Chapter 1
Literature background

The burden of malaria around the world, and the associated mortality rate is shocking. This parasitic infection results in more than 200 million clinical episodes of malaria which, in turn, results in more than 600 000 deaths per annum. Due to poor living conditions and the high socio-economic burden, sub-Saharan Africa is the most affected by malaria with > 90% of all malaria-related deaths occurring on this continent. Current statistics show that a child dies from malaria every 40 seconds, equating to an estimated 2000 children per day [1]. People living in malaria endemic areas have poor quality of life, and typically socio-economic development is poor in comparison to the rest of the world [1]. Renewed efforts have been focussing on more effective drug treatments, the development of a malaria vaccine, and alternative mosquito vector control measures with the aim of eradicating malaria. The current collection of antimalarial therapeutics is under threat of being rendered useless due to decreasing parasite susceptibility - also termed resistance development. There is still an urgent need for improved therapeutics, such as single-dose cure treatments that would improve patient compliance, as well as drugs that block transmission of the parasite [2, 3]. It has therefore become paramount to understand the parasite biology and biochemistry in order to improve prophylaxis, treatment, elimination and to stem parasite drug resistance development.

1.1 Epidemiology of malaria

1.1.1 Emergence and history

The word malaria is derived from Latin “malus” and “aria”, or Italian “mala” and “aria”, and means bad air as the disease was associated as coming from wetland or marsh conditions. Malaria is caused by an infection from parasites of the *Plasmodium* spp. and, according to earliest recorded history this parasite has plagued the world for more than 4000 years. In 2700 BC, Nei-Ching described the symptoms of malaria infections such as periodic or undulating fevers [4]. In the fourth century BC Hippocrates similarly described such symptoms and noticed that incidences occurred in wetland areas, the habitat of mosquitoes which transmit malaria [5]. In the early part of the first century AD, the Romans also recorded fevers amongst people living in wetland areas [5]. Bone tissue samples of an Egyptian mummy dating back to 1500 BC have been found to contain DNA from the malaria parasite [6]. These reported incidences suggest that the parasite has persisted for millennia. In 1880 Alphonse Laveran described *P. falciparum*

sexual blood stage forms of malaria termed gametocytes in a French soldier in Algeria [7]. During that time it was not known how these parasites were transmitted. In 1897, Ronald Ross discovered parasite oocysts in the gut of mosquitoes, establishing the developmental stages of the malaria parasite in the mosquito gut and salivary glands [8]. In 1948, Shortt and Garnham used sporozoites to infect monkeys to reveal exo-erythrocytic schizonts forms of *P. cynomolgi* and later *P. falciparum* parasites in the liver – the missing piece in the parasite's elusive life cycle [9, 10]. Evidence also suggests that malaria parasites from the genus *Plasmodium* are much older than 4 centuries and were believed to have changed hosts going from gorillas into early humans [11]. Estimations based on mutation rates of a cytochrome *b* gene have placed this host switching event to around 365 000 years ago [12]. These early ancestors of the human malaria parasites may still hold clues into pathogenesis and parasite–host interactions, and how to address parasite resistance development.

1.1.2 Transmission

Plasmodium sp. belongs to the phylum Apicomplexa, where the term 'apicomplexa' refers to the complex array of organelles located on the apical ends of these parasite invasive forms [13]. Five species of *Plasmodium* spp. can infect humans; *P. malariae*, *P. ovale*, *P. vivax*, *P. falciparum* and *P. knowlesi*. Almost 90% of deaths caused by *Plasmodium* sp. are as a result of *P. falciparum* infections [14]. *P. falciparum* infections have characteristic 48 h fever paroxysms, and cause severe and complicated malaria. The large number of fatalities and high recrudescence rate, or the re-emergence of parasites following unsuccessful clinical treatment and/or loss of protective immunity, makes *P. falciparum* malaria the most serious [15].

