

The pantothenate kinase of the human malaria parasite *Plasmodium falciparum*

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Declaration

The research presented within this thesis, except where otherwise acknowledged, is my own original work.

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Abstract

The intraerythrocytic stage of the malaria parasite *Plasmodium falciparum* has an absolute requirement for vitamin B₅ (also known as pantothenate) in order to survive. The parasite takes up extracellular pantothenate and subsequently converts it into coenzyme A (CoA) *via* a series of five universal enzymatic steps. The first enzyme of the pathway is pantothenate kinase (PanK), which phosphorylates pantothenate and commits the molecule to CoA biosynthesis. There are two putative PanK genes (designated *Pfpank1* and *Pfpank2*) in the parasite's genome, both of which are expressed in the intraerythrocytic stage of the parasite. Many antiplasmodial pantothenate analogues, including pantothenol (PanOH), CJ-15,801, *N*-substituted pantothenamides (PanAms) and PanAm derivatives have been shown to inhibit *P. falciparum* growth by targeting its CoA biosynthesis and/or utilisation, although their exact mechanism of action in the parasite remains poorly characterised.

In this study, a step-wise dose-escalating drug pressure regime with either PanOH or CJ-15,801 was used to generate resistant parasite lines. These parasite lines are cross-resistant to both PanOH and CJ-15,801, but exhibit different sensitivity profiles to the PanAm derivatives N5-trz-C1-Pan and *N*-PE- α Me-PanAm, consistent with these two groups of pantothenate analogues having different mechanisms of action. Whole-genome sequencing revealed that these parasites harbour mutations in *Pfpank1*. Some of these mutations significantly alter the activity of *PfPanK*, the parasite's requirement for pantothenate and consequently their fitness compared to the Parent line. These results are consistent with *PfPanK1* being the active PanK during this stage of the parasite's lifecycle. When analysed in conjunction with what has been reported for other organisms, the results of functional enzymatic assays performed in this study revealed important information about the modes of action of these pantothenate analogues. PanOH and CJ-15,801 are predicted to inhibit *PfPPCS* (the second enzyme of CoA biosynthesis). Conversely, N5-trz-C1-Pan and *N*-PE- α Me-PanAm are hypothesised to be metabolised into CoA analogues, which subsequently inhibit downstream CoA-utilising enzymes.

In order to characterise the activity, conformation and potential interacting partners of *Pf*PanK1 and *Pf*PanK2, green fluorescent protein (GFP)-fused copies of these proteins were expressed in *P. falciparum* parasites to enable their purification. Results of western blot and mass spectrometry analyses of immunoprecipitated *Pf*PanK are consistent with the native protein being a complex that is comprised of *Pf*PanK1, *Pf*PanK2 and the adapter protein *Pf*14-3-3I. This marks the first description of a heterodimeric PanK in nature. *In silico* analysis of the amino acid sequence of the two *Pf*PanKs and interrogation of existing phosphoproteomic studies suggest that *Pf*14-3-3I binds to *Pf*PanK2.

Taken together, the research presented in this study has extended our understanding of the *P. falciparum* PanKs and, therefore, provided further insight into the CoA biosynthesis pathway as an antimalarial drug target. Furthermore, the information generated in this study about the mechanisms of action of the pantothenate analogues will hopefully expedite the discovery of new antimalarials.

Publications

Publications arising from studies conducted during the course of this thesis:

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Abbreviations

5-FC	5-fluorocytosine
ACN	acetonitrile
ACT	artemisinin-based combination (chemo)therapy
ADP	adenosine diphosphate
Ala	alanine
AMP	adenosine monophosphate
AMPPNP	adenylyl-imidodiphosphate
Arg	arginine
ARTs	artemisinin and its semi-synthetic derivatives
ASHKA	acetate and sugar kinase/hsp70/actin
Asn	asparagine
Asp	aspartic acid
ATP	adenosine triphosphate
AtPanK1	<i>Arabidopsis thaliana</i> pantothenate kinase 1
AtPanK2	<i>Arabidopsis thaliana</i> pantothenate kinase 2
cDNA	complimentary deoxyribonucleic acid
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CI	confidence interval
CoA	coenzyme A
Cryo-EM	single-particle cryo-electron microscopy
Cys	cysteine
DAPI	4',6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DHFR	dihydrofolate reductase
DHODH	dihydroorotate dehydrogenase
DHPS	dihydropteroate synthase
DIA	data-independent acquisition
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

dNTP	deoxyribonucleotide triphosphate
DPCK	dephospho-coenzyme A kinase
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
<i>EcPanK</i>	<i>Escherichia coli</i> pantothenate kinase
EDTA	ethylenediaminetetraacetic acid
ELM	Eukaryotic Linear Motif
FA	formic acid
FACS	fluorescence-activated cell sorting
gDNA	genomic deoxyribonucleic acid
GFP	green fluorescent protein
Glu	glutamic acid
Gly	glycine
<i>hdhfr</i>	<i>human dihydrofolate reductase</i>
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase
IAA	iodoacetamide
IC₃₅	35% inhibitory concentration
IC₅₀	50% inhibitory concentration
IDA	information-dependent acquisition
IGV	Integrated Genome Viewer
Ile	isoleucine
Indel	insertions-deletion polymorphism
IRS	indoor residual spraying
ITN	insecticide-treated bed net
<i>K_m</i>	Michaelis constant
LC	liquid chromatography
LDS	lithium dodecyl sulfate
Leu	leucine
Lys	lysine
MCS	multiple cloning site
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
Met	methionine
MRA	mycoplasma removal agent

MS	mass spectrometry
<i>m/z</i>	mass-to-charge ratio
N5-Pan	<i>N</i> -pentylpantothenamide
N7-Pan	<i>N</i> -heptylpantothenamide
NLS	nuclear localisation signal
NPP	new permeability pathways
PanAm	<i>N</i> -substituted pantothenamide
PanK	pantothenate kinase
PanOH	pantothenol
<i>PbPAT</i>	<i>Plasmodium berghei</i> pantothenate transporter
<i>PbPPAT</i>	<i>Plasmodium berghei</i> phosphopantetheine adenylyltransferase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylenimine
<i>PfATP4</i>	<i>Plasmodium falciparum</i> Na ⁺ P-type adenosine triphosphatase 4
<i>PfCRT/Pfcrt</i>	<i>Plasmodium falciparum</i> chloroquine resistance transporter (protein/gene)
<i>PfDPCK</i>	<i>Plasmodium falciparum</i> dephospho-coenzyme A kinase
<i>PfEMP1</i>	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
<i>PfH3</i>	<i>Plasmodium falciparum</i> histone H3
<i>PfHK</i>	<i>Plasmodium falciparum</i> hexokinase
<i>PfHSP70</i>	<i>Plasmodium falciparum</i> heat shock protein 70
<i>PfKAHRP</i>	<i>Plasmodium falciparum</i> knob-associated histidine-rich protein
<i>PfMDR1</i>	<i>Plasmodium falciparum</i> multidrug resistant protein 1
<i>PfPanK1/Pfpank1</i>	<i>Plasmodium falciparum</i> pantothenate kinase 1 (protein/gene)
<i>PfPanK2/Pfpank2</i>	<i>Plasmodium falciparum</i> pantothenate kinase 2 (protein/gene)
<i>PfPAT</i>	<i>Plasmodium falciparum</i> pantothenate transporter
<i>PfPI3K</i>	<i>Plasmodium falciparum</i> phosphatidylinositol-3-kinase
<i>PfPI4K</i>	<i>Plasmodium falciparum</i> phosphatidylinositol-4-kinase
<i>PfPPAT</i>	<i>Plasmodium falciparum</i> phosphopantetheine adenylyltransferase
<i>PfPPCDC</i>	<i>Plasmodium falciparum</i> phosphopantothenoylcysteine decarboxylase

PfPPCS	<i>Plasmodium falciparum</i> phosphopantothenoylcysteine synthetase
Phe	phenylalanine
PKAN	pantothenate kinase-associated neurodegeneration
PPAT	phosphopantetheine adenylyltransferase
PPCDC	phosphopantothenoylcysteine decarboxylase
PPCS	phosphopantothenoylcysteine synthetase
PVDF	polyvinylidene fluoride
qPCR	quantitative polymerase chain reaction
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rsc	relative specificity constant
RT	retention-time
SaPanK	<i>Staphylococcus aureus</i> pantothenate kinase
SC₅₀	50% stimulatory concentration
Scfcu	<i>Saccharomyces cerevisiae</i> cytosine deaminase/ phosphoribosyl transferase
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
Ser	serine
SI	supplementary information
SLI	selection-linked integration
SNP	single nucleotide polymorphism
SP	sulfadoxine-pyrimethamine
Thr	threonine
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tyr	tyrosine
Val	valine
V_{max}	maximal velocity
WHO	World Health Organisation

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Chapter 1: Introduction

1.1. Malaria

Malaria is a lethal infectious disease that has historically been, and remains, one of the major causes of mortality and morbidity in the world. The World Health Organisation (WHO) estimated that there were 216 million individuals infected with malaria in 2016, which resulted in 445,000 deaths (World Health Organisation, 2017). Nearly half of the world's population (over 3 billion people) live in countries where they are at risk of contracting malaria, with pregnant women and children being particularly vulnerable. In fact, children under 5 years of age makes up 70% of all malaria deaths according to the most recent global estimates (World Health Organisation, 2017). The majority of the disease burden occurs disproportionately in sub-Saharan Africa (World Health Organisation, 2017). There is a strong correlation between poverty and malaria, and indeed the economic and social impact of malaria is also most apparent in the countries within this region (Sachs and Malaney, 2002). Although the incidence and mortality of the disease have been on a steady and substantial decline since 2010, there are worrying signs that this trend is starting to plateau or even reverse (World Health Organisation, 2017).

Malaria is caused by parasitic unicellular protozoans of the phylum *Apicomplexa* belonging to the genus *Plasmodium*. There are four species that commonly cause malaria in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. In addition, *P. ovale* has now been re-classified into two separate subspecies, *P. ovale curtisi* and *P. ovale wallikeri* (Sutherland *et al.*, 2010). Of these, *P. falciparum* is the most prevalent species in the sub-Saharan Africa region and is responsible for the biggest proportion of global mortality (World Health Organisation, 2017). It is important to note, however, that *P. vivax* is the dominant species outside of Africa (World Health Organisation, 2017). In recent years, a species that normally infects macaque monkeys, *Plasmodium knowlesi*, was identified from a large locus of human hosts in parts of Malaysian Borneo (Singh *et al.*, 2004) and, with the decline of *P. falciparum* and *P. vivax* infections, is now the most common cause of malaria in the country (William *et al.*, 2013; Yusof *et al.*, 2014; William *et al.*, 2014). Malaria

is transmitted between humans by an insect vector. A female *Anopheles* mosquito, the parasite's definitive host, becomes infected when it draws a blood meal from an infected human intermediate host. The infected vector then passes on the parasite when it feeds on its next target.

1.1.1. Malaria symptoms and pathogenesis

The initial stage of an uncomplicated malaria infection is characterised by a wide range of non-specific, often flu-like symptoms including dizziness, headache, malaise, lethargy, backache, myalgia, nausea, vomiting, anorexia and chilliness (Bartoloni and Zammarchi, 2012). The patient then develops febrile paroxysms, a hallmark of the disease. In *P. falciparum* malaria, patients often experience irregular fever initially, which can subsequently develop into a periodic quotidian, tertian or subtertian fever (Bartoloni and Zammarchi, 2012). Individuals infected with *P. falciparum* can also present symptoms of severe malaria when complications occur. Severe anaemia results due to dyserythropoiesis (Phillips *et al.*, 1986) and the accelerated destruction of uninfected erythrocytes in addition to parasite-induced erythrocyte lysis (Looareesuwan *et al.*, 1991). Metabolic disturbances such as lactic acidosis and hypoglycaemia also occur frequently (Planche and Krishna, 2006). Additionally, the ability of *P. falciparum*-infected erythrocytes to adhere to host endothelium (Udeinya *et al.*, 1981), rosette with uninfected erythrocytes (Udomsangpetch *et al.*, 1989) and clump with platelets (Pain *et al.*, 2001) allows the parasite to sequester in many of the host's organs (Miller *et al.*, 2002). This is thought to be the central factor that underlies many of the symptoms of severe *P. falciparum* malaria, including respiratory distress, renal failure, hepatic dysfunction, placental malaria and cerebral malaria, the most serious of the complications (Autino *et al.*, 2012). Cerebral malaria regularly results in seizures, coma and is nearly 100% fatal without treatment (Idro *et al.*, 2010; Dvorin, 2017). The complex pathogenesis of malaria coincides with the cyclical destruction of infected erythrocytes during the intraerythrocytic part of the parasite's lifecycle (**Section 1.1.2**) and is thought to be mediated by the systemic release of host proinflammatory cytokines and chemokines (Miller *et al.*, 1994; Clark and Schofield, 2000; Olivier *et al.*, 2014). It has been shown that haemozoin (Olivier *et al.*, 2014), a by-product of parasite haem digestion (Slater and Cerami, 1992; Pandey and Tekwani, 1996), and parasite glycosylphosphatidylinositol (Tachado *et al.*, 1997; Zhu *et al.*, 2011) activate host

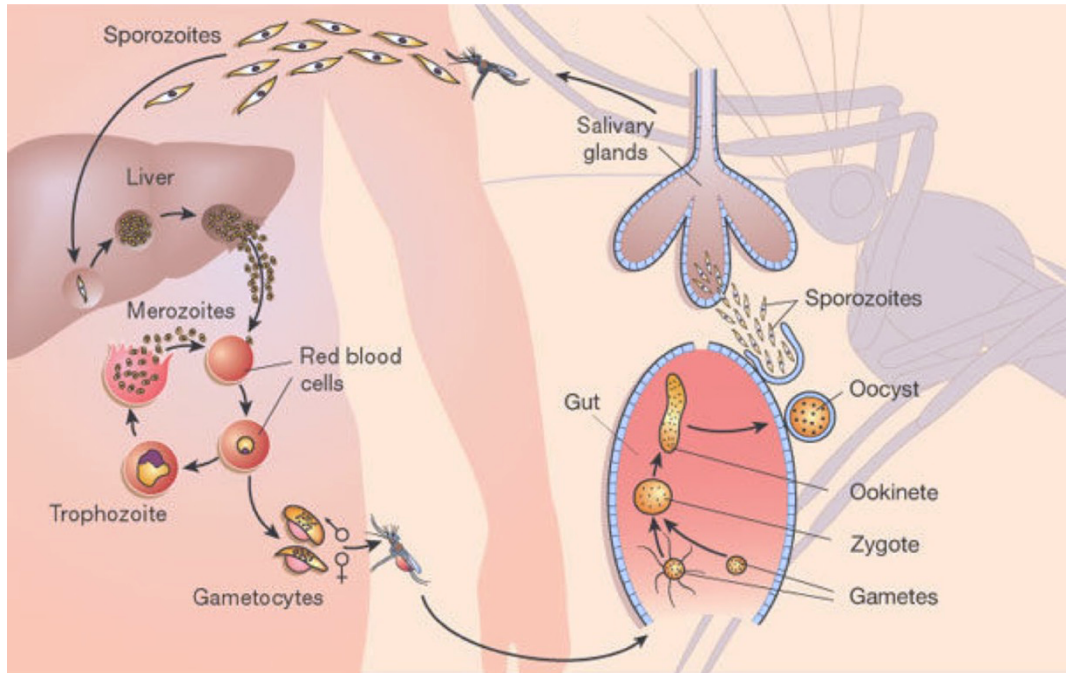
immune response and influence pathogenesis, although the exact mechanisms are still not completely understood.

1.1.2. Lifecycle of *Plasmodium falciparum*

P. falciparum has a unique and complex lifecycle that involves a human host and a mosquito vector (**Figure 1.1 a**). Parasites in the sporozoite stage are introduced into the dermis of the human host (Frischknecht *et al.*, 2004; Vanderberg and Frevert, 2004) through the proboscis of an infected female *Anopheles* mosquito when it probes for a blood meal. The motile parasites linger at the injection site (Sidjanski and Vanderberg, 1997; Yamauchi *et al.*, 2007) and gradually disperse until they encounter a blood vessel wall through which they penetrate and enter the bloodstream (Vanderberg and Frevert, 2004; Amino *et al.*, 2006). The sporozoite then travels to the liver, where it enters through Kupffer cells (resident liver macrophages) (Frevert *et al.*, 2005) and traverses through the cytosol of several hepatocytes before invading a final hepatocyte to form a parasitophorous vacuole (Mota *et al.*, 2001; Frevert *et al.*, 2005). Within each vacuole, the sporozoite dedifferentiates and undergoes intense schizogony, which culminates in the formation of thousands of infectious merozoites (Meis and Verhave, 1988; Vaughan *et al.*, 2008). These newly formed daughter cells are then delivered into the host bloodstream in structures known as merozoites (Sturm *et al.*, 2006), before they are released.

The intraerythrocytic cycle of *P. falciparum* (**Figure 1.1 b**) is the cause of all clinical symptoms associated with malaria. Each 48 h cycle is initiated when a merozoite invades a host erythrocyte. An individual merozoite attaches to the erythrocyte surface through specific ligand-receptor interactions, forms a tight junction and ultimately seals itself within a parasitophorous vacuole (Cowman *et al.*, 2017). Within this vacuole, the parasite almost immediately develops into the ring stage form (**Figure 1.1 b**). After residing within the infected erythrocyte for roughly 15 h, the parasite then progressively becomes metabolically and biosynthetically active as it transitions into the trophozoite stage (Kirk, 2001) (**Figure 1.1 b**). During this growth phase, the parasite consumes 60 to 80% of the haemoglobin in the host erythrocyte cytosol by endocytosis, which is then transported into the parasite's acidic digestive vacuole (Francis *et al.*, 1997; Krugliak *et al.*, 2002). Within this organelle, host haemoglobin is subjected to

a



b

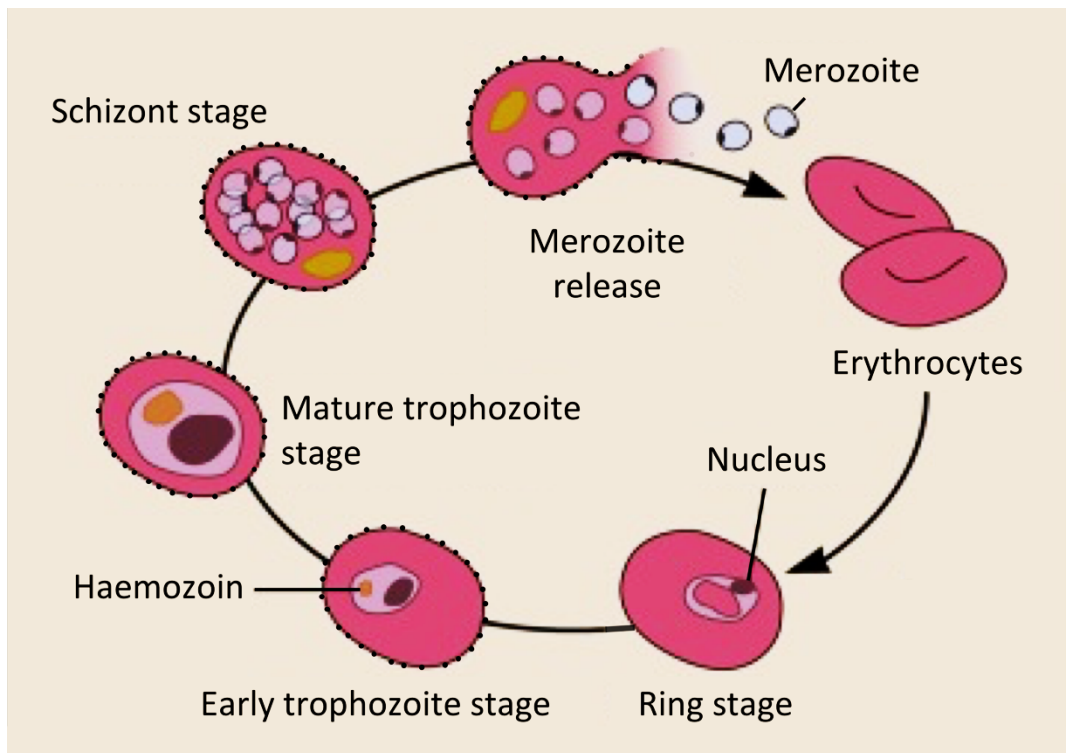


Figure 1.1. The lifecycle of *P. falciparum*. (a) The complete lifecycle of the parasite through the human (intermediate) and mosquito (definitive) hosts. Parasites (sporozoite stage) are introduced into a human host by an infected mosquito during a blood meal. They quickly traverse to the liver and infect the host cells to begin the hepatic stage. The parasites multiply asexually to produce daughter cells (merozoite stage) that are then released into the bloodstream and initiate the intraerythrocytic cycle. A small proportion of the intraerythrocytic parasites develop into male and female sexual stages (gametocytes), which can be ingested by a mosquito vector when it feeds on the infected person. These forms of the parasite mature into microgametes and macrogametes within the mosquito gut lumen and fuse to form a zygote, which then further differentiates into a motile ookinete. The parasite at this stage then penetrates the midgut wall of the mosquito where it develops into an oocyst, which then divides to generate numerous sporozoites. When the oocyst ruptures, the liberated sporozoites travel to the salivary glands of the mosquito, where they are primed to begin the lifecycle anew. Adapted from Wirth (2002). (b) A detailed representation of the intraerythrocytic stage of the parasite's lifecycle. Following a merozoite's invasion of an erythrocyte, the parasite immediately develops into an immature form (ring stage). It then goes through a metabolically active phase (trophozoite stage), rapidly consuming host cell haemoglobin and generating haemozoin crystals. The parasite finally divides to generate 16 to 32 daughter merozoites within the infected erythrocyte (schizont stage). After approximately 48 h, the infected host erythrocyte ruptures and the new merozoites are released into the bloodstream, repeating the cycle. Adapted from Lee *et al.* (2014).

proteolysis to generate small peptides (Kolakovich *et al.*, 1997), which serve as a major amino acid source for the parasite. At the same time, the toxic haem moiety, produced as a by-product of haemoglobin digestion, is converted by the parasite into chemically inert haemozoin crystals and stored within the digestive vacuole (Francis *et al.*, 1997). The parasite grows rapidly in size during the trophozoite stage, occupying approximately a third of the infected erythrocyte by 36 h (Saliba *et al.*, 1998b). As the parasite matures, it also extensively restructures the host erythrocyte that it occupies, allowing it to evade destruction by the spleen of the host through cytoadherence and to have better access to nutrients. The parasite achieves this by exporting a large number of its proteins, known as its exportome, beyond the parasite plasma membrane (Maier *et al.*, 2009). Protein traffic to the infected erythrocyte membrane is facilitated by unique, parasite-derived, membrane structures in the erythrocyte cytosol that include extensions of the parasitophorous vacuole membrane, known as the tubulovesicular network, and Maurer's clefts (Mundwiler-Pachlatko and Beck, 2013). The parasite also induces the formation of knobby protrusions on the surface of the erythrocyte membrane by expressing *Plasmodium falciparum* knob-associated histidine-rich protein (PfKAHRP) (Crabb *et al.*, 1997). These knobs act as platforms on which the parasite presents an integral membrane protein product of the *var* gene family (Smith *et al.*, 1995), PfEMP1 (*Plasmodium falciparum* erythrocyte membrane protein 1) (Baruch *et al.*, 1995), which is a key molecule involved in the cytoadherence of infected erythrocytes and parasite immune evasion due to its antigenic variability (Pasternak and Dzikowski, 2009). In addition, the parasite also enables the transport of a wide range of essential nutrients across the infected erythrocyte membrane by inducing new permeability pathways (NPP) (Ginsburg *et al.*, 1983; Ginsburg *et al.*, 1985; Kirk *et al.*, 1994; Staines *et al.*, 2006). An integral membrane protein in the host erythrocyte plasma membrane that is encoded by the parasite's *clag3* gene was found to contribute to NPP activity (Nguiragool *et al.* 2011). After around 40 – 48 h from the time of invasion (the length of this cycle varies in different species), *P. falciparum* develops into the schizont stage, during which it divides to generate 16 – 32 daughter merozoites within the host cell (**Figure 1.1 b**). These are eventually released when the host erythrocyte ruptures, with each daughter cell having the potential to repeat the cycle.

During the intraerythrocytic stage, a small proportion (0.2 – 1%) of asexual parasites undergo gametocytogenesis (Sinden, 1983) (**Figure 1.1 a**). At the start of this process, all of the merozoites from a single schizont commit themselves to develop into gametocytes of the same sex (Bruce *et al.*, 1990; Silvestrini *et al.*, 2000; Smith *et al.*, 2000). In *P. falciparum*, sexually committed parasites mature through five morphologically distinct stages of development over a period of 8 to 12 days (Baker, 2010). The mature (stage V) male and female gametocytes that circulate in the bloodstream of the human host mediate the transmission of the parasite to the mosquito vector when they are ingested during a blood meal. Parasite sexual reproduction occurs within the midgut lumen of the mosquito. The formation of the microgametes and macrogametes are triggered by the presence of xanthurenic acid, along with changes in temperature and pH (Billker *et al.*, 1997; Billker *et al.*, 1998). A zygote is produced by the fusion of the gametes, which then undergoes meiosis and develops into a motile cell known as the ookinete (Aly *et al.*, 2009). The ookinete traverses through several midgut epithelial cells before attaching to the midgut wall to begin its transformation into a sessile oocyst. In a process that takes approximately 10 to 12 days, the oocyst ultimately develops into thousands of haploid sporozoites, which are released into the mosquito host's haemolymph (Aly *et al.*, 2009). These sporozoites eventually migrate to the salivary glands of the mosquito and are primed to start the lifecycle anew.

1.2. Malaria Control

1.2.1. Malaria prevention

Current prevention efforts at controlling the spread of malaria focus mainly on three broad strategies namely vector control, preventative treatments, and the development and administration of vaccines. Two of the most commonly implemented methods aimed at preventing mosquito transmission of the parasites are the use of insecticide-treated bed nets (ITNs) and indoor residual spraying (IRS) of insecticide (World Health Organisation, 2017). These interventions played an instrumental role in reducing the global malaria burden in the past two decades, with ITNs estimated to have averted 68% of all *P. falciparum* clinical cases in Africa between 2000 and 2015 (Bhatt *et al.*, 2015). Unfortunately, the proportions of the population at risk that are being protected

by these vector control methods are either substantially lower than set targets (for ITNs) or declining (for IRS) (World Health Organisation, 2017). Furthermore, due to the limited number of insecticide classes that are being utilised in these strategies, increasing incidence of insecticide resistance (pyrethroids in particular) might jeopardise their effectiveness (Ranson and Lissenden, 2016; World Health Organisation, 2017). Current strategies also neglect the impact of outdoor transmissions. As such, future malaria vector management strategies need to integrate other tools that are currently available, such as larval control through larvicides, or strategies that are under development, such as sterile insect technique or using *Wolbachia* to generate malaria-incompatible mosquitoes, which would address these challenges (Benelli and Beier, 2017).

The WHO also recommends the prevention of malaria through drug treatments. Intermittent preventative treatment for women and infants in the form of sulfadoxine-pyrimethamine (SP; detailed in **Section 1.2.2**) are recommended in areas with moderate to high malaria transmission (Gosling *et al.*, 2010; World Health Organisation, 2017). More recently, the WHO has also recommended seasonal malaria chemoprevention, where children under five years of age are given courses of SP and amodiaquine during malaria transmission season (Coldiron *et al.*, 2017; World Health Organisation, 2017). Although most of the trials of these chemoprevention strategies indicate that they are beneficial, endemic countries have been slow to include them in their health policies. This is partly due to the problems of logistics, adherence to the treatment regime, falling levels of transmission and issues of parasite SP resistance (Gosling *et al.*, 2010; Coldiron *et al.*, 2017).

Although it is known that residents of endemic areas naturally develop immunity against malaria (albeit imperfectly), the exact underlying mechanism behind this phenomenon is not fully understood and this has been a major hurdle in the development of a practical malaria vaccine (Doolan *et al.*, 2009). Furthermore, the complex, multi-stage lifecycle of the parasite, along with the significant genetic polymorphism (and temporal switching of variant expression in the case of *PfEMP1*) of many vaccine candidates, pose additional challenges (Hill, 2011). The most advanced malaria vaccine candidate to date is RTS,S/AS01 (also known as Mosquirix). The RTS,S component consists of a recombinant protein

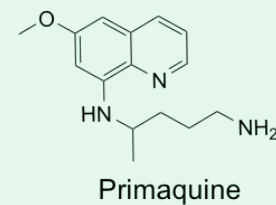
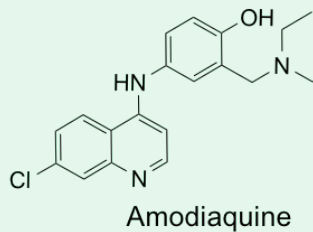
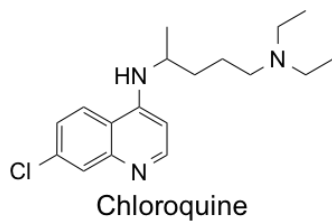
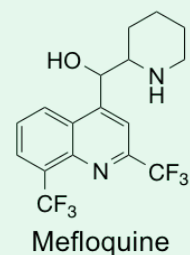
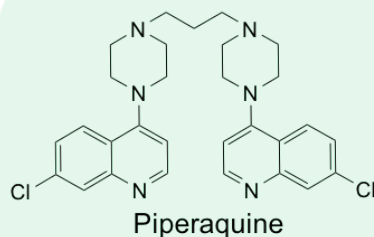
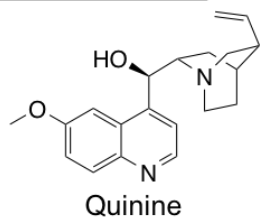
made up of the parasite's circumsporozoite protein central tandem repeat epitopes, "R", and carboxy-terminal epitopes recognised by T-cells, "T", fused to a hepatitis B surface antigen, "S", co-expressed with copious amounts of the S protein (Stoute *et al.*, 1997). When administered in conjunction with the AS01 adjuvant in a phase 2a trial, the vaccine showed 50% efficacy (Kester *et al.*, 2009). Unfortunately, the results of a large phase 3 trial that was initiated in sub-Saharan African countries in 2009 were less positive, with the vaccine having provided marginal (in infants) to moderate (young children) protection that waned rapidly, particularly in the absence of a subsequent booster dose (RTS,S Clinical Trials Partnership, 2014; RTS,S Clinical Trials Partnership, 2015). Nevertheless, the vaccine was approved for immunisation by the European Medicines Agency in mid-2015, making it the first licensed malaria vaccine, although its role in future elimination strategies remains to be seen (Gosling and Seidlein, 2016). Significant advances have been made in other malaria vaccine strategies (including whole sporozoites and transmission-blocking vaccines) although, relatively, these are still in the early stages of the clinical trials pipeline (Hoffman *et al.*, 2015).

Despite playing an important role, the future implementation of preventative strategies faces serious challenges and on its own is insufficient to effectively control malaria transmission. Furthermore, they do not benefit individuals who are currently afflicted with the disease. Therefore, chemotherapy remains an essential tool that we currently have to manage malaria morbidity and mortality.

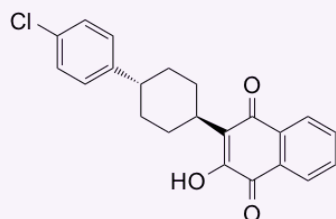
1.2.2. Malaria chemotherapy

One of the earliest treatments for malaria is an herbal remedy made from the dried bark of the cinchona tree. In 1820, quinine (**Figure 1.2**) was isolated from the bark and identified as one of the active alkaloids that give the remedy its antimalarial properties (Achan *et al.*, 2011). Quinine became the main treatment for malaria for many decades until more effective synthetic compounds were discovered. One of these synthetic replacements was a 4-aminoquinoline compound named chloroquine (**Figure 1.2**), which was found to be significantly more potent than other leading antimalarials at the time but lacking their undesired side effects and toxicity (Coatney, 1963). In addition, its ease of

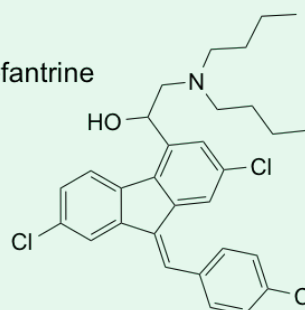
Quinolines



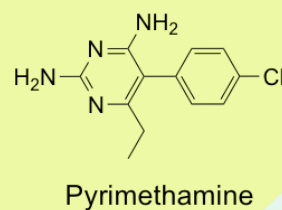
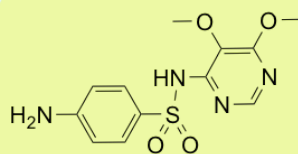
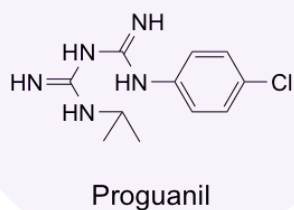
Atovaquone



Lumefantrine



Antifolates



Artemisinin (ARTs)

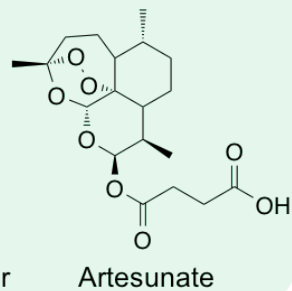
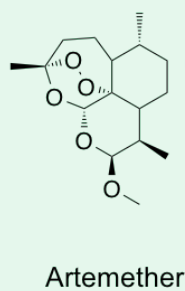
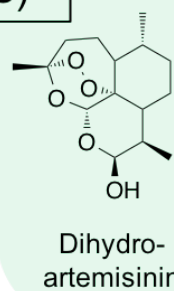
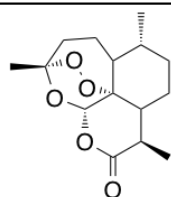


Figure 1.2. The chemical structures of antimalarials in clinical use. Quinine and chloroquine were amongst the most commonly used quinoline antimalarials, but widespread parasite resistance severely limits their effectiveness today. Most malaria parasites have also gained resistance to the antifolates proguanil, sulfadoxine and pyrimethamine. Artemisinin and its derivatives (ARTs) form the basis of the current WHO-recommended treatments for malaria. Artemisinin-based combination therapy (ACT; compounds involved are highlighted in green) is the frontline treatment against most uncomplicated malaria cases, in which a short-lived artemisinin derivative is administered together with a partner drug that possesses a longer half-life such as a quinoline, the structurally-related lumefantrine or the antifolate combination sulfadoxine-pyrimethamine (SP; highlighted in yellow). Artesunate is used to treat severe malaria. Primaquine is mainly used to prevent malaria relapse from *P. vivax*. The combination of atovaquone and proguanil (trade name Malarone; highlighted in purple) is mainly employed as prophylaxis for travellers due to its high cost, but the recent expiration of its patent could reduce its cost of production and increase its use.

administration, stability in patients, rapid parasite clearance time and relative low cost of production (AlKadi, 2007) made it a key component of the Global Malaria Eradication Programme that was launched in 1955 (Bruce-Chwatt, 1956; Mendis *et al.*, 2009). Unfortunately, due partly to its extensive and widespread implementation, parasite resistance to chloroquine was detected within 15 years of its use, initially in the Thai-Cambodian border (Harinasuta *et al.*, 1965) and two South American countries (Moore and Lanier, 1961), and eventually all other endemic countries (Mita *et al.*, 2009). This contributed to the increase in global malaria incidence and mortality, and the goal of malaria eradication was later replaced in favour of control (Mendis *et al.*, 2009; Nájera *et al.*, 2011).

Since then, an extensive amount of work has been done to determine the cause of chloroquine resistance in the parasite. Chloroquine has been shown to accumulate within the digestive vacuole of the parasite (Aikawa, 1972), within which it disrupts the biocrystallisation of haem to haemozoin either by binding to free haem and/or haemozoin (Chou *et al.*, 1980; Sullivan *et al.*, 1996; Dorn *et al.*, 1998). This leads to the accumulation of toxic haem that kills the parasite, although the exact mechanism is still unclear (Sullivan, 2002). Chloroquine-resistant parasites were found to accumulate less chloroquine in their digestive vacuoles (Saliba *et al.*, 1998a). This has been attributed to mutations in two digestive vacuole membrane transporters, namely the *P. falciparum* chloroquine resistance transporter (*PfCRT*) (Fidock *et al.*, 2000) and to a lesser extent the *P. falciparum* multidrug resistant protein 1 (*PfMDR1*) (Cowman *et al.*, 1991; Reed *et al.*, 2000). It has been shown that mutant *PfCRT* (and presumably *PfMDR1*) facilitates the leak of chloroquine from the digestive vacuole (Reed *et al.*, 2000; Sanchez *et al.*, 2005; Sanchez *et al.*, 2007; Lehane *et al.*, 2008; Martin *et al.*, 2009) and prevent the drug from accumulating to levels that are toxic to the parasite.

Other synthetic antimalarials were deployed to combat chloroquine-resistant parasites but these quickly faced the same problem. Sulfadoxine, an inhibitor of dihydropteroate synthase (DHPS), and pyrimethamine, an inhibitor of dihydrofolate reductase (DHFR), have been used for decades in combination as SP (trade name Fansidar; **Figure 1.2**) that synergistically inhibit parasite tetrahydrofolate synthesis (Sibley *et al.*, 2001). However, SP-resistant parasites

harbouring multiple mutations in the target enzymes were detected at the Thai-Cambodia border within a few years after the introduction of SP in the late 1960s and rapidly spread to other endemic regions (Mita *et al.*, 2009). As a result, SP is now mainly used as prophylaxis (Petersen *et al.*, 2011) (**Section 1.2.1**). The potent antimalarial atovaquone (**Figure 1.2**) is a hydroxynaphthoquinone analogue that inhibits the cytochrome *bc*₁ complex of the parasite (Hudson *et al.*, 1985; Fry and Pudney, 1992). This inhibition halts the parasite's electron transport chain, which in turn disrupts the function of dihydroorotate dehydrogenase (DHODH), an enzyme of the essential pyrimidine biosynthesis pathway (Painter *et al.*, 2007). Due to its current high cost of production (Nixon *et al.*, 2013) and the ease by which the parasite develops resistance through mutations in cytochrome *b* (Gil *et al.*, 2003), atovaquone is mainly used in combination with an antifolate, proguanil (trade name Malarone; **Figure 1.2**), as prophylaxis for travellers (Petersen *et al.*, 2011). However, the compound might see an increase in use as the patent for Malarone has recently expired (Nixon *et al.*, 2013). The use of other quinolones (**Figure 1.2**), including amodiaquine, mefloquine, piperazine and primaquine, and the structurally-related lumefantrine (**Figure 1.2**), were also affected by widespread parasite resistance through polymorphisms in *PfCRT* and *PfMDR1*, although they are currently still utilised in combination therapies (Petersen *et al.*, 2011; World Health Organisation, 2015).

A critical turning point for malaria chemotherapy was the rediscovery, in 1971, of a traditional Chinese herbal remedy, an extract of *Artemisia annua* (sweet wormwood), and the identification of qinghaosu (artemisinin) as the active component the following year (Klayman, 1985; Tu, 2011). Artemisinin and its semi-synthetic derivatives (ARTs; **Figure 1.2**) are sesquiterpene lactones that possess potent antiparasmodial activity, including ring stage parasites, but disappointingly short half-life (Woodrow *et al.*, 2005). By administering an ART together with another antimalarial with a longer half-life, in what is known as artemisinin-based combination therapies (ACTs; **Figure 1.2**), their efficacy was improved while simultaneously minimising the selection of drug resistant parasites (White *et al.*, 2015; Fairhurst and Dondorp, 2016). Today, the WHO recommends ACTs as frontline chemotherapies to treat uncomplicated *P. falciparum* malaria and artesunate (an ART) for severe malaria (World Health

Organisation, 2015). Although ACTs are currently still effective in most endemic areas, partial ART resistance emerged in the late 2000s in the Greater Mekong Subregion (Ménard and Dondorp, 2017). This has subsequently selected for parasite resistance to ACT partner drugs and eventually led to high rates of complete treatment failures in these countries (Ménard and Dondorp, 2017).

Despite their critical roles as frontline treatments and the rising instances of resistance, the exact mechanism of action of ARTs is not completely understood. All ARTs are converted mostly to the active metabolite dihydroartemisinin (Woodrow *et al.*, 2005) (**Figure 1.2**). It is thought that haem within the parasites cleaves the endoperoxide bridge moiety of ARTs to generate free radicals (Woodrow *et al.*, 2005; Wang *et al.*, 2015). These then bind promiscuously to and react with parasite proteins and lipids involved in essential biological processes, resulting in parasite death (Wang *et al.*, 2015; Fairhurst and Dondorp, 2016). In recent years, polymorphisms in the propeller domain of a *P. falciparum* kelch protein, *PfKelch13*, were found to correlate with parasite ART resistance (Ariey *et al.*, 2014; Ghorbal *et al.*, 2014; Straimer *et al.*, 2015). Transcriptomic studies of ART-resistant parasites found that the resistance phenotype associates with an upregulation of genes involved in the “unfolded protein stress response” pathway (Mok *et al.*, 2015; Shaw *et al.*, 2015). Subsequently, *PfKelch13* was found to facilitate protein ubiquitination that ultimately leads to their degradation by the proteasome and that reduced level of ubiquitination was observed in parasites expressing mutant *PfKelch13* (Dogovski *et al.*, 2015; Mbengue *et al.*, 2015). A model has been put forth in which *PfKelch13* negatively regulates a putative transcription factor that controls a range of genes involved in antioxidant response. A defective *PfKelch13* results in the constitutive expression of these genes that allows the parasite to respond quickly to ART (Mok *et al.*, 2015; Fairhurst and Dondorp, 2016). An alternative model was suggested by another study in which dihydroartemisinin was shown to specifically inhibit the activity of *P. falciparum* phosphatidylinositol-3-kinase (*PfPI3K*) and that a defective mutant *PfKelch13*-mediated protein ubiquitination results in an increase in the levels of *PfPI3K* and its lipid product, leading to a reduced ART sensitivity in the parasite (Mbengue *et al.*, 2015; Fairhurst and Dondorp, 2016).

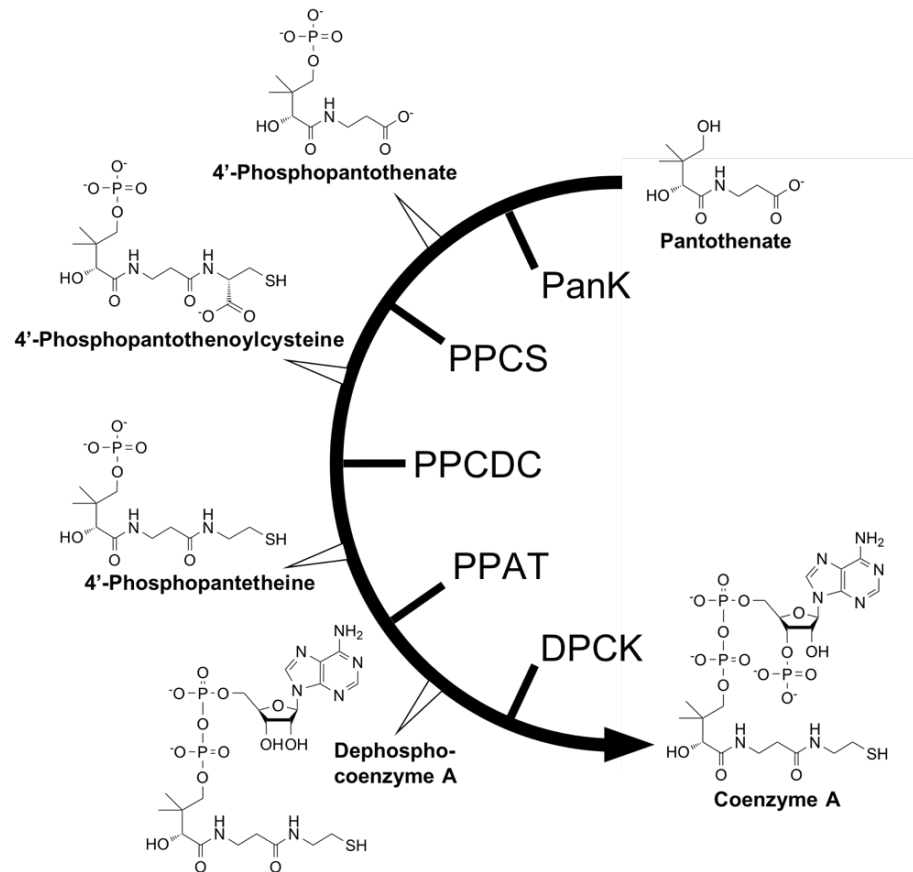
Although the *PfKelch13* polymorphisms that are associated with ART resistance in Southeast Asia are thus far absent in sub-Saharan Africa, there is a large population of low frequency *PfKelch13* alleles that could allow ART resistance to be rapidly selected in the future (Fairhurst and Dondorp, 2016; Ménard and Dondorp, 2017). Other mechanisms of ART resistance could also be involved (Sutherland *et al.*, 2017). Furthermore, the growing extent of parasite ART resistance in Southeast Asia (Ménard and Dondorp, 2017) poses a huge risk to the gains achieved in malaria control should it spread to the African continent. Hence, new strategies are needed to mitigate this risk. These could include the rotation of ACT regimes, extending the duration of current ACTs and pairing two synergistic partner drugs with an ART in triple ACTs (Ménard and Dondorp, 2017). Additionally, the identification and development of new antimalarials is an ongoing imperative in the fight against malaria. The current goal of the malaria drug discovery pipeline is to produce potent, single-exposure combination chemotherapies that utilise compounds of different chemical classes to minimise resistance generation and ideally target multiple parasite lifecycle stages (Burrows *et al.*, 2017). So far, improved rational drug design and phenotypic screening has resulted in compounds with novel mechanisms of action entering phase II clinical trials in the last few years (Wells *et al.*, 2015) including the trioxolane OZ439 (Phyo *et al.*, 2016), the spiroindolone analogue KAE609 (White *et al.*, 2014) and the imidazolopiperazine KAF156 (Leong *et al.*, 2014; White *et al.*, 2016). However, the process of drug discovery is lengthy with a low success rate (Wells *et al.*, 2015; Burrows *et al.*, 2017). Furthermore, many of the compounds that are entering the pipeline act on only a few common targets such as *P. falciparum* Na⁺ P-type adenosine triphosphatase 4 (*PfATP4*) (Lehane *et al.*, 2014) and *P. falciparum* phosphatidylinositol-4-kinase (*PfPI4K*) (Wells *et al.*, 2015). Therefore, in order to sustain the drug discovery effort, there needs to be a continuous input of compounds that represent diverse chemotypes with different mechanisms of action (Wells *et al.*, 2015; Burrows *et al.*, 2017). The intraerythrocytic stage of *P. falciparum* remains a critical target for drug discovery as it is the source of all malaria symptoms. The parasite uptake and metabolism of nutrients during this stage of its lifecycle has been a major focus of antimalarial drug development (Kirk and Saliba, 2007) and one pathway that has recently attracted renewed interest is the parasite's coenzyme A (CoA) biosynthesis pathway (Spry *et al.*, 2008).

1.3. Biosynthesis of Coenzyme A in *P. falciparum*

CoA is an essential cofactor in virtually all living organisms. It functions as an acyl group carrier and carbonyl activating group in many central metabolic reactions. CoA also acts as a source of 4'-phosphopantetheine prosthetic group for carrier proteins involved in fatty acid, polyketide and non-ribosomal peptide biosynthesis. Vitamin B₅, also known as pantothenic acid (or pantothenate when ionised), is the precursor to CoA. Plants, fungi and some bacteria are able to synthesise pantothenate *de novo* from β -alanine and α -ketoisovalerate, while animals and some species of prokaryotic and eukaryotic pathogens require an exogenous source of pantothenate (Spry *et al.*, 2008). CoA is universally synthesised from pantothenate through a series of five enzymatic steps (Genschel, 2004) (**Figure 1.3 a**). Pantothenate is first phosphorylated to 4'-phosphopantothenate by pantothenate kinase (PankK). The second enzyme, phosphopantothenoylcysteine synthetase (PPCS) catalyses the condensation of 4'-phosphopantothenate and a cysteine molecule into 4'-phosphopantothenoylcysteine. Phosphopantothenoylcysteine decarboxylase (PPCDC), the third enzyme in the pathway, then decarboxylates the cysteine moiety of 4'-phosphopantothenoylcysteine to generate 4'-phosphopantetheine. The next step involves the addition of an adenylyl group to 4'-phosphopantetheine to produce dephospho-CoA and is catalysed by phosphopantetheine adenylyltransferase (PPAT). The final enzyme, dephospho-CoA kinase (DPCK), subsequently phosphorylates dephospho-CoA at the 3'-position of the ribose ring to yield CoA.

