarial.

Host haem enzymes are required for parasite growth

Work in this thesis initially demonstrated that the host haem biosynthetic enzymes ALAD, FECH and UROS are localised in the intraerythrocytic parasite during the late stages of growth. This supports previously published work, demonstrating that host ALAD and FECH are scavenged by the parasite (Bonday et al., 2000, Dhanasekaran et al., 2004, Varadharajan et al., 2004). Questions remain about the mechanism of transport into the parasite, final parasite compartment localisation and the functional requirement of these host enzymes.

Evidence presented in this thesis established that host FECH, UROS and ALAD are essential factors for normal parasite growth. This was demonstrated using genetic models of human and mouse haem synthetic enzyme deficiency for UROS and FECH. Human red cells deficient in UROS or FECH were unable to sustain normal parasite infection, with substantial *Plasmodium* growth impairment at all stages of development compared to normal control blood. Further work in this thesis provided evidence that the parasite growth inhibitory effect in EPP cells was due to FECH deficiency, irrespective of protoporphyrin substrate levels. An important role for host FECH in malarial infection was also confirmed in a mouse model of FECH deficiency, demonstrated by the severe growth impairment of *P. chabaudi* in *Fech*^{-/-} (with 5% residual FECH activity) compared to normal wild-type control mice. Results were irrespective of sex, with both male and female *Fech*^{-/-} mice demonstrating resistance. A similar level of resistance was observed on both isogenic and mixed backgrounds, demonstrating parasite growth impairment in *Fech*^{-/-} mice is irrespective of the genetic background of the host. Furthermore, host ALAD was shown to be essential for normal parasite growth, as pharmacological inhibition of the host enzyme inhibited both parasite invasion and growth. A final question remains if the absence or inhibition of the host enzyme is having a direct effect on the mature red cell, making it inhospitable for parasite growth. This may be independent of, or in addition to

the host enzyme being scavenged by the parasite. Overall, this work has confirmed that host FECH, UROS and ALAD are vital for normal parasite growth, indicating they play an important functional role.

Implications of targeting host haem enzymes

Current antimalarials target parasite specific enzymes or processes, but many of these parasite-directed drugs still exhibit toxic side effects to the host. A host-directed therapy has been proposed here, as a novel antimalarial strategy to potentially avoid parasite resistance, however the potential side effects of targeting host enzymes must also be considered. Most of the enzymes in the mature red cell are purely residual, left over from development (i.e. the red cell is not actively synthesizing the target enzymes). Other cells that may be affected by a HDT, like hepatocytes and erythroblasts, are continually synthesising new enzymes, meaning the largest impact of such a therapy will be felt by the mature red cells. This is exemplified by griseofulvin, which inhibits FECH as a side effect to its normal antifungal activity. Human patients taking griseofulvin to treat dermatophyte skin infections can take up to one gram per day for up to several months at a time and do not display porphyric symptoms due to impaired FECH (except if patients already have a FECH deficiency). This indicates that other sites of haem synthesis, such as the liver and erythropoietic progenitor cells are not affected by griseofulvin treatment. While griseofulvin is not an irreversible inhibitor, this thesis has shown that it accumulates in red cells over a course of 500mg per day and also after a once-off 2000 mg dose. This compound has sustained activity in the red cell, as shown by the antimalarial effects still evident 48 h after the once-off dose was taken by human volunteers. This provides further evidence that a HDT targeting haem synthetic enzymes is a feasible approach to antimalarial treatment without severe side effects to the host.

Irreversible inhibitors of haem synthetic enzymes (such as SA) could also be useful antimalarials. An irreversible inhibitor will permanently inactivate its target enzyme in the red cell, meaning the red cell may be refractory to infection for its lifespan (about 120 days). This could lead to a HDT being used

intermittently (perhaps weekly or biweekly), which would mean fewer side effects, better compliance and a lower cost to the patient. In the case of SA, the potential side effects may be anticipated from people with hereditary tyrosinemia. These patients have abnormal tyrosine metabolism and produce excessive SA, resulting in ALAD inhibition. While this is an extreme example, there are side effects due to the sustained ALAD inhibition over the life of the patient. Intermittent antimalarial treatment with SA would not result in severe side effects as seen in hereditary tyrosinemia and combined with correct dosing and drug design modifications, could ideally eliminate potential side-effects.

