Coenzyme A biosynthesis and utilization in *Plasmodium falciparum*: drug targets for antimalarial chemotherapy

by

Cristiano João Macuamule

Pectora roborant cultus recti

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Supervisor: Professor Erick Strauss
Department of Biochemistry
Stellenbosch University, South Africa

Co-supervisor: Doctor Kevin J. Saliba
Research School of Biology and Medical School,
College of Medicine, Biology and Environment
The Australian National University, Australia

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Declaration

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Aos meus pais João Macuamule & Alda das Neves Quiliboi.

Ao meu irmão Arq. Domingos João Macuamule (07/08/1970 – 09/01/2010).

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Abstract

Pantothenate (also known as Vitamin B_5) is the sole precursor of the essential enzyme cofactor coenzyme A (CoA) that is required in several metabolic reactions virtually in all living organisms including the human malaria parasite *Plasmodium falciparum*. While the parasite has the capacity to generate CoA from pantothenate, it cannot produce this nutrient *de novo,* and as a result depends on external supplies. Processes in the CoA metabolic pathway have been identified as possible targets for drug development and pantothenate analogues as agents that can interfere with those processes to block parasite development.

In this dissertation it is shown that the class of pantothenate analogues known as *N*-substituted pantothenamides (PanAms) and *N*-substituted pantoyltauramides, inhibit the growth of intraerythrocytic-stage *P. falciparum* parasites at sub- and low micromolar concentrations respectively. In both cases, the compounds inhibited parasite proliferation through inhibition of pantothenate-dependent processes. It is also shown that the antiplasmodial potency of PanAms can be strengthened through structural modifications rendering the compounds less susceptible to degradation by enzymes known as pantetheinases, which occur natural and ubiquitously in mammals, particularly in the serum.

Finally it is also shown that the antiplasmodial mode of action of PanAms results from the compounds serving as alternative substrates for pantothenate kinase (PanK), the first enzyme intervening in the CoA biosynthesis pathway, thus interfering with the phosphorylation of the natural substrate – pantothenate. In addition, it negatively affects the production of functional CoA and acyl carrier proteins (ACPs) which are required in various cellular metabolic processes.

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Opsomming

Pantotenaat (ook bekend as Vitamien B_5) is die enigste voorloper van die noodsaaklike ensiemkofaktor koënsiem A (KoA) wat benodig word in verskeie metaboliese reaksies in feitlik alle lewende organismes, insluitend die malaria parasiet *Plasmodium falciparum* wat in die mens voorkom. Die parasiet het die kapasiteit om KoA te genereer vanaf pantotenaat, maar kan dié spesifieke voedingstof nie *de novo* produseer nie en moet gevolglik op eksterne bronne staatmaak. Prosesse in die KoA metaboliese padweg is geïdentifiseer as moontlike teikens vir geneesmiddelontwikkeling en pantotenaat-analoë as middels wat kan inmeng met die prosesse wat parasietontwikkeling blokkeer.

In hierdie proefskrif word daar gewys dat die klas van pantotenaat-analoë bekend as *N*-gesubstitueerde pantoteenamiede (PanAms) en *N*-gesubstitueerde pantoïeltauramiede inhibeer die groei van eritrositiese-fase *P. falciparum* by sub- en lae mikromolare konsentrasies onderskeidelik. In beide gevalle inhibeer die verbindings die verspreiding van parasiete deur pantotenaat-afhanklike prosesse te inhibeer. Dit is ook bewys dat die antiplasmodiale werking van PanAms versterk kan word deur middel van strukturele veranderinge wat die verbindings minder vatbaar maak vir afbraak deur ensieme bekend as panteteinases, wat natuurlik en alomteenwoordig in soogdiere voorkom, veral in serum.

Ten slotte is dit ook getoon dat die antiplasmodiale werking wat PanAms toon veroorsaak word deur as alternatiewe substrate vir pantotenaatkinase (PanK), die eerste ensiem in die KoA biosintese padweg, op te tree en dus so inmeng met die fosforilering van die natuurlike substraat – pantotenaat. Dit het ook 'n negatiewe invloed op die produksie van funksionele KoA en asieldraer proteïene (ACPs) wat benodig word in verskeie sellulêre metaboliese prosesse.

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Outputs

The work reported in this dissertation has contributed to the following outputs:

Papers:

1. Spry C, Macuamule C, Lin Z, Virga KG, Lee RE, Strauss E, Saliba KJ. Pantothenamides are potent, on-target inhibitors of *Plasmodium falciparum* growth when serum pantetheinase is inactivated. PLoS ONE. 2013, 8(2): e54974. doi: 10.1371/journal.pone.0054974. Epub: Feb 6, 2013.

2. de Villiers M, Macuamule C, Spry C, Hyun Y-M, Strauss E, Saliba KJ. Structural modification of pantothenamides counteracts degradation by pantetheinase and improves antiplasmodial activity. ACS Med. Chem. Lett., 2013, 4(8), pp. 784–789. doi: 10.1021/ml400180d. Epub: Jun 17, 2013.

3. Macuamule C, Barnard L, de Villiers M, Spry C, Saliba, KJ, Strauss E.. The antiplasmodial mode of action of *N*-substituted pantothenamides involves multiple targets in the CoA metabolic pathway – *Working title. Paper in preparation*.

Oral presentation:

1. Exploiting *N*-substituted pantothenamides as potential drugs for antimalarial chemotherapy.

   "Forum Lecture" presented at the Department of Biochemistry, Faculty of Science, Stellenbosch University. November 2012.

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Abbreviations

ACN	Acetonitrile
ACP	Acyl carrier protein
AcpH	ACP hydrolase
AcpS	ACP synthase
ACT	Artemisinin-based combination therapy
ANOVA	Analysis of variance
ANU	Australian National University
apo-ACP	Inactive acyl carrier protein
ASKHA	Acetate and sugar kinase/heat shock protein 70/actin superfamily
ATH	Artemether
ATP	Adenosine triphosphate
AuC	Area under the curve
b.w.	Body weight
BSA	Bovine Serum Albumin
°C	degrees Celcius
CoA	Coenzyme A
CPM	7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin
CQ	Chloroquine
CRT	Chloroquine resistance transporter
deoxy-N5-Pan	Deoxy-*N*-pentyl-normal-*N*-substituted pantothenamide
DHA	Dihydroartemisinin
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthase

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DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic acid
DPCK	Dephospho-CoA kinase
DTT	Dithiothreitol
$FADH_2$	Flavin Adenine Dinucleotide
FAS	Fatty acid synthase
G6PD	Glucose-6-phosphate dehydrogenase
GCS	γ-glutamylcysteine synthetase
h	Hour
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid
HoPanAms	Homo-*N*-substituted pantothenamides
HTC	Hepatoma cell line
IPTG	Isopropyl β-D-1-thiogalactopyranoside
IRS	Indoor residual spraying
ITN	Insecticide treated bed nets
Kg	Kilogram
Km	Michaelis-Menten constant
LB	Luria Bertani
α-MePanAms	α-methyl-*N*-substituted pantothenamides
β-MePanAms	β-methyl-*N*-substituted pantothenamides
MDR	Multidrug resistance
mg	Milligram
µg	Microgram
min	Minute
ml	Mililiter

µl	Microliter
mM	Millimole
µM	Micromolar
MMV	Medicines for Malaria Venture
MoA	Mode of Action
N5-Pan	*n*-pentylpantothenamide
n-PanAms	normal-*N*-substituted pantothenamides
NPPs	new permeation pathways
NRF	National Research Foundation
OD600	Optical density at wavelength of 600 nm
PABA	*p*-aminobenzoic acid
PAGE	Polyacrylamide gel electrophoresis
PanAms	*N*-substituted pantothenamides
α-PanAms	α-*N*-substituted pantothenamides
PanK	Pantothenate kinase
PBS	Phosphate-buffered saline
*Pf*CRT	*Plasmodium falciparum* chloroquine resistance transporter
*Pf*MDR	*Plasmodium falciparum* multidrug resistance transporter 1
*Pf*PanK	*Plasmodium falciparum* pantothenate kinase
PPAT	Phosphopantetheine adenylyltransferase
PPCDC	Phosphopantothenoylcysteine decarboxylase
PPCS	Phosphopantothenoylcysteine synthetase
PQ	Primaquine
RBC	Red Blood Cells
rcpm	Radioactivity counts per minute

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ROS	Reactive oxygen species
rpm	Revolutions per minute
SAR	Structure-activity relationship
SDS	Sodium Dodecyl Sulphate
SERCA	sarcoendoplasmic reticulum Ca^{2+}-ATPase
Sfp	*Bacillus subtilis* phosphopantetheinyl transferase
SH	Sulfhydryl
SNP	Single Nucleotide Polymorphism
SUN	Stellenbosch University
TCA	Trifluoroacetic acid
TDR	Targets Database website
TEV	Tobacco Etch Virus
Tris	Tris(hydroxymethyl)aminomethane-HCl
V*max*	Maximal rates of phosphorylation
v/v	Volume per volume
WHO	World Health Organization
w/v	Weight per volume

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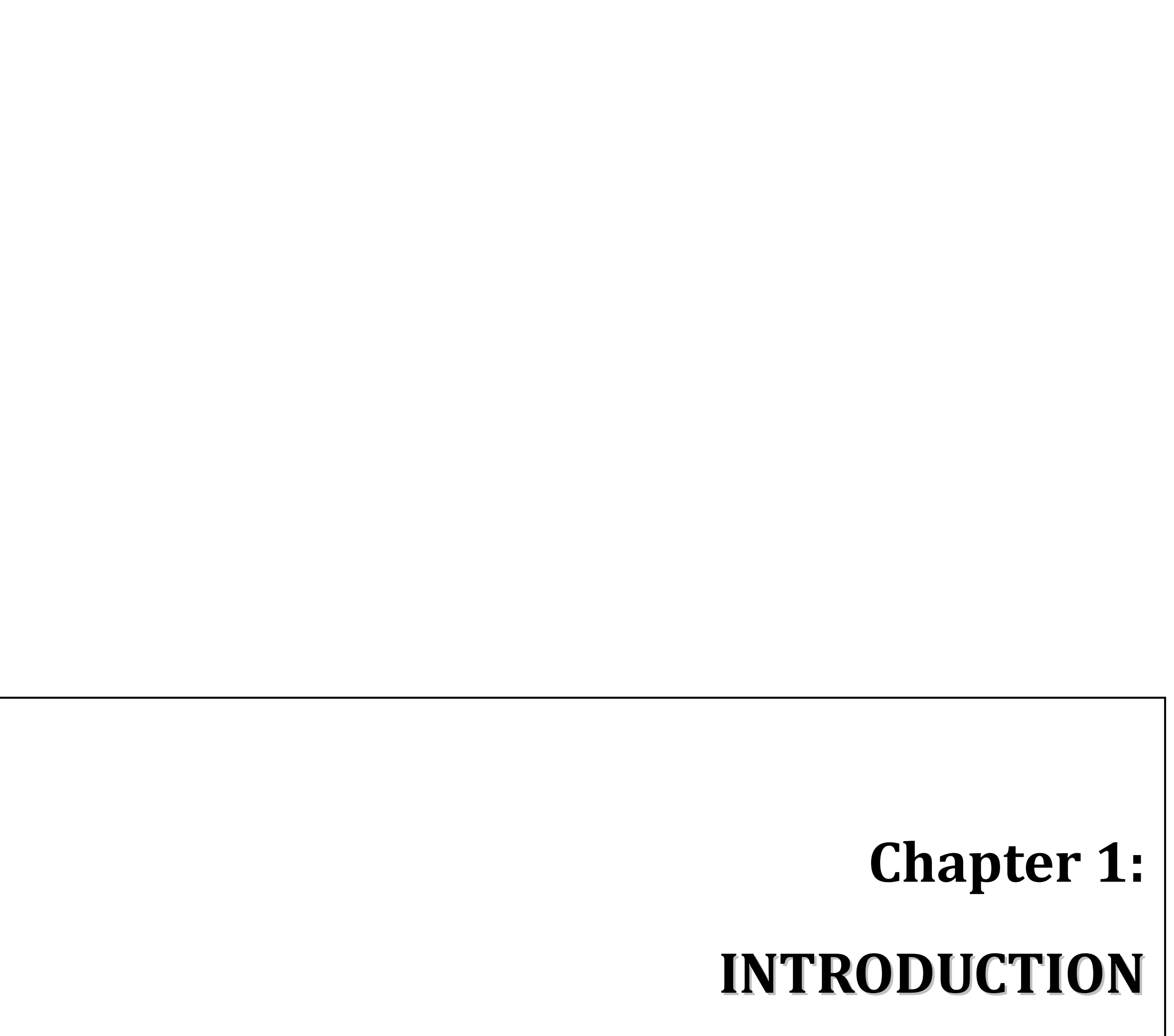

Chapter 1:

INTRODUCTION

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1. Malaria: a brief historical perspective

Malaria (also known as paludism, "chills and fever", marsh fever, or jungle fever), or a disease resembling it, was first reported over 4,000 years [1]. In 2700 BC, several characteristic symptoms of the disease were described in the *Nei Ching*, an ancient book of Medicine edited by the "Yellow Emperor" Huangdi of China. By the 4th Century BC, malaria was widely recognised in Greece where it was responsible for many deaths [1]. On a worldwide basis, the disease has probably been the most serious ailment affecting humans throughout history, with certain geographical areas, being at times rendered practically uninhabitable due to malaria [2]. Several Roman writers attributed malarial diseases to the swamps, and the most widely used term "malaria" is probably of Italian origin from the word *male* meaning "bad" and *aria* meaning "air", as it was thought that the disease was caused by "bad air" emanating from swampy lowland fields [1]. In the *Susruta* (a Sanskrit medical treatise), the symptoms of malarial fever were described and attributed to the bites of certain insects [1].

1.1. Transmission of malaria parasites

When Charles Laveran first saw malaria parasites in the erythrocytes of a patient suffering from the disease in Constantine, Algeria, then a French territory in 1880, little was known about their transmission [1-3]. In 1897, Ronald Ross discovered malaria parasites in the gut of a mosquito that had been feeding on a patient suffering from the disease. Later, using a bird malaria model, he demonstrated the role of mosquitoes in transmitting the parasites. By 9th of July 1898 Ross had established that mosquitoes served as intermediate hosts; the malaria parasites developed in the gastrointestinal tract of the insect before migrating to the salivary glands from where they were transmitted to the next vertebrate victim during succeeding blood meals [1, 3].

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1.2 Malaria epidemiology and the disease burden

Around 3.3 billion people are at risk of malaria infection every year. The World Health Organisation (WHO) estimates that 219 million clinical cases of the disease were reported in 2010, resulting in approximately 660 000 deaths [4]. Other studies using different prediction tools incorporating documented and non-documented deaths estimate that the number of malaria victims is higher, and may have reached 1.24 million [5]. The disease burden is particularly severe in sub-Saharan Africa, where approximately 90% of fatalities occur [4, 6]. Children and pregnant women — particularly during their first pregnancy — are amongst the most affected [5]. In this region of the African continent, one child dies from malaria every minute, while gestational malaria is responsible for up to 200 000 infant deaths each year [6]. Asia and Latin America are also affected, and to a lesser extent, the Middle East and Western Europe [7].

Malaria is endemic (i.e. transmission occurs naturally and continuously over a succession of years) in more than 100 countries (figure 1.1) [4], with an estimated 125 million international travellers visiting these countries yearly and about 30 000 of them contracting the disease during their journeys [8].

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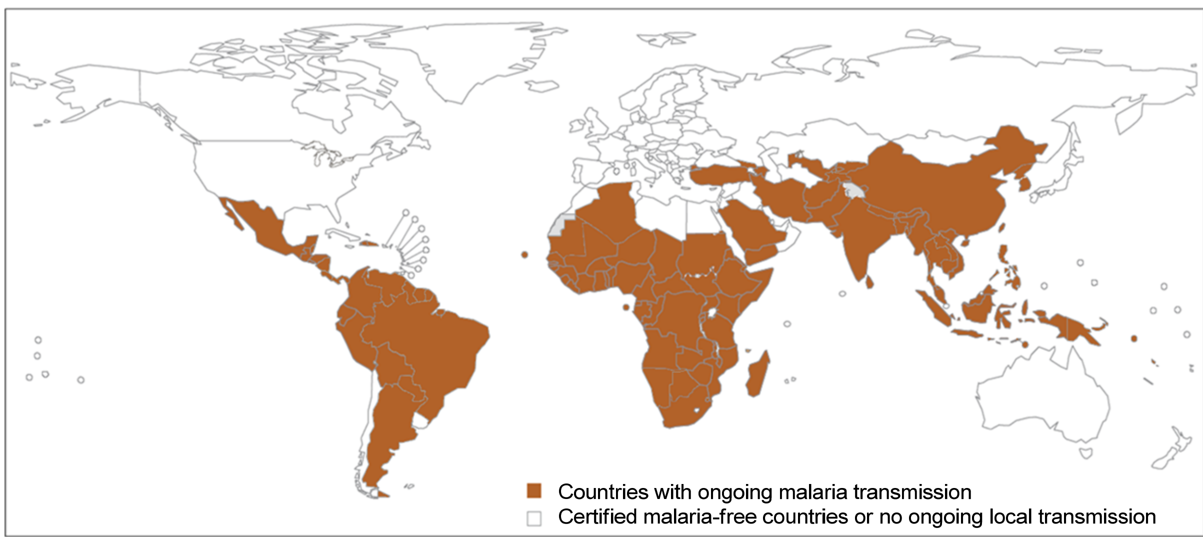

Figure 1.1 - Distribution of malaria in the world. Adapted from: World Malaria Report 2012 [4]. Malaria is essentially a disease of tropical and subtropical regions, with the natural distribution determined by the abundant rainfall and high temperatures and humidity, along with static waters providing the ideal environmental conditions for continuous mosquito breeding [9].

Malaria distribution over large geographical areas is complex. Close proximity between malaria-free and malaria-affected areas occurs [10, 11]. For example, some rural areas in the Greater Mekong sub-region (Cambodia, Lao, Myanmar, Thailand, Viet Nam and China: Yunnan Province and Guangxi Zhuang Autonomous Region) in Southeast Asia are highly affected, while several adjacent cities are essentially malaria free [12, 13]. In Africa, however, although the risk of malaria transmission is lower in major cities, the disease occurs in both rural and urban areas [13].

1.3 Life cycle of malaria parasites and disease pathogenesis

1.3.1 Life cycle of *Plasmodium spp.*

The five species of malaria parasites that infect humans naturally, i.e. *P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi*, exhibit some differences in their developmental stages. However, the life cycle of all of them include asexual and sexual

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phases occurring in both the human and the invertebrate host, the female *Anopheles* mosquito.

1.3.1.1 Asexual phase

During a blood meal, a *Plasmodium*-infected female A*nopheles* mosquito injects parasites (at this stage called sporozoites) contained in its salivary glands into the peripheral blood of the victim [14, 15]. After approximately 30 minutes, the sporozoites initiate the so-called *liver stage* as they reach the liver and penetrate its parenchyma cells (hepatocytes). It is here where they develop for between 5-10 days depending on the *Plasmodium* species. As the parasites multiply in the hepatocytes, the liver cells swell and eventually rupture releasing up to 30 000 merozoites into the circulation. The merozoites then initiate the intraerythrocytic or *blood-stage* cycle when they invade red blood cells (RBCs) within 30 seconds after their release from hepatocytes [16].

After invading the RBC, the merozoites enter a period of low metabolic activity for around 15 hours [17]; during this time, the parasite is often called "ring stage" due to its ring-shaped appearance under light microscopy. Following the apparently dormant period, the parasite initiates a vigorous metabolic phase, feeding on haemoglobin and inducing biochemical changes within the RBC while developing through the trophozoite stage and into the schizont stage, which is reached approximately 36 hours post-invasion. During the next 12 hours the schizont matures and divides, completing the intraerythrocytic stage with the rupture of the RBC and release of around 20 daughter merozoites, within approximately 48 hours post-invasion in the case of *P. falciparum*, the most virulent malaria parasite, and the one most prevalent in the African continent [16, 18-20].

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Once the newly formed merozoites are released from the ruptured RBC, most are destroyed by the host immune system, however the few that survive go on to infect fresh RBCs and reinitiate the intraerythrocytic cycle. It is important to note that, for the parasite to complete its life cycle in the mosquito, after several cycles of asexual replication – due to not yet very well understood triggers – a subset of the intraerythrocytic merozoites do not undergo schizogony (development into schizonts) but alternatively differentiate into male (microgametocyte) and female (macrogametocyte) sexual forms [16, 19] (figure 1.2).

After invading the hepatocytes some sporozoites do not develop into merozoites. Instead they remain dormant (hypnozoite stage) for weeks, months or even years, before being reactivated and resuming vigorous replication. This feature is typically found in parasites in temperate, rather than tropical areas and may be an adaptation for survival during the long winter months when transmission cannot occur [16, 19, 20].

1.3.1.2 Sexual phase

As gametocytes (transmission stage) in RBCs are ingested by female *Anopheles* mosquitoes during a blood meal, the membrane of the erythrocyte ruptures releasing the sexual forms of the parasite. In the mid gut of the mosquito, the microgametocyte fuses with the macrogametocyte to form a zygote. The zygote then differentiates into a motile form called an ookinete. After 18-24 hours, the ookinete penetrates the mosquito midgut wall where it matures into a sporozoite-filled oocyst. The oocyst then bursts releasing up to 10 000 sporozoites, which move to the lumen of the mosquito's salivary glands where they remain viable during the whole lifespan of the mosquito and ready to be injected and initiate a new infection on the next victim. Depending on the parasite

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species and environmental temperature, this phase of the *Plasmodium* life cycle is completed over a period of up to 15 days [16, 18, 19] (figure 1.2).

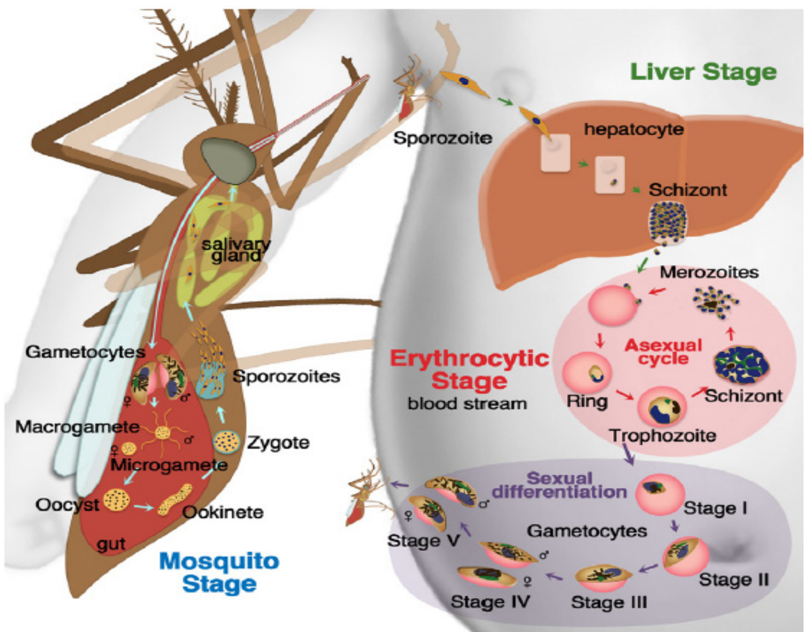

Figure 1.2 – *Plasmodium falciparum* life cycle comprising mosquito stage, liver stage and the clinically important asexual cycle taking place in red blood cells (intraerythrocytic stage). Source: Biamonte *et al*. [20].

1.3.2 Disease pathogenesis

Practically all, except cerebral malaria, clinical signs and symptoms of malaria are associated with the intraerythrocytic stage of the parasite's life cycle. During this stage, the synchronous burst of RBCs and release of merozoites as well as the discharge of massive amounts of metabolic waste (including haemozoin, also called the malaria pigment) contained in vesicular compartments (digestive vacuoles) into the blood stream, causes the clinical manifestations of the disease.

The classic symptom of malaria is paroxysm, characterized by sudden cyclical coldness followed by rigor, fever and sweating every 24 hours in *P. knowlesi* (a zoonotic malaria

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parasite naturally found in long-tailed macaque (*Macaca fascicularis*) and pig-tailed macaque (*Macaca nemestrina*)) infections; every 48 hours in *P. falciparum*, *P. vivax* and *P. ovale* infections, and every 72 hours in the case of *P. malariae* infections [21].

In the most common malaria picture, a patient presents a combination of symptoms that include fever, chills, sweating, headache, body aches, nausea and abdominal discomfort and vomiting, and general malaise [21]. Severe malaria can develop as a result of non- or inappropriate treatment or when infections are complicated by major organ, blood or metabolic impairments. In such cases the clinical manifestations of the disease may include acute severe anaemia [22] and haemoglobinuria as a result of massive haemolysis, impaired haematopoesis [23], coagulopathies [24, 25], hypotension, acute respiratory distress syndrome [25], acute renal failure [25, 26], jaundice, hypoglycaemia and the most severe condition, cerebral malaria [25], characterised by abnormal behaviours, impaired consciousness, seizures, coma and other neurological dysfunctions which are often fatal.

1.4 Control of malaria

At present, the control of malaria relies essentially on reducing the contact between humans and mosquitoes (vector control), and the use of drugs to either treat (chemotherapy) or prevent (chemoprophylaxis) the disease.

1.4.1 Vector control

The control of mosquitoes is achieved mainly through spraying of insecticides on the interior walls of homes (indoor residual spraying (IRS)) as well as use of insecticide treated bed nets (ITN) and garments. Other strategies for vector control include the less

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practiced mosquito larval source management consisting of habitat – *stagnant water* – modification, habitat manipulation, larviciding, and biological control, and the scale-up of personal protection measures including the use of insect repellents, window screens and protective clothes.

1.4.2 Vaccines

Intensive research in vaccine development is ongoing in different parts of the world. Several vaccine candidates are in different stages of development, with the first recombinant *P. falciparum* vaccine, RTS,S/AS01 currently in Phase III clinical trials [27].

Reports of the vaccine tested in Africa indicate that its efficacy has been 43.6% in the first year but was -0.4% in the fourth year. Overall, the vaccine was effective in decreasing clinical malaria in children only by 16.8% over a four year period [28]. More efficacious vaccines that can be used to prevent clinical disease caused by the most dangerous malaria parasites (*P. falciparum* and *P. vivax*) are desperately needed, as well as support for eradication efforts through induction of immunity that blocks parasite transmission.

As discussed by Birkett *et al*. [28], the challenges in malaria vaccine development are related to (i) the lack of well-characterized target immunogens and the inexistence of clear correlates of protection allowing the development of a vaccine directed to all stages of the life cycle of the most important *Plasmodium* parasites; (ii) a limited amount of effective and secure delivery systems that induce robust protective immunity; (iii) the need for vaccines designed to provide “herd protection” by targeting sexual stage

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and/or mosquito antigens; and (iv) the lack of a comprehensible clinical and regulatory pathway to licensing using non-traditional endpoints.

1.4.3 Chemotherapy, chemoprophylaxis and the development of drug resistance

Given the absence of an effective vaccine, and the fact that other malaria control strategies are not routinely practised, the use of antimalarial drugs remains the major strategy applied to treat and prevent the disease.

A number of antimalarial drugs have been used, the vast majority of which target the clinically important intraerythrocytic stage of the parasite. The most commonly used compounds belong to three major classes including quinolines, antifolates and sesquiterpene endoperoxides [29]. These classes of antimalarial drugs are briefly discussed below.

1.4.3.1 Quinolines

Quinine, the historic representative of the antimalarial quinolines, is an alkaloid extracted from the bark of the *Cinchona* tree. It was the first effective medicine used for treating malaria caused by *P. falciparum*. Quinine (**1**, figure 1.3) was the antimalarial of choice until the 1940s when more active and less toxic synthetic analogues, several of which containing the quinoline chemical scaffold, were prepared. These include chloroquine (CQ; **2**), amodiaquine (**3**) and mefloquine (**4**).

CQ was introduced into antimalarial chemotherapy also in the 1940s [30, 31] and it was the mainstay antimalarial compound for several decades. It is considered one of the most successful antimalarial drugs ever prepared, bearing in mind its low synthetic costs, chemical stability, ease of administration and safety [31]. CQ interferes with haem

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(derived from haemoglobin digestion) detoxification in *Plasmodium* parasites. The compound increases the intravesicular pH [32, 33] in parasitized erythrocytes and binds to haem, thus preventing its bio crystallization and conversion into non-toxic haemozoin crystals, which then results in an accumulation of cytotoxic haem, eventually culminating in the death of the parasite [31-35]. CQ also exhibits gametocidal activity against *P. ovale*, *P. vivax*, *P. malariae* and against immature sexual stages of *P. falciparum* [35].

The widespread use of CQ for therapeutic and prophylactic purposes eventually resulted in *P. falciparum* and, to a lesser degree, *P. vivax* developing resistance to the drug [36, 37] (Figure 1.6). *Plasmodium falciparum* resistance has been reported practically in all endemic countries [37-40], resulting in recommendations for its discontinued use as first line treatment. However, due to its low cost and lack of affordable alternatives, some countries in which malaria is endemic still rely on CQ for treatment of malaria [40, 41].

Single nucleotide polymorphisms (SNPs) in the genes encoding the *P. falciparum* CQ resistance transporter (*Pf*CRT) and multidrug resistance transporter 1 (*Pf*MDR) have been linked to reduced parasite susceptibility to CQ, other 4-aminoquinolines and related compounds [34, 42-44]. *Pf*CRT and *Pf*MDR are components of the parasite's digestive vacuole membrane and reduce the accumulation of aminoquinolines into that compartment [39, 45].

Other quinolone-based compounds, including the 8-aminoquinoline primaquine (PQ; **5**, figure 1.3) are also effective against sexual and asexual stages of *Plasmodium* parasites

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[46-48]. Newer 8-aminoquinolines such as tafenoquine (**6**) and also combinations of bulaquin (**7**) and chloroquine (**2**) are currently undergoing clinical trials [48, 49], while pamaquine (**8**) was discontinued many years ago due to toxicity issues [50]. It is expected that the new chemotherapies under development should become available soon and contribute to expanding the limited, yet most wanted repertoire of gametocidal drugs. These will play the important role of blocking transmission of parasites, which is a vital contribution towards the current agenda of malaria eradication.

Figure 1.3 – Structures of selected quinoline antimalarial drugs.

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1.4.3.2 Antifolates

Antifolate antimalarials are compounds that impair the metabolism of the vitamin B_9 (folate), an essential pathway for the survival of malaria parasites. The compounds selectively target the asexual stage of *Plasmodium* parasites by impairing the production of pyrimidine nucleotides which are required as building blocks for the synthesis of deoxyribonucleic acid (DNA) [51]. Proguanil (**9**, figure 1.4), the precursor of the antiplasmodially active metabolite cycloguanil (**10**), is a triazine chloroguanide that was introduced into clinical use in the 1940s following the development of resistance of malaria parasites to the previously used quinolone-based drugs. Cycloguanil, and also pyrimethamine (**11**), specifically target dihydrofolate reductase (DHFR), one of the key enzymes in folate metabolism that is responsible for the reduction of dihydrofolic acid to tetrahydrofolic acid [51, 52].

Sulfadoxine (**14**, figure 1.4), a sulfonamide antibiotic, is structurally related to *p*-aminobenzoic acid (PABA) and competitively inhibits another key metabolic enzyme, dihydropteroate synthase (DHPS), by limiting the utilization of PABA in the folic acid cycle.

Resistance to antifolates emerged relatively rapidly in response to drug pressure, and it is currently widespread. The mechanism of its development involves point mutations in the DHFR and DHPS enzymes that are targeted by antifolate drugs [52-54].

Although parasite resistance and consequent decrease in clinical efficacy of antifolates was reported soon after their introduction into clinical use (Figure 1.6), this class of compounds, particularly the combination of pyrimethamine (**11**) with sulfadoxine (**14**)

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(frequently called Fansidar®), was used as first-line drug combination for treatment of CQ-resistant malaria in several sub-Saharan African countries until the 1990s [38, 55, 56]. Since then, alternative antimalarial antifolates have been used, including the combination of chlorproguanil (**12**) with dapsone (**13**) (also known as LapDap®). However, similar to some aminoquinolines, this combination was withdrawn after concerns with safety in glucose-6-phosphate dehydrogenase (G6PD) deficient patients [56-58].

New antifolate chemotherapies including the Medicines for Malaria Venture (MMV)'s P218, which is effective against both CQ- and pyrimethamine-resistant as well as sensitive parasites, are currently under development [59]. The combination of antifolates with compounds acting on different targets is regarded as showing potential to greatly improve antimalarial effectiveness further while at the same time deterring the development of resistance [59-61].

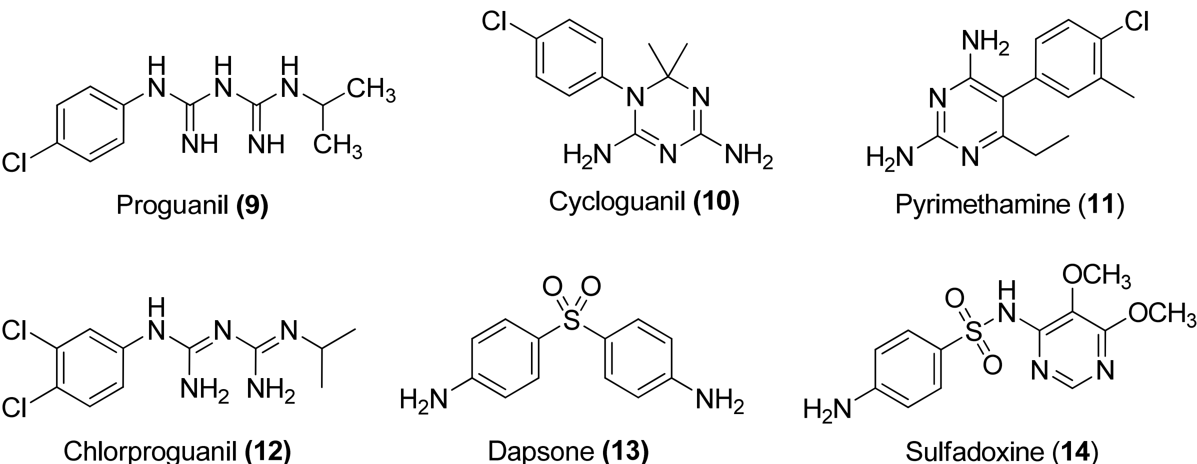

Figure 1.4 – Structures of selected antifolate antimalarial drugs.

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1.4.3.3 Sesquiterpene endoperoxides

Endoperoxides are a class of antimalarial compounds of which artemisinin (**15**, figure 1.5), also known as *qinghaosu*, is the prototype. These compounds are currently the mainstays of antimalarial treatment.

Artemisinin and its derivatives artemether (ATH; **16**), artesunate (**17**) and dihydroartemisinin (DHA; **18**), collectively called artemisinins, have a potent and fast acting activity against the early and mature intraerythrocytic stages of *P. vivax* as well as against CQ-sensitive and CQ-resistant *P. falciparum* parasites. These drugs also have gametocidal activity against early sexual stages of the parasites and therefore bear the capacity to block transmission [62].

The use of artemisinins results in rapid clearance of parasites when compared to other chemotherapies [35]. However, increased parasite recrudescence rates have been reported in association with use of these drugs, as a result of their short half-life due to rapid elimination [63, 64].

Artemisinins are prodrugs that are bioconverted into the active metabolite DHA in the parasite. The mechanism of action of this class of compounds has not been completely resolved. However, one theory proposes that when the parasite feeds on haemoglobin, within its digestive vacuole in the RBC, oxidative stress is generated by the iron that is released from the haem during haemoglobin digestion through the direct reduction of the endoperoxide bond of artemisinins. The generated reactive oxygen species (ROS) then damage the parasites' food vacuole membrane and affect mitochondrial electron transport functions, eventually resulting in parasite death [65, 66]. It is also thought that

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the antimalarial activity of artemisinins may result from the disrupted cellular redox cycling [67, 68]. Indeed, artemisinin activity depends on haemoglobin digestion [69], which is in accordance to the drug's specificity and efficacy against other haemoglobin-degrading microorganisms [70]. Other theories suggest that artemisinin oxidizes parasite Flavin Adenine Dinucleotide ($FADH_2$) and parasite redox-active flavoenzymes [71] or it is activated by reduction in the parasite mitochondria [72], both processes resulting in parasite death due to increase in ROS. Still other postulations suggest that artemisinin inhibits a homologue of the sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) in *P. Falciparum* [73]. Furthermore, pharmacologically active metabolites of artemisinin have been found to alkylate specific *P. falciparum* proteins, including a translationally controlled tumor protein homologue [74].

Artemisinin resistance has been confirmed in the Great Mekong Sub-region in Southeast Asia [75] (Figure 1.6). Further elucidation of the mechanism of action of artemisinin may improve our understanding of its resistance. However, the role of haemoglobin digestion in the mechanism of action of artemisinin [69] is consistent with one theory that suggests that artemisinin resistant parasites exhibit detained development of the ring-stage parasites - a stage in which haemoglobin digestion is just at the beginning in the forming digestive vacuole [76, 77].

Studies using microarray and biochemical analyses revealed that *P. falciparum* MDR1 and CRT gene amplification, polymorphisms at codons 241, 86, and 76 of PFE0775C, elevation of the antioxidant defence network, and increased expression of many chaperones, are involved in the mechanisms of parasite resistance to DHA and ATH [67, 78, 79]. The identification of these elements can facilitate the discovery of new antimalarial compounds that target them or other elements of the specific biochemical

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pathways involved. Promising new compounds may be able to prevent *P. falciparum* from entering an inactive stage altogether, or greatly prolong the time at which parasites recrudesce *in vitro* after artemisinin exposure. One clinical trial showed that increasing the daily dose of artemisinin from 4 mg/Kg to 6-8 mg/kg was not effective as a treatment option since the dose range did not accelerate the clearance of parasites from blood circulation and was associated with increased toxicity including temporary neutropenia [80]. Clinical findings such as this highlight the importance of identifying novel antimalarial compounds, with mechanisms of action that allow achieving similarly massive reductions in blood-circulating parasites.

Despite the emergence of resistance to artemisinins, combinations of this class of drugs and combinations of artemisinins with other antimalarial compounds including mefloquine, doxycycline or sulfadoxine/pyrimethamine (usually called artemisinin-based combination therapy, or simply ACT), remain effective and are recommended as first-line treatment for uncomplicated *P. falciparum* malaria in virtually all malaria endemic countries [81].

The demand for antimalarial compounds worldwide is very high, and although synthetic protocols for preparation of artemisinin, the sole, still largely effective, compound currently in clinical use, have been developed [82-84], the main source continues to be the plant *Artemisia annua* from which it is extracted. However, the cultivation of the plant and subsequent preparation of drugs takes at least eight months to complete; the supply is unstable and does not yield sufficient quantities for the more than 200 million clinical cases of malaria reported every year.

The potentially devastating impact of resistance to all drugs for which currently there is no replacement in sight, should it spread widely outside the current boundaries, calls for implementation of strategies to (i) extend the useful life span of artemisinin, and (ii) generate novel combination therapies not based on currently used drugs, and combinations that target essential parasite pathways and biological processes not explored by the existing compounds.

Artemisinin (**15**)

R = CH_3 - Artemether (**16**)
R = $CO(CH_2)CO_2H$ - Artesunate (**17**)
R = H - Dihydroartemisinin (**18**)

Figure 1.5 – Structures of selected sesquiterpene endoperoxide antimalarial drugs.