Unlike the avian malaria parasite *Plasmodium lophurae*, which relies on its erythrocyte host cell for the production of CoA (Bennett and Trager, 1967; Brohn and Trager, 1975; Trager and Brohn, 1975), multiple lines of evidence indicate that *P. falciparum* synthesises its own CoA using pantothenate scavenged from the human host bloodstream. The intraerythrocytic stage of *P. falciparum* parasites maintained temporarily in an axenic condition do not require exogenous CoA (Trager, 2002). On the other hand, an exogenous supply of pantothenate is necessary for intraerythrocytic *P. falciparum* to grow *in vitro* (Divo *et al.*, 1985; Saliba *et al.*, 2005). This is consistent with the absence of candidate genes of the enzymes required for *de novo* pantothenate biosynthesis in the *P. falciparum*

a



b

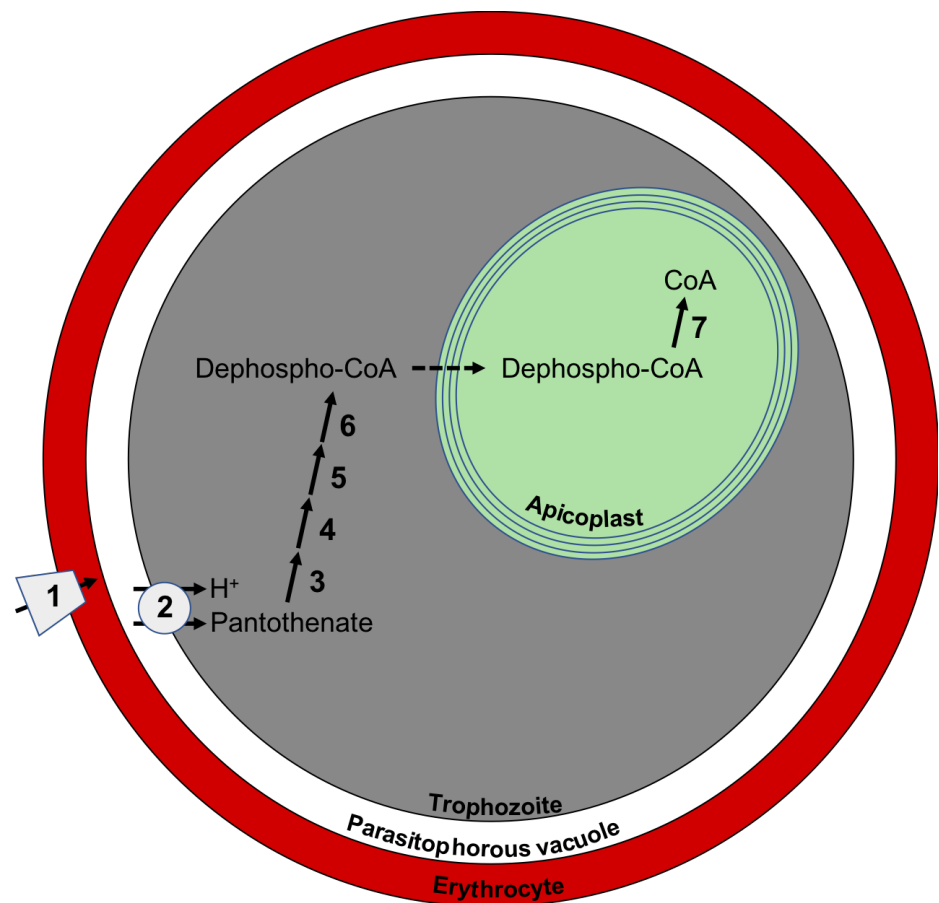


Figure 1.3. The CoA biosynthesis pathway in *P. falciparum*. (a) Pantothenate is converted into CoA through five universal steps, which are catalysed in turn by pantothenate kinase (PanK), phosphopantothenoylcysteine synthetase (PPCS), phosphopantothenoylcysteine decarboxylase (PPCDC), phosphopantetheine adenylyltransferase (PPAT) and dephospho-CoA kinase (DPCK). Adapted from Spry *et al.* (2010). (b) Pantothenate enters a *P. falciparum* trophozoite-infected erythrocyte through the parasite-induced new permeability pathways (NPP; **1**). It diffuses into the parasitophorous vacuole and is taken up into the parasite through a H⁺-coupled symporter (**2**). Once in the parasite cytosol, pantothenate is sequentially converted into dephospho-CoA by *PfPanK* (**3**), *PfPPCS* (**4**), *PfPPCDC* (**5**) and *PfPPAT* (**6**). Dephospho-CoA is then assumed to be transported into the apicoplast where the final enzyme of the pathway, *PfDPCK* (**7**), is located. The *P. falciparum* genome contains putative genes that code for these enzymes, which are all transcribed during the intraerythrocytic stage of the parasite. Multiple genes were predicted to code for *PfPanK*, *PfPPCS* and *PfPPAT*. Adapted from Spry *et al.* (2008).

genome (Müller and Kappes, 2007) and also suggest that the human erythrocyte does not contain sufficient pantothenate to support parasite growth. Pantothenate was shown to be rapidly taken up by *P. falciparum*-infected erythrocytes but not uninfected erythrocytes (Saliba *et al.*, 1998b; Spry and Saliba, 2009). Furthermore, most of the pantothenate phosphorylation detected in a *P. falciparum*-infected erythrocyte occurs inside the parasite (Saliba *et al.*, 1998b). Indeed, a more recent study found that the majority of [¹⁴C]pantothenate metabolism into soluble [¹⁴C]CoA quantified in *P. falciparum*-infected erythrocytes can be attributed to the parasite (Spry and Saliba, 2009). Additional studies have also characterised the entry of pantothenate into the parasite and its initial metabolism. Pantothenate enters the parasite-infected erythrocyte almost exclusively through the NPP (Saliba *et al.*, 1998b; Spry and Saliba, 2009) (**Section 1.1.2**) and diffuses freely into the parasitophorous vacuole presumably through low-selectivity channel in the parasitophorous vacuole membrane (Desai *et al.*, 1993) (**Figure 1.3 b**). It is then transported into the parasite cytosol by a H⁺-coupled symport mechanism with a 1:1 stoichiometry (Saliba and Kirk, 2001). A different study subsequently identified a *P. falciparum* transporter, named *PfPAT* (PF3D7_0206200), a member of the multifacilitator superfamily, and characterised its ability to transport pantothenate in yeast cells (Augagneur *et al.*, 2013). Once in the parasite cytosol, pantothenate is first phosphorylated by a high-affinity PanK with a *K_m* of 0.3 μ M (Saliba and Kirk, 2001) that is subjected to feedback inhibition by CoA and its thioesters (Lehane *et al.*, 2007), before it is converted to CoA by the next four enzymes of the pathway (**Figure 1.3 b**).

1.3.1. The pantothenate kinases of *P. falciparum*

Consistent with its ability to generate CoA from pantothenate, candidate genes predicted to encode the five enzymes of the CoA biosynthesis pathway have been found in the *P. falciparum* genome, with several of the enzymes being represented by multiple candidates (PF3D7_1420600 (*Pfpank1*) and PF3D7_1437400 (*Pfpank2*) for PanK; PF3D7_0412300 and PF3D7_1102400 for PPCS; PF3D7_0816100 for PPCDC; PF3D7_0704700 and PF3D7_1327600 for PPAT; PF3D7_1443700 for DPCK (Genschel, 2004; Pinney *et al.*, 2005; Müller and Kappes, 2007). These genes have also been shown to be transcribed during the parasite's intraerythrocytic lifecycle (Bozdech *et al.*, 2003; Aurrecoechea

et al., 2009). However, with the exception of *Pf*PanK, the activity of each of these enzymes have yet to be verified biochemically. Additionally, it is unclear why the parasite expresses multiple isoforms of the same enzyme in the same stage of its lifecycle. Although the *Pf*PanK activity in *P. falciparum* has been characterised (Saliba *et al.*, 1998b; Saliba and Kirk, 2001), it remains to be seen if both *Pf*PanK isoforms are functional. All of the PanKs that has been characterised so far can be categorised into one of three broad groups, namely type I, type II or type III (Brand and Strauss, 2005), all of which occur as homodimers (Yun *et al.*, 2000; Das *et al.*, 2006; Yang *et al.*, 2006a; Hong *et al.*, 2006; Nicely *et al.*, 2007; Hong *et al.*, 2007; Li *et al.*, 2013; Franklin *et al.*, 2015). Both *Pf*PanK1 and *Pf*PanK2 are predicted to be type II PanKs based on a structure-based sequence alignment with other prokaryotic and eukaryotic PanKs of this group (Spry *et al.*, 2010). *Pf*PanK1 was found to contain all of the conserved nucleotide-binding motifs (ADENOSINE, PHOSPHATE 1 and PHOSPHATE 2), insertion regions, and the Glu and Arg residues that are necessary for a functional PanK (Spry *et al.*, 2010). Conversely, although *Pf*PanK2 also contains the two catalytically-important amino acid residues, its PHOSPHATE 1 and 2 nucleotide-binding motifs do not align perfectly with those of other type II PanKs (Spry *et al.*, 2010). Moreover, its second PanK-specific insert is approximately twice as long in sequence compared to those of other PanKs in the group and it also contains a unique large insertion within a loop that is associated with PanK dimerisation (Hong *et al.*, 2007; Spry *et al.*, 2010). For these reasons, it is unclear whether *Pf*PanK2 is functional (Spry *et al.*, 2010). In order to investigate their activities, previous studies have attempted to express *Pf*PanKs in heterologous expression systems, including *Escherichia coli*, yeast and insect cells. However, these have yielded either insoluble (Mehlin *et al.*, 2006) or soluble but inactive proteins (Saliba Lab, unpublished data).

1.3.2. CoA biosynthesis as an antimalarial drug target

The absolute requirement of the intraerythrocytic asexual stage of *P. falciparum* for pantothenate makes its uptake and metabolism to CoA attractive targets for antimalarial drug discovery. Importantly, the enzymes involved are quite distinct from their homologues in the human host. The low-affinity (K_m of 23 mM) proton-dependent pantothenate symport system of *P. falciparum* (Saliba and Kirk, 2001) (**Figure 1.3 b**) discussed above is in stark contrast to the high affinity

(K_m of 2 – 5 μM) Na⁺:pantothenate multivitamin transporters that are present in mammalian cells (Prasad *et al.*, 1998; Prasad *et al.*, 1999; Wang *et al.*, 1999). On the other hand, mammalian PanKs that have been characterised to date bind pantothenate with affinities that are an order of magnitude lower (K_m of 6 – 25 μM) (Halvorsen and Skrede, 1982; Fisher *et al.*, 1985; Zhang *et al.*, 2005; Zhang *et al.*, 2006) than that observed for *P. falciparum* (Saliba and Kirk, 2001). Furthermore, in contrast to mammalian cells in which PanK is the key rate-limiting enzyme of CoA biosynthesis (Rock *et al.*, 2000), 4'-[¹⁴C]phosphopantothenate was observed to accumulate in [¹⁴C]pantothenate-labelled *P. falciparum*-infected erythrocytes and saponin-isolated *P. falciparum* trophozoites, observations which are consistent with PPCS being the important pathway flux control step of parasite CoA biosynthesis, at least during the intraerythrocytic stage of its lifecycle *in vitro* (Spry and Saliba, 2009). The last two enzymatic steps of CoA biosynthesis in human are performed by a bifunctional PPAT/DPCK enzyme, CoA synthetase (Daugherty *et al.*, 2002; Genschel, 2004). In *P. falciparum*, however, these functions are predicted to be catalysed by monofunctional enzymes (Genschel, 2004; Pinney *et al.*, 2005; Müller and Kappes, 2007). Additionally, *Pf*PPAT has a very low similarity to the human homologue domain (Genschel, 2004; Pinney *et al.*, 2005; Müller and Kappes, 2007), while *Pf*DPCK is predicted to localise to the apicoplast organelle (Ralph *et al.*, 2004), a relict plastid that is unique to apicomplexan parasites (McFadden *et al.*, 1996). More recently, multiple studies have characterised the CoA biosynthesis pathway in murine *Plasmodium* species to further validate it as an antimalarial drug target. The studies showed that knockout mutants of *Plasmodium berghei* and *Plasmodium yoelii* parasites lacking the orthologues of PAT (Hart *et al.*, 2014; Kenthirapalan *et al.*, 2016; Kehrer *et al.*, 2016) or PanKs (be it single (Hart *et al.*, 2016; Srivastava *et al.*, 2016; Bushell *et al.*, 2017) and double (Srivastava *et al.*, 2016) knockouts) were able to develop normal asexual intraerythrocytic parasites, consistent with these genes being dispensable during this stage of their lifecycle. However, these mutants were not able to develop any normal oocysts (or any at all) in the mosquito (Hart *et al.*, 2014; Kenthirapalan *et al.*, 2016; Kehrer *et al.*, 2016; Hart *et al.*, 2016; Srivastava *et al.*, 2016), therefore implicating these proteins in the transmission of the parasite to the vector. Specifically, *Pb*PAT was found to play a role of in the release of osmiophilic body contents required for gametocyte egress in the mosquito midgut (Kehrer *et al.*, 2016), which calls into

question the currently ascribed function of *Pf*PPAT as a pantothenate transporter (Augagneur *et al.*, 2013). The next two enzymes, PPCS and PPCDC, were also reported to be dispensable but, interestingly, PPAT and DPCK were deemed essential to the intraerythrocytic stage of these parasites (or at least important in the case of *Pb*PPAT) (Hart *et al.*, 2017; Bushell *et al.*, 2017). Taken together, these data indicate that the CoA biosynthesis pathway, and potentially the CoA requirement, of these murine *Plasmodium* species vary quite markedly from *P. falciparum*. Future studies aiming to validate potential antimalarials that target CoA metabolism as part of the drug discovery pipeline should be cautious about using these *in vivo* models of malaria.

1.3.3. Antimalarial pantothenate analogues

An array of early seminal studies investigated the antimalarial effect of pantothenate analogues on various *Plasmodium* species based on their antimicrobial properties against some species of bacteria. Multiple *N*-substituted pantoyltauramides (Brackett *et al.*, 1946; Mead and Koepfli, 1947; Winterbottom and Clapp, 1947) (**Figure 1.4**) and D-phenylpantothenone (**Figure 1.4**) analogues (Lutz and Wilson, 1947; Senear *et al.*, 1947) were found to suppress the growth of avian malaria parasites. Further studies also found several *N*-substituted pantoyltauramide analogues that were active against *P. falciparum* (Trager, 1966; Trager, 1971). Interestingly, many of these analogues have not been tested further in *P. falciparum* despite being potent (some surpass the antiplasmodial activity of quinine against avian malaria parasites) and having low toxicity (Spry *et al.*, 2008). Furthermore, their mode of action was also not investigated in detail. More recent investigations aimed at discovering compounds that inhibit *P. falciparum* growth *via* direct interference of the parasite's CoA biosynthesis and/or utilisation have identified additional classes of antiplasmodial pantothenate analogues.

Pantothenol (PanOH; **Figure 1.4**), also known as provitamin B₅, was shown by Saliba *et al.* (2005) to suppress parasite proliferation *in vitro* by competitively inhibiting pantothenate utilisation. Specifically, the compound was found to have no effect on the H⁺-coupled transport of pantothenate but was able to inhibit pantothenate phosphorylation by *Pf*PanK. Furthermore, PanOH-treated *Plasmodium vinckei vinckei*-infected mice had significantly reduced parasite

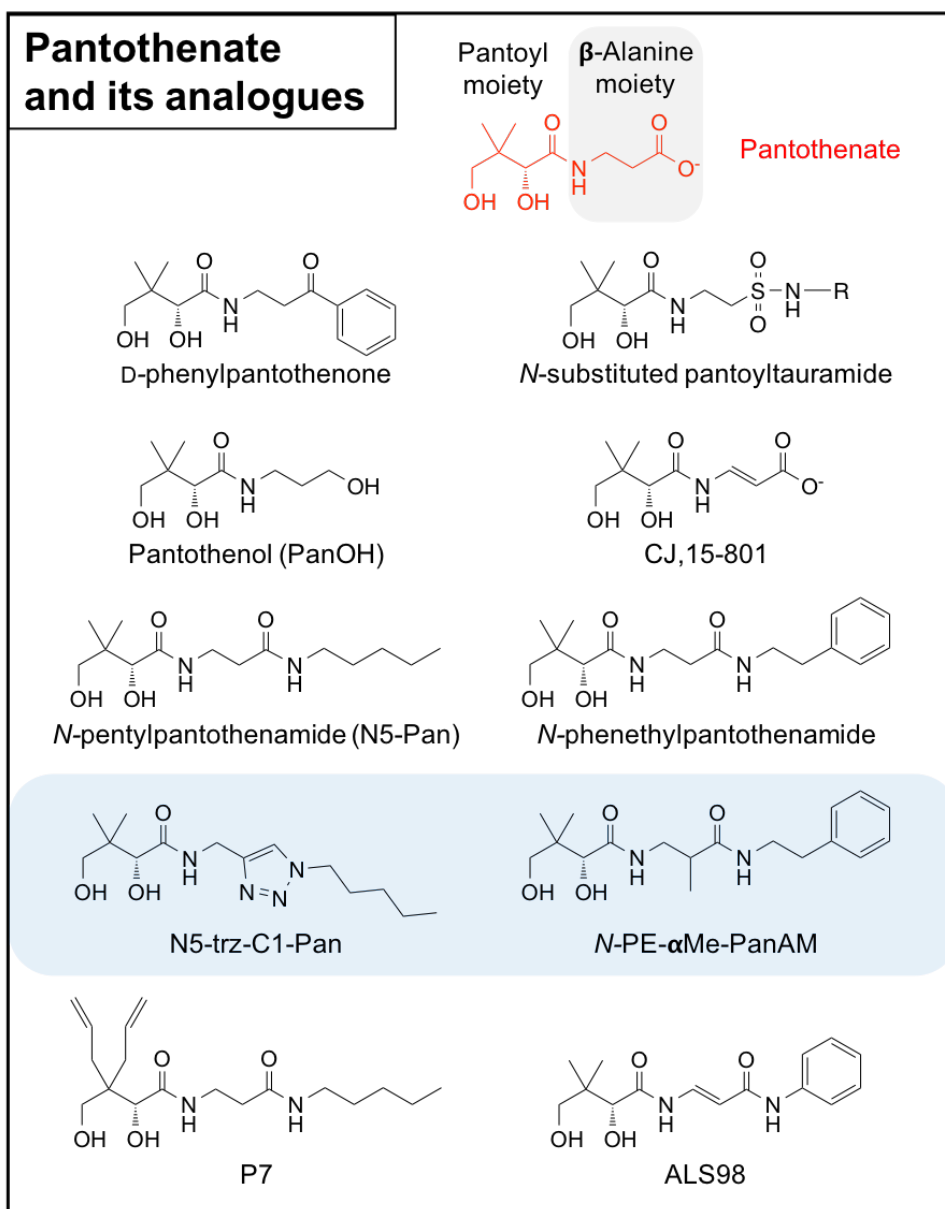


Figure 1.4. The chemical structures of pantothenate and its analogues. Pantothenate (structure in red), commonly known as vitamin B₅, is essential for the survival of the intraerythrocytic stage of *P. falciparum*. It is a precursor for the synthesis of the important cofactor coenzyme A (CoA) and is made up of a pantoyl moiety and a β -alanine moiety (highlighted in grey). Various pantothenate analogues have been shown to inhibit parasite growth *in vitro* by targeting its CoA biosynthesis and/or utilisation, but their exact mechanisms of action are still unclear. N5-trz-C1-Pan and N-PE- α Me-PanAM (highlighted in blue) are the most potent pantothenate analogues reported thus far that are resistant to serum pantetheinase degradation.

burden on day 4 and increased average survival time although the treatment did not elicit a cure (Saliba *et al.*, 2005). A follow up study investigated the uptake and metabolism of PanOH by *P. falciparum* using radiolabelled substrates (Lehane *et al.*, 2007). The transport of PanOH into parasite-infected erythrocytes (facilitated by a combination of the parasite-induced NPP and an unidentified endogenous erythrocyte mechanism and isolated parasites (transport is unaffected by pantothenate transport inhibitors) were found to be quite different from those of pantothenate (Lehane *et al.*, 2007), which support the finding of the previous study (Saliba *et al.*, 2005). PanOH was also found to be phosphorylated into 4'-phosphoPanOH by *Pf*PanK in parasite lysate, which is consistent with PanOH interacting with *Pf*PanK as a substrate (Lehane *et al.*, 2007). However, whether PanOH is metabolised further by downstream enzymes of the CoA pathway is not known.

CJ-15,801 (**Figure 1.4**) is a pantothenate analogue that was isolated from an extract of a fungal fermentation culture (*Seimatosporium* sp. CL28611), which was found to be active against three multidrug resistant strains of *Staphylococcus aureus* (Sugie *et al.*, 2001). Similar to what was observed for PanOH, CJ-15,801 was found to possess antiplasmodial activity that is antagonised by the presence of excess pantothenate (now a standard test to determine if a compound, particularly a pantothenate analogue, is targeting CoA biosynthesis and/or utilisation in the parasite) and it is able to inhibit the accumulation of pantothenate in isolated *P. falciparum* parasites (Saliba *et al.*, 2005). This is consistent with the analogue also exerting its activity by interfering with the parasite's utilisation of pantothenate. However, the exact cellular target of CJ-15,801 in the parasite is not known. In *S. aureus*, one of the few bacterial species that also expresses a type II PanK (Hong *et al.*, 2006), CJ-15,801 was found to be phosphorylated by *Sa*PanK to generate 4'-phospho-CJ-15,801 before it is catalysed by and interacts with PPCS as a tight-binding inhibitor (van der Westhuyzen *et al.*, 2012). However, it remains to be investigated if the pantothenate analogue has a similar mechanism of action in *P. falciparum*.

A subsequent study reported the synthesis and characterisation of a series of pantothenate analogues that retain the pantoyl moiety of pantothenate but vary in the group attached to their amide nitrogen (*N*-substituent; **Figure 1.4**)

(Spry *et al.*, 2005). Most of the analogues are able to inhibit pantothenate accumulation in *P. falciparum* via the inhibition of its phosphorylation by PfPanK (competitively for at least three compounds) (Spry *et al.*, 2005). Although most of these analogues only display modest antiparasmodial activity, the large structural variations between them highlight the potential for further optimisation. These data also confirm the possibility that other antibacterial pantothenate analogues could potentially be utilised as effective antiparasmodials.

One such class of pantothenate analogues are the *N*-substituted pantothenamides (PanAms), which have previously been shown to possess antibacterial activity (Clifton *et al.*, 1970; Choudhry *et al.*, 2003; Mercer *et al.*, 2008; van Wyk and Strauss, 2008; Akinnusi *et al.*, 2011; Hughes *et al.*, 2016). A recent study by Spry *et al.* (2013) investigated the antiparasmodial activity of a previously published small library of PanAms and related analogues (Virga *et al.*, 2006), including the prototypical *N*-pentylpantothenamide (N5-Pan; **Figure 1.4**) and *N*-heptylpantothenamide (N7-Pan) (Clifton *et al.*, 1970). It was found that the majority of the compounds are able to inhibit the *in vitro* growth of intraerythrocytic *P. falciparum* albeit with modest potency and also inhibit PfPanK-catalysed pantothenate phosphorylation by at least 94% (Spry *et al.*, 2013). Interestingly, the authors of the study serendipitously found that performing the *in vitro* viability assays using “Aged” medium (i.e. serum-supplemented RPMI 1640 medium that has been incubated at 37 °C for at least 40 h) vastly improved the potency of some PanAms (up to three orders of magnitude) and also modified the effect of excess pantothenate, but not those of PanOH (Spry *et al.*, 2013). The most potent PanAm in “Aged” medium is *N*-phenethylpantothenamide (**Figure 1.4**; compound 3 in (Spry *et al.*, 2013)). This PanAm-specific phenomenon was determined to be caused by pantetheinase (Spry *et al.*, 2013), an enzyme found in serum, which catalyses the hydrolysis of pantetheine into pantothenate and cysteamine (Wittwer *et al.*, 1989). PanAms (exemplified by compound 12 in the study) were found to be substrates for pantetheinase due to their structural similarity to pantetheine, a secondary amide of pantothenate, and the improvement in their antiparasmodial activity in “Aged” media was attributed to the heat-inactivation of pantetheinase found in the bovine serum albumin supplement Albumax II (Spry *et al.*, 2013). This discovery has spurred investigations into multiple strategies aimed at generating an effective antimalarial PanAm.

One such strategy involves the inhibition of serum pantetheinase activity. A study characterising a series of ketone analogues of pantetheine identified a potent inhibitor of pantetheinase activity in human and bovine serum, RR6 (Jansen *et al.*, 2013b), which was able to protect N5-Pan and N7-Pan from pantetheinase-mediated degradation and preserve their antimicrobial activity against *E. coli* and *S. aureus*, respectively (Jansen *et al.*, 2013a). Similarly, the antiplasmodial activity of PanAms (N5-Pan, N7-Pan and *N*-phenethylpantothenamide) in the presence of 10% pooled human serum *in vitro* was improved when tested in combination with CXP14.1-060, a potent pantetheinase inhibitor with poor antiplasmodial activity (so as not to occlude the true effect of vanin inhibition) (Pett *et al.*, 2015). However, as acknowledged by the authors of the study, such a drug combination is not optimal in the scheme of drug development. An alternative strategy involves generating PanAms that are resistant or immune to pantetheinase-mediated degradation. A study reported that an extension of the β -alanine moiety of PanAms protects the compounds from being broken down by pantetheine while preserving their antiplasmodial property (de Villiers *et al.*, 2013). A subsequent study showed that the addition of a methyl group to the carbon next to the β -alanine moiety carbonyl group of *N*-phenethylpantothenamide produced a PanAm derivative, *N*-PE- α Me-PanAM (**Figure 1.4, shaded blue**), that is resistant to pantetheinase degradation (**Appendix**; Macuamule *et al.*, 2015). This compound has a nanomolar “Fresh” medium activity against intraerythrocytic *P. falciparum* *in vitro* and was shown to remain specific for parasite pantothenate/CoA metabolism (**Appendix**; Macuamule *et al.*, 2015). *N*-PE- α Me-PanAM was also found to possess *in vivo* activity against the erythrocytic stage of *Plasmodium chabaudi* in mice (Chiu *et al.*, 2017). A separate investigation generated a small library of PanAm derivatives where the pantetheinase-susceptible amide group are replaced with a triazole ring (Howieson *et al.*, 2016). One of the compounds in the series, N5-trz-C1-Pan (compound 1e in Howieson *et al.* (2016); **Figure 1.4, shaded blue**), has potent antiplasmodial activity that was shown to be almost identical in the presence and absence of pantetheinase activity, consistent with the PanAm derivative being immune to degradation by the enzyme (Howieson *et al.*, 2016). There were also attempts at improving the basal antiplasmodial activity of PanAms (i.e in the absence of pantetheinase degradation). Strategies include modifying the compounds at the pantoyl moiety, such as the addition of alkyl substituents at the

geminal methyl groups (as represented by P7 in **Figure 1.4**) as previously reported for antibacterial PanAms (Akinnusi *et al.*, 2011), or combining multiple moieties of different antiparasitodal pantothenate analogues into a single compound (represented by ALS98 in **Figure 1.4**; unpublished data).

As with the other pantothenate analogues discussed above, the mechanism of action of PanAms (and their derivatives) in *P. falciparum* has not yet been elucidated. Their mechanism of action in bacteria has been characterised to a greater extent, although it is still not fully understood. The mode of action of PanAms in bacteria depends largely on the type of PanK that they possess (de Villiers *et al.*, 2014). Those expressing type III PanK are resistant to inhibition by PanAms due to the enzyme's highly selective pantothenate binding pocket (Brand and Strauss, 2005; Hong *et al.*, 2006) while type I PanKs are more promiscuous and are able to interact with diverse PanAms as their substrates (Ivey *et al.*, 2004; Li *et al.*, 2013). Indeed, N5-Pan is phosphorylated by the type I PanK in *E. coli* and is metabolised by the last two enzymes of the CoA biosynthesis pathway, PPAT and DPCK, to generate antimetabolite CoA analogue, ethyldethia-CoA, which was proposed to subsequently inhibit downstream CoA-utilising enzymes (Strauss and Begley, 2002). A separate study then showed that ethyldethia-CoA is incorporated into acyl carrier protein and inhibits its acyl-carrying function as the 4'-phosphopantothenamide group incorporated from the analogue lacks the necessary thiol group (Zhang *et al.*, 2004). The authors then showed that N5-Pan was able to inhibit fatty-acid biosynthesis (a major role of acyl carrier proteins) and proposed this as the main mode of action of PanAms in *E. coli* (Zhang *et al.*, 2004) although this has been contested (Thomas and Cronan, 2010). The same mechanism of action has also been proposed for the antibacterial activity of PanAm against *S. aureus* (Leonardi *et al.*, 2005; Virga *et al.*, 2006). On the other hand, an earlier study has found that the antistaphylococcal activity of PanAms is mediated by their inhibition of SaPanK (Choudhry *et al.*, 2003), which was further corroborated by a recent study (through co-crystallisation of SaPanK with PanAms) to be caused by the delayed release of the tightly bound 4'-phosphoPanAm product from the active site (Hughes *et al.*, 2016). It remains to be seen which of these modes of action occur in *P. falciparum* or if PanAms act through a novel mechanism in the parasite.

A strategy that has been frequently used in studying the mechanism of resistance (which in many cases reveal the mechanism of action) of antimalarials is the *in vitro* selection of drug-resistant *P. falciparum* (Nzila and Mwai, 2010; Flannery *et al.*, 2013; Corey *et al.*, 2016; Cowell *et al.*, 2018). This method was first used to generate chloroquine-resistant parasites (Nguyen-Dinh and Trager, 1978) and has since been utilised to help determine the genetic cause(s) of *P. falciparum* resistance against chloroquine (Barnes *et al.*, 1992; Lim and Cowman, 1996; Cooper *et al.*, 2002), mefloquine (Oduola *et al.*, 1988; Cowman *et al.*, 1994), pyrimethamine (Banyal and Inselburg, 1986), atovaquone (Korsinczky *et al.*, 2000), and more recently artemisinin (Witkowski *et al.*, 2010; Chavchich *et al.*, 2010) and spiroindolones (Rottmann *et al.*, 2010; Flannery *et al.*, 2015; Goldgof *et al.*, 2016). Such a study has not been attempted with antiplasmodial pantothenate analogues and could prove useful in characterising their mechanisms of action.

1.4. Aims of this thesis

The rising incidence of *P. falciparum* resistance against current frontline chemotherapies is a sobering reminder of the need for a robust antimalarial drug discovery pipeline. Interest in the parasite's CoA biosynthesis pathway as a viable antimalarial drug target has recently been revived following significant advancements in the understanding of parasite pantothenate metabolism through new biochemical techniques. In addition, multiple pantothenate analogues of various chemical classes have since been identified and validated as antiplasmodials. However, although most of these compounds have been shown to suppress parasite growth specifically *via* the inhibition of CoA biosynthesis and/or utilisation, their exact targets and mechanisms of action have not been elucidated in detail. This limits the characterisation of *P. falciparum* CoA biosynthesis pathway as an antimalarial drug target. A better understanding of the mechanism of action of antiplasmodial pantothenate analogues would enable a more targeted approach in improving the efficacy of future inhibitors.

Additionally, most of these pantothenate analogues have been proposed to interact with *Pf*PanK as substrates. Although important information about *Pf*PanK1 and *Pf*PanK2 have been derived from *in silico* analysis with other type II PanKs, their biological functions in the parasite have not been confirmed. This stems from challenges faced in the heterologous expression of these proteins. Prior to this study, it was unclear which of the two *Pf*PanKs is the active protein during the intraerythrocytic stage of the parasite's lifecycle. Furthermore, although *Pf*PanK1 and *Pf*PanK2 are both expected to form homodimers based on the crystal structures of all PanKs that have been characterised so far, their conformations in the parasite have not been experimentally verified.

This thesis therefore aimed to characterise the mechanisms of action of several antiplasmodial pantothenate analogues, and also verify the function of *Pf*PanK1 and *Pf*PanK2 in the parasite to further characterise them as drug targets. More specifically, these aims were:

- 1) To generate parasites that are resistant to the antiparasmodial pantothenate analogues PanOH and CJ-15,801 through independent dose-escalating drug pressuring regimes and subsequently use whole genome sequencing to determine the genetic changes responsible for any observed resistant phenotypes.
- 2) To characterise any drug-resistant mutants generated by:
 - a. Determining any cross-resistance to other pantothenate analogues.
 - b. Functionally characterising the mutated protein(s).
 - c. Comparing the viability of the mutants with the wild-type parent parasites and determining any difference in pantothenate requirements.
- 3) To use an immunoprecipitation approach to purify *Pf*PanK1 and *Pf*PanK2 directly from parasite extracts, functionally characterise these purified enzymes using a radiolabelled pantothenate phosphorylation assay, and also determine their conformation and interacting partners, if any, using western blot and mass spectrometry.

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Chapter 2: Materials and Methods

This chapter details the routine methods that were employed throughout the course of this study. For specific methods pertaining to a particular aspect of the study, please refer to the methods section of **Chapter 3** or **Chapter 4**.

2.1. General Methods

2.1.1. Parasite culture

The intraerythrocytic stage of *P. falciparum* parasites (3D7 strain; all subsequent transfectant and mutant cell lines were generated with this strain) were maintained in continuous *in vitro* culture using an improved adaptation (Allen and Kirk, 2010) of a previously described method (Trager and Jensen, 1976). The parasites were grown within human erythrocytes (4% haematocrit for 50 mL culture and 2% haematocrit for a 10 mL culture) suspended in RPMI 1640 medium supplemented with 11 mM glucose (final concentration of 22 mM), 200 μ M hypoxanthine, 24 μ g/mL gentamicin and 6 g/L Albumax II (defined in this study as complete medium). All cultures were maintained at 37 °C under an atmosphere of 1% oxygen, 3% carbon dioxide and 96% nitrogen and were continuously agitated at 60 rpm in a horizontally-rotating shaking incubator (Ratek). In order to replenish the nutrients consumed by the parasites (typically every 24 h for a standard 4% haematocrit culture), each culture was centrifuged ($500 \times g$ for 50 mL culture or $1,500 \times g$ for 10 mL culture, 5 min), the supernatant discarded, and the cell pellet resuspended in fresh complete medium before they were placed back into culture. On days when a culture contained predominantly trophozoite-stage parasites, dilutions were performed to decrease the parasitaemia (the percentage of parasite-infected erythrocytes) to approximately 1% and the haematocrit restored to 4% with fresh complete medium and fresh erythrocytes. The parasitaemia and parasite stage of each culture were monitored by regular examination of microscope slides containing methanol-fixed, Giemsa-stained thin blood smears of the culture.

2.1.2. Parasite synchronisation

Most experiments performed in this study required parasites of the different cultures to be at the same stage of development. Synchronisation of parasite

stage in culture was performed by selectively lysing erythrocytes infected with mature, trophozoite-staged parasites using sorbitol as described previously (Lambros and Vanderberg, 1979). Sorbitol is transported into the infected erythrocytes through NPP induced in the host erythrocyte plasma membrane by the parasite during its trophozoite stage (Kirk *et al.*, 1994; Staines *et al.*, 2006). This uptake of sorbitol is accompanied by the influx of water into the infected erythrocyte, resulting in its lysis. Briefly, the culture to be synchronised (typically when the culture consisted predominantly of ring-stage parasites) was centrifuged ($500 \times g$, 5 min) and the supernatant was discarded. The cell pellet was mixed with 10 volumes of 5% (w/v) sorbitol (to a total volume of 30 – 35 mL) and was incubated at 37 °C for 15 – 20 min with a resuspension every 5 min. The suspension was then centrifuged at $500 \times g$ for 5 min, the supernatant discarded, and the pellet resuspended in fresh complete medium. The synchronised culture was then placed into a brand-new flask or one that had been washed at least three times with 4 – 5 mL of complete medium and cultured as described above (**Section 2.1.1**).

2.1.3. Parasite isolation

In some experiments, mature trophozoite-stage parasites were isolated from the host erythrocytes *via* saponin treatment as described previously by Saliba *et al.* (1998b). Saponin is a class of detergents derived from plants that interacts with the cholesterol molecules on cell membranes to form pores. By treating a culture suspension with saponin, the cholesterol-containing host erythrocyte plasma membrane becomes permeabilised, resulting in haemolysis (Siddiqui *et al.*, 1979). Conversely, it was shown that the cholesterol-free parasite plasma membrane is left intact and the parasite remains viable for several hours following saponin treatment (Saliba and Kirk, 1999). This allows the trophozoites to be purified from most of the host materials and concentrated for downstream applications. In general, a culture of host erythrocytes infected with trophozoite-stage parasites was mixed with saponin (0.05% (w/v) final concentration) and immediately centrifuged ($2000 \times g$, 8 min). The supernatant was discarded, and parasite pellet was washed three times ($15,850 \times g$, 30 s) with standard malaria saline (125 mM NaCl, 5 mM KCl, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 mM glucose and 1 mM $MgCl_2$, pH 7.1) before it was resuspended in the appropriate buffers and utilised in experiments.

2.1.4. Parasite cryopreservation and recovery

In order to store parasites for future applications, cultures were mixed with a glycerol-based buffer and cryopreserved. First, the culture of predominantly young ring-stage parasite (only erythrocytes infected with parasites at this stage have membranes that are sufficiently robust to withstand the cryopreservation process) was centrifuged at $500 \times g$ for 5 min and the supernatant was discarded. Subsequently, three volumes of pellet were mixed with five volumes of sterile glycerolyte (0.57 g/mL glycerol, 143 mM sodium lactate, 4 mM KCl, 100 mM NaH_2PO_4 , pH 6.8). One volume of glycerolyte was first added dropwise to the pellet with constant gentle mixing and was allowed to stand for 5 min. The remaining glycerolyte was then added in the same manner before the suspension was aliquoted into cryovials (1 – 1.5 mL) and stored in -80°C .

To recover parasites from cryopreservation, the glycerol stock was first quickly thawed out at 37°C and transferred to a 50 mL conical-based centrifuge tube. Immediately, $0.2 \times$ volumes of 12% (w/v) NaCl was added dropwise with gentle mixing and the suspension allowed to stand for 5 min. Subsequently, 10 mL of 1.8% (w/v) NaCl was added to the suspension in the same manner before it was centrifuged ($500 \times g$, 5 min) and the supernatant discarded. Following this, 10 mL of a saline solution (0.9% (w/v) NaCl and 0.2% (w/v) glucose) was then added to the pellet dropwise with gentle swirling, the suspension centrifuged ($500 \times g$, 5 min) and the supernatant discarded. The pellet was then washed once in incomplete or complete medium ($500 \times g$, 5 min) before it was resuspended in the appropriate volume (typically 50 mL) of complete medium and maintained as described above (**Section 2.1.1**).

2.1.5. Cell counts and limiting dilution cloning

During the course of the study, the concentration of any erythrocyte or isolated parasite suspension was determined using an improved Neubauer haemocytometer. In addition, parasitaemia of parasite-infected erythrocyte cultures was determined by examining methanol-fixed, Giemsa-stained thin smears of the culture using a standard light microscope and counting the proportion of infected erythrocytes out of ~1000 erythrocytes.

Clonal lines of parasite cultures were generated through limiting dilution cloning according to a previously described brief method (Rosario, 1981). To clone parasites within a culture, the haematocrit and accurate parasitaemia (averaged from three Giemsa-stained smears) of the parent culture (ring-stage parasites) was determined as described above. Next, 1×10^6 cells (infected and uninfected erythrocytes) from the parent culture was added to 10 mL of complete medium and mixed thoroughly by vortexing. Subsequently, a volume of this suspension was added into a second separate suspension (10 mL complete medium with 2% haematocrit) such that 100 μ L of the second suspension (the volume aliquoted in each well of the cloning plate) would contain the desired number of parasite for cloning. The volume required of the first suspension would depend on the parasitaemia of the culture to be cloned out. For example, to get 0.5 parasites/well in the cloning plate for a parental culture with 2% parasitaemia, 25 μ L of the first suspension (this will contain 50 parasites within infected erythrocytes) was added into the second suspension and mixed well by gentle vortexing. To set up a 96-well plate for cloning, the outermost wells of the plate were filled with 200 μ L of medium to prevent evaporation and 100 μ L of the second parasite suspension described earlier was added into each of the remaining wells. Each cloning plate was then placed under an atmosphere of 1% oxygen, 3% carbon dioxide and 96% nitrogen, and incubated at 37 °C for a period of 3 – 4 weeks. Every 6 to 7 days, 80 μ L of the medium in each well was aspirated (without disturbing the red cell layer at the bottom of the well), 90 – 100 μ L of fresh complete medium was added into the well and the cells gently resuspended by pipetting. The plate was then returned to normal culture condition.

In most cases, parasites were observed in positive wells of the cloning plate after approximately 20 days in culture (this depends on the fecundity of the parental culture). To identify positive wells, 80 – 90 μ L of medium was aspirated from the well and ~1 μ L of the packed cell (aspirated from the base of the well without mixing with the remaining medium) was placed onto a microscope slide to make a thin smear. The smears of multiple wells (generally 10 wells per slide) were allowed to air dry, fixed with methanol and stained with Giemsa for microscopic examination. Positive cultures were then transferred into a suspension of 10 mL complete medium with 2% haematocrit and cultured as described above (**Section 2.1.1**). Cultures were subsequently cryopreserved when the

parasitaemia reached 2 – 5%. After the desired number of clones have been selected for propagation, the proportion of positive wells in each cloning plate (incubated for another week to allow all positive wells to grow) was determined to check that the limiting dilution was successful. This should correspond to the concentration of parasite in the suspension used for cloning or be sparser to take into account the possibility that not all of the parasites counted were viable.

2.1.6. *Mycoplasma* testing and treatment

Parasite cultures were routinely tested for *Mycoplasma* contamination using a previously described polymerase chain reaction (PCR) method with primers designed to detect conserved 16S ribosomal deoxyribonucleic acid (rDNA) regions of various common *Mycoplasma* species (Uphoff and Drexler, 2002). Briefly, the parasite culture to be tested was centrifuged ($500 \times g$, 5 min) and an aliquot of the supernatant was collected (typically 500 – 1000 μ L) and stored at -20°C . To extract *Mycoplasma* deoxyribonucleic acid (DNA) from the supernatant, samples were boiled at 95°C for 15 min and then placed on ice for 5 min. These were then centrifuged at $16,000 \times g$ for 3 min and $\geq 50 \mu\text{L}$ of each supernatant collected for PCR. A 25 μL PCR reaction using KOD Hot Start DNA polymerase (Merck Millipore) was then set up for each sample, which contained $1 \times$ KOD Hot Start DNA polymerase Buffer, 1.75 mM MgSO_4 , 400 μM of each deoxyribonucleotide triphosphate (dNTP; including deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxycytidine triphosphate (dCTP) and deoxyguanosine triphosphate (dGTP)), 0.2 μM of each 16S rDNA-specific primers described in (Uphoff and Drexler, 2002), 1 μL of sample supernatant and 1 unit of KOD Hot Start DNA polymerase. PCR was then performed with the following conditions: polymerase activation at 95°C for 2 min, 40 cycles of denaturation (95°C , 20 s), annealing (52°C , 10s) and extension (74°C , 32s) and termination (4°C , ∞). The PCR products were subsequently diluted in $5 \times$ sample loading buffer (Bioline) and loaded (typically 10 μL each) into a 1% (w/v) agarose gel in $1 \times$ TAE buffer (40 mM tris(hydroxymethyl)aminomethane (Tris), 20 mM sodium acetate, 1mM ethylenediaminetetraacetic acid (EDTA), pH 7.8) containing $1 \times$ SYBR Safe DNA gel stain alongside 5 μL of HyperLadder I DNA ladder (Bioline). Electrophoresis was performed at 110-120 V for 30 – 40 min and the DNA fragments within the gel were visualised under ultraviolet light (BioRad;

using the SYBR Safe setting). The presence of a 500 – 700 base pair PCR product indicates a positive *Mycoplasma* contamination of the culture.

Mycoplasma-infected parasite cultures were treated using mycoplasma removal agent (MRA; MP Biomedicals) according to the manufacturer's instructions. In summary, an aliquot of the infected culture (typically 5 mL) was maintained under standard parasite culture conditions (**Section 2.1.1**) in the presence of 0.5 $\mu\text{g/mL}$ of MRA for a period of approximately one week. This typically involved a medium change every two days, depending on the starting parasitaemia. No visible impairment to parasite growth was observed during this regimen. After the treatment was complete, the culture was then re-tested using the PCR test described above to ensure that the *Mycoplasma* infection had been cleared.

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Chapter 3: Mutations in the pantothenate kinase of *Plasmodium falciparum* confer diverse sensitivity profiles to antiparasmodial pantothenate analogues.

Tjhin ET, Spry C, Sewell AL, Hoegl A, Barnard L, Sexton AE, Siddiqui G, Howieson VM, Maier AG, Creek DJ, Strauss E, Marquez R, Auclair K and Saliba KJ. Mutations in the pantothenate kinase of *Plasmodium falciparum* confer diverse sensitivity profiles to antiparasmodial pantothenate analogues. *PLoS Pathog.* 2018. 14 (4): e1006918. doi: 10.1371/journal.ppat.1006918

Contributions

The work presented in this chapter are my own original work, except for the contributions detailed below.

Christina Spry generated the $\Delta Pfpank1$ -pCC-1 plasmid and performed the disruption of *Pfpank1* including the subsequent Southern blot and PCR analyses, generated the *Pfpank1*-pGlux-1 plasmid and performed the confocal microscopy of *PfPanK1*-GFP expressing cell line, generated the *PfPanK1* protein models, prepared samples for N5-trz-C1-Pan metabolism analysis, provided intellectual input into the design of the study and editing of the manuscript.

Alan L. Sewell and **Rodolfo Marquez** synthesised CJ-15,801.

Annabelle Hoegl synthesised N5-trz-C1-Pan.

Leanne Barnard synthesised *N*-PE- α Me-PanAm.

Anna E. Sexton analysed N5-trz-C1-Pan-treated parasite samples by LC-MS.

Ghizal Siddiqui analysed parasite samples using LC-MS/MS and DIA to quantify protein levels.

Vanessa M. Howieson generated the *Pfpank1*-stop-pGlux-1 plasmid.

Alexander G. Maier provided intellectual input into the parasite complementation experiment and editing of the manuscript.

Darren J. Creek provided intellectual input to the mass spectrometry analyses and the manuscript.

Erick Strauss provided analysis, editing and intellectual input to the manuscript.

Karine Auclair provided editing and intellectual input to the manuscript.

Kevin J. Saliba provided intellectual input into the design of the study, data analyses and editing of the manuscript.

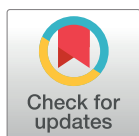
RESEARCH ARTICLE

Mutations in the pantothenate kinase of *Plasmodium falciparum* confer diverse sensitivity profiles to antiplasmodial pantothenate analogues

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Abstract

The malaria-causing blood stage of *Plasmodium falciparum* requires extracellular pantothenate for proliferation. The parasite converts pantothenate into coenzyme A (CoA) via five enzymes, the first being a pantothenate kinase (*PfPanK*). Multiple antiplasmodial pantothenate analogues, including pantothenol and CJ-15,801, kill the parasite by targeting CoA biosynthesis/utilisation. Their mechanism of action, however, remains unknown. Here, we show that parasites pressured with pantothenol or CJ-15,801 become resistant to these analogues. Whole-genome sequencing revealed mutations in one of two putative PanK genes (*Pfpank1*) in each resistant line. These mutations significantly alter *PfPanK* activity, with two conferring a fitness cost, consistent with *Pfpank1* coding for a functional PanK that is essential for normal growth. The mutants exhibit a different sensitivity profile to recently-described, potent, antiplasmodial pantothenate analogues, with one line being *hypersensitive*. We provide evidence consistent with different pantothenate analogue classes having different mechanisms of action: some inhibit CoA biosynthesis while others inhibit CoA-utilising enzymes.

Author summary

The coenzyme A (CoA) biosynthetic pathway is under investigation as a target for the development of drugs aimed at several infectious agents, including malaria parasites. To synthesise CoA, the parasite scavenges the essential precursor pantothenate (vitamin B₅). Several pantothenate analogues possess potent (nM) activity against the parasite, but their exact mechanism of action is unknown. We have generated mutant parasites that are

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resistant or hypersensitive to various pantothenate analogues. These parasites harbour mutations in a gene we now show codes for the first enzyme in the CoA biosynthesis pathway. This enzyme is not the target of the analogues, but instead converts them into anti-metabolites that, depending on the analogue, either inhibit a CoA biosynthesis enzyme or downstream CoA-utilising enzymes.

Introduction

In recent years, the effort to roll back malaria has shown encouraging progress through the increased use of insecticide-treated mosquito nets, improved diagnostics and artemisinin-based combination chemotherapies (ACTs) [1]. Evidence of this includes the decreasing worldwide malaria incidence (266 million cases in 2005 down to 212 million cases in 2015) and mortality (741,000 deaths in 2005 down to 429,000 deaths in 2015) over the past decade [1]. However, there is an alarming trend of ACT-resistant parasites emerging in multiple Asian countries where the disease is endemic [2]. Recently, there have also been multiple reports of patients contracting ACT-resistant *Plasmodium falciparum* malaria from various African countries [3,4], which exemplify the clear risk of artemisinin resistance developing in the continent. This threat to the efficacy of ACTs highlights the requirement for a new armoury of antimalarial medicines, with several compounds representing different chemotypes entering the preclinical trial stage. However, the antimalarial drug-discovery pipeline is reliant on just a few known drug targets and the probability of successfully producing a new blood-stage medicine remains low [5]. In order to manage the threat of parasite drug resistance, there needs to be a continued effort to identify new classes of antimalarials. One metabolic pathway that has garnered recent interest for drug-development is the parasite's coenzyme A (CoA) biosynthetic pathway [6,7].

Early seminal studies have shown that the asexual stage of intra-erythrocytic *P. falciparum* absolutely requires an exogenous supply of vitamin B₅ (pantothenate; Fig 1) for survival [7–9]. Pantothenate is taken up by the parasite [10,11] and converted into CoA, an essential cofactor for many metabolic processes [7]. This conversion is catalysed by a series of five enzymes, the first of which is pantothenate kinase (*PfPanK*), an enzyme that phosphorylates pantothenate to form 4'-phosphopantothenate [11]. By performing this step, the parasite traps pantothenate within its cytosol and commits it to the CoA biosynthetic pathway [10]. The additional four steps are, in turn, catalysed by phosphopantothenoylcysteine synthetase (*PfPPCS*), phosphopantothenoylcysteine decarboxylase (*PfPPCDC*), phosphopantetheine adenylyltransferase (*PfPPAT*) and dephospho-CoA kinase (*PfDPCK*) [6]. Putative genes coding for each of the enzymes in the pathway (with several enzymes having multiple putative candidates) have been identified in the *P. falciparum* genome [12,13] and have also been shown to be transcribed during the intraerythrocytic stage of the parasite's lifecycle [14]. In order to capitalise on the pathway as a potential target for drug-discovery, however, it is crucial to ascertain the exact identity of each of these putative genes. This will allow the process to become more efficient and targeted.

Investigations aimed at discovering antiplasmodial agents that act by interfering with the parasite's CoA biosynthetic pathway identified several antiplasmodial pantothenate analogues, including pantothenol (PanOH) and CJ-15,801 [9,15,16] (see Fig 1 for structures). Subsequent studies identified pantothenamides as antiplasmodial pantothenate analogues with substantially increased potency [17–19]. Unfortunately pantothenamides are unstable *in vivo* because they are degraded by the serum enzyme pantetheinase [17]. Recent reports of structural

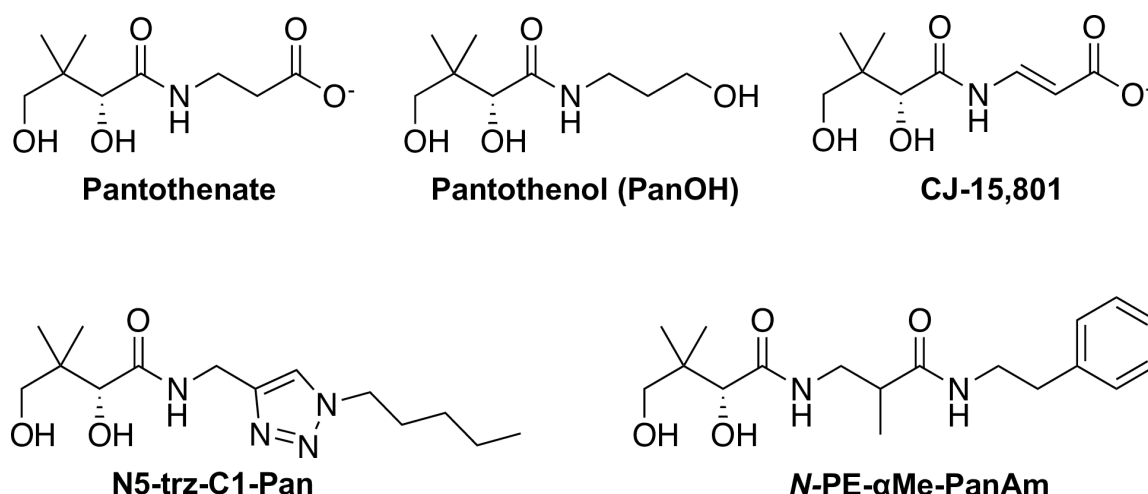


Fig 1. Structures of pantothenate and the pantothenate analogues used in this study.

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optimisations of lead pantothenamides have described two compounds, N5-trz-C1-Pan (compound 1e in Howieson *et al.* [20]) and N-PE-αMe-PanAm (see Fig 1 for structures), that are potent antiparasmodials (with nanomolar IC₅₀ values) and also resistant to pantetheinase-mediated degradation [20,21]. However, although these compounds have been shown to target CoA biosynthesis or utilisation, their exact mechanism(s) of action has not been elucidated.

In this study, we have used continuous drug-pressuring with PanOH or CJ-15,801 to generate a number of *P. falciparum* parasite lines that are several-fold resistant to these pantothenate analogues. Whole-genome sequencing revealed mutations in one of the two putative *Pfpank* genes of all of the clones, *Pfpank1*. Complementation experiments confirmed that these mutations are responsible for the resistance phenotypes. Characterisation of the effects of the mutations on parasite growth in culture and also *PfPanK* function, generated data consistent with the mutated gene coding for an active PanK in *P. falciparum* and with the gene being essential for normal parasite development during the intraerythrocytic stage of its lifecycle. Additional characterisation of the PanOH and CJ-15,801-resistant lines revealed that antiparasmodial pantothenate analogues have at least two distinct mechanisms of action, targeting CoA biosynthesis or utilisation. Both of these mechanisms can be influenced by the *Pfpank1* mutations identified here. Furthermore, our study provides genetic evidence validating the importance of the metabolic activation of pantothenate analogues in the antiparasmodial activity of these compounds.