A potential role for host haem enzymes in the liver stage of malarial infection

Apart from red cells, the liver is the other site of *Plasmodium* infection in the human host. Coincidentally, the liver is also the other main site of haem synthesis, where haem is required as a cofactor for several cytochromes. Like in the red cell, the parasite may also scavenge and use the host haem synthetic enzymes to supplement its own metabolic pathways during exoerythrocytic development. While *P. chabaudi* was used in this study to investigate *in vivo* blood stage infection, another murine model of malaria, *P. berghei*, may serve as a useful tool to investigate the potential role host haem enzymes play in the liver stage of infection. *P. berghei* is widely used as a rodent model of the liver stage in malaria. Future experiments using both *P. berghei* and the variety of available haem deficient mouse models and inhibitors described in this thesis may open up a new line of investigation into the importance of host haem enzymes in the liver stage of malarial infection. If the parasite relies on scavenging host haem enzymes during the liver stage of infection, targeting host enzymes in this stage may eliminate the progression of infection prior to the symptomatic red cell stage.

Over 700 other potential red cell targets for a host-directed therapy

Alongside the haem synthetic enzymes investigated in this thesis, there are a multitude of other potential HDT candidates in the red cell. Several other red cell proteins have been demonstrated to be required for parasite invasion, growth or egress. These include superoxide dismutase, peroxiredoxin-2, calpain-1, erythrocyte G protein, host protein kinases and basigin (Fairfield et al., 1983, Koncarevic et al., 2009, Chandramohanadas et al., 2009, Murphy et al., 2006, Crosnier et al., 2011, Sicard et al., 2011). Any of these proteins could be potential host-targets for a novel antimalarial therapy. Additionally, several proteomic studies have predicted around 700 other enzymes (Alvarez-Llamas et al., 2009, Goodman et al., 2007, Kakhniashvili et al., 2004, Pasini et al., 2006, Roux-Dalvai et al., 2008) in the mature red cell. Future work could take a candidate approach to investigate the importance of other host red cell enzymes in malarial infection. In a technique recently described, each of these proteins in mature red cells could be systematically immunodepleted, resealed and infected *in vitro* with *P. falciparum*, with subsequent growth kinetics investigated (Chandramohanadas et al., 2009, Murphy et al., 2006). Further investigations into the role of host red cell enzymes may reveal new insights into host-parasite biology in addition to the identification of potential new antimalarial targets.

An additional technique to investigate novel potential host targets is that of chemical mutagenesis screens. N-ethyl-N-nitrosurea (ENU) is a chemical agent inducing random mutations in the germ-line of mice. Progeny of ENU-treated mice may be screened for malaria susceptibility (to *P. chabaudi*) and red cell phenotype. Mice that demonstrate malarial resistance can be sequenced to identify the responsible gene. This is a powerful unbiased approach currently being undertaken in the Foote laboratory that may be useful in identifying new host targets for a novel HDT.

Conclusions

Work described in this thesis has demonstrated that the host haem synthetic enzymes ALAD, UROS and FECH are localised in the *Plasmodium* parasite during

intraerythrocytic growth. Genetic knockdown of enzyme activity and pharmacologic targeting of the host haem synthetic enzymes resulted in impaired *Plasmodium* growth, indicating their requirement as essential host factors during intraerythrocytic parasite development. This work has also identified griseofulvin as a potential new antimalarial compound. Griseofulvin is an FDA and TGA-approved drug that may be a useful new generation “off the shelf” antimalarial agent. Overall, this work has validated a host-directed therapy as a novel antimalarial strategy, paving the way for host-directed antimalarial treatments as a new therapeutic field, which may avoid parasite resistance common to the current range of antimalarials.

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