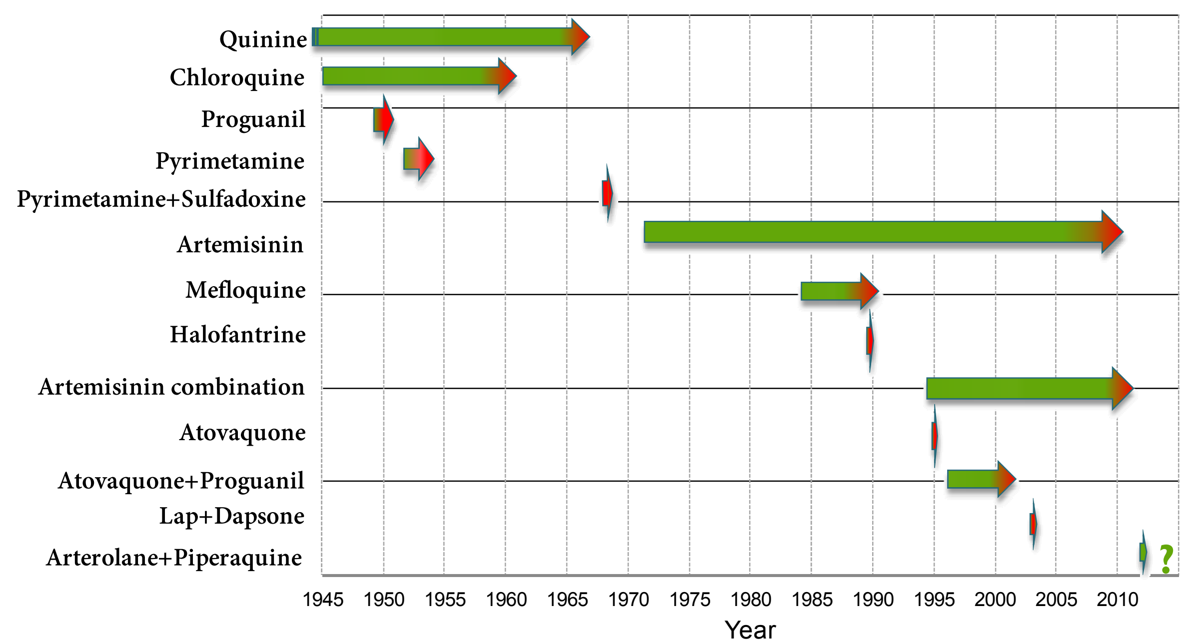

Figure 1.6 – Approximate date of introduction of selected chemotherapies (green shading) and the observation of antimalarial drug resistance (red shading). Modified and updated from: Hyde [85].

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1.4.4 Searching for new antimalarial drugs

Several antimalarial chemotherapy development approaches have explored previously identified targets as well as existing pharmacophores (defined as ensembles of steric and electronic features that are necessary to ensure the optimal intermolecular interactions with specific biological targets and to trigger (or block) its biological response) [86].

Therapy optimization using new dosing regimens of existing drugs has also been applied to develop new chemotherapies. Among other examples, this approach has been explored to produce combinations of Amodiaquine/Sulfadoxine/Pyrimethamine [87]; Amodiaquine/Artesunate [88]; Chlorproguanil/Dapsone [89] and more recently, to produce the child-friendly, dispersible ACT formulation which is a combination of Artemether and Lumefantrine (**19**, figure 1.7) [90], commercially known as Coartem®.

Lumefantrine (**19**)

Figure 1.7 –Structure of lumefantrine (**19**), a compound used in combination with other chemotherapeutics for improved antimalarial activity of formulations including Coartem® or Riamet®.

Natural products, mainly derived from plants, constitute another approach for developing antimalarial chemotherapies [91]. Screening of natural products known to possess antipyretic and other pharmacological properties can be the starting point for

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medicinal chemistry endeavours [92, 93]. Natural products are the sources of the two most important drugs used for antimalarial chemotherapy; quinine and artemisinin. However, as discussed previously, the supply of plant-derived compounds is generally unstable and costly.

Using drugs that have been developed for other diseases is also a possibility that has been explored for antimalarial drug development. Such drugs might be active against malaria parasites by acting against orthologs of their targets in other systems, or their antiplasmodial activity may be through different mechanisms [29]. Exploring such an approach has the advantage of making use of compounds — regardless of their mechanism of action — that have already been developed and approved for use in humans, which translates into reduced costs of development and less time spent in product development prior to introduction into the market [29]. This approach can also make use of inexpensive drugs, particularly after production restrictions associated for example with patents have expired, as has been observed with some antibiotics including folate antagonists [94] and atovaquone [95].

The use of combinations of drugs to which the parasites have developed resistance with "resistance reverser" compounds is another approach used in antimalarial drug development. A number of clinically used drugs, including the antihypertensive verapamil (**20**, figure 1.5) [96, 97] and the antidepressant desipramine (**21**) [98], have been shown to reverse the resistance of *P. falciparum* parasites to CQ, opening the possibility of co-administration of CQ with resistance modulators for antimalarial treatment.

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Verapamil (**20**)

Desipramine (**21**)

Trifluoperazine (**22**)

Figure 1.8 – Structures of selected examples of antimalarial drug resistance reversers

It is important to note that the use of a single resistance reverser drug often requires administration of extremely high concentrations of the compound in order to achieve a positive effect. However, when combinations of resistance modulators including verapamil, desipramine and trifluoperazine (**22**, figure 1.7) are applied, clinically practical concentrations can be used to achieve better drug resistance reversing and antimalarial efficacy [98-100].

Synthesis of analogues of existing known antimalarial compounds to achieve improved activity is probably the mostly exploited approach in antimalarial drug development. Under this strategy (as well as the ones discussed previously), knowledge of the mechanism of action or the biological target(s) of the compound is not necessarily required. This approach has successfully been applied, for example, to prepare more effective quinine analogues including CQ (2), amodiaquine (3), primaquine (5), and mefloquine (4) (Figure 1.3) and also to prepare more effective antifolates and

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artemisinins, based on the chemical structures of their parent compounds pyrimethamine (**11**) and artemisinin (15), respectively [101-103].

It is known that antimalarial drugs are based on only about 30 different chemical scaffolds of which only around 10 are clinically useful [103]. In addition to the limited diversity of scaffolds, the drawbacks of chemical modifications of existing compounds — particularly those to which parasites have developed resistance — are that such modified chemical agents, being structurally related to their parent drugs, may be affected by the phenomenon of cross-resistance (resistance that results from exposure to a similarly acting and/or chemically related substance). Therefore, the development of new chemical entities with novel targets and novel mechanisms of action is desirable to allow the effective control of malaria and the ultimate goal of eradicating the disease.

1.4.5 Identifying novel antimalarial drug targets

Possibly the most innovative and also challenging approach in antimalarial drug development is identifying new targets and then designing compounds that act on such targets. Nevertheless, the readily accessible TDR - Targets Database website (http://tdrtargets.org) that integrates genomic information with functional data (including, for example, data on gene expression, phylogeny and essentiality, that has been collected from various sources), associated with our current knowledge of the parasite biology and biochemistry and the availability of technologies for genetic manipulation, constitute some of the necessary tools for identification and characterisation of drug targets and development of effective chemotherapies.

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A drug target can be a single gene, a gene product or a molecular mechanism identified based on genetic analysis or biological observations [104]. Targets that are unique to the parasite, as well as those that are shared with humans but exhibit important differences that allow for selectivity, can be exploited for drug development. For obvious reasons, targets that are exclusive to the parasite are preferred as they allow the possibility of designing agents with maximum selectivity towards the parasite and minimum risk of toxicity to the human host.

At least 60 biological targets for antimalarial drug development have been identified in *P. falciparum* [103, 105] and, as discussed by Jana and Paliwal [106], these include phospholipid biosynthesis, the hexose transporter on the parasite's plasma membrane; parasite proteases including plasmepsins and falcipains; the 5-enolpyruvyl shikimate 3-phosphate synthase enzyme; isoprenoid biosynthesis; the thioredoxin reductase and γ-glutamylcysteine synthetase (GCS) redox system; purine and pyrimidine metabolism among others.

It is important to point out that one disadvantage of using target-based drug discovery is that predicting which targets are essential for the parasite while in the human host is often difficult. Additionally, well-validated molecular targets do not always yield new classes of compounds, and resistance may emerge more rapidly for compounds that interact with a single cellular target. For these reasons, and also considering that often a very potent compound against a specific target may not exhibit any activity against the whole parasite (because, for example, it cannot enter the parasite cell), several current drug development endeavours are shifting and/or incorporating whole cell phenotypic screening. This has resulted in the identification of several thousands of potent

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antimalarial compounds [103, 105-108], most of which are particularly active against the clinically important asexual intraerythrocytic-stage of the parasite life-cycle, although some are also active against the liver-stage and sexual forms.

Although whole-cell phenotypic drug screening has so far been very successful in identifying new antiplasmodial compounds, the challenge remains to elucidate the mode of action (MoA) of these compounds in order to facilitate, through medicinal chemistry, the improvements required for better antimalarial activity as well as better pharmacokinetics profiles.

One metabolic process that has been identified through biological observations as a potential drug target in several microorganisms — including malaria parasites — is the biosynthesis of Coenzyme A (CoA).

1.4.5.1 Pantothenate and the universal biosynthesis of CoA

Pantothenate (**23**, Scheme 1.1), the ionized form of pantothenic acid, also known as vitamin B_5, is a water-soluble compound with a name derived from the Greek word *pantothen* meaning "from everywhere". It gets this name as a result of its ubiquitous presence in small quantities in virtually all natural food sources [109] – consequently, vitamin B_5 deficiency is very rare, although it has been reported in cases of severe malnutrition in World War II prisoners [109]. When occurring, B_5 avitaminosis results in symptoms that include fatigue, allergies, nausea, numbness, painful burning and tingling in the feet, and abdominal pain; all of which are reversed by pantothenic acid supplementation [109].

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Certain bacteria, fungi and plants have the ability to synthesise pantothenate *de novo* while animals and eukaryotes (and some prokaryotes) must acquire this nutrient from external sources. Following uptake or *de novo* synthesis, pantothenate is used to synthesize the essential enzyme cofactor CoA through a universal five-step enzymatic process depicted in scheme 1.1 [110].

Scheme 1.1 - Biosynthesis of Coenzyme A from pantothenate and its cellular utilization. Dashed arrows represent movement into the apicoplast (grey square, a vestigial plastid organelle derived from a secondary endosymbiosis, which is part of an apical structure designed to aid entry of the parasite into a host cell and also hosts several metabolic processes including lipid, isoprenoid, haem, amino-acids, ion-sulphur cluster synthesis among other functions) – in the case of malaria parasites – where CoA synthesis is completed and the incorporation of the 4'-phosphopantetheine prosthetic group (in blue colour) into the acyl carrier protein (ACP) takes place.

The first step in the pathway is the pantothenate kinase (PanK)-catalysed phosphorylation of pantothenate, resulting in 4'-phosphopantothenic acid (**24**). Next, a cysteine molecule is condensed with 4'-phosphopantothenic acid in a reaction catalysed

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by phosphopantothenoylcysteine synthetase (PPCS) to form 4'-phosphopantothenoylcysteine (**25**). Following this, the cysteine portion of 4'-phosphopantothenoylcysteine is decarboxylated in a reaction catalysed by phosphopantothenoylcysteine decarboxylase (PPCDC) resulting in 4'-phosphopantetheine (**26**). In the fourth step, phosphopantetheine adenylyltransferase (PPAT) catalyses the attachment of an adenylyl group to 4'-phosphopantetheine resulting in the formation of dephospho-CoA (**27**). In the final step, dephospho-CoA kinase (DPCK) catalyses the phosphorylation of dephospho-CoA at the 3'-position of the ribose moiety culminating in the production of CoA (**28**) [111]. The 4'-phosphopantetheine prosthetic group is then covalently attached to a conserved serine residue in a pre-formed inactive acyl carrier protein (*apo*-ACP) converting it into an active *holo*-ACP form in a reaction catalysed by ACP synthase (AcpS). ACP hydrolase (AcpH) is then responsible for 4'-phosphopantetheine prosthetic group recycle [112, 113].

It has been shown that only the dextrorotatory (D)-(-) isomer of pantothenate has biological activity, while the levorotatory (L)-(+) form may act as an antagonist of its enantiomer [114, 115].

1.5. Pantothenate utilization and CoA metabolism as drug targets in *P. falciparum*

CoA is essential in many, if not all, living organisms. All genome sequences known to date encode enzymes that utilize CoA or its thioesters as substrates. Among other activities, CoA functions as the universal acyl group carrier and carbonyl-activating group in several critical reactions in cellular metabolism. It is the source of the 4'-phosphopantetheine prosthetic group as already discussed. ACP and CoA play a critical

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role in fatty acids, poliketide and non-ribosomal peptide synthesis [116-118], and ACP is also involved in tetrahydrofolate and lysine metabolism [119, 120].

A study by Saliba and co-workers has shown that the CoA biosynthetic pathway is vital for the survival of *P. falciparum* parasites [121]. Processes in this metabolic pathway have been identified as specific targets for drug development including (i) the uptake of pantothenate, which is the essential starting material in the pathway; (ii) pantothenate phosphorylation by PanK, which is the first and obligatory step in the pathway and (iii) CoA utilization in fatty acid metabolism and other processes that rely on CoA-thioesters as co-substrates.

1.5.1. Pantothenate uptake as a drug target

In 1976 Trager and Jensen [122] developed a method for continuous *in vitro* cultivation of intraerythrocytic-stage malaria parasites in human RBCs. Since then, their groundbreaking discovery has aided the completion of other remarkable studies and proved to be an essential tool for antimalarial drug research. For instance, while investigating the nutritional requirements for *P. falciparum* grown in *in vitro* continuous cultures, Divo and co-workers [123] demonstrated that the parasite exhibits an absolute requirement for pantothenate. In their studies the authors showed that parasite proliferation was inhibited by 82% when all water-soluble vitamins were omitted from the culture medium. On the other hand, parasite proliferation was normal when each of the vitamins were omitted one at a time except for pantothenate, which resulted in inhibition of parasite proliferation by 80%, clearly demonstrating its essentiality for the intraerythrocytic-stage multiplication of the parasite.

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Under physiological conditions the membrane of normal, uninfected human erythrocytes can be considered to be impermeable to pantothenate, since the vitamin crosses the membrane at a very slow rate [124]. However, it has been demonstrated that pantothenate avidly traverses the membrane of *P. falciparum*-infected RBCs, and the rapid pantothenate uptake results from the fact that the maturing parasite inside the host RBC induces the formation of so called “new permeation pathways” (NPPs) on the membrane of the erythrocyte to serve as gateways through which it imports the nutrients that it requires and excretes various metabolic waste products. NPPs are permeable to a wide range of small molecules including neutral and inorganic ions, and uninfected RBCs do not exhibit NPPs [17, 124-127].

An in depth analysis of the mechanism of transport of pantothenate across the membrane of the parasite and the membrane of the RBC has revealed several important differences. First, *P. falciparum* parasites take up pantothenate through a H^+:pantothenate symporter system, which means that the transport of pantothenate throughout the membrane is dependent on H^+ as opposed to the Na^+-dependent pantothenate transporter system in mammalian cells. Once pantothenate is taken up and reaches the cytosol of the erythrocyte it enters the parasitophorous vacuole (a vacuole that is formed during erythrocyte invasion and where the parasite resides) by diffusion or through channels present on the membrane of the vacuole. Afterwards it is transported across the parasite plasma membrane by the H^+-coupled transporter which has a 1 to 1 stoichiometry, meaning that the transport of one H^+ ion down the electrochemical gradient is associated with the transport of a single pantothenate molecule into the parasite cytosol [128]. Second, the mammalian pantothenate transporter system is characterised by high substrate affinity, exhibiting a Michaelis-

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Menten constant (K_m) of 2-5 μM [129-131] compared to the low substrate affinity (K_m of ~23 mM) symporter of *P. falciparum* [128].

Although some molecular details of the pantothenate transport systems remain to be elucidated, it is accepted that since the pantothenate requirements of infected erythrocytes differ compared to their normal healthy counterparts, such disparities could potentially be exploited to design antimalarial agents that specifically disrupt pantothenate uptake by the parasite, while only minimally affecting mammalian cells.

1.5.2 Phosphorylation of pantothenate by PanK and its potential as a drug target

Once pantothenate reaches the cytosol, the *P. falciparum* PanK (*Pf*PanK) — which has been shown to have an exceptionally high affinity (K_m of ~ 0.3 μM) for the vitamin, rapidly phosphorylates it resulting in its trapping inside the parasite. The enzyme requires adenosine triphosphate (ATP) to convert pantothenate into 4'-phosphopantothenic acid [124, 128].

Although phosphorylation of pantothenate has been reported in the erythrocyte cytosol, the rate at which the reaction occurs in the human cell is negligible when compared to the phosphorylation mediated by *Pf*PanK in the parasite [128].

It has been reported that the pantothenate phosphorylation product, 4'-phosphopantothenate, accumulates in infected RBCs as well as in isolated parasites, but not in healthy uninfected erythrocytes. This is an indication that CoA biosynthesis is regulated differently in the parasite *versus* in the host RBCs. Similar to what occurs in the parasite cytosol, *Pf*PanK activity in parasite lysates is inhibited by, CoA indicating

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that *Pf*PanK, similar to what has been reported for most other organisms with a type II PanK, is susceptible to feedback inhibition by CoA or its thioesters [132].

Although limited information is currently available with regards to the other four enzymes required to complete the CoA biosynthesis pathway in *P. falciparum*, candidate genes for these enzymes have been identified and data consistent with their expression during the intraerythrocytic-stage of the parasite's life cycle has been generated [132].

Contrary to what is known about *Pf*PanK, with biochemical activity that has been demonstrated in *P. falciparum*, no biochemical evidence of the activity of the other CoA biosynthesis pathway enzymes has been demonstrated in the parasite. Nevertheless, taking into consideration the presence of candidate genes, and the demonstrated capacity of the parasite to synthesise CoA [125], it is postulated that PPCS, PPCDC and PPAT sequentially convert 4'-phosphopantothenic acid into 3'-dephospho-CoA within the parasite cytosol [133]. Dephospho-CoA is then transported to the apicoplast where it is converted into CoA in a reaction catalysed by DPCK.

DPCK has been shown to possess an apicoplast amino-terminal bipartite leader sequence, the first part of which is a signal peptide that targets the protein to the secretory pathway and the second part, called the transit peptide, directs proteins from the secretory pathway across the various membranes surrounding the organelle [133-135].

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The presence of an apicoplast leader sequence on DPCK is indicative of its expression outside the organelle and subsequent importation to the organelle where the final step of the CoA biosynthesis is presumed to take place (Scheme 1.1).

Mammalian PanKs also belong to type II pantothenate kinases, the best characterized of which is the murine PanK. Type II PanKs are members of the ribonuclease H-like family of kinases, which is related to the acetate and sugar kinase/hsp70/actin superfamily (ASKHA) [136, 137].

Since the biosynthesis of CoA depends on PanK as the initiating step, it is reasonable to consider targeting this enzyme in endeavours for antimalarial drug development. Although both the human and *Pf*PanK belong to the same type II pantothenate kinases and share some common aspects as already discussed, they also exhibit major differences based on their low sequence similarity, which makes this enzyme very attractive for exploitation as potential antimalarial drug target.

1.5.3 CoA utilisation and its potential as a drug target

It has been reported that the apicoplast contains ACP and all the enzymes required for CoA utilization in fatty acid biosynthesis [134, 135, 138]. Fatty acids are precursors of essential structural and metabolic components including, among others, phospholipids, which are necessary for the formation and function of cell membranes as well as for plasma lipoproteins. Fatty acids are synthesized from malonyl-CoA, which is formed by the carboxylation of acetyl-CoA. Malonyl-CoA serves as a universal precursor for elongation of acyl chains under the action of a complex of associated enzymes known as

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the fatty acid synthase (FAS). The synthesis occurs in a repetitive cycle of condensation, reduction, dehydration and reduction reactions.

Until a few years ago, it was thought that *P. falciparum* lacked the ability for *de novo* fatty acid biosynthesis and that it relied on scavenging lipids from the host RBC. However, upon completion of the genome sequencing of *P. falciparum* and the discovery of the apicoplast with its metabolic machinery that includes ACP and genes coding for a type II FAS system, this initial hypothesis has been questioned, particularly since the encoded proteins show primary sequence homology to orthologues involved in *de novo* fatty acid synthesis in bacteria and plants [134, 139].

While *P. falciparum* bears genes coding for a type II FAS – a synthetic system in which each reaction in the fatty acids biosynthesis is catalysed by a discrete enzyme [140], humans harbour a type I FAS system in which a large multifunctional polypeptide contains all the enzymes required for all the reactions of the *de novo* fatty acid biosynthesis [134, 139, 141]. Considering the differences in fatty acids biosynthesis in *P. falciparum versus* human cells, and the large amounts of fatty acids required by the parasite, either from *de novo* synthesis (which is said not to be essential during the intraerythrocytic stage of the parasite's life cycle while particularly important in the liver-stage development phase [142]) or scavenged from the host, the utilisation of CoA as the central enzyme cofactor seems to be a very suitable drug target for antimalarial drug development.

A consequence of the attractiveness of the CoA metabolic pathway as a target for antiplasmodial drug development as discussed above is that over the years, numerous

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compounds with chemical structures resembling that of pantothenate have been synthesised and tested against different malaria parasites. Some of the compounds prepared were observed to have parasite proliferation promoting activity while others seemed not to affect the parasite multiplication. Encouragingly, some compounds exhibited potent inhibitory properties against clinically important malaria parasites in both *in vitro* and *in vivo* systems.

The compounds prepared and tested against malaria parasites and other microorganisms since the 1940s until recently have been extensively reviewed by Spry *et al*. [134]. A selection of some of the more important compounds for antimalarial drug development has been made and these are discussed below.

1.6 Pantothenate analogues as antimalarial agents

1.6.1 Pantoytaurine and related compounds

The observation that calcium pantothenate promoted the development of intraerythrocytic stage *P. lophurae* in cultures, grasped the interest of scientists who then began to examine the activity of pantothenate analogues as antimicrobial agents [143].

Pantoyltaurine (**29**, figure 1.9) and pantoyltauramide (**30**) – pantothenic acid analogues in which the carboxylic acid group is replaced by a sulphonic group – which have been previously synthesized and tested against some pathogenic bacteria, were among the first pantothenic acid related compounds examined for their activity against malaria parasites [144]. The activity of these compounds was tested *in vivo* against the avian

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malaria parasites *P. lophurae*, *P. gallinaceum* and *P. relictum* in ducks, chickens and canaries, respectively.

When incorporated in the ration used to feed the birds, both compounds were essentially inactive against the parasites. Nevertheless, D-pantoyltauramide at doses of 2 g/kg body weight (b.w.) per day or 400 mg/kg b.w./day administered intravenously inhibited the proliferation of *P. gallinaceum* in chickens [145]. Cantrell [146] also demonstrated that the antiplasmodial activity of pantoyltauramide was antagonised by the simultaneous administration of calcium pantothenate, indicative of the activity of the compound resulting from interference with pantothenate utilization.

Encouraged by the results on the *in vivo* antiplasmodial activity of D-pantoyltauramide, a large series of *N*-substituted pantoyltauramides was prepared aimed at improving their pharmacokinetics profile, particularly their absorption/excretion characteristics relative to the parent compound, and hence to be more active after oral administration. The newly prepared compounds resulted also in better antiplasmodial activity against *P. gallinaceum* in chickens and against the simian parasite *P. coatneyi* as well as the human *P. falciparum* in monkey and human erythrocytes respectively in *in vitro* experiments [147]. Some compounds, including compounds **31**a-e, (figure 1.9), were even more active than quinine, the gold standard antimalarial drug at the time. The most active, compound (**31d**) was four times more potent than quinine when tested against a blood-induced infection of *P. gallinaceum* in which peak parasitaemia was reached 4 days post-infection [144] and, sixteen times more potent against a blood-induced infection with the same parasite, in which peak parasitaemia was reached 7 days post-infection. It is relevant to point out here that the latter test is a more sensitive test for the

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antiplasmodial activity of slower acting compounds [144]. In addition to their improved pharmacokinetic and antiplasmodial activity, the *N*-substituted analogues of pantoytauramide proved to be better tolerated (less toxic) than quinine after administration to *rhesus* monkeys [148]. Consistent with their action being through interference with pantothenate utilization by the parasite, their co-administration with large amounts of that nutrient resulted in reduced antiplasmodial activity.

Pantoyltaurine **(29)**

Pantoyltauramide **(30)**

31

31a R = Me **31b** R = **31c** R = **31d** R = **31e** R =

Figure 1.9 - Structures of pantoyltaurine (**29**), pantoyltauramide (**30**) and *N*-substituted pantoyltauramides (**31a-e**).

1.6.2 Phenylpantothenone and related analogues

After having shown some antimicrobial activity against several pathogenic bacteria *in vitro*, the pantothenate analogue phenylpantothenone (**32**, figure 1.10) was tested against various malaria parasites. It is interesting to note that when tested *in vivo* against *P. lophurae* in ducks, phenylpantothenone failed to impair parasite proliferation. However, when screened against the same parasite and also against *P. gallinaceum* in chickens, the compound exhibited an antiplasmodial activity equivalent or greater than that of quinine [144]. As had been observed with the previous compounds, the activity of

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D-phenylpantothenone was decreased by the concomitant administration of pantothenate.

As D-phenylpantothenone had demonstrated some antiplasmodial activity against various malaria parasites with varying potency in different hosts, structural modifications were sketched and a number of analogues prepared. The new compounds demonstrated improved activity against the parasites and among these, compound **33** (figure 1.10) exhibited greater antiplasmodial activity compared to the parent drug D-phenylpantothenone. This particular compound was threefold more effective against *P. gallinaceum* than quinine, and its activity was suppressed when co-administered with pantothenate [144].

After development of methods for *in vitro* cultivation of various species of malaria parasites — including the avian parasites *P. lophurae* and *P. gallinaceum*, the monkey parasite *P. coatneyi* and the clinically important human parasite *P. falciparum* — for at least one life cycle, the antiplasmodial activity of the compounds was screened again *in vitro*. All the previous *in vivo* results were confirmed in the *in vitro* experiments with some of the compounds demonstrating even higher antiplasmodial potency [147, 149].

Interestingly, although some D-phenylpantothenone analogues and *N*-substituted pantoyltauramides were active against several malaria parasites in different *in vivo* models and were less toxic than quinine, none of them have been tested for their antimalarial activity in humans or other primates.

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D-phenylpantothenone (32) 33

Figure 1.10 - Structures of D-phenylpantothenone (**32**) and D-*para*-chloro-2-methyl-phenylpantothenone (**33**).

1.6.3 Pantothenol and related compounds

Pantothenol (**34**, figure 1.11), the alcohol analogue of pantothenate, has more recently been investigated *in vitro* for its antiplasmodial activity against *P. falciparum*. The compound exhibited an IC_{50} (the concentration that inhibits parasite proliferation by 50%) of 60 μM against the CQ-resistant parasites of the FAF6 strain [121]. When administered orally at 1.4 g/kg b.w./day for 4 days to mice infected with *P. vinckei vinckei*, pantothenol was active, significantly reducing parasitaemia in the treated animals, although not resulting in cure. Not surprisingly, the *in vitro* antiplasmodial activity of this compound, similar to previously reported with other pantothenate analogues, was antagonised by the simultaneous administration of pantothenate [121].

Pantothenol is a provitamin that in mammalian cells is oxidised by the enzyme alcohol dehydrogenase, resulting in the production of pantothenate [150]. Considering the metabolic pathway targeted by these compounds, it can be deduced that the *in vivo* conversion of pantothenol to pantothenate not only will reduce the concentration of active drug (pantothenol) but also produce the very same compound (pantothenate) that antagonises its antiplasmodial activity; a possible reason for the observed inability of pantothenol to cure *P. vinckei vinckei*-infected mice [121].

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Despite the poor *in vivo* growth inhibition results obtained, it is relevant to point out that *in vitro* experiments demonstrated that pantothenol at the concentrations required for inhibition of parasite proliferation was selective towards the parasite and did not affect the proliferation of mammalian T-lymphocyte cells (Jurkat cell line). Saliba and co-workers [121] also demonstrated that pantothenol inhibited parasite proliferation through inhibition of the phosphorylation of pantothenate by the parasite's PanK enzyme.

Pantothenol **(34)** **35** **36**

37 **38** **39**

Figure 1.11 - Structure of pantothenol (**34**) and analogue compounds (**35-39**).

Other pantothenate analogues known as *N*-pantoyl-substituted amines — including compounds **35-39** (figure 1.11) with chemical structures related to pantothenol — which had demonstrated antibacterial activity, have also been tested against malaria parasites, revealing *in vitro* growth inhibitory activity at concentrations of 10-200 μM [126, 151].

Other compounds which like pantothenol, result from simple modifications of the β-alanine moiety of pantothenate have been prepared and tested against different pathogenic microorganisms. Some of these, including CJ-15,801 (**40**, figure 1.9), a

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metabolic product of the fungus *Seimatosporium* sp., have also been tested *in vitro* against the human malaria parasite *P. falciparum* [152]. The compound inhibited parasite proliferation with an IC_{50} of 39 ± 3 μM in the presence of standard concentrations (~1 μM) of pantothenate in the culture medium. At a concentration of ~250 μM under standard parasite growth conditions, CJ-15,801 completely inhibited parasite proliferation. However, when the concentration of pantothenate in the culture medium was increased to 100 μM or 500 μM, the antiplasmodial activity was terminated, again indicating that CJ-15,801 interfered with pantothenate utilization by the parasite.

CJ-15,801 was also found to be selective towards the parasite as it did not inhibit mammalian rat hepatoma cell line (HTC) proliferation when administered at the antiplasmodially effective concentrations [152].

HO OH H N OH O O

CJ-15,801 **(40)**

Figure 1.12 - Structure of compound CJ-15,801 (**40**).

1.6.4 *N*-substituted pantothenamides

N-substituted pantothenamides (PanAms) are pantothenate analogues in which the carboxylate group of pantothenate has been replaced by a secondary or tertiary amide group (figure 1.13).

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Secondary or tertiary Amide

Carboxylate

Pantothenate

N-substituted pantothenamides

Figure 1.13 - Structures highlighting the differences between pantothenate and *N*-substituted pantothenamides.

A number of such compounds have been reported to inhibit the *in vitro* proliferation of various bacteria [153-157] including *Escherichia coli* and *Staphylococcus aureus*. In those microorganisms, the most studied and class representative PanAm, *n*-pentylpantothenamide (N5-Pan) (**41,** Scheme 1.2) has been reported to serve as a substrate for PanK and, as a consequence, inhibit the phosphorylation of pantothenate resulting in the production of a 4'-phosphopantothenamide [156, 158, 159]. This is then further processed by the other enzymes of the CoA biosynthetic pathway to yield a CoA analogue; ethyldethia-CoA [160].

The 4'-phosphopantothenamide moiety of ethyldethia-CoA is incorporated into *apo*-ACP by AcpS, converting the carrier protein into a non-functional ACP (called *crypto*-ACP) by the virtue of lacking the sulfhydryl (-SH) group of natural CoA, which is required for the protein to function as a transporter of acyl groups from one reaction to another in fatty acids biosynthesis [161, 162]. *Crypto*-ACP is recycled back to *apo*-ACP through a hydrolytic reaction catalysed by AcpH (scheme 1.2). However, it has not been determined whether the induction of stasis in bacterial replication caused by this class of compounds results from the inhibition of CoA biosynthesis, the inhibition of fatty acid

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biosynthesis, the inhibition of other CoA-dependent process(es) or a combination of multiple factors.

Scheme 1.2 - In bacteria *N*-substituted pantothenamides (such as N5-Pan) act as antimetabolites by forming coenzyme A analogues (ethyldethia CoA) lacking the essential terminal thiol (-SH) group of natural CoA.

Encouraged by the current knowledge on the antiplasmodial activity of pantothenic acid analogues against *P. falciparum*, previous researchers in our laboratory developed a method for simultaneous synthesis and purification of PanAm [157]. Using this method, a mini-series of 147 PanAms was prepared and tested *in vitro* against different pathogenic bacteria as well as against *P. falciparum* parasites. Until the recent publication of a study [163] done in collaboration with our co-workers at the Australian National University (ANU) – part of which is described in this dissertation, no research specifically investigating the activity of PanAms against malaria parasites had been published in the literature.

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1.7 Project aims

Considering the rich history of research on pantothenate and coenzyme A biosynthesis as potential drug targets for antimalarial chemotherapy, as discussed above, and the fact that our laboratory has recently generated a mini-library of PanAms that were demonstrated in previous studies to be active against various pathogenic bacteria as well as against malaria parasites, this project was initiated with two major aims:

1. To improve the antiplasmodial activity of PanAms and expand the current repertoire of pantothenic acid analogues with antiplasmodial activity.

2. To investigate the mode of action (MoA) of PanAms against intraerythrocytic-stage *P. falciparum* parasites. Specifically, it was aimed to determine whether the antiplasmodial activity of this class of compounds results from:
   (i) Inhibition of CoA biosynthesis by virtue of interference with the phosphorylation of pantothenate by *Pf*PanK, or;
   (ii) Reduction of CoA and/or ACP-levels, leading to the inhibition of processes dependent on these entities.

1.8 Dissertation outline

In the first chapter of this dissertation (**Chapter 1**) a general introduction to malaria, the CoA metabolic pathway and a literature review of relevant work done on pantothenate analogues as potential antimalarial drugs is presented. However, the work that resulted in pursuit of the aims stated above is reported in the following four chapters, as outlined below.

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Chapter 2 consists of a detailed description of the materials and methods used to generate and analyse all the results presented in this dissertation.

In **Chapter 3** the results obtained with the *in vitro* antimalarial growth assays performed with PanAms that were chemically modified to improve their antiplasmodial activity are reported, and includes a structure activity analysis to tease out how best potency/stability can be achieved.

In **Chapter 4** the work done towards elucidating the MoA of PanAms is discussed, specifically, the experiments and their results, performed to determine whether the antiplasmodial activity of *N*-substituted pantothenamides resulted from inhibition of the parasite CoA metabolism through interference with the pantothenate phosphorylation by *Pf*PanK, followed by the results obtained on the assays performed to determine if PanAms interfere with CoA-dependent processes downstream of *Pf*PanK — particularly in relation to the parasites' ACP.

To complete this dissertation, **Chapter 5** contains a summary of the results obtained and provides a general discussion of the outcomes of the project, followed by prospects for future research on PanAms as antimalarial agents.

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Chapter 2:

MATERIALS AND METHODS

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2. Material and Methods

2.1 Materials

2.1.1 General chemicals and drugs

All general chemicals, reagents, culture media and chloroquine diphosphate salt used during this study were purchased from Sigma-Aldrich. Antibiotics and Albumax II for cell culturing and SYBR Safe DNA gel stain (10 000 × concentrate in Dimethyl Sulphoxide (DMSO)) for fluorescence assays were purchased from Invitrogen. Scintillation fluids (Microscint O and Microscint 40) were obtained from Perkin-Elmer Inc. All the compounds used were of the highest quality available and the solvents were of CHROMASOLV HPLC grade.

2.1.2 *N*-substituted pantothenamides

A number of PanAms were synthesised previously by Dr. Marianne de Villiers, following a method developed by van Wyk and Strauss [1]. These compounds were prepared from pantothenic acid or one of two structural analogues in which its β-alanine moiety was replaced with either glycine (to give α-pantothenic acid) or γ-aminobutyric acid (to give homopantothenic acid). A range of amines representing four chemical motifs (i.e. primary alkyl amines, primary heteroatom-containing aliphatic amines, primary amines with substituents containing aromatic groups and secondary cyclic amines) was used to introduce the amide moiety into each of these acids in parallel. In this manner three sets of PanAms were prepared, referred to as α-pantothenamides (α-PanAms), normal-pantothenamides (*n*-PanAms, where *n* signifies "normal") and homopantothenamides (HoPanAms) respectively (Table 2.1). The compounds were dissolved in DMSO as 200 mM stock solutions and stored at -20°C until required.

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Table 2.1 Chemical structures of *N*-substituted pantothenamides tested *in vitro* against intraerythrocytic-stage *P. falciparum* parasites

α-PanAm (**a**) *n*-PanAm (**b**) HoPanAm (**c**)

Entry #	R group	Entry #	R group	Entry #	R group
41	NH	**46**	NH, S	**51**	NH, OCF_3
42	NH	**47**	NH	**52**	NH, CF_3
43	NH	**48**	NH	**53**	NH, CF_3
44	NH	**49**	NH, OMe	**54**	NH, NH, S, O O, NMe_2
45	NH	**50**	NH, OMe	**55**	NH, N

Pantoyltauramides (Table 2.2)[1] as well as α-methyl pantothenamides (α-MePanAms) and β-methyl pantothenamides (β-MePanAms) (Table 2.3)[1] were also dissolved in DMSO as 200 mM stocks and stored at -20°C until used [2].

Table 2.2 Structures of *N*-substituted pantoyltauramides tested *in vitro* against intraerythrocytic-stage *P. falciparum* parasites

Entry #	R group	Entry #	R group	Entry #	R group
56	NH	**57**	NH, S	**58**	NH

[1] The synthesis of these compounds was performed by Mr. Collins Jana as part of his MSc studies.

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Table 2.3 Structures of *N*-substituted α-methyl and β-methyl pantothenamides tested *in vitro* against intraerythrocytic-stage *P. falciparum* parasites

α-MePanAm (**d**) β-MePanAm (**e**)

Entry #	R group	Entry #	R group
41		52	
42		53	
43		59	
44		60	
45		61	
46		62	
47		63	
48		64	
49		65	
50		66	

Additional stocks of PanAms **41b, 41d**, **44b**, **48b** (table 2.1), **79** and **83** (figure 2.1) were synthesised by Ms. Leanne Barnard and were dissolved either in DMSO or in 75%:25% v/v Acetonitrile (ACN): H_2O as 50 – 300 mM stock solutions and stored at -20°C until required.

(79) (83)

Figure 2.1 - Chemical structures of deoxy-N5-Pan (**79**) and deoxy-*N*-phenethyl *n*-PanAm (**83**).

2.1.3 Radioactive compounds

Sodium D-[1-^{14}C]pantothenate with specific activity of 50 mCi/mmol was purchased from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA and was used in initial experiments. Subsequently, sodium D-[2,3-^{14}C]pantothenate was prepared by Dr. David Choveaux from β-[2,3-^{14}C]alanine with specific activity of 110 mCi/mmol (also purchased from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA) using a previously described method [3]. Radiolabelled versions of selected PanAms, namely [^{14}C]*N*-hexyl α-PanAm (**43a***[1]), [^{14}C]*N*-hexyl *n*-PanAm (**43b***), [^{14}C]*N*-hexyl HoPanAm (**43c***) and [^{14}C]*N*-benzyl *n*-PanAm (**48b***), were synthesised by Prof. Erick Strauss using [1-^{14}C]*n*-hexylamine hydrochloride and [7-^{14}C]benzylamine hydrochloride (both purchased from American Radiolabeled Chemicals, Inc. St. Louis, MO, USA at 50 mCi/mmol) as radioactive starting material and the protocol described by van Wyk and Strauss [1]. The compounds were stocked as 0.178 - 1.99 mM in DMSO and stored at -20˚C until used.

2.1.4 Parasites

The *P. falciparum* 3D7 (a parasite used in this study was available in the Laboratory of Dr. Kevin Saliba at the Research School of Biology, The Australian National University (for experiments conducted at ANU), and was obtained from Prof. Jacky Snoep at the

[1] An asterisk combined with a number identifying a structure denotes a radioactive compound.

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Department of Biochemistry, Stellenbosch University (SUN), for experiments performed at SUN.

2.1.5 Bacteria

E. coli strain BL21* (DE3) was obtained from Invitrogen.

2.1.6 Culture medium

RPMI 1640, Luria Bertani (LB) agar and broth media were purchased in powder form from Sigma-Aldrich for experiments performed at SUN, and RPMI 1640 ready-made in liquid form from Invitrogen™, for experiments performed at ANU.

2.1.7 Human erythrocytes

The human erythrocytes used during the study (typically Group O, Rh^+) were provided by the Western Cape Blood Bank, South Africa (for experiments performed at SUN) or by the Australian Red Cross Blood Service, Canberra, Australia (for experiments done at ANU).

2.1.8 Human pantetheinase

Recombinant human pantetheinase (Vanin) was purchased in a lyophilised powder form from Novoprotein Scientific, Inc., and was reconstituted as 100 ng/ml stocks in phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) and stored at -20°C until required.

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2.1.9 Plasmids

Plasmids used for the expression of *Bacillus subtilis* phosphopantetheinyl transferase (Sfp) (pET29-Sfp), *E. coli* PanK (pET28a-*Ec*CoaA), PPAT (pET28-*Ec*CoaD) and DPCK (pET28-*Ec*CoaE), all carrying a kanamycin resistance gene (Kan^R) and encoding for N-terminal hexahistidine tag (6×His-tag) fusion proteins were available in our laboratory. Plasmids used for the expression of a truncated version of *Pf*ACP (residues S57-Q137) with an N-terminal 6×His-tag (pSPr22, described in Du et *al*. [4]), and a non-tagged *Pf*ACP (pJGa7, described in Gallagher and Prigge [5]), both based on the pMAL-cHT expression vector described in Muench et *al*. [6] and therefore carrying an ampicilin resistance gene (AmpR), as well as the constitutively tobacco etch virus (TEV) protease producing vector pRK586 (KanR), were gifts from Prof. Sean T. Prigge of the Johns Hopkins Malaria Research Institute, Johns Hopkins University, Baltimore, MD, USA.