Materials and methods

Parasite culture and lysate preparation

P. falciparum parasites were maintained in RPMI 1640 media supplemented with 11 mM glucose (final concentration of 22 mM), 200 μM hypoxanthine, 24 μg/mL gentamicin and 6 g/L Albumax II (referred to as complete medium) as previously described [22]. Clonal parasite populations were generated through limiting dilution cloning as reported previously [23], with modifications. Parasite lysates were prepared from saponin-isolated mature trophozoite-stage parasites as described previously [10].

Plasmid preparation and parasite transfection

Several plasmid constructs were generated through the course of this study to be used for different lines of investigations. The strategies used to generate the *Pfpank1*-pGlux-1, *Pfpank1*-stop-pGlux-1 and Δ *Pfpank1*-pCC-1 plasmids are detailed in the SI and the primers used in these strategies are listed in S1 Table. The constructs were transfected into ring-stage parasites and positive transfectants were selected by introducing WR99210 (10 nM) [24].

Compound synthesis

The pantothenate analogues CJ-15,801 [25], N5-trz-C1-Pan [26] and *N*-PE- α Me-PanAm [21], used in this study were synthesised as reported previously.

SYBR Safe-based parasite proliferation assay

The effect of various compounds on the *in vitro* proliferation of the different parasite lines were tested using a previously-reported SYBR Safe-based fluorescence assay [17], with minor modifications (SI). *In vitro* pantothenate requirement experiments were performed similarly (SI), except instead of a test compound, ring stage-infected erythrocytes were incubated in pantothenate-free complete RPMI 1640 medium (made complete as described above; Athena Enzyme Systems) supplemented with 2-fold serial dilutions of pantothenate. IC₅₀ and SC₅₀ (defined in the Results section) values were determined from the sigmoidal curves fitted to each data set using nonlinear least squares regression (SI).

Drug pressuring

Two independent drug-pressuring cultures were initiated for each of the pantothenate analogues, PanOH and CJ-15,801. Pressuring was initiated by exposing synchronous ring-stage Parent line parasites (10 mL culture of 2 or 4% parasitaemia and 2% haematocrit) to either analogue at the IC₅₀ values obtained for the Parent line at the time (PanOH = 400 μ M and CJ-15,801 = 75 μ M). Parasites were then exposed to cycles of increasing drug-pressure that lasted about 2–4 weeks each (SI). When the pressured parasites became approximately 8 $\times$ less sensitive than the Parent line to the selecting analogues, they were cloned and cultured in the absence of the analogues for the remainder of the study.

Competition assay

In order to compare the fitness of the mutant clones with that of the Parent, we set up three competition cultures, each containing a mixture of one mutant line and the Parent line. Equal number of parasites (5×10^8 cells in the first experiment and 2.5×10^8 cells in the second experiment) from each line were mixed into a single culture. Aliquots (3 to 5 mL) of these cultures were immediately used for a PanOH SYBR Safe-based parasite proliferation assay (to generate Week 0 data) as described above. The cultures were then maintained under standard conditions as detailed above for a period of 6 weeks before they were used to perform another PanOH proliferation assay (to generate Week 6 data).

Whole genome sequencing and variant calling

Next generation whole genome sequencing was performed by the Biomolecular Resource Facility at the Australian Cancer Research Foundation, the Australian National University. Samples were sequenced with the Illumina MiSeq platform with version 2 chemistry (2 $\times$ 250 base pairs, paired-end reads) Nextera XT Kit (Illumina). To determine the presence of any single nucleotide polymorphisms (SNPs) in the genomes of the drug-pressured clones, the

genomic sequencing data were analysed using an integrated variant calling pipeline, PlaTyPus, as previously described [27], with minor modifications to resolve operating system incompatibility. As PlaTyPus does not detect insertions-deletions polymorphisms (“indels”), the Integrated Genome Viewer (IGV) software (Broad Institute) was used to manually inspect the gene sequences of all putative enzymes in the CoA biosynthetic pathway for indels.

Quantitative polymerase chain reaction (qPCR)

To determine the proportions of parasites (i.e. Parent versus mutant clone) that make up each competition culture, we performed qPCR to measure the amount of Parent and mutant genomic DNA (gDNA) at week 0 and week 6 of the competition assay. gDNA was extracted using a DNeasy Plant Mini Kit (QIAGEN) or QIAamp DNA Blood Midi Kit (QIAGEN) following the manufacturer’s instructions, unless otherwise specified (detailed in S1). Six different primer sets were designed, each to detect the specific variants (SNPs or indel) in the wild-type and mutant *Pfpank1* alleles (S1 Table). The qPCRs were performed using QuantiTect 2 × SYBR Green PCR Master Mix (QIAGEN) essentially as reported previously [28] with the following specifications: The reactions (20 μL final volume) for “Parent vs PanOH-A” and “Parent vs PanOH-B” contained 2 μL of 10 ng/μL gDNA stocks while that for “Parent vs CJ-A” contained 4 μL of 10 ng/μL gDNA stocks. The PCR program for “Parent vs PanOH-A” and “Parent vs CJ-A” entailed an initial DNA polymerase activation step (95°C for 15 min) followed by 40 cycles of denaturation (94°C for 15 s), annealing (60 or 55°C, respectively, for 30 s) and extension (72°C for 30 s) with the detection step (green channel) set during the extension step of each cycle. For “Parent vs PanOH-B” the PCR was programmed as above except that it was set for 45 cycles, the annealing step was carried out at 58°C and included an additional detection step (65°C for 15 s, green channel) in each cycle. Following each reaction, a melt curve analysis was performed starting with a temperature that is 1°C lower than the annealing temperature and increased to 99°C at 1°C/s. Control reactions that each contained defined proportions of the Parent and mutant gDNA as templates, set at the same final DNA concentration as detailed above, were included in each qPCR experiment to enable preparation of a standard curve. The standard curve was generated by plotting the threshold cycle values obtained for the control reactions against the corresponding log₁₀ gDNA amount and fitting a straight line ($y = y_0 + ax$, where y denotes threshold cycle value, x denotes log₁₀ DNA amount and a denotes primer efficiency). The concentration of Parent and mutant gDNA in each sample was determined by comparing their threshold cycle against the appropriate standard curve. All reactions were performed in duplicate.

Generation of *PfPanK1* model

The structure of *PfPanK1* minus its parasite-specific inserts was predicted by homology modeling using the AMPPNP and pantothenate-bound human PanK3 structure (PDB ID: 5KPR [29]) as a template. *PfPanK1* shares 28% sequence identity with human PanK3 over the parts of the protein that have been modeled. The model was generated using the one-to-one threading module of the Phyre2 webserver (available at <http://www.sbg.bio.ic.ac.uk/phyre2>) [30].

Confocal microscopy

Erythrocytes infected with trophozoite-stage 3D7 strain *P. falciparum* parasites expressing *PfPanK1*-GFP were observed and imaged either with a Leica TCS-SP2-UV confocal microscope (Leica Microsystems) using a 63 × water immersion lens or a Leica TCS-SP5-UV

confocal microscope (Leica Microsystems) using a 63 × oil immersion lens. The parasites were imaged as fixed or live cells as described in the **SI**.

Attempted disruption of *Pfpank1*

The *Pfpank1* disruption plasmid, $\Delta Pfpank1$ -pCC-1 (**SI**), was transfected into wild-type 3D7 strain *P. falciparum*, and positive transfectants were selected as described above. *P. falciparum* parasites have previously been shown to survive equally well in a pantothenate-free complete RPMI 1640 medium supplemented with $\geq 100 \mu\text{M}$ CoA as compared to standard complete medium, consistent with them having the capacity to take up exogenous CoA, hence bypassing the need for any *PfPanK* activity [7]. Therefore, to support the growth of any *Pfpank1* gene-disrupted parasites generated with the $\Delta Pfpank1$ -pCC-1 construct, parasites were continuously maintained in complete medium supplemented with $100 \mu\text{M}$ CoA following transfection. Positive and negative selection steps (with WR99210 and 5-fluorocytosine (5-FC), respectively) were performed to isolate $\Delta Pfpank1$ -pCC-1-transfectant parasites in which the double cross-over homologous recombination had occurred (detailed in **SI**).

Southern blot analysis

gDNA samples ($\sim 2 \mu\text{g}$) extracted from $\Delta Pfpank1$ -pCC-1-transfectant parasites isolated through the positive and negative selection steps were digested with the restriction enzyme *Afl*III (New England Biolabs), before being analysed by Southern blotting using the digoxigenin (DIG) system (Roche) according to the Roche DIG Applications Manual for Filter Hybridisation. The results from the Southern blot were confirmed with PCR (the primers used are shown in **S1 Table**).

[^{14}C]Pantothenate phosphorylation by parasite lysate

The phosphorylation of [^{14}C]pantothenate by parasite lysates prepared from the Parent and mutant clonal lines was measured using Somogyi reagent (which precipitates phosphorylated compounds from solution) as outlined previously [31], with some modifications (detailed in **SI**).

Metabolism of N5-trz-C1-Pan

Cultures of predominantly trophozoite-stage *P. falciparum*-infected erythrocytes (Parent line) were concentrated to $>95\%$ parasitaemia using magnet-activated cell sorting as described elsewhere [32]. Following recovery, trophozoite-infected erythrocytes were treated with N5-trz-C1-Pan ($1 \mu\text{M}$) or a solvent control (0.01% v/v DMSO) before the metabolites in these samples were extracted and processed for liquid chromatography-mass spectrometry (LC-MS) analysis. Metabolite samples were analysed by LC-MS, using a Dionex RSLC U3000 LC system (ThermoFisher) coupled with a high-resolution, Q-Exactive MS (ThermoFisher), as described previously [33] (detailed in **SI**). LC-MS data were analysed in a non-targeted fashion using the IDEOM workflow, as described elsewhere [34]. Unique features identified in N5-trz-C1-Pan-treated samples were manually assessed by visualising high resolution accurate mass LC-MS data with Xcalibur Quanbrowser (ThermoFisher) software.

PfPanK1 protein levels

To measure *PfPanK1* levels, parasite samples were prepared from Parent and mutant cultures as previously described with minor modifications [35]. Briefly, mature trophozoites were saponin-isolated from a culture of infected erythrocytes (10% parasitaemia, 2% haematocrit in 30

mL) by resuspending the pellet in $1 \times$ phosphate buffered saline (PBS; Sigma-Aldrich) containing 0.05% (w/v) saponin, $1 \times$ complete mini protease inhibitor cocktail (Roche), 20 mM sodium fluoride and 0.1 mM sodium orthovanadate. The isolated trophozoites were pelleted ($2,000 \times g$, 8 min), washed ($15,850 \times g$, 30 s, supernatant discarded after each wash) once in 1 mL of the above solution excluding saponin, and twice with 1 mL $1 \times$ PBS. The trophozoite pellet was then stored at -80°C until required. For sample processing, a total of 500 μg of protein, accurately determined using the Pierce BCA protein assay kit (ThermoFisher), was incubated overnight with sequencing-grade trypsin (1:50 dilution; Promega). On the following day, trypsin activity was quenched using 5% formic acid (FA) and the detergent (sodium deoxycholate) used for protein solubilisation was removed. The samples were then dried and resuspended in 20 μL of 2% (v/v) acetonitrile (ACN) and 0.1% (v/v) FA for LC-MS/MS analysis. For facilitating retention-time (RT) alignments among samples, a RT kit [36] (iRT kit, Biognosys) was spiked into all samples (1:20 dilution).

LC-MS/MS was carried out as described previously [35], with minor modifications. Briefly, samples were loaded at a flow rate of 15 $\mu\text{L}/\text{min}$ onto a reversed-phase trap column ($100 \mu\text{m} \times 2 \text{ cm}$; Acclaim PepMap media, Dionex), which was maintained at a temperature of 40°C . Peptides were then eluted from the trap column at a flow rate of 0.25 $\mu\text{L}/\text{min}$ through a reversed-phase capillary column ($75 \mu\text{m} \times 50 \text{ cm}$; LC Packings, Dionex). The HPLC gradient was set to 158 min using a gradient that reached 30% ACN after 123 min, then 34% ACN after 126 min, 79.2% ACN after 131 min and 2% ACN after 138 min, at which it was maintained for a further 20 min. The mass spectrometer was operated in a data-independent acquisition (DIA) mode with a 25-fixed-window setup of 24 m/z effective precursor isolation over the m/z range of 375–975 Da.

For Spectronaut processing, raw files were loaded into Spectronaut (version 11, Biognosys) with an in-house *P. falciparum*-infected red blood cell library and default settings. Briefly, RT prediction type was set to dynamic iRT and the correction factor for the window was set to one. Mass calibration was set to local mass calibration. Interference correction was on MS2 level. The false discovery rate was set to 1% at peptide precursor level. For quantification, the interference correction was activated and a cross run normalisation was performed using the total peak area as the normalisation base. A significance filter of 0.01 was used. The peptides used in the identification and quantitation of *Pf*PanK1 and the four control proteins are listed in S2 Table.

Statistical analysis

Statistical analysis of means was carried out with unpaired, two-tailed, Student's *t* tests using GraphPad 6 (GraphPad Software, Inc) from which the 95% confidence interval of the difference between the means (95% CI) was obtained. All regression analysis was done using Sigma-Plot version 11.0 for Windows (Systat Software, Inc).

Results

Pfpank1 mutations mediate parasite resistance to PanOH and CJ-15,801

The 3D7 *P. falciparum* strain was cloned through limiting dilution, and a single parasite line (henceforth referred to as the Parent line) was used to generate all of the subsequent lines tested in this study (unless otherwise specified). This was done to ensure that all of the parasite lines generated during the course of this study would share a common genetic background. Using the Parent line, three independent drug-pressuring cultures were set up (two with PanOH and one with CJ-15,801). When these parasites had attained approximately 8-fold decrease in sensitivity (~ 11 – 13 weeks of continuous pressuring), they were subsequently

cloned by limiting dilution and maintained in the absence of drug pressure. In this manner, three parasite clones were generated: PanOH-A and PanOH-B were generated from the two independent PanOH-pressured cultures while CJ-A was generated from the CJ-15,801-pressured culture. The clones are significantly resistant (95% confidence interval (CI) exclude 0) to the pantothenate analogues they were pressured with. The 50% inhibitory concentration (IC_{50}) values of PanOH against PanOH-A and PanOH-B, and the IC_{50} value of CJ-15,801 against CJ-A are approximately 7–8-fold higher than those measured against the Parent line (Fig 2A and 2B and S3 Table). Significant cross-resistance towards the other pressuring analogue was observed for these clones, as compared to the Parent line (95% CI exclude 0). The PanOH-A and PanOH-B lines were found to be 4–6-fold less sensitive to CJ-15,801 while CJ-A was found to be 13-fold less sensitive to PanOH (Fig 2A and 2B and S3 Table). To ensure that the clones did not develop a general drug-resistance phenotype during the selection process, we tested their sensitivity to chloroquine, an antiparasmodial with a mechanism of action that is unrelated to the parasite's CoA biosynthetic pathway [37]. We found that all of the drug-pressured lines have chloroquine IC_{50} values that are indistinguishable from that of the Parent line (95% CI include 0; Fig 2C and S3 Table).

The PanOH and CJ-15,801 resistance phenotypes observed in the clones were stable for several months of continuous culture in the absence of the pressuring analogue (≥ 3 months), consistent with a genetic alteration in these parasites. To determine the mutation(s) responsible for these phenotypes, gDNA was extracted from each clone and subjected to whole genome sequencing. All of the drug-resistant clones were found to harbour a unique mutation in the putative pantothenate kinase gene, *Pfpank1* (PF3D7_1420600), as shown in Fig 3A. Other non-synonymous mutations were detected for each clone (S4 Table) but we did not find another gene that is mutated in all three clones. The mutation found in the *Pfpank1* of PanOH-A results in the substitution of Asp507 for Asn. The other two drug-resistant clones have a mutation at position 95 of the protein: the *Pfpank1* of PanOH-B harbours a deletion of an entire codon leading to a loss of Gly from the *PfPanK1* protein, while the *PfPanK1* of CJ-A has a Gly to Ala substitution. Since the structure of *PfPanK1* has not yet been resolved, we generated a three-dimensional model in order to map the mutations within the enzyme. Fig 3B shows a *PfPanK1* model structure (pink) based on the solved structure of human PanK3 in complex with adenylyl-imidodiphosphate (AMPPNP) and pantothenate (PDB ID: 5KPR), overlaid on this structure (blue). The spheres shown in the model indicate the positions of the mutated residues, while the bound AMPPNP and pantothenate indicate the active site of the enzymes. Although the mutations are far apart in the primary amino acid sequence of *PfPanK1*, they are positioned in closer proximity to each other in the folded protein and are situated adjacent to the active site.

To confirm that the resistance phenotypes observed for the clones are directly caused by the mutations in *Pfpank1*, each clone was transfected with an episomal plasmid (*Pfpank1*-stop-pGlux-1) that enables the parasites to express the wild-type copy of *Pfpank1* (in addition to the endogenous mutated copy). These complemented lines are indicated with a superscripted “+”-WTPanK1”. From Fig 4 (and S3 Table), it can be observed that the complemented mutant clones (grey bars) are significantly less resistant to PanOH (Fig 4A) and CJ-15,801 (Fig 4B) compared to the non-complemented mutant clones (black bars; 95% CI exclude 0). As expected, the relative sensitivity of the mutant clones to chloroquine is unchanged by the presence of the *PfPanK1*-encoding plasmid (95% CI include 0; Fig 4C). Transfection of the *PfPanK1*-encoding plasmid into the Parent line did not alter its sensitivity to PanOH, CJ-15,801 or chloroquine (95% CI include 0; S3 Table). These data are consistent with the mutations observed in *Pfpank1* being responsible for the resistance phenotype observed in the mutant clones. An alternative strategy to confirm that the mutant *PfPanK1* is responsible for the

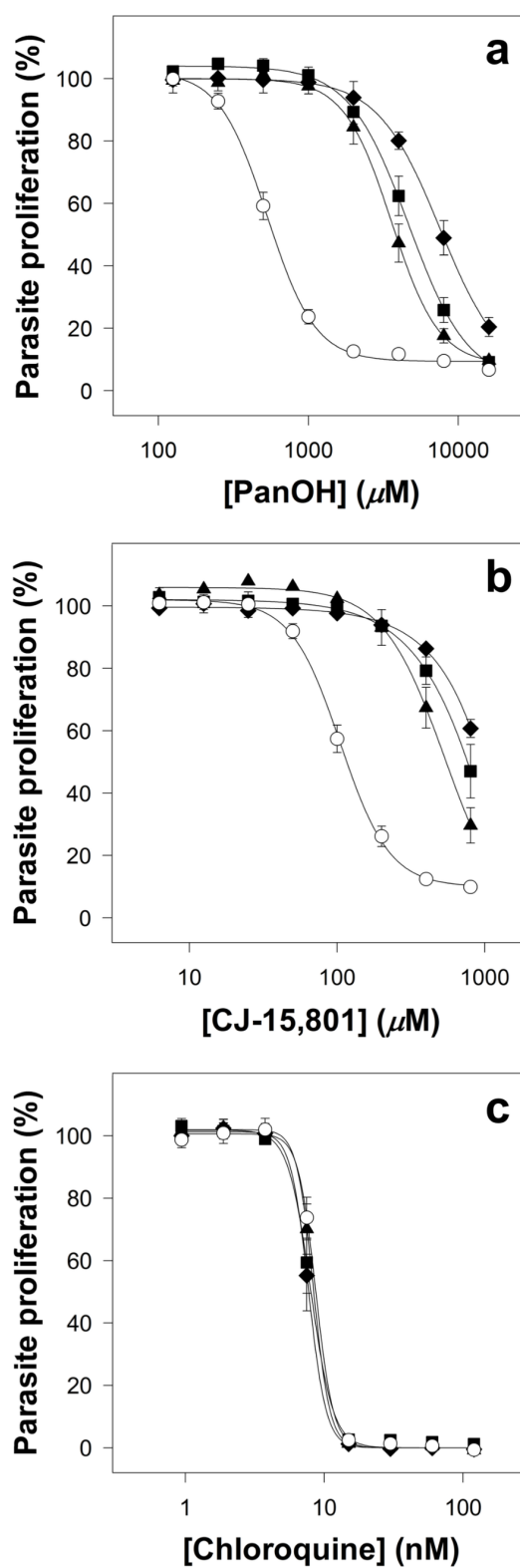


Fig 2. Percentage proliferation of parasites from the Parent (white circles), PanOH-A (black triangles), PanOH-B (black squares) and CJ-A (black diamonds) lines in the presence of (a) PanOH, (b) CJ-15,801 or (c) chloroquine. Drug-pressured lines were generated by exposing Parent line parasites to 11 – 13 weeks of continuous drug-pressuring with either PanOH (for PanOH-A and PanOH-B) or CJ-15,801 (for CJ-A), followed by limiting dilution cloning. Values are averaged from ≥ 4 independent experiments, each carried out in triplicate. All error bars represent SEM. Error bars are not visible if smaller than the symbols.

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a	Parasite line	Nucleotide change in <i>Pfpank1</i> coding sequence	Amino acid change in <i>PfPanK1</i>
	PanOH-A	C $\rightarrow$ T	D507N
	PanOH-B	Codon deletion	Δ G95
	CJ-A	C $\rightarrow$ G	G95A

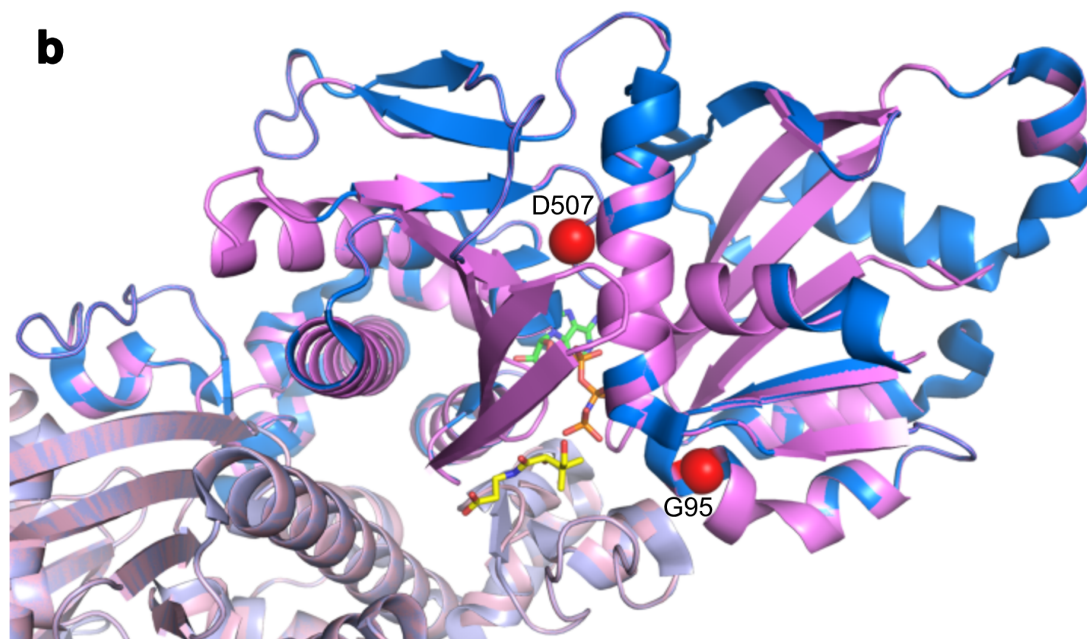


Fig 3. Mutations in *Pfpank1* and the affected residues within the *PfPanK1* protein. (a) The single nucleotide polymorphisms detected in the *Pfpank1* gene (accession number: PF3D7_1420600) of PanOH-A, PanOH-B and CJ-A, and the corresponding amino acid changes in the *PfPanK1* protein. (b) A three-dimensional homology model of the *PfPanK1* protein (pink) based on the solved structure of human PanK3 (PDB ID: 5KPR), overlaid on the human PanK3 structure in its active conformation (blue), with an ATP analogue (AMPPNP; carbon atoms coloured green) and pantothenate (carbon atoms coloured yellow) bound. *PfPanK1* shares 28% sequence identity with human PanK3 over the parts of the protein that have been modeled. Red spheres indicate the residues (G95 and D507) affected by the mutations in the parasite proteins. Human PanK3 has been shown to exist as a dimer. Here, individual monomers are shown in different shades of pink and blue.

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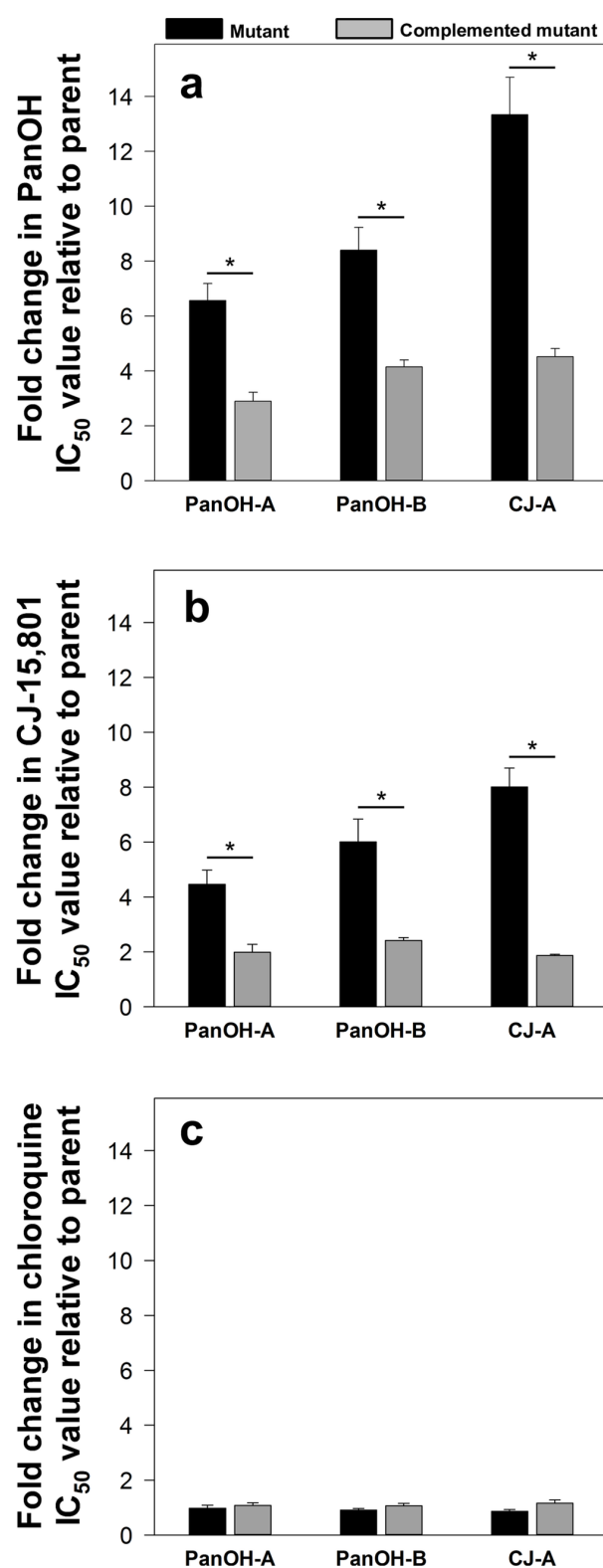


Fig 4. The fold change in (a) PanOH, (b) CJ-15,801 and (c) chloroquine IC₅₀ values of PanOH-A, PanOH-B and CJ-A parasite lines, in the absence (black bars) or presence (grey bars) of wild-type *Pf*PanK1 complementation, relative to that of their corresponding parental lines. Each fold change value is averaged from ≥ 3 independent experiments and error bars represent SEM. An asterisk indicates that the fold change in sensitivity of the mutant is significantly altered by complementation (PanOH fold change 95% CI: PanOH-A = -5.29 to -2.05, PanOH-B = -6.25 to -2.26 & CJ-A = -13.00 to -4.62; CJ-15,801 fold change 95% CI: PanOH-A = -4.17 to -0.79, PanOH-B = -6.14 to -1.06 & CJ-A = -8.05 to -4.22). No change in chloroquine sensitivity was observed (95% CI: PanOH-A = -0.23 to 0.43, PanOH-B = -0.09 to 0.40 & CJ-A = -0.01 to 0.59). The fold change in the sensitivity to CJ-15,801 for PanOH-B and CJ-A before and after complementation was calculated using IC₃₅ values.

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observed phenotypes would be to express the mutant forms of *Pf*PanK1 in the Parent line. This has not, however, been attempted.

Pfpank1 mutations impair parasite proliferation

We performed a competition assay to determine whether the *Pfpank1* mutations impart a fitness cost to the parasite clones. Each of the competition cultures was set up by mixing an equal number of parasites from the Parent line and one of the mutant clonal lines, and was maintained under standard conditions for a period of 6 weeks (Fig 5A). The sensitivity of each competition culture to PanOH was tested on the day the lines were mixed (Week 0) and again at the end of the 6-week period (Week 6). As expected, each Week 0 (dashed line) PanOH dose-response curve is between those obtained for the Parent and the respective mutant clone (dotted lines). A shift of the dose-response curve obtained at week 6 (solid line) towards the dose-response curve of the Parent line would indicate that the mutant *Pf*PanK1 imparts a fitness cost on the clone. As shown in Fig 5B, the Week 6 curve for the PanOH-A competition culture only exhibits a marginal leftward shift from Week 0, whereas those for PanOH-B (Fig 5C) and CJ-A (Fig 5D) exhibits a more substantial shift, almost reaching the dose-response curve of the Parent line (dotted line, white circles). These trends are supported by qPCR analysis designed to determine the proportions of mutant versus Parent gDNA at week 0 and week 6 (S1 Fig). These results are consistent with the mutations at position 95 in the *Pf*PanK1 of PanOH-B and CJ-A having a greater negative impact on the *in vitro* growth of the parasites. We also investigated the importance of *Pf*PanK1 expression for parasite growth by attempting to disrupt the *Pfpank1* locus in wild-type 3D7 parasites through homologous recombination (S2A Fig). However, using Southern blots, we failed to detect the presence of transfectants with the expected gene-knockout integration event (S2B Fig), consistent with this gene being essential during the parasite's intraerythrocytic stage. The lack of integration of the knockout construct into the *Pf*PanK1 locus, even in a small sub-population of the parasite culture, was confirmed using PCR (S2C Fig).

*Pf*PanK1 is a functional pantothenate kinase located within the parasite cytosol

The phosphorylation of radiolabelled pantothenate by lysates prepared from each of the mutant clones and the Parent line was measured to determine if the mutations in the putative *Pfpank1* gene affect PanK activity, and hence whether the gene codes for a functional PanK. As shown in Fig 6, at the end of the 75 min time-course, the lysate prepared from PanOH-A phosphorylated approximately $3 \times$ more [¹⁴C]pantothenate than the lysate prepared from the Parent line, while the lysate of PanOH-B generated about $3 \times$ less phosphorylated [¹⁴C]pantothenate compared to the Parent line. By comparison, the lysate prepared from CJ-A only produced a small amount of phosphorylated [¹⁴C]pantothenate in the same time period. The inset in Fig 6 demonstrates that PanK activity could be detected in CJ-A lysates when the

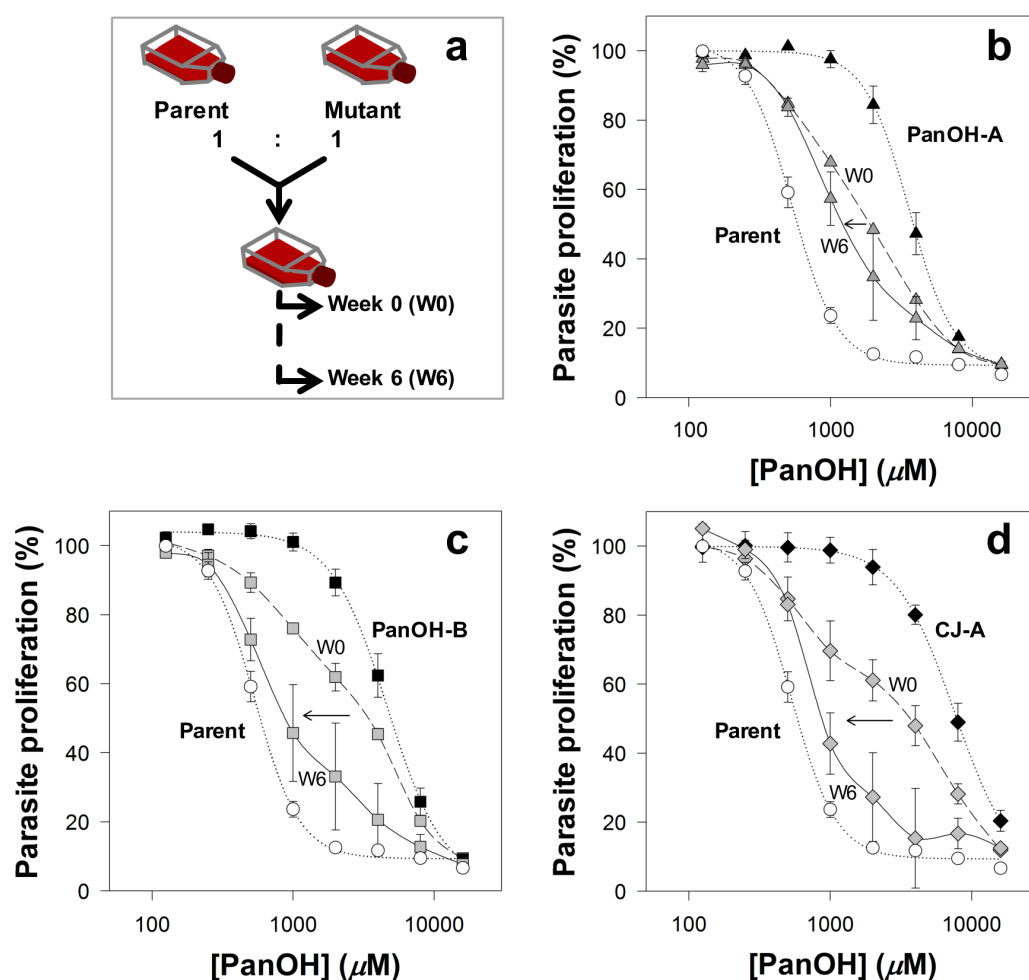


Fig 5. The fitness of the different mutant lines generated in this study relative to the Parent line as determined from parasite competition assays. (a) A flow chart illustrating how the competition assay was performed. For each competition culture, an equal number of parasites from the Parent and a mutant line were combined into a single flask. These mixed cultures were maintained for a period of 6 weeks. The fitness cost associated with the *Pfpank1* mutations was assessed by determining the PanOH sensitivity of the mixed cultures: (b) Parent vs PanOH-A (grey triangles), (c) Parent vs PanOH-B (grey squares) and (d) Parent vs CJ-A (grey diamonds), at Week 0 (W0; dashed lines) and Week 6 (W6; solid lines). It was expected that the greater the fitness cost to the mutant, the greater the shift of its mixed culture PanOH dose-response curve toward the Parent line curve after 6 weeks. Arrows indicate this shift between W0 and W6. The parasite proliferation curves (dotted lines) of the respective mutant clones (black symbols) and Parent line (white circles) are also shown for comparison. Values for the mixed cultures are averaged from 2 independent experiments, each carried out in triplicate. Error bars represent SEM ($n \geq 4$) for the individual cultures and range/2 for the mixed cultures, and are not visible if smaller than the symbols.

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experiment was carried out in the presence of a 100-fold higher pantothenate concentration ($200 \mu\text{M}$) and for an extended time (420 min). These observations provide strong evidence that the *Pfpank1* gene codes for a functional PanK. Further, we generated from the wild-type 3D7 strain a transgenic parasite line that episomally expresses a GFP-tagged copy of *PfPanK1* in order to localise the protein within the parasite. We found that *PfPanK1* is largely localised throughout the cytosol of trophozoite-stage parasites, and is not excluded from the nucleus (Fig 7).

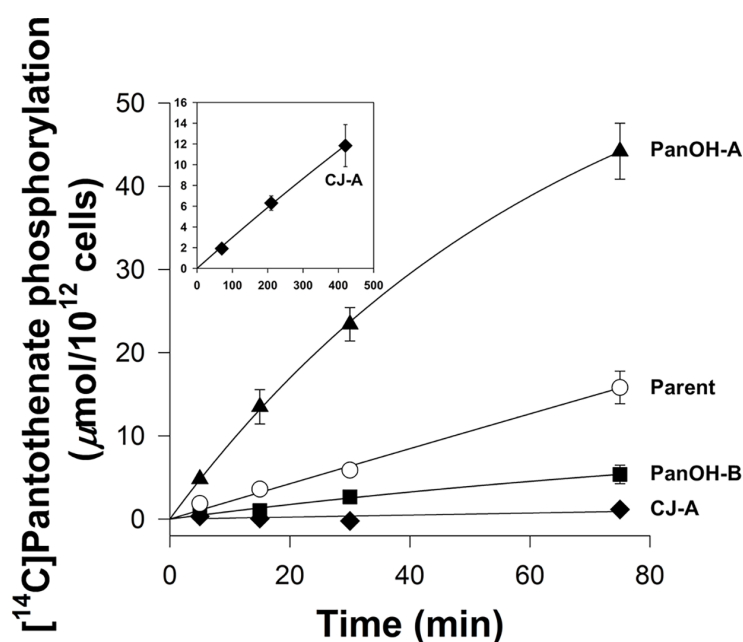


Fig 6. The phosphorylation of [^{14}C]pantothenate ($2\ \mu\text{M}$) over time (in minutes) by lysates generated from Parent (white circles), PanOH-A (black triangles), PanOH-B (black squares) and CJ-A (black diamonds) parasites. Inset shows [^{14}C]pantothenate ($2\ \mu\text{M}$) supplemented with non-radioactive pantothenate to a total pantothenate concentration of $200\ \mu\text{M}$) phosphorylation by CJ-A parasite lysates measured over 420 min. Values are averaged from 3 independent experiments, each performed with a different batch of lysate and carried out in duplicate. Error bars represent SEM and are not visible if smaller than the symbols.

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To investigate further the effects of the *Pf*PanK1 mutations present in the PanOH- and CJ-15,801-resistant clones, we analysed the PanK activity profiles using lysates prepared from each mutant and the Parent, and determined their kinetic parameters from the Michaelis-Menten equation (Fig 8). The apparent pantothenate K_m values of the mutant clones are 26–609-fold higher (95% CI exclude 0) than that of the Parent line. The maximal velocity ($V_{\max}$) of pantothenate phosphorylation by lysates prepared from PanOH-A, PanOH-B and CJ-A are also significantly higher (95% CI exclude 0) than that of the Parent. To eliminate the possibility that the elevated $V_{\max}$ values are due to increased *Pf*PanK1 levels in the mutant parasites, we used DIA-MS to compare the *Pf*PanK1 level in each of the mutant lines with that of the Parent parasite. We found that *Pf*PanK1 levels are unchanged in the CJ-A line (95% CI include 0) and are only elevated by $26 \pm 5\%$ and $27 \pm 4\%$ (mean $\pm$ SEM) in PanOH-A and PanOH-B, respectively (S3 Fig). This modest (or lack of an) increase in *Pf*PanK1 levels is insufficient to explain the larger increases in $V_{\max}$ values observed in lysates prepared from the mutant lines, as the increase in protein levels would need to be ~ 20 -fold for PanOH-A and ~ 2 -fold for PanOH-B to account for the altered $V_{\max}$ values. We have therefore not adjusted the observed $V_{\max}$ values to account for these small increases in the *Pf*PanK1 levels. We also calculated the *Pf*PanK relative specificity constant for each parasite line, which indicates the catalytic efficiency of each variant of *Pf*PanK relative to that of the Parent line. The relative specificity constant obtained for PanOH-A (0.74 ± 0.04 , mean $\pm$ SEM) is not significantly different (95% CI include 0) from that of the Parent. However, those of PanOH-B (0.058 ± 0.004 , mean $\pm$ SEM) and CJ-A (0.019 ± 0.005 , mean $\pm$ SEM) are significantly lower (95% CI exclude 0). These data are consistent with all three *Pf*PanK1 mutations observed reducing the enzyme's affinity for

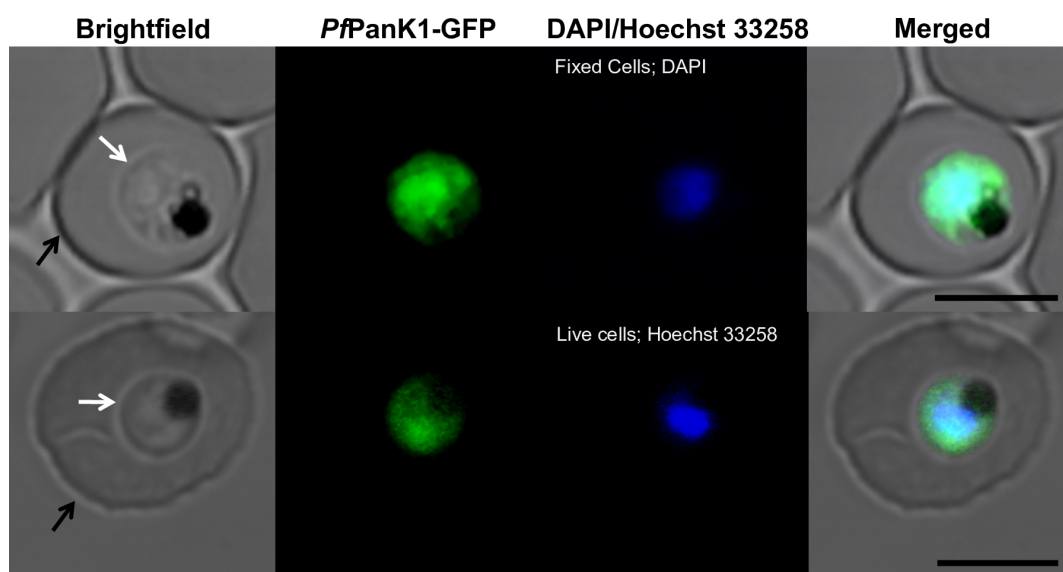


Fig 7. Confocal micrographs showing the subcellular location of GFP-tagged *PfPanK1* in 3D7 strain parasites harbouring the *Pfpank1*-pGlux-1 episomal plasmid. From left to right: Brightfield, GFP-fluorescence, DAPI- (fixed cells; top) or Hoechst 33258- (live cells; bottom) fluorescence, and merged images of erythrocytes infected with trophozoite-stage *P. falciparum* parasites expressing *PfPanK1*-GFP. Arrows indicate the plasma membranes of either the erythrocytes (black) or the parasites (white). Scale bar represents 5 μ m.

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pantothenate, although the associated increase in the enzyme V_{max} compensates for the reduced affinity: fully in the PanOH-A clone, but to a much lesser extent in PanOH-B and CJ-A (resulting in a 17-fold and 52-fold reduction in the enzyme's catalytic efficiency, respectively).

CJ-A requires a higher extracellular pantothenate concentration

An extracellular supply of pantothenate is essential for the *in vitro* proliferation of the intraerythrocytic stage of *P. falciparum* [8]. Given the impact that the *PfPanK1* mutations have on PanK activity (Fig 8), we set out to determine whether a higher extracellular concentration of pantothenate is required to support the proliferation of the different mutant clones relative to that required by the Parent line. As observed in Fig 9A, the proliferation of the Parent line (white circles) increased as the extracellular pantothenate concentration was increased, reaching the 100% control level (parasites maintained in the presence of 1 μ M pantothenate, the concentration of pantothenate in the complete RPMI medium used to maintain all of the parasite cultures, which is within the physiologically relevant range in human blood [38,39]) at approximately 100 nM. In order to compare the extracellular pantothenate requirement between the different lines, we determined the SC_{50} (50% stimulatory concentration; i.e. the concentration of pantothenate required to support parasite proliferation to a level equivalent to 50% of the control level) values for the mutants (with and without complementation) and Parent. From Fig 9B, it can be seen that the SC_{50} values of PanOH-A and PanOH-B are not different from that of the Parent line (95% CI include 0). Conversely, as illustrated by the rightward shift in its dose-response curve (black diamonds, Fig 9A), the pantothenate SC_{50} of CJ-A is approximately 3-fold higher than that of the Parent (Fig 9B; 95% CI = 2.7 to 30.9). Furthermore, consistent with the data from the complementation experiments (Fig 4), the SC_{50} value

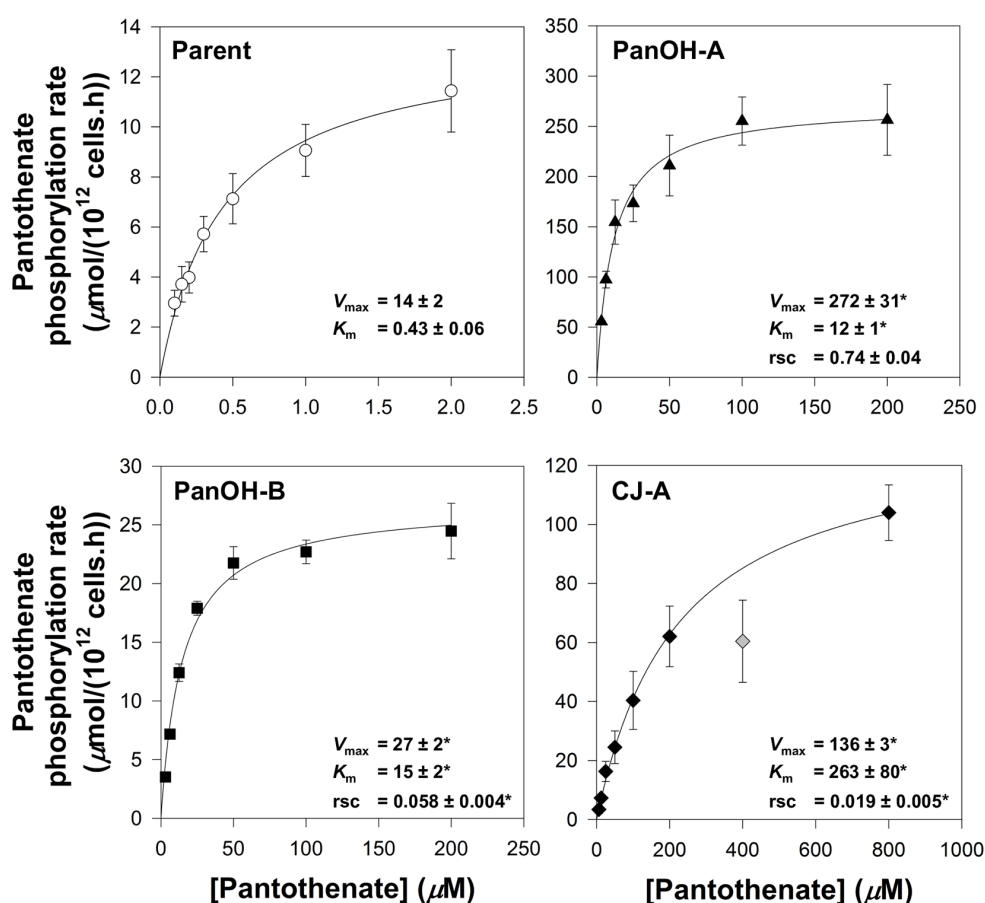


Fig 8. *PfPanK* activity profiles derived from the initial rates of pantothenate phosphorylation by lysates generated from Parent (white circles), PanOH-A (black triangles), PanOH-B (black squares) and CJ-A (black diamonds) parasites at various pantothenate concentrations. The grey diamond indicates a data point that was outside the 95% confidence band when it was included in the non-linear regression. This data point was therefore deemed an outlier and was omitted from the data set used to generate the fitted curve shown. Values are averaged from 3 independent experiments, each performed with a different batch of lysate and carried out in duplicate. Error bars represent SEM and are not visible if smaller than the symbols. Relative specificity constant (rsc) values express the specificity constants obtained for each cell line relative to that of the Parent. The V_{max} (μmol/(10¹² cells.h)), K_m (μM) and rsc values determined from the curves are shown within each box. Errors represent SEM (n = 3). An asterisk indicates that the value is significantly different compared to the Parent line (V_{max} 95% CI compared to Parent: PanOH-A = 173 to 344, PanOH-B = 5.2 to 21.6 & CJ-A = 112 to 133; K_m 95% CI compared to Parent: PanOH-A = 8.8 to 13.3, PanOH-B = 8.3 to 20.7 & CJ-A = 42 to 484; rsc 95% CI compared to Parent: PanOH-B = -1.366 to -0.519 & CJ-A = -1.404 to -0.557). No significant difference was observed between the rsc of the Parent and PanOH-A (95% CI compared to Parent: -0.698 to 0.179).

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of CJ-A^{+WTPfPanK1} (grey diamonds, Fig 9A) is comparable to that of the Parent line and also the control line, Parent^{+WTPfPanK1} (Fig 9B; 95% CI include 0), indicating that the episomal expression of wild-type *PfPanK1* is sufficient to reverse the phenotypic effects of the mutation.

N5-trz-C1-Pan and N-PE-αMe-PanAm have a different mechanism of action to PanOH and CJ-15,801

To determine whether the resistance of the clones to PanOH and CJ-15,801 extends to other pantothenate analogues, we tested the mutant clones against the two recently-described,

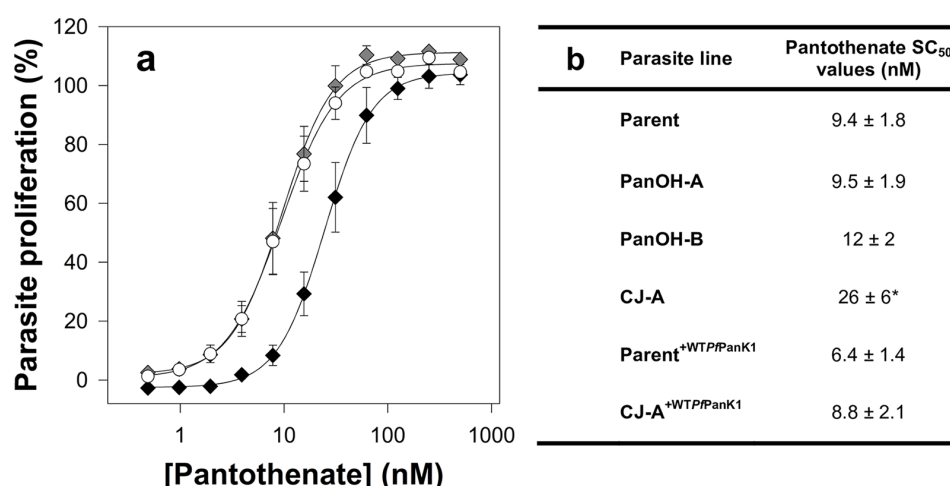


Fig 9. The pantothenate requirement of the different parasite lines observed in this study. (a) Percentage proliferation of parasites grown in different pantothenate concentrations. Values are averaged from ≥ 3 independent experiments, each carried out in triplicate. Error bars represent SEM and are not visible if smaller than the symbols. For clarity, only data from the Parent (white circles), CJ-A (black diamonds), and CJ-A^{+WTPPanK1} (grey diamonds) lines are shown. (b) The pantothenate stimulatory concentration 50 (SC₅₀) values obtained for Parent, PanOH-A, PanOH-B, CJ-A, Parent^{+WTPPanK1} and CJ-A^{+WTPPanK1} line parasites, which is the concentration of pantothenate required in the medium to support parasite proliferation by 50% (with 100% set to parasites grown in complete medium containing 1 μ M pantothenate). Errors represent SEM ($n \geq 3$). An asterisk indicates that the value is significantly different from that obtained for the Parent line (CJ-A SC₅₀ value 95% CI compared to Parent: 2.7 to 30.9). No significant difference was observed between the SC₅₀ value of the Parent and those of PanOH-A, PanOH-B, Parent^{+WTPPanK1} and CJ-A^{+WTPPanK1} (95% CI compared to Parent: PanOH-A = -6.8 to 7.1, PanOH-B = -3.8 to 9.3, Parent^{+WTPPanK1} = -9.3 to 3.3 & CJ-A^{+WTPPanK1} = -7.8 to 6.6).