Infections by other *Plasmodium* species are becoming increasingly prevalent. *P. knowlesi* was previously thought to only infect macaque monkeys, however increased incidence of near-fatal human infections from Malaysia and Thailand highlighted the host switching capability of the parasite [16]. In part, poor diagnosis of *P. knowlesi* in humans was attributed to the inability of rapid diagnostic tests detecting this parasite, retarding diagnosis and complicating treatment [16]. Infections with *P. malariae* characteristically cause 72 h fever paroxysms [17]. Such *P. malariae* infections are relatively mild compared to *P. falciparum* infections, and rarely result in death [17-19]. P. vivax malaria has 48 h recurring paroxysms associated with fever, sweating and headaches, reflecting the asexual reproductive life cycle within the human hosts. *P. vivax* is not restricted to tropical climates and is more prevalent in South and Central America as well as in Asia [19-21]. Infections from this parasite account for more than 50% of infections outside of

Africa [19-21]. After successful treatment of *P. vivax* malaria a relapse can occur, which arises when dormant liver stage forms of the parasite or hypnozoites reactivate and reinvade [22, 23]. For this reason *P. vivax* is more resilient and more difficult to control [23]. There are a number of factors that determine the latency period of the hypnozoites, which can range between a few weeks up to 9 months, and these include geographic location, climate, and the initial levels of parasite inoculation [22, 23]. An increase in mortality rates due to *P. vivax* infections and mixed co-infections with *P. falciparum* has been observed [24]. Moreover, increased spread of drug resistant *P. vivax* parasites has highlighted the increasing burden of malaria caused by this parasite specie [24].

Malaria is transmitted by female *Anopheles* mosquitoes, although the genus contains more than 500 species only a select few can transmit *P. falciparum* parasites. These include *An. gambiae*, *An. funestus* and *An. arabiensis*, and are predominant in Africa [25]. These mosquitoes are habitually anthropophilic – they prefer human blood above other sources. These mosquitoes often seek shelter inside houses and have nocturnal feeding habits [25]. Mosquitoes that are infected with *P. falciparum* parasites have increased feeding intervals and consume greater volumes of blood during feeding [26]. The period of blood feeding followed by maturation of eggs in mosquito ovaries and then oviposition, known as the gonotrophic cycle, can be as short as 2 days in tropical climates [20]. The short gonotrophic cycle and increasing feeding behaviour in tropical climates determine the rate of transmission of malaria. Not surprisingly, events of feeding are also determined by the "attractiveness" of the host; *An. gambiae* mosquitoes have olfactory receptors that respond to 1-octen-3-ol, excreted from human skin, and are also capable of detecting 2,3-butanedione, a by-product produced by skin bacteria [27]. Some individuals may therefore be more likely to get bitten by mosquitoes. Skin microorganism diversity, and the ability of the microbiota to produce volatile odorants, is also believed to play a role in host attractiveness, where individuals with lower diversity are more readily targeted by the mosquitoes [28].

1.1.3 Life cycle of *P. falciparum* parasites

As outlined by Figure 1.1, *P. falciparum* parasites are introduced into the human host whilst an infected mosquito takes a blood meal. Specialised invasive forms termed sporozoites migrate towards the liver, where a single sporozoite is all that is required to establish an infection within the host [29, 30]. After successful invasion of hepatic tissues, sporozoites undergo exoerythrocytic schizogony and multiply to form merozoites [29].