2.1.10 Plastic and glassware

The plastic and glassware used were typically purchased from Sigma-Aldrich, from Lasec SA or from Merck & Co., Inc. Falcon 15-ml tubes, 50-ml tubes and 250-ml ventilated and sealed tissue culture flasks were used for all cell culture and *in vitro* assays. Nunc cell culture clear 96-well plates (flat-bottomed) were used for parasite growth assays as well as SYBR green-based fluorescence measurements. Nunc flat-bottomed black 96-well plates were used for fluorescamine-based fluorescence assays. Whatman OptiPlate™ 96 and UNIFILTER™ 350 white opaque 96-well plates and TopSeal-A Press-on Adhesive Sealing Film purchased from Perkin-Elmer Inc. were used for phosphorylation assays with radiolabeled compounds.

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2.2 Methods

2.2.1 *In vitro* cultivation of *P. falciparum* parasites

All experiments were performed using the 3D7 strain of *P. falciparum* parasites. The parasites were maintained in synchronous continuous cultures essentially as described by Allen and Kirk [7]. The parasites were cultured in human erythrocytes suspended in RPMI-1640 culture medium supplemented with 25 mM 2-[4-(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES), 11 mM glucose, 200 μM hypoxanthine, 24 μg/ml gentamycin and 0.6% w/v Albumax II, pH 7.4 (Albumax-complete RPMI) to a final hematocrit of ~5%. Cultures were grown at 37°C in sealed tissue culture flasks gassed with a low O_2 gas mixture (1% O_2, 3% CO_2, in N_2) and constant rotary shaking (~50 rpm) to promote synchronized growth of the parasites [7].

One to five days before using cultures for experiments the cultures were treated with 5% (w/v) D-sorbitol solution for 10 min at 37°C to ensure that cultures were synchronous [8]. The culture medium was changed daily and parasitised erythrocytes were diluted with uninfected erythrocytes on days when parasites were mainly in the trophozoite stage. The parasitaemia (the percentage of RBCs infected with the parasites) was assessed by light microscopy and maintained at 5-15%.

2.2.2 Preparation of erythrocytes for use in *P. falciparum* parasite cultures

In preparation for use of RBCs for cultivation of *P. falciparum* parasites, fresh blood – collected in heparinised plastic blood bags and maintained at 4°C for a maximum of two weeks after collection from healthy donors – was washed to ensure minimal contamination with leukocytes and coagulated proteins. For this purpose, the blood was centrifuged in 50-ml sterile Falcon tubes at 1500×g for 8 min after which the

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supernatant, the cells at the interface between the supernatant and the RBCs, and occasional blood clots were removed. Next, the RBCs were washed three times by adding one equivalent volume of RPMI 1640 incomplete medium – which differs from complete medium by lacking Albumax II – followed by centrifugation and removal of the supernatant. Washed RBCs were maintained in an equivalent volume of RPMI 1640 complete medium at 4˚C for a maximum of two weeks before use in experiments.

2.2.3 Parasite isolation

For experiments performed with parasites isolated from their host RBCs, mature trophozoite-stage *P. falciparum* were isolated from the erythrocytes by treatment with saponin (0.05% w/v), a plant-derived glycoside detergent that interacts with the cholesterol contained in the membranes of erythrocytes resulting in lytic pores. Saponin also interacts with the cholesterol in the membrane of the parasitophorous vacuole rendering it permeable to large solutes including proteins, however it does not damage the parasite plasma membrane which lacks cholesterol [9]. Immediately after adding saponin to *P. falciparum* parasite culture suspension, the mixture was briefly mixed then centrifuged for 8 min at 2000×g at room temperature. The supernatant (containing haemoglobin, other components of RBCs cytosol and RBCs plasma membranes) was discarded. The resultant pellet of parasites was washed by re-suspension in malaria saline buffer (25 mM HEPES, 125 mM NaCl, 5 mM KCl, 20 mM glucose and 1 mM $MgCl_2$, pH 7.1) followed by centrifugation at 14000×g for 30 s and discarding of the supernatant. The washing steps were repeated (typically 3-4 times) until the supernatant was clear.

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2.2.4 *P. falciparum* parasites lysate preparation

Parasite lysates were prepared from washed, saponin-isolated intact *P. falciparum* trophozoites as described above (section 2.2.3). The parasites were suspended in cold 10 mM tris(hydroxymethyl)aminomethane-HCl (Tris), pH 7.4 buffer. A sample, typically 10 µl of the trophozoite suspension was set aside to perform parasite counts. Next, the parasites were triturated by repeated (10 times) aspiration and vigorous flushing through a 25-gauge injection needle. The lysate was then centrifuged at 2000×g for 30 min at 4˚C after which the supernatant containing the parasite proteins was retrieved and transfered into a fresh microcentrifuge (eppendorf) tube. This process was repeated typically for three times before the parasite lysate was stored at -20˚C until required.

2.2.5 Cell counts

The number of trophozoites in parasite lysates was determined by counting in an improved Neubauer haemocytometer under light microscopy at a magnification of 100×. Parasitaemia in *P. falciparum* cultures was determined by counting infected *versus* non infected RBCs using Giemsa stained smears also under light microscopy at a magnification of 1000×.

2.2.6 Preparation of complete medium for *in vitro* growth assays

The antiplasmodial activity of test compounds was assessed in two complete culture media that had been subjected to different treatment conditions prior to use. “Fresh” medium refers to RPMI 1640 complete medium that was maintained at 4˚C for a maximum of 48 h after preparation, and incubated at 37˚C for a maximum of 1 h, prior to use. “Aged” medium refers to RPMI-1640 complete medium that was incubated at 56˚C for at least 18 h before incubation at 37˚C prior to use.

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2.2.7 *In vitro P. falciparum* growth assays

Parasite proliferation was assessed using the Smilkstein *et al*. [10] fluorescence-based SYBR green assay with minor modifications. Experiments were carried out in 96-well plates starting with parasites in the ring stage, a haematocrit of 1% and a parasitaemia at 1%. Parasites were cultured in standard RPMI-1640 complete medium (which contains 1 μM pantothenate) or RPMI-1640 complete medium supplemented with pantothenate to a final concentration of 100 μM. Two-fold serial dilutions of PanAms (from 200 μM to 0.0195 μM) were made in triplicate in final volumes of 200 μl. CQ-treated parasites at 5 μM were included in the experiments to estimate 0% parasite proliferation, while parasites cultured in the absence of any test compounds served to estimate 100% parasite proliferation.

After loading the 96-well plates with the respective test cultures, the plates were placed in a desiccator, flushed with low oxygen gas mixtures (1% O_2, 3% CO_2, in N_2) and incubated at 37˚C for 96 h to allow the parasites to complete two intraerythrocytic-stage cycles. Following the 96 h incubation time, the plates were immediately frozen at -80˚C until processed for determining parasite proliferation. To quantify parasite multiplication, 100 μl of SYBR Safe solution consisting of 2 μl of SYBR Safe DNA gel stain (10 000 × concentrate in DMSO) in 10 ml lysis solution (20 mM Tris, 5 mM EDTA, 0.008% w/v saponin, 0.08% v/v Triton X-100, pH 7.5) were added per well on new 96-well plates – final DMSO concentration of 0.001%. Next, the frozen plates containing the cultures were thawed at room temperature and their contents were resuspended thoroughly by up-and-down pipetting followed by transfer of 100 μL of the suspension into the wells of the plate containing the SYBR Safe solution. Fluorescence was then measured immediately with Excitation and Emission wavelengths of 490 nm and 520

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nm, respectively, using a FLUOROstar OPTIMA multidetection microplate reader (BMG Labtech GmbH; for experiments conducted at ANU) or a Varioskan multimode reader (Thermo Fisher Scientific Inc.; for experiments done at SUN). The background fluorescence (determined from wells containing cultures treated with 5 μM CQ) was subtracted from the fluorescence measured in each well.

2.2.8 *In vitro* phosphorylation assays

2.2.8.1 Time-course phosphorylation of [14C]Pantothenate and [14C]Pantothenamides by *P. falciparum* pantothenate kinase from parasite lysates

To assess the ability of *Pf*PanK in parasite lysates prepared from saponin-isolated trophozoites to phosphorylate [^{14}C]Pan (**23***), [^{14}C]*N*-hexyl α-PanAm (**43a***), [^{14}C]*N*-hexyl *n*-PanAm (**43b***), [^{14}C]*N*-hexyl HoPanAm (**43c***) and [^{14}C]*N*-benzyl *n*-PanAm (**48b***), time-course phosphorylation assays were performed using the Somogyi reagent (combination of $ZnSO_4$ and $Ba(OH)_2$)-based method described by Saliba *et al*., [11], with some modifications.

Phosphorylation reactions were performed at 37°C using parasite lysates at a final concentration of 2.0–2.5×10^7 parasite cells/ml. The lysates had been previously prepared and stored at -20°C and briefly thawed on ice before being added to kinase buffer (50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, final concentrations, pH 7.4) containing the radiolabelled compounds tested.

At pre-determined time intervals up to 120 min, 50 μl samples of the phosphorylation reactions were retrieved in duplicate and added to designated wells of Whatman 96-well UNIFILTER™ 350 plates (with 0.45 μm PP filter bottom and short drip directors)

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that had been pre-loaded with 50 µl of 150 mM $Ba(OH)_2$. The samples were quickly mixed with the $Ba(OH)_2$ in the wells by up-and-down pipetting to terminate the phosphorylation reaction as a result of protein precipitation. After completing sample retrieval, 50 µl of 150 mM $ZnSO_4$ was added to each well and the plates were left standing for 10 min at room temperature to allow further precipitation out of solution, of any phosphorylated compounds by the Somogyi reagent. Subsequently, non-phosphorylated compounds in solution were removed by means of applying vacuum to the bottom of the plates, leaving only the radiolabelled phosphorylated compounds (solid) trapped on the filter in each well. The plates were subsequently washed twice with water (200 µl in each well) and once with 200 µl 95% (v/v) ethanol and kept under vacuum for further ~30 min before placed in an incubator to dry overnight at 37°C. Next, 30 µl of Microscint-O was added to each well and immediately sealed at the bottom and at the top side with a TopSeal-A Press-on Adhesive Sealing Film. The radioactivity in each well was measured immediately by scintillation counting in a TopCount Microplate Scintillation and Luminescence Counter (Packard).

To determine total radioactivity so as to allow the conversion of radioactivity counts per minute (rcpm) values into molar concentrations, two 50 µl aliquots of the phosphorylation reaction mixtures were added to two wells of a 96-well OptiPlate™ 96 plate. Next, 150 µl of Microscint-40 was added into each well and the plate was sealed at the top side with TopSeal-A Press-on Adhesive Sealing Film before the amount of radioactivity in each well was counted in the same Scintillation and Luminescence Counter as described above.

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2.2.8.2 Determining *P. falciparum* pantothenate kinase kinetic parameters for pantothenate and for pantothenamides

To determine whether PanAms are inhibitors or alternative substrates of *Pf*PanK, the phosphorylation of radioactively-labelled [^{14}C]Pan (**23***) and [^{14}C]PanAms (**43a-c*** and **48b***) were performed as described above (section 2.2.8.1) with the test compounds at concentrations between 0.0391 and 5.0 µM. Kinetic parameters (K_m and V_{max}) were determined by fitting the Michaelis-Menten equation to the curves generated from the initial rate data obtained in this manner.

2.2.8.3 [^{14}C] Pantothenate phosphorylation inhibition by pantothenamides

Concentration-response curves for the inhibition of [^{14}C]Pan phosphorylation by various PanAms were determined from reactions containing 0.2 µM [^{14}C]Pan (**23***) in kinase buffer and the specific PanAm in concentrations ranging between 0.1 and 100 µM. After equilibration at 37°C for 10 min, the reaction was initiated by adding an appropriate volume (typically 10 µl) of parasite lysate that had been thawed on ice. Phosphorylation was then allowed to continue for 30 min (a time period during which the phosphorylation reaction rate increased linearly under control conditions) before 50 µl samples were collected in duplicate and placed into Whatman 96-well UNIFILTER™ 350 plates treated and processed as described in section 2.2.8.1.

Reactions where equivalent volumes of 10 mM Tris buffer, pH 7.4 were added instead of parasite lysate served as blanks (0% phosphorylation), while reactions in which no PanAms were added served as 100% phosphorylation controls. Concentration-response curves were then generated as percentages in relation to the 100% phosphorylation control.

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2.2.9 Protein expression and purification

2.2.9.1 Preparation of BL21*(DE3) competent *E. coli* cells

Escherichia coli BL21*(DE3) cells (only referred to as BL21 cells for the remainder of this dissertation) were made chemically competent for uptake of DNA by streaking the cells on LB agar plate without any antibiotic and grown overnight at 37°C. Next, one colony was picked from the agar plate and inoculated in 5 ml of LB broth medium without any antibiotics and incubated overnight with shaking at 200 revolutions per minute (rpm) at 37°C. The culture was then diluted 1:100 (5 ml culture suspension in 500 ml culture medium) and incubated at 37°C with shaking at 200 rpm until the culture's optical density at wavelength of 600 nm (OD_{600}) as measured on an "Implen OD_{600} DiluPhotometer", reached 0.5-0.6 (typically after 1.5 to 2 hours). At this stage the cell suspension was centrifuged at 4000 × g at 4°C for 10 min. The supernatant was then discarded and the cells maintained on ice. Next, the cells were resuspended in 100 ml of ice cold 100 mM $MgCl_2$ and mixed by pipetting up-and-down then incubated on ice for 30 min. Following this, the suspension was centrifuged at 4000 × g at 4°C for 10 min and the supernatant was discarded. Finally, the cells were resuspended in 10 ml of 100 mM $CaCl_2$ with 15% glycerol and aliquots of 170 µl were prepared in sterile pre-cooled microcentrifuge tubes and stored at -80°C until required.

2.2.9.2 Transformation of plasmids into BL21 competent cells

All plasmids were transformed into BL21 cells for expression. For each plasmid, a volume of 1 µl was aseptically transferred to a cold sterile microcentrifuge tube. Next, 80 µl of BL21 competent cells were added into each microcentrifuge tube and incubated on ice for 30 min. Following this, the mixture was heated at 42°C for 45 s then immediately cooled on ice for 5 min. Nine hundred µl of LB broth medium without any antibiotics

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was then added into the microcentrifuge tube and the cell suspension incubated at 37°C for 1 h with shaking at 200 rpm. Following this incubation, the cells were centrifuged at 2000×g for 4 min and 850 µl of the supernatant were discarded. The cell pellet was then resuspended in the remaining ~150 µl of supernatant and plated in an agar plate containing 50 µg/ml kanamycin (for the pET-based plasmids) or 100 µg/ml ampicilin (for the plasmids encoding *Pf*ACP). Sterilized glass beads were placed onto the agar and rotated to spread the cell suspension before incubation with the lid down at 37°C overnight.

To introduce the TEV protease-encoding plasmid into BL21 cells that were already transformed with the pSPr22 plasmid, a single bacterial colony growing on the agar plate was picked and inoculated in 5 ml of LB broth containing 100 µg/ml ampicilin. The culture suspension was incubated at 37°C overnight with shaking at 200 rpm. Following overnight incubation, the 5 ml culture suspension was centrifuged at 4000×g on a bench centrifuge for 5 min. The supernatant was then discarded and the cells made chemically competent and transformed with the second plasmid, pRK586 (KanR) as described above (section 2.2.9.1), except that the LB broth culture medium used contained 100 µg/ml ampicilin instead of an antibiotic-free culture medium. An LB agar plate with both 50 µg/ml kanamicin and 100 µg/ml ampicilin was used instead of an agar plate containing only 100 µg/ml ampicilin or 50 µg/ml kanamicin. The second transformation with the TEV protease-encoding plasmid allowed *in vivo* cleavage and release of mature *Pf*ACP without any additional vector derived amino acids.

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The BL21 cells which were doubly transformed with the plasmids pSPr22 and pRK586, as well as the ones into which the plasmid pJGa7 or the bacterial plasmids were transformed, were then used for protein overexpression.

2.2.9.3 Protein overexpression in BL21 cells

For protein expression trials, a single isolated colony was picked from each agar plate and inoculated in 5 ml of LB broth containing corresponding antibiotics in composition and quantities as specified above (section 2.2.9.2). These starter cultures were incubated at 37°C with shaking at 200 rpm until they became slightly cloudy. The cultures were then diluted 1:100 in LB medium with the respective antibiotics in 2 L conical flask (final culture volume of 500 ml). The cultures were incubated at 37°C with shaking at 200 rpm until they reached an OD_{600} of ~0.6. At this stage 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to induce protein expression. After IPTG-induced protein expression, cultures were maintained overnight at 37°C with shaking at 200 rpm. After this incubation period, the cultures were poured into centrifuge bottles and the cells harvested by centrifugation at 10 000×g for 30 min at 4°C. After discarding the supernatant, the wet cell mass was determined and cells were used immediately for protein purification or were stored at -20°C until required. Different incubation temperatures (20°C, 25°C, 37°C) pre- and post-IPTG-induction of protein expression were trialled for all proteins in attempts to optimise the expression conditions.

2.2.9.4 Protein extraction, purification and concentration determination

The harvested cells from the IPTG-induced cultures were resuspended in 10 ml binding buffer (20 mM Tris, 0.05% w/v sodium azide, 5 mM imidazole, 500 mM NaCl, pH 7.9) per gram of wet cell pellet and maintained at temperatures below 10°C to minimise

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protein degradation by proteases. The cells were kept on ice, lysed by sonication on a high intensity ultrasonic processor working at 20 kHz, with a 6.5 mm microdip, at an amplitude of 50% and pulser cycles of 60 s ON and ~5 min OFF to ensure the cells and the lysate remained at a temperature bellow 10˚C.

Next, the lysate was centrifuged at 20 000×g for 20 min at 10˚C to pellet the cells debris. The supernatant was retrieved and filtered using Acrodisc® Premium 25 mm syringe filter with GxF/0.4 µm pore size GHP Membrane (Pall Life Sciences) before injection into an ÄKTAprime-system. The crude cell extract was loaded into 1.0 ml GE Healthcare Life Sciences HiTrap™ HP column preloaded with Ni^{2+}. After washing steps and column equilibration at a flow rate of 0.5 ml/min, the entire filtered protein extract was injected slowly into the system allowing the binding of the 6×His-tagged protein to the column preloaded with Ni^{2+}. Before retrieving the protein trapped in the Ni^{2+} column, a wash step with 15% elution buffer (20 mM Tris, 0.05% w/v sodium azide, 500 mM imidazole, 500 mM NaCl, pH 7.9) and 85% binding buffer was initiated to remove any non-specifically bound proteins. Elution of the target protein was achieved with stepwise increase of the concentration of imidazole by increasing the proportion of elution buffer while monitoring with UV absorption at 280 nm. The elution buffer – with high concentrations of imidazole and salts – containing the purified protein was then exchanged with a no-imidazole and low salts containing gel filtration buffer (25 mM Tris, 5 mM $MgCl_2$, pH 8.0) using the same ÄKTAprime-system, now with a HiTrap Desalting 5 ml column, pre-packed with Sephadex G-25 Superfine (GE Healthcare Life Sciences).

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The protein solution was then treated with glycerol at a final concentration of 10% v/v to allow protein stabilisation for cryopreservation. Protein concentrations were estimated using the Bradford method [11], and bovine serum albumin (BSA) was used to prepare standards of known concentration for reference purposes.

2.2.9.5 Analysis of protein purity

Purified bacterial protein samples were visualized using sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE). Appropriate volumes of the proteins were mixed with equal volumes of 2×SDS-PAGE sample loading buffer (0.125 M Tris-HCl, 4.0% SDS, 30% glycerol, 1.5% β-mercaptoethanol and 0.02% bromophenol blue, pH 6.8), heated at 95°C for 5.0 min, loaded on 12% gel and ran in SDS-PAGE running buffer (0.025 M Tris-HCl, 0.1% SDS, 0.192 M glycine, pH 6.8).

Purified (6×His-tagged) and crude extracts (non-tagged) of *Pf*ACP were visualised using conformation-sensitive non-denaturing PAGE. Appropriate volumes of protein samples were mixed with equal volumes of 2× sample loading buffer without SDS and β-mercaptoethanol. The samples were loaded on a 15% gel with 4 M urea and analysed in native running buffer (25 mM Tris-HCl, 192 mM Glycine, pH 8.3).

The gels were then stained with Coomassie Brilliant-Blue stain (45% methanol, 40% H_2O, 10% acetic acid and 5% w/v Coomassie Brilliant-Blue) and destained with destaining solution (45% methanol, 45% H_2O, 10% acetic acid) to visualise protein bands migrated in the gel. Protein identification was made through estimation of the molecular weight of protein bands migrated on the gel in comparison with proteins of

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known molecular weight (Ultra-Low Range or ColorBurst™ Markers, Sigma-Aldrich) migrated on the same gel.

2.2.10 Fluorescent labelling of CoA and *Pf*ACP

Commercial CoA trilithium salt (Sigma-Aldrich) and CoA from *P. falciparum* parasite lysates, as well as *Pf*ACP overexpressed in BL21 and from *P. falciparum* parasite lysates, were labelled with 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM). This compound is a profluorescent coumarin maleimide derivative that becomes fluorescent upon reaction with thiols [12]. Commercial CoA or BL21 overexpressed *Pf*ACP were reacted with CPM dissolved to 2 mM in DMSO, in argon-purged buffer at final concentrations of 10 mM Tris, 0.5 mM CoA (or *Pf*ACP) and 0.5 mM CPM, pH 7.4, incubated at room temperature for 1.5 h. For labelling of *P. falciparum* culture-derived CoA or *Pf*ACP, homogenous cultures with the parasites synchronised at ring stage, were split into three equal parts and, two of the parts were treated with N5-Pan (**46b**) to final concentrations of 50μM and 100μM. An equivalent volume of DMSO – the solvent used to prepare the PanAm – was added to the third part of the split culture and served as a control, ensuring that the quantity of DMSO in any of the cultures never exceeded 0.001% v/v.

After cultures were grown following standard protocols for 24 h and 72 h – allowing the parasites to mature to the 1st and 2nd generation of trophozoites post treatment respectively, the parasitized RBCs were retrieved by centrifugation and parasites isolated from the RBCs as described under section 2.2.3.

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Following parasite isolation, thophozoites were counted as described previously (section 2.2.5) and lysates were prepared from parasite suspensions containing varying numbers of parasites (~1.0×10^5 to ~2.5×10^{12} parasites/ml). Next, volumes of 10-25 µl of CPM-labelled parasite lysates were injected onto a High Pressure Liquid Chromatography (HPLC) column and analysed as detailed below, allowing the amount of ~2.5×10^7 trophozoites per ml of suspension to be established as the detection limit for CPM-labelled *Pf*ACP. Following determination of the minimum number of parasites per ml required for detection of *Pf*ACP, lysates were prepared from parasite suspensions with ~2.5×10^{10} trophozoites, this time allowing adequate peak sizes of *Pf*ACP and CoA labelled with CPM to be obtained.

Following parasite isolation, the cells were resuspended in buffer (10 mM Tris, 0.5 mM CPM, pH 7.4 with 0.5 mM CPM final concentration) before being flushed with argon and the tube tightly closed. The cells were then triturated through vigorous vortexing at 35 Hz in a Velp Scientifica 2X^3 mixer for 3-5 min or through repeated (10 times) vigorous passage through a 25-gauge injection needle. Next, the CoA and ACP present in the parasite lysates were labelled with excess CPM (0.5 mM final concentration) under an argon atmosphere to allow complete fluorescent labelling of all thiol groups present in the lysate. The mixture was then allowed to stand for 1.5 h at room temperature before centrifugation at 2000×g for 30 min permit recovery of the supernatant containing CPM-labelled CoA and parasite proteins in solution.

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2.2.11 HPLC screening of CPM-labelled CoA

CPM-labelled CoA samples were analysed on a Hewlett-Packard series 1100 HPLC system using an analytical reverse-phase Luna HPLC C18 column 250 x 4.6 mm, 5 µm particle size (Phenomenex) associated to a guard column with the same pore size.

The fluorescence was monitored with the FLD detector at excitation and emission wavelengths of 387 and 465 nm, respectively, with chromatographic peaks at retention time of ~8.9 min corresponding to the elution of CoA-CPM. Samples (10 µl) were injected into the column after equilibration with 80% v/v solvent A (ammonium acetate 10 mM pH 5.0) and 20% v/v solvent B (ACN) at a flow rate of 1 ml/min. The following program was used to detect the CPM-labelled CoA: 5 min at 80% solvent A/20% solvent B; 1 min linear gradient from 80% solvent A/20% solvent B to 60% solvent A/40% solvent B; 15 min isocratic at 60% solvent A/solvent 40% B; 2 min linear gradient from 60% solvent A/40% solvent B to 40% solvent A/60% solvent B; 7 min isocratic at 40% solvent A/60% solvent B was then used with the flow rate at 1 ml/min.

2.2.12 HPLC screening of CPM-labelled and non-labelled *Pf*ACP

Screening of CPM-labelled or non-labelled *Pf*ACP was performed on a Hewlett-Packard series 1100 HPLC system using an analytical reverse-phase HPLC C18 peptide column 4.6 x 250 mm, 5 µm particle size, 300 Å (Phenomenex) associated to a guard column with the same pore size.

For non-labelled *Pf*ACP samples, absorbance was monitored at 220 nm while for CPM-labelled protein fluorescence was monitored at excitation and emission wavelengths of 387 and 465 nm, respectively, with chromatogram peaks corresponding to the elution of

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*Pf*ACP at ~19.8 min retention time. Samples of 10 µl were injected into the column after being equilibrated with 80% v/v solvent A (0.1% v/v trifluoroacetic acid (TFA) in distilled and deionised H_2O)/20% v/v solvent B (0.1% TFA in ACN) at a flow rate of 1 ml/min. The following program adapted from Chan and Thomas [13] was used to separate non-labelled *apo*- and *holo*- forms as well as CPM-derivatized forms of *Pf*ACP: 5 min at 80% solvent A/20% solvent B; 20 min linear gradient from 80% solvent A/20% solvent B to 20% solvent A/80% solvent B; 10 min isocratic at 20% solvent A/solvent 80% B; 1 min linear gradient from 20% solvent A/80% solvent B to 80% solvent A/20% solvent B; 10 min isocratic at 80% solvent A/20% solvent B was then used, with the flow rate at 1 ml/min.

2.2.13 Fluorescamine-based *in vitro* pantothenamides degradation assessment

Hydrolysis of α-PanAms, *n*-PanAms and HoPanAms was measured using a modification of a fluorescence-based assay described previously for measurement of *N*-acetyl-1-D-myoinosityl-2-amino-2-deoxy-a-D-glucopyranoside deacetylase activity [14]. Recombinant human pantetheinase (Novoprotein Scientific, Inc.) at a final concentration of 100 ng/ml or an equivalent volume of phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) or Albumax II (at a final concentration 0.6%, w/v) was added to solutions (at 37 °C) of 100 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol (DTT), 0.01% (w/v) BSA, 0.1% (v/v) DMSO, with or without pantothenamides at a final concentration of 200 µM. At appropriate time points, aliquots of 30 µL of the reaction mixtures were retrieved in duplicate and mixed with 10 µl 20% (v/v) TCA to terminate the reaction. Following removal of the precipitated protein by centrifugation (14 000×g, 15 min), 25 µl of each supernatant was transferred to the wells of a black 96-well plate and 75 µl 1 M borate (pH 9.0) followed

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by 30 µl 10 mM fluorescamine in ACN was added to each well. The plate was then incubated at 37°C for 10 min before fluorescence was measured using a Varioskan spectral scanning multimode reader (Thermo Scientific) with excitation and emission wavelengths of 390 nm and 485 nm, respectively for experiments performed at SUN or using a FLUOROstar OPTIMA multidetection microplate reader (BMG Labtech GmbH) for experiments conducted at ANU. The background fluorescence (determined from wells containing samples with no enzyme) was subtracted from the fluorescence measured in each well.

2.2.14 Statistical analysis

To test for statistically significant differences between the means of two groups, two-tailed student *t* tests were performed, paired or unpaired as appropriate. To test for significant differences between the means of more than two groups, one-way analysis of variance (ANOVA) was performed. Pairwise comparisons were made post-hoc with Tukey's multiple comparisons test. ANOVA was performed using Instat 3 (GraphPad Software, Inc). Linear and non-linear regression analysis was performed using SigmaPlot for Windows, version 11.0 (Systat software).

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Chapter 3:

SEARCHING FOR PANTOTHENAMIDES WITH IMPROVED ANTIPLASMODIAL ACTIVITY

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Parts of the results in this chapter have been published in the following papers:

1. Spry C, Macuamule C, Lin Z, Virga KG, Lee RE, Strauss E, Saliba KJ. Pantothenamides are potent, on-target inhibitors of *Plasmodium falciparum* growth when serum pantetheinase is inactivated. *PLoS ONE*. 2013, 8(2): e54974.
doi: 10.1371/journal.pone.0054974. Epub: Feb 6, 2013.
[Reprint included as Appendix A].

2. de Villiers M, Macuamule C, Spry C, Hyun Y-M, Strauss E, Saliba KJ. Structural modification of pantothenamides counteracts degradation by pantetheinase and improves antiplasmodial activity. *ACS Med. Chem. Lett.*, 2013, 4(8), pp. 784–789.
doi: 10.1021/ml400180d. Epub: Jun 17, 2013.
[Reprint included as Appendix B].

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3.1 Introduction

The search for new antimalarial agents is an ongoing pursuit that has intensified during the last several years since the release of reports indicating that *P. falciparum*, the most virulent of the malaria parasites that cause the disease in humans, has become resistant to all chemotherapeutic agents currently in use, and also in response to the current malaria eradication research agenda [1-3].

The pipeline for antimalarial compounds is currently robust, particularly for the intraerythrocytic stage parasites [2]. However, as discussed in chapter 1 section 1.4.4, most of the compounds under investigation target the same molecules and/or pathways and belong to a limited diversity of chemical scaffolds. Being chemically related to drugs to which the parasites have already developed resistance, most of the compounds under investigation may soon become ineffective as they are affected by the phenomenon of cross-resistance. Therefore, new, more potent and non-expensive chemotherapeutic agents targeting novel essential molecules and pathways in the parasite are required.

One promising set of targets that has been investigated for antimalarial drug discovery and development encompasses the processes and pathways dependent on pantothenic acid as detailed in the previous chapters of this dissertation. Pantothenic acid serves as the biosynthetic precursor of the essential metabolic cofactor and universal acyl group carrier, CoA [4] and, as discussed before, *P. falciparum* parasites have absolute requirement for an exogenous supply of this nutrient – at least during its intraerythrocytic stage [5] – and a sensitivity to compounds that interfere with the parasite's ability to utilize the nutrient. Structural analogues of pantothenic acid have

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been shown to be promising compounds in this regard, with many inhibiting the growth of the intraerythrocytic parasite [6, 7].

As detailed in chapter 2, PanAms are a class of pantothenic acid analogues in which the carboxylic acid group (β-alanine moiety) of pantothenic acid is replaced by a secondary or a tertiary amide group. These compounds have been demonstrated to possess antibacterial [8-15] and antimalarial [16] activity, with their mode of action most likely dependent on the inhibition of CoA biosynthesis, or CoA-dependent processes.

Growth inhibition experiments performed by previous researchers in our laboratory at SUN indicated that PanAms had only modest *in vitro* antiplasmodial activity against intraerythrocytic-stage *P. falciparum* parasites with the most potent compound (**54c**, table 2.1) showing IC_{50} of 1.1 ± 0.2 μM [16]. It was then suspected that the poor antiplasmodial potency was due to some degree of inactivation or degradation of the compounds during growth inhibition experiments. Later on, while examining the antiplasmodial activity of a series of PanAms against intraerythrocytic-stage *P. falciparum* parasites, our collaborators at ANU serendipitously discovered that the antiplasmodial potency of the compounds was enhanced considerably when the parasite culture medium being used – which contains the commonly used serum substitute Albumax II [17] or human serum – was pre-incubated at 37°C for a prolonged period (aged medium). Growth experiments performed with the aged medium allowed observation of the sub-micromolar (IC_{50} = 20 ± 2 nM) potency of PanAms that had shown no antiplasmodial effect when tested under standard culturing conditions in freshly prepared culture medium (fresh medium).

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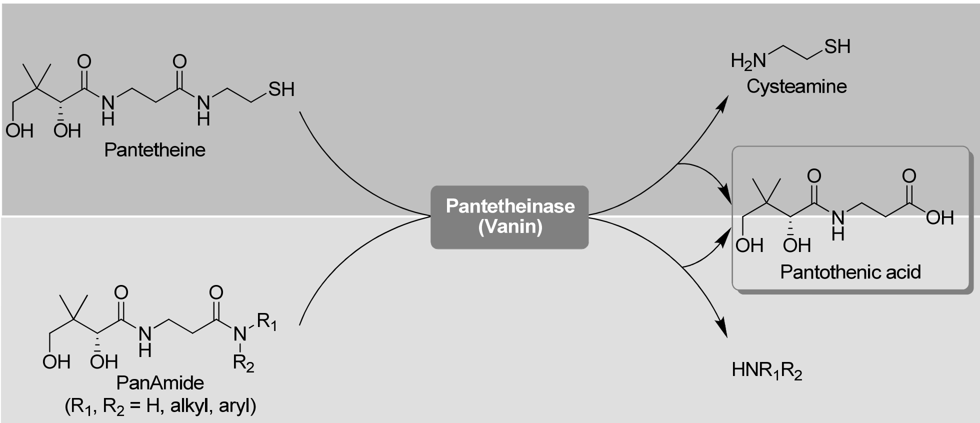
The final piece of the puzzle fell into place with the realisation of the presence of temperature-sensitive enzymes with pantetheinase activity in parasite culture medium and that this might explain the altered PanAms antiplasmodial activity in aged *versus* fresh medium. Pantetheinase activity is associated with the Vanin proteins, which are members of the nitrilase superfamily that occur ubiquitously in soluble or membrane bound forms in various tissues. The proteins play the physiological role of recycling pantothenic acid and the antioxidant cysteamine using an invariant Glu-Lys-Cys catalytic triad to hydrolyze the CoA metabolite pantetheine [18, 19]. Being promiscuous, the enzymes tolerate a wide range of structural modifications at the cysteamine terminal, explaining their ability to also hydrolyze compounds other than their natural substrate, including PanAms (Scheme 3.1) [20, 21].



Scheme 3.1 - Schematic representation of pantetheinase-mediated hydrolysis of pantetheine and pantothenamides. The natural function of the pantetheinase enzymes is to hydrolyze the CoA metabolite pantetheine to pantothenic acid and cysteamine (reaction in dark grey box). Pantothenamides are amide analogues of pantothenic acid that are also susceptible to degradation by Vanin (reaction in light grey box).

Their susceptibility to degradation by the naturally occurring Vanin pantetheinases, present particularly in the serum of mammals [19], severely hampered the further development of PanAms as antiplasmodial agents. One approach through which this limitation could be addressed would be to co-administer PanAms with pantetheinase

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inhibitors, as promoted by Ruan *et al*. [22] and by Jasen *et al*. [23]. However, such a strategy would eventually require a separate optimization of the properties of two compound sets, and possibly result in adverse effects associated with inhibition of the physiological function of pantetheinases [19]. Therefore, an alternative approach was investigated in which the molecular structures of the PanAms were modified to render them less susceptible to pantetheinase degradation while maintaining or improving their antiplasmodial potency. To achieve this, a previously-described series of PanAms [24] was investigated to better understand the antiplasmodial activity of the modified compounds. The series was prepared from pantothenic acid or one of two structural analogues in which the β-alanine moiety was replaced with either glycine (to form α-pantothenic acid) or γ-aminobutyric acid (to result in homopantothenic acid), followed by introduction of the amide moiety via condensation with a range of amines representing four chemical motifs into each of the three acids in parallel. With such a strategy, three sets of PanAms were prepared as detailed in the previous chapter (chapter 2, section 2.1.2).

It is important to note that the structural modification of the β-alanine moiety of pantothenic acid (either by removal or addition of a methylene group to form α- and HoPanAms respectively) results in alterations of the pantothenoyl moiey and, since previous studies showed that pantetheinase relies on the pantothenoyl moiety of its substrate for recognition [20, 21] it was expected that the modifications would reduce the scissile amide bond's susceptibility to attack by the Cys residue of the enzyme's catalytic triad and thereby prevent or reduce the rate of the pantetheinase-mediated degradation of the α-PanAms and HoPanAms series relative to that of the *n*-PanAms counterparts. Consequently, α-PanAms and HoPanAms should act as pantetheinase-

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resistant antiplasmodial agents if the structural modifications did not affect their target specificity or mode of action.

In this chapter, the experiments that were performed to explore the merits of this strategy, as well as the potential of the α-PanAms, HoPanAms and other pantothenic acid analogues, namely the α-MePanAms, β-MePanAms as well as pantoyltauramides for having improved resistance to pantetheinase-mediated degradation as antiplasmodial agents, are described.

3.2 Results and discussion

3.2.1 *In vitro* pantetheinase-mediated degradation of pantothenamides

Whether α-PanAms and HoPanAms were more resistant to pantetheinase-mediated degradation compared to their *normal* counterparts under *in vitro* conditions was investigated first. This was tested directly by incubating five sets of α-, *n*- and HoPanAms, each with a different type of amide substituent, in the presence of recombinant human pantetheinase. The amount of amine released under these conditions was then determined periodically using a fluorescamine-based fluorescence assay as detailed in chapter 2, section 2.2.13. After 24 h, the hydrolysis of the *n*-PanAm compounds was found to be complete, as can be demonstrated for the selected *n*-PanAm **44b** as an example (figure 3.1). The percentage of PanAm degraded and consequently resulting in release of the amine detected was determined by comparing the fluorescent signal to that obtained from a standard curve prepared using the pure amine that is expected to be released upon hydrolysis. On the other hand, under the same conditions, the hydrolysis of members of the α-PanAm and HoPanAm series was only between 5-15% complete, except in the case of the compounds **44a** and **44c** where it was

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approximately 25%. These results demonstrate that modifying the pantothenoyl moiey of the *n*-PanAm series confers resistance to degradation by pantetheinases, or at least reduces the rate of its degradation (figure 3.2) due to reduced or lack of substrate recognition by the enzyme.

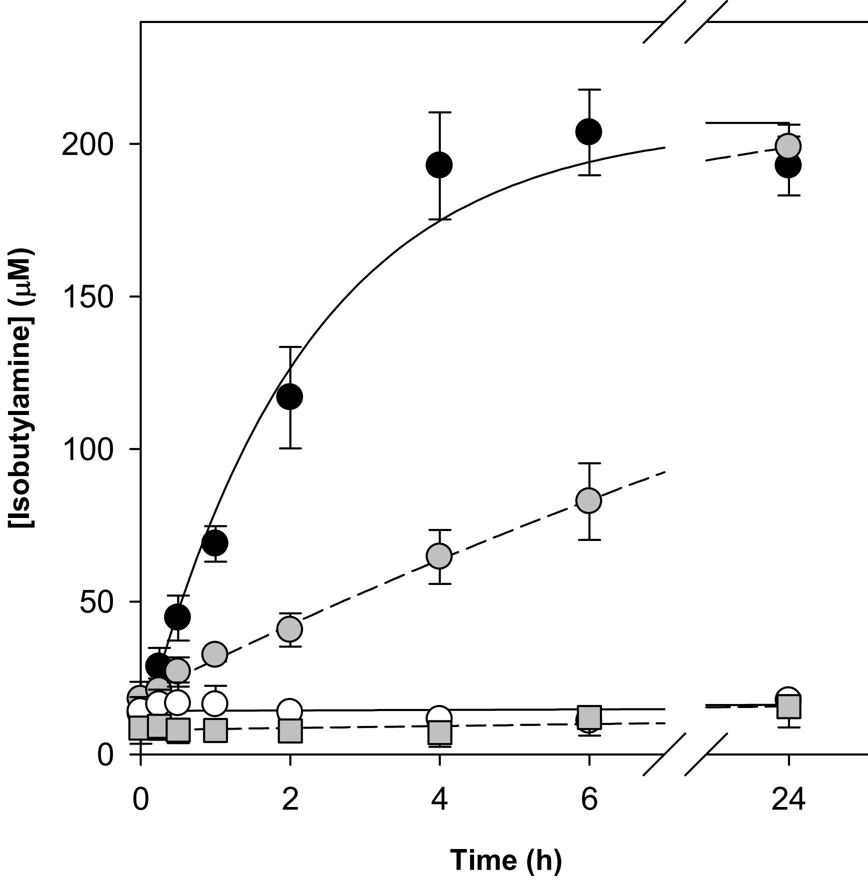

Figure 3.1 - Hydrolysis of pantothenamide **44b** in the presence of recombinant human pantetheinase and Albumax II. The time courses show the concentration of isobutylamine – the amine product of degradation of pantothenamide **44b** – detected during its incubation with recombinant human pantetheinase (100 ng/ml; black circles), Albumax II (0.6%, w/v; grey circles), or an equivalent volume of water (white circles), and during incubation of Albumax II (0.6%, w/v) in the absence of PanAm **44b** (grey squares). At each time-point, the amount of primary amine was measured using a fluorescamine-based fluorescence assay. Fluorescence measurements were converted to isobutylamine concentrations using a standard curve generated using isobutylamine samples of known concentration that had been processed in the same manner as the test samples. The data are from four independent experiments, each performed in duplicate, and error bars represent SEM. Where not visible, the error bars are smaller than the symbols.