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modified pantothenamides with potent antiparasmodial activities, namely N5-trz-C1-Pan [20] and N-PE- α Me-PanAm [21]. We found that PanOH-A is 3-fold more sensitive to N5-trz-C1-Pan, PanOH-B is 2-fold more resistant and CJ-A is 9-fold more resistant when compared to the Parent line (95% CI exclude 0; Fig 10A and S5 Table, left side). Similarly, we found that relative to the Parent line, PanOH-A was more sensitive to N-PE- α Me-PanAm (~2-fold), while CJ-A is 2-fold more resistant (95% CI exclude 0; Fig 10B and S5 Table, left side). The sensitivity of PanOH-B to N-PE- α Me-PanAm was statistically indistinguishable from that of the Parent line (95% CI = -0.046 to 0.005; Fig 10B and S5 Table, left side). These results indicate that *Pf*PanK1 can influence the sensitivity of the parasite to multiple antiparasmodial pantothenate analogues. Remarkably, the mutation at position 507 of the *Pf*PanK1 in PanOH-A makes the parasite resistant to the antiparasmodial activity of certain pantothenate analogues (PanOH and CJ-15,801) whilst at the same time *hyper-sensitises* the parasite to pantothenate analogues of a different class (modified pantothenamides, N5-trz-C1-Pan and N-PE- α Me-PanAm).

Previous work has shown that, in bacteria, pantothenamides are metabolised by the CoA biosynthetic pathway to form CoA antimetabolites [40], consistent with PanK activity being important for metabolic activation of pantothenamides. Additionally, it has been reported recently that pantothenamides are also phosphorylated by the PanK in *P. falciparum* [41], in line with their metabolic activation in bacteria. We therefore set out to determine whether the modified, pantothenase-resistant, pantothenamides are metabolised and to what extent. In order to do so, we exposed intact Parent line *P. falciparum*-infected erythrocytes to N5-trz-C1-Pan (at $\sim 10 \times$ the IC₅₀ for 4 h) and subjected lysates from the treated (and control) samples

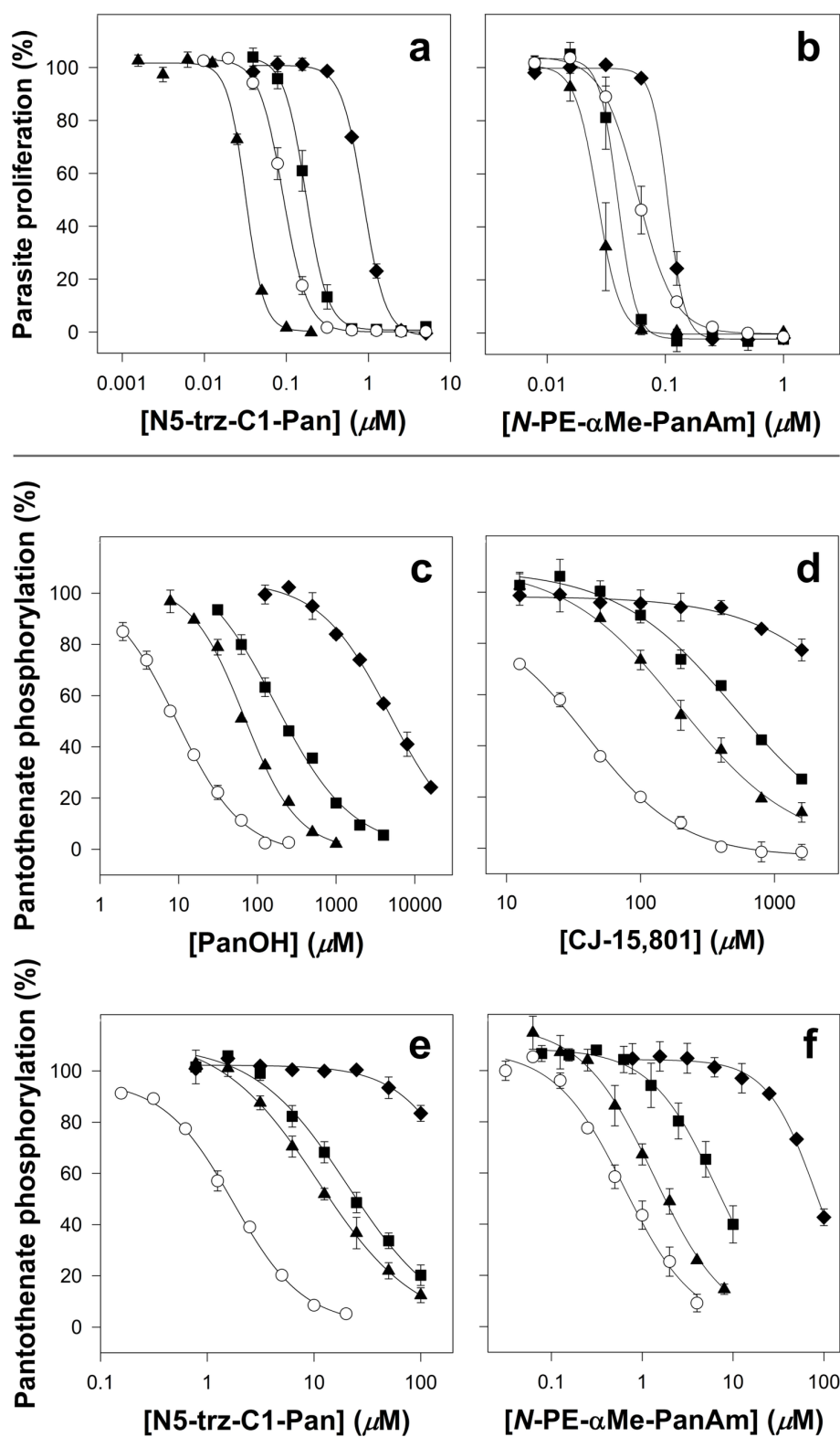


Fig 10. Percentage parasite proliferation of the different lines in the presence of the pantothenate analogues (a) N5-trz-C1-Pan and (b) N-PE- α Me-PanAm, and inhibition of [14 C]pantothenate phosphorylation in parasite lysates by various pantothenate analogues: (c) PanOH, (d) CJ-15,801, (e) N5-trz-C1-Pan and (f) N-PE- α Me-PanAm. Symbols represent Parent (white circles), PanOH-A (black triangles), PanOH-B (black squares) and CJ-A (black diamonds) parasite lines. The Y-axes of c–f indicate the percentage of total pantothenate phosphorylation. Values are averaged from ≥ 3 independent experiments, each carried out in triplicate for the parasite proliferation assays and duplicate for the phosphorylation assays. Error bars represent SEM and are not visible if smaller than the symbols.

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to untargeted LC-MS. Among the metabolites extracted from parasite-infected erythrocytes treated with N5-trz-C1-Pan (but not untreated control samples) were molecules with masses corresponding to phosphorylated N5-trz-C1-Pan ($[M-H]^-$ m/z 378.1668), a dephospho-CoA analogue of N5-trz-C1-Pan ($[M-2H]^{2-}$ m/z 353.6099) and a CoA analogue of N5-trz-C1-Pan ($[M-H]^-$ m/z 787.1858) as shown in [S4 Fig](#). This is consistent with N5-trz-C1-Pan being metabolised within infected erythrocytes to generate a CoA antimetabolite.

Lastly, we investigated the ability of the different pantothenate analogues to inhibit the phosphorylation of [14 C]pantothenate by parasite lysates prepared from the various mutant clones. These data are shown in [Fig 10C–10F](#) and [S5 Table](#), right side. All of the analogues tested are significantly less effective (95% CI exclude 0) at inhibiting pantothenate phosphorylation by lysates generated from the mutant clones compared to their ability to inhibit pantothenate phosphorylation by lysates prepared from the Parent line. The exception being N-PE- α Me-PanAm, which did not reach statistical significance when tested against lysate prepared from PanOH-A. Additionally, the effectiveness of the analogues at inhibiting pantothenate phosphorylation by lysates prepared from the mutant lines all observe the following order: PanOH-A > PanOH-B > CJ-A.

Discussion

The putative *Pfpank1* gene codes for a functional pantothenate kinase

When *Pf*PanK1 is compared to type II PanKs from other organisms in a multiple protein sequence alignment, the three nucleotide-binding motifs characteristic of this superfamily can be seen to be conserved in *Pf*PanK1, consistent with it being a functional PanK [7]. However, biochemical confirmation of its putative function as a PanK has not been demonstrated. In this study, we show that mutations in the putative *Pf*PanK1 lead to substantial changes in pantothenate kinase activity ([Fig 8](#)) providing, for the first time, biochemical evidence that the cytosolic *Pf*PanK1 ([Fig 7](#)) is a functional pantothenate kinase. Furthermore, the fact that multiple independent experiments aimed at generating parasites resistant to PanOH and CJ-15,801 always selected for mutations in *Pf*PanK1 ([Fig 3A](#) and [S4 Table](#)) is consistent with the kinase being the primary PanK involved in the metabolic activation of pantothenate analogues, at least during the intraerythrocytic stage. Although it is intriguing that *Pf*PanK1 is not excluded from the nucleus, this phenomenon has been reported for some PanKs in other organisms [42].

Although it is clear that the *Pf*PanK1 residues at position 95 and 507 are required for normal *Pf*PanK1 activity ([Fig 8](#)), and the *Pf*PanK1 model structure ([Fig 3B](#)) shows that both residues are situated adjacent to the enzyme active site, their exact role(s) in modifying the activity of the protein is less obvious. The Gly residue at position 95 is conserved in eukaryotic PanKs [7] and is the residue at the cap of the $\alpha 2$ -helix in the inactive conformation of the protein ([S5A Fig](#)). One possibility is that the change to Ala at this position could affect the structure of the helix and consequently the overall stability of the protein (at least when the protein is in the inactive state), as Gly has been shown to be much better than Ala at conferring structural

stability when located at the caps of helices [43]. Alternatively, Gly residues have been shown to be present at a higher frequency in the active sites of some enzymes where they likely confer the flexibility to alternate between open and closed conformations [44]. Although the Gly95 residue is not part of the *Pf*PanK1 active site *per se*, it is within close proximity to the site (Fig 3B), and the α 2-helix certainly undergoes a conformational change when PanK switches from the inactive conformation to the active one [29]. This conformational change is demonstrated in S5A Fig, which shows an overlay of the human PanK3 crystal structures in the active [29] and inactive [45] state (Gly95 in *Pf*PanK1 is equivalent to Gly117 in the human enzyme). It is worth noting that acetyl-CoA (an inhibitor of the enzyme) can only be accommodated in the binding site when the enzyme is in the inactive state, as when the enzyme is in the active state, the α 2-helix encroaches on the space occupied by acetyl-CoA (S5A Fig). This lends further support to the importance of this helix for the enzyme to transition from the inactive to the active state (and *vice versa*) and, therefore, to the role that Gly95 could play in this process. Either way, these suggestions may explain why a mutation at this position has a greater impact on *Pf*PanK1 function than the mutation at position 507. The Asp residue at position 507 is replaced by a different amino acid (Glu) in most other eukaryotic PanKs, although they are both negatively-charged [7]. A substitution to the uncharged Asn could disrupt any important salt-bridges or hydrogen bonds with the residue at this position. In human PanK3, the amino acid equivalent to Asp507 is Glu354. In the enzyme's inactive state, Glu354 is within ionic bonding distance of Arg325, which in turn interacts with the 3'-phosphate of acetyl-CoA (S5B Fig) [45] and may therefore stabilise the inactive state of the enzyme. Conversely, in the enzyme's active state, Glu354 and Arg325 are not within ionic bonding distance (S5B Fig). If Asp507 in *Pf*PanK1 plays a similar role to that proposed for Glu354 in human PanK3, the change at position 507 to an Asn (abolishing the negative charge) could prevent stabilisation of the inactive state, providing an explanation for the increased activity observed in PanOH-A (Fig 6). Determining the crystal structure of *Pf*PanK1 bound with pantothenate may provide a better understanding of the roles these residues play in PanK function.

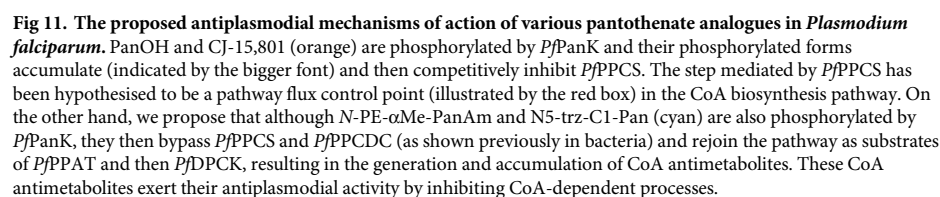
Pf*PanK1 is essential for normal intraerythrocytic proliferation of *P. falciparum

It has been established that almost all of the 4'-phosphopantothenate found in *P. falciparum*-infected erythrocytes is generated within the parasite by *Pf*PanK as part of its metabolism into CoA [10], which is in line with *Pf*PanK activity being essential for the parasite's survival. In the present study, our inability to knock out *Pf*PanK1 (S2 Fig) is consistent with the protein being essential for the intraerythrocytic stage of *P. falciparum*, although we cannot exclude the unlikely possibility that the regions we targeted for the required double-crossover recombination event are genetically intractable. Our observation that clones PanOH-B and CJ-A, which harbour mutations at position 95 of *Pf*PanK1, can be outcompeted by the Parent parasites in competition assays over approximately 20 intraerythrocytic cycles (Fig 5) is consistent with the mutations incurring a fitness cost. This, in turn, indicates that *Pf*PanK1 is essential for normal parasite development, at least during the blood stage of its lifecycle. It was also found here that clone CJ-A requires an approximately 3-fold higher extracellular pantothenate concentration in order to survive (Fig 9), coinciding with this clone having the *Pf*PanK with the highest K_m . This is not surprising given the reported importance for the substrate concentration to exceed the enzyme K_m for optimal enzyme efficiency [46,47]. More importantly, the requirement by this clone for a higher concentration of extracellular pantothenate is also congruent with *Pf*PanK1 being essential for the normal development of *P. falciparum* during its asexual blood stage.

Our observation that *PfPanK1* is essential for normal parasite growth during the blood stage is at odds with recent reports that show both PanK1 and PanK2 from *Plasmodium yoelli* and *Plasmodium berghei* are dispensable during the blood stage of those parasites [48–50]. However, both of these murine malaria parasite species preferentially infect reticulocytes [51,52]. Unlike the mature erythrocytes preferred by *P. falciparum*, reticulocytes have been shown to provide a rich pool of nutrients for the parasite, allowing the murine parasites to survive metabolic or genetic changes that would have been deleterious in *P. falciparum* [53]. It is therefore conceivable that unlike the condition faced by *P. falciparum*, the reticulocyte-residing parasites are able to salvage sufficient CoA or CoA intermediates from the host cell for their survival, rendering the two PanK proteins dispensable during their intraerythrocytic stage, a possibility acknowledged by the authors of the *P. berghei* study [49].

PanOH and CJ-15,801 share a common mechanism of action

We have presented data consistent with the observed *PfPanK1* mutations being the genetic basis for the PanOH and CJ-15,801 resistance phenotypes observed in all of the drug-pressured clones we generated (Fig 4). As shown by the data presented in Fig 10, both PanOH and CJ-15,801 inhibited pantothenate phosphorylation by the mutated *PfPanK1* proteins less effectively than their inhibition of pantothenate phosphorylation by the wild-type *PfPanK1* (the order of their IC₅₀ values is Parent < PanOH-A < PanOH-B < CJ-A). Importantly, this order is also reflected in the level of resistance of the mutant clones to these two analogues, although the magnitude is not preserved (Fig 2 and S3 Table). These data are consistent with *PfPanK1* being involved in the antiparasmodial activity of these analogues either as a target or a metabolic activator. Previous studies have demonstrated that the antiparasmodial activity of both PanOH and CJ-15,801 involves the inhibition of pantothenate phosphorylation by *PfPanK* [9,15]. More specifically, PanOH has been shown to inhibit *PfPanK*-mediated pantothenate phosphorylation by serving as its substrate [54]. CJ-15,801 is also likely to be a substrate of *PfPanK*, especially since it has been shown to be phosphorylated by the *S. aureus* PanK [55], another type II PanK [56]. In addition, the same study showed that the second enzyme in the CoA biosynthetic pathway, PPCS, subsequently accepts phosphorylated CJ-15,801 as a substrate, and performs the first step of the PPCS reaction (cytidylylation) on it. However, unlike what happens with 4'-phosphopantothenate, this cytidylylated phospho-CJ-15,801 acts as a tight-binding, dead-end inhibitor of the enzyme [55]. A separate study also concluded that PanOH targets the PPCS enzyme in *Escherichia coli* and *Mycobacterium tuberculosis* [57]. Based on the data generated in this study and the published reports that PanOH and CJ-15,801 both inhibit PPCS in other systems, we propose a similar mechanism of action for these compounds in *P. falciparum*, whereby they are phosphorylated by *PfPanK1* and subsequently block *PfPPCS* as dead-end inhibitors (Fig 11). The overlapping pattern of cross-resistance between the two compounds (Fig 2) is also in line with them having a similar mechanism of action. We propose that the observed resistance to PanOH and CJ-15,801 is due to the mutated *PfPanK1* having a reduced capacity to phosphorylate these analogues relative to pantothenate. This would have the effect of reducing the amount of phosphorylated PanOH or CJ-15,801 generated relative to 4'-phosphopantothenate, thereby allowing the parasites to survive at higher concentrations of the drugs. Furthermore, our observation that the mutant clones have comparable levels of PanOH and CJ-15,801 resistance (Fig 2 and S3 Table), despite having *PfPanK1* proteins of vastly different efficiency (Fig 8), is likely due to the pathway flux control at the *PfPPCS*-mediated step in the CoA biosynthetic pathway of *P. falciparum*, as shown in a previous study [32].



N5-trz-C1-Pan is converted into an antiplasmodial CoA analogue – N-PE- α Me-PanAm likely shares the same mechanism of action

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therefore expected to bypass the *PfPPCS* and *PfPPCDC* steps of the CoA biosynthetic pathway on its way to being converted into the CoA antimetabolite version of N5-trz-C1-Pan (Fig 11).

Furthermore, the antiparasmodial activity rank order of N5-trz-C1-Pan against the various mutant clones is very similar to that of *N*-PE- α Me-PanAm – with PanOH-A being hypersensitive to both compounds, CJ-A resistant to both and PanOH-B, by comparison, exhibiting only small changes in sensitivity to the two compounds (Fig 10A and 10B) – and is starkly different to those of PanOH and CJ-15,801 (Fig 2). These are congruent with (i) the antiparasmodial mechanism of action of *N*-PE- α Me-PanAm being similar to that of N5-trz-C1-Pan and (ii) the antiparasmodial mechanism of action of *N*-PE- α Me-PanAm and N5-trz-C1-Pan being different to that of PanOH and CJ-15,801.

The order of antiparasmodial activity of N5-trz-C1-Pan and *N*-PE- α Me-PanAm against the mutant clones can be explained on the basis of (i) the difference in the rate of *PfPanK1* activity in the various clones at the concentrations of pantothenate and N5-trz-C1-Pan / *N*-PE- α Me-PanAm used (Fig 6), (ii) the fact that the *PfPPCS*-mediated step imposes pathway flux control [32] and (iii) the fact that this pathway flux control is bypassed by N5-trz-C1-Pan (and almost certainly also by *N*-PE- α Me-PanAm) *en route* to its conversion into a CoA antimetabolite. As seen in Fig 6, in the presence of 2 μ M pantothenate (a similar concentration to the 1 μ M present in the antiparasmodial assay), the pantothenate phosphorylation rate of the different clones has the following rank order: PanOH-A > Parent > PanOH-B > CJ-A, approximately the inverse of the antiparasmodial IC₅₀ values of N5-trz-C1-Pan and *N*-PE- α Me-PanAm (described above). Therefore, PanOH-A, for example, would be expected to generate more 4'-phosphopantothenate and phosphorylated N5-trz-C1-Pan (or *N*-PE- α Me-PanAm), based on the assumption that the mutation also leads to increased phosphorylation activity towards the pantothenate analogues (point i). Whilst the increased levels of 4'-phosphopantothenate would not be expected to result in a concomitant increase in CoA (due to the pathway flux control at *PfPPCS*; point ii), the increased production of phospho-N5-trz-C1-Pan would result in increased levels of the N5-trz-C1-Pan CoA antimetabolite as the flux control step is bypassed (point iii). This would explain the increased sensitivity of PanOH-A to both N5-trz-C1-Pan and *N*-PE- α Me-PanAm and also the sensitivity rank order of the other parasite lines.

In conclusion, our study confirms for the first time that *PfPanK1* functions as the active pantothenate kinase in the asexual blood stage of *P. falciparum*. Our data show that the sites of mutation in *PfPanK1* reported here are important residues for normal *PfPanK* function and are essential for normal intraerythrocytic parasite growth, although further structural and functional studies are required to elucidate their exact role(s). Furthermore, we propose that following phosphorylation by *PfPanK1*, PanOH and CJ-15,801 compete with 4'-phosphopantothenate and serve as dead-end inhibitors of *PfPPCS* (depriving the parasite of CoA). In contrast, *N*-PE- α Me-PanAm and N5-trz-C1-Pan are further metabolised (by *PfPPAT* and *PfDPCK*) into CoA analogues that kill the parasite by inhibiting CoA-utilising metabolic processes. Finally, we provide the first genetic evidence consistent with pantothenate analogue activation being a critical step in their antiparasmodial activity.

Supporting information

S1 Materials and methods. Additional information about materials and methods used in the study.

(DOCX)

S1 Table. List of oligonucleotides used in this study. MCS: Multiple Cloning Site.

(DOCX)

S2 Table. Sequences of peptides used to identify and quantify the five proteins of interest shown in S3 Fig. ^aCertainty of peptide identification.

(DOCX)

S3 Table. Inhibition of the proliferation of Parent, PanOH-A, PanOH-B, CJ-A, Parent^{WT}PanK1, PanOH-A^{WT}PanK1, PanOH-B^{WT}PanK1 and CJ-A^{WT}PanK1 parasites by PanOH, CJ-15,801 and chloroquine, as represented by IC₅₀ values. Errors represent SEM (n ≥ 3). The averaged CJ-15,801 IC₅₀ value for PanOH-B (n = 3) includes a single extrapolated value, because in one experiment the highest concentration tested (800 μM) did not inhibit parasite growth by ≥ 50%. An asterisk indicates that the IC₅₀ value of the mutant line is significantly different from that obtained for the Parent line (PanOH IC₅₀ 95% CI compared to Parent IC₅₀: PanOH-A = 2664 to 3432, PanOH-B = 3553 to 4552 & CJ-A = 6046 to 7456; CJ-15,801 IC₅₀ 95% CI compared to Parent IC₅₀: PanOH-A = 296 to 436, PanOH-B = 495 to 717 & CJ-A = 688 to 823). The chloroquine IC₅₀ values of the different lines are indistinguishable (95% CI compared to Parent IC₅₀: PanOH-A = -2.023 to 1.646, PanOH-B = -2.114 to 0.492 & CJ-A = -2.507 to 0.158).

(DOCX)

S4 Table. List of non-synonymous single nucleotide polymorphisms found in the PanOH-A, PanOH-B and CJ-A parasite lines as determined by whole genome sequencing and variant calling using the PlaTyPus integrated pipeline. Uppercase letters in the “Codon Change” column denotes the base within the codon that has been altered in the coding sequence before/after the mutation. The deletion in PanOH-B at position 95 is not included here because PlaTyPus is unable to detect insertions-deletions polymorphisms.

(DOCX)

S5 Table. Inhibition of the proliferation of Parent, PanOH-A, PanOH-B and CJ-A parasites by N5-trz-C1-Pan and N-PE-αMe-PanAm, and the inhibition of [¹⁴C]pantothenate phosphorylation in parasite lysates (generated from the same lines) by the pantothenate analogues PanOH, CJ-15,801, N5-trz-C1-Pan and N-PE-αMe-PanAm, as represented by IC₅₀ values. Errors represent SEM (n ≥ 3). An asterisk indicates that the value is significantly different from that obtained for the Parent line (95% CI of N5-trz-C1-Pan proliferation inhibition IC₅₀ compared to Parent: PanOH-A = -0.088 to -0.038, PanOH-B = 0.047 to 0.121 & CJ-A = 0.715 to 0.824; 95% CI of PE-αMe-PanAm proliferation inhibition IC₅₀ compared to Parent: PanOH-A = -0.060 to -0.008 & CJ-A = 0.024 to 0.076; 95% CI of PanOH phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 46 to 69, PanOH-B = 71 to 263 & CJ-A = 4531 to 5300; 95% CI of CJ-15,801 phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 10 to 309 & PanOH-B = 330 to 621; 95% CI of N5-trz-C1-Pan phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 6.5 to 13 & PanOH-B = 9.8 to 30; 95% CI of N-PE-αMe-PanAm phosphorylation inhibition IC₅₀ compared to Parent: PanOH-B = 3.6 to 9.0 & CJ-A = 75 to 97).

(DOCX)

S1 Fig. Quantitative PCR analysis of gDNA samples extracted from the three competition assay cultures generated in the study, Parent vs PanOH-A, Parent vs PanOH-B and Parent vs CJ-A (Fig 5). Data are shown as the proportions of mutant gDNA (out of a 100% total) at week 0 and week 6. Values are averaged from two independent competition assays, each analysed by qPCR in duplicate. Error bars represent range/2.

(TIF)

S2 Fig. The attempt at knocking-out *Pfpank1* in wild-type 3D7 parasites through homologous integration. (a) Schematic representations of the $\Delta Pfpank1$ -pCC-1 construct and the wild-type *Pfpank1* gene locus before and after either a homologous double crossover integration of the *hdhfr* cassette of $\Delta Pfpank1$ -pCC-1 or a 5'/3' single crossover homologous integration of the $\Delta Pfpank1$ -pCC-1 construct. The positions of *Afl*III restriction sites are indicated. The 5' *Pfpank1* and 3' *Pfpank1* homologous flanks are indicated by translucent blue boxes. (b) Southern blot of *Afl*III-digested gDNA extracted from wild-type parasites and from $\Delta Pfpank1$ -pCC-1-transfectants resistant to WR99210 or both WR99210 and 5-fluorocytosine (5-FC) after one or two rounds of WR99210 cycling. (i) and (ii) represent independently-selected drug-resistant cultures. The blot was probed with the 3' *Pfpank1* flank (as indicated by the black bar in (a)). The probe hybridised to fragments that correspond to the 3D7 wild-type (3.3 kb) and the plasmid (8.9 kb). The fragment that is consistent with a homologous double crossover-disrupted locus (3.8 kb) was not detected in either independent culture. *hdhfr*: human dihydrofolate reductase—conveys resistance to WR99210. *Scfcu*: *Saccharomyces cerevisiae* cytosine deaminase/phosphoribosyl transferase—conveys sensitivity to 5-FC. (c) PCR confirmation that a *Pfpank1* knockout event cannot be detected, even in a small sub-population of parasites. Samples from the same parasite populations shown in (b) were used to generate DNA templates that were then used with primers selected to amplify a product of 1.9 kb from the wild-type sequence and a 3.6 kb product from parasites with the *Pfpank1* gene knocked out (and replaced with the *hdhfr* gene). A PCR product consistent with the size expected for amplification from the wild-type sequence was observed (black arrow). The identity of this product was confirmed by sequencing. All the reactions generated an approximately 1.1 kb PCR product and some reactions, under certain conditions, produced fainter PCR products likely due to non-specific primer binding. Importantly, no product of a size consistent with that expected for a *Pfpank1* knockout (3.6 kb) was observed in any of the samples. These data, therefore, are consistent with those presented in the Southern blot (b). (TIF)

S3 Fig. Abundance of (a) *PfPanK1* and (b) various control proteins (*PfPanK2*, *PfHSP70*, *PfHK* and *Pf α -tubulin) in Parent, PanOH-A, PanOH-B and CJ-A line trophozoites.* Protein levels are determined by LC-MS/MS followed by DIA analysis. Values are averaged from ≥ 3 independent parasite preparations and error bars represent SEM. An asterisk indicates that the protein peak intensity measured for a mutant line is significantly different from that obtained for the Parent line (95% CI of *PfPanK1* protein level compared to Parent: PanOH-A = 3.87×10^3 to 1.18×10^5 & PanOH-B = 8.86×10^3 to 1.18×10^5). The protein abundance of *PfPanK1* in CJ-A (95% CI compared to Parent = -3.38×10^4 to 1.01×10^5) and those of the housekeeping proteins in all three mutant lines are indistinguishable from the Parent line levels (95% CI for *PfPanK2* level compared to Parent: PanOH-A = -1.81×10^4 to 2.53×10^4 , PanOH-B = -1.52×10^4 to 2.25×10^4 & CJ-A = -3.43×10^4 to 1.99×10^4 ; 95% CI for *PfHSP70* level compared to Parent: PanOH-A = -8.98×10^7 to 9.72×10^7 , PanOH-B = -6.92×10^7 to 6.27×10^7 & CJ-A = -1.45×10^8 to 1.45×10^8 ; 95% CI for *PfHK* level compared to Parent: PanOH-A = -4.41×10^5 to 2.79×10^6 , PanOH-B = -1.18×10^5 to 2.47×10^6 & CJ-A = -2.42×10^6 to 2.13×10^6 ; 95% CI for *Pf α -tubulin level compared to Parent: PanOH-A = -5.57×10^6 to 7.45×10^6 , PanOH-B = -4.47×10^6 to 4.25×10^6 & CJ-A = -1.18×10^7 to 2.22×10^6). (TIF)*

S4 Fig. Extracted ion chromatograms (i) and mass spectra (ii) of N5-trz-C1-Pan (a) and downstream metabolites (b–d). Extracted ion chromatograms (i) show the relative intensity of each LC peak. Red lines represent metabolites extracted from N5-trz-C1-Pan-treated parasites and blue lines represent those extracted from DMSO-treated parasite control samples,

each carried out in quadruplicate. High resolution mass spectra (ii) of each compound ionised in negative mode: (a) N5-trz-C1-Pan ($C_{14}H_{26}N_4O_3$). Theoretical $m/z = 297.1932$. Observed $m/z = 297.1942$. $\Delta ppm = 3.36$. (b) Phosphorylated N5-trz-C1-Pan ($C_{14}H_{27}N_4O_6P$). Theoretical $m/z = 377.1595$. Observed $m/z = 377.1594$. $\Delta ppm = -0.27$. (c) Dephospho-CoA N5-trz-C1-Pan analogue ($C_{24}H_{39}N_9O_{12}P_2$). Theoretical $m/z = 352.6024$. Observed $m/z = 352.6024$. $\Delta ppm = 0.0$. (d) CoA N5-trz-C1-Pan analogue ($C_{24}H_{40}N_9O_{15}P_3$). Theoretical $m/z = 786.1784$. Observed $m/z = 786.1780$. $\Delta ppm = -0.51$. Data shown are from a single experiment representative of two independent experiments.

(TIF)

S5 Fig. The human PanK3 protein structure in its inactive conformation (yellow; PDB ID: 3MK6) overlaid onto its active conformation (blue; PDB ID: 5KPR). The amino acid residues of the human protein at positions 117 (indicated by the spheres in a) and 354 (side chains shown in b) correspond to the mutated residues of *Pf*PanK1 reported in this study (at positions 95 and 507, respectively). (a) The red arrow indicates the change in the conformation of the $\alpha 2$ -helix between the inactive and active states. The altered configuration in the active state transitions the mutated glycine that corresponds to position 117 away from the end cap of the $\alpha 2$ -helix. (b) Dashed grey lines (inactive conformation) and the solid arrow (active conformation) represent distances between residue side chains and/or acetyl-CoA (in Å) in PanK3. A relay of interactions between Glu354, Arg325 and the 3'-phosphate of acetyl-CoA may stabilise the inactive state of the enzyme. However, in the protein's active conformation, Glu354 and Arg325 are not within bonding distance (≥ 4.4 Å).

(TIF)

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Supporting Information - Materials and Methods

Plasmid preparation and nucleic acid purification

The *Pfpank1*-pGlux-1 plasmid consists of the wild-type *Pfpank1*-coding sequence inserted within multiple cloning site III of pGlux-1, which contains the human dihydrofolate reductase (*dhfr*) gene that confers resistance to WR99210 as a positive selectable marker. This places *Pfpank1* under the regulation of the *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) promoter, and upstream of the GFP-coding sequence. This plasmid construct allows the parasite to express a GFP-tagged *PfPanK1* and was used to localise the protein within the parasite. The *Pfpank1*-stop-pGlux-1 plasmid is identical to the *Pfpank1*-pGlux-1 construct but contains two stop codons between the *Pfpank1*-specific sequence and the GFP-coding sequence. This enables the parasite to express wild-type *PfPanK1* that is not GFP-tagged and was used to complement the mutant lines with wild-type *PfPanK1*. The Δ *Pfpank1*-pCC-1 construct was generated using the pCC-1 plasmid [1] as the backbone (**Figure S2 a**). This plasmid contains the *dhfr* gene cassette as a positive selectable marker. Two *Pfpank1*-specific sequences were cloned into sites flanking the *dhfr* cassette to allow for homologous integration into the native *Pfpank1* locus of the transfected parasite. The plasmid also contains the *Saccharomyces cerevisiae* cytosine deaminase/phosphoribosyl transferase (*Scfcu*) gene that confers sensitivity to 5-fluorocytosine (5-FC) as a negative selectable marker. Parasites that integrate the *dhfr* gene cassette into *Pfpank1* by homologous recombination and no longer carry the Δ *Pfpank1*-pCC-1 construct as an episome should lose the *Scfcu* gene and therefore be resistant to 5-FC, while parasites that maintain the episomal Δ *Pfpank1*-pCC-1 would be killed due to the toxic metabolite generated in the presence of *Scfcu*. The genomic regions for homologous integration were selected such that if the construct is successfully integrated into the *P. falciparum* genome by double crossover homologous recombination, the *dhfr* gene cassette will replace a 500 base-pair region of the first exon of *Pfpank1*, and hence will disrupt the gene.

The *Pfpank1* sequence used to generate the *Pfpank1*-pGlux-1 and *Pfpank1*-stop-pGlux-1 plasmids was amplified from parasite RNA. The RNeasy Mini Kit (QIAGEN) was used to purify total RNA from saponin-isolated *P. falciparum* parasites (typically 2×10^7 cells) according to the manufacturer's protocol for purifying total RNA from animal cells. An optional 15 min DNase I incubation was included in the procedure to eliminate residual genomic DNA (gDNA). Complementary DNA (cDNA) was synthesised from total RNA using SuperScript II Reverse Transcriptase (ThermoFisher), with Oligo(dT)₁₂₋₁₈ primer, and included an optional incubation with RNaseOUT Recombinant Ribonuclease Inhibitor, as per manufacturer's instructions. When required, parasite gDNA was extracted from saponin-isolated trophozoite-stage parasites using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's instructions. For quantitative polymerase chain reaction (qPCR), parasite gDNA was extracted from frozen (-80°C) parasite infected-erythrocyte pellets using a QIAamp DNA Blood Midi Kit (QIAGEN) following the manufacturer's instructions, and included an additional RNase A digestion (1 mg/mL final concentration). When necessary, an additional phenol-chloroform extraction step was performed after gDNA extraction as follows: equal volumes (250 μL each) of phenol and chloroform were added to each sample and gently vortexed to mix. Samples were centrifuged ($16,300 \times g$, 5 min) to separate the different phases. The aqueous phase was extracted and any residual transition phase removed by another centrifugation ($16,300 \times g$, 5 min). The purified gDNA was then precipitated overnight ($2.5 \times$ volumes of absolute ethanol, 0.3 M sodium acetate, pH 5.2, -20°C), washed once with ice-cold 70% ethanol and resuspended in 100 μL of 10 mM Tris, 0.5 mM ethylenediaminetetraacetic acid (EDTA), pH 9.0.

Pfpank1-specific sequences were subsequently amplified from cDNA (to generate the *Pfpank1*-pGlux-1 and *Pfpank1*-stop-pGlux-1 constructs) or gDNA of wild-type 3D7 strain *P. falciparum* (to generate the Δ *Pfpank1*-pCC-1 construct) using either KOD Hot Start

DNA polymerase (Merck Millipore) or Platinum *Pfx* DNA polymerase (ThermoFisher), with the oligonucleotide primers listed in **Table S4**, following each manufacturer's protocol. To generate the *Pfpank1*-stop-pGlux-1 and *Pfpank1*-pGlux-1 constructs, the In-Fusion cloning (Clontech) method was employed to insert the *Pfpank1*-coding sequence into pGlux-1, with and without the introduction of stop codons prior to the GFP-coding sequence, respectively. Before the cloning step, the pGlux-1 plasmid was linearised by sequential digestions with *XhoI* (ThermoFisher) and then *KpnI* (New England Biolabs), according to each manufacturer's recommendation. The In-Fusion reactions were set up with either the In-Fusion Dry-Down PCR Cloning Kit (for *Pfpank1*-pGlux-1) or the In-Fusion HD EcoDry Cloning Kit (for *Pfpank1*-stop-pGlux-1) essentially as described in the manufacturer's protocol. To generate the Δ *Pfpank1*-pCC-1 construct, the *Pfpank1* flank fragments (amplified from parasite gDNA to contain specific restriction sites; **Table S4**) were digested with their respective restriction enzymes (*SacII* and *SpeI* (both New England Biolabs) for the 5' *Pfpank1* flank, and *EcoRI* (ThermoFisher) and *AvrII* (New England Biolabs) for the 3' *Pfpank1* flank) according to each manufacturer's protocol. Subsequently, the digested 5' *Pfpank1* flank was ligated into a linearised pCC-1 plasmid (following *SacII* and *SpeI* digestion performed as before) using a Quick T4 DNA ligase (New England Biolabs), following manufacturer's protocol, to generate an intermediate plasmid. This intermediate plasmid was then linearised by *EcoRI* and *AvrII* digestion performed as above, and was ligated with the 3' *Pfpank1* flank as described above to generate the final Δ *Pfpank1*-pCC-1 plasmid (**Figure S2 a**).

The purity and concentration of all DNA and RNA samples was determined using a NanoDrop ND-1000 spectrophotometer (Nanodrop Technologies). Sanger sequencing of DNA samples was performed at the Australian Genome Research Facility (Sydney or Brisbane) to confirm the accuracy of DNA sequences.

SYBR Safe-based parasite proliferation assay

The *in vitro* inhibition experiments carried out in this study were performed as described previously [2]. Briefly, synchronous ring-stage parasite-infected erythrocytes were suspended within wells of a 96-well plate, in a final volume of 200 μ L complete medium (0.5% parasitaemia and 1% haematocrit) in the presence of the test compound dissolved in water or DMSO, with the highest concentration of DMSO not exceeding 0.1% v/v. Parasite-infected erythrocytes incubated in 500 nM chloroquine were used as a no-proliferation control, while those incubated in the absence of inhibitory compounds were used as a 100% parasite proliferation control. The parasite suspensions were then incubated at 37 °C for 96 h under a low-oxygen atmosphere (96% nitrogen, 3% carbon dioxide and 1% oxygen). At the end of the incubation, each 96-well plate was frozen at -20 °C (to lyse the cells) and thawed before being processed as follows: 100 μ L was aliquoted from the content of the wells of each test plate into the corresponding wells of a new 96-well plate. Subsequently, 100 μ L of lysis buffer (20 mM Tris, pH 7.5, 5 mM EDTA, 0.008% w/v saponin and 0.08% v/v Triton X-100) containing SYBR Safe DNA gel stain (1 $\times$ final concentration in 200 μ L) was added to each well. The SYBR Safe fluorescence signal in each well was then measured with a FLUOstar OPTIMA plate-reader with 490 nm excitation and 520 nm emission wavelengths, after setting the gain on a 100% parasite proliferation control well.

The *in vitro* pantothenate requirement experiments were performed as described above with the following modifications. All solutions were prepared in complete pantothenate-free medium and cells used for these experiments were washed twice in complete pantothenate-free medium (10 mL, 1,500 $\times$ g for 5 min) to remove any pantothenate. Ring stage-infected erythrocyte suspensions were then placed in the final suspension containing the desired concentration of pantothenate (pantothenate-free complete RPMI 1640 medium supplemented with 2-fold serial dilutions of pantothenate). In these experiments, parasite-infected erythrocytes incubated in the absence of pantothenate served as a no-proliferation control, while those incubated in 1 μ M pantothenate (the

pantothenate concentration normally present in complete medium used for *in vitro* parasite culture) served as a 100% parasite proliferation control.

Each data set was fitted with sigmoidal curves (typically either $y = y_0 + \frac{ax^b}{c^b + x^b}$ or $y = \frac{ax^b}{c^b + x^b}$, where y represents percentage parasite proliferation, y_0 represents minimum percentage parasite proliferation, a represents the range of percentage parasite proliferation, b represents the Hill coefficient, c represents the IC₅₀ or SC₅₀ value and x represents compound concentration) using a nonlinear least squares regression. The IC₅₀ and SC₅₀ values for these experiments were then determined from these sigmoidal curves and averaged from several independent experiments. The fold change, relative to the Parent line, in the sensitivity of each mutant clone to a particular compound was calculated as the ratio between the IC₅₀ value obtained for the mutant clone from a single experiment and the average Parent IC₅₀ value. The same was done for the corresponding *Pf*PanK1-complemented lines. IC₃₅ values (obtained by the same method described above) were used to determine the CJ-15,801 fold change for PanOH-B and CJ-A because $\geq 50\%$ inhibition of growth was not obtained for all of the experiments performed for these clones.

Drug pressuring

Parasite drug-pressuring cultures with PanOH and CJ-15,801 were initiated by exposing synchronous ring-stage Parent line parasites (10 mL culture of 2 or 4% parasitaemia and 2% haematocrit) to either analogue at the IC₅₀ values obtained for the Parent line at the time (PanOH = 400 μ M and CJ-15,801 = 75 μ M). Parasites were then exposed to cycles of increasing drug-pressure that lasts about 2 – 4 weeks each. During each cycle, each culture was maintained continuously in the presence of a fixed concentration of the pressuring analogue, with a medium replacement every 48 h and fresh erythrocytes (100 μ L) added at least once a week. Whenever the parasitaemia for each culture reached approximately 5 – 10%, a parasite proliferation assay was performed with predominantly ring-stage parasites as described above. If the IC₅₀ value obtained for the assay was equal to or greater than the pressuring concentration, aliquots of the culture were cryopreserved, and the concentration of the pantothenate analogue was increased. The cycle was then repeated, each time increasing the compound concentration in a step-wise fashion (multiples of 2 from the initial pressuring concentration) until the pressured parasites became approximately 8 $\times$ less sensitive than the Parent line to the selecting compounds. This process took 11 – 13 weeks. The drug-resistant parasites were subsequently cultured and cloned in the absence of pantothenate analogue pressure for at least 3 months. The clones were then confirmed to have retained resistance to the pantothenate analogue used in the selection process, and were subsequently maintained in a drug-free environment for the remainder of the study.

Confocal microscopy

To prepare coverslip-bound cells, parasite-infected erythrocytes (5 – 10% parasitaemia) were washed once (centrifuged at 500 $\times g$, 5 min) and resuspended at approximately 2% haematocrit in phosphate buffered saline (PBS), before 1 – 2 mL was added to a polyethylenimine (PEI)-coated coverslip placed within a well of a 6-well plate. Plates were incubated with shaking for 15 min at room temperature, after which unbound cells were washed off the coverslips with PBS (2 mL per well, with a 2 min shaking incubation followed by aspiration).

For imaging of fixed cells, unstained coverslip-bound cells were fixed in 1 mL of PBS containing 4% (w/v) paraformaldehyde (Electron Microscopy Services) and 0.0075% (w/v) glutaraldehyde for 30 min at room temperature without shaking. The fixative was then removed and the coverslips washed in PBS three times as described above, before they were rinsed in water and dried. A drop of SLOWFADE (Invitrogen)

containing the nuclear stain 4',6-diamino-2-phenylindole (DAPI) was added to the centre of the coverslip, which was then inverted onto a microscope slide and sealed with nail polish, before being used for imaging.

Live cells were stained with Hoechst 33258 nuclear stain and bound to a coverslip for imaging. Briefly, parasite-infected erythrocytes were prepared in PBS (~4% haematocrit) as described above, pelleted ($2,000 \times g$, 30 s), and resuspended in PBS containing 20 $\mu\text{g/mL}$ Hoechst 33258. Cells were incubated in the dye for 20 min at room temperature with shaking, pelleted and washed three times with PBS ($2,000 \times g$, 30 s), before being resuspended in PBS (~2% haematocrit) and bound to PEI-coated coverslips as described above. Shortly prior to imaging, 5 – 10 μL of PBS was added to the centre of the coverslip, before it was inverted onto a microscope slide and sealed with nail polish.

Attempted disruption of *Pfpank1*

The *Pfpank1* disruption plasmid, $\Delta Pfpank1$ -pCC-1, was transfected into wild-type 3D7 strain *P. falciparum*, and positive transfectants were selected as described previously [3].

WR99210-resistant $\Delta Pfpank1$ -pCC-1-transfectant parasites were subsequently maintained in the absence of WR99210 for three weeks to promote the loss of episomal copies of the construct. WR99210 (10 nM) was re-introduced to predominantly ring-stage $\Delta Pfpank1$ -pCC-1-transfectant cultures. Complete medium and WR99210 were replaced daily after the re-introduction of WR99210, and fresh erythrocytes added when appropriate. Parasites from this culture (1 cycle off and on WR99210) were then either subjected to negative selection or maintained in the absence of WR99210 for a second three-week period to promote further loss of episomal copies of the $\Delta Pfpank1$ -pCC-1 construct. The second WR99210 cycle was carried out exactly as the first.

Negative selection was performed to isolate parasites in which the *hdhfr* selection cassette of the $\Delta Pfpank1$ -pCC-1 construct had been integrated into the genome by double crossover homologous recombination. In these parasites, the *Scfcu* gene that confers sensitivity to 5-FC should have been lost. Negative selection was initiated with parasites that had been through either 1 or 2 rounds of WR99210 cycling. 5-FC (232 nM) was introduced to cultures of predominantly trophozoite-stage parasites, while selection with WR99210 (10 nM) was maintained and the medium was replaced daily. When parasites were no longer observable in Giemsa-stained culture smears, the medium (along with WR99210 and 5-FC) was replaced every second day and fresh erythrocytes (100 μL) were added once a week. Once WR99210- and 5-FC-resistant parasites were observed in culture, the medium (containing WR99210 and 5-FC) was replaced daily, with fresh erythrocytes added as appropriate.

[^{14}C]Pantothenate phosphorylation by parasite lysate

In the [^{14}C]pantothenate phosphorylation time course, each reaction contained a final concentration of 50 mM Tris-HCl (pH 7.4), 5 mM ATP, 5 mM MgCl_2 and 2 μM (0.1 $\mu\text{Ci/mL}$) [^{14}C]pantothenate (American Radiolabeled Chemicals). The phosphorylation reactions were initiated by the addition of parasite lysate (at a concentration corresponding to $\sim 2.0 \times 10^7$ cells/mL) and were maintained at 37 °C throughout the experiment. For each condition, a reaction with identical components to the corresponding test reaction but lacking parasite lysate was prepared and this served as the negative (no phosphorylation) control. Reactions were terminated by transferring 50 μL aliquots (in duplicate) into wells of a 96-well white polystyrene microplate with either 0.45 μm polypropylene filter bottom wells and short drip directors (Whatman), or 0.2 μm hydrophilic PVDF membrane filter bottom wells (Corning), which had been pre-loaded with 50 μL 150 mM barium hydroxide. To determine total radioactivity, 50 μL aliquots of each phosphorylation reaction were transferred, in duplicate, and mixed thoroughly with

150 μ L Microscint-40 by pipetting the mixture at least 50 times in the wells of an OptiPlate-96 microplate (Perkin-Elmer). All of these samples were then processed, measured and analysed as described previously [4].

Activity profiles of the *Pf*PanK enzymes from the different mutant clones and the Parent line were determined across the following ranges of pantothenate concentrations, with the range of [14 C]pantothenate concentrations in brackets: 0.1 – 2 μ M (0.005 – 0.1 μ Ci/mL) for the Parent, 3.125 – 200 μ M (0.1 μ Ci/mL) for PanOH-A and (0.156 – 0.313 μ Ci/mL) PanOH-B, and 6.25 – 800 μ M (0.3 – 0.313 μ Ci/mL) for CJ-A. The pantothenate phosphorylation reactions in these kinetic experiments were set up as described previously [4], with the following minor modifications. Each mL of the reactions contained lysates prepared from $\sim 1.0 \times 10^8$ cells. All of the reactions were designed such that the amount of pantothenate phosphorylated by the lysate increased linearly with time during the experiment (15 – 40 min for Parent, 13 – 90 min for PanOH-A, 0.5 – 4.2 h for PanOH-B and 1 – 28 h for CJ-A). The initial rate of each reaction was determined from the gradient of its individual linear regression. These rates were then plotted as a function of pantothenate concentration, and the Michaelis-Menten equation " $y = \frac{ax}{b+x}$ " was fitted to the data, where y represents V_0 , a represents $V_{\max}$, b represents K_m and x represents substrate concentration. The Michaelis constant (K_m) and maximal velocity ($V_{\max}$) parameters of the *Pf*PanK variants were then determined from each fitted curve. The specificity constant of each *Pf*PanK variant was calculated by dividing its $V_{\max}$ value by the corresponding K_m value. The relative specificity constant was in turn calculated by dividing the specificity constant value obtained for each variant in each independent repeat to the average Parent specificity constant value.

Pantothenate phosphorylation experiments were also performed in the presence of antiplasmodial pantothenate analogues to evaluate what effects the different *Pf*PanK1 mutations have on the inhibitory activities of these analogues. The reactions (each containing lysates equivalent to 5.0×10^7 cells/mL and 0.1 μ Ci/mL of [14 C]pantothenate) were set up essentially as described previously [4] and were incubated at 37 °C for 10 min (PanOH-A), 40 min (Parent), 2 h (PanOH-B) or 5 h (CJ-A), during which the amount of pantothenate phosphorylated increased linearly with time in the absence of analogue. The pantothenate analogues were dissolved in water or DMSO, with the highest concentration of DMSO not exceeding 0.1% v/v. The amount of pantothenate phosphorylated in each reaction was expressed as a percentage of total pantothenate phosphorylation (wells without any test analogue). The data was then plotted as a

function of analogue concentration and a suitable sigmoidal curve (either $y = y_0 + \frac{ax^b}{c^b+x^b}$

or $y = \frac{ax^b}{c^b+x^b}$, where y represents percentage pantothenate phosphorylation, y_0 represents minimum percentage pantothenate phosphorylation, a represents the range of percentage pantothenate phosphorylation, b represents the Hill coefficient, c represents the IC_{50} value and x represents analogue concentration) fitted to determine the IC_{50} of each analogue.

Metabolism of N5-trz-C1-Pan

Cultures of predominantly trophozoite-stage *P. falciparum*-infected erythrocytes (Parent line) were first concentrated to > 95% parasitaemia using magnet-activated cell sorting as described elsewhere [5]. Following a 1 h-recovery at 37 °C in complete medium and an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen, N5-trz-C1-Pan (1 μ M) or an equivalent volume of the solvent control (0.01% v/v DMSO) was added in triplicate or quadruplicate to suspensions of the *P. falciparum*-infected erythrocytes in complete medium. After a 4 h exposure to N5-trz-C1-Pan and/or DMSO at 37 °C (again under an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen), 3.0×10^7 cells from each sample were pelleted by centrifugation at $650 \times g$ for 3 min. The supernatant was discarded and the cells washed in ice-cold culture medium (1 mL, $650 \times g$, 3 min, 4 °C) followed by ice-cold PBS (500 μ L, $650 \times g$, 3 min, 4 °C). To extract

metabolites, the washed cells were resuspended in ice-cold methanol containing internal standards (1 μ M Tris and 1 μ M CHAPS) and incubated on a vortex mixer for 1 h at 4 °C. The samples were clarified by centrifugation (at 17,000 $\times g$, 10 min, 4 °C) before being processed for liquid chromatography-mass spectrometry (LC-MS) analysis.