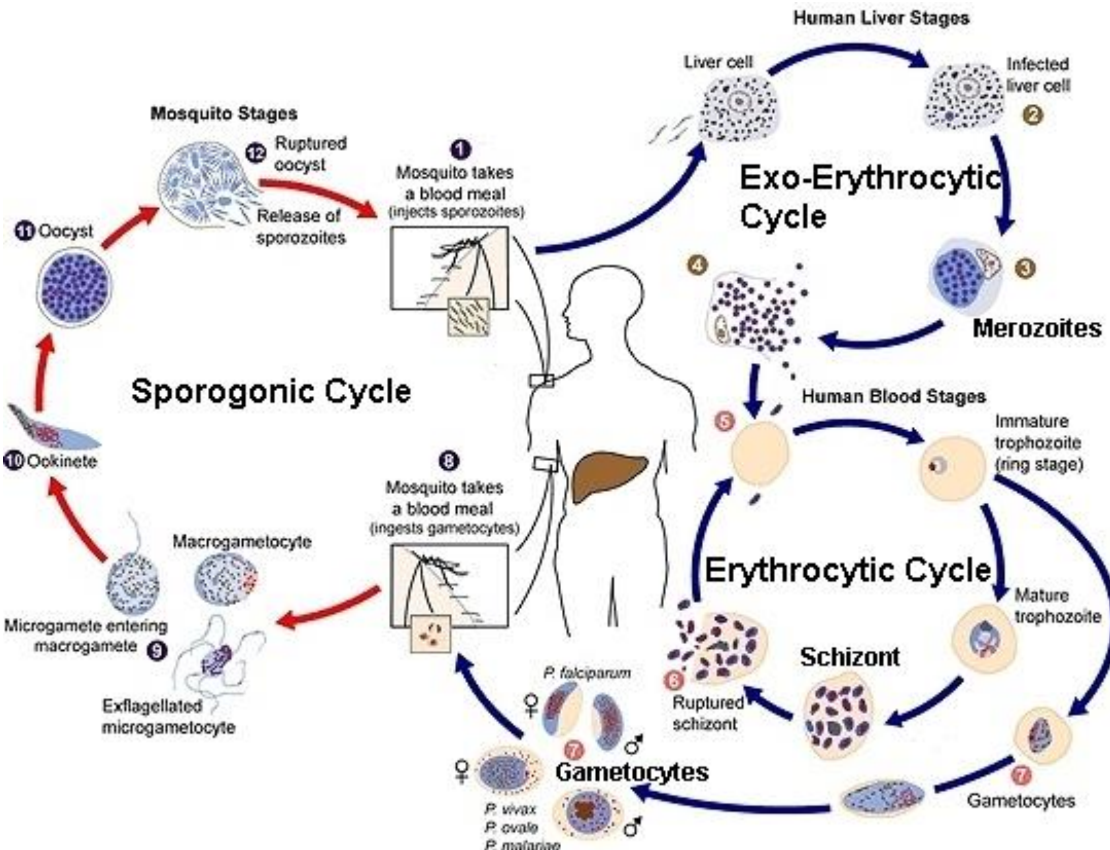

Figure 1.1: The life cycle of *P. falciparum*. Sporozoites, from an infected mosquito, invade the human skin (1), and migrate toward the liver to infect hepatic tissues (2). These parasites mature in the liver (3), and rupture to release merozoites (4). Merozoites invade erythrocytes (5) and mature to become schizonts. Mature schizonts rupture to release merozoites, which reinvade erythrocytes (5). Sexually-destined schizonts become gametocytes (7), which can be consumed by a mosquito during a blood-meal (8). Gametocytes mature in the midgut of the mosquito, and eventually rupture to release sporozoites, which invade the salivary glands of the infected mosquito. Figure adapted from the Centers for Disease Control (CDC) [31].

These merozoites invade human erythrocytes thereby initiating asexual erythrocytic schizogony, also termed the intra-erythrocytic development cycle (IDC). Merozoites are pear-shaped invasive forms of the parasite, which contain apically located organelles that facilitate attachment and invasion of the erythrocyte [29, 32]. Erythrocyte entry involves membrane protein interactions between parasites and erythrocytes, followed by invasion and the formation of the protective parasitophorous vacuole [29]. The IDC is composed of ring, trophozoite and schizont forms, in which the parasites survive by proteolytically digesting one of the most abundant host proteins within the erythrocyte – haemoglobin (Hb) [33]. The schizont stage marks the development of several daughter merozoites that explosively egress from the erythrocyte to invade new erythrocytes. Sexual development or gametocytogenesis involves the invasion and maturation of sexually committed merozoites [34]. This results in the formation of gametocytes that are accessible to mosquitoes during feeding, completing the cycle of disease transmission. Gametocytes are then ingested by the female mosquitoes during a blood meal taken from a *P.*

falciparum infected individual, thereby initiating the sporogonic stages of the parasite's life cycle. After recombination of gametocytes (male and female micro- and macro-gametes) within the mosquito's midgut lumen, the parasite matures into ookinetes, which migrate into the midgut epithelium and develops into oocysts [35]. These maturing oocysts, which start to bulge from the midgut epithelium into the mosquito haemocoelic cavity, eventually ruptures giving rise to millions of sporozoites that go on to invade the salivary glands in the mosquito and are available to reinitiate the invasion cycle [35].