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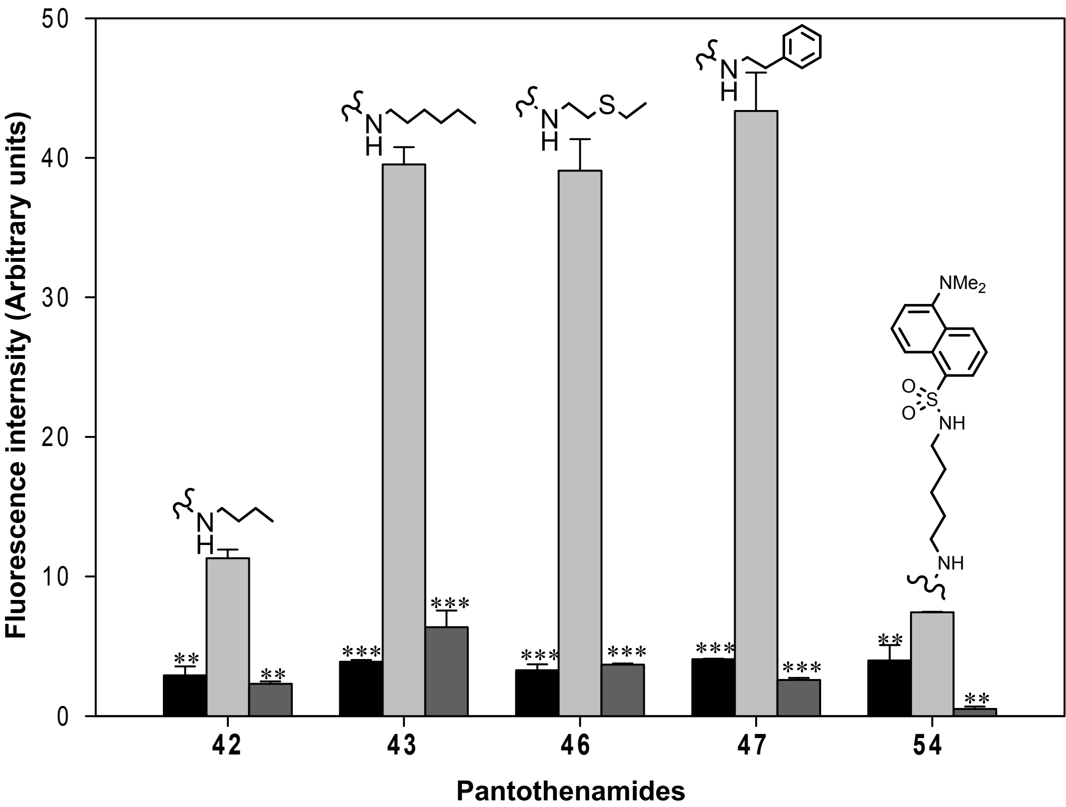

Figure 3.2 - Recombinant human pantetheinase-mediated hydrolysis of *N*-substituted pantothenamides. Five sets of α-PanAms (black bars), *n*-PanAms (light grey bars) and HoPanAms (dark grey bars) – structure identifiers indicated below the bars, and structure of amide substituent shown above – were treated with recombinant human pantetheinase. After 24 h, the amount of amine released was determined by means of a fluorescamine-based fluorescence assay. Values represent the mean from three independent experiments, each performed in duplicate; the error bars represent SEM. Where not visible, the error bars are small and are contained within the column plots. The asterisks above the error bars represent the significance of the difference between the values determined for the respective α-PanAm and HoPanAm series members vs. the *n*-PanAm in a given set (** $P < 0.005$; *** $P < 0.001$, one way ANOVA). Note that since the fluorescence intensity of the fluorescamine-amine conjugates for different complexes is dependent on the structure of the amine, the fluorescence intensity is not necessarily comparable between the different sets.

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3.2.2 *In vitro* antiplasmodial activity of selected *N*-substituted pantothenamides against intraerythrocytic-stage *P. falciparum* parasites

3.2.2.1 Antiplasmodial activity in standard ("fresh") culture medium

Following the demonstration of resistance of α-PanAms and HoPanAms to hydrolysis by pantetheinases, relative to their *normal* counterparts, we set out to determine whether this feature would translate into increased antiplasmodial potency in the presence of pantetheinase. The inhibitory activity of 15 sets of α-PanAm, *n*-PanAm and HoPanAm compounds was determined *in vitro* against intraerythrocytic *P. falciparum* parasites (strain 3D7) in 96 h growth assays initiated with parasites predominantly in the ring stage as described in chapter 2 section 2.2.7. For these experiments, freshly prepared complete culture medium was used to ensure the presence of pantetheinase activity. From the IC_{50} values obtained in this manner (Table 3.1), it is evident that modification of the β-alanine moiety in an *n*-PanAm significantly improves their antiplasmodial activity ($P < 0.05$ and $P < 0.01$ for α-PanAm and HoPanAm members respectively compared to their *n*-PanAm counterparts, unpaired t-test).

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Table 3.1 *In vitro* antiplasmodial activity of *N*-substituted pantothenamides against intraerythrocytic-stage *P. falciparum* parasites

Entry	R group	IC_{50} (µM)* Fresh Medium‡			IC_{50} (µM)* Aged Medium		
		α-PanAm (a)	*n*-PanAm (b)	HoPanAm (c)	α-PanAm (a)	*n*-PanAm (b)	HoPanAm (c)
41		18 ± 3	> 200	2.8 ± 1.4	14. ± 2	7.5 ± 6.2	2.2 ± 0.1
42		5.3 ± 3.2	> 200	10 ± 3	ND	ND	ND
43		7.5 ± 0.3	> 200	10 ± 6	7.9 ± 1.8	0.6 ± 0.01	14 ± 7
44		18 ± 2	100 ± 2.8	11 ± 5	17 ± 1	0.8 ± 0.7†	2.2 ± 0.2
45		161 ± 40	> 200	7.5 ± 0.7	140 ± 40	>200	3.3 ± 0.5
46		12 ± 1	> 200	1.7 ± 0.3	11 ± 1	0.3 ± 0.02	1.9 ± 0.1
47		3.4 ± 0.8	53 ± 11	2.1 ± 0.1	4.2 ± 0.2	0.020±0.003†	2.7 ± 0.3
48		110 ± 13	> 200	3.1 ± 1.6	127 ± 16	> 200	4.7 ± 0.5
49		> 200	> 200	23 ± 1	71 ± 8	> 200	10 ± 1
50		>200	> 200	24 ± 1	> 200	> 200	22 ± 1
51		81 ± 12	> 200	5.7 ± 0.2	64 ± 16	136 ± 34	4.9 ± 1.3
52		47 ± 1	116 ± 12	2.8 ± 1.4	45 ± 6	117 ± 12	2.0 ± 0.1
53		145 ± 44	> 200	2.1 ± 0.9	116 ± 21	156 ± 22	1.9 ± 1.0
54		155 ± 27	> 200	1.1 ± 0.5	6.7 ± 0.5	38 ± 6	ND
55		>200	> 200	> 200	> 200	> 200	> 200

ND – Not determined
* - The IC_{50} values were calculated from data collected from three independent experiments performed in triplicate as detailed in chapter 2, section 2.2.7. Error values represent SEM.
‡ - Data generated at SUN after publication of the paper in appendix B.
† - Data published in the papers in appendix A and B was generated by Zhiyang Lin at ANU.

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When analyzing the results of the growth assays performed in fresh medium, it is evident that while the α-PanAms show variable antiplasmodial activity, practically all the HoPanAms exhibit IC_{50} values below 20 μM, with 9 out of 15 exhibiting IC_{50} values below 10 μM. The most potent (compound **54c**) with an IC_{50} of 1.1 ± 0.5 μM (mean ± SEM; n = 4). These results highlight the relevance of elongation of the β-alanine moiety of the *n*-PanAms as the structural modification of choice to counteract pantetheinase-mediated degradation of the compounds and improved antiplasmodial activity.

3.2.2.2 Probing for an improved method for pantetheinase inactivation in culture medium

Following the observation that the antiplasmodial potency of PanAms in culture medium that had been continuously incubated at 37°C for 40 h was considerably enhanced when compared to the potency of the compounds tested in fresh medium and, the fact that further incubation of the medium for a total of 80 h improved the antiparasitic activity of compound **44b** (*N*-isobutyl-*n*-PanAm) even further in previous studies [25], other methods for ageing the culture medium to eliminate as much pantetheinase activity as possible were investigated. This aged medium would then be used to examine the intrinsic antiplasmodial activity of PanAms, without the impairment caused by residual pantetheinase-mediated degradation. Freshly prepared culture medium was split into five different 50 ml-centrifuge tubes followed by different treatments. Tube Nr. 1 was incubated at room temperature (~25°C) under UV irradiation for 2 hours then kept refrigerated (4°C) for ~20 h, tube Nr. 2 was incubated at 56°C for 4 hours, tube Nr. 3 was incubated at 37°C for 40 h, tube Nr. 4 was incubated at 56°C overnight (12-16 h) and tube Nr. 5 was kept refrigerated at 4°C for a maximum of 22 h before use. The culture

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media were then used to perform growth inhibition assays with PanAm **44b**, following standard procedures as described in chapter 2, section 2.2.7.

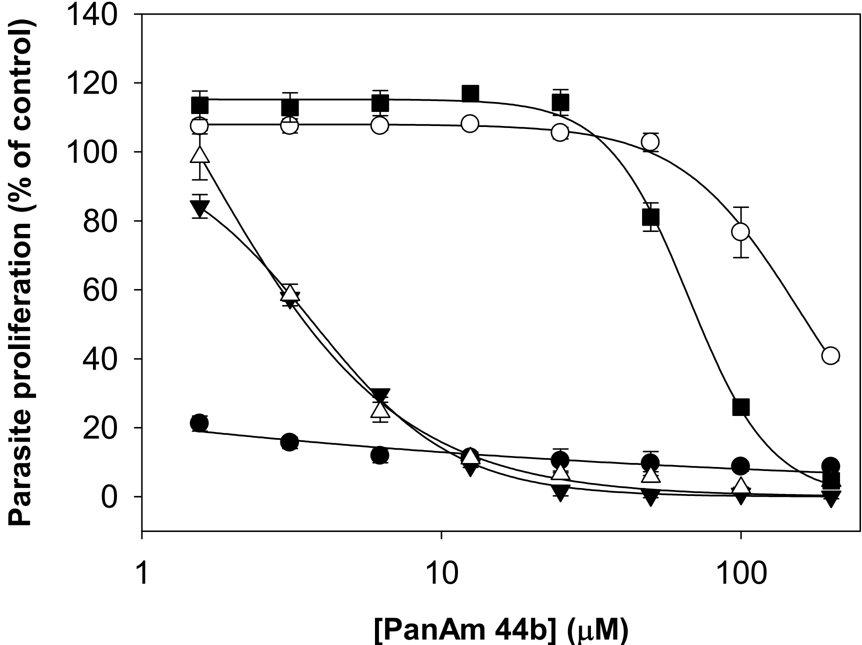

Figure 3.3 - Antiplasmodial activity of PanAm **44b** in culture media pre-incubated at different temperatures to inactivate pantetheinase activity. The concentration response curves show the effect of pre-incubating complete culture medium at room temperature (~25°C) for 2 hours under UV light (black squares); 37°C for 40 hours (white triangles); 56°C for 4 hours (black triangles); 56°C overnight (black circles) and culture medium maintained refrigerated at 4°C for a maximum of 22 hours before use (white circles) on the proliferation of intraerythrocytic-stage *P. falciparum* parasites cultured for 96 h, as measured using the fluorescent SYBR Green I-based assay. The data were collected from two independent experiments performed in duplicate and error bars represent range/2.

As shown in figure 3.3, the continuous incubation of the culture medium at 56°C for 12-16 hours resulted in PanAm **44b** exhibiting the best antiplasmodial activity (IC_{50} = 0.8 ± 0.7 μM), followed by incubation at 37°C for 40 h (IC_{50} = 3.6 ± 1.2 μM) or incubation at 56°C for 4 hours (IC_{50} = 3.7 ± 2.6 μM) which practically revealed similar growth inhibitory activities. Although the incubation of culture medium at 25°C under UV light (IC_{50} = 27.2 ± 2.2 μM) did exhibit some effect, the improvement was smaller compared to

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the other culture ageing methods used and the antiplasmodial activity of the compound in that medium was enhanced by ~4-fold compared to the activity observed in fresh medium maintained at 4°C (IC_{50} = 99.9 ± 2.8 μM). These results influenced the decision to use the incubation of complete culture medium at 56°C for 18 h as the method of choice for *P. falciparum* growth assays in aged medium.

3.2.2.3 Antiplasmodial activity of pantothenamides in aged medium

To further confirm that the higher potency of α- and HoPanAms was in fact due to their resistance to pantetheinase degradation, the antiplasmodial activity of the 15 sets of pantothenamides was tested in culture medium that had most of the pantetheinase activity inactivated through prolonged incubation at 56°C (aged medium) as described in chapter 2, section 2.2.6 and compared to the antiplasmodial activity in fresh medium with pantetheinase activity. The results show that while the potency of the *n*-PanAms improved dramatically under pantetheinase-void conditions, the activity of the α-PanAms and HoPanAms determined in the presence or absence of pantetheinase activity was practically not affected for most of the compounds ($P > 0.05$, unpaired t-test). This provides further evidence that the structural modification strategy achieved its goal of imparting resistance to pantetheinase degradation.

Importantly, seven out of thirteen *n*-PanAms that had IC_{50} values higher than 200 μM in fresh culture medium were found to be more potent in “aged” medium. Under such conditions the IC_{50} values determined for these compounds were at least 1.3-fold (PanAm **53b**) to ~700-fold (PanAm **46b**) lower than the IC_{50} values determined when the compounds were tested in fresh medium. In addition, the phenethyl substituted *n*-PanAm **47b** with an IC_{50} of 53 ± 11 in fresh medium was revealed to be very potent in

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aged medium, exhibiting an IC_{50} value of 20 ± 3 nM (mean ± SEM; n = 3). This effect is graphically shown for a selected set of α-, *n*- and HoPanAms (compounds **43a-c**) in aged and in fresh medium (figure 3.4). While the activity of the *n*-PanAm was significantly higher ($P < 0.001$, one way ANOVA) in aged medium compared to the activity in fresh medium, with growth inhibition of 99.3% *versus* 7.5% at a concentration of 10 μM for example in aged medium and in fresh medium, respectively (figure 3.4B), the activity of its *α*- and HoPanAm counterparts was unaffected by the age of the culture medium (P = 0.32, unpaired t-test; figure 3.4A and C).

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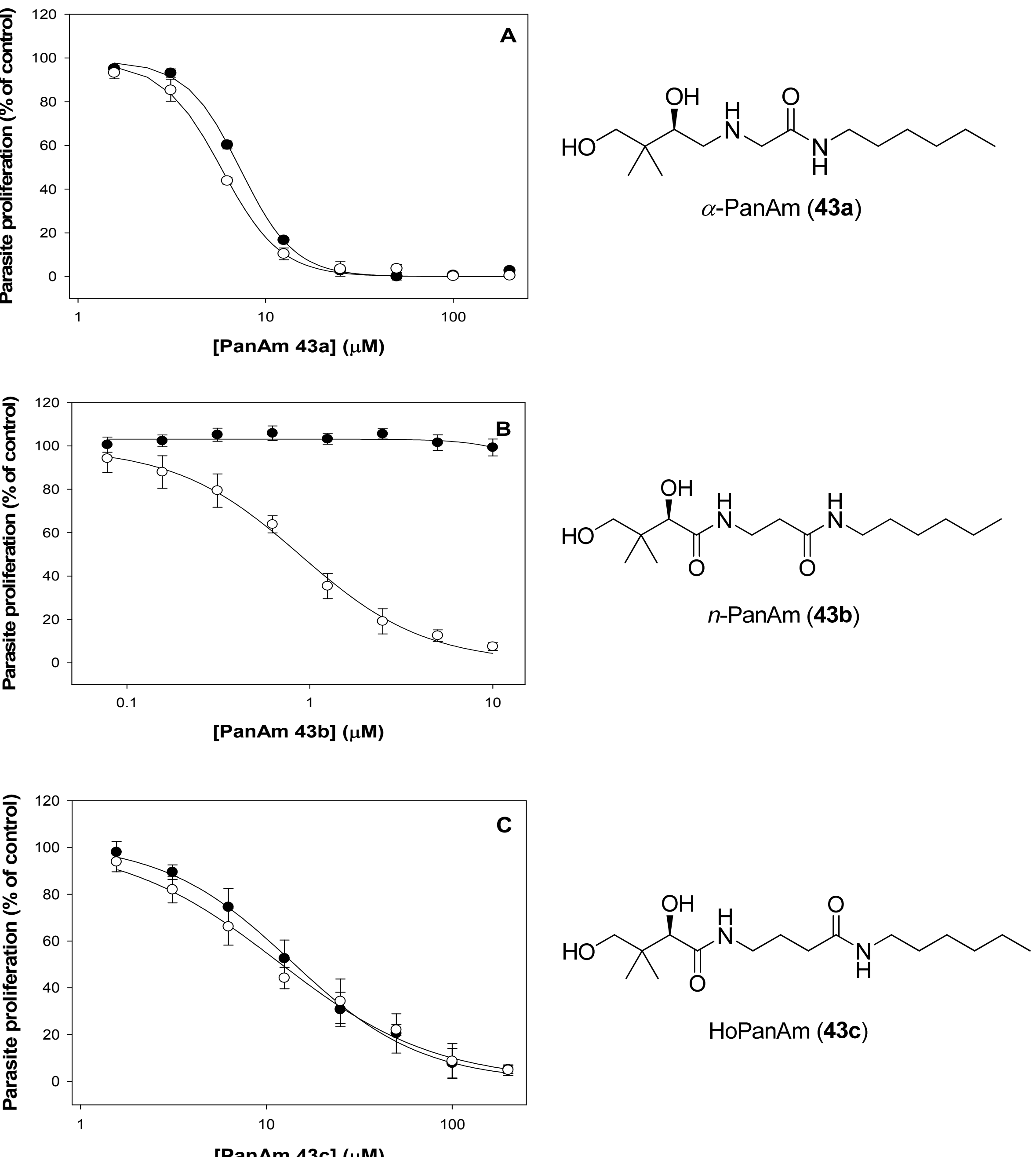

Figure 3.4 - Antiplasmodial activity of PanAms in fresh (closed circles) and in aged (open circles) medium. The concentration response curves show the effect of increasing concentrations of α-PanAm **43a** (Panel A), *n*-PanAm **43b** (Panel B) and HoPanAm **43c** (Panel C) on proliferation of intraerythrocytic-stage *P. falciparum* parasites cultured for 96 h in medium containing 1 μM pantothenate, as measured using the fluorescent SYBR Green I-based assay. The data were collected from three or four (fresh medium) independent experiments performed in triplicate and error bars represent SEM.

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These results confirm that structural modification of *n*-PanAms is a viable approach to confer resistance to pantetheinase degradation while at the same time preserving antiplasmodial activity.

3.2.2.4 Structure-activity relationship analysis of pantothenamides antiplasmodial activity

As described above, the main finding of the growth inhibition tests conducted in fresh and aged medium was that the α- and HoPanAm compounds show good antiplasmodial activity even in the presence of pantetheinase. However, when the result of the tests in both fresh and aged medium are considered together, it is clear that the *n*-PanAm series still show the best potency. Among all the compounds tested, the phenethyl-substituted *n*-PanAm **47b** with an IC_{50} of 20 ± 3 nM was the most potent, followed by the linear thioether-substituted *n*-PanAm **46b** with an IC_{50} of 300 ± 20 nM. In contrast, the most potent modified pantothenamide was HoPanAm **54c** that had an IC_{50} of 1.1 ± 0.5 μM. This indicates that although structural modification imparts pantetheinase-resistance, it does so at the cost of losing antiplasmodial potency – most probably because of the specificity of the as yet unknown target of these compounds, or of the enzymes that transform these compounds into their inhibitory forms.

Another important observation is that there does not seem to be any obvious structure-activity relationship between the α-, *n*- and HoPanAm counterparts in relation to the amide substitution. This is not surprising if it is assumed that all three series of compounds interact with the same target, since the shortening or elongation of the β-alanine moiety by one methylene group would displace the amide substituent relative to the pantoyl moiety that all three hold in common. Conversely, it can then be predicted

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that compounds with modification in the amide substituent that is opposite to those in the β-alanine moiety would counteract these effects, and should show some correlation in potency. One case to illustrate this point is the phenethyl-substituted *n*-PanAm **47b** that shows an IC_{50} of 20 ± 3 nM (Table 3.1) in "aged" medium. The displacement of its phenyl group (as in the case of *n*-PanAm **48b**) leads to loss of antiplasmodial activity as indicated by the IC_{50} of more than 200 µM. In contrast, the corresponding HoPanAm **48c** which has an extension on the β-alanine moiety that causes the phenyl group to return to the same relative position as in *n*-PanAm **47b** has an IC_{50} of 4.7 ± 0.5 µM. Similar trends can be observed for the *n*-PanAm **43b** and HoPanAm **41c** suggesting that all three series of compounds exert their antiplasmodial activity through interaction with the same target(s).

PanAms with a linear thioether substituent were among the most potent structurally-modified compounds tested in aged medium, with HoPanAm **46c** exhibiting the lowest IC_{50} value of these at 1.9 ± 0.1 µM. This compound and its *alpha*- and *normal*-counterparts **46a** and **46b** were found to be equipotent with the corresponding alkyl-substituted pantothenamides **41c**, **41a** and N5-Pan (**41b**) respectively, suggesting that linear, non-polar amide substituents are preferred in general. This is confirmed by the poor potency of PanAms **55a-c** – all of which contain an ionisable tertiary amine – which were demonstrated to be the poorest inhibitors of *P. falciparum* parasite proliferation *in vitro*.

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3.2.3 Antiplasmodial activity of *N*-substituted α-methyl and β-methyl pantothenamides

The results detailed above confirmed that structural modification of the β-alanine moiety of the pantothenamide core structure conferred resistance to pantetheinase-mediated degradation, but that divergence from the β-alanine core structure also caused a loss in potency. Therefore a new series of compounds was synthesised in which the basic *n*-PanAm structure was retained, but a methyl group was introduced as a substituent on either of the α- or β-carbons of its β-alanine. The goal of such a structural modification was to increase the steric bulk in the vicinity of the amide carbonyl group, with the rationale that such a modification would increase their biological stability as it would prevent or retard their enzymatic hydrolysis by pantetheinase activity. At the same time we hypothesized that the retention of the *n*-PanAm core structure would still allow for high specificity interactions, ensuring potency similar to that previously seen for the *n*-PanAm compounds.

Scheme 3.2 - Schematic representation of the parallel synthesis of α- and β-methylated *N*-substituted pantothenamides.

To test this proposal, α- and β-methylated analogues of the *n*-PanAms that had demonstrated the most potent growth inhibition of *P. falciparum* parasites in previous experiments were synthesized. For preparation of these methylated pantothenamides, a method previously developed in our laboratory and described elsewhere [24] for the

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parallel synthesis and purification of any number of pantothenamides was employed. In this method, α- and β-methylated pantothenic acid molecules are prepared and then converted to their corresponding activated thioesters. Following the preparation of the thioesters, aminolysis reactions were performed with the appropriate amines to result in the desired α-methyl and β-methyl PanAms (α-MePanAms and β-MePanAms respectively) (Scheme 4.2). These newly prepared α- and β-MePanAms were then assessed for their antiplasmodial profile in fresh, as well as in aged medium, following the same procedures as described before (chapter 2, section 2.2.7).

As expected, the antiplasmodial activity of the α- and β-MePanAms determined in fresh medium was, in general, significantly improved compared to the values determined for the *n*-PanAms under the same conditions. Eight of the eleven β-MePanAms tested exhibited IC_{50} values significantly lower than their normal counterparts ($P < 0.01$, unpaired t-test, n =3), with two of these compounds exhibiting IC_{50} values below 20 μM. Similarly, nine of the eleven α-MePanAms tested exhibited IC_{50} values lower than their equivalent *normal*-counterparts ($P < 0.01$, unpaired t-test, n=3), with four of them demonstrating antiplasmodial activity at IC_{50} values below 20 μM. In general, the α-MePanAm compounds showed increased potency compared to their β-methylated counterparts, suggesting that placement of the methyl group is an important consideration for the retention of activity. Of all the compounds tested, the improvement on the antiplasmodial activity of the phenethyl-substituted pantothenamide was most striking, with the α-MePanAm **47d** having an IC_{50} that is more than 880-fold lower compared to its *n*-PanAm equivalent, decreasing from an IC_{50} of 53 ± 11 μM to 0.06 ± 0.02 μM (mean ± SEM, n=3).

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To confirm whether the enhanced antiplasmodial activity of these compounds resulted from their improved resistance to pantetheinase degradation, five α- and β-MePanAms containing the same amine substituents as the PanAms previously tested were selected for pantetheinase-mediated degradation and incubated in the presence of recombinant human pantetheinase as detailed before (chapter 2, section 2.2.13). The amount of amine released under these conditions was then determined using the described fluorescamine-based fluorescence assay. The hydrolysis of the compounds was assessed after 24 h. As predicted, the *n*-PanAms were completely hydrolyzed in the presence of vanin, their α- and β-methylated counterparts were only marginally degraded (figure 3.5).

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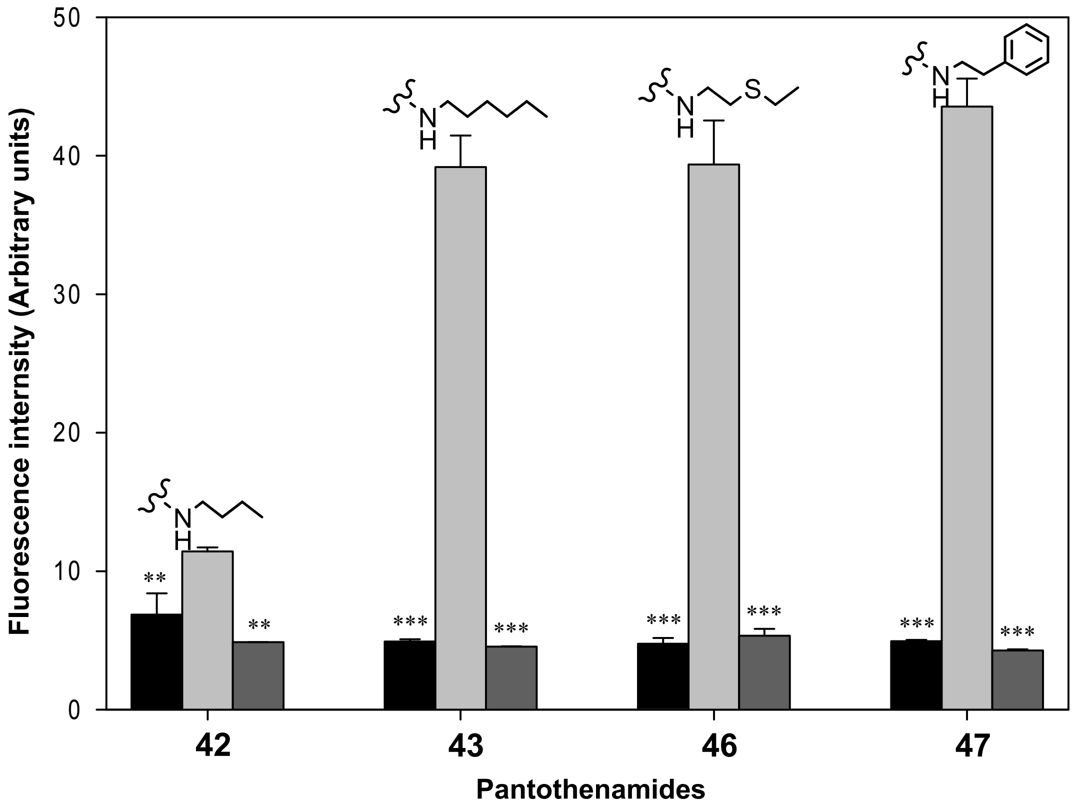

Figure 3.5 - Hydrolysis of α- and β-methylated pantothenamides by recombinant human pantetheinase. Four sets of selected α-MePanAms (black bars), *n*-PanAms (light grey bars) and β-MePanAms (dark grey bars) – structure identifiers indicated below the bars, and structure of amide substituent shown above – were treated with recombinant human pantetheinase. After 24 h, the amount of amine released was determined by means of a fluorescamine-based fluorescence assay. Values represent the mean from three independent experiments, each performed in duplicate; the error bars represent SEM. Where not visible, the error bars are right on the edge of the column. The asterisks above the error bars represent the significance of the difference between the values determined for the respective α-MePanAm and β-MePanAm series members *versus* the *n*-PanAms in a given set (** $P < 0.005$; *** $P < 0.001$, one way ANOVA). Note that since the fluorescence intensity of the fluorescamine-amine conjugates for different complexes is dependent on the structure of the amine, the fluorescence measurements are not necessarily comparable between the different sets.

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Table 3.2 ***In vitro*** **antiplasmodial activity of α-methyl and β-methyl pantothenamides against intraerythrocytic-stage** ***P. falciparum*** **parasites**

α-MePanAm β-MePanAm

		IC_{50} (μM) in Fresh Medium		
Entry	**R group**	***n*-PanAm (b)**	**α-MePanAm (d)**	**β-MePanAm (e)**
41		182 ± 17	47 ± 1	117 ± 7
42		> 200	> 200	66 ± 4
43		134 ± 6	23 ± 1	32 ± 1
45		117 ± 14	13 ± 1	69 ± 3
46		143 ± 5	10 ± 2	37 ± 3
47		53 ± 11	0.06 ± 0.02	14 ± 1
48		> 200	13 ± 1	18 ± 1
59		199 ± 53	27 ± 1	> 200
60		> 200	76 ± 2	108 ± 6
61		> 200	20 ± 2	33 ± 2
63		> 200	> 200	41 ± 2

These results clearly demonstrate that the introduction of the methyl group on the α- or β-carbons of the β-alanine moiety of *n*-PanAms, which increases the steric bulk in the vicinity of the amide carbonyl group, does indeed decrease the susceptibility to pantetheinase-mediated degradation of these compounds.

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3.2.4 Antiplasmodial activity of *N*-substituted pantoyltauramides

Following the positive and extremely encouraging results obtained with the structural modification of *n*-PanAms to render them resistant to degradation by pantetheinases, it was decided to explore the antiplasmodial activity of other pantothenic acid analogues that have been synthesized and tested against avian malaria parasites in the 1940s, namely the pantoyltauramides [26, 27]. To achieve this objective, a set of *N*-substituted pantoyltauramides was prepared in our laboratory.[1] Three of these (compounds **56**, **57** and **58**) were assessed for their antiplasmodial activity against intraerythrocytic-stage *P. falciparum* parasites in standard (fresh) culture medium. The results obtained indicated that the pantoyltauramides also possess good antiplasmodial activity, with the most potent compound (**56**) exhibiting an IC_{50} of 5.7 ± 0.2 μM (mean ± SEM, n=3) (Table 3.3).

[1] The synthesis of these compounds was performed by Mr. Collins Jana as part of his MSc studies.

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Table 3.3 Structures and antiplasmodial activity of pantoyltauramides against *P. falciparum* parasites in fresh and aged medium and the antagonistic effect of increasing the extracellular pantothenate concentration from 1 µM to 100 µM

		IC_{50} (µM)*					
		1 µM Pantothenate		100 µM Pantothenate		Fold Shift[2]	
Entry	R group	Fresh Medium	Aged Medium	Fresh Medium	Aged Medium	Fresh Medium	Aged Medium
56		5.7 ± 0.2	4.8 ± 0.1	113 ± 2	125 ± 7	20	26
57		8.2 ± 0.2	11 ± 0.2	278 ± 14	257 ± 7	34	25
58		35 ± 2	42 ± 0.4	295 ± 33	290 ± 35	8	7

* The IC_{50} values were calculated from data collected from two independent experiments performed in triplicate as detailed in chapter 2, section 2.2.7. Error values represent range/2.

When comparing the antiplasmodial activity of pantoyltauramides (table 3.3) with that of *N*-substituted PanAms (table 3.1), the results suggest that the latter are better growth inhibitors of intraerythrocytic-stage *P. falciparum* parasites as demonstrated if comparing compounds with the same amine substituent. For example, compounds **47a-e** exhibited IC_{50} values ranging between 0.06 ± 0.02 µM and 53 ± 11 µM in fresh medium, compared to the IC_{50} value of 35 ± 2 for the pantoyltauramide **58.** In fact, among the pantothenamides, only *n*-PanAm **47b** with a poor IC_{50} of 53 ± 11 in fresh medium exhibited poorer antiplasmodial activity when compared to the pantoytauramide bearing the same amine substituent.

To assess the antiplasmodial activity of pantoyltauramides in a culture medium with reduced pantetheinase activity, the three selected compounds were tested in aged

[2] Magnitude of change in IC_{50} value when growth assays are performed in medium with 1 µM *versus* 100 µM pantothenate.

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medium following the same protocols as detailed previously. As shown in Table 3.3, the presence or absence of pantetheinases did not significantly ($P > 0.05$, one way ANOVA) affect the antiplasmodial activity of the pantoyltauramides, suggesting that these compounds too, are resistant to pantetheinase-mediated degradation and that their antiplasmodial activity exhibited in fresh medium reveals their intrinsic activity against intraerythrocytic-stage *P. falciparum* parasites.

While the pantoyltauramides are structurally very similar to the pantothenamides, the exchange of an amide for a sulphonamide group could have important effects on target selectivity, which could explain their comparatively lower potency. Subsequently, it was set out to investigate whether pantoyltauramides exerted their antiplasmodial activity "on-target", i.e. like the pantothenamides, affect processes in *P. falciparum* parasites that are dependent on pantothenic acid [25]. This was done by investigating the activity of the three selected compounds in the presence of excess pantothenic acid (100 μM), compared to the ~1 μM that is present in standard culture medium [28], similar to the quantity of the vitamin under normal conditions in circulating whole blood of mammals, ~1.57 to 2.66 μM [29]. By means of concentration-response assays that were conducted in both fresh and aged culture medium (table 3.3), a significant ($P < 0.01$, n=3, unpaired t-test) increase in the IC_{50} values was observed for all the compounds tested, with the potency reduced by 7 to 34-fold. Pantoyltauramide **58** exhibited the smallest fold shift when tested in culture medium containing excess pantothenate both in fresh and in aged medium.

Although a detailed explanation of the mechanisms through which the observed shifts occur remain to be elucidated, the results confirm that pantoyltauramides exert their

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antiplasmodial inhibitory effects by affecting processes that depend on pantothenate. The lower antiplasmodial potency of these compounds relative to the *n*-PanAms with the same amide substituent when tested in aged medium is therefore not due to them acting on a different target, but is more likely the consequence of poor preference of the target to pantoyltauramides as a result of its high specificity or the specificity of one or more of the enzymes that activate these compounds to their inhibitory forms.

3.3 Conclusion

In these studies it was shown that pantothenate analogues – including *N*-substituted pantothenamides and *N*-substituted pantoyltauramides – inhibit growth of intraerythrocytic-stage *P. falciparum* parasites, with the former analogues doing so at sub-micromolar and the latter at low micromolar concentrations. In both cases, these compounds act through inhibition of pantothenate-dependent processes, most likely CoA biosynthesis and utilization. It was also demonstrated that the antiplasmodial activity of pantothenamides can be improved through structural modifications that render them less susceptible to degradation by pantetheinases, while at the same time maintaining their "on-target" antiplasmodial activity.

While additional studies on the potential rescue effect that addition of pantothenate could have in cultures that had been treated with PanAms or pantoyltauramides are warranted, the observation that the antiplasmodial activity of PanAms and pantoyltauramides could be negated (as shown by the increase in the IC_{50} values) in the experiments where culture medium was supplemented with pantothenate clearly indicates that the pantothenate analogues interfere with pantothenate-dependent process(s) in the intraerythrocytic-stage *P. falciparum*. parasites.

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Overall, this work has provided significant insight towards understanding the pantothenamide class of antimicrobials, and affords new avenues that can be investigated for improvement of the antiplasmodial activity of this class of compounds.

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Chapter 4:

INVESTIGATING THE ANTIPLASMODIAL MODE OF ACTION OF PANTOTHENAMIDES

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4.1 Introduction

N-substituted pantothenamides have been shown to possess antibacterial activity *in vitro* against *E. coli* and *S. aureus* among other susceptible bacteria [1-5]. In those microorganisms, the compounds bind to and serve as substrates for bacterial PanK, the first enzyme in the CoA biosynthesis pathway, and as a consequence competitively inhibit PanK-catalysed pantothenate phosphorylation [4, 6-9]. Upon phosphorylation, the resulting 4'-phosphopantothenamides are further transformed by PPAT and DPCK into CoA analogues [8], inhibiting CoA-utilising enzymes and other proteins, including ACP [7, 10]. This protein requires the CoA-derived 4′-phosphopantetheine moiety for its maturation from an inactive *apo*-ACP to the active *holo*-ACP form, which functions as an acyl group carrier in fatty acid biosynthesis. While it has been established that PanAms cause bacteriostasis in susceptible bacteria, it is still not known whether their activity results from inhibition of CoA biosynthesis [11], fatty acid biosynthesis [10] or other CoA-utilizing process(es), or a combination of the above factors, and whether the same mode of action is operational in each case.

The results reported in the previous chapter of this dissertation (chapter 3), demonstrated that some PanAms are potent antiplasmodial compounds with IC_{50} values comparable to that of chloroquine, the benchmark for sensitive parasites. Moreover, it was shown that chemical modifications that render PanAms resistant to pantetheinase degradation while maintaining their antiplasmodial activity can be exploited for development of this class of compounds as potential antimalarial medications. The “on-target” activity, revealed by demonstrating that the IC_{50} of PanAms was increased when concentration response assays were performed in growth media with excess pantothenate concentration impelled further investigation to elucidate the exact

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molecular target(s) with which these compounds interact in the parasite, and thereby their MoA. This chapter provides a description of the work undertaken to complete this aim.

The first experiments were directed at determining the manner in which PanAms interact with *Pf*PanK, i.e. as inhibitors (which would highlight *Pf*PanK as the target of their inhibitory activity or as substrates (which would suggest that they are transformed into antimetabolites in a manner similar to that seen in many bacteria. Concurrently, it was set out to investigate the reason why (as shown in chapter 3) *n*-PanAms exhibited better antiplasmodial activity compared to their α- and HoPanAm counterparts with the same amide substitution when tested in culture medium that was devoid of pantetheinase activity. The second set of experiments where designed to investigate the potential targets of the PanAms in the event that they acted as antimetabolites, i.e. exerting an effect downstream of *Pf*PanK-catalysed phosphorylation. This was done by investigating two potential targets: (i) CoA levels, and (ii) the *holo*-ACP pools in *P. falciparum* parasite cultures.