Metabolite samples were analysed by LC-MS as follows. Samples (10 μ L) were separated over a ZIC-pHILIC column (5 μ m, 4.6 $\times$ 150 mm; Merck) using 20 mM ammonium carbonate and acetonitrile as the mobile phases. The Q-Exactive MS operated at a mass resolution of 35,000 from m/z 85 to 1,050 and was fitted with a heated electrospray source that switched between positive and negative ionisation modes. Pooled technical replicates were analysed using data-dependent tandem mass spectrometry (MS/MS) to confirm the identity of unique features in downstream analysis.

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Primer Name Primer Sequence (5'-3')	Function
<i>Pfpank1</i> -pGlux-1-5'primer TTACATATAA CTCGAG ATGAGAAAGTATAAAAAACG	PCR amplification of <i>Pfpank1</i> flanked by a 16-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>XhoI</i> restriction site (underlined). The transcription start codon is highlighted in blue.
<i>Pfpank1</i> -pGlux-1-3'primer TCTTCTCCTT ACTGGTACC GGCAAGGAAAAACATCCAAGAGC	PCR amplification of <i>Pfpank1</i> flanked by a 20-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>KpnI</i> restriction site (underlined).
5' <i>Pfpank1</i> flank-5'primer GACTCCGCGGTAACCTTTTATTATCATCG	PCR amplification of a <i>SacII</i> / <i>SpeI</i> restriction site-flanked 5' <i>Pfpank1</i> flank that includes the <i>SacII</i> restriction site (underlined).
5' <i>Pfpank1</i> flank-3'primer GATC ACTAG TTTCATCATACAGATATTTCG	PCR amplification of a <i>SacII</i> / <i>SpeI</i> restriction site-flanked 5' <i>Pfpank1</i> flank that includes the <i>SpeI</i> restriction site (underlined).
3' <i>Pfpank1</i> flank-5'primer GATCGAATTCAGCTATTGGGGAGGCACC	PCR amplification of an <i>EcoRI</i> / <i>AvrII</i> restriction site-flanked 3' <i>Pfpank1</i> flank that includes the <i>EcoRI</i> restriction site (underlined).
3' <i>Pfpank1</i> flank-3'primer GACTCCTAGGAAAGATAACTATTTCATGTC	PCR amplification of an <i>EcoRI</i> / <i>AvrII</i> restriction site-flanked 3' <i>Pfpank1</i> flank that includes the <i>AvrII</i> restriction site (underlined).
pCC-1MCS1-5'primer TGGAACTACTAAATATATATCCAATGGCCCTT	Sanger sequencing of pCC-1 clones with the 5' <i>Pfpank1</i> flank inserted into MCS one.
pCC-1MCS1-3'primer CCAATAGATAAAATTGTAG	Sanger sequencing of pCC-1 clones with the 5' <i>Pfpank1</i> flank inserted into MCS one.
pCC-1MCS2-5'primer CCTAATCATGTAATCTTAAATTTTC	Sanger sequencing of pCC-1 clones with the 3' <i>Pfpank1</i> flank inserted into MCS two.
pCC-1MCS2-3'primer GTACTGAGAGTGACCATATGCGG	Sanger sequencing of pCC-1 clones with the 3' <i>Pfpank1</i> flank inserted into MCS two.
5' <i>Pfpank1</i> flank-internal-3'primer1 ACGTACCTCCAATATCTAGCG	Sanger sequencing of pCC-1 clones with the 5' <i>Pfpank1</i> flank inserted into MCS one.
5' <i>Pfpank1</i> flank-internal-3'primer2 ATCCTTCTTCTCGAGGACATTCTG	Sanger sequencing of pCC-1 clones with the 5' <i>Pfpank1</i> flank inserted into MCS one.
3' <i>Pfpank1</i> flank-internal-3'primer1 GAATTAACCCCTTGCTACC	Sequencing of pCC-1 clones with the 3' <i>Pfpank1</i> flank inserted into MCS two.
<i>Pfpank1</i> -internal-5'primer ATAAGTTAACAGGAGGAGG	Sanger sequencing of <i>Pfpank1</i> -pGlux-1 clones.
<i>Pfpank1</i> -internal-3'primer1 TGAATTAAACCTTGGGTACC	Sanger sequencing of <i>Pfpank1</i> -pGlux-1 clones.
<i>Pfpank1</i> -internal-3'primer2 ACTCATGTGTAAGTGATTCC	Sanger sequencing of <i>Pfpank1</i> -pGlux-1 clones.
<i>Pfpank1</i> -stop-pGlux-1-5'primer TTACATATAA CTCGAG ATGAGAAAGTATAAAAAACGAAT	PCR amplification of <i>Pfpank1</i> flanked by a 16-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>XhoI</i> restriction site (underlined). Blue: transcription start.
<i>Pfpank1</i> -stop-pGlux-1-3'primer TCTTCTCCTT ACTGGTACC CTACTAGGCAAGGAAAAACA	PCR amplification of <i>Pfpank1</i> flanked by a 20-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>KpnI</i> restriction site (underlined). Red: transcription stops.
<i>Pfpank1</i> -stop-internal-5'primer CAGGAGGAGGGGACACATA	Sanger sequencing of <i>Pfpank1</i> -stop-pGlux-1 clones.
<i>Pfpank1</i> -stop-internal-3'primer AGGATGGAAAGGAGAGGTATT	Sanger sequencing of <i>Pfpank1</i> -stop-pGlux-1 clones.
<i>Pfpank1</i> -stop-external-3'primer GCATCACCTTCACCCCTCTCC	Sanger sequencing of <i>Pfpank1</i> -stop-pGlux-1 clones.
<i>Pfpank1</i> -PvPanOHA-Wt-5'primer CCAAGTTTATTTCCTAAGCACG	qPCR of Parent vs PanOH-A gDNA. Forward primer binds preferentially to the wild-type <i>Pfpank1</i> sequence at the site responsible for the D507N mutation in PanOH-A.
<i>Pfpank1</i> -PvPanOHA-Mut-5'primer CCAAGTTTATTTCCTAAGCAC A	qPCR of Parent vs PanOH-A gDNA. Forward primer binds preferentially to the mutant <i>Pfpank1</i> sequence at the site responsible for the D507N mutation in PanOH-A (red: discerning nucleotide).
<i>Pfpank1</i> -PvPanOHA-3'primer ATTTGAATGATAAAGTAGGCAAGG	qPCR of Parent vs PanOH-A gDNA. Common reverse primer.
<i>Pfpank1</i> -PvPanOHB-5'primer GACATATCTAAGTTAGATGACACTTGA	qPCR of Parent vs PanOH-B gDNA. Common forward primer.
<i>Pfpank1</i> -PvPanOHB-Wt-3'primer TATTTATGTGCCCTCCTC	qPCR of Parent vs PanOH-B gDNA. Reverse primer binds preferentially to the wild-type <i>Pfpank1</i> sequence at the site responsible for the ΔG95 mutation in PanOH-B.
<i>Pfpank1</i> -PvPanOHB-Mut-3'primer TATTTATGTGCCCTCCT G	qPCR of Parent vs PanOH-B gDNA. Reverse primer binds preferentially to the wild-type <i>Pfpank1</i> sequence at the site responsible for the ΔG95 mutation in PanOH-B (red: discerning nucleotide).
<i>Pfpank1</i> -PvCJA-Wt-5'primer AATAACGTTAACAGGAGGAGG	qPCR of Parent vs CJ-A gDNA. Forward primer binds preferentially to the wild-type <i>Pfpank1</i> sequence at the site responsible for the G95A mutation in CJ-A.
<i>Pfpank1</i> -PvCJA-Mut-5'primer AATAACGTTAACAGGAGGAG C	qPCR of Parent vs CJ-A gDNA. Forward primer binds preferentially to the mutant <i>Pfpank1</i> sequence at the site responsible for the G95A mutation in CJ-A (red: discerning nucleotide).
<i>Pfpank1</i> -PvCJA-3'primer GCCTTTATTTATTCATTCC	qPCR of Parent vs CJ-A gDNA. Common reverse primer.
<i>Pfpank1</i> -KO-determination-5'primer TTACATATAA CTCGAG ATGAGAAAGTATAAAAAACGAATTAAACA	Determination of <i>Pfpank1</i> knockout when paired with <i>Pfpank1</i> -KO-determination-3'primer. A 1.9 kb product is expected when using template from wild-type parasites and a 3.6 kb product is expected with template from <i>Pfpank1</i> knockout parasites. Bold: sequence homologous with gene.
<i>Pfpank1</i> -KO-determination-3'primer TCTTCTCCTT ACTGGTACC CTACTAGGCAAGGAAAAACATCC	Determination of <i>Pfpank1</i> knockout when paired with <i>Pfpank1</i> -KO-determination-5'primer. A 1.9 kb product is expected when using template from wild-type parasites and a 3.6 kb product is expected with template from <i>Pfpank1</i> knockout parasites. Bold: sequence homologous with gene.

S1 Table. List of oligonucleotides used in this study.

Protein	Peptide (start and end position, [+16] oxidation)	Score ^a	m/z	Mass (Da)	Retention time (min)
PfPanK1	296'_IAGTAIGGGTLMGLAK_'311	95.07	715.9051	1429.796	78.2
PfPanK1	6'_NELNISNVLEK_'16	90.7	636.843	1271.672	72
PfPanK2	257'_FMPTNLYYFTK_'267	64.2	712.849	1423.684	89.9
PfPanK2	393'_DLTASFFGNAQHIEENVK_'409	48.1	945.9696	1889.925	83.2
PfHSP70	172'_DAGTIAGLNVN[+16]R_'183	81.2	617.31643	1232.6183	61.1
PfHSP70	172'_DAGTIAGLNVMR_'283	75.8	609.31966	1216.6248	76.4
PfHSP70	325'_DTLIPVEK_'332	91.1	457.763	913.5115	57.4
PfHSP70	611'_EAESVCAPIMSK_'622	114.7	661.30978	1320.605	48.4
PfHSP70	573'_EKLQPAEIETCMK_'585	77.8	788.8898	1575.765	45.12
PfHSP70	141'_ENAEAFGLK_'149	79.15	489.7481	977.4817	49.6
PfHSP70	315'_FEELCIDYFR_'324	161.2	696.31842	1390.6223	93.4
PfHSP70	472'_FHLDGIPPAPR_'482	144.8	610.3331	1218.652	59.5
PfHSP70	472'_FHLDGIPPAPR_'482	89	407.2242	1218.651	58.8
PfHSP70	89'_FTESSVQSDM[+16]K_'99	103.4	610.3331	1218.652	25.2
PfHSP70	89'_FTESSVQSDMK_'99	85.8	629.7854	1257.556	38.4
PfHSP70	100'_HWPFTVK_'106	173.9	457.7479	913.481	53.6
PfHSP70	184'_IINEPTAAAIAYGLHK_'199	109.8	841.4695	1680.924	67.2
PfHSP70	184'_IINEPTAAAIAYGLHK_'199	47.5	561.3134	1680.918	69.1
PfHSP70	88'_KFTESSVQSDMK_'99	234.8	693.8322	1385.65	29.2
PfHSP70	88'_KFTESSVQSDMK_'99	88.1	462.8904	1385.649	29.2
PfHSP70	125'_LFHPEEISSM[+16]VLQK_'138	46.8	837.4352	1672.856	65.4
PfHSP70	125'_LFHPEEISSM[+16]VLQK_'138	59.3	558.6239	1672.85	64.7
PfHSP70	125'_LFHPEEISSMVLQK_'138	190.3	829.4345	1656.855	77.9
PfHSP70	125'_LFHPEEISSMVLQK_'138	136.01	553.2919	1656.854	77.89
PfHSP70	249'_LVNFCVEDFK_'258	113.9	635.81022	1269.6059	80.6
PfHSP70	249'_LVNFCVEDFKR_'259	121.4	713.86108	1425.7076	64.9
PfHSP70	139'_M[+16]KENAEAFGLK_'140	119.4	627.3124	1252.61	34.7
PfHSP70	139'_M[+16]KENAEAFGLK_'140	89.3	418.5446	1252.612	34.7
PfHSP70	139'_MKENAEAFGLK_'140	101.4	619.3164	1236.618	41.5
PfHSP70	139'_MKENAEAFGLK_'140	48.9	413.231	1236.617	41.4
PfHSP70	153'_NAVITVPAYFNDSQR_'167	159.2	847.92906	1693.8436	80.43
PfHSP70	34'_NENVDIANDQGNR_'47	128.2	786.3726	1570.731	43
PfHSP70	73'_NPENTVFDAK_'82	125.7	567.775	1133.536	39.3
PfHSP70	73'_NPENTVFDAKR_'83	74.6	645.8253	1289.636	29.6
PfHSP70	553'_NSLENYCYGVK_'563	104.4	673.80534	1345.5961	49.1
PfHSP70	107'_SGVDEKPM[+16]IEVTYQGEK_'123	184.8	963.4614	1924.908	46.2
PfHSP70	107'_SGVDEKPM[+16]IEVTYQGEK_'123	138.9	642.6434	1924.908	46.2
PfHSP70	107'_SGVDEKPMIEVTYQGEK_'123	152.01	955.464	1908.913	53.9
PfHSP70	107'_SGVDEKPMIEVTYQGEK_'123	141.1	637.3119	1908.9	53.9
PfHSP70	107'_SGVDEKPMIEVTYQGEK_'124	95.2	685.3418	2053.004	36.9
PfHSP70	438'_SQIFTTYADNPQGVLIQVYEGER_'460	57.2	876.7689	2627.285	102.5
PfHSP70	343'_SVHEVVLVGGSTR_'355	79.9	670.3708	1338.727	37.7
PfHSP70	343'_SVHEVVLVGGSTR_'355	79.7	447.2489	1338.725	37.7
PfHSP70	586'_TITTILEWLEK_'596	111.63	673.8821	1345.75	119.4

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<i>Pf</i> HK	337'_FMVNVLQSACSK_'348	112.8	692.34118	1382.6678	72.7
<i>Pf</i> HK	429'_FTCGVDGSLFVK_'440	104.2	665.32943	1328.6443	74
<i>Pf</i> HK	475'_GAAITAAVIALNADIPQLP_'493	21.7	607.0155	1818.0247	136.7
<i>Pf</i> HK	475'_GAAITAAVIALNADIPQLP_'493	81.7	910.02112	1818.0277	136.7
<i>Pf</i> HK	96'_GTYYAIDFGGTNFR_'109	99.7	791.37023	1580.7259	85.8
<i>Pf</i> HK	398'_ICEAVYNR_'405	161.7	512.74789	1023.4812	31.5
<i>Pf</i> HK	288'_IINIEFGNFDK_'298	98.2	655.34292	1308.6713	87.6
<i>Pf</i> HK	221'_LMNDAFVR_'228	96.1	483.24716	964.47977	53.3
<i>Pf</i> HK	81'_M[+16]LDSCIANIPTGQEK_'95	142.2	846.90042	1691.7863	60.1
<i>Pf</i> HK	324'_MISGAYLGEIVR_'335	97.5	654.85283	1307.6911	80.6
<i>Pf</i> HK	81'_MLDSCIANIPTGQEK_'95	150.8	838.90303	1675.7915	66.7
<i>Pf</i> HK	406'_SAALAAGTIAAIK_'419	127.8	614.86649	1227.7184	68.9
<i>Pf</i> HK	454'_VILADKAENLIIPADDGSGK_'474	81.8	718.0673	2151.1801	85.1
<i>Pf</i> HK	162'_YIMGEFNDLNDK_'173	111.9	729.83211	1457.6497	74.2
<i>Pf</i> α-tubulin	44'_ANDDAFNTFFSETGAGK_'60	88.2	896.3956	1790.777	91.5
<i>Pf</i> α-tubulin	65'_CVFVDLEPTVVDEVR_'79	97.8	888.94593	1775.8773	96.8
<i>Pf</i> α-tubulin	327'_DVNAAVATIK_'336	90.3	501.28487	1000.5552	50
<i>Pf</i> α-tubulin	423'_EDLAALEK_'430	80.2	444.73726	887.45996	45.5
<i>Pf</i> α-tubulin	113'_EVIDVCLDR_'121	134.2	559.77937	1117.5442	64.02
<i>Pf</i> α-tubulin	244'_FDGALNVDVTEFQTNLVPYPR_'264	14.1	799.06999	2394.1881	112.9
<i>Pf</i> α-tubulin	395'_FDLMYAK_'401	99.2	444.22022	886.42589	65.9
<i>Pf</i> α-tubulin	265'_JHFMLSSYAPVVSARE_'280	81.7	889.9615	1777.908	78.5
<i>Pf</i> α-tubulin	265'_JHFMLSSYAPVVSARE_'280	89.9	593.6431	1777.908	78.6
<i>Pf</i> α-tubulin	230'_LIAQVISSLTASLR_'243	138.2	736.44601	1470.8775	119
<i>Pf</i> α-tubulin	216'_NLDIERPTYTNLNR_'229	156.2	573.63288	1717.8768	54.5
<i>Pf</i> α-tubulin	85'_QLFHPEQLISGK_'96	182.5	698.88302	1395.7515	64.5

S2 Table. Sequences of peptides used to identify and quantify the five proteins of interest shown in S3 Fig. ^aCertainty of peptide identification.

Parasite line	Parasite proliferation inhibition IC ₅₀ values (μM)		
	PanOH	CJ-15,801	Chloroquine
Parent (3D7 strain)	547 ± 35	106 ± 8	0.009 ± 0.001
PanOH-A	3,595 ± 338*	471 ± 54*	0.009 ± 0.001
PanOH-B	4,600 ± 453*	712 ± 110*	0.008 ± 0.001
CJ-A	7,298 ± 745*	>800	0.008 ± 0.001
Parent ^{+WTP/PanK1}	672 ± 37	127 ± 7	0.010 ± 0.001
PanOH-A ^{+WTP/PanK1}	1946 ± 222	252 ± 37	0.010 ± 0.001
PanOH-B ^{+WTP/PanK1}	2786 ± 170	357 ± 23	0.010 ± 0.001
CJ-A ^{+WTP/PanK1}	3036 ± 203	302 ± 29	0.011 ± 0.001

S3 Table. Inhibition of the proliferation of Parent, PanOH-A, PanOH-B, CJ-A, Parent^{+WTP/PanK1}, PanOH-A^{+WTP/PanK1}, PanOH-B^{+WTP/PanK1} and CJ-A^{+WTP/PanK1} parasites by PanOH, CJ-15,801 and chloroquine, as represented by IC₅₀ values. Errors represent SEM (n ≥ 3). The averaged CJ-15,801 IC₅₀ value for PanOH-B (n = 3) includes a single extrapolated value, because in one experiment the highest concentration tested (800 μM) did not inhibit parasite growth by ≥ 50%. An asterisk indicates that the IC₅₀ value of the mutant line is significantly different from that obtained for the Parent line (PanOH IC₅₀ 95% CI compared to Parent IC₅₀: PanOH-A = 2664 to 3432, PanOH-B = 3553 to 4552 & CJ-A = 6046 to 7456; CJ-15,801 IC₅₀ 95% CI compared to Parent IC₅₀: PanOH-A = 296 to 436, PanOH-B = 495 to 717 & CJ-A = 688 to 823). The chloroquine IC₅₀ values of the different lines are indistinguishable (95% CI compared to Parent IC₅₀: PanOH-A = -2.023 to 1.646, PanOH-B = -2.114 to 0.492 & CJ-A = -2.507 to 0.158).

List of non-synonymous mutations in PanOH-A

Chromosome	Position	Gene Name	Gene Description	Ref Base	Alt Base	Codon Change	Amino Acid Change
Pf3D7_12_v3	283230	PF3D7_1206300	conserved protein, unknown function	C	T	Gaa/Aaa	E2025K
Pf3D7_14_v3	854135	PF3D7_1420600	pantothenate kinase, putative (PANK)	C	T	Gat/Aat	D507N
Pf3D7_08_v3	1080639	PF3D7_0824900	conserved protein, unknown function	C	T	Gat/Aat	D785N
Pf3D7_11_v3	704658	PF3D7_1118500	nucleolar protein 56, putative	G	A	aGa/aAa	R495K

List of non-synonymous mutations in PanOH-B

Chromosome	Position	Gene Name	Gene Description	Ref Base	Alt Base	Codon Change	Amino Acid Change
Pf3D7_12_v3	283209	PF3D7_1206300	conserved protein, unknown function	C	T	Gaa/Aaa	E2032K
Pf3D7_01_v3	318038	PF3D7_0107600	serine/threonine protein kinase, putative	A	C	Aat/Cat	N1141H

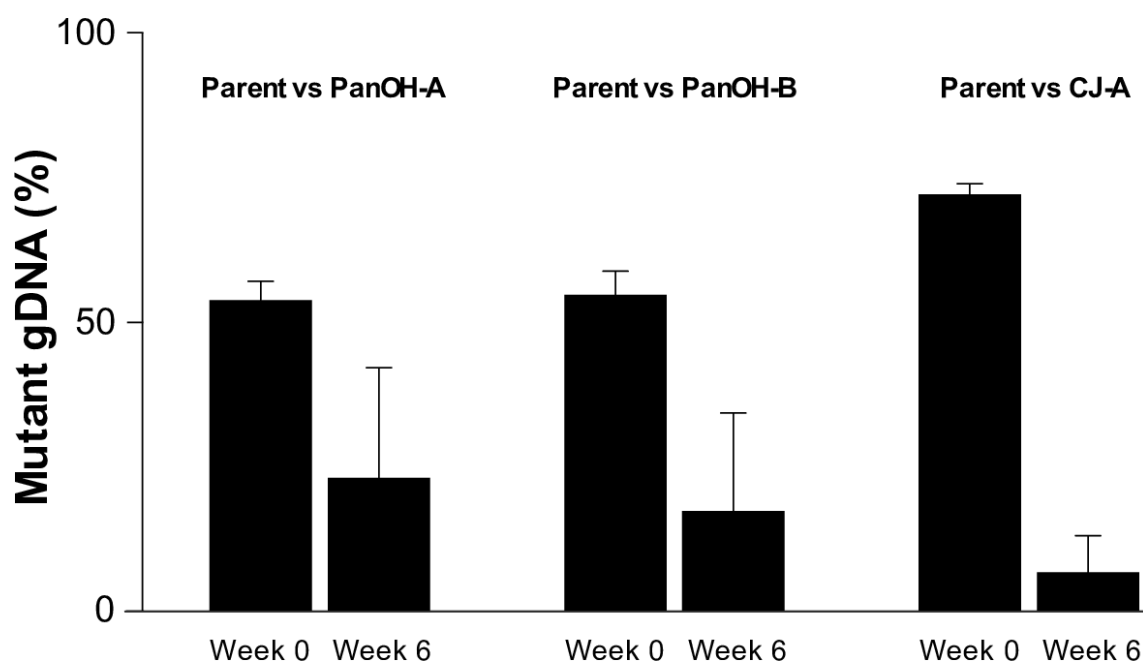
List of non-synonymous mutations in CJ-A

Chromosome	Position	Gene Name	Gene Description	Ref Base	Alt Base	Codon Change	Amino Acid Change
Pf3D7_09_v3	762391	exon		G	T	tGt/tTt	C394F
Pf3D7_14_v3	855680	PF3D7_1420600	pantothenate kinase, putative (PANK)	C	G	gGg/gCg	G95A
Pf3D7_10_v3	1493497	PF3D7_1037600	DNA repair helicase RAD25, putative	A	G	Aaa/Gaa	K594E

S4 Table. List of non-synonymous single nucleotide polymorphisms found in the PanOH-A, PanOH-B and CJ-A parasite lines as determined by whole genome sequencing and variant calling using the PlaTyPus integrated pipeline. Uppercase letters in the “Codon Change” column denotes the base within the codon that has been altered in the coding sequence before/after the mutation. The deletion in PanOH-B at position 95 is not included here because PlaTyPus is unable to detect insertions-deletions polymorphisms.

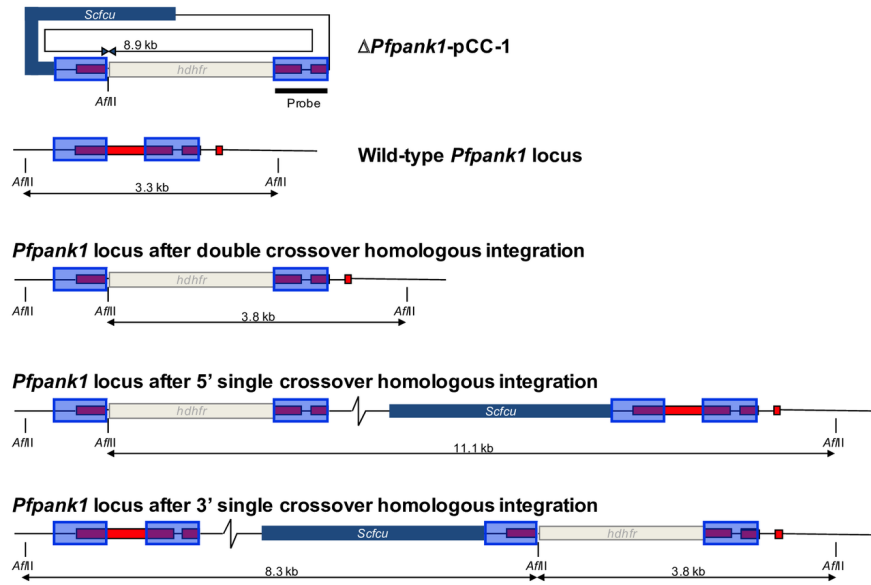
Parasite line	Parasite proliferation inhibition IC ₅₀ values (μM)		Pantothenate phosphorylation inhibition IC ₅₀ values (μM)			
	N5-trz-C1-Pan	N-PE-αMe-PanAm	PanOH	CJ-15,801	N5-trz-C1-Pan	N-PE-αMe-PanAm
Parent	0.095 ± 0.007	0.060 ± 0.007	9.6 ± 0.7	37 ± 7	1.7 ± 0.1	0.70 ± 0.13
PanOH-A	0.032 ± 0.001*	0.026 ± 0.004*	67 ± 4*	196 ± 53*	11 ± 1*	1.4 ± 0.2
PanOH-B	0.179 ± 0.018*	0.040 ± 0.004	176 ± 35*	512 ± 52*	22 ± 4*	7 ± 1*
CJ-A	0.864 ± 0.028*	0.110 ± 0.004*	4925 ± 138*	>1600	>100	81 ± 6*

S5 Table. Inhibition of the proliferation of Parent, PanOH-A, PanOH-B and CJ-A parasites by N5-trz-C1-Pan and N-PE-αMe-PanAm, and the inhibition of [¹⁴C]pantothenate phosphorylation in parasite lysates (generated from the same lines) by the pantothenate analogues PanOH, CJ-15,801, N5-trz-C1-Pan and N-PE-αMe-PanAm, as represented by IC₅₀ values. Errors represent SEM (n ≥ 3). An asterisk indicates that the value is significantly different from that obtained for the Parent line (95% CI of N5-trz-C1-Pan proliferation inhibition IC₅₀ compared to Parent: PanOH-A = -0.088 to -0.038, PanOH-B = 0.047 to 0.121 & CJ-A = 0.715 to 0.824; 95% CI of PE-αMe-PanAm proliferation inhibition IC₅₀ compared to Parent: PanOH-A = -0.060 to -0.008 & CJ-A = 0.024 to 0.076; 95% CI of PanOH phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 46 to 69, PanOH-B = 71 to 263 & CJ-A = 4531 to 5300; 95% CI of CJ-15,801 phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 10 to 309 & PanOH-B = 330 to 621; 95% CI of N5-trz-C1-Pan phosphorylation inhibition IC₅₀ compared to Parent: PanOH-A = 6.5 to 13 & PanOH-B = 9.8 to 30; 95% CI of N-PE-αMe-PanAm phosphorylation inhibition IC₅₀ compared to Parent: PanOH-B = 3.6 to 9.0 & CJ-A = 75 to 97).

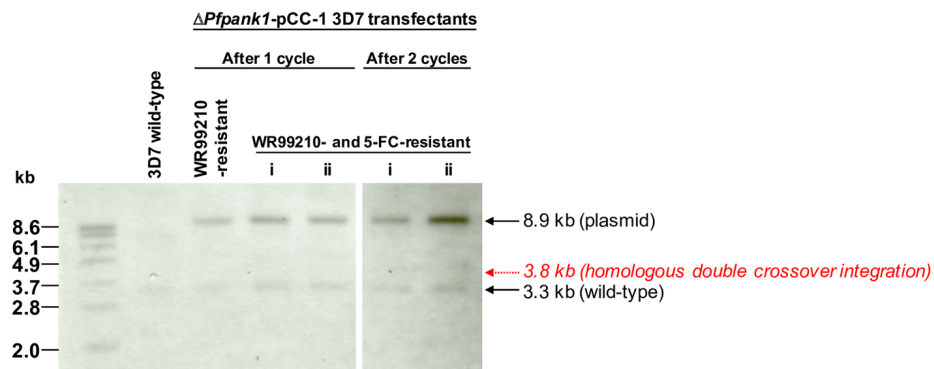


S1 Fig. Quantitative PCR analysis of gDNA samples extracted from the three competition assay cultures generated in the study, Parent vs PanOH-A, Parent vs PanOH-B and Parent vs CJ-A (Fig 5). Data are shown as the proportions of mutant gDNA (out of a 100% total) at week 0 and week 6. Values are averaged from two independent competition assays, each analysed by qPCR in duplicate. Error bars represent range/2.

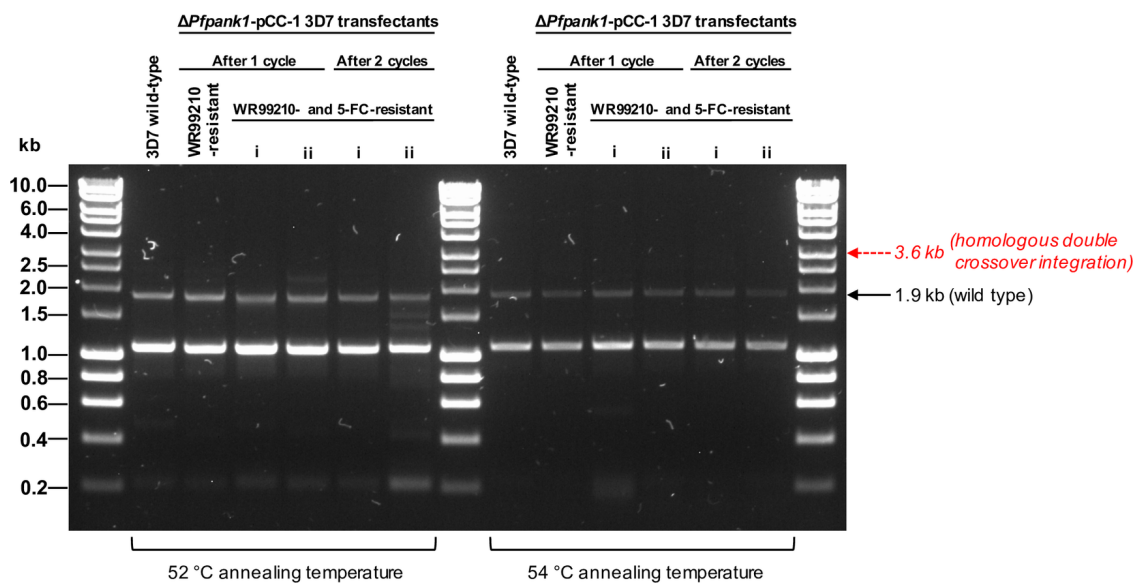
a



b

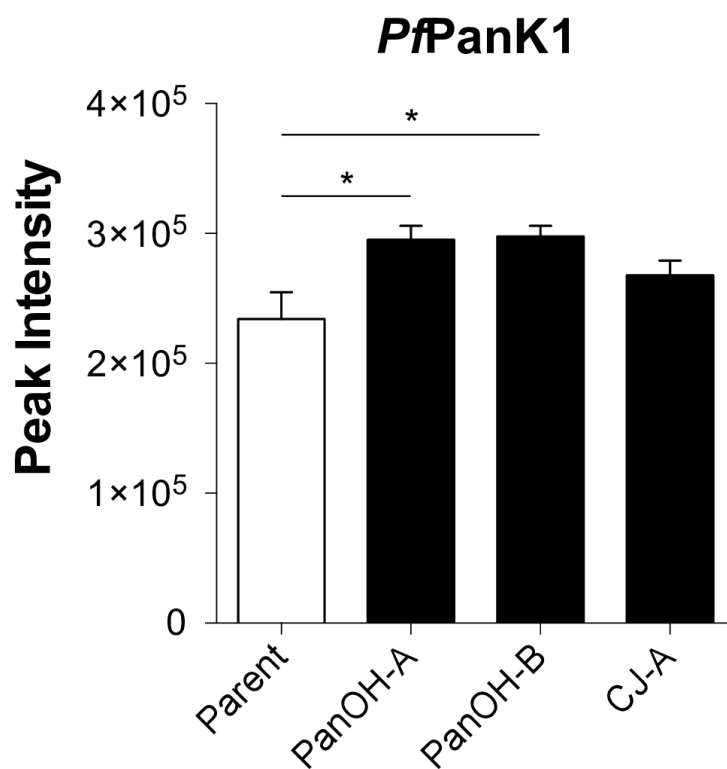


c

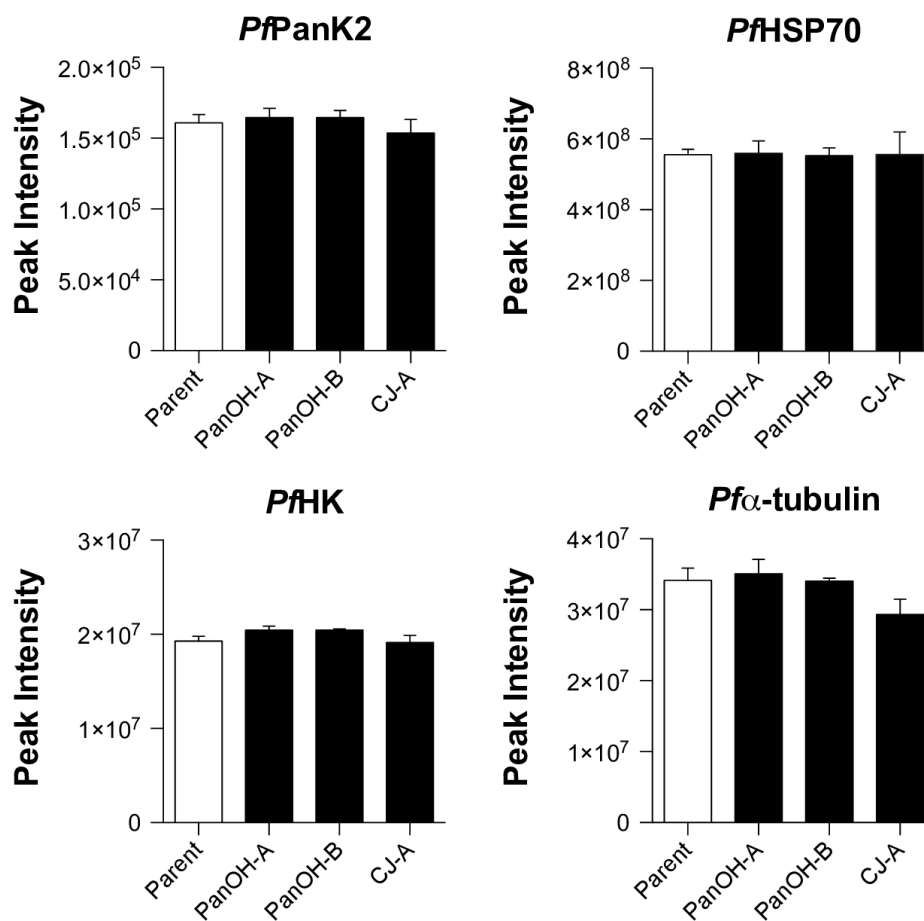


S2 Fig. The attempt at knocking-out *Pfpank1* in wild-type 3D7 parasites through homologous integration. (a) Schematic representations of the $\Delta Pfpank1$ -pCC-1 construct and the wild-type *Pfpank1* gene locus before and after either a homologous double crossover integration of the *hdhfr* cassette of $\Delta Pfpank1$ -pCC-1 or a 5'/3' single crossover homologous integration of the $\Delta Pfpank1$ -pCC-1 construct. The positions of *Afl*III restriction sites are indicated. The 5' *Pfpank1* and 3' *Pfpank1* homologous flanks are indicated by translucent blue boxes. (b) Southern blot of *Afl*III-digested gDNA extracted from wild-type parasites and from $\Delta Pfpank1$ -pCC-1-transfectants resistant to WR99210 or both WR99210 and 5-fluorocytosine (5-FC) after one or two rounds of WR99210 cycling. (i) and (ii) represent independently-selected drug-resistant cultures. The blot was probed with the 3' *Pfpank1* flank (as indicated by the black bar in (a)). The probe hybridised to fragments that correspond to the 3D7 wild-type (3.3 kb) and the plasmid (8.9 kb). The fragment that is consistent with a homologous double crossover-disrupted locus (3.8 kb) was not detected in either independent culture. *hdhfr*: human dihydrofolate reductase—conveys resistance to WR99210. *Scfcu*: *Saccharomyces cerevisiae* cytosine deaminase/phosphoribosyl transferase—conveys sensitivity to 5-FC. (c) PCR confirmation that a *Pfpank1* knockout event cannot be detected, even in a small sub-population of parasites. Samples from the same parasite populations shown in (b) were used to generate DNA templates that were then used with primers selected to amplify a product of 1.9 kb from the wild-type sequence and a 3.6 kb product from parasites with the *Pfpank1* gene knocked out (and replaced with the *hdhfr* gene). A PCR product consistent with the size expected for amplification from the wild-type sequence was observed (black arrow). The identity of this product was confirmed by sequencing. All the reactions generated an approximately 1.1 kb PCR product and some reactions, under certain conditions, produced fainter PCR products likely due to non-specific primer binding. Importantly, no product of a size consistent with that expected for a *Pfpank1* knockout (3.6 kb) was observed in any of the samples. These data, therefore, are consistent with those presented in the Southern blot (b).

a

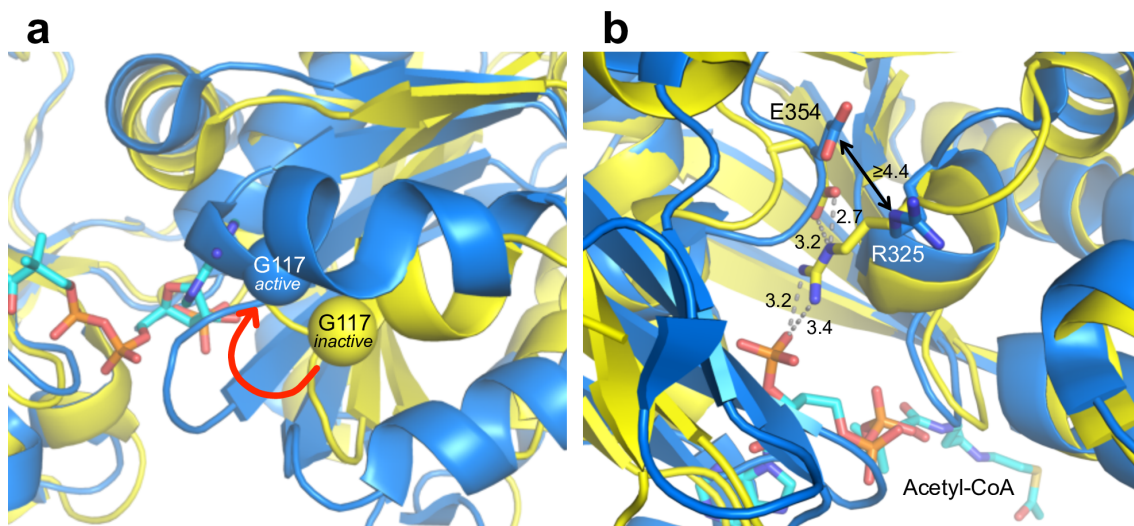


b



S3 Fig. Abundance of (a) PfPanK1 and (b) various control proteins (PfPanK2, PfHSP70, PfHK and Pfα-tubulin) in Parent, PanOH-A, PanOH-B and CJ-A line trophozoites. Protein levels are determined by LC-MS/MS followed by DIA analysis. Values are averaged from ≥ 3 independent parasite preparations and error bars represent SEM. An asterisk indicates that the protein peak intensity measured for a mutant line is significantly different from that obtained for the Parent line (95% CI of *PfPanK1* protein level compared to Parent: PanOH-A = 3.87×10^3 to 1.18×10^5 & PanOH-B = 8.86×10^3 to 1.18×10^5). The protein abundance of *PfPanK1* in CJ-A (95% CI compared to Parent = -3.38×10^4 to 1.01×10^5) and those of the housekeeping proteins in all three mutant lines are indistinguishable from the Parent line levels (95% CI for *PfPanK2* level compared to Parent: PanOH-A = -1.81×10^4 to 2.53×10^4 , PanOH-B = -1.52×10^4 to 2.25×10^4 & CJ-A = -3.43×10^4 to 1.99×10^4 ; 95% CI for *PfHSP70* level compared to Parent: PanOH-A = -8.98×10^7 to 9.72×10^7 , PanOH-B = -6.92×10^7 to 6.27×10^7 & CJ-A = -1.45×10^8 to 1.45×10^8 ; 95% CI for *PfHK* level compared to Parent: PanOH-A = -4.41×10^5 to 2.79×10^6 , PanOH-B = -1.18×10^5 to 2.47×10^6 & CJ-A = -2.42×10^6 to 2.13×10^6 ; 95% CI for *Pfα-tubulin* level compared to Parent: PanOH-A = -5.57×10^6 to 7.45×10^6 , PanOH-B = -4.47×10^6 to 4.25×10^6 & CJ-A = -1.18×10^7 to 2.22×10^6).

S4 Fig. Extracted ion chromatograms (i) and mass spectra (ii) of N5-trz-C1-Pan (a) and downstream metabolites (b–d). Extracted ion chromatograms (i) show the relative intensity of each LC peak. Red lines represent metabolites extracted from N5-trz-C1-Pan-treated parasites and blue lines represent those extracted from DMSO-treated parasite control samples, each carried out in quadruplicate. High resolution mass spectra (ii) of each compound ionised in negative mode: (a) N5-trz-C1-Pan ($C_{14}H_{26}N_4O_3$). Theoretical m/z = 297.1932. Observed m/z = 297.1942. Δppm = 3.36. (b) Phosphorylated N5-trz-C1-Pan ($C_{14}H_{27}N_4O_6P$). Theoretical m/z = 377.1595. Observed m/z = 377.1594. Δppm = -0.27. (c) Dephospho-CoA N5-trz-C1-Pan analogue ($C_{24}H_{39}N_9O_{12}P_2$). Theoretical m/z = 352.6024. Observed m/z = 352.6024. Δppm = 0.0. (d) CoA N5-trz-C1-Pan analogue ($C_{24}H_{40}N_9O_{15}P_3$). Theoretical m/z = 786.1784. Observed m/z = 786.1780. Δppm = -0.51. Data shown are from a single experiment representative of two independent experiments.



S5 Fig. The human Pank3 protein structure in its inactive conformation (yellow; PDB ID: 3MK6) overlaid onto its active conformation (blue; PDB ID: 5KPR). The amino acid residues of the human protein at positions 117 (indicated by the spheres in a) and 354 (side chains shown in b) correspond to the mutated residues of *PfPank1* reported in this study (at positions 95 and 507, respectively). (a) The red arrow indicates the change in the conformation of the $\alpha 2$ -helix between the inactive and active states. The altered configuration in the active state transitions the mutated glycine that corresponds to position 117 away from the end cap of the $\alpha 2$ -helix. (b) Dashed grey lines (inactive conformation) and the solid arrow (active conformation) represent distances between residue side chains and/or acetyl-CoA (in Å) in Pank3. A relay of interactions between Glu354, Arg325 and the 3'-phosphate of acetyl-CoA may stabilise the inactive state of the enzyme. However, in the protein's active conformation, Glu354 and Arg325 are not within bonding distance (≥ 4.4 Å).

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Chapter 4: *Plasmodium falciparum* pantothenate kinase activity is mediated by a unique *PfPanK1*, *PfPanK2* and *Pf14-3-3I* complex.

The work in this chapter is presented in the form of a manuscript to be submitted for publication.

Manuscript prepared as:

Tjhin ET, Spry C, van Dooren GG and Saliba KJ. *Plasmodium falciparum* pantothenate kinase activity is mediated by a unique *PfPanK1*, *PfPanK2* and *Pf14-3-3I* complex.

Contributions

The work presented in this chapter is my own original work, except for the contributions detailed below.

Christina Spry generated the *Pfpank2*-pGlux-1 plasmid, performed the confocal microscopy of the *PfPanK2*-GFP expressing cell line and provided intellectual input into the design of the study.

Giel G. van Dooren provided intellectual input into the design of the study.

Kevin J. Saliba provided intellectual input into the design of the study, data analyses and editing of the manuscript.

4.1. Introduction

The malaria parasite *P. falciparum* takes up pantothenate from its host (Saliba *et al.*, 1998b; Saliba and Kirk, 2001) and converts it into CoA (Spry and Saliba, 2009). This process has been shown to be essential for the parasite's survival, at least in the asexual intraerythrocytic stage of its lifecycle (Divo *et al.*, 1985; Saliba *et al.*, 2005; Spry *et al.*, 2010). The CoA biosynthetic pathway is catalysed, in turn, by five enzymes (Spry *et al.*, 2008), several of which have been shown to be coded by multiple putative genes in the parasite's genome (Genschel, 2004; Pinney *et al.*, 2005). All of these enzymes have also been found to be expressed during the intraerythrocytic stage of the parasite's lifecycle (Bozdech *et al.*, 2003). *PfPank* is the first enzyme in the CoA biosynthetic pathway and it catalyses the phosphorylation of pantothenate into 4'-phosphopantothenate within the parasite (Saliba *et al.*, 1998b), the first step in committing the molecule towards CoA biosynthesis. Two putative genes have been identified in the parasite's genome as possible Pank enzymes, namely PF3D7_1420600 (*Pfpank1*) and PF3D7_1437400 (*Pfpank2*). Our recent investigation (Tjhin *et al.*, 2018) has generated data that are consistent with *PfPank1* being the active Pank in the asexual stage of the parasite. However, the function of *PfPank2* during this stage of the parasite's lifecycle, if any, remains unclear.

PanKs are categorised into three distinct types based on their primary sequences and enzyme kinetics, and all eukaryotic PanKs that have been characterised so far belong to a group of type II PanKs (Yang *et al.*, 2006a). Based on their protein folding topology, eukaryotic PanKs are classified as part of the ASHKA (acetate and sugar kinase/hsp70/actin) superfamily, members of which possess three distinct conserved motifs (ADENOSINE, PHOSPHATE 1 and PHOSPHATE 2) (Cheek *et al.*, 2005). A structure-based sequence alignment of *P. falciparum* PanKs with other type II PanKs by Spry *et al.* (2010) predicted that *PfPank1* and, to a lesser extent, *PfPank2* also belong to this group of type II PanKs. *PfPank1* was found to contain all of the conserved ASHKA superfamily nucleotide-binding motifs, insertion regions, and the Glu and Arg residues that are essential for Pank function (Spry *et al.*, 2010). Based on this structural alignment, *PfPank1* was predicted to be a functional Pank. Although *PfPank2* was shown to also contain the aforementioned catalytically-important residues, two of its nucleotide-binding

motifs do not align perfectly with those of other eukaryotic PanKs, and its second PanK insert is approximately twice the size of those found in other type II PanKs (Spry *et al.*, 2010). Furthermore, *PfPanK2* also contains a unique large insert in a loop associated with dimerisation of PanKs in their native conformation (Hong *et al.*, 2007; Spry *et al.*, 2010). Due to these reasons, it was thought that *PfPanK2* is unlikely to be active.

To date, all PanKs with a solved protein structure have been shown to exist as homodimers (Yun *et al.*, 2000; Das *et al.*, 2006; Yang *et al.*, 2006a; Hong *et al.*, 2006; Nicely *et al.*, 2007; Hong *et al.*, 2007; Li *et al.*, 2013; Franklin *et al.*, 2015). Although both *PfPanK1* and *PfPanK2* are expected to also exist as dimers, their native conformations remain unknown as their structures are yet to be solved. Attempts to express *PfPanK1* heterologously in various expression systems, including *E. coli*, yeast and insect cells, have yielded either insoluble (Mehlin *et al.*, 2006) or soluble but inactive proteins (Saliba Lab, unpublished data).

In this study, a co-immunoprecipitation approach was used to isolate both *PfPanK1* and *PfPanK2* from parasite lysate. This allowed the characterisation of their native conformations and potential interacting partners. The results from western blots, mass spectrometry and enzyme activity analyses are consistent with both *PfPanK1* and *PfPanK2* being part of the same protein complex, making *PfPanK* a unique heterodimeric enzyme, unlike all other PanKs that have been characterised to date.

4.2. Materials and Methods

4.2.1. Parasite culture

P. falciparum parasites were maintained in RPMI 1640 media supplemented with 11 mM glucose (to a final concentration of 22 mM), 200 μ M hypoxanthine, 24 μ g/mL gentamicin and 6 g/L Albumax II (referred to as complete medium) as described previously (Allen and Kirk, 2010).

4.2.2. Plasmid preparation and parasite transfection

The *Pfpank2*-pGlux-1 plasmid was generated and transfected into wild-type 3D7 strain *P. falciparum* to generate the PanK2-GFP line. The same construct was also transfected into the Parent line and each of the mutant clones described previously by Tjhin *et al.* (2018). The strategy used to generate the *Pfpank2*-pGlux-1 plasmid is detailed in the **SI** and the primers used are listed in **Table 4.S1**. The construct was transfected into ring-stage parasites and successful transformants were subsequently selected and maintained using WR99210 (10 nM) as described previously (Tjhin *et al.*, 2013).

4.2.3. Confocal microscopy

Fixed *PfPanK2*-GFP-expressing 3D7 strain *P. falciparum* parasites within infected erythrocytes were observed and imaged with either a Leica TCS-SP2-UV confocal microscope (Leica Microsystems) using a 63 $\times$ water immersion lens or a Leica TCS-SP5-UV confocal microscope (Leica Microsystems) using a 63 $\times$ oil immersion lens as described in the **SI**.

4.2.4. Protein gel electrophoresis and western blot

Parasite samples were run through either denaturing or blue native gels when the presence and abundance of a single protein of interest, or protein(s) of interest in their native conformation was to be detected, respectively. Briefly, mature trophozoite-stage parasites were isolated from infected erythrocytes by saponin lysis, as described previously (Saliba *et al.*, 1998b). Saponin-isolated parasites were then pelleted and lysed in the appropriate buffers before the supernatant generated was used for gel electrophoresis in precast NuPAGE or NativePAGE gels (ThermoFisher) according to the manufacturer's instructions

(detailed in **SI**). Gel contents were transferred to the appropriate membranes (nitrocellulose or polyvinylidene fluoride (PVDF)) and blocked (detailed in **SI**) before immunoblotting.

Blocked membranes were exposed (1.5 – 2 h) to specific primary and secondary antibodies to allow for the detection of the protein of interest. All antibodies were diluted in 4% skim milk in PBS (referred to herein as blotto). The primary antibodies used in this study were an anti-green fluorescent protein (GFP) mouse monoclonal antibody (0.4 $\mu\text{g/mL}$ final concentration; Roche) and a pan-specific anti-14-3-3 rabbit polyclonal antibody (0.25 $\mu\text{g/mL}$ final concentration; Abcam). The secondary antibodies used were an anti-mouse goat horseradish peroxidase (HRP)-conjugated antibody or an anti-rabbit goat HRP-conjugated antibody (both 0.08 $\mu\text{g/mL}$ final concentration; Santa Cruz Biotechnology). After each incubation, membranes were washed at least three times (10 min each) in fresh 0.1% v/v Tween20 in PBS (PBST). To visualise the protein band(s), membranes were incubated in Pierce ECL Plus Substrate (ThermoFisher) according to the manufacturer's instructions and subsequently exposed to X-ray films.