1.1.4 Pathogenesis and clinical features

During the IDC, malaria disease symptoms become visible as characterised by periodic paroxysms, anaemia leading to hypoxia, hypoglycaemia, hyperlactaemia or metabolic acidosis, impaired consciousness and respiratory distress [36]. Septicaemia, other bacterial infections and febrile diseases can make accurate diagnosis of malaria difficult. The onset of severe malaria is characterised as severe anaemia and obstruction of brain microvasculature by parasites, and eventually results in death [37]. Symptoms of severe malaria are compounding, for instance the reduced oxygen carrying capacity (hypoxia) due to anaemia that leads to anaerobic metabolism and the accumulation of lactic acid, metabolic acidosis or hyperlactaemia [37]. Acidosis stimulates respiratory distress in which the human body tries to rid itself of excess CO_2 through rapid breathing to increase blood pH [36]. Cerebral malaria is caused by modification of the erythrocyte membrane by adhesion proteins including *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1) and enables parasites to adhere to epithelial and endothelial vascular tissues, eventually leading to microvascular obstructions, impaired consciousness, coma and death [33].

1.1.5 Distribution

Globally, more than 36% of the world's population live in areas of malaria areas and in 2011 the World Health Organization (WHO) reported that malaria transmission occurs in more than 100 countries worldwide [38, 39]. Malaria is endemic to tropical and sub-Saharan regions of the world and in 2010 it was estimated that 91% of all malaria infections occurred in Africa [39]. Geographic elevation as well as annual rainfall patterns affects mosquito breeding habitats, which in turn determines the intensity of malaria transmission. There are therefore large differences in the risk of malaria infection throughout Africa [38]. Interestingly, red blood cell protein polymorphisms confer a degree of resistance against malaria infections and correlate to

the distribution of the parasite. The most striking of these genetically maintained resistance markers are haemoglobinopathies such as thalassemia, HbC, HbE, and sickle cell disease (SCD) or HbS [40]. The mechanism of SCD resistance to malaria involves diminished parasite invasion and poor parasite growth within the erythrocyte, with increased phagocytosis of sickle cell infected erythrocytes [40]. Although SCD protects from severe malaria, this polymorphism results in anaemia, which reduces oxygen delivery to tissues leading to fatigue and increased risk to bacterial infections.

1.1.6 Incidence rates (frequency)

The WHO has reported that during 2010 there were more than 216 million cases of malaria, with around 655 000 deaths of which 91% occurred in Africa [39]. However, malaria mortality rates are believed to be underestimated, and instead Murray *et al.* suggested that there were 1.2 million recorded fatalities in 2010 [41]. It is estimated that 714 000 (58%) of these deaths were children under the age of 5 [41], which is in contrast to the WHO reports that estimate around 86% of all malaria deaths to be children aged 5 and below [39]. Some estimations on the cumulative probability of dying from malaria have been proposed by Murray *et al.* [41]. The mortality risk is the highest in central as well as western sub-Saharan Africa, as outlined in endemicity maps of *P. falciparum* in 2010 (Figure 1.2) [41]. Dramatic reductions in malaria-related deaths have been noted in Zambia, Kenya, Tanzania and Ethiopia. However these regions are still associated with high malaria mortality rates [41].