4.2 Results and Discussion

4.2.1 Interaction of *N*-substituted pantothenamides with *Pf*PanK: pantothenate phosphorylation inhibition

A simple, yet effective test to establish whether the relative potency observed for the various PanAms series correlates with their interaction with *Pf*PanK is to test these compounds for their ability to inhibit pantothenate phosphorylation. Compounds that have a high affinity for *Pf*PanK – be it as actual inhibitors, or as alternative substrates that effectively compete with pantothenate for the enzyme – will show potent inhibition,

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while those that have a low affinity or poor interaction will not. To assess this, 22 selected PanAms of the α-, *n*- and HoPanAm series that represent some chemical diversity in their amide moieties were selected based on the *in vitro* antiplasmodial activity in standard (fresh) as well as in aged culture medium (chapter 3, section 3.2.2).

The PanAms were tested at concentrations ranging between 0.1 and 100 μM for their ability to interfere with the phosphorylation of 0.2 μM [^{14}C]pantothenate by parasite lysates which contain *Pf*PanK. Parasite lysates where used since *Pf*PanK has not been successfully expressed and purified. The activity of the enzyme was determined by measuring the amount of [^{14}C]pantothenate phosphorylated over 30 min (a time period during which pantothenate phosphorylation increased linearly over time under control conditions) in the presence or absence of the test compounds using the Somogyi-based precipitation assay detailed in chapter 2, section 2.2.8. From the data generated, a concentration-response profile was then established for each compound. Representative concentration-response profiles are shown in figure 4.1 for the set of *N*-phenethyl PanAms (**47a-c**) and *N*-isopropyl PanAms (**80a-c**).

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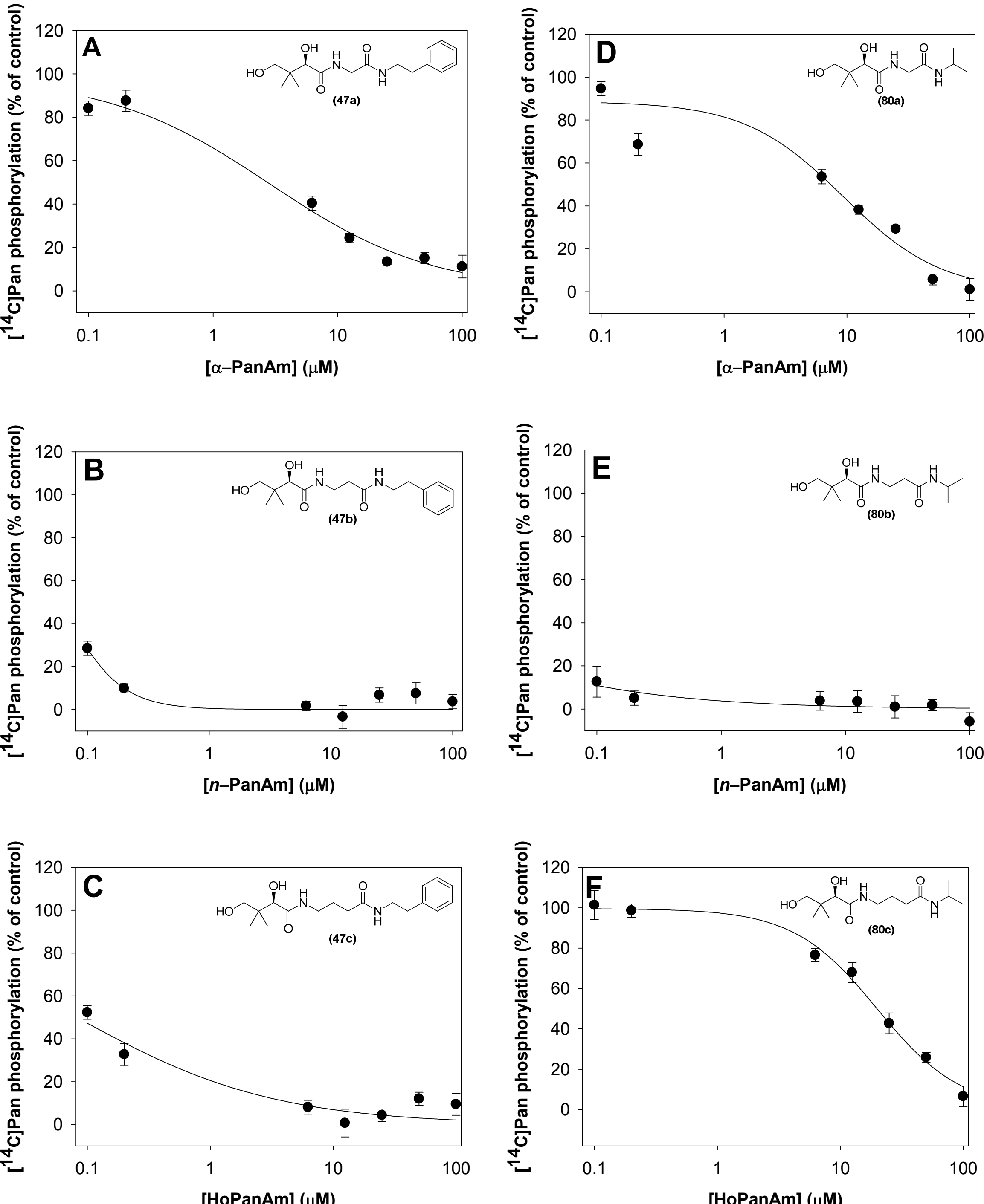

Figure 4.1 Effect of PanAms on the *in vitro* phosphorylation of [^{14}C]pantothenate by *Pf*PanK containing trophozoite-stage *P. falciparum* parasite lysates. Concentration-response experiments were performed for all the compounds shown in Table 4.1. The plots are examples of the profiles generated for the set of *N*-phenethyl PanAms (**47a-c**), panels A, B and C and *N*-isopropyl PanAms (**80a-c**), panels D, E and F. The data were generated from two repeat experiments performed in duplicate. Data were calculated as percentage of control reactions in which no PanAm was added (i.e. 100% phosphorylation) and, error bars indicate range/2.

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In figure 4.1 it can be observed that an increase in the concentration of the PanAms directly correlates with a decrease in the amount of the pantothenate phosphorylated. The concentration-response profiles were subsequently used to calculate the concentration of each PanAm that results in 50% inhibition (IC_{50}) of pantothenate phosphorylation. This was done by fitting a Sigmoidal, Logistic, 3 Parameter dose-response/variable slope to the data, using nonlinear least squares regression and averaging the IC_{50} estimates from at least two independent experiments. Most of the *n*-PanAms were found to be so potent that the IC_{50} fell below the lowest concentration tested. In those cases the reported value is an estimate of the upper limit of the true IC_{50} value and is shown in Table 4.1.

Table 4.1 – Comparison of the *N*-substituted PanAm-mediated inhibition of [^{14}C]Pantothenate phosphorylation by *Pf*PanK containing trophozoite-stage *P. falciparum* parasite lysates and the antiplasmodial activity of PanAms *in vitro*

		IC_{50} (µM Inhibition of Pan Phosphorylation			IC_{50} (µM) antiplasmodial activity in Fresh Medium			IC_{50} (µM) antiplasmodial activity in Aged Medium		
Entry #	**R group**	**α-PanAm**	***n*-PanAm**	**Ho-PanAm**	**α-PanAm**	***n*-PanAm**	**Ho-PanAm**	**α-PanAm**	***n*-PanAm**	**Ho-PanAm**
41		1.2 ± 1.6	0.2 ± 0.1	< 0.1	18 ± 3	> 200	2.8 ± 1.4	14. ± 2	7.5 ± 6.2	2.2 ± 0.1
43		31 ± 6	< 0.1	0.3 ± 0.1	7.5 ± 0.3	> 200	10 ± 6	7.9 ± 1.8	0.6 ± 0.01	14 ± 7
46		1.3 ± 0.2	< 0.1	0.2 ± 0.02	12 ± 1	> 200	1.7 ± 0.3	11 ± 1	0.3 ± 0.02	1.9 ± 0.1
47		2.9 ± 0.8	< 0.1	0.2 ± 0.1	3.4 ± 0.8	53 ± 11	2.1 ± 0.1	4.2 ± 0.2	0.020±0.003†	2.7 ± 0.3
48		0.3 ± 0.2	ND	1.7 ± 1.9	110 ± 13	> 200	3.1 ± 1.6	127 ± 16	> 200	4.7 ± 0.5
80		9.3 ± 3.4	< 0.1	20 ± 2	ND	ND	ND	ND	ND	ND
81		22 ± 5	< 0.1	6.9 ± 1.9	ND	ND	ND	ND	ND	ND
82		< 0.1	ND	< 0.1	ND	ND	ND	ND	ND	ND

ND - Not determined due to limited stock of compounds

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When analysing the data, the most consistent structure-activity relationship (SAR) that can be deduced from the results is that the *n*-PanAms are significantly more potent inhibitors of parasite lysate-mediated pantothenate phosphorylation than their α- and HoPanAm counterparts. This suggests that possibly the *n*-PanAms have the strongest interaction with the *Pf*PanK in the parasite lysates, either as inhibitors or as substrates. This is not surprising considering that pantothenate (the natural substrate for PanK) and the *n*-PanAms have the intact β-alanine moiety in common. The *n*-PanAms therefore have a molecular structure more suitable for interaction with the active site of the enzyme than their two structurally modified counterparts. Moreover, the results of the *in vitro* antiplasmodial activity tests against intraerythrocytic-stage *P. falciparum* parasites in aged medium (i.e. with inactivated pantetheinase), as reported in the section 3.2.2 of the previous chapter (and shown again in table 4.1 for selected compounds), showed that the *n*-PanAms also exhibited the most potent antiplasmodial activity compared to their shortened and lengthened counterparts. Taken together, these results indicate that the interaction of PanAms with *Pf*PanK contained in trophozoite-stage parasite lysates is a key element in the MoA of these compounds in intraerythrocytic-stage *P. falciparum* parasites.

A SAR analysis of the amide substituents suggests that some groups do show greater potency across the three sets of PanAms tested than others. For example, the α- and HoPanAm with amide substituents prepared from secondary amines (such as **80a**, **c** and **81a**, **c**) are poor inhibitors of *Pf*PanK-mediated pantothenate phosphorylation in comparison to compounds with amide substituents prepared from primary unbranched aliphatic (**41a**, **c**) and heteroatom-containing aliphatic amines (**46a**, **c**), and primary amines with substituents containing aromatic groups (**47a**, **c** and **48a**, **c**) and aliphatic

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carbocycles (**82a**, **c**). However, the correlation between potency and the length of the β-alanine moiety of these compounds still far outweighs any of the amide substituent relationships.

4.2.2 *In vitro* phosphorylation activity of pantothenamides by *Pf*PanK

While the phosphorylation inhibition results discussed above highlight the *n*-PanAms as the most potent inhibitors of parasite lysates *Pf*PanK's-mediated pantothenate phosphorylation activity, it cannot distinguish whether this is due to the compounds acting as inhibitors of the enzyme's activity, or if the *n*-PanAms in fact act as preferred alternative substrates and therefore can effectively compete with pantothenate for the enzyme's active site. To investigate if any of the PanAms served as substrate for the enzyme contained in parasite lysates, phosphorylation reactions with *Pf*PanK containing parasite lysates were performed with a range of concentrations of each of four selected [^{14}C]-labelled PanAms, namely *N*-hexyl α-PanAm, *N*-hexyl *n*-PanAm and *N*-hexyl HohexylPanAm (compounds **43a-c**) and *N*-benzyl *n*-PanAm (compound **48b**) as substrates.

These compounds were selected on the basis of their *in vitro* antiplasmodial activity in fresh and aged medium as reported previously (chapter 3, section 3.2.2.1) and also to include compounds representing all three PanAm types (with the same aliphatic amide substitution) and at least one PanAm with an aromatic amide substitution. A limited but representative number of compounds were used for these tests due to restrictions on the availability of their radiolabelled versions.

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Phosphorylation reactions were also performed with pantothenate under the same *in vitro* conditions for comparison. The reactions were assayed using the Somogyi reagent-based precipitation method detailed in chapter 2, section 2.2.8. From the time-course data at each concentration ranging from 0.005 to 0.31 μM for [^{14}C]*N*-benzyl *n*-PanAm (**48b***), 0.04 to 2.5 μM for [^{14}C]Pan (**23***), [^{14}C]*N*-hexyl *n*-PanAm (**43b***) and [^{14}C]*N*-hexyl HoPanAm (**43c***), and from 0.08 to 5 μM for [^{14}C]*N*-hexyl α-PanAm (**43a***), initial rates were determined – an example of time-course phosphorylation reaction of Pan and PanAms by *Pf*Pank containing parasite lysates is shown in figure 4.2. From the initial rates, the activity profiles shown in figure 4.3 were then obtained.

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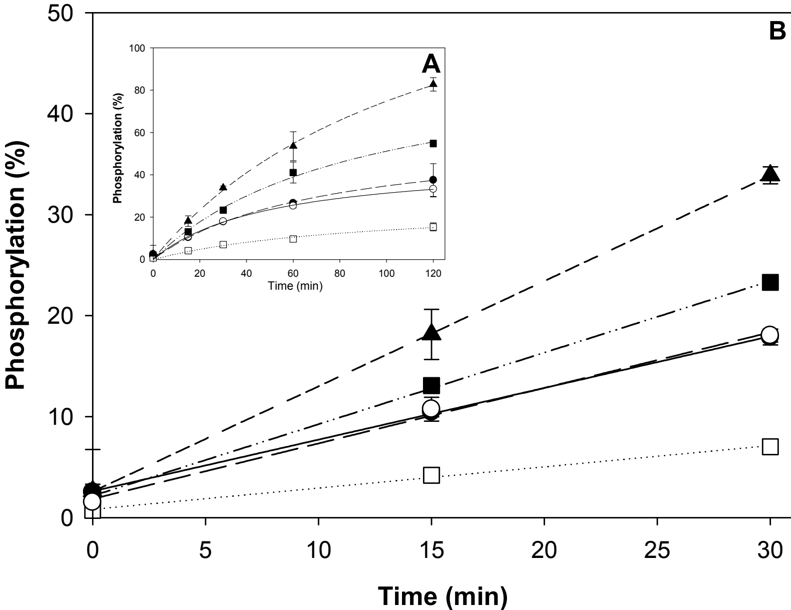

Figure 4.2 Time-course phosphorylation of Pan and PanAms. [14C]labelled compounds were assayed at concentrations with specific activity of 0.1 μCi to determine the period of time during which the phosphorylation of [14C]Pan (**23***, black triangles), [14C]*N*-hexyl α-PanAm (**43a***, black circles), [14C]*N*-hexyl *n*-PanAm (**43b***, white circles), [14C]*N*-hexyl HoPanAm (**43c***, black square) and [14C]*N*-benzyl *n*-PanAm (**48b***, white squares) by *Pf*Pank from parasite lysates at 37°C in kinase buffer (50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, pH 7.4) increased linearly. At pre-determined time intervals, up to 120 minutes, samples of the phosphorylation reaction mixtures were retrieved and treated as described in chapter 2, section 2.2.8.1. Panel **A** (inset) shows the time-course phosphorylation of the tested compounds during the whole period assayed (120 min). Panel **B** is a magnification of the initial 30 min, a time period during which the phosphorylation of the compounds was observed to increase linearly. Symbols represent the mean of two independent experiments performed in duplicate. Error bars show range/2. Where not visible, error bars are smaller than the symbol.

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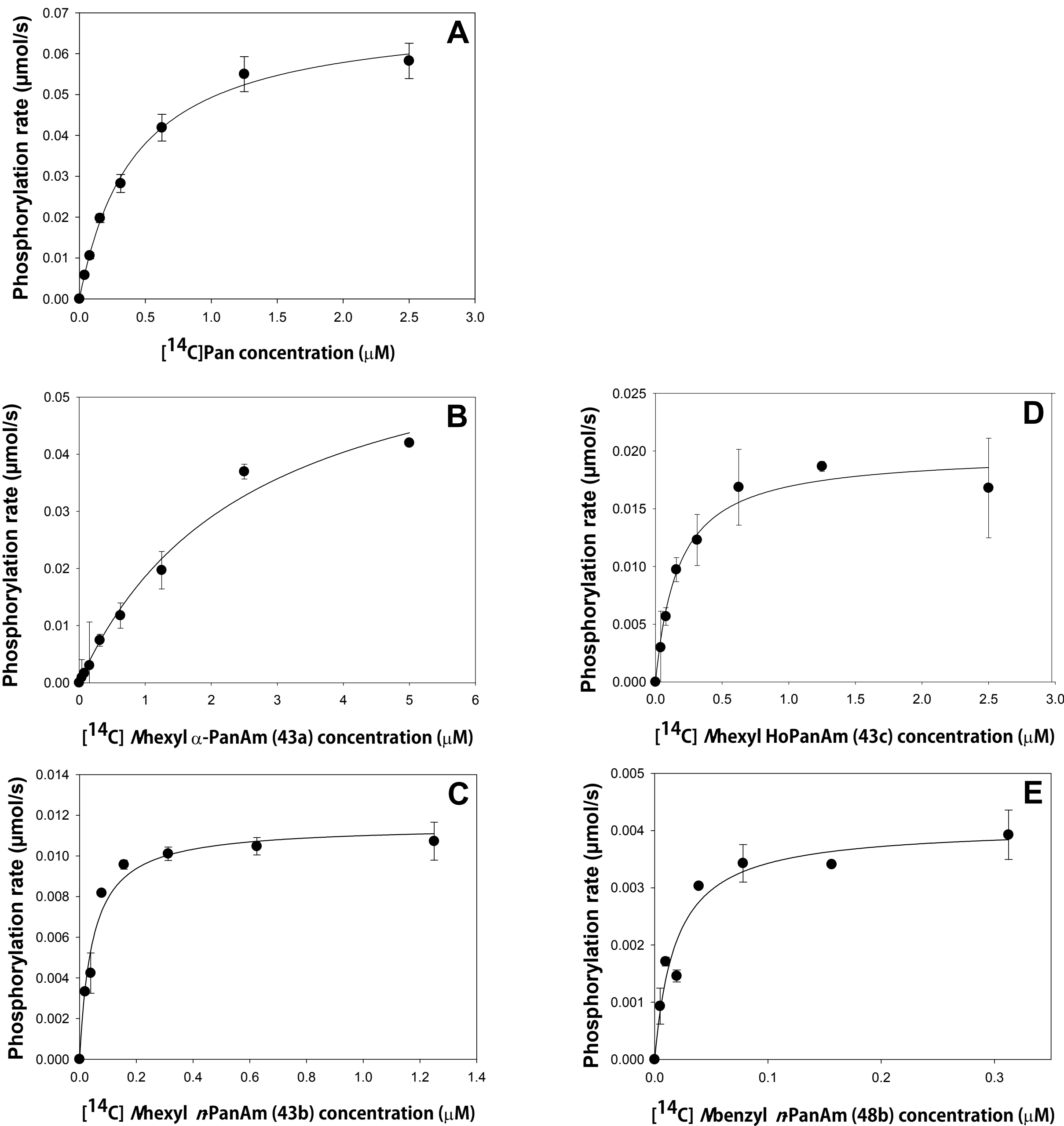

Figure 4.3 - Phosphorylation of pantothenate and selected pantothenamides by *Pf*PanK in parasite lysates. Activity profiles of selected PanAms at increasing substrate concentrations. Panel **A** indicates the phosphorylation of pantothenate and Panels **B**, **C**, **D** and **E** show the phosphorylation of *N*-hexyl α-PanAm (**43a**), *N*-hexyl *n*-PanAm (**43b**), *N*-hexyl HoPanAm (**43c**) and *N*-benzyl *n*-PanAm (**48b**) respectively. The phosphorylation reactions were carried out in kinase buffer (50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, pH 7.4) using a modified and miniaturised Somogyi reagent-based assay as described in chapter 2, section 2.2.8.2. Initial rates were determined from the time course data at each concentration. Symbols represent the mean of two independent experiments and the error bars show range/2. The solid curves show the best fit of the Michaelis-Menten equation to the data, from which the kinetic parameters (V_{max} and K_m) were determined.

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As can be seen from the activity profiles, all of the PanAms tested were taken by the *Pf*PanK enzyme contained in parasite lysates as substrates, showing phosphorylation activity (V_{max} of 0.01 ± 0.01 to 0.06 ± 0.01 μmol.s^{-1}/10^7 cells) in a range similar to that exhibited for the natural substrate, pantothenate (V_{max} of 0.07 ± 0.02 μmol.s^{-1}/10^7 cells). These results are consistent with previous studies in which the pantothenic acid analogue pantothenol was demonstrated to serve as a substrate for *Pf*PanK in parasite lysates [12]. To quantify *Pf*PanK's phosphorylation efficiency of these compounds, the Michaelis-Menten equation $v=a*V_{max}[S]/(K_m+[S])$ was fitted to the profile data, allowing the kinetic parameters V_{max} and K_m to be obtained for each compound. The results are summarized in table 4.2.

Table 4.2 Kinetic parameters for *Pf*PanK towards selected PanAms.

Entry #	Compound	V_{max} (μmol.s^{-1}/10^7 cells)	K_m (μM)
23		0.07 ± 0.02	0.4 ± 0.04
43a		0.06 ± 0.01	2.6 ± 0.01
43b		0.01 ± 0.01	0.05 ± 0.02
43c		0.02 ± 0.01	0.2 ± 0.05
48b		0.01 ± 0.01	0.02 ± 0.01

Comparing the kinetic parameters, it appears that, in general, the maximal rates of phosphorylation (V_{max}) of the PanAms (regardless of structure) are generally lower than that of pantothenate (*Pf*PanK's natural substrate) except for *N*-hexyl α-PanAm (**43a**) with a V_{max} of 0.06 ± 0.01 μmol.s^{-1}/10^7 cells. The normal PanAms *N*-hexyl *n*-PanAm (**43b**) and *N*-benzyl n-PanAm (**48b**) showing maximal rates of phosphorylation ~6-fold lower

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than the phosphorylation of pantothenate where, among the compounds tested, the ones that exhibited the lowest phosphorylation rates. A comparison of the K_m values of the compounds tested reveals that both the *n*-PanAms tested (Compounds **43b** and **48b**) exhibit values at least an order of magnitude lower than the K_m obtained for pantothenate. The K_m for the HoPanAm **43c** is ~2.5-fold lower, while the α-PanAm **43a** is the only compound with a K_m higher than that for pantothenate (~5-fold higher). These findings suggest that the *n*-PanAms act as preferred substrates of the *Pf*PanK contained in the complex matrix that parasite lysates are and, that the potent inhibition of pantothenate phosphorylation observed may be a result of these compounds effectively competing with pantothenate for the enzyme's active site. Moreover, based on the available data, there is indication that PanAms are not inhibitors of parasite lysate contained *Pf*PanK's phosphorylation activity, providing evidence against the hypothesis that the enzyme acts as the main target for the observed antiplasmodial activity of these compounds.

4.2.3 Deoxy-N5-Pan is a poor inhibitor of pantothenate phosphorylation, and of *P. falciparum* parasite proliferation

To further confirm that the PanAms (and particularly the *n*-PanAms) mainly inhibit *P. falciparum* by acting as antimetabolites, i.e. that they act as substrates, and not inhibitors of *Pf*PanK, the PanAm analogue deoxy-N5-Pan (compound **79**) was prepared (figure 4.3). This compound has the same amide substitution as N5-Pan (compound **41b**) but lacks its 4'-hydroxyl group and as such cannot be phosphorylated by *Pf*PanK but it should retain all the other important binding interactions with the enzyme. Since N5-Pan acts as an excellent substrate of *Pf*PanK, it was therefore expected that deoxy-N5-Pan should act as a competitive inhibitor of the enzyme with potency similar to that seen

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for N5-Pan in the inhibition tests described previously (section 4.2.1). However, inhibition tests done under similar conditions as all the other PanAms (50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, pH 7.4) revealed that deoxy-N5-Pan (**79**) has an IC_{50} value for inhibition of 0.2 μM pantothenate phosphorylation greater than 100 μM; much larger than the value of 0.2 ± 0.1 μM determined for N5-Pan (figure 4.4).

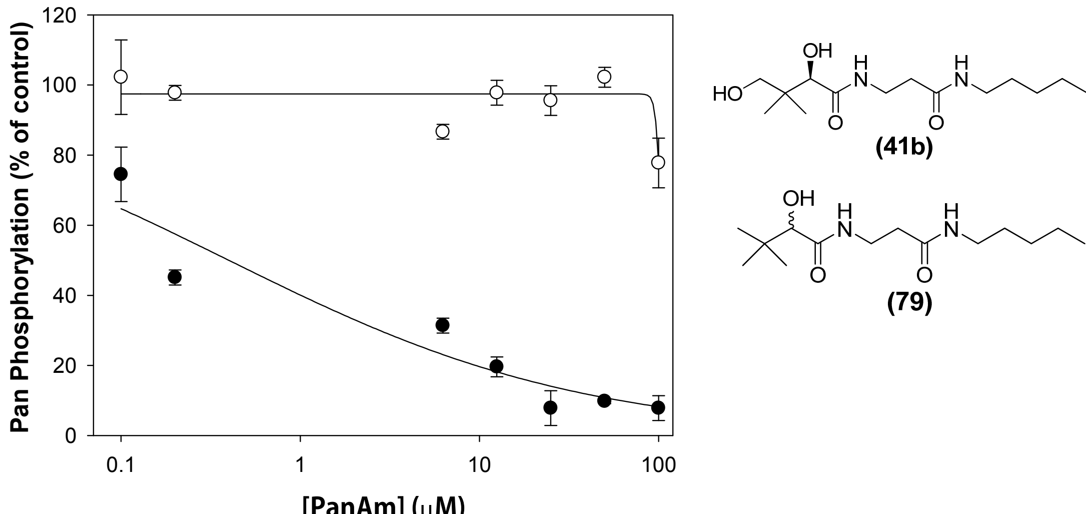

Figure 4.4 - Inhibition of pantothenate phosphorylation by N5-Pan (41b, black circles) and deoxy-N5-Pan (**79**, white circles). The concentration response curves show the effect of increasing concentrations of N5-Pan and deoxy-N5-Pan on inhibition of phosphorylation of 0.2 μM pantothenate by *Pf*PanK from lysates prepared from trophozoite-stage parasites. The data were obtained from two independent experiments each carried out in duplicate (error bars represent range/2). Where not shown, error bars are smaller than the symbol.

Deoxy-N5-Pan's poor inhibition of pantothenate phosphorylation indicates that the 4'-OH that accepts the phosphoryl group forms crucial binding interactions with the *Pf*PanK enzyme. This suggests that the enzyme is predisposed to the binding of PanAms that can act as alternate substrates, and consequently that serves as a gateway to their metabolic activation as CoA antimetabolite inhibitors. This conclusion was further confirmed by the results of tests in which deoxy-N5-Pan (**79**) and also deoxy-*N*-phenethyl *n*-PanAm (**83**) – the 4'-deoxy analogue of *N*-phenethyl *n*-PanAm (**47b**), which

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showed the most potent antiplasmodial activity in the tests described in the previous chapter – were evaluated for their ability to inhibit parasite proliferation (figure 4.5). These results showed that neither compound is sufficiently potent to cause *P. falciparum* parasite growth inhibition below 50% of the control, even at the highest concentration (200 μM) tested, while the two parent compounds showed potent antiplasmodial activities with IC_{50} values of 7.5 ± 6.2 μM and 0.020 ± 0.003 μM for N5-Pan (**41b**) and *N*-phenethyl *n*-PanAm (**47b**) respectively.

These findings clearly show that the *in vitro* antiplasmodial potency of the PanAms correlates with their *Pf*PanK-mediated phosphorylation activity, and they do not rule out the possibility that, in addition to initially interfering with pantothenate phosphorylation, PanAms act through interaction with a target downstream of *Pf*PanK after their activation through phosphorylation by the enzyme.

4.2.4 Evaluating the effect of PanAms on targets downstream of *Pf*PanK in cultured intraerythrocytic-stage *P. falciparum* parasites

Having demonstrated that the PanAms act as alternative substrates of *Pf*PanK, and most likely are activated by it, the effect of these compounds on potential targets downstream of *Pf*PanK in intraerythrocytic-stage *P. falciparum* parasites was investigated. Specifically, the aim was to determine whether the compounds have any effect on the CoA levels in the parasite, and/or if they affected the active ACP pools (i.e. the levels of *holo*-ACP).

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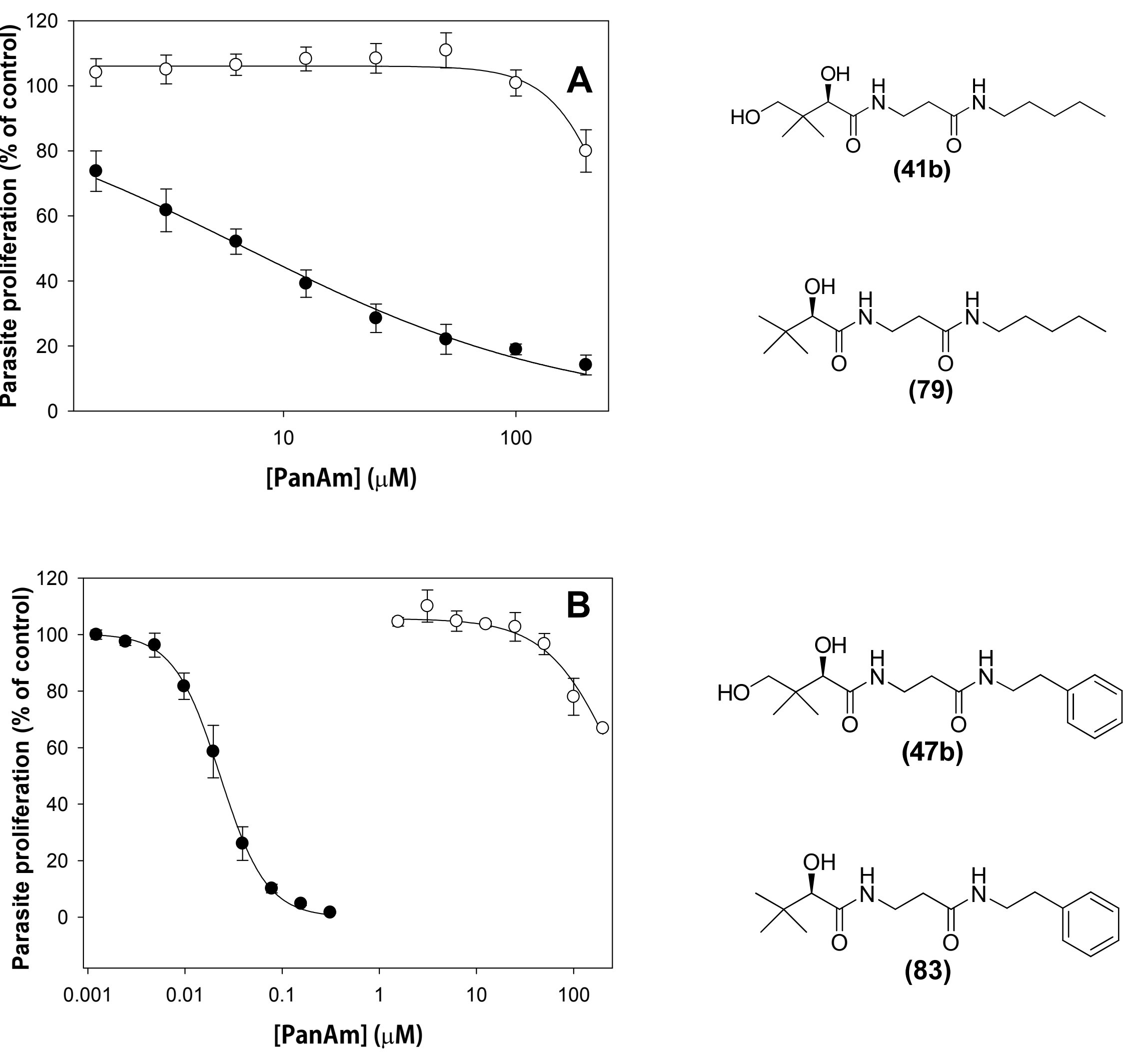

Figure 4.5 - Antiplasmodial activity of selected normal pantothenamides (black circles) and deoxy-pantothenamides (white circles) in culture medium treated at 56°C for 18h to remove pantetheinase activity. Panel **A** and panel **B** show the concentration response curves as measured using the SYBR Green I-based growth assay, for increasing concentrations of N5-Pan (**41b**) and deoxy-N5-Pan (**79**), and for *N*-phenethyl *n*-PanAm (**47b**) and deoxy-*N*-phenethyl *n*-PanAm (**83**) respectively. Symbols represent the mean of two independent experiments, each performed in triplicate and error bars indicate range/2. Where not shown, error bars are smaller than the symbol.

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4.2.4.1 Effect of PanAm treatment on the CoA levels in *P. falciparum* parasite cultures

To determine the effect of PanAm treatment on the CoA levels of *P. falciparum* parasites, cultures in aged medium with the parasites synchronised at ring stage were split into three equal parts. Two of the cultures splits were treated with N5-Pan (**41b**) to final concentrations of 50 μM and 100 μM respectively, while an equivalent volume of DMSO – the solvent used to dissolve the PanAm – was added to the third culture to serve as control (DMSO final concentration never exceeded 0.05% v/v in parasite cultures).

The parasites cultures were then incubated following standard protocols for 24 h (to allow the parasites to mature from ring stage to the first generation of trophozoites post treatment) and 72 h (to allow the parasites to grow to the second generation of trophozoites post treatment with the PanAm). Cultures were then retrieved and, after isolating the parasites at trophozoite stage from infected RBCs as described in chapter 2, section 2.2.3, parasites were counted as detailed in chapter 2, section 2.2.5 before lysates were prepared in parasite suspensions containing ~2.5×10^{10} cells/ml, following the procedures described in chapter 2, section 2.2.4. Next, the CoA present in the parasite lysates was labelled with excess (0.5 mM final concentration) 7-diethylamino-3-(4-maleimidylphenyl)-4-methylcoumarin (CPM) under argon atmosphere to allow complete fluorescence labelling of all free thiol groups in the parasite lysate. This was followed by analysis of the derivatized lysate using a reverse-phase HPLC C18 columns as detailed in chapter 2, section 2.2.12 (figure 4.6). The area under the curve (AuC) of the peaks corresponding to fluorescently-labelled CoA (identified by comparison to a known standard) was subsequently used to generate quantitative data for comparison between the samples (figure 4.7).

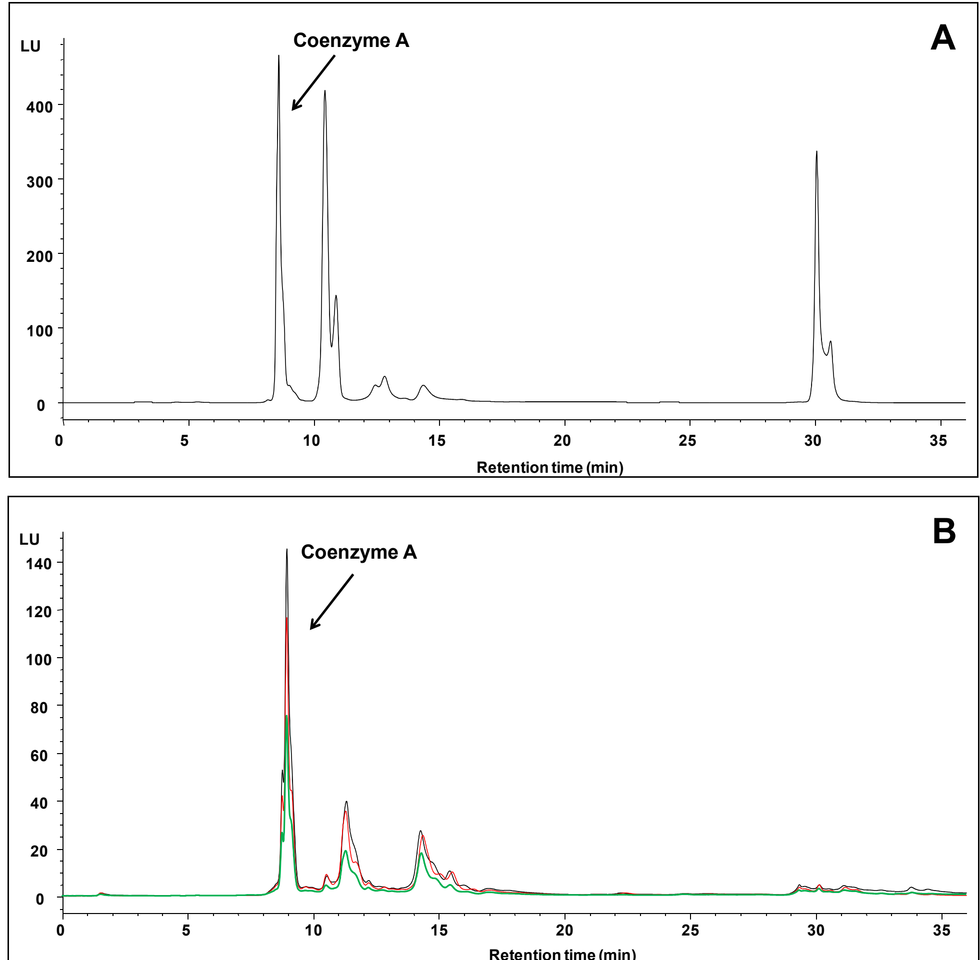

Figure 4.6 – HPLC chromatograms depicting the effect of N5-Pan (**41b**) on CoA levels in parasite cultures. Ring stage parasites were treated with the test compound and incubated for 24 hours followed by lysate preparation and analysis by a C18 reverse phase HPLC column. Panel **A** represents a chromatogram of a standard of commercial CoA (Sigma-Aldrich) after fluorescent labelling with CPM at 0.5 mM in ice-cold 10 mM Tris buffer, 1 mM TCEP, pH 7.4. The peaks other than the one at 9 min which corresponds to CoA in the standard commercial CoA chromatogram, possibly represent TCEP adducts of CPM. The CoA peak is skewed by approximately 1.5 min possibly as a result of CPM labelling. Panel **B** shows the chromatograms of the control culture (black line), cultures treated with 50 μM N5-Pan (red line) and cultures treated with 100 μM N5-Pan (green line) for 24 h post-treatment analysed in a similar manner. Lysates were standardarised for parasite number prior to analysis (see text for details). The peaks at 9 min represent the fluorescently labelled CoA and the lower and broader peaks possibly correspond to thiol-group containing CoA derivatives or other thiol containing metabolites equally affected by N5-Pan treatment to the cultures.

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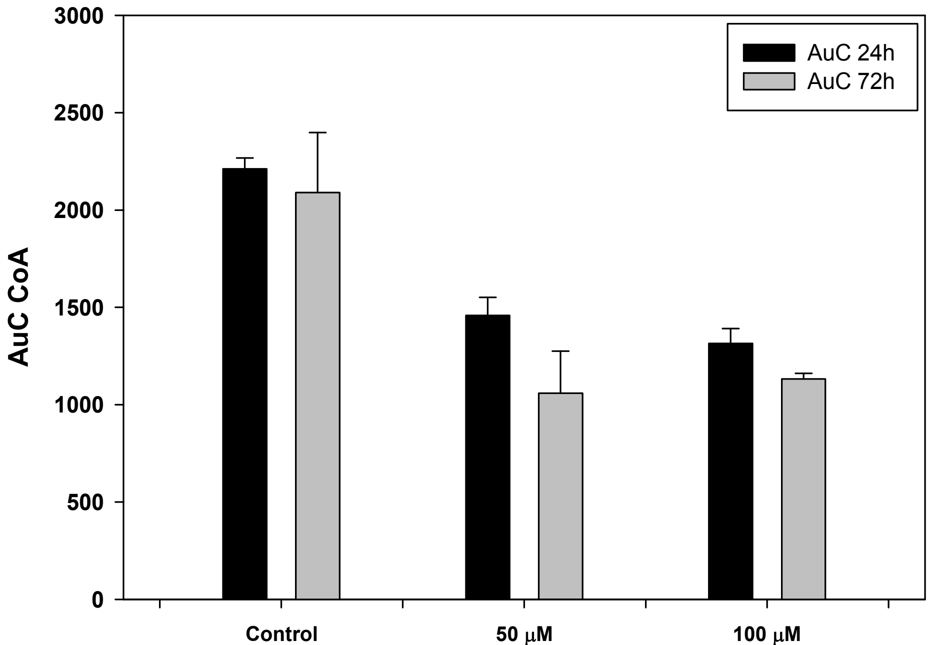

Figure 4.7 – Testing the effect of different concentrations of N5-Pan (**41b**) on the levels of CoA in *P. falciparum* parasite cultures incubated for 24 h and 72 h post-treatment. The bars were generated from HPLC data similar to that shown in figure 4.6 by determining the AuC of the peaks corresponding to fluorescently labelled CoA. The error bars indicate range/2 of the average data of two independent experiments, each carried out in duplicate.