4.2.5. Fluorescence-activated cell sorting

Saponin-isolated mature trophozoites from 3D7 wild-type, Parent⁺*PfPank2-GFP*, PanOH-A⁺*PfPank2-GFP*, PanOH-B⁺*PfPank2-GFP* and CJ-A⁺*PfPank2-GFP* cultures were subjected to fluorescence-activated cell sorting (FACS) analysis to determine the proportion of GFP-positive cells in each cell line. Aliquots of each isolated parasite suspension were diluted in a saline solution (125 mM NaCl, 5 mM KCl, 25 mM HEPES, 20 mM glucose and 1 mM MgCl_2 , pH 7.1) to a concentration of $\sim 10^6 - 10^7$ cells/mL in 1.2 mL Costar polypropylene cluster tubes (Corning) and sampled for FACS analysis (in measurements of 100,000 cells, low sampling speed) with the following settings: forward scatter = 450 V (log scale), side scatter = 350 V (log scale) and Alexa Fluor 488 = 600 V (log scale). The 3D7 wild-type result from the first analysis was used to establish a gating strategy that defined a threshold below which parasites were deemed to be auto-fluorescent. This strategy was then applied in all subsequent analyses to determine the proportion of cells in each cell line that was GFP-positive (i.e. above the defined threshold).

4.2.6. Co-immunoprecipitation

In order to isolate GFP-tagged proteins from parasite lysates, co-immunoprecipitation was performed using GFP-trap (high affinity anti-GFP alpaca nanobody bound to agarose beads; Chromotek). Parasite lysate was prepared from saponin-isolated trophozoites as described previously (Saliba *et al.*, 1998b). In experiments where the amount of co-immunoprecipitated proteins was to be standardised across cell lines and biological repeats, the number of GFP-positive cells required for lysate preparation was calculated by a combination of haemocytometer count and FACS. Co-immunoprecipitation was then performed essentially as recommended in the manufacturer's protocol (detailed in **SI**). All co-immunoprecipitation fractions from Parent^{+PfPank2-GFP}, PanOH-A^{+PfPank2-GFP}, PanOH-B^{+PfPank2-GFP} and CJ-A^{+PfPank2-GFP} cell lines contained proteins bound from 5×10^7 GFP-positive cells (divided into two equal aliquots, one used in the [¹⁴C]pantothenate phosphorylation assay and the other for denaturing western blot).

When an aliquot of the co-immunoprecipitation fraction (beads that have bound proteins from $\sim 10^6 - 10^7$ GFP-positive cells) was required for western blot, the bead suspension was centrifuged ($2,500 \times g$, 2 min), the supernatant removed, and the beads resuspended in 50 μ L sample buffer containing 2 $\times$ NuPAGE lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher) and 2 $\times$ NuPAGE sample reducing agent (ThermoFisher). In some experiments, 10 μ L aliquots of the total and unbound lysate fractions were each mixed with 10 μ L of the same sample buffer. These samples were then boiled (95 °C, 10 min) and 10 μ L of each was then used in a denaturing gel western blot as described above.

4.2.7. [¹⁴C]Pantothenate phosphorylation by co-immunoprecipitated fraction

In order to determine the Pank activity of the protein(s) isolated in the co-immunoprecipitation assays, the bound fractions were used to perform a [¹⁴C]pantothenate phosphorylation time course. The bead suspension containing the co-immunoprecipitated proteins was centrifuged ($2,500 \times g$, 2 min), the supernatant removed, and the beads resuspended in 500 μ L of buffer containing 100 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 7.4), 10 mM adenosine

triphosphate (ATP) and 10 mM MgCl₂ (i.e. twice the concentration required for the phosphorylation reaction) in 1.5 mL microcentrifuge tubes. Each time course was then initiated by the addition of 500 μ L of 4 μ M (0.2 μ Ci/mL) [¹⁴C]pantothenate in water, which was warmed to 37 °C, to the bead suspension (1 mL final reaction). The beads within the reactions were resuspended by pipetting every 10 – 15 min throughout the time course. Aliquots of each reaction (50 μ L each) was terminated at pre-determined time points by mixing with 50 μ L 150 mM barium hydroxide preloaded within the wells of a 96-well, 0.2 μ m hydrophilic PVDF membrane filter bottom plate (Corning). All phosphorylated compounds in each well were then precipitated by the addition of 50 μ L 150 mM zinc sulfate to generate the Somogyi reagent (Somogyi, 1945), the wells processed, and the radioactivity in the plate determined as detailed previously (Spry *et al.*, 2014). Total radioactivity in each phosphorylation reaction was determined by mixing 50 μ L aliquots of each reaction (in duplicate) thoroughly with 150 μ L Microscint-40 (PerkinElmer), pipetting the mixture at least 50 times, in the wells of an OptiPlate-96 microplate (PerkinElmer) (Spry *et al.*, 2014).

4.2.8. Mass spectrometry of co-immunoprecipitated fractions

The identities of the proteins that had been co-immunoprecipitated from parasite lysates prepared from PanK1-GFP, PanK2-GFP and Free-GFP cultures were determined by mass spectrometry. Aliquots of bead-bound co-immunoprecipitated fractions were resuspended in 2 $\times$ NuPAGE LDS sample buffer and 2 $\times$ NuPAGE sample reducing agent and sent (at ambient temperature, within 24 h) to the Australian Proteomics Analysis Facility (Sydney) for mass spectrometry processing (detailed in **SI**).

4.2.9. Prediction of 14-3-3 binding site(s)

The amino acid sequences of *Pf*PanK1, *Pf*PanK2 and *P. falciparum* histone H3 (*Pf*H3) were scanned for any potential 14-3-3 binding motifs using three online prediction software programs. Eukaryotic Linear Motif (ELM) is available at <http://elm.eu.org/>. The sequences were analysed at Scansite 3.0 (available at <http://scansite3.mit.edu/#proteinScan;method=input>) using the mammalian motif class and low stringency (the level of stringency that allowed the detection of the

validated 14-3-3 binding sites in *PfH3* (Dastidar *et al.*, 2013)). 14-3-3-Pred is available at <http://www.compbio.dundee.ac.uk/1433pred/>.

4.2.10. Prediction of nuclear localisation signal in *PfPank2*

The amino acid sequences of *PfPank1* and *PfPank2* were analysed for any potential nuclear localisation signals (NLSs) using several online prediction tools. cNLS Mapper predicts any importin α -dependent NLSs (Kosugi *et al.*, 2009) and is available at http://nls-mapper.iab.keio.ac.jp/cgi-bin/NLS_Mapper_form.cgi. SeqNLS (Lin *et al.*, 2012) is available at <http://mleg.cse.sc.edu/seqNLS/>. PSORTII is available at <https://psort.hgc.jp/form2.html>.

4.3. Results

4.3.1. *Pf*PanK1-GFP and *Pf*PanK2-GFP are part of similarly-sized complexes with PanK activity

Two cell lines, namely PanK1-GFP (first generated in a previous study (Tjhin *et al.*, 2018)) and PanK2-GFP, were used to investigate the interacting partners of the parasite's putative pantothenate kinases. These cell lines have each been transformed with an episomal construct (pGlux-1 plasmid backbone) that contains the wild-type sequence of *Pfpank1* or *Pfpank2*, upstream of a GFP-coding sequence. From **Figure 4.1 a**, as observed for *Pf*PanK1-GFP (Tjhin *et al.*, 2018), it can be seen that the GFP-fluorescence in the trophozoite-stage PanK2-GFP cell line is mainly localised throughout the cytosol and is not excluded from the nucleus of the parasite. As seen from the anti-GFP western blots of PanK1-GFP and PanK2-GFP denaturing protein gel in **Figure 4.1 b**, each cell line was found to express a GFP-tagged protein with molecular weights that are consistent with *Pf*PanK1-GFP (~87 kDa) and *Pf*PanK2-GFP (~118 kDa), respectively. For reference, the molecular weight of GFP is ~27 kDa. Interestingly, under non-denaturing native conditions, both *Pf*PanK1-GFP and *Pf*PanK2-GFP were found to be part of a complex of a similar molecular weight, as indicated by the common bands that are positioned just above the 242 kDa protein standard (**Figure 4.1 c**). In addition, a smaller complex that is > 146 kDa in size was also found in the PanK1-GFP cell line (**Figure 4.1 c**).

In order to further examine the complexes that were detected in the native western blots of PanK1-GFP and PanK2-GFP parasites, co-immunoprecipitation was performed to isolate any *Pf*PanK1-GFP or *Pf*PanK2-GFP-associated proteins from parasite lysates generated from these cell lines. A third cell line, termed Free-GFP (kindly provided by Assoc. Prof. Alex Maier), which expresses unbound GFP protein, was included as a control. A denaturing western blot was then performed with the different fractions generated from the co-immunoprecipitation. As shown in **Figure 4.2 a**, the reduction in the intensity of protein bands in the unbound fractions as compared to the total lysate fractions (the amount loaded for both fractions was based on the same number of cells) indicate that the co-immunoprecipitation has purified most of the GFP-tagged proteins in the PanK1-GFP and PanK2-GFP cell lines.

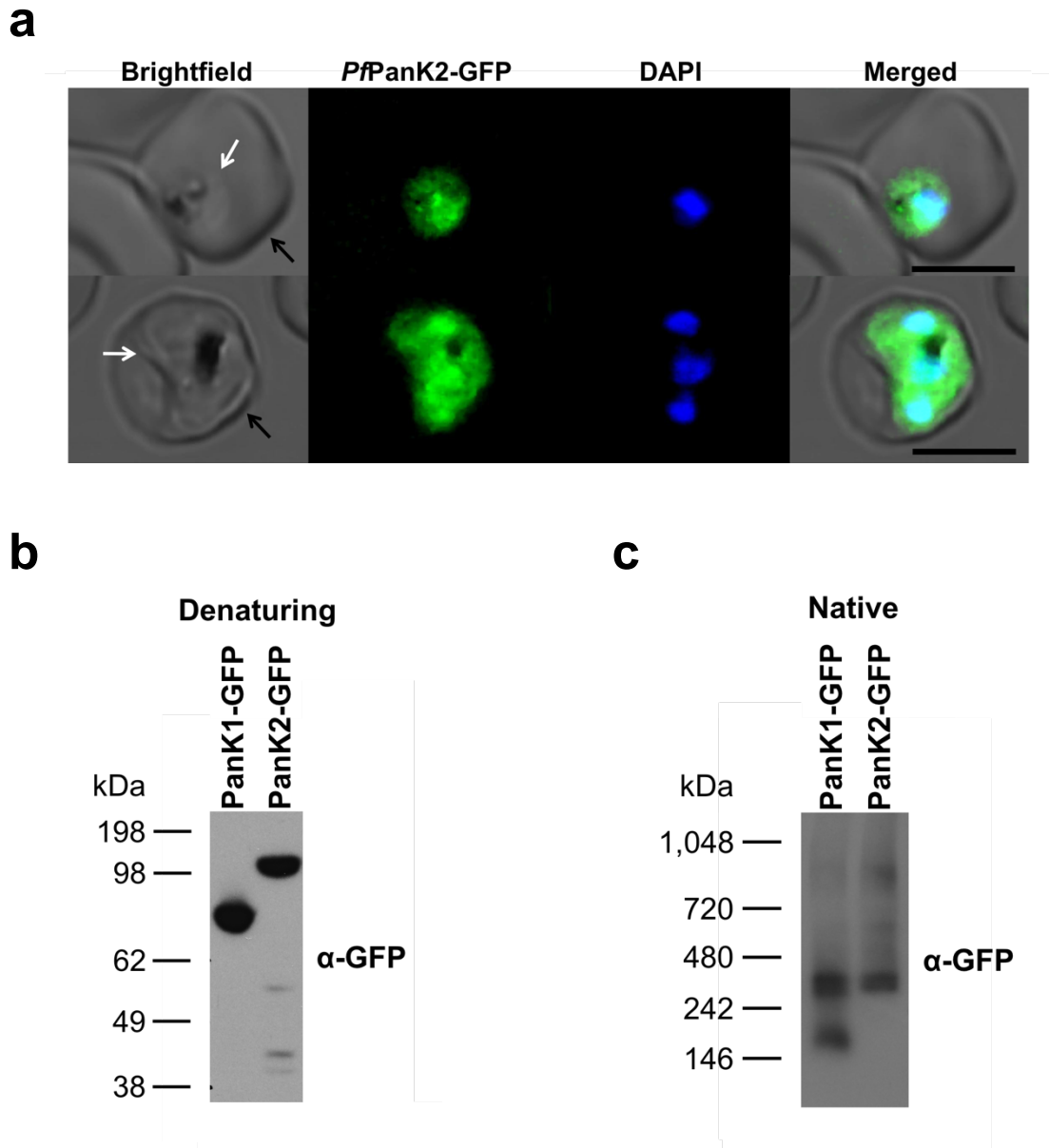


Figure 4.1. *Pf*PanK1 and *Pf*PanK2 form protein complexes of similar molecular weight. (a) Confocal micrographs showing the subcellular location of *Pf*PanK2-GFP in PanK2-GFP parasites. From left to right: Brightfield, GFP-fluorescence, DAPI-fluorescence, and merged images of erythrocytes infected with trophozoite-stage *P. falciparum* parasites expressing *Pf*PanK2-GFP. Arrows indicate the plasma membranes of the erythrocytes (black) or the parasites (white). Scale bar represents 5 μ m. Images were obtained from Dr. Christina Spry (unpublished data). (b) Denaturing and (c) native western blot analyses of the GFP-tagged proteins present in PanK1-GFP and PanK2-GFP parasites. The expected sizes of the proteins are ~87 kDa for *Pf*PanK1-GFP and ~118 kDa for *Pf*PanK2-GFP. The molecular weight of GFP is ~27 kDa. Western blots were performed with anti-GFP antibodies and each blot shown is a representative of three independent experiments, each performed with a different batch of parasites. Protein molecular weight standards are shown in kDa.

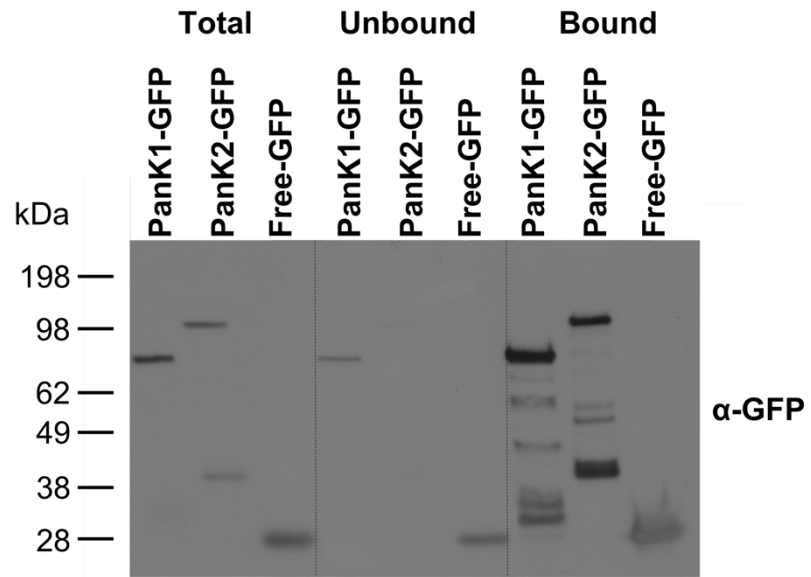
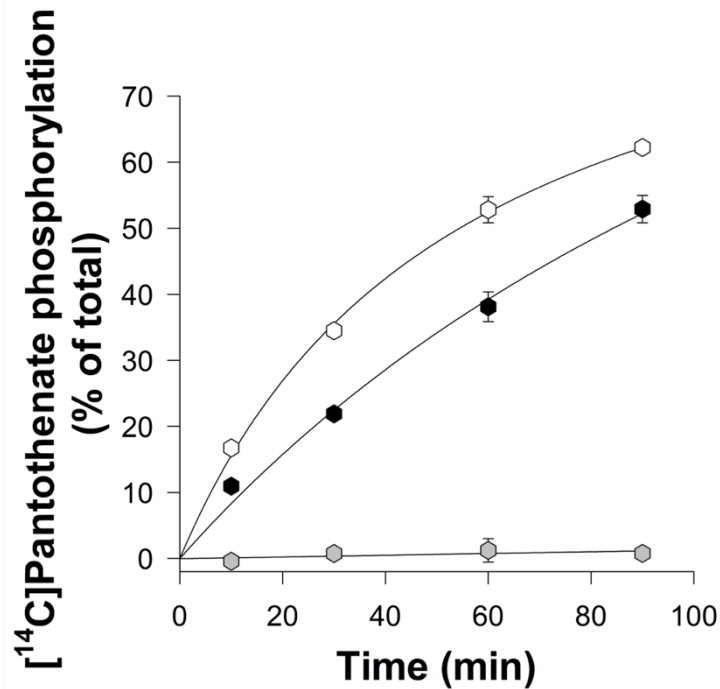
a**b**

Figure 4.2. GFP-trap co-immunoprecipitated fractions of PanK1-GFP and PanK2-GFP are able to phosphorylate pantothenate. (a) Denaturing western blot analysis of the GFP-tagged proteins present in the total lysate, unbound and GFP-trap-bound co-immunoprecipitated fractions of PanK1-GFP, PanK2-GFP and Free-GFP parasites. Western blots were performed with anti-GFP antibodies and the blot shown is a representative of three independent experiments each performed with a different batch of parasites. Protein molecular mass standards are shown in kDa. (b) The phosphorylation of [^{14}C]pantothenate ($2\ \mu\text{M}$) over time (min) by GFP-trap co-immunoprecipitated fractions that were purified from lysates of PanK1-GFP (black hexagons), PanK2-GFP (white hexagons) and Free-GFP (grey hexagons). Data shown are representative of two independent experiments, each performed with a different batch of parasites and carried out in duplicate. Error bars represent range/2 and are not shown if smaller than the symbols.

In the bound fractions of PanK1-GFP and PanK2-GFP, the most intense protein bands correspond to *Pf*PanK1-GFP and *Pf*PanK2-GFP, respectively, although other less intense bands of lower molecular weight were also detected (**Figure 4.2 a**). These smaller protein bands could be breakdown products of *Pf*PanK2. A single band that is consistent with the molecular weight of GFP (~27 kDa) was detected in all three fractions of Free-GFP (**Figure 4.2 a**). The relatively large amount of GFP present in the unbound fraction of the Free-GFP line indicate that the amount of GFP present in the cell lysate exceeded what could be bound through the co-immunoprecipitation.

To test if any of the isolated proteins or protein complexes possess PanK activity, a [¹⁴C]pantothenate phosphorylation assay was performed using the bound fractions purified from all three cell lines. In **Figure 4.2 b**, it can be observed that the bound fractions from both PanK1-GFP and PanK2-GFP were able to phosphorylate pantothenate, reaching 50 – 60% of total phosphorylation by 90 min, consistent with both fractions containing an active PanK. Conversely, the bound fraction of Free-GFP did not display PanK activity (**Figure 4.2 b**).

4.3.2. *Pf*PanK1 and *Pf*PanK2 form a single complex that includes a *Pf*14-3-3I dimer

To identify the proteins that were purified in the co-immunoprecipitation, the bound fractions from PanK1-GFP, PanK2-GFP and Free-GFP (included as a negative control) parasites were subjected to mass spectrometry analysis. From **Table 4.1**, it can be observed that both *Pf*PanK1 (43% coverage on average; **Figure 4.S1**) and *Pf*PanK2 (39% coverage on average; **Figure 4.S2**) were unequivocally detected in the bound fractions of both PanK1-GFP and PanK2-GFP. Additionally, *Pf*14-3-3I (55% coverage on average; **Figure 4.S3**) was the third protein with the most abundant number of unique peptide detected in both bound fractions (**Table 4.1**). Other proteins, including M17 leucyl aminopeptidase, were also detected in the mass spectrometry analysis, albeit with a comparatively fewer number of peptides (**Table 4.1**, **Table 4.S2** and **Table 4.S3**).

In order to further confirm that *Pf*PanK1 and *Pf*PanK2 are part of a single protein complex, the Parent and mutant lines, PanOH-A, PanOH-B and CJ-A, that were generated in a previous study (Tjhin *et al.*, 2018) were transfected with the same

Protein detected	No of peptides detected (> 95% confidence)	
	PanK1-GFP co-immunoprecipitation	PanK2-GFP co-immunoprecipitation
<i>Pf</i> PanK2, putative	24	77
<i>Pf</i> PanK1	23	49
14-3-3 protein (<i>Pf</i> 14-3-3I)	14	41
M17 leucyl aminopeptidase	4	8

Table 4.1. Mass spectrometry of PanK1-GFP and PanK2-GFP GFP-trap co-immunoprecipitated fractions identified *Pf*PanK1, *Pf*PanK2 and *Pf*14-3-3I as being most abundant. Only proteins with three or more peptides detected and that are present in all of the co-immunoprecipitations performed are shown. Proteins detected in the Free-GFP or wild-type 3D7 co-immunoprecipitations (negative controls) were removed. Data shown are a representative of two independent analysis, each performed with a different batch of parasites.

episomal plasmid used to generate the PanK2-GFP parasites, allowing for the expression of *Pf*PanK2-GFP in these lines. These transfectant lines are designated with a “+*Pf*PanK2-GFP” superscript. Co-immunoprecipitation was then carried out on the lysate produced from these transfectants and the bound fractions were used to perform additional [¹⁴C]pantothenate phosphorylation assays. Previously, it was reported by Tjhin *et al.* (2018) that the mutations in the *Pf*PanK1 of PanOH-A, PanOH-B and CJ-A lines have altered the *Pf*PanK activity of these parasites in the following rank order relative to the Parent line: PanOH-A > Parent > PanOH-B > CJ-A (**Figure 4.3 a**). It can be observed from **Figure 4.3 b** that the bound fractions of the transfected mutant lines also phosphorylated pantothenate with the same rate rank order: PanOH-A^{+*Pf*PanK2-GFP} > Parent^{+*Pf*PanK2-GFP} > PanOH-B^{+*Pf*PanK2-GFP} > CJ-A^{+*Pf*PanK2-GFP}. Steps were taken to ensure that the difference in pantothenate phosphorylation rates was not caused by variations in the amount of proteins in the bound fractions used for the assays. The same number of GFP-positive cells, as determined by a combination of haemocytometer cell counts and FACS analysis (**Figure 4.S4**), were used for each co-immunoprecipitation. Furthermore, an aliquot of each bound fraction used in the phosphorylation assays were analysed on denaturing western blots. As shown in **Figure 4.3 c**, all of the bound fractions used in each independent repeat of the phosphorylation assay contained similar amounts of protein (as indicated by the amount of *Pf*PanK2-GFP present). In addition to the native western blot (**Figure 4.1 c**) and mass spec (**Table 4.1**) data, these results are consistent with both *Pf*PanK1 and *Pf*PanK2 being part of the same complex, and that this complex is responsible for the pantothenate phosphorylation activity observed in *P. falciparum*.

To confirm that *Pf*14-3-3I is indeed part of the PanK complex in the parasite, a pan-specific anti-14-3-3 antibody was used to probe the western blots presented in **Figure 4.1 c** and **Figure 4.2 a**. In the anti-14-3-3 native western blot presented in **Figure 4.4 a**, an intense band that is just under the 66 kDa molecular weight standard was detected for PanK1-GFP, PanK2-GFP and Free-GFP cell lines, consistent with *P. falciparum* 14-3-3 protein(s) forming a dimer/dimers. A less intense band just above the 242 kDa molecular weight marker was detected in PanK1-GFP and PanK2-GFP but was absent in Free-GFP (**Figure 4.4 a**). Furthermore, when a denaturing anti-14-3-3 western blot was performed with the

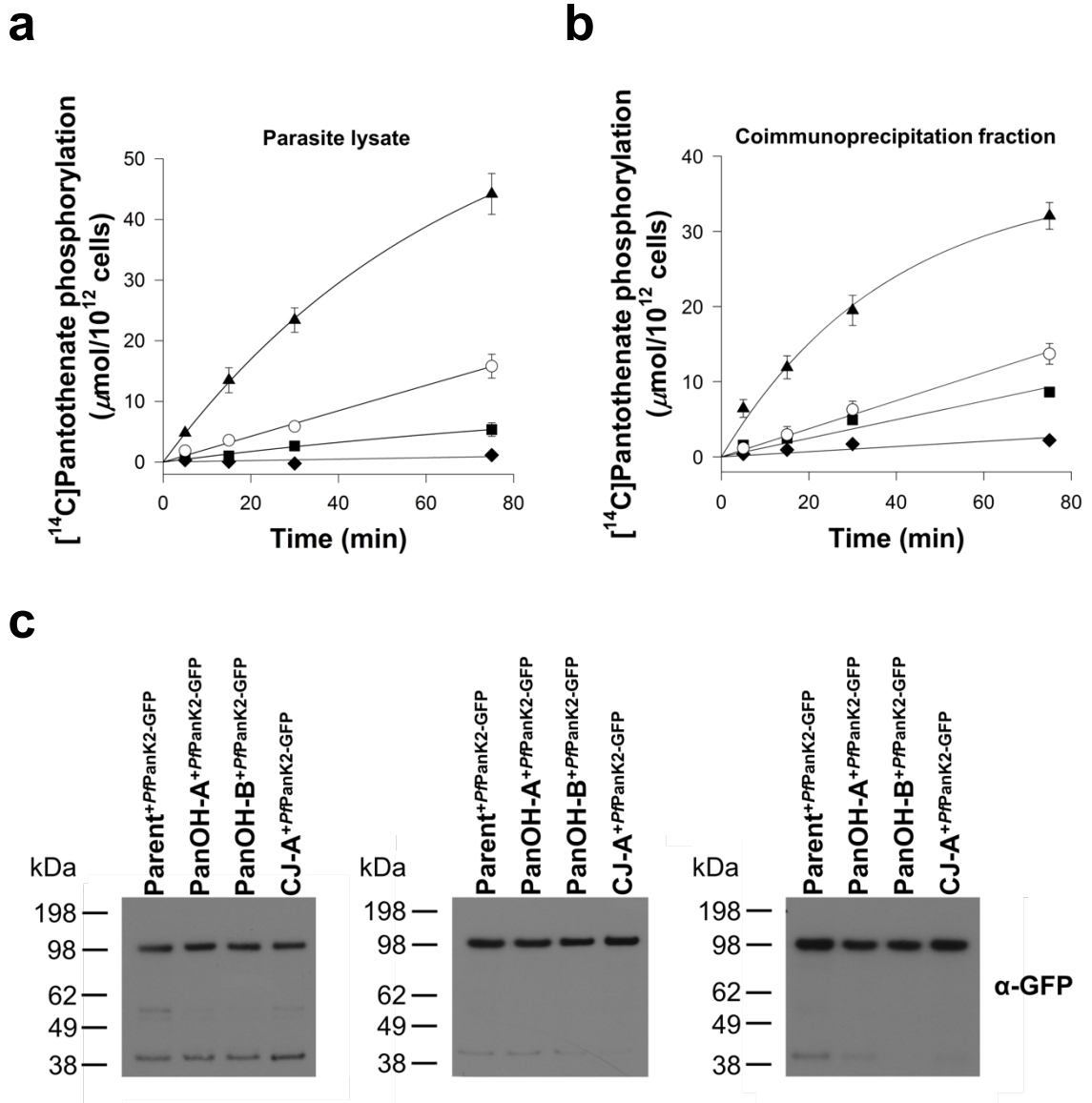


Figure 4.3. PanK2-GFP co-immunoprecipitated fraction of *PfPanK1* mutants exhibit the same pantothenate phosphorylation rate profile as parasite lysates prepared from the mutants. The phosphorylation of $[^{14}\text{C}]\text{pantothenate}$ ($2 \mu\text{M}$) over time (min) by (a) lysates generated from Parent (white circles), PanOH-A (black triangles), PanOH-B (black squares) and CJ-A (black diamonds) parasites and (b) GFP-trap co-immunoprecipitated fractions that were purified from lysates of Parent⁺*PfPanK2-GFP* (white circles), PanOH-A⁺*PfPanK2-GFP* (black triangles), PanOH-B⁺*PfPanK2-GFP* (black squares) and CJ-A⁺*PfPanK2-GFP* (black diamonds) parasites. Figure (a) was reproduced from Tjhin *et al.* (2018). Values in (b) are averaged from three independent experiments, each performed with a different batch of parasites and carried out in duplicate. Error bars represent SEM and are not shown if smaller than the symbols. (c) Denaturing western blot analysis of *PfPanK2-GFP* in the GFP-trap co-immunoprecipitated fractions that were used in the $[^{14}\text{C}]\text{pantothenate}$ phosphorylation assay in (b). Western blots were performed with anti-GFP antibodies and each blot represents the analysis of the fractions used in a single independent experiment of the phosphorylation time course. The same volume of samples ($10 \mu\text{L}$ per lane) was used for all three experiments. Protein molecular weight standards are shown in kDa.

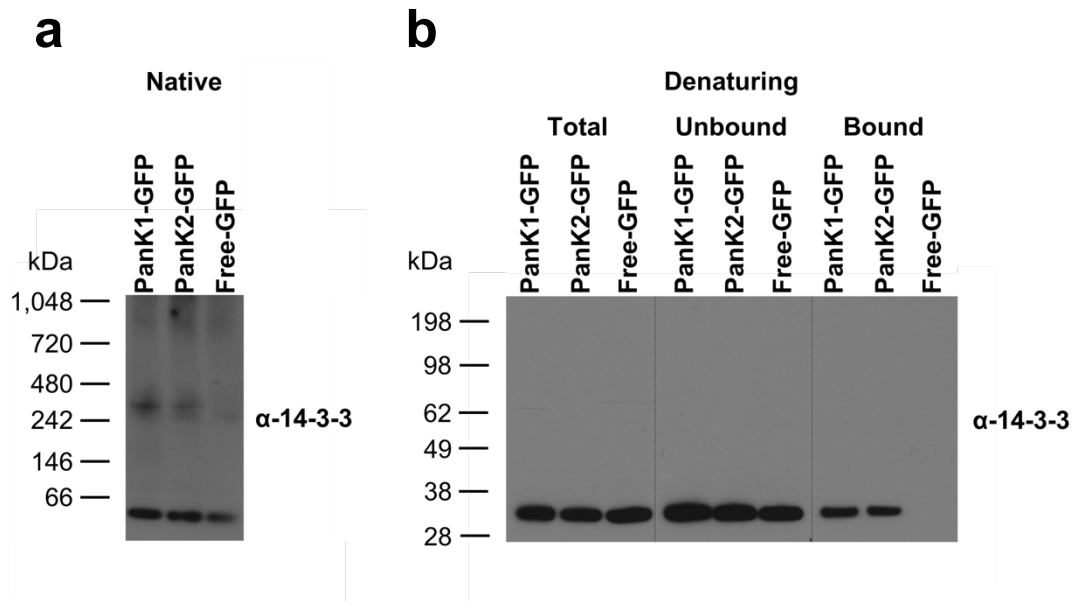


Figure 4.4. The *Pf*14-3-3I dimer associates with the *Pf*PanK heterodimer complex. (a) Native and (b) denaturing protein gel electrophoresis followed by western blot analyses of the GFP-tagged proteins present in PanK1-GFP, PanK2-GFP and Free-GFP parasite fractions. Western blots were performed with pan-specific anti-14-3-3 antibodies (previously shown to detect *Plasmodium* 14-3-3 (Lalle *et al.*, 2011)) and each blot shown is a representative of two independent experiments each performed with a different batch of parasites. Protein molecular weight standards are shown in kDa.

total lysate, unbound and bound fractions from the co-immunoprecipitation of PanK1-GFP, PanK2-GFP and Free-GFP, a single ~30 kDa band (consistent with the size of the Pf14-3-3I monomer) was detected in all fractions except for the bound fraction of Free-GFP (**Figure 4.4 b**). These data are consistent with a 14-3-3 molecule being part of the PanK complex of the parasite. Specifically, the complex includes Pf14-3-3I rather than one of the other isoforms because only peptides belonging to this isoform were detected in the mass spectrometry analysis (**Table 4.1**, **Table 4.S2**, **Table 4.S3** and **Figure 4.S3**).

To explore the possibility that PfPanK1 and/or PfPanK2 contain 14-3-3 binding motif(s), their amino acid sequences were analysed using several online prediction software, namely Eukaryotic Linear Motif (ELM) resource (Dinkel *et al.*, 2016), Scansite (Obenauer *et al.*, 2003) and 14-3-3-Pred (Madeira *et al.*, 2015). The amino acid sequence of *P. falciparum* histone H3 (PfH3) protein (PF3D7_0610400) was also analysed as a positive control because Pf14-3-3I has been shown experimentally to bind to the H3S28ph residue of the protein (Dastidar *et al.*, 2013). As annotated in **Figure 4.5 a**, only 14-3-3-Pred detected binding sites in PfPanK1, with each site predicted only by a single method. On the other hand, as shown in **Figure 4.5 b & c**, all three prediction tools indicated that at least one 14-3-3 binding site is present in both PfPanK2 and PfH3. More importantly, the experimentally-verified Pf14-3-3I binding site in PfH3 that involves the H3S28ph histone modification site (Dastidar *et al.*, 2013) was detected by all prediction tools (**Figure 4.5 c**). In other organisms, 14-3-3 binding requires its target to possess a phosphoserine/threonine residue (Yaffe *et al.*, 1997). Therefore, several existing *P. falciparum* phospho-proteomic libraries (Treeck *et al.*, 2011; Solyakov *et al.*, 2011; Lasonder *et al.*, 2012; Pease *et al.*, 2013) were interrogated for phospho-peptides belonging to PfPanK1, PfPanK2 and PfH3. All four studies detected phosphorylation sites for PfPanK2, specifically at positions Ser55, Ser516, Ser569, Ser571 and Ser621 (**Figure 4.5 b**). However, it is important to note that none of these phosphorylated residues were predicted to be a 14-3-3 binding site. Conversely, no phosphorylation sites were found for PfPanK1 (**Figure 4.5 a**). Interestingly, the PfH3 phosphorylation site required for Pf14-3-3I binding (denoted here as Ser29) was only detected in two of the four phospho-proteome studies (Treeck *et al.*, 2011; Lasonder *et al.*, 2012) (**Figure 4.5 c**).

a

PfPanK1 (PF3D7_1420600)

1	MRKYKNELNISNVLE	KKDDCCSLDIG	GTLIKVVYVN	HKYIHDDNIKENTEHLMIKMNGNK	60
61	NIYLTFFDISKLDDTLYFLLRNN	LIKKKI	TLTGG	GAHKYFYHVLEK	120
121	NKIYVSKYLYDEKLSIFSIFVCSTKSNDQNKND	DINNFSKI	IEGQVLF	DKKFVDFKPSD	180
181	TIKIYVEKRYFDKNGNDDNNNDDDDNNDD	NNNDDNNNDNY	MILTC	SRKDEMNCIMNG	240
241	IHTLFSV	PKSFFRYERFLNVKVPVKI	ISPFH	PFIIANIGSGISILKSNGYDSYQRIAGTA	300
301	IGGGTLMGLAKIILDNISFEELIKCAE	DKNKNI	SFDLK	MKHIIGDAPVDGCTHANTLASC	360
361	FGCLKNILKEIKENNGHNKTIHHEVAK	GLIQMV	SYNIG	YMVYLLSKMHNVKRIFFSGKYI	420
421	SNNEYIMESLTHGVYYYYLHFNSK	MNGVDK	INDINLKN	NIAKNTIKRELYNVKDIHFNDK	480
481	DMNSYLSYHYKLQDKEILPQV	LFPKHDGFLGALGCFFLA			519

b

PfPanK2 (PF3D7_1437400)

1	MGNTLGIECSFNYVHVTTVLI	NKKLIKES	NNDSKNEK	DIREEKEK	LEKNNVSIPSN	DNKL	60
61	NMNVHLSWLKEKYKKEYINLEEDVSKSDEYNNCDDDY	IKMKKN	TFSYI	LDMQHI	IDS	SDVQ	120
121	VFSLKKNSEKNVHTHISINELYKCFHEDDNLEKKFIKYL	N	EVKYHK	L	DISQIDTVNIQHL		180
181	YDDEIAEIIYFWSFKLKYLD	ECILKYYKKNIN	YMF	INVTGKNKN	LIKKKFLQITGKN	NIFQ	240
241	HNEIKCINNSICFLKRFMP	TNLYYFTKYNEE	NERASCT	SQND	EDDEK	KKKKKKNLLYQSFI	300
301	NKEEVSEIKSYVIVNMKRAVCYHLVNEQN	LIERIG	TLVVG	FRTVMGLFLL	ITGRPC	SLQR	360
361	ICQLAKNGT	NRTFDM	TVQDI	YGTSYSNAGLCKDLTASFFGNAQHI	ENVKNILNTYDEEKN		420
421	INIKEEDDKLMNVYEYENESSCYENVSSCMSTEISECQE	IFETE	ECIGFEVEKNNINYYR				480
481	NKYSEI	SKDKTKKLLVSNKNFNVDNCKIPIFNFN	S	SDSYCNMNSFKSKEKLFKHNIEEQ			540
541	QKQEEGYKQKEDDNFLMNDIDNYLSIKHSL	S	DNEINMYEYH	RKNYNL	KKSF	LKKLFKKQY	600
601	LNENGKIIHNINLSIQKEVSSFILKNKK	RFSYK	TYDKQI	VKKACKS	ITLKNV	CSETNKNK	660
661	MDSNLKDKTIVKYN	TKMCDLS	RSLLSMA	IFTTVYLSYIHCNLYNADHI	FTGYNFEDDVC		720
721	KELFQIIIKFLSHNRQKIYFAKISKYISSLGS	AIELINWERIHIDN					766

c

PfH3 (PF3D7_0610400)

1	MARTKQTARKS	TACKAPRK	Q	LASKAAR	RKS	API	SAGIKKP	HRRYRPG	TVALRE	IRRYQK	STD	60
61	LLI	IRKLPFQRLVREIAQDYKTDL	RFQSSAVMAL	Q	EAAEAYLVGLFEDTNLCAIHAKRV	TI						120
121	MPKDIQLARR	IRGERS										136

Figure 4.5. *In silico* analysis of 14-3-3 binding sites in PfPanK1, PfPanK2 and PfH3. The amino acid sequences of (a) PfPanK1, (b) PfPanK2 and (c) PfH3 are shown with the position of the first and last residue of each line indicated by the flanking numbers. Highlighted residues indicate the 14-3-3 binding motif predicted by ELM (red), Scansite 3.0 (grey) and 14-3-3-Pred (yellow = 1 method; blue = 2 methods; green = 3 methods), with the phosphoserine/threonine residue required for binding indicated in bold. Residues with more than one highlight were detected by more than one prediction software. “*” indicates a phosphorylated residue that has been detected in one or more of the following *P. falciparum* phosphoproteomic studies: Solyakov *et al.* (2011), Treeck *et al.* (2011), Lasonder *et al.* (2012) or Pease *et al.* (2013). “!” indicates a parasite-specific phosphorylated residue that was not detected in the phosphoproteome studies but was detected in purified *P. falciparum* histone H3 (PfH3) by Dastidar *et al.* (2013). Black boxes indicate residues of two inserts that are specific to type II eukaryotic PanKs. Residues of parasite-specific inserts in PfPanK1 and PfPanK2 are contained in red and blue boxes, respectively. Residues shown in orange are predicted to be nuclear localisation signals (NLSs) of PfPanK2.

4.4. Discussion

4.4.1. *P. falciparum* PfPanK complex

All PanKs with a crystal structure have been found to form homodimers (Yun *et al.*, 2000; Das *et al.*, 2006; Yang *et al.*, 2006a; Hong *et al.*, 2006; Nicely *et al.*, 2007; Hong *et al.*, 2007; Li *et al.*, 2013; Franklin *et al.*, 2015). Even though both PfPanK1 and PfPanK2 are expected to also form homodimers, this has not been investigated prior to the current study. Here we present data that are consistent with the two parasite PanKs forming a heterodimer (**Figure 4.1 c**, **Table 4.1** and **Figure 4.3 b**), a unique phenomenon that has never been reported for PanKs in nature. The PfPanK heterodimer complex has a molecular weight that approximates the combined molecular weight of PfPanK1, PfPanK2 and a Pf14-3-3I dimer (**Figure 4.1 c**). Due to the limited resolution of the native gel, it is difficult to obtain an accurate estimate of the molecular weight of the complex. Therefore, it is possible that the complex also contains other proteins, such as M17 leucyl aminopeptidase and heat shock protein 70-2 (**Table 4.1**, **Table 4.S2** and **Table 4.S3**). However, since peptides from these proteins were not detected at the same abundance as PfPanK1, PfPanK2 and Pf14-3-3I, their presence in the functional PanK is less clear. The parasite's M17 leucyl aminopeptidase has been shown to function as a homo-hexamer with a combined molecular weight of 320 kDa (Stack *et al.*, 2007; McGowan *et al.*, 2010), which makes it an unlikely component of the PanK complex. The M17 leucyl aminopeptidases is thought to regulate the amino acid pool within the parasite mainly through haemoglobin digestion (Stack *et al.*, 2007; McGowan *et al.*, 2010), although their counterpart in plants have been shown to moonlight as molecular chaperones (Scranton *et al.*, 2012). *P. falciparum* heat shock protein 70-2 is an endoplasmic reticulum-based chaperone protein that is postulated to be involved in protein secretion and degradation processes within the organelle (Przyborski *et al.*, 2015). As other proteins involved in protein degradation (e.g. polyubiquitin) were also detected in the mass spectrometry analysis (**Table 4.S2** and **Table 4.S3**) it is conceivable that both M17 leucyl aminopeptidase and heat shock protein 70-2 are involved in this process, likely because PfPanK1-GFP and PfPanK2-GFP are expressed in addition to the native proteins in the respective cell lines. An additional band (~150 kDa) was also detected in the anti-GFP native blot for PfPanK1 (**Figure 4.1 c**), which could correspond to either a homodimer of PfPanK1 or a degraded

*Pf*PanK heterodimer complex. Additional data presented in this study show that the *Pf*PanK heterodimer exhibits PanK activity and that its ability to phosphorylate pantothenate is influenced by the mutations present in *Pf*PanK1 (**Figure 4.2 b** and **Figure 4.3 b**). It remains unclear if the *Pf*PanK1 mutations reported previously (Tjhin *et al.*, 2018) affects the *Pf*PanK1 monomer specifically or the *Pf*PanK heterodimer activity as a whole.

4.4.2. *P. falciparum* 14-3-3 proteins

In this study, we have presented data that are consistent with *Pf*14-3-3I forming an association with the *Pf*PanK complex (**Table 4.1** and **Figure 4.4 a & b**). The 14-3-3 protein family is a group of highly conserved proteins of ~30 kDa that occur over a wide array of eukaryotic organisms ranging from mammals to model organisms such as *Saccharomyces cerevisiae*, *Arabidopsis* and *Drosophila* (van Hemert *et al.*, 2001; Rosenquist *et al.*, 2000). Multiple isoforms of 14-3-3 have been found in every organism that expresses the protein (Rosenquist *et al.*, 2000). The 14-3-3 proteins are involved in a large range of cellular functions involving cell cycle regulation, signal transduction and apoptosis (reviewed in van Hemert *et al.* (2001)). They carry out these functions by modifying their target protein's trafficking/targeting (reviewed in Muslin and Xing (2000)), conformation, co-localisation, and/or activity (reviewed in Bridges and Moorhead (2005)). Unsurprisingly, 14-3-3 proteins have also been previously identified in several *Plasmodium* species (Al-Khedery *et al.*, 1999; Di Girolamo *et al.*, 2008; Lalle *et al.*, 2011; Dastidar *et al.*, 2013). In *Plasmodium berghei*, *Pb*14-3-3 was one of the proteins detected in the lipid rafts of parasite-infected erythrocytes (Di Girolamo *et al.*, 2008) and was also found to bind to internalised erythrocyte host dematin protein (Lalle *et al.*, 2011). Another study identified three putative 14-3-3 genes in *P. falciparum* and characterised two isoforms, *Pf*14-3-3I and *Pf*14-3-3II, specifically with regards to their ability to bind parasite histone H3 protein (Dastidar *et al.*, 2013). Both *Pf*14-3-3I and *Pf*14-3-3II were found to bind purified parasite histones, although only the *Pf*14-3-3I isoform bound synthetic histone H3 peptides with phosphorylated Ser29 residue (one of the histone H3 residues recognised by 14-3-3 in other organisms when phosphorylated) (Dastidar *et al.*, 2013). Similar to what was observed for *Pb*14-3-3 (Lalle *et al.*, 2011), *Pf*14-3-3I was found to localise to both the cytoplasm and nucleus of asexual stage parasites, which is reflective of its pleiotropic functions (Dastidar *et al.*, 2013).

Most 14-3-3 proteins commonly occur as dimers, with some isoforms shown to form both homo- and heterodimers (Jones *et al.*, 1995; Chaudhri *et al.*, 2003; Yang *et al.*, 2006b). Consistent with these data, the ~60 kDa band detected in the native anti-14-3-3 blot (**Figure 4.4 a**) and the ~30 kDa band detected in the denaturing blot (**Figure 4.4 b**) likely correspond to the 14-3-3 dimer(s) and monomer, respectively. Although the mass spectrometry analysis of the co-immunoprecipitated fractions of *Pf*PanK1 and *Pf*PanK2 only detected peptides unique to *Pf*14-3-3I, we cannot exclude the possibility that the *Pf*14-3-3II isoform was also present in the total and unbound fractions of the denaturing blot and as homo/heterodimers in the native blot due to their similar molecular weight (30.2 for *Pf*14-3-3I and 31.8 kDa for *Pf*14-3-3II (Aurrecoechea *et al.*, 2009)).

4.4.3. *Pf*14-3-3I binding site and function

In general, 14-3-3 proteins bind to phosphorylated Ser/Thr residues in proteins that harbour two canonical motifs: RSXpS/TXP (mode I) and RXY/FXpS/TXP (mode II), where pS/T represents a phosphoserine or phosphothreonine (Yaffe *et al.*, 1997). A third motif, pS/T-X(1-2)-COOH, was subsequently introduced to classify another group of interaction that requires a phosphorylated Ser/Thr residue close to the carboxy terminus (Coblitz *et al.*, 2006). More recent bioinformatics surveys have further refined these consensus motifs (Johnson *et al.*, 2010; Panni *et al.*, 2011). It was also found that most 14-3-3 binding sites occur within disordered regions of target proteins (Bustos and Iglesias, 2006). In this study, several 14-3-3 binding sites were predicted for both *Pf*PanK1 and *Pf*PanK2, although more were found in *Pf*PanK2 (**Figure 4.5 a & b**). Phosphoproteome studies found that only *Pf*PanK2 harbour phosphorylated residues (Treeck *et al.*, 2011; Solyakov *et al.*, 2011; Lasonder *et al.*, 2012; Pease *et al.*, 2013), as annotated in **Figure 4.5 b**, although these did not coincide with the 14-3-3 binding sites predicted for *Pf*PanK2. However, it is worth noting that only two of the *P. falciparum* phospho-proteome analyses detected the Ser29 phosphorylation site in *Pf*H3 required for 14-3-3 binding (Treeck *et al.*, 2011; Lasonder *et al.*, 2012) and subsequent analysis of purified *Pf*H3 found three additional parasite-specific phosphorylation sites (Dastidar *et al.*, 2013). Therefore, it is possible that there are additional undetected phosphorylation sites in *Pf*PanK2 that correspond to a 14-3-3 binding motif. Taking into account the

predicted 14-3-3 binding sites (**Figure 4.5 a & b**), the size of the *Pf*PanK complex (**Figure 4.1 c**) and the result of the mass spectrometry analysis (**Table 4.1**, **Table 4.S2** and **Table 4.S3**), it is likely that the *Pf*14-3-3I dimer binds solely to *Pf*PanK2 although the possibility of the *Pf*14-3-3I dimer acting as a bridge/scaffold between *Pf*PanK2 and *Pf*PanK1 (or another protein) cannot be completely excluded. Upon binding to *Pf*PanK2, the *Pf*14-3-3I dimer likely acts as a “molecular anvil”, so called because a series of interactions between the numerous α -helices of the 14-3-3 molecule confer it an exceptionally rigid structure (Yaffe, 2002). It was hypothesised that in many cases the 14-3-3I dimer binds to tandem sites on a single target protein and acts as a stable support on which the target can be reshaped or stabilised (Yaffe, 2002). Many proteins containing multiple 14-3-3 binding sites have previously been identified (Johnson *et al.*, 2010) and there are multiple lines of evidence that support this type of 14-3-3 interaction being important to the target protein’s activity (Tzivion *et al.*, 1998; Obsil *et al.*, 2001; Obsilova *et al.*, 2008; Kosteleccky *et al.*, 2009). However, due to the large range of cellular functions that 14-3-3 proteins have been implicated in, the exact site(s) and purpose of *Pf*14-3-3I binding to *Pf*PanK2 is not immediately obvious.

For the control protein *Pf*H3, all three 14-3-3 binding site-prediction tools detected the experimentally-determined *Pf*14-3-3I binding site at Ser29 (**Figure 4.5 c**; (Dastidar *et al.*, 2013)). For *Pf*PanK2, two predicted 14-3-3 binding sites that are positioned close to each other, Tyr336 and Ser357, were also detected by multiple binding site prediction tools (**Figure 4.5 b**). Interestingly, Tyr336 is positioned close to the Arg333 residue that was predicted to be important for pantothenate binding in PanKs (Hong *et al.*, 2007; Spry *et al.*, 2010). In addition, *Pf*PanK2 is the only PanK amongst those analysed by Spry *et al.* (2010) to have a Tyr at position 336 instead of a Gly. Binding of the *Pf*14-3-3I dimer to these sites could, therefore, influence *Pf*PanK2 binding to pantothenate and potentially affect the function of the heterodimer. Both *Pf*PanK1 and *Pf*PanK2 contain two inserts that are commonly found in all type II eukaryotic PanKs in addition to insert regions that are typical of *P. falciparum* proteins (**Figure 4.5 a & b**; (Spry *et al.*, 2010)). All of the 14-3-3 prediction tools detected binding sites in the inserts of *Pf*PanK2 (**Figure 4.5 b**). Thr278 is located within the second eukaryotic insert of *Pf*PanK2. The eukaryotic inserts in PanKs have been postulated to be important for CoA feedback inhibition of the enzyme (Hong *et al.*, 2006), although it should

be noted that insert 2 of *PfPank2* is almost twice the size of other type II PanKs (Spry *et al.*, 2010). On the other hand, Ser484, Ser631, Ser646 and Thr648 are all located within the *PfPank2*-specific insert (**Figure 4.5 b**). Intriguingly, this insert occurs in a loop that has been implicated in dimer formation in PanKs (Hong *et al.*, 2007; Spry *et al.*, 2010). Therefore, it is possible that binding of the *Pf14-3-3I* dimer here functions to modulate the formation of the heterodimer.

The 14-3-3 site at Ser52 overlaps with one of the predicted NLSs of *PfPank2* (**Figure 4.5 b**), which are all located within potentially disordered regions of the protein (data not shown). Binding of *Pf14-3-3I* at this site could potentially influence the cellular localisation of the *PfPank* complex. Humans and mice express four functional isoforms of type II PanKs, namely PanK1 α , PanK1 β , PanK2 and PanK3, each possessing a different N-terminus that directs the protein to a specific subcellular compartment (Alfonso-Pecchio *et al.*, 2012). It was postulated that the different isoforms sense the CoA requirement and allow the regulation of CoA biosynthesis within their specific compartment (Alfonso-Pecchio *et al.*, 2012). In particular, human PanK2 has been found to localise to both the mitochondria and the nucleus (Hörtnagel *et al.*, 2003; Alfonso-Pecchio *et al.*, 2012) and mutations in human PanK2 that result in the loss of its function have been linked to a neurodegenerative disorder known as pantothenate kinase-associated neurodegeneration (PKAN) (Zhou *et al.*, 2001). It is worth noting that both *PfPank1*-GFP and *PfPank2*-GFP were detected in the nucleus of the parasite (**Figure 4.1 a** and Tjhin *et al.* (2018)). It is, therefore, possible that the parasite expresses a heterodimeric *PfPank* to allow for CoA homeostasis in different cellular compartments, and *Pf14-3-3I* dimer binding to *PfPank2* at Ser52 could regulate the localisation of the heterodimer to the nucleus.