A comparison of the levels of CoA between the treated cultures incubated for 24 h or 72 h and the control cultures shows considerable differences. However, the difference between the treated cultures either incubated for 24 h or 72 h is less pronounced. It is noteworthy that the parasites from treated cultures after 72 hours incubation appeared morphologically slightly altered with an apparent smaller size compared to the parasites in the control culture. For a more precise evaluation of the effect of PanAms, future studies will need to also consider parasite pellet size - as opposed to measurements based on the number of parasites alone – and also measuring an independent metabolite that is not affected by the PanAms treatment, to rule out the possibility of non-specific effects of the compounds.

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4.2.4.2 Effect of PanAms on *Pf*ACP levels in *P. falciparum* parasite cultures

Once it was established that N5-Pan decreased the levels of free CoA in treated parasites, the effect of the compound on the ACP pools was investigated. To examine this, parasite lysates prepared from control cultures and cultures treated with N5-Pan as described for the CoA level analyses, were examined by conformation-sensitive PAGE and by HPLC as detailed in chapter 2, sections 2.2.9.5 and 2.2.12. The identity of the ACP forms from cultured parasites was confirmed by comparison with heterologously-expressed and purified *Pf*ACP standards; the identity of which were confirmed by LC-ESI-MS analysis, and by migration of the protein in native, conformation-sensitive gel electrophoresis after labelling with the thiol-reactive fluorescent CPM probe (figure 4.8).

Following the establishment of the ACP standards, the samples prepared from cultures growing in aged medium and prepared for CoA analysis as described above were analysed by HPLC and conformation-sensitive gel to investigate the effect of the PanAm on the ACP pools. In these experiments, only the *holo*-ACP form could be fluorescently labelled with CPM by the virtue of presenting a thiol group with which CPM could react. The immature carrier protein, *apo*-ACP as well as the *crypto*-ACP (reporter-modified ACP) forms which lack a thiol group on their structures could not be labelled with CPM. The competitive production and accumulation of immature *apo*-ACP and *crypto*-ACP forms results in decreased levels of the mature and functional *holo*-ACP that can be labelled with CPM. The levels of the *holo*-ACP forms in N5-Pan-treated cultures should therefore decrease as a function of increasing the dose of N5-Pan administered to the cultures.

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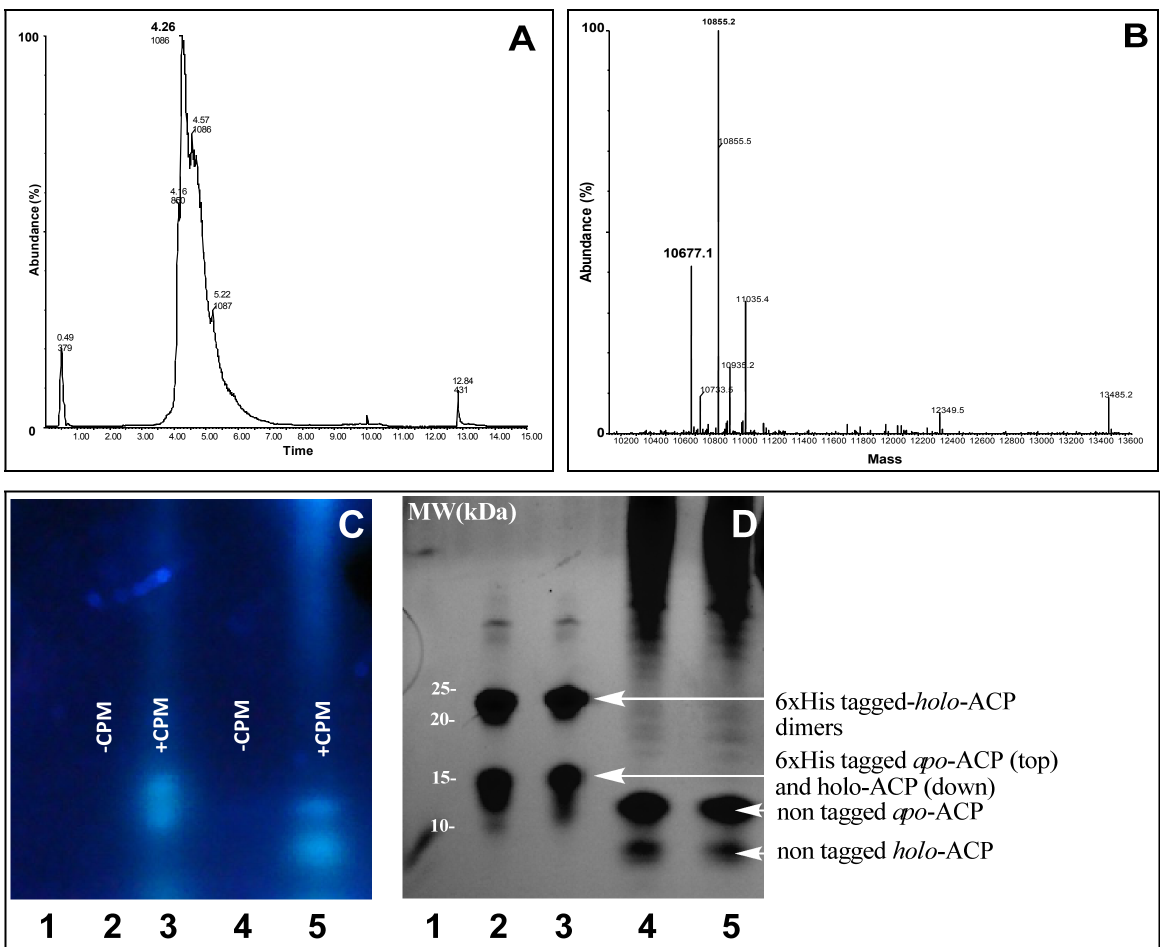

Figure 4.8 - Confirmation of the integrity of heterologously expressed *Pf*ACP by LC-ESI-MS and conformation-sensitive PAGE. Panel **A**: HPLC chromatogram of purified 6×His-PfACP as analysed by MS. The major peak at 4.26 min represents PfACP as indicated by its mass shown on panel B. Panel **B**: Mass spectrum of the peak at 4.26 min; the base mass of 10677.1 Da is consistent with the ExPASY computed mass of 10676.899 Da for the mature holo-ACP (i.e. including the 6×HisTag and the 4'-phosphopantetheine prosthetic group). Panel **C**: Conformation-sensitive gel electrophoresis analysis of CPM-labelled *Pf*ACP as visualized under UV light (to illuminate CPM-labelled protein bands). Note the large, typically double fluorescent bands on the conformation-sensitive PAGE of both the standard heterologously expressed and purified as well as the culture-derived CPMlabelled PfACP. The lower fluorescent band on lane 5 appears to indicate a larger amount of fluorescently labelled ACP. Some fluorescence on that lane is at the front of the gel and may derive from a number of small molecule adducts in the non-purified crude extract. Panel **D**: The same gel as shown in panel C, but after Coomassie blue staining. Lanes 2 and 3 were loaded with heterologously expressed and purified 6×His-PfACP as described in chapter 2, section 2.2.9.4. Lanes 4 and 5 were loaded with crude extracts of E. coli cells heterologously expressing PfACP without any added tag, and not purified by any means. The upper bands on this panel, visualised only after Coomassie Brilliant Blue staining correspond to dimeric forms of holo-ACP. These forms cannot be fluorescently-labelled with CPM since the probe reacts only with free thiols.

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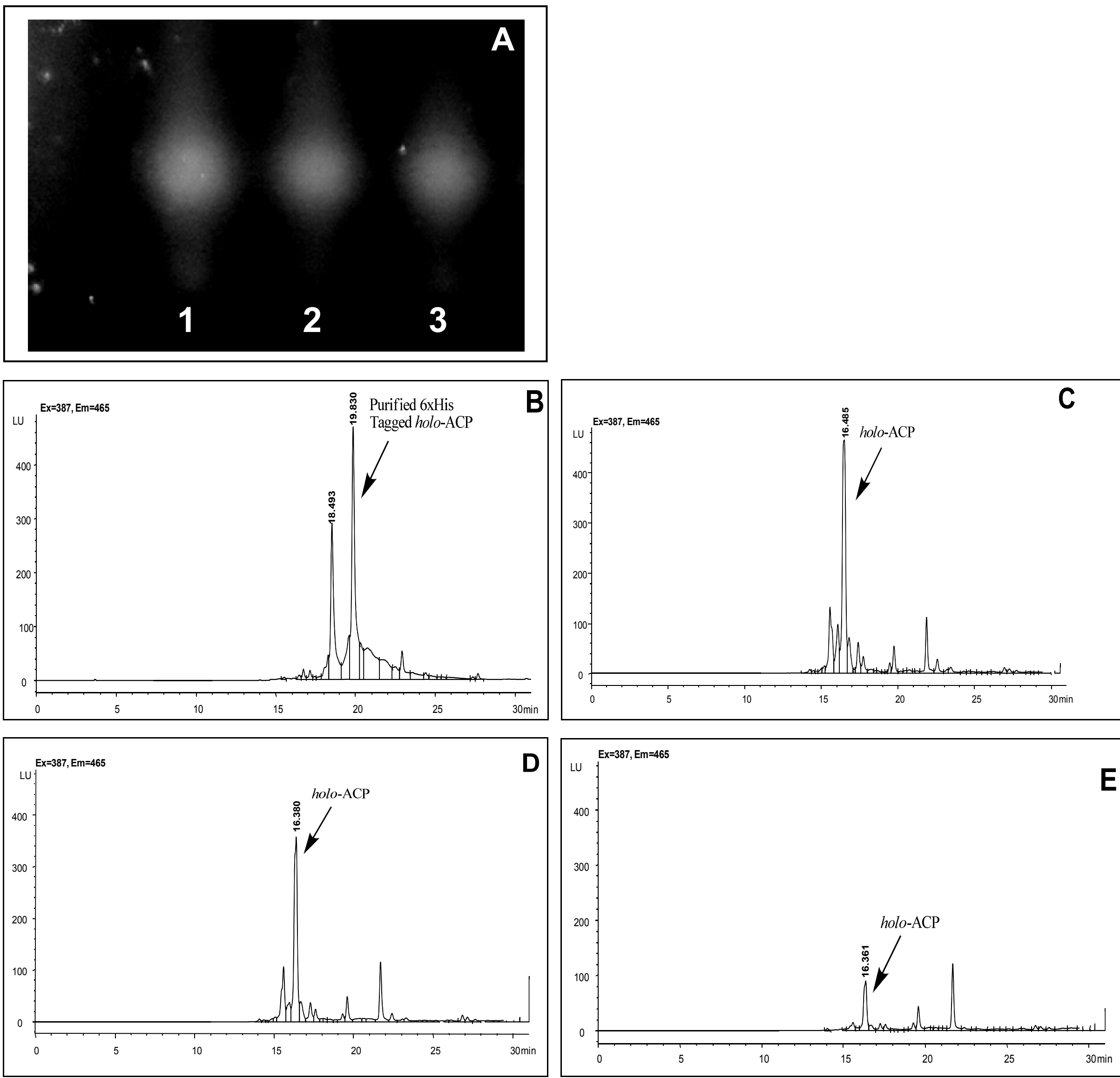

Figure 4.9 – Effect of N5-Pan treatment on *holo*-ACP levels in *P. falciparum* parasites. Ring stage parasite cultures were treated with N5-Pan and incubated for 24 hours followed by lysate preparation and analysis by conformation-sensitive polyacrylamide gel electrophoresis and HPLC. Panel **A** shows a gel of samples prepared from control cultures (lane 1), cultures treated with N5-Pan at 50 µM (lane 2) and 100 µM (lane 3) and incubated for 24 h as described in chapter 2, section 2.2.4. Panel **B** shows the standard HPLC chromatogram for the heterologously expressed and purified *holo*-ACP, and panels **C**, **D** and **E** show the HPLC chromatograms for the control culture, culture treated with 50 µM N5-Pan for 24 h and culture treated with 100 µM N5-Pan for 24 h, respectively. CPM-labelled *Pf*ACP runs as double and/or large fluorescent bands on conformation-sensitive PAGE and shows double HPLC peaks that are both decreased by the treatment of cultures with N5-Pan. Note that the 6×His tagged CPM-labelled ACP has a retention time of ~19.8 min with a second minor peak at ~18.5 min while the CPM-labelled ACP without the six histidine tag shows a retention time at ~16.6 min with the minor peak also present, now at ~15.3 min.

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Analysis by conformation-sensitive PAGE of cultures treated with 50 µM and 100 µM N5-Pan did not show clear differences in the levels of the CPM-labelled *holo*-ACP compared to control cultures (figure 4.9, Panel A), possibly due to poor dynamic range offered by this means of analysis (i.e. camera sensor is already saturated at low fluorescence levels).

However, when the samples were analysed by HPLC, the quantity of *holo*-ACP – as indicated by the AuC of the corresponding peaks on HPLC chromatograms – in the treated cultures incubated for 24 hours post-treatment was lower than the amount of the protein in the control cultures (figure 4.10, Panel **A**). Similar results were observed for the parasite cultures that were analysed 72 hours post-treatment, as shown on Panel **B** of figure 4.10.

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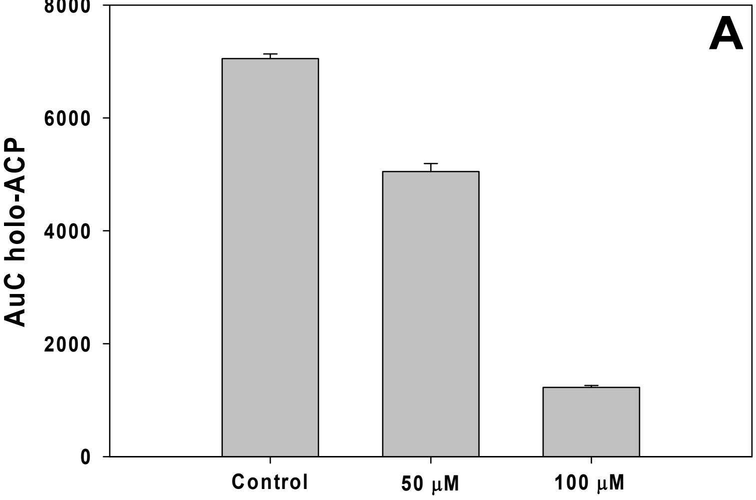

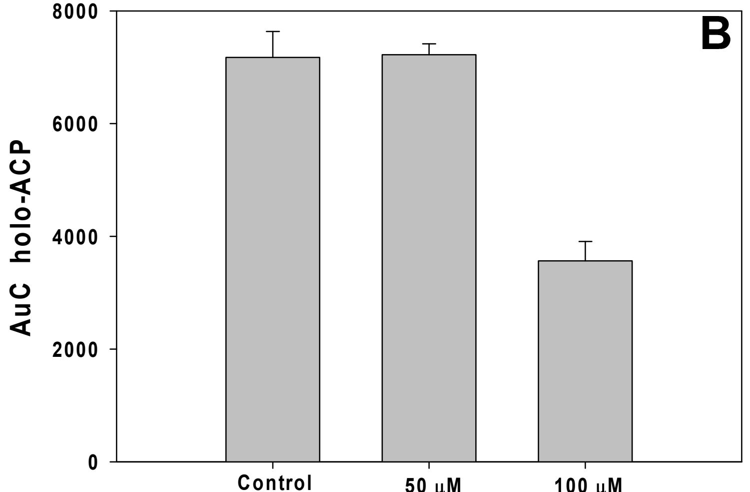

Figure 4.10 - Effect of increasing concentrations of N5-Pan on *holo*-ACP levels in *P. falciparum* parasites incubated for 24 h (Panel **A**) and 72 h (Panel **B**) post-treatment in culture medium treated at 56°C for 18 h to remove pantetheinase activity[1]. The bars were generated from data representing the AuC of CPM fluorescently-labelled peaks corresponding to *holo*-ACP on HPLC chromatograms from two independent experiments. The difference between the treated cultures (50 μM and 100 μM) and the control cultures at 24 hours post-treatment (Panel **A**) and between the cultures treated at 100 μM and the control cultures after incubation for 72 hours (Panel **B**) are noteworthy while the difference between the cultures treated at 50 μM and the control cultures incubated for 72 h are negligible. Error bars represent range/2.

[1] The effect of N5-Pan (**41b**) and the fluorescent PanAm *N*-dansylcadaverine *n*-PanAm (**54b**) on the ACP pools was initially investigated in fresh medium (which contains active pantetheinase). The results obtained (not shown) indicated no difference in the levels of CPM-labelled holo-ACP in treated and non-treated cultures after 24 h or 72 h incubation following standard culture procedures – as would be expected if the compounds are degraded by pantetheinase activity.

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Since the 4'-phosphopantheteine prosthetic group of *holo*-ACP is derived from CoA, the observed low levels of *holo*-ACP is most likely the result of the higher levels of the antimetabolite ethyldethia-CoA (the product of N5-Pan being transformed by the CoA biosynthetic enzymes) compared to CoA; under such circumstances the prosthetic group derived from the PanAm is preferentially transferred to *apo*-ACP which results in decreased levels of free *holo*-ACP. Note that after 72 h, there is practically no difference between the levels of CPM-labelled *holo*-ACP in the cultures treated at 50 µM N5-Pan compared to the control culture. This is possibly a result of N5-Pan being consumed or degraded over time resulting in the levels of CoA rising again and, as a consequence, the levels of *holo*-ACP also increasing and tending to reach same levels as in the control culture.

It has been suggested that fatty acid biosynthesis is not essential for intraerythrocytic-stage *Plasmodium spp* parasites survival and replication *in vitro* [14]. However, the findings described here suggest that *Pf*ACP is actively being produced during this stage of the parasite's life cycle. Whether ACP plays an important role in fatty acids biosynthesis or any other functions during intraerythrocytic-stage *P. falciparum* parasites has not been established [13-15]. Nevertheless, the fact that intraerythrocytic-stage parasites from which the apicoplast had been removed could be cultivated indefinitely *in vitro* – provided that the culture medium was supplemented with isopentenyl pyrophosphate, a product of the non-mevalonate isoprenoid precursor biosynthesis pathway – suggests that fatty acids metabolism is not essential [16]. Although the results reported in the present section of this dissertation suggest that CoA and ACP are targeted by PanAms and play an important role in the MoA of PanAms,

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additional studies are needed to unequivocally inform on the role and importance of the ACP in intraerythrocytic-stage *P. falciparum* parasites.

4.3 Conclusion

In this part of the study, the target(s) of the antiplasmodial action of PanAms was investigated, specifically focusing on the basis of the apparent selectivity between the α-, *n*-, and HoPanAm series. Compounds belonging to all three series were found to serve as alternative substrates for *Pf*PanK based on their inhibition of pantothenate phosphorylation by the enzyme present in parasite lysates and on the results of the *in vitro* phosphorylation assay studies. This was found to be especially the case for the *n*-PanAms, which revealed to interact more actively with the enzyme and as such could effectively compete with pantothenate for the enzyme's activity. This indicates that while the *n*-PanAms are present at high concentrations, they can competitively inhibit pantothenate phosphorylation resulting in decreased quantities of 4'-phosphopantothenic acid and therefore reduced CoA biosynthesis. Subsequent experiments confirmed that indeed both CoA and *holo*-ACP levels were reduced upon PanAm treatment.

Taking the factors discussed above into consideration, it seems reasonable to assume that the PanAms act through a combination of factors which may include pantothenate phosphorylation inhibition (in the period immediately after PanAm administration), the reduction of CoA and *holo*-ACP levels, and the formation of CoA antimetabolites (and ACPs loaded with inactive prosthetic groups). However, only the first two of these factors were studied here, and further studies are required to confirm that the third takes place, and to allow definite conclusions to be drawn. Nonetheless, the experiments

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reported here clearly suggest that polypharmacology may be involved in the mode of action of PanAms, with the compounds likely to be interacting with and inhibiting more than just a single enzyme or process. This is of utmost importance as it may reduced the risk of the parasites developing resistance to the drug since when a compound targets a single molecule, a single mutation can result in development of resistance to the drug, a phenomenon that is less likely to occur when multiple targets are involved in the MoA of the drug.

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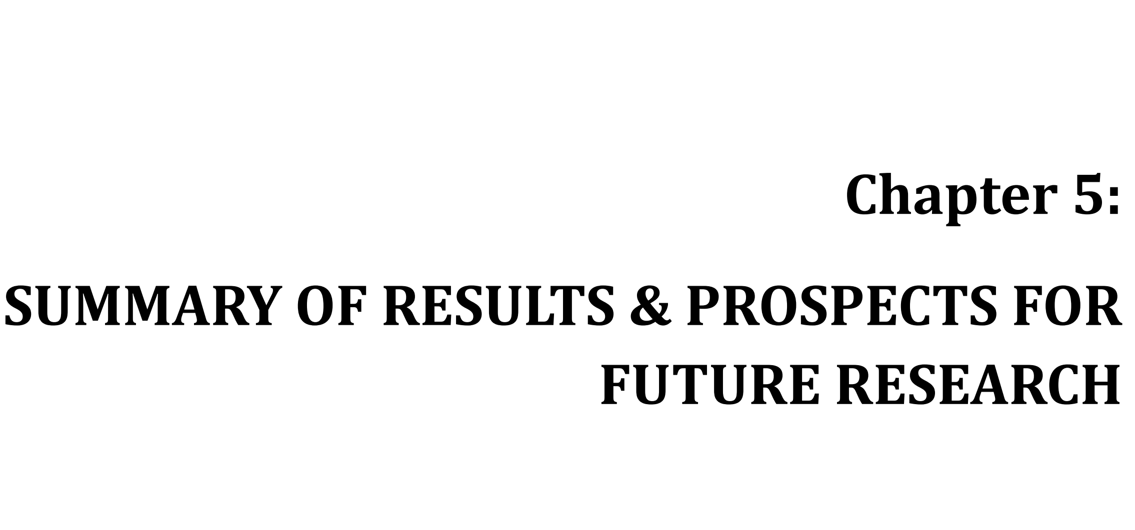

Chapter 5:

SUMMARY OF RESULTS & PROSPECTS FOR FUTURE RESEARCH

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5.1 Pantothenate utilization and CoA biosynthesis as drug targets

As discussed in chapter 1, pantothenate and pantothenate analogues have been under scrutiny for development of antimalarial drugs since the 1940's [1], with particular intensity during the last several years since the discovery that *P. falciparum*, the parasite that causes the most widespread and deadly form of malaria in humans, has developed resistance to virtually all the drugs currently in use [2, 3].

It has been known for some time that PanAms have antimicrobial activity, particularly in the case of bacteria where it has been shown that the inhibition results from the ability of these compounds to interfere with the utilization of pantothenate which is an essential nutrient required for the biosynthesis of coenzyme A in basically all living organisms [4]. In this study it was demonstrated that in the malaria parasite *P. falciparum,* the first enzyme of the CoA biosynthetic pathway (pantothenate kinase) also has the ability to catalyse the transformation of PanAms, which then impact negatively on the CoA and functional ACP levels in the parasite.

The utilization of metabolic pathways as targets for antimicrobial chemotherapy is an already well-established approach. For example, since many bacteria require para-aminobenzoic acid (PABA) for the synthesis of folic acid as they are unable of utilizing the acid from exogenous sources, PABA analogues have been very successfully used as antimicrobials. The sulphonamides, which competitively inhibit the utilization of PABA by bacteria, were the first antimicrobial agents used systemically for the prophylaxis and therapy of human bacterial infections [5]. Sulphonamides are, until now, still successfully used as antibacterial chemotherapies, particularly in combination with other drugs as discussed in the introductory chapter of this dissertation. Sulphonamides

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are also well established as antimalarial compounds and sulphadoxine, a sulphonamide used in combination with pyrimethamine, inhibits a second key reaction in the synthesis of folic acid. The Sulphadoxine/Pyrimethamine combination, commonly known as Fansidar®, has been for long time as an antimalarial combination and for some time it was second only to chloroquine as the most commonly administered antimalarial [6]. The importance of PABA analogues and of the sulphonamides in particular in the treatment of microbial infections during around 70 years from their institution validates the approach of exploiting the mechanisms involved in the utilization of essential nutrients as targets for antimicrobial agents. Thus, the uptake mechanisms and the enzymes involved in the metabolism of nutrients identified as essential for the development of pathogenic microorganisms constitute important targets for development of future effective antimicrobial chemotherapies.

5.1.1 Pantothenamides are potent antiplasmodial agents susceptible to degradation under physiological conditions

While investigating the antiplasmodial activity of PanAms, researchers in a collaborator's laboratory at the Australian National University noticed that the antiplasmodial activity of PanAms when measured using freshly prepared culture medium was lower compared to when it was measured in medium that had been repeatedly incubated at 37°C for prolonged periods. This phenomenon was subsequently traced to the presence of a thermo-labile enzyme with pantetheinase activity present in the serum substitute (Albumax II) used to prepare the culture medium.

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The experiments performed and reported in this thesis revealed that the modest *in vitro* antiplasmodial activity of the compounds tested in freshly-prepared medium resulted from their degradation by these enzymes with pantetheinase activity. The enzymes are members of the nitrilase superfamily and play an important physiological role by recycling pantothenic acid from the CoA metabolite pantetheine, and producing the antioxidant cysteamine as a secondary product [7, 8]. In this study it was shown that these enzymes are promiscuous and degrade PanAms (regardless of the structure of their amide substituents) to pantothenic acid and the respective amine, rendering the compounds inactive against *P. falciparum* parasites [9].

To continue the study of pantothenic acid and CoA utilization as drug targets for antimalarial chemotherapy, and of PanAms as potential antiplasmodial agents, a method was developed for improved and consistent inactivation of proteins with pantetheinase activity present in the parasite culture medium. This method, consisting of continuous incubation of complete culture medium (i.e. containing Albumax II) at 56°C for 18 hours (reducing the required incubation period of 40 hours at 37°C) proved to be efficient for inactivating more pantetheinase activity compared to the previously used method, and resulted in expected improvement in the antiplasmodial activity of the *n*-PanAms, the series of compounds that contain the same number of methylene groups in the β-alanine moiety as pantothenic acid [9].

5.1.2 Structural modification of pantothenamides confers resistance to pantetheinase-mediated degradation

To try and identify PanAms that are resistant to pantetheinase-mediated hydrolysis, a set of modified PanAms previously prepared by other researchers in our laboratory [10]

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were tested for their resistance to pantetheinase degradation and also for their antiplasmodial activity. It was found that compounds that had a modified β-alanine moiety (i.e. α- and HoPanAms, in which the scissile amide bond was displaced relative to their normal counterparts) proved to be resistant to degradation by pure pantetheinase and by the enzyme present in Albumax II [9]. Unfortunately, this resistance to pantetheinase degradation came at a price, as the modified PanAms showed decreased antiplasmodial activity when compared to that of the corresponding *n*-PanAm.

To overcome this problem, selected *n*-PanAms were modified by addition of a methyl group to either the α- or β- position of the β-alanine moiety to prevent or retard their degradation. These new PanAms exhibited resistance to pantetheinase degradation and therefore showed improved antiplasmodial activity compared to their n-PanAms counterparts when tested in culture media in which the enzyme was present. This demonstrated that PanAm modification, particularly when maintaining the length of the β-alanine moiety, is a promising path for exploration and development of novel antimalarial drugs active against intraerythrocytic-stage parasites.

Another class of potentially pantetheinase-resistant pantothenate analogues that was investigated for their antiplasmodial activity is the class of *N*-substituted pantoyltauramides. These compounds also inhibited *P. falciparum* parasite proliferation through the inhibition of pantothenate-dependent processes – as confirmed by the increase in the IC_{50} when the compounds were tested in culture medium with excess pantothenate.

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5.1.3 Polypharmacology is involved in the mode of action of pantothenamides

In addition to investigating the antiplasmodial activity, the MoA of PanAms was investigated in this study. In this respect, the effect of selected PanAms on the activity of *Pf*PanK containing parasite lysates was investigated under *in vitro* standardised conditions. All compounds tested, which included members of the α-, *n*- and HoPanAms, were found to serve as substrates of the enzyme. Among the different sets of α-, *n*- and HoPanAms with the same amine substituent tested, the normal (*n*-) compounds exhibited the highest interaction with the *Pf*PanK enzyme in parasite lysates and also the highest potency for inhibition of the enzyme's activity in phosphorylating pantothenate. These results correlated well with the compounds' high *in vitro* antiplasmodial activity when tested in the absence of pantetheinase enzymes.

Further investigation of the MoA of PanAms revealed that treatment of *P. falciparum* parasite culrures with these compounds results in lower CoA levels (probably due to their conversion to non-functional CoA antimetabolites) and lower functional *holo*-ACP levels. Thus, as discussed in chapter 4, the results indicate that the antiplasmodial activity of PanAms is through a combination of factors including interference with pantothenate phosphorylation by the first enzyme in the CoA metabolic pathway in the parasites, the formation of non-functional CoA analogues and ACPs loaded with inactive 4'-phospho-PanAm prosthetic groups, which then impact on CoA and ACP levels.

As already indicated, although further studies are required to unequivocally define the mode of antiplasmodial action of PanAms, the experiments reported in this dissertation indicate that polypharmacology may be involved in the MoA of these compounds, with

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the tested PanAms interacting with more than just a single enzymatic step in the pantothenate utilization and CoA biosynthesis in the parasite.

5.2 Prospects for future research

5.2.1 Expansion of the library of pantothenamides

The results described in this dissertation indicate that one of the major hindrances to development of PanAms as antimalarial drugs is the susceptibility of the compounds to pantetheinase-mediated degradation. Having been demonstrated in this dissertation and by de Villiers et al. [11] that structural modifications can render the compounds resistant to the deleterious effect of the pantetheinase enzymes, one of the crucial steps towards improving the antiplasmodial activity of PanAms will be to modify these compounds to, not only (i) confer resistance to pantetheinase-mediated degradation, but also (ii) improve their antiplasmodial activity and potentially (iii) prevent plasma protein binding.

Increased chemical stability, i.e. resistance to pantetheinase-mediated degradation and possibly an improved pharmacokinetic profile, could potentially be achieved by replacement of the PanAms' key hydrolysable amide bond with bioisosteric groups. In such an approach, *n*-PanAms with amide substituents related to the most potent antiplasmodial activity should be considered for modification and evaluation of the compounds' antiplasmodial activity. Such a strategy is important for circumventing pantetheinase-mediated hydrolysis *in vivo*, which is crucial for the future development of PanAms as antimicrobial agents suitable for systemic administration, since stability in serum will be required.

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Some authors have suggested the combination of PanAms with known pantetheinase inhibitors [12, 13]. However, such a strategy would eventually require the optimization of the properties of two compound sets, and possibly result in complications related to adverse drug interactions and/or inhibition of the physiological function of pantetheinases.

5.2.2 Further investigation of the mode of action of pantothenamides

The studies described in this dissertation showed that PanAms are potent antiplasmodial agents when their *in vitro* degradation by serum pantetheinase is negated. It was also demonstrated that the mode of action of these compounds is through interference with the parasite's utilization of pantothenate by hijacking its PanK enzyme to initiate their (PanAms) transformation into inhibitory CoA antimetabolites and also the so-called *crypto*-ACPs that are modified with non-functional 4'-phospho-PanAm moieties instead of the normal 4'-phosphopantetheine. However, to establish the exact basis for its inhibition, it will be important to determine the major form of the antimetabolite in the intraerythrocytic-stage parasite – i.e. whether it is the 4'-phospho-PanAm form, the dephospho-CoA or CoA antimetabolite forms, or whether it is all mainly trapped on the ACP – as this is most likely to be the form that interacts with target (or targets) that cause the observed inhibition of parasite proliferation. This could be done by radioactively or fluorescently tracking the transformation of radioactivity labelled or fluorescent PanAm after uptake, and evaluating the variation in the levels of its different biotransformed species over time.

Furthermore, the investigation of the mode of action of PanAms against intraerythrocytic-stage malaria parasites should also examine the effect of non-

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pantothenate analogue compounds on the levels of CoA and ACP on treated cultures. While the studies reported in this dissertation clearly showed effects on CoA and ACP levels upon pantothenamide treatment, it is possible that this could have been the result of treatment stress (dying parasites showing decreased metabolic activity). To rule this out, additional control experiments will have to be performed.

5.2.3 Investigation of the antiplasmodial activity of pantothenamides in *in vivo* systems and against liver-stage parasites

A number of pantothenate analogues have been shown to possess *in vivo* antimalarial activity in mice, monkeys, or birds [1, 14, 15]. In light of the identified methods to overcome the pantetheinase-mediated degradation of PanAms during the course of this study, such approaches should be used and, the PanAms evaluated *in vivo* to ascertain their efficacy against malaria parasites in animal (mouse) models of the disease. The *in vivo* experiments would provide information on the actual/practical activity of the compounds; provide indications of their *in vivo* pharmacokinetics and toxicity profile *in vivo*, which would then allow the introduction of further improvements on the chemical structures for better antiplasmodial activity.

In vivo studies are opportune and relevant at this stage of the PanAms investigation as antimalarial agents. In addition to their high antiplasmodial potency in *in vitro* systems as shown in this dissertation and in previously published work [9, 11], it has been shown in concentration−response profile studies against Jurkat cells (a human leukemic T-cell line) that although no obvious trend was apparent for selected α- and HoPanAms, the selectivity indexes (the ratio of the IC_{50} values measured against Jurkat cells to the IC_{50} values for the inhibition of *P. falciparum* growth) of tested HoPanAm ranged from

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40 to >100, indicating that at least for those PanAms the toxicity to mammalian cells is significantly lower than to parasite cells [11]. This justifies the prospect that the most potent α-, Ho- and α-MePanAms should now be tested in *in vitro* studies against the mouse model of malaria.

Additionally, investigating the antiplasmodial activity of PanAms against drug-resistant *P. falciparum* parasite strains as well as against other important species of the malaria parasites including the second most important parasite species, *P. vivax* is of paramount importance since new antimalarial drugs are constantly needed due to the development of resistance of the parasites to currently chemotherapies.

Finally, considering the current malaria research eradication agenda in which antimalarial compounds that are active against more than one stage of the parasite's life cycle, or compounds that can block the transmission of parasites from one human victim to another are preferred, it will be crucial to investigate PanAms as inhibitors of malaria parasites at different developmental stages including gametocyte development and also against liver-stage parasites. Liver-stage parasite studies are particularly relevant since, as presented in this dissertation, PanAms clearly affect CoA and ACP levels; therefore, these compounds would be predicted to be active against that stage of the parasites' life cycle since *de novo* fatty acid synthesis – which depend on both CoA and ACP – is more important for the development of the liver-stage [16] than the intraerythrocytic-stage parasites.

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Appendix A

Spry C, Macuamule C, Lin Z, Virga KG, Lee RE, Strauss E, Saliba KJ. Pantothenamides are potent, on-target inhibitors of *Plasmodium falciparum* growth when serum pantetheinase is inactivated.

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Pantothenamides Are Potent, On-Target Inhibitors of *Plasmodium falciparum* Growth When Serum Pantetheinase Is Inactivated

Christina Spry[1¤], Cristiano Macuamule[3], Zhiyang Lin[1], Kristopher G. Virga[4,5], Richard E. Lee[5,6], Erick Strauss[3], Kevin J. Saliba[1,2*]

1 Research School of Biology, College of Medicine, Biology and Environment, The Australian National University, Canberra, Australian Capital Territory, Australia, **2** Medical School, College of Medicine, Biology and Environment, The Australian National University, Canberra, Australian Capital Territory, Australia, **3** Department of Biochemistry, Stellenbosch University, Matieland, Stellenbosch, South África, **4** Department of Pharmaceutical and Administrative Sciences, Presbyterian College School of Pharmacy, Clinton, South Carolina, United States of America, **5** Department of Pharmaceutical Sciences, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, **6** Department of Chemical Biology and Therapeutics, St. Jude Children's Research Hospital, Memphis, Tennessee, United States of America

Abstract

Growth of the virulent human malaria parasite *Plasmodium falciparum* is dependent on an extracellular supply of pantothenate (vitamin B_5) and is susceptible to inhibition by pantothenate analogues that hinder pantothenate utilization. In this study, on the hunt for pantothenate analogues with increased potency relative to those reported previously, we screened a series of pantothenamides (amide analogues of pantothenate) against *P. falciparum* and show for the first time that analogues of this type possess antiplasmodial activity. Although the active pantothenamides in this series exhibit only modest potency under standard *in vitro* culture conditions, we show that the potency of pantothenamides is selectively enhanced when the parasite culture medium is pre-incubated at 37°C for a prolonged period. We present evidence that this finding is linked to the presence in Albumax II (a serum-substitute routinely used for *in vitro* cultivation of *P. falciparum*) of pantetheinase activity: the activity of an enzyme that hydrolyzes the pantothenate metabolite pantetheine, for which pantothenamides also serve as substrates. Pantetheinase activity, and thereby pantothenamide degradation, is reduced following incubation of Albumax II-containing culture medium for a prolonged period at 37°C, revealing the true, sub-micromolar potency of pantothenamides. Importantly we show that the potent antiplasmodial effect of pantothenamides is attenuated with pantothenate, consistent with the compounds inhibiting parasite proliferation specifically by inhibiting pantothenate and/or CoA utilization. Additionally, we show that the pantothenamides interact with *P. falciparum* pantothenate kinase, the first enzyme involved in converting pantothenate to coenzyme A. This is the first demonstration of on-target antiplasmodial pantothenate analogues with sub-micromolar potency, and highlights the potential of pantetheinase-resistant pantothenamides as antimalarial agents.

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* E-mail: kevin.saliba@anu.edu.au

¤ Current address: Department of Chemistry, University of Cambridge, Cambridge, United Kingdom

Introduction

Every day approximately half of the world's population is at risk of contracting malaria, a lethal infectious disease estimated to have claimed 655 000 lives [1] (if not more [2]) in 2010. New chemotherapeutic agents are desperately needed to combat malaria as *Plasmodium falciparum*, the most virulent of the parasites that cause the disease in humans, has developed resistance to all antimalarial agents in clinical use [3].

Plasmodium falciparum has an absolute requirement for exogenous pantothenate (vitamin B_5; Figure 1) [4,5,6], a precursor of the essential enzyme cofactor coenzyme A (CoA). Analogues of pantothenate that interfere with the utilization of pantothenate by *P. falciparum* have been shown to inhibit growth of *Plasmodium* parasites *in vitro* and *in vivo* [5,7,8], raising interest in pantothenate utilization as a potential antimalarial drug target, and pantothenate analogues as the chemical entities to strike this target [6,9]. The structure of pantothenol, one antiplasmodial pantothenate analogue identified previously, is shown in Figure 1.

Pantothenamides, pantothenate analogues in which the carboxyl group of pantothenate is replaced with an amide group (Figure 1), have been shown to possess antibacterial activity *in vitro* [10,11,12,13,14]; *Escherichia coli* and *Staphylococcus aureus* are among the bacteria demonstrated to be susceptible to inhibition by these compounds. Pantothenamides have been shown to serve as substrates of pantothenate kinase (PanK), the first enzyme in the CoA biosynthesis pathway, and as a consequence inhibit PanK-catalysed pantothenate phosphorylation [13,15,16,17,18]. The resultant 4'-phosphopantothenamides are further metabolized by

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Pantothenate

Pantothenol

Pantothenamide
(R_1, R_2 = H, alkyl, aryl)

Pantetheine

Figure 1. Chemical structures of pantothenate and related compounds. The hydroxy analogue of pantothenate, pantothenol, inhibits growth of *P. falciparum in vitro* and *in vivo*. Amide analogues of pantothenate, pantothenamides (for which the core structure is shown), possess antibacterial activity *in vitro*. Pantetheine, a naturally occurring pantothenate derivative, is hydrolyzed to pantothenate (and cysteamine) by the enzyme pantetheinase.
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the CoA biosynthesis pathway of bacteria to yield analogues of CoA [17]. Such CoA analogues have been shown to be incorporated by, and inhibit the function of, acyl carrier protein [16,19], a protein involved in fatty acid biosynthesis that requires the 4′-phosphopantetheine moiety of CoA for activation. Whether the mechanism that ultimately results in bacteriostasis is inhibition of CoA biosynthesis [20], fatty acid biosynthesis [19] or another CoA-utilizing process, or a combination of the above, remains to be resolved.

In this study, the effect of a series of pantothenamides (see Figure 2) on the growth of erythrocytic stage *P. falciparum* parasites was investigated. We show for the first time that under standard *in vitro* culture conditions pantothenamides inhibit parasite growth, albeit with modest potency. Serendipitously, however, we discovered that the antiplasmodial potency of pantothenamides is enhanced considerably when the parasite culture medium used for growth assays (which contains the commonly used serum substitute Albumax II [21] or human serum) is pre-incubated at 37°C for a prolonged period. Consequently, sub-micromolar concentrations of pantothenamides that have no effect in freshly prepared medium inhibit parasite growth effectively in the pre-incubated medium. We present evidence that links this finding to the presence in parasite culture medium of pantetheinase activity, the activity of an enzyme that catalyzes the hydrolysis of pantetheine (Figure 1) to pantothenate and cysteamine. In animals, pantetheinase activity is typically linked with the Vanin proteins [22], soluble or membrane bound proteins that belong to the nitrilase superfamily, the members of which share an invariant Glu-Lys-Cys catalytic triad [23]. We show, using an *in vitro* primary amine detection assay, that a pantothenamide selected from the series tested here is hydrolyzed in the presence of Albumax II, demonstrating Albumax II to be a source of pantetheinase activity. Furthermore, we show that recombinant human pantetheinase (Vanin-1) reduces the antiplasmodial potency of the pantothenamide in the pre-incubated medium *in vitro*, and, that the attenuating effect of the pantetheinase is alleviated by incubation of the pantetheinase-supplemented medium at 37°C. Together these data are consistent with pantetheinase-mediated pantothenamide degradation occurring in medium freshly supplemented with Albumax II or serum under *in vitro* culture conditions, lowering the effective pantothenamide concentration, and thereby masking the sub-micromolar antiplasmodial potency of pantothenamides.

Importantly, we demonstrate that the potent antiplasmodial effect of the pantothenamides in the medium pre-incubated at 37°C is alleviated with pantothenate, and therefore results specifically from inhibition of pantothenate and/or CoA utilization. We also show that all of the pantothenamides in this series inhibit *P. falciparum* PanK-catalysed pantothenate phosphorylation (and hence serve as substrates or inhibitors of *P. falciparum* PanK). The data presented here provide additional validation of pantothenate and CoA utilization as potential antiplasmodial drug targets.

Materials and Methods

Reagents

The pantothenamides tested were synthesized and purified as described previously [18]. Each pantothenamide was dissolved in dimethyl sulfoxide (DMSO) to a final concentration of 400 mM before being diluted in the solution pertinent to the experiment. For *in vitro* growth assays, the concentration of DMSO introduced into cultures never exceeded 0.05% (v/v), in phosphorylation assays the DMSO concentration present was 0.025% (v/v), and in fluorescamine assays the concentration of DMSO never exceeded 0.15% (v/v). Albumax II was purchased from Life Technologies (Mulgrave, Victoria, Australia), dissolved to a concentration of 20% (w/v) in water, filter sterilized and stored frozen (−20°C). SYBR Safe DNA gel stain (10 000× stock concentration) was also from Life Technologies. Recombinant human pantetheinase (Vanin-1) was purchased from Novoprotein Scientific Inc. (Short Hills, New Jersey, USA) and was reconstituted to a concentration of 200 μg/mL in sterile phosphate-buffered saline and stored at −20°C for no longer than 3 weeks before use. [^{14}C]Pantothenate (55 mCi/mmol) was purchased from American Radiolabelled Chemicals, Inc., and pantothenol and fluorescamine were from Sigma-Aldrich.

Cell Culture

Erythrocytic stage *P. falciparum* parasites (strain 3D7) were maintained within human erythrocytes in continuous culture essentially as described previously [24,25]. *P. falciparum*-infected erythrocytes were routinely cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 20 mM D-glucose, 200 μM hypoxanthine, 24 mg/L gentamycin and Albumax II (0.6%, w/v), which, hereafter is referred to as Albumax-complete RPMI. For several days prior to carrying out the assays testing the effect of compounds on the growth of *P. falciparum* in medium supplemented with human serum (instead of Albumax II), the parasites were cultured in the same medium mentioned above, except that human serum (10%, v/v, pooled from different blood donors) was used rather than Albumax II. This medium is hereafter referred to as human serum-complete RPMI.

In vitro Growth Assays

The *in vitro* antiplasmodial activity of test compounds was determined using the malaria SYBR Green I-based fluorescence assay described by Smilkstein *et al.* [26], with minor modifications. Briefly, *P. falciparum*-infected erythrocytes were incubated in culture medium containing two-fold dilutions of the test compounds in 96-well microtiter plates. Assays were initiated

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Compound	R	IC_{50} against *P. falciparum* (μM) Fresh medium 1 μM pantothenate	IC_{50} against *P. falciparum* (μM) Aged medium 1 μM pantothenate	Inhibition of pantothenate phosphorylation at 100 μM (%) 0.2 μM pantothenate
1 (N5-Pan)		124 ± 15	2.0 ± 0.2	99 ± 1
2 (N7-Pan)		> 200	7.7 ± 3.5	99 ± 1
3		72 ± 24	0.020 ± 0.002	100
4		> 200	> 200	100
5		> 200	> 200	94 ± 3
6		> 200	> 200	96 ± 2
7		> 200	> 200	98 ± 2
8		> 200	> 200	94 ± 5
9		> 200	50 ± 3	99 ± 1
10		> 200	> 200	100
11		134 ± 22	0.34 ± 0.01	100
12		133 ± 12	1.1 ± 0.1	95 ± 5
13		66 ± 4	150 ± 5	100
14		> 200	> 200	100
15		> 200	> 200	100
16		> 200	73 ± 15	100
17		44 ± 5	0.067 ± 0.002	95 ± 5
18		76 ± 12	0.23 ± 0.06	98 ± 2
19		49 ± 8	0.27 ± 0.06	96 ± 4
20		> 200	49 ± 8	94 ± 6
21		166 ± 18	1.9 ± 0.5	98 ± 2
22		162 ± 20	1.4 ± 0.1	100

Figure 2. Effect of pantothenamides on proliferation of *P. falciparum* and *P. falciparum* lysate-catalysed [^{14}C]pantothenate phosphorylation. The 50% inhibitory concentrations (IC_{50} values) measured against *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI containing 1 μM pantothenate, as determined using the SYBR Green I-based growth assay are shown. Assays were performed using Albumax-

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complete RPMI prepared within 48 h of the assay, stored at 4°C, and incubated at 37°C for a maximum of 1 h (fresh) or Albumax-complete RPMI incubated continuously at 37°C for 40 h immediately after preparation (aged). The IC_{50} values shown for parasites cultured in fresh Albumax-complete RPMI are averages from between two and eight independent experiments each performed in duplicate or triplicate. Where the IC_{50} values determined were below 200 μM, they are presented as the mean ± SEM from between three and eight independent experiments. The IC_{50} values shown for parasites cultures in aged medium are averages from between two and three independent experiments each performed in triplicate. Where the IC_{50} values determined were below 200 μM, they are presented as the mean ± range/2 or SEM as appropriate. The percentage inhibition of [^{14}C]pantothenate phosphorylation by PanK in *P. falciparum* lysate caused by pantothenamides (when tested at a concentration of 100 μM) in the presence of 0.2 μM pantothenate are also shown. The percentage inhibition was calculated from the measured amounts of [^{14}C]pantothenate phosphorylated during a 10 min incubation in the presence of pantothenamide and in the presence, instead, of the corresponding concentration of DMSO only. Data are averages ± range/2 from two independent experiments, each performed in duplicate. A value of 100 indicates complete inhibition of [^{14}C]pantothenate phosphorylation was observed in both independent experiments. The amount of [^{14}C]pantothenate phosphorylated by *P. falciparum* lysate was significantly lower in the presence of all pantothenamides ($P<0.0001$, ANOVA).
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with ring-stage *P. falciparum*-infected erythrocytes at a hematocrit and parasitemia of 1%. Wells containing infected erythrocytes in the presence of chloroquine (0.25–2 μM) served as zero growth controls and wells containing infected erythrocytes in the absence of chloroquine or test compounds served as 100% parasite growth controls. Plates were incubated at 37°C under an atmosphere of 96% nitrogen, 3% carbon dioxide and 1% oxygen for 96 h, before 100 μL from each well was mixed with 100 μL of SYBR Safe DNA gel stain (0.2 μL/mL) in 20 mM Tris, pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 0.008% (w/v) saponin, 0.08% (v/v) Triton X-100, in a second 96-well microtitre plate. Fluorescence was measured using a FLUOstar OPTIMA multi-detection microplate reader from BMG LABTECH with excitation and emission wavelengths of 490 and 520 nm, respectively. The 50% inhibitory concentration (IC_{50}) of each test compound under each test condition was calculated by fitting the data to a sigmoidal curve (typically $y = a/(1+ (x/x_0)^b)$ using nonlinear least squares regression (SigmaPlot, Systat Software) and averaging the IC_{50} estimates from independent experiments.

Preparation of Culture Medium for in vitro Growth Assays

The antiplasmodial activity of test compounds was assessed in media that had been subjected to different pre-incubations. "Fresh" medium refers to medium that was stored for a maximum of 48 h at 4°C, and incubated at 37°C for a maximum of 1 h, prior to use. "Aged" medium refers to medium that was stored for a minimum of one week at 4°C and incubated intermittently at 37°C, or medium incubated continuously at 37°C for 40 h soon after preparation. As indicated at the relevant positions within the "Results" section, for some experiments the fresh medium used was, more specifically, medium used immediately following preparation, and the aged medium used was, more specifically, medium that was incubated at 37°C for 40 h immediately prior to use.

[^{14}C]Pantothenate Phosphorylation Assays

To assess the effect of the pantothenamides on pantothenate phosphorylation by *P. falciparum* PanK, the phosphorylation of [^{14}C]pantothenate by *P. falciparum* lysates was measured in the presence and absence of a single concentration of each pantothenamide. *P. falciparum* lysate was prepared from trophozoite stage parasites "isolated" from their host erythrocyte by treatment with 0.05% (w/v) saponin (essentially as described by Saliba *et al.* [27]), and washed (five times) in HEPES-buffered saline (125 mM NaCl, 5 mM KCl, 20 mM D-glucose, 25 mM HEPES, 1 mM $MgCl_2$, pH 7.1). Lysates were prepared as described by van Schalkwyk *et al.* [28], except that 10 mM potassium phosphate, pH 7.4 (rather than 10 mM Tris, pH 7.4), was used to lyse the cells. Aliquots of lysate were stored at −20°C. The concentration of cell lysates was determined from cell counts made using an improved Neubauer hemocytometer.

[^{14}C]Pantothenate phosphorylation was assayed using the Somogyi reagent [29] essentially as described by Saliba *et al.* [27], except that pantothenate phosphorylation was terminated by heat denaturation (rather than acid denaturation) of the protein in reaction aliquots. Briefly, *P. falciparum* lysate was added to solutions (at 37°C) of 50 mM Tris, 5 mM ATP, 5 mM $MgCl_2$, at pH 7.4, containing [^{14}C]pantothenate (at a final concentration of 0.01 μCi/mL, or 0.2 μM), and a pantothenamide (at a final concentration of 100 μM) or an equivalent volume of solvent. Typically, lysate prepared from $5.4–6.5\times10^7$ parasites was present in each mL of reaction solution. Zero phosphorylation control reactions were prepared by adding a corresponding volume of 10 mM potassium phosphate instead of parasite lysate. Following a ten minute incubation at 37°C (a period during which pantothenate phosphorylation increased linearly with time in the absence of inhibitors) 200 μL aliquots of the [^{14}C]pantothenate phosphorylation reactions were transferred in duplicate to microcentrifuge tubes, and immediately incubated at 95°C for 10 min to terminate phosphorylation. Terminated reaction samples were centrifuged at 15 800×*g* for 10 min to pellet the denatured protein, before two aliquots of each supernatant (typically 80 μL) were transferred to new microcentrifuge tubes. To one aliquot, 500 μL Somogyi reagent was added, and to the other, 500 μL water was added. The samples were processed for determination of phosphorylated [^{14}C]pantothenate as described previously [27].

In vitro Fluorescamine-based Assay

Hydrolysis of compound **12** to pantothenate and isobutylamine was measured using a modification of a fluorescence-based assay described previously for the measurement of *N*-acetyl-1-D-*myo*-inositylৎ-2-amino-2-deoxy-α-D-glucopyranoside deacetylase activity [30,31]. The assay utilizes fluorescamine (4-phenylspiro-[furan-2(3*H*),1-phthalan]-3,3′-dione; a non-fluorescent molecule that reacts with primary amines to form a fluorescent product [32]) for the detection of primary amines. Briefly, recombinant human pantetheinase (at a final concentration of 100 ng/mL), Albumax II (at a final concentration 0.6%, w/v), or an equivalent volume of water, was added to solutions (at 37°C) of 100 mM potassium phosphate, pH 7.5, 0.5 mM dithiothreitol, 0.01% (w/v) bovine serum albumin, 0.1% (v/v) DMSO, with or without pantothenamide (at a final concentration of 200 μM). At the appropriate time points, aliquots (30 μL) of the reaction mixtures were removed in duplicate and mixed with 10 μL 20% (v/v) trichloroacetic acid to terminate the reaction. Following removal of the precipitated protein by centrifugation (14 000×*g*, 15 min), 25 μL of each supernatant was transferred to the wells of a black 96-well microtitre plate and 75 μL 1 M borate (pH 9.0) followed by 30 μL 10 mM fluorescamine was added to the wells. The plate was then

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incubated at 37°C for 10 min before fluorescence was measured using a FLUOstar OPTIMA multidetection microplate reader from BMG LABTECH with excitation and emission wavelengths of 390 and 485 nm, respectively. The fluorescence detected was converted to the corresponding isobutylamine concentration using an isobutylamine standard curve. In each experiment, the isobutylamine standard curve was generated from aliquots (30 μL) of isobutylamine solutions (0.02–200 μM) processed in parallel with the reaction aliquots as described above.

Statistical Analysis

To test for a statistically significant difference between the means of two groups, two-tailed student *t* tests were performed. To test for statistically significant differences between the means of more than two groups, one-way analysis of variance (ANOVA) was performed. Pairwise comparisons were made post-hoc with Tukey's multiple comparisons test (when comparing all means) or Dunnett's multiple comparisons test (when comparing all means with a control mean). P values reported are multiplicity adjusted. ANOVA was performed using GraphPad 6 (GraphPad Software, Inc).

Results

Antiplasmodial Activity of Pantothenamides under Standard *in vitro* Culture Conditions

A series of 22 previously published [18] pantothenamides was tested *in vitro* for antiplasmodial activity against erythrocytic stage *P. falciparum* parasites (strain 3D7) in 96 h growth assays initiated with parasites predominantly in the ring stage. The series of pantothenamides (see Figure 2 for structures) was composed of 21 secondary amides of pantothenate, each with a different amide substituent, as well as a lone tertiary amide. The series included the prototypical pantothenamides N5-Pan and N7-Pan (compounds **1** and **2**, respectively). All of the pantothenamides tested were stereochemically pure, with *R*-configuration at the chiral carbon of the 2,4-dihydroxy-3,3-dimethylbutanamide moiety. As shown in Figure 2, when tested in "fresh" Albumax-complete RPMI (Albumax-complete RPMI stored at 4°C for a maximum of 48 h, and incubated at 37°C for a maximum of 1 h, prior to use), ten of the 22 pantothenamides inhibited proliferation of *P. falciparum* parasites with IC_{50} values below 200 μM. With the exception of compound **2** (N7-Pan; the pantothenamide with the longest linear alkyl chain), the pantothenamides with simple alkyl (linear or branched) or alkenyl amide substituents were among the ten active pantothenamides. Compound **3** (with a phenethyl substituent) was the only pantothenamide containing a carbocycle with an IC_{50} value below 200 μM.

The potency of pantothenamides with acyclic heteroatom-containing substituents varied. Three pantothenamides with linear thioether substituents were tested for antiplasmodial activity; compounds **17** and **18** were among the most active of the series, while compound **14** had little effect even at a concentration of 200 μM. Compound **17** was also significantly more active than N5-Pan, the corresponding alkyl pantothenamide ($P = 0.02$, ANOVA). Among the four pantothenamides with linear ether substituents, compound **19** was the most active ($P < 0.0001$, ANOVA), compounds **21** and **22** were also active, while compound **15** lacked antiplasmodial activity at the concentrations tested. The less polar thioether pantothenamides **17** and **18** were significantly more active ($P < 0.003$, ANOVA) than the corresponding ether pantothenamides **22** and **21**, respectively. Compounds **16** and **20** (both with terminal hydroxyl groups in the substituent) were without effect even at the highest concentration tested.

Albumax II-complete RPMI contains 1 μM pantothenate, a concentration close to the normal whole blood pantothenate concentration [33]. When the ten pantothenamides with IC_{50} values below 200 μM were tested against *P. falciparum* parasites in fresh Albumax-complete RPMI to which 100 μM pantothenate was added, the IC_{50} values measured were between 1 ± 0.1 (compound **13**; mean ± range/2) and 1.7 ± 0.4 (compound **19**; mean ± range/2) times higher than those measured against parasites in Albumax-complete RPMI containing 1 μM pantothenate. The minor attenuating effect (or lack of an effect) of pantothenate supplementation on the antiplasmodial activity of the pantothenamides, contrasts with the dramatic attenuating effect of pantothenate on the antiplasmodial activity of previously reported antiplasmodial pantothenate analogues such as pantothenol and CJ-15,801 [5,7,8].

Antiplasmodial Activity in Albumax-complete Medium Pre-incubated at 37°C for a Prolonged Period

During the course of screening pantothenamides for antiplasmodial activity, it was serendipitously discovered that a prolonged 37°C pre-incubation of the Albumax-complete RPMI used for a growth assay has a dramatic effect on the antiplasmodial activity of pantothenamides. As shown in Figure 2, nine of the ten pantothenamides that inhibit growth of *P. falciparum* with IC_{50} values below 200 μM in fresh Albumax-complete RPMI were found to be more potent in Albumax-complete RPMI incubated at 37°C for 40 h prior to use ("aged" Albumax-complete RPMI) than in fresh Albumax-complete RPMI. When tested in aged medium, the IC_{50} values determined for these nine compounds were between one and three orders of magnitude lower than the IC_{50} values determined when the pantothenamides were tested in fresh medium. Additionally, four of the 12 pantothenamides that did not inhibit growth of *P. falciparum* with IC_{50} values below 200 μM in fresh Albumax-complete RPMI (compounds **2**, **9**, **16** and **20**) did so in aged Albumax-complete RPMI. The effect of increasing concentrations of three selected pantothenamides (compounds **3**, **12**, and **19**) on the growth of *P. falciparum* in fresh and aged Albumax-complete RPMI is shown in Figure 3A–C. The activity of pantothenol was, by contrast with the activity of many of the pantothenamides, comparable in aged and fresh medium ($P = 0.32$, unpaired *t* test; Figure 3D). Hence, the potency of pantothenamides is selectively enhanced in aged medium.

Among the most potent pantothenamides in aged medium was the phenethyl substituted pantothenamide, compound **3** ($IC_{50} = 20 \pm 2$ nM; mean ± SEM; $n = 3$). One other carbocycle-bearing pantothenamide (compound **9**; a pantothenamide with a methoxy-substituted benzyl substituent) was also observed to inhibit parasite growth in aged medium with an IC_{50} value below 200 μM, but was significantly less potent ($P = 0.0009$, ANOVA). When tested in aged medium, an IC_{50} value below 200 μM was measured for compound **2** (N7-Pan), however the pantothenamide remained less active than the pantothenamides with shorter alkyl amide substituents (compounds **1**, **11** and **12**). As observed when the compounds were tested in fresh medium, in aged medium: (i) compounds **17** and **18** (two thioether substituted pantothenamides) were among the most potent pantothenamides, and the pantothenamide with the shorter thioether substituent lacked appreciable activity even at 200 μM; and (ii) the activity of the pantothenamides with ether substituents increased with increasing chain length, with compound **19** being among the most potent pantothenamides. Compounds **16** and **20** (both with terminal hydroxyl groups in the substituent) demonstrated an inhibitory

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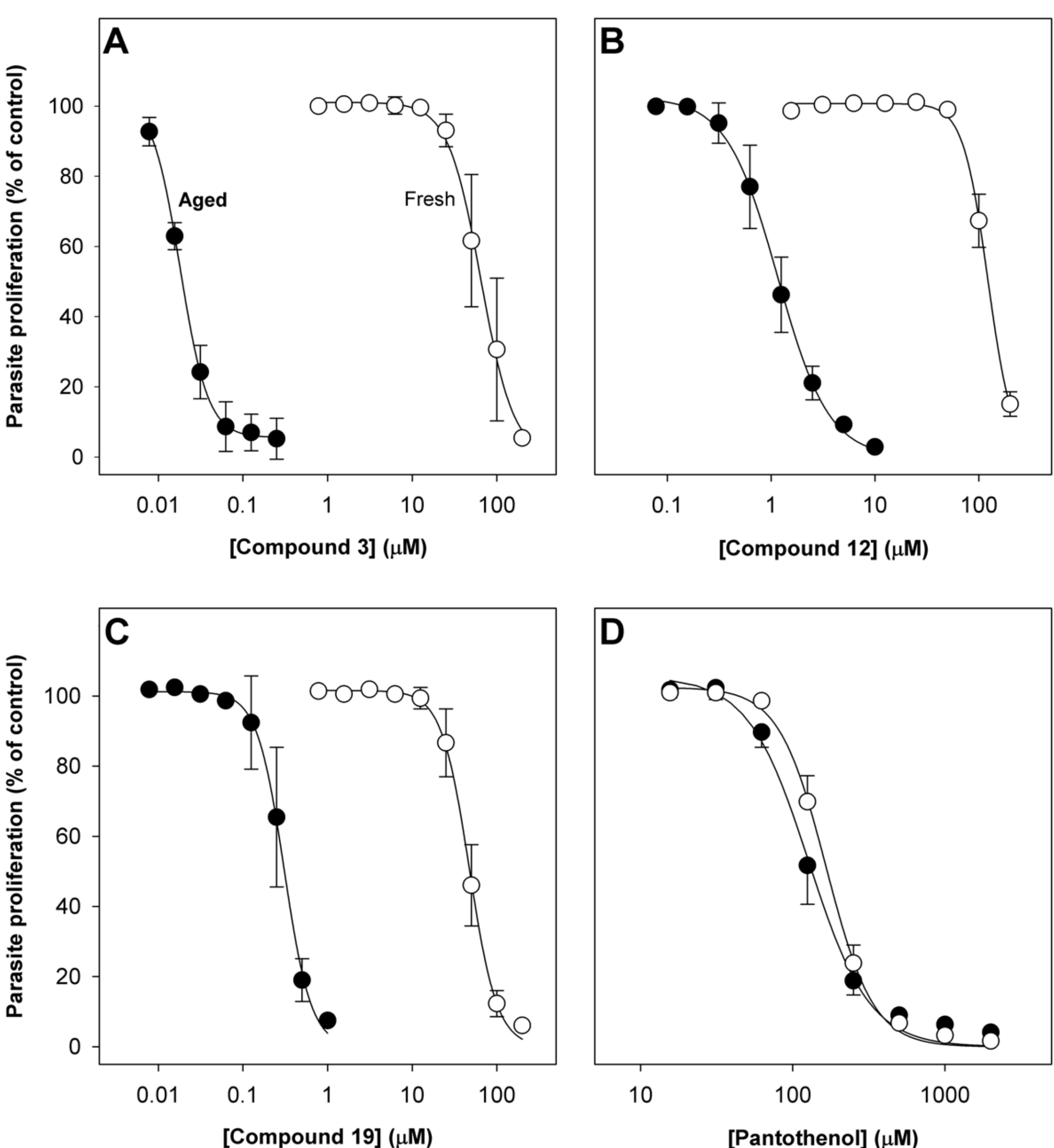

Figure 3. Antiplasmodial effect of pantothenamides and pantothenol in fresh and aged Albumax-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound **3** (**A**), compound **12** (**B**), compound **19** (**C**), and pantothenol (**D**) on proliferation of *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI containing 1 μM pantothenate, as measured using the SYBR Green I-based growth assay. Assays were performed using Albumax-complete RPMI stored for a maximum of 48 h at 4°C, and incubated at 37°C for a maximum of 1 h (fresh; open circles) or Albumax-complete RPMI incubated continuously at 37°C for 40 h immediately after preparation (aged; closed circles). The data obtained with parasites cultured in fresh Albumax-complete RPMI are from between three and eight independent experiments performed in duplicate or triplicate and error bars represent SEM. The data obtained with parasites cultured in aged Albumax-complete RPMI are from between two and three independent experiments performed in duplicate or triplicate and error bars represent range/2 or SEM. For clarity, in **D**, the concentration-response curves represented by the closed circles are shown with negative error bars only, and the concentration-response curves represented by the open circles are shown with positive error bars only. Where not shown, error bars are smaller than the symbol.
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effect in aged medium, however, they remained among the least potent pantothenamides.

Not only did the potency of pantothenamides in fresh and aged Albumax-complete RPMI differ, the effect of pantothenate supplementation on pantothenamide potency differed depending on whether the compounds were tested in fresh or aged medium; the effect of supplementation with 100 μM pantothenate was greater when compounds were tested in aged medium than in fresh. For example, as shown in Figure 4 (closed bars), the IC_{50} values determined for compounds **12** and **19** against parasites cultured in aged Albumax-complete medium supplemented with 100 μM pantothenate were 21 ± 1 (mean ± SEM; n = 3) and 24 ± 1 (mean ± range/2; n = 2) times higher, respectively, than the corresponding IC_{50} values against parasites cultured in aged Albumax-complete RPMI containing 1 μM pantothenate; by comparison, the IC_{50} values determined for compounds **12** and

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19 against parasites cultured in fresh Albumax-complete RPMI supplemented with 100 μM pantothenate were only 1.3±0.2 and 1.7±0.4 (mean ± range/2; n = 2) times higher, respectively, than the corresponding IC_{50} values against parasites cultured in fresh Albumax-complete RPMI containing 1 μM pantothenate (Figure 4, open bars). These data are consistent with the inhibition of growth in aged medium resulting from inhibition of pantothenate and/or CoA utilization. Pantothenate was found to antagonize the antiplasmodial activity of pantothenol effectively in both fresh and aged medium (Figure 4). Taken together, these data are consistent with (i) the pre-incubation of Albumax-complete RPMI at 37°C effecting a change in the medium that specifically and reproducibly enhances the antiplasmodial activity of pantothenamides; and (ii) pantothenamides acting via an effect on pantothenate and/or CoA utilization under these conditions.

Inhibition of Pantothenate Phosphorylation

To explore the mechanism of action of pantothenamides, we investigated whether, like antiplasmodial pantothenate analogues reported previously [5,7,8], pantothenamides inhibit *P. falciparum* PanK-catalyzed pantothenate phosphorylation. As shown in Figure 2, in the presence of each pantothenamide, the amount of [^{14}C]pantothenate phosphorylated by PanK present in *P. falciparum* lysate during a 10 min period (in which pantothenate phosphorylation increased linearly with time under control conditions) was significantly reduced relative to the amount phosphorylated in the absence of pantothenamides ($P<0.0001$, ANOVA). At the pantothenamide concentration tested (100 μM, a concentration 500-fold higher than the concentration of pantothenate present in the assay), each of the pantothenamides inhibited [^{14}C]pantothenate phosphorylation by ≥94% (Figure 2). These data are consistent with all of the pantothenamides tested here interacting with *P. falciparum* PanK, either as alternate (competitive) substrates or as inhibitors of its phosphorylating activity.

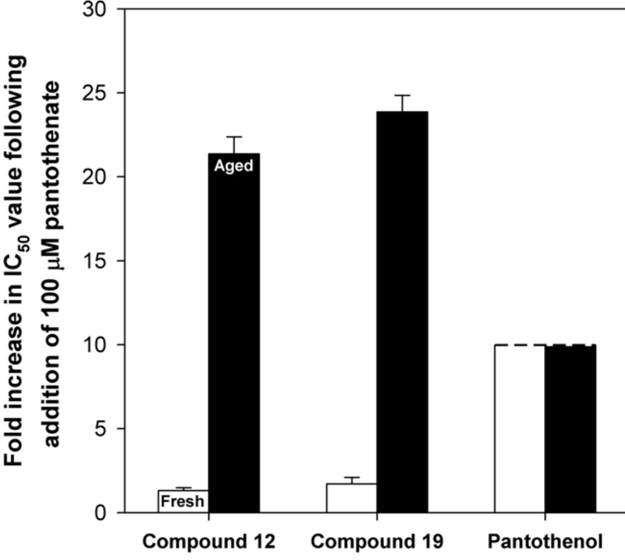

Figure 4. Effect of pantothenate supplementation on pantothenamide and pantothenol potency in fresh and aged Albumax-complete RPMI. The bars represent the fold-increases in the IC_{50} values of compound 12, compound 19 and pantothenol in Albumax-complete RPMI following supplementation with 100 μM pantothenate. The fold-increases in IC_{50} values were calculated by dividing each IC_{50} value measured against *P. falciparum* cultured in the presence of 100 μM pantothenate by the corresponding IC_{50} value measured against *P. falciparum* cultured in the presence of 1 μM pantothenate. The fold-increases in IC_{50} values were determined in assays performed using Albumax-complete RPMI stored for a maximum of 48 h at 4°C, and incubated at 37°C for a maximum of 1 h (fresh; open bar) or Albumax-complete RPMI stored for a minimum of one week at 4°C and incubated intermittently at 37°C, or, soon after preparation, incubated continuously at 37°C for up to 40 h (aged; closed bar). The fold-increases in IC_{50} values are averaged from between two and four independent experiments in which test compounds were tested in Albumax-complete RPMI containing 1 μM pantothenate and Albumax-complete RPMI supplemented with 100 μM pantothenate in parallel. Each experiment was performed in duplicate or triplicate, and error bars represent SEM or range/2. Pantothenol bars are shown with a broken edge to indicate that only a lower limit on the fold-increase in IC_{50} could be determined. This is because less than 50% inhibition of growth was observed at the highest pantothenol concentration tested (2 mM).
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Effect of Albumax II-supplementation on the Antiplasmodial Activity of Pantothenamides in Aged Culture Medium

The data presented thus far are consistent with there being a heat-labile component in Albumax-complete RPMI that antagonizes the activity of pantothenamides. In an attempt to identify such a component, the activity of a selected pantothenamide (compound **12**) in aged Albumax-complete RPMI supplemented immediately prior to the assay with various components of Albumax-complete RPMI, was investigated. A component of the medium that was found to antagonize the activity of pantothenamides was Albumax II, the lipid-rich bovine serum albumin preparation used as a serum substitute [21]. As shown in Figure 5A (open circles), the addition of Albumax II (0.6%, w/v) to aged Albumax-complete medium reduced the activity of compound **12**. In the presence of the additional Albumax II, compound **12** had little-to-no effect on parasite growth even at a concentration of 200 μM, a concentration that inhibits parasite growth completely in aged Albumax-complete RPMI without additional Albumax. Furthermore, the activity of pantothenol was unaffected by supplementation with the additional Albumax II (Figure 5B), consistent with Albumax II specifically influencing the potency of pantothenamides. To investigate whether the attenuating effect of Albumax II could be linked to the increased potency of pantothenamides in Albumax-complete RPMI incubated for a prolonged period at 37°C, we determined whether the attenuating effect of the additional Albumax II could be alleviated by incubating the aged medium to which additional Albumax II had been added for a further 40 h at 37°C. The pantothenamide was indeed more potent (>65-fold; Figure 5A, grey circles) when tested in aged Albumax-complete RPMI supplemented with Albumax II and aged a second time. The possibility that this increased potency was due to further inactivation of an independent component of the aged medium was investigated by testing the pantothenamide in aged medium incubated for the same total length of time as the aged Albumax-complete RPMI supplemented with Albumax II and then incubated. Compound **12** was slightly more active in the Albumax-complete RPMI subjected to two 40 h incubations at 37°C than in the Albumax-complete RPMI subjected to a single 40 h incubation (Figure 5A, closed squares). This demonstrated that a component of the Albumax-complete RPMI had not been fully depleted/inactivated after the initial 40 h and hence that the pantothenamide had not reached a maximum potency in this medium after the initial 40 h incubation. Nonetheless, the increase in potency was far less than the increase in potency observed following incubation of the aged medium supplemented with additional Albumax II, consistent with the Albumax II being sensitive to heat treatment. Taken

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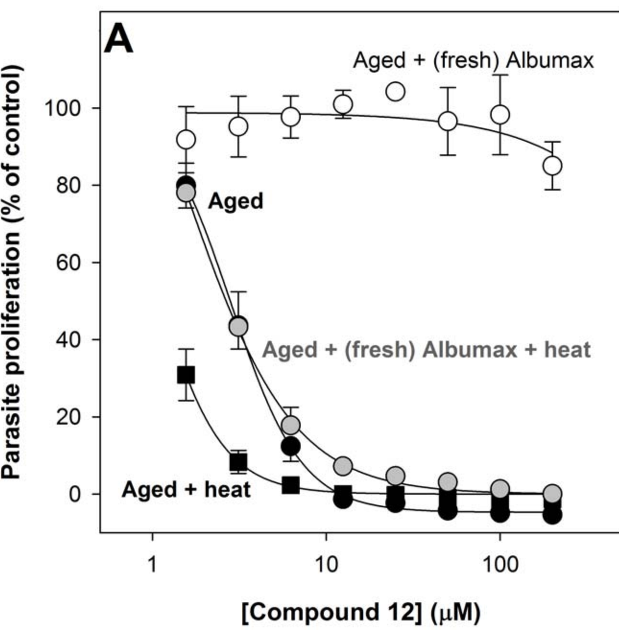

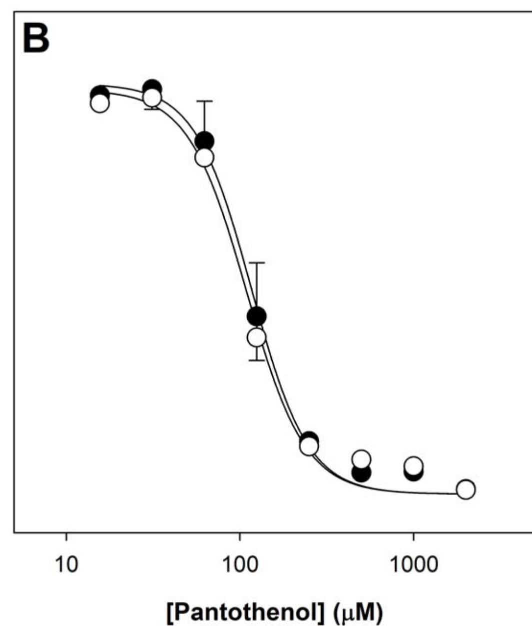

Figure 5. Effect of Albumax II supplementation on pantothenamide and pantothenol potency in aged Albumax-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound **12** (**A**) and pantothenol (**B**) on proliferation of *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI as measured using the SYBR Green I-based growth assay. Assays were performed using (i) Albumax-complete RPMI incubated immediately after preparation at 37°C for 40 h (aged; closed circles); (ii) aged Albumax-complete RPMI supplemented with additional Albumax II (0.6%, w/v) immediately prior to the assay (aged+(fresh) Albumax; open circles); (iii) aged Albumax-complete RPMI supplemented with additional Albumax II (0.6%, w/v) and heated at 37°C for 40 h immediately prior to the assay (aged+(fresh) Albumax+heat; grey circles); and (iv) aged Albumax-complete RPMI heated at 37°C for 40 h immediately prior to the assay (aged+heat; closed squares). The data presented in **A** are averaged from three independent experiments, each performed in triplicate, and error bars represent SEM. The data presented in **B** are averaged from two independent experiments, performed in duplicate or triplicate, and error bars represent range/2. For clarity, in **A**, the concentration-response curves represented by the closed circles are shown with negative error bars only, and the concentration-response curves represented by the grey circles are shown with positive error bars only. In **B**, the concentration-response curves represented by the closed circles are shown with positive error bars only, and the concentration-response curves represented by the open circles are shown with negative error bars only. Where not shown, error bars are smaller than the symbol.
doi:10.1371/journal.pone.0054974.g005

together, these data implicate Albumax II as the labile factor in fresh Albumax-complete RPMI that influences the potency of pantothenamides.

Antiplasmodial Activity in Human Serum-complete Medium

To investigate whether the pantothenamide-attenuating property of Albumax II was unique to this bovine serum albumin preparation or whether the property was shared with human serum, the activity of compound **12** in medium containing human serum (10%, v/v), rather than Albumax II, was determined. In human serum-complete RPMI prepared immediately prior to growth assays (fresh human serum-complete RPMI), compound **12** had no effect on parasite growth even at a concentration of 200 μM, the highest concentration tested (Figure 6A, open circles). In human serum-complete RPMI that had been incubated at 37°C for 40 h (aged human serum-complete RPMI), however, compound **12** inhibited proliferation of *P. falciparum* with an IC_{50} value of 22±10 μM (mean ± range/2; $n = 2$; Figure 6A, closed circles). Pantothenol, on the other hand, inhibited parasite growth with similar activity in fresh and aged human serum-complete RPMI (Figure 6B). These data demonstrate that pantothenamide potency is selectively enhanced in human serum-complete RPMI following incubation of the medium at 37°C for 40 h, as it is in Albumax-complete RPMI following incubation, consistent with Albumax II and human serum having in common a labile component that decreases pantothenamide potency.

Pantetheinase-mediated Pantothenamide Degradation *in vitro*

The activity of pantetheinases, enzymes that catalyze the hydrolysis of pantetheine to pantothenate and cysteamine, has previously been detected in human serum [34]. Since pantetheine is a secondary amide of pantothenate it more closely resembles the pantothenamides (Figure 1) than pantothenol and other previously reported [7,8] antiplasmodial pantothenate analogues do. In light of this, we considered the possibility that pantothenamides in this series are pantetheinase substrates and that Albumax II may be a source of pantetheinase activity. To explore this hypothesis we adapted a fluorescence-based assay used previously to measure *N*-acetyl-1-D-*myo*-inositYl-2-amino-2-deoxy-α-D-glucopyranoside deacetylase activity [30,31] to measure any breakdown of compound **12** to pantothenate and isobutylamine (a primary amine). This assay utilizes fluorescamine, a molecule which is itself non-fluorescent, but generates a fluorescent product upon reaction with primary amines. Using an isobutylamine standard curve, fluorescence measurements were converted to isobutylamine concentrations. As shown in Figure 7 (closed circles), when compound **12** (200 μM) was incubated with recombinant human pantetheinase (100 ng/mL), primary amine was generated, and the concentration increased approximately linearly with time before reaching a maximum after ~4 h. The maximum reached corresponded to an isobutylamine concentration of ~200 μM, consistent with all of the pantothenamide present having been hydrolyzed. By contrast, pantothenamide hydrolysis was not

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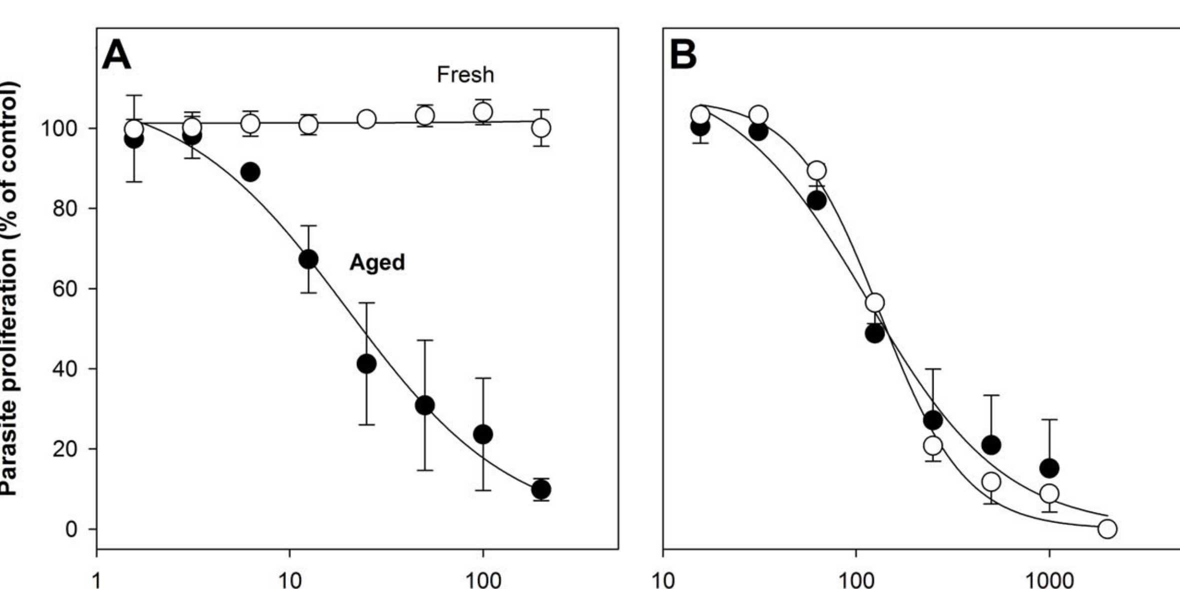

Figure 6. Antiplasmodial effect of a pantothenamide and pantothenol in fresh and aged human serum-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound **12 (A)** and pantothenol (**B**) on the proliferation of *P. falciparum* parasites cultured (for 96 h) in human serum-complete RPMI as measured using the SYBR Green I-based growth assay. Assays were performed using human serum-complete RPMI prepared immediately prior to experimentation (fresh; open circles) or human serum-complete RPMI heated at 37°C for 40 h immediately following preparation (aged; closed circles). The data obtained with parasites cultured in fresh human serum-complete RPMI are from three independent experiments, each performed in duplicate or triplicate, and error bars represent SEM. The data obtained with parasites cultured in aged human serum-complete RPMI are from two independent experiments, each performed in duplicate or triplicate, and error bars represent range/2. For clarity, in **B**, concentration-response curves represented by the closed circles are shown with positive error bars only, and the concentration-response curves represented by the open circles are shown with negative error bars only. Where not shown, error bars are smaller than the symbol.
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observed when compound **12** was incubated under the same conditions in the absence of pantetheinase (Figure 7, open circles). Hence, these data are consistent with pantetheinase mediating the hydrolysis of pantothenamides in addition to pantetheine. Primary amine was also generated during incubation of compound **12** with Albumax II (0.6%, w/v) (Figure 7, grey circles), consistent with Albumax II (when present at a concentration equivalent to that present in Albumax-complete medium) mediating hydrolysis of the pantothenamide. Furthermore, incubation of Albumax II in the absence of pantothenamide did not result in primary amine generation (Figure 7, grey squares), which eliminated the possibility that the amine generated resulted from degradation of the Albumax II and not the pantothenamide. In the presence of Albumax II, over one third of the pantothenamide present was hydrolyzed during a 6 h incubation, and pantothenamide hydrolysis appeared to reach completion within 24 h.