In conclusion, this study presents data that are consistent with *PfPank1* and *PfPank2* forming a functional heterodimeric *PfPank* complex in the parasite, a unique phenomenon that hitherto has not been observed for other PanKs in nature. The data presented also indicate that the *PfPank* complex interacts with the *Pf14-3-3I* dimer by binding to *PfPank2*, which may function to modulate the localisation, enzymatic activity or the formation of the heterodimer complex.

4.5. Supplementary Information

4.5.1. Plasmid preparation

The *Pfpank2*-pGlux-1 construct has the *Pfpank2*-coding sequence inserted within multiple cloning site (MCS) III of pGlux-1. This plasmid backbone contains the human dihydrofolate reductase (*hdhfr*) gene that confers resistance to WR99210 as a positive selectable marker. As a result, *Pfpank2* is placed under the regulation of the *Plasmodium falciparum* chloroquine resistance transporter (*PfCRT*) promoter, and upstream of the GFP-coding sequence. This construct enables the transfected parasites to express a GFP-tagged *PfPANK2*, which allows for the localisation of the protein within the parasite. This also allows the isolation and determination of any proteins that associate with *PfPANK2*.

The *Pfpank2* sequence used to generate the *Pfpank2*-pGlux-1 construct was initially amplified from parasite ribonucleic acid (RNA). Total RNA was purified from saponin-isolated *P. falciparum* parasites (typically 2×10^7 cells) using the RNeasy Mini Kit (QIAGEN) according to the protocol for purifying total RNA from animal cells. An optional 15 min DNase I incubation was included to eliminate residual genomic DNA. Complementary DNA (cDNA) was synthesised from this total RNA sample using SuperScript II Reverse Transcriptase (ThermoFisher) with Oligo(dT)₁₂₋₁₈ primer and included an optional incubation with RNaseOUT Recombinant Ribonuclease Inhibitor (ThermoFisher), all according to the manufacturer's protocol. The *Pfpank2*-specific sequence was then amplified from cDNA using either KOD Hot Start DNA polymerase (Merck Millipore) or Platinum *Pfx* DNA polymerase (ThermoFisher) with the oligonucleotide primers listed in **Table 4.S1** according to each manufacturer's instructions. The *Pfpank2*-pGlux-1 construct was generated by inserting the *Pfpank2*-coding sequence into pGlux-1 using the In-Fusion cloning (Clontech) method. Before cloning, the pGlux-1 plasmid was linearised by sequential digestions with *Xho*I (ThermoFisher) and subsequently *Kpn*I (New England Biolabs), according to each manufacturer's recommendation. The In-Fusion reaction was set up with the In-Fusion Dry-Down PCR Cloning Kit, essentially as described in the manufacturer's protocol.

4.5.2. Confocal microscopy

Coverslip-bound cells were first prepared by washing (centrifuged at $500 \times g$, 5 min) parasite-infected erythrocytes (5 – 10% parasitaemia) once and resuspending them at ~2% haematocrit in 137 mM NaCl, 2.7 mM KCl, 10 mM phosphate buffer, pH 7.4 (phosphate buffered saline; PBS). Next, 1 – 2 mL of the suspension was added to a polyethylenimine (PEI)-coated coverslip placed within a well of a 6-well plate. Plates were incubated (with shaking) for 15 min at room temperature and unbound cells were subsequently washed off the coverslips with PBS (2 mL per well, with a 2 min shaking incubation followed by aspiration). Cells were then fixed with 1 mL of PBS containing 4% (w/v) paraformaldehyde (Electron Microscopy Services) and 0.0075% (w/v) glutaraldehyde (30 min at room temperature). The fixative was then aspirated, and the coverslips washed in PBS three times as described above, before they were rinsed in water and dried. A drop of SLOWFADE (Invitrogen) containing the nuclear stain 4',6-diamidino-2-phenylindole (DAPI) was added to the centre of the coverslips. Finally, each coverslip was inverted onto a microscope slide, sealed with nail polish and used for confocal imaging.

4.5.3. Denaturing protein gel electrophoresis

Denaturing gels were used when the presence and abundance of a single protein of interest was to be detected. Mature, trophozoite-stage parasites were isolated from infected erythrocytes by saponin lysis as described previously (Saliba *et al.*, 1998b). Saponin-isolated parasites (typically $\sim 10^8$ cells) were centrifuged ($15,850 \times g$, 30 s) and the supernatant was removed. The pellet was resuspended in 200 μ L of lysis buffer (1 $\times$ mini cOmplete protease inhibitor cocktail (Roche), 1 $\times$ NuPAGE LDS sample buffer (ThermoFisher), 1 $\times$ NuPAGE sample reducing agent (ThermoFisher), 50 – 60 units of benzonase nuclease (Novagen) and 7.5 – 10 mM of $MgCl_2$), mixed well by vortexing and then boiled at 95 °C for 10 min. The sample was then centrifuged ($16,000 \times g$, 30 min) to pellet the haemozoin before the supernatant was used for gel electrophoresis. 10 μ L of each sample was subsequently loaded into separate wells of a NuPAGE 4 – 12% Bis-Tris protein gel (1.0 mm, 12 wells; ThermoFisher) alongside 5 μ L of SeeBlue Plus2 pre-stained protein standards (ThermoFisher). Electrophoresis was performed in 1 $\times$ NuPAGE 2-(*N*-morpholino)ethanesulfonic acid (MES)

sodium dodecyl sulfate (SDS) running buffer (ThermoFisher) at 200 V for 30 – 35 min. The gel contents were transferred (35 V, 1.5 h) to a nitrocellulose membrane (ThermoFisher) in 1 × NuPAGE transfer buffer with 10% v/v methanol. The membrane was then blocked in 4% blotto with shaking, either overnight at 4 °C or for 2 h at room temperature.

4.5.4. Native protein gel electrophoresis

In order to detect the presence and abundance of protein(s) of interest in their native conformation, parasite samples were subjected to blue native gel electrophoresis. Briefly, saponin-isolated trophozoite-stage parasites ($4 - 8 \times 10^8$ cells) were centrifuged ($15,850 \times g$, 30 s) and the supernatant removed from the pellet. The parasites were then resuspended well by vortexing in 200 μ L of lysis buffer containing 1 × mini cOmplete protease inhibitor cocktail (Roche), 1 × NativePAGE sample buffer (ThermoFisher), 0.5% w/v digitonin, 2 mM EDTA, 50 – 60 units of benzonase nuclease (Novagen) and 7.5 – 10 mM of $MgCl_2$, and incubated with tumbling end-over-end at 4 °C. The lysis preparation was then centrifuged at $16,000 \times g$ for 30 min at 4 °C and the supernatant immediately used for gel electrophoresis. Prior to electrophoresis, NativePAGE 5% G-250 sample additive (ThermoFisher) was added to each sample supernatant to a final concentration of 0.125% and mixed well by vortexing. Samples (typically 10 μ L) were then loaded into the wells of a NativePAGE 4 – 16% Bis-Tris protein gel (1.0 mm, 10 wells; ThermoFisher). 5 μ L of NativeMark unstained protein standards (ThermoFisher) was loaded into the gel alongside the samples to allow for protein mass determination. Electrophoresis was carried out at 4 °C according to the manufacturer's protocol. In brief, electrophoresis was initially performed at 150 V for approximately 40 min with ~200 mL Dark Blue cathode buffer (1 × NativePAGE running buffer and 1 × NativePAGE cathode additive; ThermoFisher) in the inner buffer chamber and ~600 mL of anode buffer (1 × NativePAGE running buffer) in the outer buffer chamber. After the dye front had migrated through 1/3rd of the gel length, the Dark Blue cathode buffer was aspirated from the inner buffer chamber and was replaced with ~200 mL of Light Blue cathode buffer (1 × NativePAGE running buffer and 0.1 × NativePAGE cathode additive; ThermoFisher). Electrophoresis was then resumed at 250 V for the remainder of the run (40 – 60 min). At the end of the run, the proteins within

the gel were transferred as described above to a primed 0.45 μ m PVDF membrane (GE healthcare; soaked prior to use for 30 s in absolute methanol, rinsed briefly in water and soaked in 1 $\times$ NuPAGE transfer buffer with 10% v/v methanol for several minutes). At the end of the transfer, proteins were fixed to the membrane by a 15 min incubation in 10% v/v acetic acid and briefly rinsed in water. In order to visualise the ladder, the membrane was very briefly (~5 s) de-stained in absolute methanol and rinsed in water before it was blocked overnight in 4% blotto at 4 °C with shaking.

4.5.5. Co-immunoprecipitation

GFP-tagged proteins were purified from parasite lysate using bead-bound GFP-trap (a proprietary recombinant alpaca anti-GFP antibody; Chromotek) following the manufacturer's instructions. Briefly, saponin-isolated trophozoites were resuspended in 500 μ L of lysis buffer containing 1 $\times$ mini cOmplete protease inhibitor cocktail (Roche) or 1 $\times$ Halt protease inhibitor cocktail (EDTA-free; ThermoFisher), GFP-trap wash buffer (10 mM Tris/Cl, pH 7.5, 150 mM NaCl and 0.5 mM EDTA), 0.5% w/v digitonin, 50 – 60 units of benzonase nuclease (Novagen) and 3 – 4 mM of $MgCl_2$. The pellet was resuspended well by vortexing and the suspension was incubated (30 – 60 min) with tumbling end-over-end at 4 °C. Subsequently, the suspension was centrifuged (16,000 $\times g$, 30 min, 4 °C) and the supernatant used for GFP-trap binding. Prior to co-immunoprecipitation, 25 μ L (for each lysate) of GFP-trap-agarose bead slurry was primed by three washes (2,500 $\times g$, 2 min, 4 °C) in 500 μ L of GFP-trap wash buffer. The supernatant was removed from the beads at the end of the third wash and 450 – 500 μ L of the lysate generated in the parasite lysis step was applied to the beads. In some experiments, 50 μ L of the total lysate was collected for western blot. This suspension was then incubated for 1 h at 4 °C with tumbling end-over-end. At the end of the incubation, the bead suspension was centrifuged (2,500 $\times g$, 2 min, 4 °C) and the supernatant was discarded. In some experiments, 50 μ L of this supernatant was collected to be used in western blots as the unbound fraction. The proteins bound to the beads were washed 3 $\times$ (2,500 $\times g$, 2 min, 4 °C) in GFP-trap wash buffer with or without 1 $\times$ mini cOmplete protease inhibitor cocktail or 1 $\times$ Halt protease inhibitor cocktail (EDTA-free). After removing the supernatant at the end of the third wash, the beads were resuspended in GFP-

trap wash buffer (typically 200 – 300 μL) and aliquots of these were used for downstream experiments.

4.5.6. Mass spectrometry of co-immunoprecipitated fractions

The co-immunoprecipitated proteins purified through GFP-trap binding were processed and identified through mass spectrometry analysis at the Australian Proteomics Analysis Facility. First, the loading buffer in the samples were separated from the proteins through a short one-dimension gel electrophoresis. The samples were denatured at 95 °C for 10 min and 2 $\times$ 15 μL of each sample was loaded to the lanes of a 12% iGel protein gel (1.0 mm, 12-well; NuSep). The gel was run at 15 mA for 25 min and subsequently washed with a solution containing 10% v/v methanol and 7% v/v acetic acid for 15 min. The gel was then washed with a fixant for 90 min and stained overnight in Coomassie. The band corresponding to the proteins was subsequently excised and de-stained with ammonium bicarbonate/acetonitrile (ACN). The protein samples were then reduced with 25 mM dithiothreitol (DTT) at 60 °C for 30 min and alkylated with 55 mM iodoacetamide (IAA) before an in-gel digestion of the gel slice was performed overnight using 200 ng trypsin to generate protein fragments. The peptides generated were extracted from the gel with bath sonication and ACN/formic acid (FA), dried and then reconstituted in 30 μL of loading buffer.

The peptide samples were then subjected to 1D NanoLC ESI MS/MS analysis. Sample (10 μL) was injected onto a peptide trap (Halo C18, 150 $\mu\text{m} \times 5$ cm) for pre-concentration and desalted with 0.1% v/v FA, 2% v/v ACN at 4 $\mu\text{L}/\text{min}$ for 10 min. The peptide trap was then switched into line with the analytical column. Peptides were subsequently eluted from the column using a linear solvent gradient, with steps, from $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (98:2; + 0.1% v/v FA) to $\text{H}_2\text{O}:\text{CH}_3\text{CN}$ (2:98; + 0.1% v/v FA) with constant flow (600 nL/min) over an 80 min period. The LC eluent was subjected to positive ion nanoflow electrospray MS analysis in an information-dependent acquisition mode (IDA). In the IDA mode, a time-of-flight mass spectrometry survey scan was acquired (m/z 350-1500, 0.25 s), with twenty largest multiply charged ions (counts > 150) in the survey scan sequentially subjected to MS/MS analysis. MS/MS spectra were accumulated for 100 ms (m/z 100 – 1500) with rolling collision energy.

The results of the mass spectrometry analysis were identified by comparing the peptides' amino acid sequences against an annotated protein database for *P. falciparum* 3D7 strain (version 28; PlasmoDB) using the ProteinPilot Software (version 4.2; SCIEX) at a detection threshold of >1.30 (95.0% confidence).

Primer Name Primer Sequence (5'-3')	Function
<i>Pfpank2</i> -pGlux-1-5' primer TTACATATAA CTCGAG ATCGGTAATACATTAGGTATTG	PCR amplification of <i>Pfpank2</i> flanked by a 16-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>Xho</i> I restriction site (underlined). The transcription start codon is highlighted in blue.
<i>Pfpank2</i> -pGlux-1-3' primer TCTTCTCCTTTACT GGTACC ATTGTCTATGTGAATTCGTTCC	PCR amplification of <i>Pfpank2</i> flanked by a 20-base sequence homologous with the linearisation site of pGlux-1 (in bold) that includes the <i>Kpn</i> I restriction site (underlined).
<i>Pfpank2</i> -internal-5' primer1 TCTGATGAATATAATAACTGTGATGACG	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.
<i>Pfpank2</i> -internal-5' primer2 ATGAAAGAGCTAGCTGTACTAGCC	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.
<i>Pfpank2</i> -internal-3' primer1 TCTTCGTCATTTTGGCTAGTACAGC	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.
<i>Pfpank2</i> -internal-3' primer2 TCACTAATTCTGTACTCATACAAGTGC	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.
<i>Pfpank2</i> -internal-3' primer3 ACCATTCCTATTAGATATTGC	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.
<i>Pfpank2</i> -internal-3' primer4 TCTGGATAAATCACATTTTGG	Sanger sequencing of <i>Pfpank2</i> -pGlux-1 clones.

Table 4.S1. List of oligonucleotides used in this study.

Proteins detected in first mass spec analysis of PanK1-GFP GFP-trap co-immunoprecipitation	No of peptides (>95% confidence)	Proteins detected in first mass spec analysis of PanK2-GFP GFP-trap co-immunoprecipitation	No of peptides (>95% confidence)
<i>Pf</i> PanK1	20	<i>Pf</i> PanK1	19
<i>Pf</i> PanK2, putative	10	<i>Pf</i> PanK2, putative	16
<i>Pf</i> 14-3-3I	7	<i>Pf</i> 14-3-3I	16
heat shock protein 70-2	3	M17 leucyl aminopeptidase	5
M17 leucyl aminopeptidase	3	karyopherin beta	4
karyopherin beta	2	heat shock protein 70-2	4
60S ribosomal protein L12, putative	2	ubiquitin-60S ribosomal protein L40	3
HSP40, subfamily A, putative	2	polyubiquitin	3
protein SIS1	1	6-phosphofructokinase	3
ubiquitin-60S ribosomal protein L40	1	40S ribosomal protein S11	3
polyubiquitin	1	HSP40, subfamily A, putative	3
tubulin beta chain	1	glutamate--tRNA ligase, putative	3
40S ribosomal protein S25	1	40S ribosomal protein S18, putative	3
60S ribosomal protein L23, putative	1	60S ribosomal protein L12, putative	2
DSK2, putative	1	40S ribosomal protein S25	2
60S ribosomal protein L13, putative	1	endoplasmic reticulum-resident calcium binding protein	1
glutamate--tRNA ligase, putative	1	60S ribosomal protein L13, putative	1
40S ribosomal protein S18, putative	1	tubulin beta chain	1
40S ribosomal protein S11	1	60S ribosomal protein L18, putative	1
60S ribosomal protein L18, putative	1	60S ribosomal protein L23, putative	1
6-phosphofructokinase	1	DSK2, putative	1
endoplasmic reticulum-resident calcium binding protein	1	protein SIS1	1

Table 4.S2. List of proteins identified in the first mass spectrometry analysis of PanK1-GFP and PanK2-GFP GFP-trap co-immunoprecipitated fractions. Only proteins that are present in both co-immunoprecipitations and are absent in the negative control co-immunoprecipitations (using Free-GFP and 3D7 wild-type lysates) are shown.

Proteins detected in second mass spec analysis of PanK1-GFP GFP-trap co-immunoprecipitation	No of peptides (>95% confidence)	Proteins detected in second mass spec analysis of PanK2-GFP GFP-trap co-immunoprecipitation	No of peptides (>95% confidence)
<i>Pf</i> PanK2, putative	24	<i>Pf</i> PanK2, putative	77
<i>Pf</i> PanK1	23	<i>Pf</i> PanK1	49
<i>Pf</i> 14-3-3I	14	<i>Pf</i> 14-3-3I	41
elongation factor 1-gamma, putative	6	elongation factor 2	14
M17 leucyl aminopeptidase	4	T-complex protein 1 subunit alpha	14
elongation factor 2	3	heat shock protein 70-2	10
heat shock protein 70-2	2	elongation factor 1-gamma, putative	9
26S protease regulatory subunit 8, putative	2	M17 leucyl aminopeptidase	8
ubiquitin-60S ribosomal protein L40	2	26S protease regulatory subunit 8, putative	7
polyubiquitin	2	tubulin beta chain	6
protein DJ-1	2	26S protease regulatory subunit 7, putative	6
26S protease regulatory subunit 7, putative	1	60S ribosomal protein L12, putative	5
26S protease regulatory subunit 4, putative	1	26S protease regulatory subunit 4, putative	5
V-type proton ATPase catalytic subunit A	1	phosphoethanolamine N-methyltransferase	4
alpha tubulin 1	1	40S ribosomal protein S20e, putative	4
alpha tubulin 2	1	60S ribosomal protein L1, putative	3
tubulin beta chain	1	ubiquitin-60S ribosomal protein L40	3
60S ribosomal protein L12, putative	1	polyubiquitin	3
plasmepsin IV	1	alpha tubulin 1	2
phosphoethanolamine N-methyltransferase	1	alpha tubulin 2	2
T-complex protein 1 subunit alpha	1	26S proteasome regulatory subunit RPN8, putative	2
early transcribed membrane protein 10.2	1	40S ribosomal protein S12, putative	2
26S proteasome regulatory subunit RPN8, putative	1	V-type proton ATPase catalytic subunit A	1
40S ribosomal protein S20e, putative	1	endoplasmic reticulum-resident calcium binding protein	1
karyopherin beta	1	early transcribed membrane protein 10.2	1
40S ribosomal protein S12, putative	1	karyopherin beta	1
plasmepsin I	1	plasmepsin IV	1
endoplasmic reticulum-resident calcium binding protein	1	protein DJ-1	1
60S ribosomal protein L1, putative	1	plasmepsin I	1
deoxyribose-phosphate aldolase, putative	1	deoxyribose-phosphate aldolase, putative	1

Table 4.S3. List of proteins identified in the second mass spectrometry analysis of PanK1-GFP and PanK2-GFP GFP-trap co-immunoprecipitated fractions. Only proteins that are present in both co-immunoprecipitations and are absent in the negative control co-immunoprecipitations (using Free-GFP and 3D7 wild-type lysates) are shown.

PfPanK1 coverage from PanK1-GFP co-immunoprecipitation

1	MRKYKNELNISNVLEKKDDCCSLDIGGTLIKVVYVNHKYIHDDNIKENTEHLMIKMNGNK	60
61	NIYLTFFDISKLDDTLYFLLRNNLIKKKITLTGGGAHKYFYHVLEKALYHKLGMINKGD	120
121	NKIYVSKYLYDEKLSIFSIFVCSTKSNDQKNDDINNFNSKIIEGQVLFDKKFVDFKPSD	180
181	TIKIYVEKRYFDKNGNDDNNDDDDNNDDNNDDNNDDNYMILTCR KDEMNCIMNG	240
241	IHTLFSVDKSFFRYERFLNVKVPVKITSPFHPFIIANIGSGISILKSNGYDSYQRIAGTA	300
301	IGGGTLMGLAKIILDNISFEELIKCAEDKNKNISFDLKMKHIIGDAPVDGCTHANTLASC	360
361	FGCLKNILKEIKENNGHNKTIHHEVAKGLIQMVSYNIGYMVYLLSKMHNVKRIFFSGKYI	420
421	SNNEYIMESLTHGVYYYYLHFNSKMNGVDKINDINLKNNIAKNTIKRELYNVKDIHFNDK	480
481	DMNSYLSYHYKLQDKEILPQVLFPHDGLGALGCFFLA	519

Coverage = 36.4%

PfPanK1 coverage from PanK2-GFP co-immunoprecipitation

1	MRKYKNELNISNVLEKKDDCCSLDIGGTLIKVVYVNHKYIHDDNIKENTEHLMIKMNGNK	60
61	NIYLTFFDISKLDDTLYFLLRNNLIKKKITLTGGGAHKYFYHVLEKALYHKLGMINKGD	120
121	NKIYVSKYLYDEKLSIFSIFVCSTKSNDQKNDDINNFNSKIIEGQVLFDKKFVDFKPSD	180
181	TIKIYVEKRYFDKNGNDDNNDDDDNNDDNNDDNNDDNYMILTCR KDEMNCIMNG	240
241	IHTLFSVDKSFFRYERFLNVKVPVKITSPFHPFIIANIGSGISILKSNGYDSYQRIAGTA	300
301	IGGGTLMGLAKIILDNISFEELIKCAEDKNKNISFDLKMKHIIGDAPVDGCTHANTLASC	360
361	FGCLKNILKEIKENNGHNKTIHHEVAKGLIQMVSYNIGYMVYLLSKMHNVKRIFFSGKYI	420
421	SNNEYIMESLTHGVYYYYLHFNSKMNGVDKINDINLKNNIAKNTIKRELYNVKDIHFNDK	480
481	DMNSYLSYHYKLQDKEILPQVLFPHDGLGALGCFFLA	519

Coverage = 49.5%

Figure 4.S1. Mass spectrometry coverage of *PfPanK1*. *PfPanK1* peptides detected in the mass spectrometry analysis of PanK1-GFP and PanK2-GFP co-immunoprecipitation using GFP-trap. Residues in green were detected with $\geq 95\%$ confidence, while residues in orange were detected with $\geq 90\%$ confidence. Percentage coverages were calculated using only the residues labelled green.

PfPanK2 coverage from PanK1-GFP co-immunoprecipitation

1	MGNTLGIECSFNIVHVTTLINKKLIKESNNDSKNEKDIREEKEKKLPKNVSI ^{PS} NDNKL	60
61	NMNVHLSWLKEKYKKEY ^{IN} LEEDVSKSDEYNNCDDDIYKMKKNTFSYILDMQHIIDS ^{SD} VQ	120
121	VFSLKKNSEKNVHTHISINELYKCFHEDDNLEKKFIKYLNFVKYHKLDISQIDTVNIQHL	180
181	YDDEIAEIIYFWSFKLKY ^{LD} ECILKYYKKNINYMFINVTGKNKNLIK ^{KKFL} QITGKNNIFQ	240
241	HNEIKCINNSICFLKR ^{FM} PTNLYYFTKYNEENERASCTSQNDEDEK ^{KKKK} KNLLYQSFI	300
301	NKEEVSEIKSYVIVNMKRAVCYHLVNEQN ^{LIER} IGTLYVG ^{FRT} VMGLFLLITGRPCSLQR	360
361	ICQLAKNGTNRTFDMTVQDIYGTSYSNAGLCKDLTASFFGNAQH ^{IE} NVKNILNTYDEEKN	420
421	INIKEEDDKLMNVYEYENESSCYENVSSCMSTEISECQEIFETEECIGFEVEKNNINYYR	480
481	NKYSFLSKDKTKKLLVSNKNFN ^{VY} DNCKIPIFNFNSDSYCNMNSFKSKEKLFKH ^{NIE} EQ	540
541	QKQEEGYKQKEDDNFLMNDIDNYLSIKHSLSDNEINMYEYHRKNYNLKK ^{SFL} KKLFKKQY	600
601	LNENGKIIHNINLSIQKEVSSFILKNKKRFSYKTYDKQIVKKACKSITLKNVCSETNKNK	660
661	MDSNLKDKTIVKYNTKMCDLSRSLLSMAIFTTVYLSYIHCNLYNADHIFTGYNFEDDVC	720
721	KELFQIIIKFLSHNRQKIYFAKISKYISSLGSAIELINWERIHIDN	766

Coverage = 29.2%

PfPanK2 coverage from PanK2-GFP co-immunoprecipitation

1	MGNTLGIECSFNIVHVTTLINKKLIKESNNDSKNEKDIREEKEKKLPKNVSI ^{PS} NDNKL	60
61	NMNVHLSWLKEKYKKEY ^{IN} LEEDVSKSDEYNNCDDDIYKMKKNTFSYILDMQHIIDS ^{SD} VQ	120
121	VFSLKKNSEKNVHTHISINELYKCFHEDDNLEKKFIKYLNFVKYHKLDISQIDTVNIQHL	180
181	YDDEIAEIIYFWSFKLKY ^{LD} ECILKYYKKNINYMFINVTGKNKNLIK ^{KKFL} QITGKNNIFQ	240
241	HNEIKCINNSICFLKR ^{FM} PTNLYYFTKYNEENERASCTSQNDEDEK ^{KKKK} KNLLYQSFI	300
301	NKEEVSEIKSYVIVNMKRAVCYHLVNEQN ^{LIER} IGTLYVG ^{FRT} VMGLFLLITGRPCSLQR	360
361	ICQLAKNGTNRTFDMTVQDIYGTSYSNAGLCKDLTASFFGNAQH ^{IE} NVKNILNTYDEEKN	420
421	INIKEEDDKLMNVYEYENESSCYENVSSCMSTEISECQEIFETEECIGFEVEKNNINYYR	480
481	NKYSFLSKDKTKKLLVSNKNFN ^{VY} DNCKIPIFNFNSDSYCNMNSFKSKEKLFKH ^{NIE} EQ	540
541	QKQEEGYKQKEDDNFLMNDIDNYLSIKHSLSDNEINMYEYHRKNYNLKK ^{SFL} KKLFKKQY	600
601	LNENGKIIHNINLSIQKEVSSFILKNKKRFSYKTYDKQIVKKACKSITLKNVCSETNKNK	660
661	MDSNLKDKTIVKYNTKMCDLSRSLLSMAIFTTVYLSYIHCNLYNADHIFTGYNFEDDVC	720
721	KELFQIIIKFLSHNRQKIYFAKISKYISSLGSAIELINWERIHIDN	766

Coverage = 48.6%

Figure 4.S2. Mass spectrometry coverage of *PfPanK2*. *PfPanK2* peptides detected in the mass spectrometry analysis of PanK1-GFP and PanK2-GFP co-immunoprecipitation using GFP-trap. Residues in green were detected with $\geq 95\%$ confidence, while residues in orange were detected with $\geq 90\%$ confidence. Percentage coverages were calculated using only the residues labelled green.

Pf14-3-3l coverage from PanK1-GFP co-immunoprecipitation

1	MATSEELKQLRCDCTYRSKLAEQAERYDEMADAMRTLVEQCVNNDKDELTVEERNLLSVA	60
61	YKNAVGARASWRIISSVEQKEMSKANVHNKNVAATYRKKVEEELNNICQDILNLLTKKL	120
121	IPNTSESESKVFYYKMKGDYYRYISEFSCDEGKKEASNCAQEAYQKATDIAENELPSTHP	180
181	IRLGLALNYSVFFYEILNQPHQACEMAKRAFDDAITEFDNVSEDSYKDSTLIMQLLRDNL	240
241	TLWTSDLQGDQTEEKSKDEGLE	262

Coverage = 43.1%

Pf14-3-3l coverage from PanK2-GFP co-immunoprecipitation

1	MATSEELKQLRCDCTYRSKLAEQAERYDEMADAMRTLVEQCVNNDKDELTVEERNLLSVA	60
61	YKNAVGARASWRIISSVEQKEMSKANVHNKNVAATYRKKVEEELNNICQDILNLLTKKL	120
121	IPNTSESESKVFYYKMKGDYYRYISEFSCDEGKKEASNCAQEAYQKATDIAENELPSTHP	180
181	IRLGLALNYSVFFYEILNQPHQACEMAKRAFDDAITEFDNVSEDSYKDSTLIMQLLRDNL	240
241	TLWTSDLQGDQTEEKSKDEGLE	262

Coverage = 66.8%

Figure 4.S3. Mass spectrometry coverage of *Pf14-3-3l*. *Pf14-3-3l* peptides detected in the mass spectrometry analysis of PanK1-GFP and PanK2-GFP co-immunoprecipitation using GFP-trap. Residues in green were detected with $\geq 95\%$ confidence, while residues in orange were detected with $\geq 90\%$ confidence. Percentage coverages were calculated using only the residues labelled green.

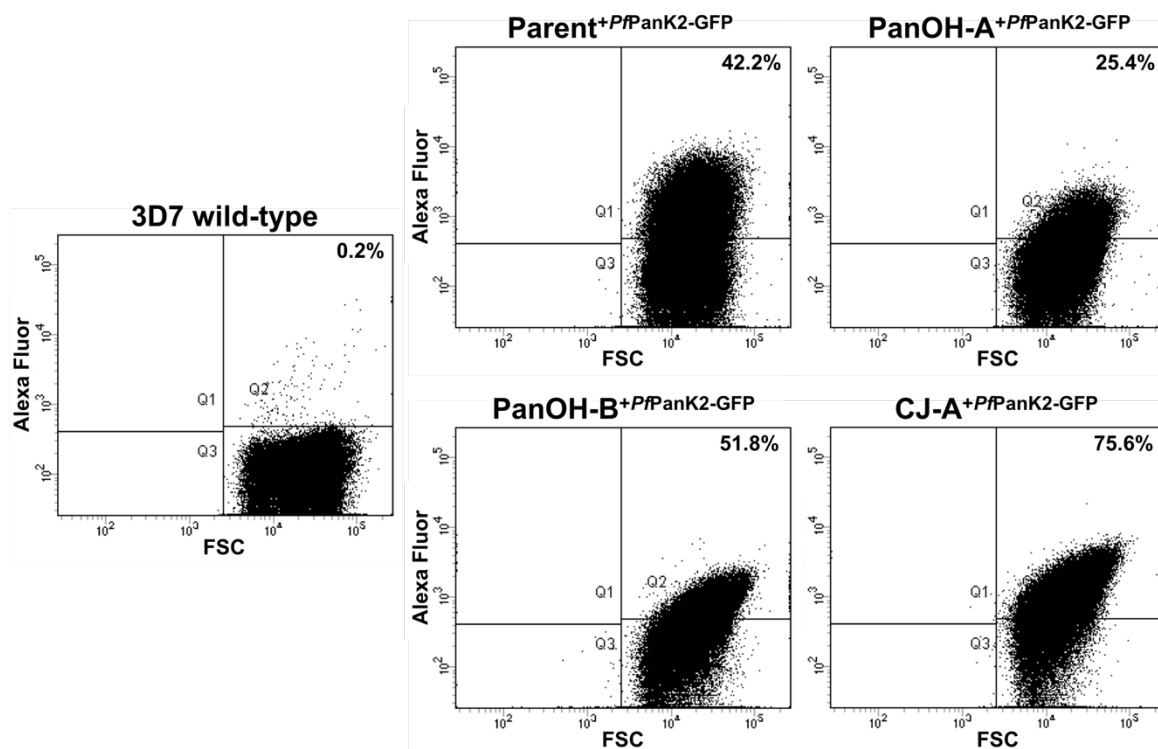


Figure 4.S4. Proportion of GFP-positive cells in *PfPanK2*-GFP-expressing Parent and mutant cell lines. FACS analysis of saponin-isolated 3D7 wild-type, Parent^{+*PfPanK2*-GFP}, PanOH-A^{+*PfPanK2*-GFP}, PanOH-B^{+*PfPanK2*-GFP} and CJ-A^{+*PfPanK2*-GFP} trophozoites. The forward scatter (FSC) intensity on each x-axis corresponds to increasing cell size and the Alexa Fluor intensity on each y-axis corresponds to increasing GFP fluorescence. The proportion of GFP-positive cells in each transfectant line was determined by setting-up a gating strategy using 3D7 wild-type trophozoites as a threshold below which parasites were defined to be auto-fluorescent. The percentage value in the upper-right quadrant of each plot indicates the proportion of GFP-positive cells for that line. Data shown are representative of three independent experiments, each analysed prior to generating the co-immunoprecipitated fractions used in the [¹⁴C]pantothenate phosphorylation assay presented in Figure 4.3 b.

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Chapter 5: General Discussion

5.1. The *P. falciparum* PanK Complex

5.1.1. Summary of results

The malaria parasite, *P. falciparum*, takes up extracellular pantothenate from its host and converts it into CoA through a series of five universal enzymatic steps. Candidate genes predicted to code for these enzymes are present in the parasite's genome and are transcribed during the intraerythrocytic stage of its lifecycle. *PfPanK*, the enzyme that catalyses the first step of *P. falciparum* CoA biosynthesis, is one of three enzymes in the pathway that are encoded by multiple candidate genes. One of the aims of this thesis was to validate the functions of *PfPanK1* and *PfPanK2*, and determine which of these enzymes is the active PanK in the intraerythrocytic stage of *P. falciparum*. In **Chapter 3**, it was shown that parasites resistant to the pantothenate analogues PanOH and CJ-15,801 harbour mutations in *PfPanK1*, consistent with *PfPanK1* being the active PanK during this stage of the parasite's lifecycle. Results presented in **Chapter 4** subsequently showed that *PfPanK* exists as a *PfPanK1*-*PfPanK2* heterodimer. The *PfPanK* dimer was also shown to associate with another dimeric protein, *Pf14-3-3I*, which belongs to a class of proteins known to perform a plethora of functions in other eukaryotic organisms.

5.1.2. The active sites of the *PfPanK* heterodimer and other roles of *PfPanK2*

In this study, it was shown that *PfPanK1* associates with *PfPanK2* to form a heterodimeric *PfPanK* complex which also interacts with *Pf14-3-3I*. However, the specific role(s) that each protein performs remains to be elucidated. Mutations of the *PfPanK1* residues reported in **Chapter 3**, Gly95 and Asp507, are able to directly influence the efficiency of *PfPanK* at phosphorylating pantothenate and also the ability of various pantothenate analogues to inhibit this activity. The Gly95Ala mutation or deletion is predicted to impact on the ability of the $\alpha 2$ -helix to change its conformation when the enzyme changes from an inactive to an active state, while the Asp507Glu mutation is proposed to hinder the enzyme from stabilising its inactive conformation. Both of these *PfPanK1* mutations are not expected to cause any changes to *PfPanK2* or affect the ability of *PfPanK1* to dimerise with *PfPanK2* as they are not located within the predicted dimerisation

sites, although these possibilities cannot be completely excluded. Furthermore, no mutations were induced in *Pf*PanK2 from drug-pressuring the parasites. While these results are consistent with *Pf*PanK1 being responsible for phosphorylating pantothenate, they raise questions about the role of *Pf*PanK2 in the complex.

Comparing the sequences of *Pf*PanK1 and *Pf*PanK2 with those of other type II PanKs that have solved structures, such as human PanK3, gives useful information that support the formation of a heterodimeric *Pf*PanK and also reveal the possible function of each protein. As discussed in **Chapter 4**, all of the PanKs that have been characterised so far form homodimers with two functional active sites (Yun *et al.*, 2000; Das *et al.*, 2006; Yang *et al.*, 2006a; Hong *et al.*, 2006; Nicely *et al.*, 2007; Hong *et al.*, 2007; Li *et al.*, 2013; Franklin *et al.*, 2015). In human PanK3, a representative of a type II PanK, the active sites can be forced into an inactive/open conformation by the binding of the allosteric inhibitor acetyl-CoA or stabilised into an active/closed conformation by the binding of ATP and pantothenate (Subramanian *et al.*, 2016). When the enzyme binds acetyl-CoA, the adenine ring of the inhibitor acts as a wedge to prevent the closure of the enzyme's ATP binding domain (Leonardi *et al.*, 2010). This places the critical Glu138 catalytic residue in the wrong position and maintains the enzyme in its inactive conformation (Leonardi *et al.*, 2010). When the tightly-bound acetyl-CoA is displaced by AMPPNP (a non-hydrolysable ATP analogue introduced in **Chapter 3**) and pantothenate, the adenine ring of AMPPNP is rotated $\sim 180^\circ$ compared to that of acetyl-CoA, allowing the nucleotide binding domain to close and interact with AMPPNP, and repositions the Glu138 residue to perform its catalytic function (Subramanian *et al.*, 2016). Each active site is formed by different parts of the identical monomers (Hong *et al.*, 2007; Leonardi *et al.*, 2010; Subramanian *et al.*, 2016). More specifically, the pantothenate binding site is formed between the $\beta 10$ - $\beta 11$ strands of the monomer that binds ATP·Mg²⁺ (referred to herein as monomer A) and the loop between $\alpha 7'$ - $\beta 13'$ from the opposite monomer (referred to herein as monomer B, residues of which are annotated with an apostrophe), with the latter forming a rigid "lid" in the active conformation of the enzyme (Leonardi *et al.*, 2010; Subramanian *et al.*, 2016) (**Figure 5.1 a**). Several residues on both monomers have been shown to be important for the interaction between pantothenate and PanK3. The monomer

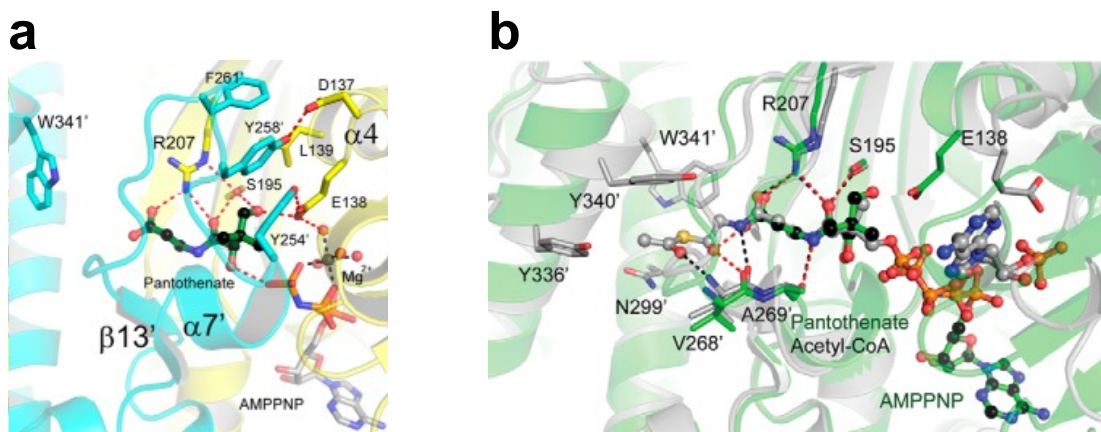


Figure 5.1. Interactions between substrates and the active site of human PanK3. (a) The active site structure of the PanK3-AMPPNP-Mg²⁺-pantothenate complex (PDB ID: 5KPR). The active site residues derived from the ATP-binding monomer (monomer A) is shown in yellow and the residues of the opposite monomer (monomer B) is shown in cyan. (b) Superimposed structures of the PanK3-AMPPNP-Mg²⁺-pantothenate complex (green) and the PanK3-acetyl-CoA complex (grey; PDB ID: 3MK6). The overlapping sections of pantothenate and acetyl-CoA interact with the same PanK3 active site residues. Hydrogen bonds in the PanK3-AMPPNP-Mg²⁺-pantothenate and PanK3-acetyl-CoA complexes are shown as red and black dashed lines, respectively. Figures are reproduced from Subramanian *et al.* (2016).

A residues Glu138, Ser195 and Arg207, and the monomer B residues Val268' and Ala269' have been shown to form hydrogen bonds with the pantothenate molecule (**Figure 5.1 a & b**). As discussed above, Glu138 has also been confirmed to be the critical catalytic residue required for the activation of the hydroxyl in pantothenate for attack on the phosphate of ATP (Subramanian *et al.*, 2016). The interactions between other residues on monomer A (Asp137, Glu138 and Leu139) and monomer B (Tyr254', Tyr258' and Phe261') were found to stabilise the "lid" and also the bound pantothenate substrate (**Figure 5.1 a**). In particular, the hydrogen bond that forms between Glu138 and Tyr254' plays an important role in the allosteric activation of the enzyme (Subramanian *et al.*, 2016). All of these residues are perfectly conserved in the other human PanK isoforms except for PanK4 (**Figure 5.2**), which has been reported to be inactive (Zhang *et al.*, 2007). A previous structure-based sequence alignment analysis comparing *Pf*PanK1 and *Pf*PanK2 with other type II PanKs found that the PHOSPHATE 1 and PHOSPHATE 2 motifs were conserved in *Pf*PanK1 but not *Pf*PanK2, which suggests that the latter's ability to bind ATP (i.e. to act as monomer A) could be greatly impeded (Spry *et al.*, 2010) (**Figure 5.2**). Most of the residues identified in human PanK3 to be important for pantothenate binding (Glu138, Arg207 and Ala269') are conserved in both *Pf*PanKs (blue boxes in **Figure 5.2**). The Ser195 residue in monomer A of human PanK3 also remains unchanged in *Pf*PanK1 but is replaced with a Cys in *Pf*PanK2 (**Figure 5.2**). Although the thiol group in the side chain of Cys could, in theory, still form hydrogen bonds, they would be longer than those formed by hydroxyl groups due to the larger size of the sulphur atom (Gregoret *et al.*, 1991). When this Ser residue was mutated to a Val in human PanK3, the bulkier side chain of Val increased the enzyme's K_m for both pantothenate and pantetheine, and made it refractory to inhibition by acetyl-CoA (Leonardi *et al.*, 2010). How the change to Cys at this position impacts *Pf*PanK2's ability to bind pantothenate remains to be seen. The monomer B Val268' residue of human PanK3 is replaced with Leu in *Pf*PanK1 and Thr in *Pf*PanK2 (**Figure 5.2**). This is not expected to abolish the hydrogen bond between the residue and pantothenate as it is the carbonyl backbone of the residue that forms the important interaction (Leonardi *et al.*, 2010; Subramanian *et al.*, 2016). However, it is worth noting that *Pf*PanK1 is the only type II PanK analysed with Leu at this position (**Figure 5.2**). Interestingly, when either of the neighbouring Ala residues (Ala267' and Ala269') were replaced with Phe in human PanK3, the much bulkier

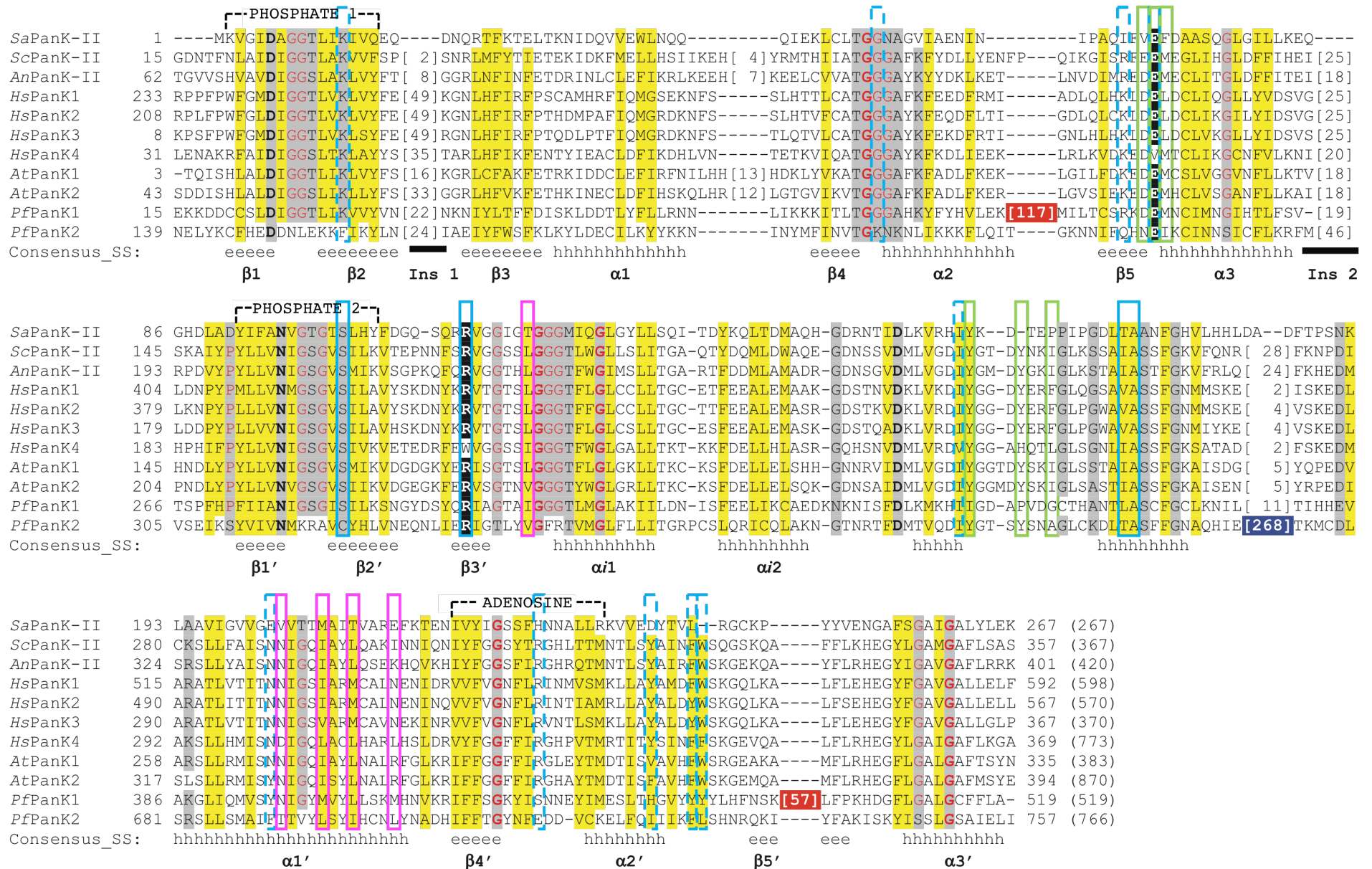


Figure 5.2. Structure-based multiple sequence alignment of representative type II PanK enzymes. The conserved PHOSPHATE 1, PHOSPHATE 2, and ADENOSINE motifs of the ASKHA superfamily of kinases are labelled at the top of the alignment. The Glu residue involved in catalysis and the Arg residue involved in positioning the substrate are annotated on a black background. Residues that were found to interact with pantothenate and acetyl-CoA in human PanK3 are marked within blue boxes, and those that interact specifically with acetyl-CoA are marked within dashed blue boxes. Monomer A and monomer B residues that were found to interact to form the human PanK3 active site are marked within green boxes. The catalytic Glu residue is marked within a box with blue and green line as it is involved in both the interaction with pantothenate and the stabilisation of the active site through interaction with a Tyr residue of the opposite monomer. Residues contained within pink boxes were found to be important for human PanK3 dimerisation. Conserved residues are highlighted as follows: identical = bold, uncharged = yellow, charged/polar/small = grey, and Gly = red. The two insertion regions (Ins 1 and Ins 2) common in eukaryotic type II PanKs but are absent in prokaryotic PanKs (represented by SaPanK-II) are indicated by the black bars, while the PfPanK1- and PfPanK2-specific inserts are highlighted on a red and blue background, respectively. Consensus secondary structure elements (Consensus_SS; e = β -strand, h = α -helix) are marked below the alignment. The numbers at the start and end of each sequence indicate the position of the first and last residue, respectively. The lengths of insertions are specified within the square brackets and the total lengths of protein sequences are shown in the parentheses. Species names are abbreviated as follows: Sa = *Staphylococcus aureus*, Sc = *Saccharomyces cerevisiae*, An = *Aspergillus nidulans*, Hs = *Homo sapiens*, At = *Arabidopsis thaliana* and Pf = *Plasmodium falciparum*. Figure is adapted from Spry *et al.* (2010).

benzene ring of Phe resulted in a catalytically inactive enzyme despite it being correctly folded (Leonardi *et al.*, 2010). The residues found to be important for stabilising the pantothenate binding site in human PanK3 are conserved to different extents in *Pf*PanK1 and *Pf*PanK2 (green boxes in **Figure 5.2**). The Asp137 residue of monomer A is conserved in *Pf*PanK1 but is substituted to an uncharged Asn in *Pf*PanK2 (Spry *et al.*, 2010). The Leu139 of human PanK3 monomer A is not conserved in either *Pf*PanKs. In *Pf*PanK1, however, it is replaced with a Met, which is also the case in the type II PanKs of *Aspergillus nidulans*, *Arabidopsis thaliana* and *S. cerevisiae* (**Figure 5.2**) that have been shown to be functional (Calder *et al.*, 1999; Tilton *et al.*, 2006; Olzhausen *et al.*, 2009). On the other hand, *Pf*PanK2 is the only PanK in the analysis with an Ile at this position (**Figure 5.2**). Interestingly, the monomer B Tyr254' and Tyr258' residues of human PanK3 are perfectly conserved in *Pf*PanK2, and all other functional type II PanKs in the analysis, but *not Pf*PanK1 where they have been replaced with the hydrophobic amino acids Ile and Phe, respectively (**Figure 5.2**). Both Ile and Phe lack the hydroxyl group in their side chains required to form the hydrogen bonds with the Asp137 (or Asn in the case of *Pf*PanK2 as discussed above) and Glu138 of monomer A to stabilise the active site (**Figure 5.1 a**). The Phe261' residue is not conserved in either *Pf*PanKs (**Figure 5.2**). In summary, relative to the other type II PanKs included in the analysis, there is a greater degree of conservation for the critical monomer A residues in *Pf*PanK1, while the important monomer B residues are surprisingly better-conserved in *Pf*PanK2. Taken together, the results generated in this study and these observations are consistent with *Pf*PanK1 performing the role of monomer A (i.e. the catalytic pocket in *Pf*PanK1 being the main, if not only, active site of the *Pf*PanK heterodimer), while *Pf*PanK2 functions as monomer B (i.e. its $\alpha 7'$ - $\beta 13'$ loop forms the rigid "lid" over the active site in the active conformation of the *Pf*PanK heterodimer).