Pantetheinase-mediated Pantothenamide Degradation under *in vitro* Culture Conditions

To establish whether Albumax II-mediated pantothenamide hydrolysis could explain the reduced potency of pantothenamides in fresh Albumax-complete RPMI, we compared the activity of compound **12** in aged Albumax-complete RPMI with and without recombinant human pantetheinase. As shown in Figure 8 (open circles), 100 ng/mL recombinant human pantetheinase (when added at the start of the assay) alleviated the antiplasmodial effect of compound **12** in aged Albumax-complete RPMI. This result is consistent with pantetheinase-mediated pantothenamide degradation occurring under *in vitro* culture conditions and, in turn, attenuating the antiplasmodial effect of pantothenamides. Importantly, the activity of compound **12** in aged Albumax-complete RPMI supplemented with recombinant human pantetheinase increased after the medium was incubated at 37°C for 40 h (Figure 8; grey circles). Moreover, the pantothenamide was more potent in this medium than in aged Albumax-complete medium incubated in parallel but to which the recombinant human pantetheinase was added only after the second incubation at 37°C (i.e. immediately prior to the assay; Figure 8; grey squares). The latter provides evidence that the increase in pantothenamide activity is largely due to inactivation of pantetheinase and not a result of further inactivation of an independent component of the medium. Taken together these data demonstrate that the antagonizing effect of pantetheinase in parasite culture medium can be alleviated by incubating the medium at 37°C, and are consistent with inactivation of pantetheinase occurring during the incubation. Hence, inactivation of Albumax II-derived pantetheinase during prolonged incubation at 37°C, can explain the increase in pantothenamide potency observed when Albumax-complete medium is incubated for a prolonged period at 37°C.

Discussion

That pantothenamides possess antibacterial activity has been known for some time [12]. In this study we show for the first time that pantothenamides also possess antiplasmodial activity. Additionally, we show there to be a labile serum-derived factor common to Albumax II and human serum that specifically antagonizes the antiplasmodial activity of pantothenamides (Figures 5 and 6) and thereby masks their potency. We

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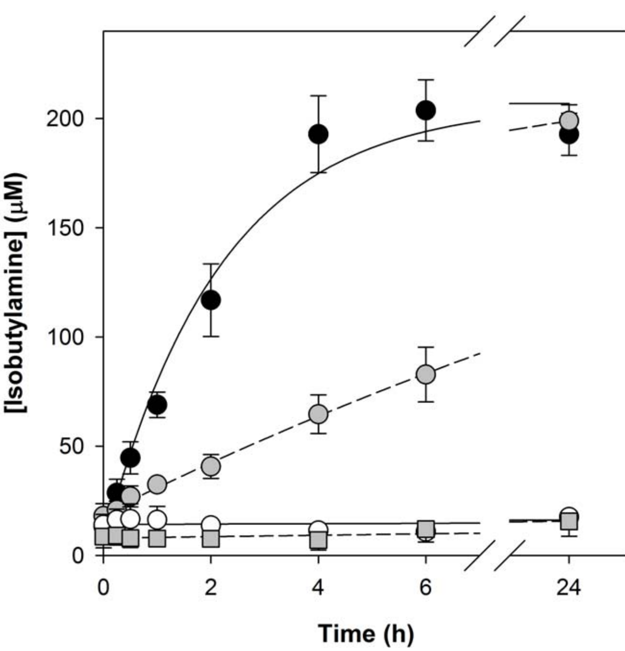

Figure 7. Hydrolysis of a pantothenamide in the presence of recombinant human pantetheinase and Albumax II. The time-courses show the concentration of isobutylamine (a product of compound **12** hydrolysis) detected during incubation of compound **12** with recombinant human pantetheinase (100 ng/mL; closed circles), Albumax II (0.6%, w/v; grey circles), or an equivalent volume of water (open circles), and during incubation of Albumax II (0.6%, w/v) in the absence of compound **12** (grey squares). At each time-point, the amount of primary amine was measured using a fluorescamine-based fluorescence assay. Fluorescence measurements were converted to isobutylamine concentrations using a standard curve generated using isobutylamine samples of known concentration that had been processed in the same manner as the test samples. The data are from three or four independent experiments, each performed in duplicate, and error bars represent SEM. For clarity, the time-courses represented by the open circles are shown with positive error bars only, and the time-courses represented by the grey squares are shown with negative error bars only. Where not shown, error bars are smaller than the symbol.
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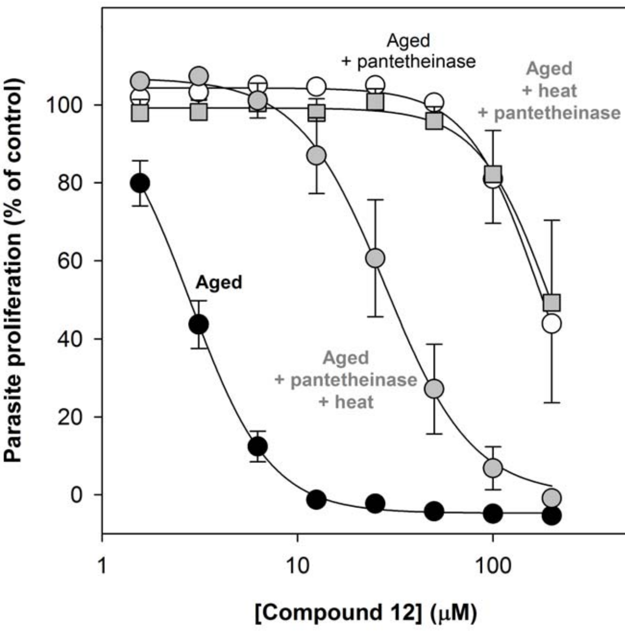

Figure 8. Effect of pantetheinase supplementation on the potency of a pantothenamide in aged Albumax-complete RPMI. The concentration-response curves show the effect of increasing concentrations of compound **12** on proliferation of *P. falciparum* parasites cultured (for 96 h) in Albumax-complete RPMI as measured using the SYBR Green I-based growth assay. Assays were performed using (i) Albumax-complete RPMI incubated immediately after preparation at 37°C for 40 h (aged; closed circles); (ii) aged Albumax-complete RPMI supplemented with recombinant human pantetheinase (100 ng/mL) immediately prior to the assay (aged+pantetheinase; open circles); (iii) aged Albumax-complete RPMI supplemented with recombinant human pantetheinase (100 ng/mL) and heated at 37°C for 40 h immediately prior to the assay (aged+pantetheinase+heat; grey circles); and (iv) aged Albumax-complete RPMI heated at 37°C for 40 h before being supplemented with recombinant human pantetheinase (100 ng/mL) immediately prior to the assay (aged+heat+pantetheinase; grey squares). The data are from three independent experiments, each performed in triplicate, and error bars represent SEM. For clarity, the time-courses represented by the open circles are shown with negative error bars only, and the time-courses represented by the grey squares are shown with positive error bars only. Where not shown, error bars are smaller than the symbol.
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demonstrate this factor to be pantetheinase, an enzyme that hydrolyzes pantetheine to form pantothenate and cysteamine. This conclusion is based on several findings: (i) that recombinant human pantetheinase (Vanin-1) also mediates hydrolysis of an antiplasmodial pantothenamide (compound **12**), a finding consistent with earlier reports of secondary amides of pantothenate other than pantetheine serving as pantetheinase substrates [35,36,37]; (ii) that the pantothenamide is hydrolyzed in the presence of Albumax II (Figure 7), consistent with Albumax II, like serum, being a source of pantetheinase activity; and (iii) that the antiplasmodial potency of the pantothenamide is reduced in the presence of recombinant human pantetheinase and that this attenuating effect is alleviated by incubating the pantetheinase-supplemented culture medium at 37°C (Figure 8). Taken together these results provide strong evidence that in fresh medium, pantetheinase-mediated pantothenamide degradation masks the antiplasmodial potency of pantothenamides. Moreover, we show that in aged culture medium, pantothenamides inhibit growth of *P. falciparum* with potency unparalleled by antiplasmodial pantothenate analogues identified previously.

Five pantothenamides were found to inhibit growth of *P. falciparum* in aged culture medium with sub-micromolar IC_{50} values; for one pantothenamide (compound **3**), an IC_{50} value as low as 20 nM was determined (Figure 2). Furthermore, the finding that the potency of at least one pantothenamide (compound **12**) was greater in medium incubated for 80 h than in medium pre-incubated for 40 h (the standard incubation period employed in this study to "age" medium; Figure 5) is consistent with there being residual pantetheinase activity in medium pre-incubated for 40 h, and indicates that a maximum pantothenamide potency has not been reached in this medium. To determine the maximum (true) potency of pantothenamides, it will be important to test the pantothenamides under conditions where pantetheinase-mediated degradation cannot take place. This, however, is not a trivial task. Serum albumin is required for growth of *P. falciparum* [38,39] and so to achieve strictly pantetheinase-free conditions, an albumin preparation from which all pantetheinase activity has been removed is needed. Whether there is pantetheinase activity associated with preparations of human erythrocytes (required for growth of erythrocytic-stage *P. falciparum*) will also need to be

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determined. Based on homology searches conducted using the human pantetheinase sequences, the parasite genome appears devoid of putative pantetheinase-encoding genes. This observation, and the finding that the parasite is sensitive to pantothenamide-mediated inhibition, suggests that the parasite is incapable of pantetheine (and pantothenamide) hydrolysis. An alternative strategy for determining the true potency of pantothenamides is to test them in the presence of known pantetheinase inhibitors [36]. However, as some pantetheinase inhibitors are also reported to inhibit parasite growth (e.g. [40,41]) (presumably via non-pantetheinase related mechanisms; e.g. [42]), such a strategy may prove problematic.

The observed attenuating effect of pantothenate on the antiplasmodial activity of pantothenamides in aged medium is consistent with the growth inhibition resulting from inhibition of pantothenate and/or CoA utilization, whether it be via inhibition of (i) the uptake of pantothenate; (ii) the biosynthetic conversion of pantothenate to CoA; or (iii) CoA-utilizing enzymes (by pantothenamide-derived CoA analogues). Notably, this is the first report of compounds that inhibit growth of *P. falciparum* at sub-micromolar concentrations by such a mechanism and, as such, this study provides important further validation of pantothenate and CoA utilization as potential antimalarial drug targets. Addition of 100 μM pantothenate was observed to alleviate growth inhibition in the presence of pantothenamides to a much lesser extent than in aged medium (Figure 4). To reconcile this finding, it is important to consider that one of the products of pantothenamide hydrolysis is pantothenate. For this reason, where pantothenamide hydrolysis has occurred, the pantothenate concentration will have increased from the initial 1 μM present in the medium. This will not only attenuate the antiplasmodial effect of the pantothenamides, but will mean that addition of 100 μM pantothenate will increase the pantothenate concentration less than 100-fold. We did investigate the possibility that the antiplasmodial effect observed in fresh medium in the presence of pantothenamides was due to the primary amines generated upon pantothenamide hydrolysis, however, at least for the amines tested (those that should result from hydrolysis of compounds **12**, **17** and **19**), little-to-no inhibition was observed at a concentration of 200 μM (data not shown).

We showed that all 22 pantothenamides tested inhibit *P. falciparum* PanK-catalysed pantothenate phosphorylation, consistent with the pantothenamides either inhibiting or serving as alternate substrates of *P. falciparum* PanK. From the data presented it is not, however, possible to discriminate between these two possibilities. It has been shown previously that *P. falciparum* PanK accepts pantothenol as a substrate for phosphorylation [43]. If pantothenamides also serve as *P. falciparum* PanK substrates, it should be investigated whether the resultant 4'-phosphopantothenamides are metabolized by *P. falciparum* CoA biosynthesis enzymes downstream of PanK as they are by bacterial CoA biosynthesis enzymes [17], and whether this has an effect on CoA levels in the parasite. Inhibition of fatty acid biosynthesis (as a result of inactivation of acyl carrier protein with pantothenamide derived CoA analogues) has been implicated as a primary cause of pantothenamide toxicity in *E. coli* [19]. As fatty acid biosynthesis is not required during the pantothenamide-susceptible erythrocytic stage of *P. falciparum* development [44,45] it is unlikely that the key target of pantothenamides in *P. falciparum* is fatty acid synthesis.

For the SAR generated in this study (Figure 2) to inform future pantothenamide design, it will be important to determine the extent to which they reflect (i) relative efficacy against the target, (ii) relative cell permeabilities (and/or susceptibility to efflux), (iii) relative rates of pantetheinase-mediated hydrolysis, and (iv) inactivation by other mechanisms including serum binding. In light of the demonstration in this study that pantothenamides are hydrolyzed by serum pantetheinase *in vitro*, it is likely that they will also be subject to pantetheinase-mediated hydrolysis *in vivo*, and thereby rendered ineffective as antiplasmodial agents *in vivo*. Consistent with this, compound **12** had little-to-no effect on parasite growth at concentrations up to 200 μM in the presence of human serum (Figure 6A). Therefore to exploit the antiplasmodial potency of pantothenamides it will be important to consider strategies for circumventing pantetheinase-mediated hydrolysis *in vivo*. This is also crucial for the future development of pantothenamides as antibacterial agents, as serum stability will be required for all but topical applications. One strategy is to develop antiplasmodial (or antibacterial) pantothenamide analogues that are resistant to degradation by pantetheinases by, for example, using a bioisosteric replacement strategy [46,47] to replace the key hydrolyzable amide bond. Another strategy for circumventing pantetheinase-mediated pantothenamide hydrolysis *in vivo* is to simultaneously inhibit host pantetheinase. Recently, however, genetic studies in mice have provided evidence that a reduction in pantetheinase activity increases susceptibility to malaria, perhaps as a result of modulation of the inflammatory response [48]. The design of pantetheinase-resistant pantothenamides may therefore be a preferable strategy for circumventing pantetheinase-mediated degradation.

In conclusion, in this study we present, for the first time, analogues of pantothenate that inhibit growth of *P. falciparum* at sub-micromolar concentrations through inhibition of pantothenate and/or CoA utilization, and propose the identification of pantetheinase-resistant pantothenamide analogues as a viable strategy for the discovery of antimalarial agents.

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Author Contributions

Interpreted data and discussed experimental design: CS CM ZL KGV REL ES KJS. Conceived and designed the experiments: CS CM ES KJS. Performed the experiments: CS CM ZL KJS. Analyzed the data: CS CM ZL KJS. Contributed reagents/materials/analysis tools: KGV REL ES. Wrote the paper: CS ES KJS.

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Appendix B

De Villiers M, Macuamule C, Spry C, Hyun Y-M, Strauss E, Saliba KJ. Structural modification of pantothenamides counteracts degradation by pantetheinase and improves antiplasmodial activity.

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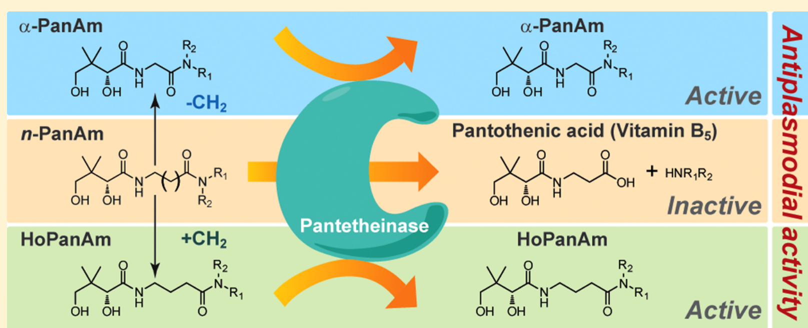
Structural Modification of Pantothenamides Counteracts Degradation by Pantetheinase and Improves Antiplasmodial Activity

Marianne de Villiers,[†,∥] Cristiano Macuamule,[†] Christina Spry,[‡,⊥] Yoo-Min Hyun,[‡] Erick Strauss,[*,†] and Kevin J. Saliba[*,‡,§]

[†]Department of Biochemistry, Stellenbosch University, Stellenbosch 7600, South Africa

[‡]Research School of Biology and [§]Medical School, College of Medicine, Biology, and Environment, The Australian National University, Canberra, Australian Capital Territory 0200, Australia

Supporting Information



ABSTRACT: Pantothenamides are secondary or tertiary amides of pantothenic acid, the vitamin precursor of the essential cofactor and universal acyl carrier coenzyme A. A recent study has demonstrated that pantothenamides inhibit the growth of blood-stage *Plasmodium falciparum* with submicromolar potency by exerting an effect on pantothenic acid utilization, but only when the pantetheinase present in the growth medium has been inactivated. Here, we demonstrate that small modifications of the pantothenamide core structure are sufficient to counteract pantetheinase-mediated degradation and that the resulting pantothenamide analogues still inhibit the in vitro proliferation of *P. falciparum* by targeting a pantothenic acid-dependent process (or processes). Finally, we investigated the toxicity of the most potent analogues to human cells and show that the selectivity ratio exceeds 100 in one case. Taken together, these results provide further support for pantothenic acid utilization being a viable target for antimalarial drug discovery.

KEYWORDS: *Pantothenamide, antimalarial, pantothenic acid, coenzyme A, pantetheinase, drug metabolism*

Nearly half the world's population is at risk of contracting malaria, a lethal infectious disease that is estimated to have caused the deaths of more than half a million people in 2010 alone.[1,2] Consequently, the search for new antimalarial agents is an ongoing pursuit that has intensified since the release of reports that *Plasmodium falciparum*, the most virulent of the protozoan parasites that cause the disease in humans, has become resistant to all chemotherapeutic agents currently in use.[3]

One set of targets that has shown promise for antimalarial drug discovery and development encompasses the processes and pathways dependent on pantothenic acid (vitamin B_5), which serves as the biosynthetic precursor to the essential metabolic cofactor and universal acyl carrier coenzyme A (CoA).[4] This is due to *P. falciparum* showing an absolute requirement for exogenous pantothenic acid,[5] and a sensitivity to compounds that interfere with its ability to utilize this vitamin. Structural analogues of pantothenic acid have shown the most promise in this regard, with many being characterized as growth inhibitors of the blood-stage parasite.[6−8] However, their exact point of action remains to be elucidated.

The pantothenamides are one class of such pantothenic acid analogues that we have recently investigated for antiplasmodial activity.[9] These compounds are prepared by transforming the carboxylic acid group of pantothenic acid to a secondary or tertiary amide and have previously been studied as antibacterial agents.[10−17] While our initial growth inhibition experiments seemed to indicate that these analogues only had modest in vitro antiplasmodial activity against blood-stage *P. falciparum*, we found this to be due to their degradation by enzyme(s) present in the human serum (or the serum substitute, Albumax II) that is used as a necessary supplement to the parasite culture medium. The Vanin proteins, which are members of the nitrilase superfamily, were identified as the probable responsible agent.[9] These proteins have pantetheinase activity, hydrolyzing

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the CoA metabolite pantetheine by means of an invariant Glu–Lys–Cys catalytic triad to form pantothenic acid and the antioxidant cysteamine.[18,19] However, they tolerate a wide range of structural modifications on the cysteamine moiety, thereby explaining their ability to also degrade pantothenamides other than pantetheine (Scheme 1).[20,21]

Scheme 1. Pantothenamides and Their Degradation by the Enzyme Pantetheinase (Vanin)[a]

pantothenamide (R_1,R_2 = H, alkyl, aryl)

pantetheine

Pantetheinase (Vanin)

HNR_1R_2

H_2N SH

pantothenic acid

[a]Pantothenamides are amide analogues of pantothenic acid that are susceptible to degradation by pantetheinase. The native function of this enzyme is to hydrolyze the CoA metabolite pantetheine to pantothenic acid and cysteamine (reaction in grey box).

We found the pantothenamides' antiplasmodial potency to be significantly enhanced when growth inhibition experiments were performed using culture medium that had been preheated (or aged) to remove most of the pantetheinase activity present, with five of the compounds tested showing submicromolar IC_{50} values under these conditions. Among these, *N*-phenethyl pantothenamide displayed the highest potency with an IC_{50} of 20 ± 2 nM,[9] comparable to the IC_{50} of chloroquine when measured against parasite strains sensitive to the drug.[22] Moreover, we found the inhibitory effects of the pantothenamides to be attenuated in the presence of increased concentrations of pantothenic acid, confirming that these compounds act on pathways or processes dependent on this vitamin.

The further development of pantothenamides as antiplasmodials is therefore currently hampered by the ubiquitous presence of Vanin proteins, especially by those found in serum.[19] One approach whereby this shortcoming can be addressed is to combine pantothenamides with known pantetheinase inhibitors.[23,24] However, such a strategy would eventually require the separate optimization of the properties of two compound sets and may result in complications due to adverse interactions between them, and/or inhibition of the physiological function of pantetheinases.[19] We therefore decided to investigate an alternative approach in which the pantothenamides are structurally modified in a manner that would counteract pantetheinase-mediated degradation without compromising their antiplasmodial activity.

A previously described series of modified pantothenamides seemed ideally suited to test such an approach.[16] This series was prepared from pantothenic acid or one of two structural analogues in which its *β*-alanine moiety is replaced with either glycine (to give *α*-pantothenic acid) or *γ*-aminobutyric acid (to give homopantothenic acid). A range of amines representing four chemical motifs (i.e., primary alkyl amines, primary heteroatom-containing aliphatic amines, primary amines with substituents containing aromatic groups, and secondary cyclic amines) was used to introduce the amide moiety into each of these acids in parallel. In this manner, three sets of pantothenamides were formed, referred to as *α*-pantothenamides (*α*-PanAm), *n*-pantothenamides (*n*-PanAm, where *n* signifies normal), and homopantothenamides (HoPanAm), respectively (Tables 1 and S1, Supporting Information). Importantly, the structural modifications cause a displacement in the pantothenamide amide bond in the *α*-PanAm and HoPanAm series relative to its position in the *n*-PanAm series by either the removal or addition of a methylene group. Since previous studies showed that pantetheinase relies on the pantothenoyl moiety of its substrate for recognition,[20,21] we expected that this displacement would reduce the scissile amide bond's susceptibility to attack by the Cys residue of the enzyme's catalytic triad and thereby prevent or reduce the rate of the pantetheinase-mediated degradation of the *α*-PanAm and HoPanAm series relative to that of the *n*-PanAm series.

We decided to determine whether members of the *α*-PanAm and HoPanAm series are more resistant to pantetheinase-mediated degradation compared to their *n*-PanAm counterparts under in vitro conditions. This was tested directly by incubating four sets of pantothenamides, each with a different type of amide substituent, in the presence of recombinant human pantetheinase. The amount of amine released under these conditions was then determined periodically using a previously developed fluorescamine-based fluorescence assay (Figure 1).[9] After 24 h, the hydrolysis of the *n*-PanAm compounds was found to be complete (data not shown), in agreement with our previous findings.[9] However, under the same conditions, the hydrolysis of members of the *α*-PanAm and HoPanAm series was between 5 and 15% complete, except in the case of the PanAm-2 set where it approached ~25%. This demonstrates that displacing the pantothenamide amide bond in the *n*-PanAm series confers resistance to pantetheinase degradation.

Next, we set out to determine whether the increased pantetheinase resistance of the *α*-PanAm and HoPanAm members (relative to the corresponding *n*-PanAm members) also translated into increased antiplasmodial potency in the presence of pantetheinase. The inhibitory activity of 47 sets of *α*-PanAm, *n*-PanAm, and HoPanAm compounds was determined in vitro against intraerythrocytic *P. falciparum* (strain 3D7) in 96 h growth assays initiated with parasites predominantly in the ring stage. For these tests, fresh Albumax-complete RPMI culture medium was used, to ensure the presence of pantetheinase activity.[9] From the IC_{50} values obtained in this manner (data for compounds showing IC_{50} values below 200 μM are given in Table 1; the complete data set is given in Table S1, Supporting Information), it is clear that displacement of the scissile amide bond in a *n*-PanAm significantly improves its antiplasmodial activity ($P < 0.05$ and $P < 0.01$ for *α*-PanAm and HoPanAm members, respectively, compared to *n*-PanAm members; unpaired *t* test) as predicted. To confirm that the increased potency of the *α*-PanAm and HoPanAm members is due to their resistance to pantetheinase degradation, the antiplasmodial activity of 11 selected sets of *α*-PanAm, *n*-PanAm, and HoPanAm

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Table 1. Inhibitory Activity of Selected Pantothenamides on the in Vitro Proliferation of *P. falciparum*

α-PanAm — n-PanAm — HoPanAm

PanAm Entry	R_{Am}	IC_{50} (μM) in fresh medium[a]			IC_{50} (μM) in aged medium[a]		
		α-PanAm	*n*-PanAm	HoPanAm	α-PanAm	*n*-PanAm	HoPanAm
1		13 ± 1	199 ± 53	13 ± 1	ND[b]	ND	ND
2		5.3 ± 2.1	>200	11 ± 1	ND	ND	ND
3		7.0 ± 1.4	182 ± 17	3.9 ± 0.4	14 ± 2	7.5 ± 6.2	2.2 ± 0.1
4		4.7 ± 0.1	134 ± 6	8.0 ± 0.7	7.9 ± 1.8	0.55 ± 0.01	14 ± 7
5		46 ± 3	>200	11 ± 1	ND	ND	ND
9		18 ± 2	200 ± 1	11 ± 2	ND	1.1 ± 0.1[c]	ND
11		12 ± 3	>200	8.9 ± 0.3	ND	ND	ND
14		6.4 ± 0.7	149 ± 7	12 ± 1	ND	ND	ND
15		164 ± 2	>200	2.1 ± 0.2	140 ± 40	>200	3.3 ± 0.5
18		10 ± 1	87 ± 13	2.0 ± 0.1	11 ± 1	0.28 ± 0.02	1.9 ± 0.1
20		38 ± 7	143 ± 5	7.7 ± 0.1	ND	0.23 ± 0.06[c]	ND
29		3.4 ± 0.8	53 ± 11	2.1 ± 0.1	ND	0.020 ± 0.002[c]	ND
30		122 ± 28	>200	6.1 ± 0.5	127 ± 16	>200	4.7 ± 0.5
31		26 ± 6	>200	9.5 ± 0.7	71 ± 8	>200	10 ± 1
32		>200	>200	21 ± 1	>200	>200	22 ± 1
34		53 ± 17	144 ± 13	2.4 ± 0.4	ND	ND	ND
35		71 ± 9	>200	6.3 ± 1.1	64 ± 16	136 ± 34	4.9 ± 1.3
36		48 ± 8	107 ± 2	3.5 ± 0.2	45 ± 6	117 ± 12	2.0 ± 0.1
37		89 ± 10	>200	4.0 ± 0.6	116 ± 21	156 ± 22	1.9 ± 1.0
38		155 ± 27	>200	1.1 ± 0.1	ND	ND	ND

[a]Inhibition of the proliferation of *P. falciparum* cultured (for 96 h) in either fresh (i.e., freshly prepared) or aged (i.e., heat-treated to reduce pantetheinase activity) Albumax-complete RPMI containing 1 μM pantothenic acid (see Supporting Information for details). Values represent the mean ± SEM (or range/2) from at least two independent experiments, each performed in triplicate. [b]ND, not determined. [c]Values taken from ref 9.

pantothenamides was tested in aged medium (i.e., medium subjected to prolonged heat-treatment to reduce the pantetheinase activity). The results show that, while the potency of the *n*-PanAm members improve under these conditions (consistent with our previous results[9]), the activity of the α-PanAm and HoPanAm members determined in the

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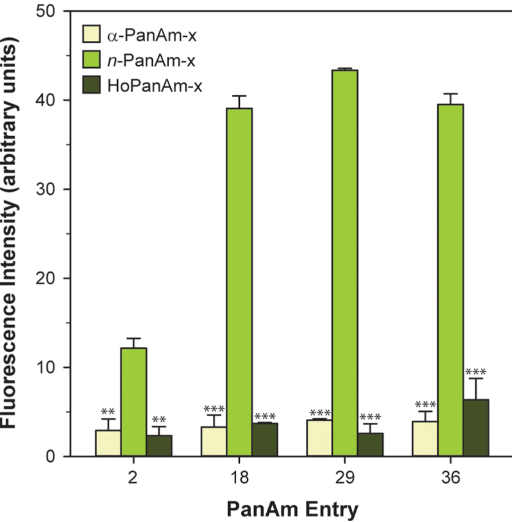

Figure 1. Pantetheinase-mediated hydrolysis of the α-PanAm, n-PanAm, and HoPanAm series of pantothenamides. Four sets of α-PanAm, n-PanAm, and HoPanAm compounds (see Table 1 for structures) were treated with recombinant human pantetheinase. After 24 h, the amount of amine released was determined by means of a fluorescamine-based fluorescence assay. Values represent the mean from three independent experiments, each performed in duplicate; the error bars represent SEM. Asterisks above the bars indicate the significance of the difference between the values determined for the respective α-PanAm and HoPanAm series members and the n-PanAm in a given set (** $P < 0.005$; *** $P < 0.001$, one way ANOVA). Note that, since the fluorescence intensity of fluorescamine−amine conjugates is dependent on the structure of the amine, the fluorescence measurements are not necessarily comparable between the different sets.

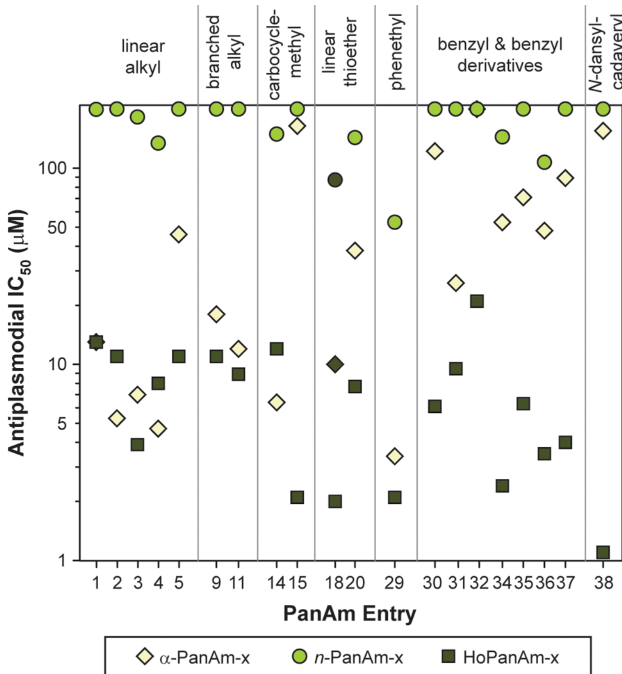

Figure 2. Structure−activity relationship analysis of the inhibitory activity of selected pantothenamides on the in vitro proliferation of *P. falciparum* determined in fresh medium. Graphical representation of the data given in Table 1, highlighting the differences between the activities of the α-PanAm, n-PanAm, and HoPanAm members, and the influence of the structure of the amide substituent (structural group descriptions are given above the graph) on potency. Symbols touching the top of the graph represent compounds with IC_{50} values > 200 μM.

presence and absence of pantetheinase is comparable ($P > 0.05$; unpaired t test). Taken together, these results confirm that the structural modification of pantothenamides is a viable approach to confer resistance to pantetheinase degradation while at the same time preserving antiplasmodial activity.

A comparison of the IC_{50} values determined for the various pantothenamides in fresh medium reveals several interesting trends (Figure 2). First, while the α-PanAm members show variable antiplasmodial potencies, the majority of inhibitory HoPanAm members have IC_{50} values below 10 μM, with the most potent compound (HoPanAm-38) having an IC_{50} value of 1.1 ± 0.1 (mean ± SEM; $n = 4$). This highlights the elongation of the β-alanine moiety of the n-PanAm series as the preferred structural modification to counteract pantetheinase degradation while maintaining antiplasmodial activity. Second, there does not seem to be a clear structure−activity relationship between the α-PanAm, n-PanAm, and HoPanAm series from the perspective of an analysis that focuses on the amide substituent. This would not be unexpected if all three series interacted with the same target since the shortening or elongation of the β-alanine moiety by one methylene unit would displace the amide substituent relative to the pantoyl moiety that all three hold in common. Therefore, one might predict that compounds that have a similar but opposite modification in the amide substituent would counteract these effects and should show some correlation in potency. A case in point is the phenethyl-substituted n-PanAm-29 that shows potent (20 ± 2 nM; mean ± SEM; $n = 3$)[9] activity in aged medium. Displacement of the phenyl group of n-PanAm-29 (as in the case of n-PanAm-30) leads to a complete loss of activity (IC_{50} > 200 μM); in contrast, the corresponding HoPanAm-30, which has an extension that returns the phenyl group to the same relative position as in n-PanAm-29, has an IC_{50} value of 4.7 ± 0.5 μM (mean ± range/2; $n = 2$). Similar examples can be found among the alkyl-substituted pantothenamides (Table 1, entries 3 and 4), suggesting that all three series of pantothenamides interact with the same target(s).

We next set out to confirm that the α-PanAm and HoPanAm members are on-target and, like n-PanAm members, affect processes in *P. falciparum* dependent on pantothenic acid.[9] This was done by investigating the antimalarial activity of these compounds in the presence of excess pantothenic acid (100 μM, compared to the 1 μM that is normally present in culture medium). Concentration−response assays were conducted in fresh culture medium with 25 compounds selected on the basis of their antiplasmodial activity and structural diversity (Table 2). A significant increase ($P < 0.01$, unpaired t test) in IC_{50} values was observed for nearly all the compounds tested, with potency reduced by >95-fold in one case. The only exceptions were three α-PanAm members with aromatic substituents (α-PanAm-36, -37, and -38) that showed significant but small ($P \approx 0.01$) or negligible shifts ($P > 0.1$). While the exact basis for this difference is not clear, for the other α-PanAm and HoPanAm members tested these results indicate that these compounds, like the n-PanAm pantothenamides, exert their inhibitory effects by affecting targets dependent on pantothenic acid. This conclusion is strengthened further by the fact that the HoPanAm members, which in general showed the highest potency in fresh medium (Table 1), also showed the largest fold shifts when the concentration of pantothenic acid was

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Table 2. Effect of Pantothenic Acid Supplementation on Pantothenamide Potency in Fresh Medium[a]

	IC_{50} (μM) in fresh medium with added pantothenic acid[a]		fold shift	
PanAm entry	α-PanAm	HoPanAm	α-PanAm	HoPanAm
3	142 ± 16	195 ± 5	20	50
4	104 ± 4	199 ± 6	22	25
14	183 ± 8	ND[b]	29	ND
15	ND	>200	ND	>95
18	200 ± 19	169 ± 8	19	85
29	135 ± 6	139 ± 10	40	68
30	>200	>200	>2	>33
31	189 ± 12	>200	7	>21
32	ND	>200	ND	>10
34	>200	88 ± 24	>4	37
35	>200	>200	>3	>32
36	57 ± 4	59 ± 14	1	17
37	177 ± 8	169 ± 24	2	42
38	157 ± 19	38 ± 1	1	35

[a]Inhibition of proliferation of *P. falciparum* cultured (for 96 h) in fresh (i.e., in the presence of active pantetheinase) Albumax-complete RPMI containing 100 μM pantothenic acid (see Supporting Information for details). Fold shift gives the ratio of the IC_{50} values determined in fresh medium (Table 1) to those determined in the presence of added pantothenic acid. Values represent the mean ± range/2 from two independent experiments, each performed in triplicate. [b]ND, not determined.

increased. A discussion on the possible identity of these targets is provided in the Supporting Information.

We finally also explored the potential utility of the 10 most potent α-PanAm and HoPanAm members identified in this study as antimalarial drugs by investigating their toxicity to mammalian cells. This was done by determining their concentration−response profiles against Jurkat cells (a human leukemic T-cell line). The toxicity results (Table 3) show that although no obvious trend was apparent for these compounds, the selectivity indexes (the ratio of the IC_{50} values measured against Jurkat cells to the IC_{50} values for the inhibition of *P. falciparum* growth) of HoPanAm-3, -15, -18, -29, and -37 ranged from 40 to >100, indicating that at least these pantothenamides should be considered for in vivo antimalarial activity studies in a mouse model of malaria.

Table 3. Toxicity Assessment of the Most Potent α-PanAm and HoPanAm Series Members

PanAm entry	IC_{50} (μM) vs Jurkat cells[a]	selectivity index
α-PanAm-4	126 ± 13	27
α-PanAm-29	53 ± 5	16
HoPanAm-3	192 ± 5	49
HoPanAm-15	113 ± 6	54
HoPanAm-18	>200	>100
HoPanAm-29	99 ± 16	47
HoPanAm-34	23 ± 4	9
HoPanAm-36	13 ± 1	4
HoPanAm-37	161 ± 23	40
HoPanAm-38	31 ± 12	28

[a]Jurkat cells were cultured for 96 h in medium containing 1 μM pantothenic acid. Values represent means ± SEM from three independent experiments performed in triplicate. The selectivity index for each compound indicates the ratio of the IC_{50} values obtained for the inhibition of Jurkat cell growth and in vitro parasite proliferation in fresh medium.

In conclusion, we have demonstrated in this study that antiplasmodial pantothenamides can be structurally modified to counteract their susceptibility to degradation by pantetheinase while at the same time maintaining their on-target antiplasmodial activity. The good selectivity for parasite growth inhibition (compared to human cell inhibition) of the most potent pantetheinase-resistant pantothenamides identified lends further support for continued focus on these antivitamins as potential new antimalarials.

■ ASSOCIATED CONTENT

Supporting Information

Full set of inhibition results (Table S1), supplementary discussion, and all experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: estrauss@sun.ac.za (E.S.); kevin.saliba@anu.edu.au (K.J.S.).

Present Addresses

‖Department of Cell Biology, Radiation, and Stress Cell Biology, University Medical Center Groningen, University of Groningen, 9713 AV Groningen, The Netherlands.

⊥Department of Chemistry, University of Cambridge, Cambridge, CB2 1EW, U.K.

Author Contributions

M.d.V. and C.M. performed the experiments and data analysis. Y.H. provided initial data that contributed to the conclusions. M.d.V., C.M., C.S., E.S., and K.J.S. contributed to experimental design and data interpretation. M.d.V., E.S., and K.J.S. wrote the paper with input from all the authors. All authors have approved the final version of the manuscript.

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Notes

The authors declare no competing financial interest.

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