In addition to its postulated function as monomer B in the enzyme's active site, *Pf*PanK2 could play other roles as part of the *Pf*PanK heterodimer. As shown in **Chapter 3** and **4**, neither *Pf*PanK1 nor *Pf*PanK2 are excluded from the nucleus of the parasite. Moreover, it is postulated in **Chapter 4** that *Pf*PanK2, which possesses three predicted NLSs in its sequence, could play a role in directing the complex into the parasite's nucleus. While the specific purpose of having the

PfPank dimer in the nucleus is not immediately clear, it was suggested that the enzyme could be involved in the CoA homeostasis within the organelle (**Chapter 4**), a function that was postulated for the nucleus-bound isoform of human Pank2 (Hörtnagel *et al.*, 2003; Alfonso-Pecchio *et al.*, 2012). *PfPank2* could also function as the regulator of *PfPank* activity. As discussed earlier, the human Pank3 homodimer undergoes a conformational change when it transitions from the inactive/open state to the active/closed state (Subramanian *et al.*, 2016). Importantly, this conformational change is synchronised between both active sites by the $\alpha 10$ helix of each monomer. The amino terminus of the helix is situated next to the ATP-binding site of one monomer, while its carboxyl end is adjacent to the acetyl-CoA binding pocket of the opposite monomer (Subramanian *et al.*, 2016). Binding of acetyl-CoA to one active site induces a conformational change in the $\alpha 10$ helix that makes it conducive for another acetyl-CoA molecule to bind to the opposite active site, while the binding of ATP and pantothenate results in a different conformational change that promotes both active sites to switch into the active/closed state (Subramanian *et al.*, 2016). Moreover, introducing a Gly19Val mutation into one of the monomer abrogated the cooperative binding of the enzyme to ATP but did not appear to affect its synchronised binding to acetyl-CoA (Subramanian *et al.*, 2016). If a similar cooperative regulatory mechanism occurs in the *PfPank* heterodimer, then *PfPank2* could act as an “off switch” as it is (likely) unable to bind ATP but is potentially able to bind to acetyl-CoA through its pantothenate binding site; *PfPank* may be more prone to bind its allosteric inhibitors than ATP because of *PfPank2*. Interestingly, one of the *Pf14-3-3I* binding sites identified in **Chapter 4**, Tyr336, is adjacent to the pantothenate binding site of *PfPank2*. Binding of *Pf14-3-3I* at this site could prevent acetyl-CoA from binding *PfPank2* and potentially “switch on” the enzyme. A previous study has shown that the activity of *PfPank* can be inhibited by CoA and its thioesters (acetyl-CoA and malonyl-CoA) (Lehane *et al.*, 2007). However, many of the human Pank3 residues identified to be involved in the specific binding to acetyl-CoA (Lys24, Gly116, Lys135, Ile253', Tyr254', Asn299', Arg325, Tyr336', Tyr340' and Trp341') (Leonardi *et al.*, 2010; Subramanian *et al.*, 2016) are not conserved in *PfPank1* or *PfPank2* (blue boxes with dashed line in **Figure 5.2**). It is interesting to note that one of these residues (Gly116 in human Pank3) is positioned next to the Gly95 residue in *PfPank1* that was found to be important for normal *PfPank*

function in **Chapter 3**, further implicating Gly95 in the allosteric regulation of *PfPank*. All in all, it is unclear if these differences in amino acid residues ultimately influence the binding of CoA and its thioesters to either *PfPank* and/or the synchronised regulatory mechanism, if it is indeed present in the enzyme, and this warrants further investigation (discussed in **Section 5.1.3**).

Although the results of this study are consistent with *PfPank1* and *PfPank2* forming the *PfPank* heterodimer, it is not obvious whether the two monomers achieve this by associating in the same manner as the human PankS. The residues that were identified to interact in the dimerisation interface of human Pank3 (Hong *et al.*, 2007) are not perfectly conserved in both *PfPank*s, although most have side chains of similar properties and/or share conservancy with other type II PankS (pink boxes in **Figure 5.2**). Furthermore, it remains unclear whether the *PfPank2*-specific 268 amino acid insert near its dimerisation interface (Spry *et al.*, 2010) or the *Pf14-3-3I* dimer (**Chapter 4**) play any role in how *PfPank1* and *PfPank2* associate to form the dimer. However, given that no convincing 14-3-3 binding site was predicted for *PfPank1* and that it was not detected in any of the phosphoproteomic studies (Treeck *et al.*, 2011; Solyakov *et al.*, 2011; Lasonder *et al.*, 2012; Pease *et al.*, 2013) discussed in **Chapter 4**, *Pf14-3-3I* is unlikely to play a role in the dimerisation of *PfPank*.

5.1.3. Verifying the roles of *PfPank1*, *PfPank2* and *Pf14-3-3I*

In this study, several key residues have been identified (for *PfPank1* in **Chapter 3**) or predicted (for *PfPank2* in **Chapter 4** and **Section 5.1.2**) to be important for the functions of the *PfPank* monomers. The importance of these residues could be verified through site-directed mutagenesis, a strategy that was used to confirm and characterise the critical residues of human Pank3 (Hong *et al.*, 2007; Leonardi *et al.*, 2010; Subramanian *et al.*, 2016) discussed in the previous section. For example, the catalytically-important Glu243 residue of *PfPank2* could be mutated and the impact this has on the activity of the *PfPank* dimer could be determined using the [¹⁴C]pantothenate phosphorylation assay that was used in this study. This would indicate if the *PfPank2* monomer has a functional active site. The same mutagenesis strategy could also be used in conjunction with western blot and/or mass spectrometry analysis to determine how the dimerisation of *PfPank* or its ability to bind to the *Pf14-3-3I* dimer is

affected when the residues predicted to be important for these functions are replaced. However, it would be time-consuming to perform site-directed mutagenesis on all of the important residues that were identified. Furthermore, not all of the residues can be functionally characterised in this manner. To give an example, the Val268' and Ala269' residues of monomer B was shown to interact with acetyl-CoA and pantothenate through their backbone carbonyl (Leonardi *et al.*, 2010; Subramanian *et al.*, 2016). These interactions cannot be studied by replacing the amino acid residues at these positions as all of them possess the same carbonyl group.

Ultimately, the most informative way to characterise these residues, the binding of *Pf*PanK to its substrates and inhibitors, and the interactions between the *Pf*PanK complex constituents in general would be to solve the structure of the *Pf*PanK complex through either X-ray crystallography or single-particle cryo-electron microscopy (cryo-EM). The latter technique is particularly attractive as it results in structural information that better reflects the protein in its native conformation (Vénien-Bryan *et al.*, 2017). It is also better suited for large protein complexes and also proteins that have multiple conformational states (Vénien-Bryan *et al.*, 2017). Additionally, the amount of sample required for cryo-EM is smaller than what is needed for X-ray crystallography (Wang and Wang, 2017), although sample preparation remains a critical aspect of cryo-EM, with sample homogeneity being especially important (Vénien-Bryan *et al.*, 2017). The two strategies that can be employed to obtain pure *Pf*PanK for structural determination were introduced in **Chapter 4**, each with their own limitations. The *Pf*PanK complex can be generated through a heterologous expression system. However, previous attempts at using this strategy to produce *Pf*PanK1 and *Pf*PanK2 generated either insoluble (Mehlin *et al.*, 2006) or soluble but inactive proteins (Saliba Lab, unpublished data). Based on the results of this study, the latter is likely due to the fact that *Pf*PanK is a heterodimeric enzyme, with the association between *Pf*PanK1 and *Pf*PanK2 predicted to be essential for its activity. With this in mind, a functional *Pf*PanK complex could be generated by assembling together the independently-expressed monomers, and potentially the *Pf*14-3-3I dimer. It is important to note, however, that the *Pf*PanK2 monomer would need to be phosphorylated if the *Pf*PanK complex does require the *Pf*14-3-3I dimer to function. The advantage of this strategy, if a functional *Pf*PanK

can be generated, is that relatively large quantities of each component can be generated through heterologous expression. Alternatively, the *Pf*PanK complex can also be purified through a co-immunoprecipitation approach that was employed in **Chapter 4**. In order to eliminate the possibility of the GFP epitope tag having any effect on the structure of the *Pf*PanK complex, a proteolytic site could be introduced in between the tag and *Pf*PanK1/*Pf*PanK2 amino acid sequence, which will allow for the GFP protein to be removed from the complex after co-immunoprecipitation. Although it is shown in this study that the co-immunoprecipitation method generates a functional *Pf*PanK complex **Chapter 4**, it could be challenging to obtain sufficient amount of the protein complex for cryo-EM. If the co-immunoprecipitation approach does generate sufficient amount of material for structural analysis, this strategy could be a framework for the characterisation of downstream CoA biosynthesis pathway enzymes, which remain poorly studied. Firstly, the epitope tagging would enable the localisation and purification of the protein of interest. Secondly, when used in conjunction with enzyme coupled assays that allow the quantification of ATP consumption (Koresawa and Okabe, 2004), adenosine diphosphate (ADP) production (Tai *et al.*, 2011) or pyrophosphate production (Strauss *et al.*, 2004), this strategy allows for the functional characterisation of the immunoprecipitated proteins.

As discussed in this study, several NLSs are predicted in the sequence of *Pf*PanK2 (**Chapter 4**), which could play a role in localising some of the *Pf*PanK to the parasite's nucleus (**Chapter 3 and 4**). A transgenic reporter system similar to what was used to localise *Pf*PanK1 and *Pf*PanK2 in this study could be employed to verify if these NLSs are functional. For example, the NLSs in an episomal copy of an epitope-tagged *Pf*PanK2 could be mutated to render them non-functional and any impact these would have on the localisation of the *Pf*PanK complex could be determined. Alternatively, if any of these sequences are prohibitively difficult to mutate, a different strategy can be used. This involves fusing multiple copies (as proteins < 40 kDa can freely diffuse into and accumulate in the nucleus (Terry *et al.*, 2007)) of the reporter protein sequence directly to the 5' and/or 3' end(s) of the *Pf*PanK2 NLS sequence. The localisation of this fusion protein when expressed in the parasite would indicate whether the NLS is able to facilitate the transport of *Pf*PanK into the nucleus. In *P. falciparum*, this method was first used to confirm the function of a N-terminal bipartite leader

sequence as an apicoplast targeting sequence (Waller *et al.*, 2000). A similar strategy has since been used to verify predicted NLS sequences of various *P. falciparum* proteins by expressing the NLS-reporter fusion protein in the parasite (Russo *et al.*, 2009) or other eukaryotic expression systems (Chan *et al.*, 2006; Lin *et al.*, 2009; Babar *et al.*, 2016). Future experiments could also explore the role of the *Pf*PanK complex in the nucleus. This could be achieved by generating and characterising the phenotypes of parasites that express an endogenous mutant *Pf*PanK2 lacking the functional NLS. However, as previous attempts at knocking-out *Pf*PanK1 (**Chapter 3**) have shown, this could be challenging if the presence of the *Pf*PanK dimer in the nucleus is essential for the survival of the parasite in its intraerythrocytic stage. A promising alternative strategy that can be employed is called “knock sideways”, a technique that is now coupled with a recently described selection-linked integration (SLI) method that allows for a more effective and rapid genomic integrations of constructs (Birnbaum *et al.*, 2017). The knock sideways strategy makes use of a chemically-induced dimerisation system in which two proteins, termed FKBP and FRB*, bind in the presence of a rapalog ligand (Birnbaum *et al.*, 2017). By tagging *Pf*PanK2 with FKBP in a parasite that expresses a cytosolic or membrane-bound FRB*, the presence of rapalog would shuttle the *Pf*PanK complex out of the nucleus. This conditional removal would therefore allow for the characterisation of the *Pf*PanK complex function in the nucleus without the need to modify the native *Pf*PanK2 protein sequence.

5.1.4. Heterodimeric PanKs in other organisms

The finding of this study that *Pf*PanK exists as a heterodimer (**Chapter 4**) raises the possibility that other organisms could possess PanKs with a heterodimeric conformation. As introduced in previous chapters, PanKs can be broadly categorised into three groups, namely type I, II or III (Brand and Strauss, 2005). Most bacterial species express the type I PanK (as exemplified by the CoaA enzyme in *E. coli* (Song and Jackowski, 1992)), while type II PanKs are found mainly in eukaryotic organisms although some are found in prokaryotes (for example the *S. aureus* PanK (Leonardi *et al.*, 2005)) (Brand and Strauss, 2005). The third type is a group of PanKs in several species of pathogenic bacteria that do not share any sequence similarity to type I and type II PanKs (Brand and Strauss, 2005). Interestingly, some bacterial species carry multiple types of PanK.

For example, some species of *Bacillus* (Yocum *et al.*, 2004; Nicely *et al.*, 2007; Ogata *et al.*, 2014), *Mycobacterium* (Awasthy *et al.*, 2010) and *Streptomyces* (Nicely *et al.*, 2007) possess both type I and type III PanKs. On the other hand, *Bacillus anthracis* (Nicely *et al.*, 2007; Paige *et al.*, 2008) and *Bacillus cereus* (Nicely *et al.*, 2007) express both type II and type III PanKs. This phenomenon is not unique to prokaryotes. The yeast *S. cerevisiae* expresses a functional PanK, termed CAB1 (Olzhausen *et al.*, 2009), that shares some sequence similarity to a type II PanK in another fungal species, *Aspergillus nidulans* (Calder *et al.*, 1999). However, *S. cerevisiae* also expresses another protein that is structurally similar to the *E. coli* type I PanK, indicating that it could function as a PanK, although this is yet to be biochemically verified (Li de La Sierra-Gallay *et al.*, 2004). In *Bacillus subtilis*, the two PanKs that are expressed appear to play a redundant function (Yocum *et al.*, 2004). However, the type II PanK in *B. anthracis* (Nicely *et al.*, 2007) and the type III PanK in *Mycobacterium tuberculosis* (Awasthy *et al.*, 2010) have been reported to lack PanK activity. In these cases, it is unclear why these organisms express PanKs of multiple types despite some of them appearing to be non-functional. It is possible that, like PfPanK1 and PfPanK2, these different PanK types need to form heterodimers in order to carry out their function.

Although eukaryotes express almost exclusively type II PanKs, many higher eukaryotes have been found to express multiple PanK isoforms encoded by different genes. For example, *Arabidopsis thaliana* expresses two isoforms of PanKs, AtPanK1 and AtPanK2, that are functionally complementary (Kupke *et al.*, 2003; Tilton *et al.*, 2006). On the other hand, mice express four catalytically active PanK isoforms (PanK1 α , PanK1 β , PanK2 and PanK3) that exhibit varying levels of expression in different tissues and also different sensitivities to inhibition by CoA and its thioesters (Rock *et al.*, 2000; Rock *et al.*, 2002; Kuo *et al.*, 2005; Zhang *et al.*, 2005; Leonardi *et al.*, 2007b). Orthologues of these proteins are also present in humans (Zhou *et al.*, 2001; Ni *et al.*, 2002; Hörtnagel *et al.*, 2003; Ramaswamy *et al.*, 2004), with the human PanK2 being uniquely targeted to the mitochondria (Hörtnagel *et al.*, 2003). Although these mammalian PanK isoforms have been shown to exist as homodimers (Hong *et al.*, 2007), it has never been investigated whether they also associate into heterodimers *in vivo*. The conservation of most of the critical residues required for dimerisation and active site formation across these functional PanK isoforms (**Figure 5.2**) is certainly

consistent with them being able to form heterodimers. In addition, mammals also express another PanK isoform, designated as PanK4, which is most abundantly expressed in muscle (Zhou *et al.*, 2001). PanK4 has been reported to be catalytically inactive (Zhang *et al.*, 2007), likely due to the fact that it does not possess the critical Glu and Arg residues required for PanK activity (**Figure 5.2**). Surprisingly, however, the expression of human PanK4 was able to rescue the growth of *E. coli* (Hört nagel *et al.*, 2003) and survival of *Drosophila* (Wu *et al.*, 2009) that had their endogenous PanK knocked out. It therefore remains unclear whether human PanK4 possesses PanK activity (as suggested by the results of these complementation assays) and whether the protein heterodimerises with other human PanKs in order to function.

Alternatively, it is also possible that the heterodimeric PanK is a phenomenon unique to *Plasmodium* species or apicomplexans in general. Although PanKs have been shown to be dispensable during the intraerythrocytic stages of *P. berghei* and *P. yoelii*, knockout mutant parasites lacking *either* PanK1 or PanK2 showed defective oocyst development in mosquitoes (Hart *et al.*, 2016; Srivastava *et al.*, 2016). This indicates that it is essential for *both* PanKs to be expressed during this developmental stage of these murine *Plasmodium* species, which is consistent with the two PanKs in these parasites either performing distinct functions or coming together to perform the same function as a heterodimer. As of the writing of this thesis, little is known about the PanKs of other apicomplexans. There are two proteins in the genome of the closely related *Toxoplasma gondii* that have been annotated as PanKs (Gajria *et al.*, 2008) (one is annotated as a fumble protein, which is the sole PanK in *Drosophila* (Afshar *et al.*, 2001)). Intriguingly, both of these PanKs (TGME49_235478 and TGME49_307770) have been shown to be important for the cellular fitness of *T. gondii* tachyzoites (mean phenotypic scores of -6 and -4, respectively, with a more negative score denoting a greater contribution to parasite fitness) (Sidik *et al.*, 2016), despite one of the PanK (TGME49_235478) lacking the conserved PHOSPHATE1 and PHOSPHATE2 motifs (data not shown) as seen in *Pf*PanK2.

5.2. The mechanisms of action of antiplasmodial pantothenate analogues

5.2.1. Summary of results

The spread of ACT-resistant *P. falciparum* strains is a growing concern and it highlights the need for new drugs to combat malaria. During its intraerythrocytic stage, *P. falciparum* absolutely requires an exogenous supply of pantothenate, which it uses to synthesise CoA. This dependency on the host for pantothenate makes the CoA biosynthesis pathway of *P. falciparum* an attractive drug target, especially because the enzymes involved are distinct from their counterpart in the human host (Spry *et al.*, 2008). Many pantothenate analogues, including PanOH (Saliba *et al.*, 2005), CJ-15,801 (Saliba and Kirk, 2005), PanAms (Spry *et al.*, 2013) and PanAm derivatives (Macuamule *et al.*, 2015; Howieson *et al.*, 2016) have been shown to inhibit the growth of *P. falciparum* by interfering with its CoA biosynthesis and/or utilisation. However, their exact mechanisms of action have not been characterised in detail. It is shown in **Chapter 3** that PanOH and CJ-15,801 share a common mechanism of action, which is distinct from that of N5-trz-C1-Pan and N-PE- α Me-PanAm. It is also shown that N5-trz-C1-Pan is phosphorylated by PfPanK before it is metabolised in turn by PfPPAT and PfDPCK into a CoA analogue.

5.2.2. Validating the targets of PanOH, CJ-15,801 and PanAm derivatives

In **Chapter 3**, PanOH and CJ-15,801 are postulated to kill *P. falciparum* by inhibiting PfPPCS, the second enzyme of the CoA biosynthesis pathway. A previous study has shown that PanOH is phosphorylated by PfPanK upon entering the parasite (Lehane *et al.*, 2007). Although no direct evidence is available, CJ-15,801 likely undergoes the same step as it is a known substrate of another type II PanK in *S. aureus* (van der Westhuyzen *et al.*, 2012). These phosphorylated compounds are expected to subsequently accumulate within the parasite, as PfPPCS was previously shown to limit the flux of substrates through the parasite's CoA biosynthesis pathway (Spry and Saliba, 2009), before binding and inhibiting PfPPCS. However, how this inhibition is achieved remains to be elucidated. The inhibition of *E. coli* and *S. aureus* PPCS by 4'-phospho-CJ-15,801 have been characterised in detail by van der Westhuyzen *et al.* (2012).

4'-Phospho-CJ-15,801 was shown to be cytidylylated by PPCS to generate 4'-phospho-CJ-15,801-cytidine monophosphate (as bacteria uses cytidine triphosphate instead of ATP for this reaction), which then acts as a reversible, but tight-binding, terminal inhibitor of the enzyme (van der Westhuyzen *et al.*, 2012). A separate study has also presented data that are consistent with 4'-phospho-PanOH inhibiting the *M. tuberculosis* PPCS enzyme, although the mechanism and kinetic parameters of this inhibition was not characterised in detail (Kumar *et al.*, 2007). It remains unknown, however, whether these pantothenate analogues also inhibit *Pf*PPCS in a similar manner. There are several methods that can be employed to elucidate their exact mode of inhibition in *P. falciparum*. The same mass spectrometry analysis performed in **Chapter 3** could be used to determine the CoA biosynthesis pathway metabolites generated by parasites that have been exposed to either PanOH or CJ-15,801. Not only would this confirm whether these pantothenate analogues are phosphorylated by *Pf*PanK *in situ*, it would also show if they are metabolised further along the CoA biosynthesis pathway. In addition, the specific activity of *Pf*PPCS, purified either through heterologous expression or the immunoprecipitation strategy used in this study, could be characterised using an enzyme-coupled assay, such as one that is linked to the production of pyrophosphate employed by van der Westhuyzen *et al.* (2012). This would then allow for the kinetic characterisation of *Pf*PPCS inhibition by the single-step reaction intermediates of PanOH and CJ-15,801 (4'-phospho-PanOH-adenosine monophosphate (-AMP) and 4'-phospho-CJ-15,801-AMP, respectively) if these analogues do inhibit the enzyme. Furthermore, this would also determine which of the two *Pf*PPCS enzymes expressed by the parasite (PF3D7_0412300 and PF3D7_1102400) is the active enzyme in the intraerythrocytic stage of its lifecycle.

It is also proposed in **Chapter 3** that the CoA analogue of N5-trz-C1-Pan exerts its antiparasmodial activity downstream of the CoA biosynthesis pathway enzymes. N-PE- α Me-PanAm is also expected to have the same mechanism of action although this needs to be confirmed using the same mass spectrometry analysis that was performed for N5-trz-C1-Pan. Previous studies have shown that PanAms, such as the classical N5-Pan, exhibit different mechanisms of action in different species of bacteria. In *E. coli*, N5-Pan has been shown to be phosphorylated by *Ec*PanK before being converted into the CoA antimetabolite

ethyldethia-CoA (Strauss and Begley, 2002), which was found to inhibit acyl carrier proteins (Zhang *et al.*, 2004). Although this same mode of antibacterial action was also proposed for *S. aureus* (Leonardi *et al.*, 2005; Virga *et al.*, 2006), a more recent finding (Hughes *et al.*, 2016) supports an alternative mechanism in which the antibacterial activity of PanAms is thought to be mediated by the direct inhibition of SaPanK (Choudhry *et al.*, 2003). The findings of this study (the fact that N5-trz-C1-Pan is converted into a CoA analogue, that the profile of PfPanK inhibition by the PanAm derivatives does not match their antiplasmodial activity profile and that a *P. falciparum* strain expressing a catalytically-defective PfPanK can become resistant to these PanAm derivatives) are consistent with these PanAm derivatives acting as substrates for PfPanK but not as inhibitors of the enzyme, unlike what was proposed in a recent study (Chiu *et al.*, 2017). This hypothesis is supported by a recent investigation of the mechanism of action of PanAms, in which 4'-phospho-N-phenethylpantothenamide was shown to be a poor inhibitor of PfPanK despite the compound still exhibiting antiplasmodial activity (de Villiers *et al.*, 2017). However, the exact target(s) of the CoA analogues of these PanAms and its derivatives remains unclear. One strategy that could be used to distinguish the exact targets of these PanAms and derivatives would be to generate resistant parasites via the dose-escalating step-wise *in vitro* drug-pressuring performed in this study (**Chapter 3**). A study that was recently deposited in the pre-print repository bioRxiv has found that a group of PanAm analogues (these compounds are PanAms that has their labile amide bond inverted) are converted within *P. falciparum* parasites into CoA-conjugates, which inhibit acetyl-CoA synthetase activity (Schalkwijk *et al.*, 2018). Moreover, the *in vitro* drug-pressuring of *P. falciparum* using one of these PanAm analogues, CXP18.6-052, generated resistant parasites that harbour mutations in acetyl-CoA synthetase and another CoA-utilising enzyme, acyl-CoA synthetase 11 (Schalkwijk *et al.*, 2018). Although the authors of this study performed a complementation study for the mutation in acetyl-CoA synthetase, the same experiment has not been done for the acyl-CoA synthetase enzyme. In addition, these mutant proteins have not been functionally characterised. Whether PanAms and the derivatives tested in this study also target these enzymes remains to be investigated. An alternative strategy would be to overexpress a putative target enzyme, for example the acyl-CoA synthetase, in the parasite and determine if they consequently develop resistance to the PanAms. This method

has previously been used to validate potential drug targets in *P. falciparum* (Gardiner *et al.*, 2006; Slavic *et al.*, 2010; Deplaine *et al.*, 2011; Chan *et al.*, 2013; Tjhin *et al.*, 2013).

5.2.3. Novel ways to target parasite CoA biosynthesis using *Pf*PanK inhibitors

The findings of this study reveal that while *Pf*PanK is not the target of the pantothenate analogues tested, the enzyme plays a critical role in activating these compounds in order for them to exert their antiplasmodial properties. This is in line with the conclusion of a recent study that looked at characterising the mode of action of PanAms as potential antimalarials (de Villiers *et al.*, 2017). Unfortunately, when considered in conjunction with the fact that the parasite is still able to grow (albeit at a reduced rate) even when its *Pf*PanK activity is diminished, this conclusion undermines the attractiveness of *Pf*PanK as a drug target, especially because most of the current compounds being developed to target the parasite's CoA biosynthesis are substrates of the enzyme. Despite this caveat, it remains important to develop antiplasmodials with different mechanisms of action. A tight-binding or irreversible competitive inhibitor of *Pf*PanK, similar to that observed for PanAms against *Sa*PanK (Hughes *et al.*, 2016), could in theory still inhibit the growth of the parasite. Furthermore, a *bona fide* *Pf*PanK inhibitor can be used in conjunction with inhibitors of one or more of the other *P. falciparum* CoA biosynthesis pathway enzymes as part of a novel chemotherapeutic strategy. A recent series of investigation that used a high throughput CoA rescue approach to screen two compound libraries and their subsequent hit expansions have identified several chemically-diverse compounds that were suggested to inhibit CoA biosynthesis (Fletcher and Avery, 2014; Weidner *et al.*, 2017). Most of these compounds were predicted to inhibit *Pf*PPAT or *Pf*DPCK because their inhibitory effects on the intraerythrocytic stage of the parasite could be reversed by the addition of dephospho-CoA or CoA (Fletcher *et al.*, 2016; Weidner *et al.*, 2017), although it remains to be seen if the rescue effect is mediated by the intact molecules or metabolites generated from their breakdown. Nevertheless, co-administration of a *Pf*PanK inhibitor and an inhibitor that does not need to be activated through *Pf*PanK-mediated phosphorylation, such as these potential *Pf*PPAT/*Pf*DPCK inhibitors, could be synergistic in nature as both compounds act to deplete the parasite's pool of

available CoA. In **Chapter 3**, the different parasite clones that express mutant versions *PfPanK1* were shown to exhibit different sensitivity profiles against different types of pantothenate analogues. More specifically, all of the mutants are resistant to the antiplasmodial effects of PanOH and CJ-15,801 (predicted to inhibit *PfPPCS*) but are inhibited differently (PanOH-A is more sensitive, PanOH-B is as sensitive as the wild-type, and CJ-A is resistant) by N5-trz-C1-Pan and *N*-PE- α Me-PanAm (predicted to inhibit CoA-utilising enzymes). These mutants also exhibit different sensitivity profiles to ALS98 and P7 (Saliba Lab, unpublished data), which indicate that these pantothenate analogues have unrelated mechanisms of action to the compounds tested in **Chapter 3**. These examples show that the sensitivity profiles of these clones could be used as an indicator of the mechanisms of action of future pantothenate analogues. Furthermore, they could be used in conjunction with the metabolite rescue experiments described by Fletcher *et al.* (2016) to more efficiently screen for and determine the mode of action of future *P. falciparum* CoA biosynthesis pathway inhibitors.

There is currently no known compound in the literature has been shown to be a true inhibitor of *PfPanK* (i.e. being able to inhibit the enzyme's activity without acting as its substrate). One strategy that could be used to generate a *PfPanK* inhibitor is to modify its substrates in such a way that prevents them from being metabolised. For example, de Villiers *et al.* (2017) generated versions of N5-Pan and *N*-phenethylpantothenamide that lack their 4'-hydroxyl group, which is required for *PfPanK* phosphorylation. However, this modification reduced the ability of these PanAms at inhibiting *PfPanK* in these instances (de Villiers *et al.*, 2017), although this could be due to the fact that the 4'-hydroxyl group was shown to be important in stabilising the binding of pantothenate (and presumably its analogues) within the active site of human PanK3 (**Figure 5.1 a**) (Subramanian *et al.*, 2016). Therefore, replacing the 4'-hydroxyl group of pantothenate analogues with another polar group that cannot participate in the phosphorylation could, in theory, turn the substrates into inhibitors of *PfPanK*. Alternatively, additional *PfPanK* inhibitors can also be derived from their allosteric inhibitors. As discussed in **Section 5.1.2**, human PanKs are regulated by CoA and its thioesters through allosteric inhibition (Zhang *et al.*, 2005; Leonardi *et al.*, 2007a; Subramanian *et al.*, 2016). This feedback regulation is also present for *PfPanK* (Lehane *et al.*, 2007). Furthermore, previous reports have shown that

intraerythrocytic *P. falciparum* parasites are able to survive in a pantothenate-free media that has been supplemented with CoA (Spry *et al.*, 2010), and that CoA and dephospho-CoA are able to rescue the parasite from inhibition by compounds that target CoA biosynthesis and/or metabolism (Fletcher and Avery, 2014; Fletcher *et al.*, 2016; Weidner *et al.*, 2017). These data are consistent with the parasite having a means by which to take up CoA (and potentially its analogues) from its surroundings. Therefore, analogues of CoA that have been designed to inhibit *Pf*PanK could potentially enter the parasite through a similar pathway and exert their activity. Additionally, a previous study on human PanK3 that screened a large library of biologically active molecules identified two classes of compounds, thiazolidinediones and sulfonylureas, that inhibit human PanK3 by docking in its acetyl-CoA binding site (Leonardi *et al.*, 2010). Although these compounds are not potent inhibitors of the human enzyme (Leonardi *et al.*, 2010), their effectiveness on *Pf*PanK could be different given that the latter is a heterodimer. These compounds could also be the scaffold upon which future *Pf*PanK inhibitors are designed. Moreover, the discovery of *Pf*PanK inhibitors, whether they are pantothenate analogues, CoA analogues or compounds of novel structures, would be greatly facilitated once the structure of *Pf*PanK is determined. Identifying the critical residues required for the interactions between *Pf*PanK and its substrates would streamline the design of *Pf*PanK inhibitors.

5.3. Conclusions

The initial aim of this study was to determine the modes of action of PanOH and CJ-15,801 and this was achieved by generating resistant *P. falciparum* parasites through a drug pressuring regime. Whole genome sequencing of the resistant parasites revealed the genetic cause of resistance to be mutations in the gene coding for *Pfpank1*. Information about the mechanisms of action of several key pantothenate analogues were determined by profiling the sensitivities of these parasite lines to these compounds and functionally characterisation the mutant *Pf*PanK1. PanOH and CJ-15,801 are hypothesised to be inhibitors of *Pf*PPCS, while N5-trz-C1-Pan and N-PE- α Me-PanAm are predicted to be metabolised further by enzymes of the CoA biosynthesis pathway enzymes into CoA analogues and inhibit downstream CoA-utilising processes. These would require further examination through functional characterisation of purified enzymes,

generation of additional drug-resistant parasites and/or metabolic analysis of drug-treated parasites. Although none of the pantothenate analogues tested in this study are inhibitors of *Pf*PanK, such compounds could be paired with non-conventional CoA biosynthesis pathway inhibitors in future chemotherapy strategy. The fact that mutations in *Pf*PanK1 directly affect *Pf*PanK activity is consistent with *Pf*PanK1 acting as the functional PanK during the intraerythrocytic stage of the parasite's lifecycle. However, further characterisation of both *Pf*PanKs through western blot and mass spectrometry analysis of co-immunoprecipitated enzymes revealed that *Pf*PanK is a heterodimeric complex consisting of *Pf*PanK1 and *Pf*PanK2 that associates with the *Pf*14-3-3I dimer. By comparing the amino acid sequences of *Pf*PanK1 and *Pf*PanK2 to that of human PanK3 and analysing the differences in critical residues required for PanK function, it is hypothesised that *Pf*PanK1 likely contains the sole functional catalytic site of the *Pf*PanK complex. Parts of *Pf*PanK2 is thought to interact with *Pf*PanK1 to form the complete and stable active site. *In silico* analysis of the *Pf*PanK2 sequence indicate that the protein is likely the binding site of the *Pf*14-3-3I dimer, although the exact functions of these two proteins remain unknown. Possible roles of *Pf*PanK2 include the allosteric regulation of *Pf*PanK and the translocation of *Pf*PanK into the parasite nucleus. Further characterisation of the interactions between the protein components of the *Pf*PanK complex, and those between the complex and its substrates/inhibitors would require the structure of the complex to be determined, for example through cryo-EM. This study has also developed strategies and resources that can be applied in future investigations. The *Pf*PanK1 mutant parasites generated in this study can be used in future investigations to characterise the enzyme and the mechanisms of action of new CoA biosynthesis pathway inhibitors. The co-immunoprecipitation approach employed in this study also acts as a proof of principle that such a strategy can be used to purify and characterise the function, localisation, conformation and interacting partners of the remaining putative enzymes of the parasite's CoA biosynthesis pathway.

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Appendix

Macuamule CJ*, **Tjhin ET***, Jana CE, Barnard L, Koekemoer L, de Villiers M, Saliba KJ and Strauss E. A pantetheinase-resistant pantothenamide with potent, on-target, and selective antiplasmodial activity. *Antimicrob Agents Chemother.* 2015. 59 (6): 3666–3668. doi: 10.1128/AAC.04970-14

*Contributed equally to this study.

Contribution:

I performed and analysed the data for all of the *P. falciparum* (Fig. 2a) and the human foreskin fibroblast (Fig. 2c) growth assays presented in the paper, provided intellectual input into the design of the study, and contributed to the editing of the manuscript.

A Pantetheinase-Resistant Pantothenamide with Potent, On-Target, and Selective Antiplasmodial Activity

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Pantothenamides inhibit blood-stage *Plasmodium falciparum* with potencies (50% inhibitory concentration [IC₅₀], ~20 nM) similar to that of chloroquine. They target processes dependent on pantothenate, a precursor of the essential metabolic cofactor coenzyme A. However, their antiplasmodial activity is reduced due to degradation by serum pantetheinase. Minor modification of the pantothenamide structure led to the identification of α -methyl-*N*-phenethyl-pantothenamide, a pantothenamide resistant to degradation, with excellent antiplasmodial activity (IC₅₀, 52 $\pm$ 6 nM), target specificity, and low toxicity.

One-half of the world's population (~3.4 billion people) is at risk of contracting malaria, with pregnant women and children <5 years of age being especially vulnerable. In 2013, the WHO estimated that malaria caused ~584,000 deaths globally, with the majority occurring in Africa (1). Although efforts to control and to eliminate malaria in the past 15 years have saved an estimated 3.3 million lives (1), drug-resistant parasites continue to emerge (2). This places the progress in the fight against the disease under pressure, especially since there is no effective vaccine against malaria (3). Several new drug targets have been identified in recent years (4); however, these targets now need to be exploited through the development of directed treatments.

We are interested in targeting the biosynthesis of the essential cofactor coenzyme A (CoA) from the water-soluble vitamin B₅ (pantothenate, compound 1 in Fig. 1) for antimalarial drug development (5, 6). It has been shown that extracellular pantothenate is essential for intracellular malaria parasites (7), which indicates that *Plasmodium falciparum* does not utilize exogenous CoA but must synthesize CoA *de novo* (8).

Pantothenate analogues interfere with the ability of *P. falciparum* to utilize the vitamin, with many analogues being characterized as growth inhibitors of the blood-stage parasites (9–11). Furthermore, a recent study showed that CoA biosynthesis can be targeted by a chemically diverse set of inhibitors that do not resemble pantothenate, the most potent of which had a 50% inhibitory concentration (IC₅₀) of 120 nM against blood-stage parasites (12). These studies support pantothenate utilization (and therefore CoA biosynthesis and CoA-dependent processes) as an antiplasmodial target.

Recently we showed that *N*-substituted pantothenamides (PanAms), a specific class of pantothenate analogues, have excellent antiplasmodial activity. Among these, *N*-phenethyl-pantothenamide (*N*-PE-PanAm) (compound 2 in Fig. 1) exhibited an IC₅₀ of 20 nM (13); this potency is comparable to that of chloroquine (14, 15). In practice, however, the antiplasmodial activity of the PanAms is decreased since they are degraded by pantetheinase (13), a ubiquitous enzyme of the Vanin protein family that is present in serum (16, 17). Pantetheinase normally catalyzes the hydrolysis of pantetheine (a CoA-derived metabolite) to form

pantothenate and cysteamine (18, 19), but it also acts on compounds with a wide range of variations in the cysteamine moiety, including the PanAms (Fig. 1) (13). In a previous study, we found that replacement of the β -alanine moiety of the PanAms with either glycine or γ -aminobutyric acid gave rise to pantetheinase-resistant variants, due to displacement of the scissile amide bond (20). Unfortunately, these structural modifications also reduced the potency of the resulting PanAms (IC₅₀ values of ≥ 1 μ M), indicating that their target (or targets) requires the pantothenate core structure to be retained for optimal inhibition.

In light of this finding, we set out to develop a pantetheinase-resistant PanAm in which the β -alanine core was retained. This was achieved by adding a methyl group to the carbon adjacent to the amide carbonyl group, thereby increasing the steric bulk at this center. We predicted that this modification would reduce the rate of pantetheinase-mediated hydrolysis by limiting the access of the enzyme's cysteine nucleophile to the scissile amide bond. The methylated version of *N*-PE-PanAm, i.e., α -methyl-*N*-PE-PanAm (α -Me-*N*-PE-PanAm) (compound 3 in Fig. 1), was prepared by condensing D,L-3-amino-isobutyrate to pantolactone, followed by partial purification by cation-exchange chromatography. The product, α -methyl-D-pantothenate, was purified by flash column chromatography (FCC) before being coupled to *N*-phenethylamine using diphenylphosphoryl azide in the presence of triethylamine. After purification by FCC, α -Me-*N*-PE-PanAm was

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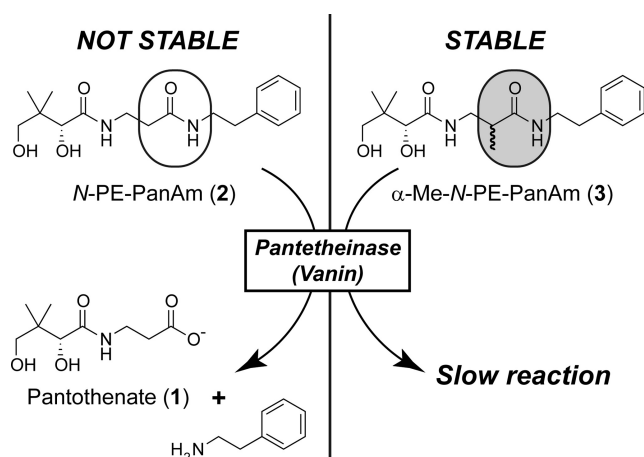


FIG 1 (Left) Structure of *N*-PE-PanAm (compound 2), which can be degraded by pantetheinase (Vanin) to form pantothenate (compound 1) and phenethylamine. (Right) Structure of α -Me-*N*-PE-PanAm (compound 3), which shows limited degradation by pantetheinase. The vulnerable (scissile) amide bond is indicated by the oval on the left, while the shaded oval on the right shows how it is modified by introduction of the methyl group to increase stability.

obtained in a final overall yield of 45%, as a mixture of two epimers (see the supplemental material for more details).

The antiplasmodial activity of α -Me-*N*-PE-PanAm against the chloroquine-sensitive *P. falciparum* strain 3D7 (chloroquine IC_{50} , 11 ± 1 nM [mean $\pm$ standard error of the mean {SEM}]; $n = 3$) was determined in “aged medium” (i.e., medium in which pantetheinase had been inactivated by incubation at 37°C for 40 h), in a manner similar to that used previously for *N*-PE-PanAm (13, 20). Under these conditions, α -Me-*N*-PE-PanAm showed excellent antiplasmodial activity, with an IC_{50} of 29 ± 2 nM (mean $\pm$ SEM; $n = 3$), a value that is only slightly greater than that of *N*-PE-PanAm (Fig. 2a). Furthermore, α -Me-*N*-PE-PanAm demonstrated exceptional resistance to degradation by pantetheinase, compared to *N*-PE-PanAm, as can be seen from its antiplasmodial activity in normal medium (i.e., with active pantethei-

nase), with an IC_{50} of 52 ± 6 nM (mean $\pm$ SEM; $n = 3$) (Fig. 2a), compared to the *N*-PE-PanAm IC_{50} of $\sim 6,200$ nM (13, 20). Performing the same test with a chloroquine-resistant strain (strain Dd2; chloroquine IC_{50} , 173 ± 5 nM [mean $\pm$ range/2]; $n = 2$) gave an IC_{50} of 129 ± 4 nM (mean $\pm$ range/2; $n = 2$); based on currently available data, it is unclear whether this difference is related to chloroquine resistance or is merely a variation in strain sensitivity. More importantly, resistance to pantetheinase degradation did not come at a cost in target specificity, since addition of excess extracellular pantothenate (100 μ M) to the medium antagonized the antiplasmodial activity of α -Me-*N*-PE-PanAm against the 3D7 strain (IC_{50} , 860 ± 102 nM [mean $\pm$ SEM]; $n = 3$; $P = 0.01$) (Fig. 2a).

To confirm the stability of α -Me-*N*-PE-PanAm, we also tested its *in vitro* degradation by recombinant pantetheinase (human VNN1) (Fig. 2b). This was done by incubating substrate (500 μ M *N*-PE-PanAm or α -Me-*N*-PE-PanAm; 500 μ M phenethylamine was used as a reference, i.e., equivalent to 100% product formation) in 100 mM HEPES (pH 7.6) containing 500 μ M dithiothreitol (DTT) and 0.05 μ g/ μ l bovine serum albumin (BSA), at 37°C. The reaction (in a final volume of 300 μ l) was initiated by the addition of pantetheinase (1.6 μ g/ μ l), and the mixture was incubated for 24 h. The amount of amine produced was determined by quenching 30 μ l of the reaction mixture with 10 μ l of *N*-ethylmaleimide (6 μ M), followed by incubation (for 10 min at 37°C) with 2 mM fluorescamine in 517 mM borate (pH 9), in a final volume of 145 μ l. Fluorescence was subsequently measured using a Thermo Varioskan multiplate spectrofluorimeter (excitation wavelength, 395 nm; emission wavelength, 485 nm). We were able to confirm that α -Me-*N*-PE-PanAm was more resistant to pantetheinase-mediated degradation than *N*-PE-PanAm, as it showed only $26\% \pm 2\%$ (mean $\pm$ range/2; $n = 2$) hydrolysis (normalized to the control, which represented 100% phenethylamine formed) after 24 h, compared to $96\% \pm 9\%$ (mean $\pm$ range/2; $n = 2$) for *N*-PE-PanAm under the same conditions.

The activity of α -Me-*N*-PE-PanAm was tested against a human cell line (human foreskin fibroblasts [HFF]) to determine its selectivity (21). The cells were exposed to α -Me-*N*-PE-PanAm

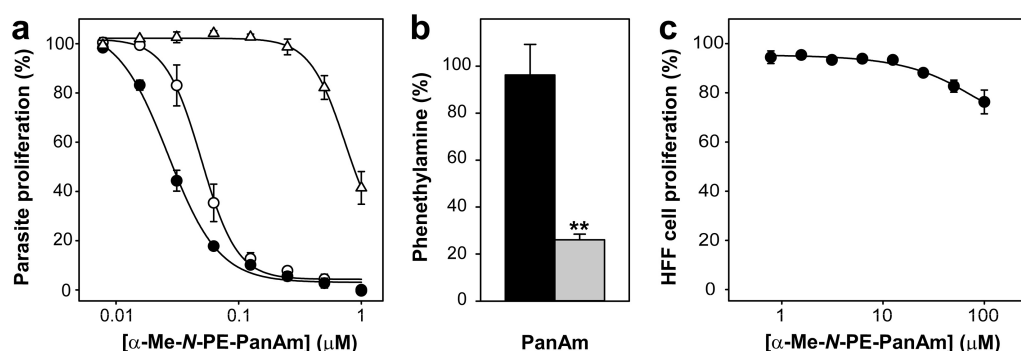


FIG 2 (a) Inhibition of proliferation of *P. falciparum* parasites (chloroquine-sensitive strain 3D7) by α -Me-*N*-PE-PanAm. Parasites were cultured for 96 h in medium with (○) or without (●) pantetheinase activity; the inhibition was antagonized when the extracellular pantothenate concentration in the medium with pantetheinase was increased from the usual 1 μ M to 100 μ M (Δ), consistent with the compound being on target. Values represent the mean $\pm$ SEM of three independent experiments, each performed in triplicate. (b) Pantetheinase-mediated hydrolysis of *N*-PE-PanAm (black bar) and α -Me-*N*-PE-PanAm (gray bar) *in vitro* after treatment with recombinant human pantetheinase for 24 h. The amount of phenethylamine released was determined by derivatization with fluorescamine. Values represent the mean from two independent experiments, each performed in triplicate; the error bars represent range/2. **, $P < 0.001$, Student's *t* test. (c) HFF proliferation in the presence of α -Me-*N*-PE-PanAm after 96 h. Values represent the mean $\pm$ SEM of three independent experiments, each performed in triplicate.

(0.781 to 100 μ M) for 4 days to reach confluence, and plates were stored at -80°C prior to exposure to SYBR Safe, as was done for the parasite experiments. We found that α -Me-*N*-PE-PanAm had limited cytotoxicity for HFF cells, with a selectivity index (SI) greater than 1,500 (Fig. 2c), rivalling the SI of chloroquine ($\sim$ 1,300) determined using a similar cell line (15).

With these promising findings, we unveil α -Me-*N*-PE-PanAm as the first pantothenate analogue with excellent potential as a new lead compound for antimalarial drug development, based on its potent inhibition of blood-stage parasites in the presence of serum pantetheinase, lack of activity against human cells, and desirable physicochemical characteristics (22) (see the supplemental material for details). Future work will focus on determining whether the two epimers show a difference in activity, performing tests on other stages of the parasite's life cycle, and determining the pharmacokinetic properties and *in vivo* efficacy of α -Me-*N*-PE-PanAm, to establish its long-term potential for development as an antimalarial.

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SUPPLEMENTAL MATERIAL

A pantetheinase-resistant pantothenamide with potent, on-target and selective antiplasmodial activity

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MATERIALS

All materials used were as described before (1, 2).

STATISTICAL ANALYSES

The statistical significance for the IC₅₀ values and the pantetheinase assay was determined using a two-tailed Student's t-test for paired samples.

SYNTHESES

α -Methyl pantothenic acid (mixture of epimers)

D,L-3-Amino isobutyric acid (500 mg, 4.85 mmol) was dissolved in 1 M aqueous sodium hydroxide (4.80 mL, 4.85 mmol) and lyophilized, after which pantolactone (700 mg, 5.28 mmol) was added. The mixture was stirred under an inert atmosphere overnight at 130 °C. The resulting sticky oil was dissolved in water and loaded onto a prewashed Amberlite IR120 (H⁺-form) ion exchange resin. The free acid was eluted with water and lyophilized before purification by flash column chromatography (5:2:1:1 ethyl acetate: methanol: water: acetonitrile) afforded the product (0.97 g, 86%) as a white powder. *R*_f = 0.47. δ_{H} (400 MHz; D₂O; 25 °C) 0.76 (3H, s, -CH₃), 0.79 (3H, s, -CH₃), 1.01 (3H, d, *J* = 7.1 Hz, -CH₃), 2.47–2.59 (1H, m, -CH-), 3.21–3.25 (2H, m, -CH₂-), 3.26 (1H, d, *J* = 11.1 Hz, -CH-), 3.38 (1H, d, *J* = 11.1 Hz, -CH-), 3.85 (1H, s, -CH-, epimer A) and 3.86 (1H, s, -CH-, epimer B). δ_{C} (100 MHz; D₂O; 25 °C) 14.9, 19.8, 21.1, 39.2, 40.2, 40.3, 42.2, 42.3, 69.1, and 76.5; (MS-ESI) [M+H]⁺ 234.14 (Calculated [C₁₀H₂₀NO₅]⁺ = 234.13), [M-H]⁻ 232.09 (Calculated [C₁₀H₁₈NO₅]⁻ = 232.12).

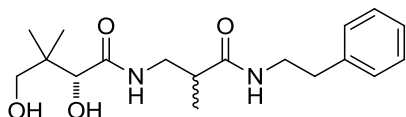
α -Me-N-PE-PanAm (mixture of epimers)

Phenethylamine (163 μ L, 1.29 mmol) and diphenylphosphoryl azide (278 μ L, 1.29 mmol) were added to a solution of α -methyl pantothenic acid (250 mg, 1.07 mmol) in dimethylformamide (4 mL) at RT. After cooling the mixture to 0 °C, triethylamine (180 μ L, 1.29 mmol) was added. The reaction mixture was stirred for an additional 2 h at 0 °C and left to stir overnight at RT. Dimethylformamide was removed *in vacuo* and Amberlite IR400 (OH⁻-form) resin was added. The reaction mixture was filtered and lyophilized before purification by flash column chromatography (10% methanol/dichloromethane) afforded the target compound (188 mg, 52%) as a yellow oil. *R*_f = 0.24. δ_{H} (600 MHz; CDCl₃; 25 °C) 0.90 (3H, s, -CH₃, epimer A), 0.91 (3H, s, -CH₃, epimer B), 0.99 (3H, s, -CH₃, epimer A), 1.00 (3H, s, -CH₃, epimer B), 1.10 (3H, d, *J* = 7.1 Hz, -CH₃), 2.47–2.59 (1H, m, -CH-), 2.80 (2H, t, *J* = 7.1 Hz, -CH₂-), 3.28–3.28 (2H, m, -CH₂-), 3.42–3.49 (2H, m, -CH₂-), 3.51–3.58 (2H, m, -CH₂-), 3.98 (1H, apparent t, *J* = 5.1 Hz, -CH-, both epimers), 5.98 (1H, br s, -NH-), 7.17 (2H, d, *J* = 7.4 Hz, arom), 7.22

(1H, d, $J = 7.3$ Hz, arom), 7.25–7.27 (1H, br s, -NH-) and 7.30 (2H, dd, $J = 7.3, 7.4$ Hz, arom). δ_C (150 MHz; CDCl₃; 25 °C) 15.7, 20.4, 20.4, 35.5, 40.6, 40.7, 42.1, 48.7, 70.8, 77.5, 126.6, 126.6, 128.7, 128.7, 128.7, 138.7, 173.7, and 175.0; (HRMS) $[M+H]^+$ 337.2124 (Calculated $[C_{18}H_{29}N_2O_4]^+ = 337.2127$).

PHYSICOCHEMICAL PROPERTIES

α -Me-N-PE-PanAm



Molecular weight: 336.42 g.mol⁻¹

Calculated lipophilicity (ClogP): 0.62

Number of H-bond donors: 4

Number of H-bond acceptors: 2

Polar surface area (PSA): 98.66 Å² at pH = 7.40

Distribution (logD): 0.62 at pH = 7.40

Number of rotatable bonds (NRotB): 9

Fraction of sp^3 carbons (F_{sp^3}): 0.556

Values of ClogP, PSA, logD and NRotB were calculated with calculator plugins in the program MarvinSketch 6.0.6, 2013 from ChemAxon (Budapest, Hungary). Calculations used equal weights of VG, KLOP, and PHYS methods, and used electrolyte concentrations (Na⁺, K⁺ and Cl⁻) set to 0.1 mol.dm⁻³. Tautomerization were not considered in the calculations (see <https://www.chemaxon.com/marvin-archive/5.11.3/marvin/help/calculations/partitioning.html>).

REFERENCES

1. **De Villiers M, Macuamule C, Spry C, Hyun Y-M, Strauss E, Saliba KJ.** 2013. Structural modification of pantothenamides counteracts degradation by pantetheinase and improves antiplasmodial activity. *ACS Med. Chem Lett.* **4**:784-789.
2. **Spry C, Macuamule C, Lin Z, Virga KG, Lee RE, Strauss E, Saliba KJ.** 2013. Pantothenamides are potent, on-target inhibitors of *Plasmodium falciparum* growth when serum pantetheinase is inactivated. *PLoS One* **8**:e54974.