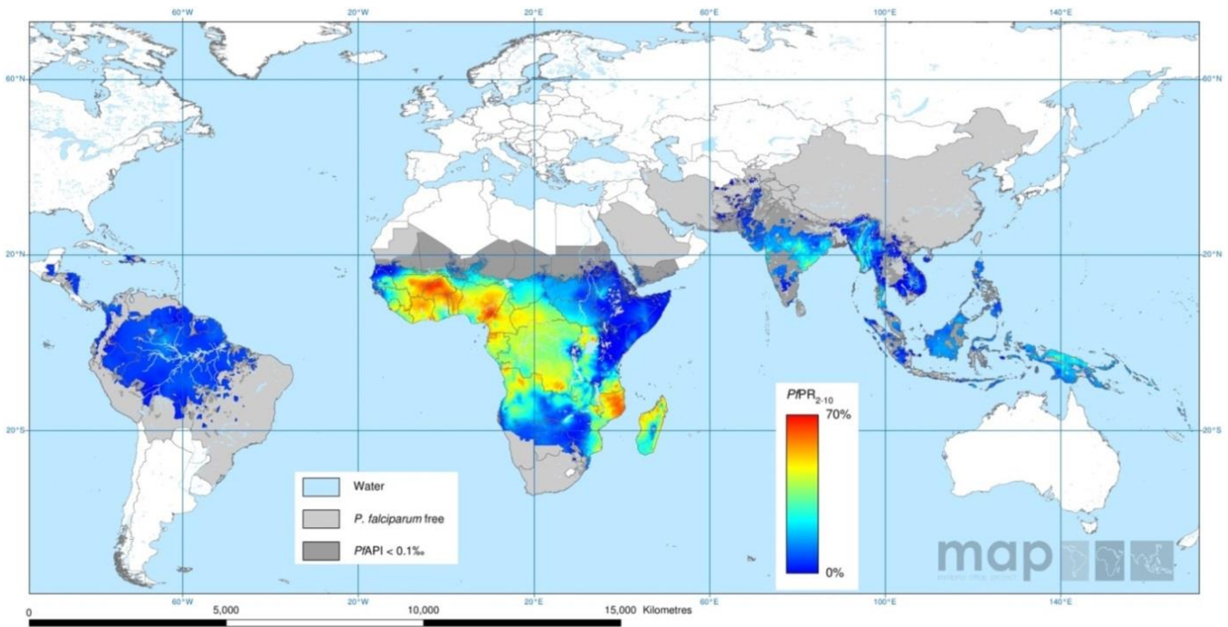

Figure 1.2: The spatial distribution of *P. falciparum* endemicity in 2010. The burden of malaria is shown as an intensity value of age-standardised *P. falciparum* parasite rate ($PfPR_{2\text{-}10}$). This value describes the estimated proportion of children between the ages of 2 and 10 that were infected with *P. falciparum* malaria during 2012. The figure was adapted as obtained from the Malaria Altas Project free resource database (http://www.map.ox.ac.uk). Maps were generated as described by Gething *et al.* [42].

1.2 Control and treatment strategies

Several control measures have been implemented with the goal of reducing and eliminating malaria transmission [3]. These include indoor residual spraying (IRS) of insecticides, the distribution of insecticide treated bed nets (ITN) and the use of antimalarial chemotherapeutics. Other means of control involve the development of a malaria vaccine. Two types of vaccine are feasible for malaria control and eradication; vaccines that block initial sporozoite invasion and transmission blocking vaccines which target sexual gametocyte forms. Global frameworks such as the Roll Back Malaria Partnership (RBM), launched in 1998 by the WHO and the United Nations Children's Fund (UNICEF), amongst others, aim to reduce malaria mortality by implementing preventative measures together with effective mosquito control programs. The Bill and Melinda Gates foundation Global Health Program also focus on developing malaria vaccines and more effective therapeutics and insecticides to reduce the spread of the parasites. The RBM initiated The Global Malaria Action Plan (GMAP, http://www.rbm.who.int/gmap/index.html) which aims to reduce the number of deaths caused by malaria to 0 in 2015. GMAP outlines three main goals for defeating malaria; control malaria to reduce the burden, eliminate malaria systematically, and develop tools to support global control and elimination. These ambitious goals are challenged by several factors including management of parasite resurgence and transmission from endemic areas. Moreover, the cost of elimination and eradication exceeds that

of treatment, and implementation of eradication schemes needs to be tailored for resource-limited areas.

1.2.1 Vaccines

Several stages of the parasite life cycle can be targeted using vaccines. Vaccines against the pre-erythrocytic sporozoites parasites are the most attractive. This is due to the fact that a small number of sporozoites (5-50) invade the human, compared to the large number present during the IDC, and therefore does not overwhelm immune responses. Humans do not exhibit any clinical symptoms of infection during sporozoite invasion and are not immune compromised, increasing the likelihood of parasite elimination during this stage of infection. Maturation of sporozoites typically takes around 6 days, allowing time for facilitated elimination of the parasites from the host [43-45]. Immunization with attenuated *P. falciparum* sporozoites has been effective in conferring protection, however, immunity is short lived (36-42 weeks), requires repetitive immunizations and is species specific [46, 47].

With the aim of developing a sporozoite vaccine the RTS,S/AS vaccine was designed to target circumsporozoite (CS) protein, a dominant membrane protein of the sporozoite. The antigen candidate consists of C-terminal conserved repeat regions in CS protein (termed RT) linked to a hepatitis B surface protein (termed S), which assemble into virus-like particles once expressed [47]. In Phase II clinical trials the vaccine had between 30% - 50% efficacy, protecting against malaria infections and the onset of severe malaria for up to 18 months. This is the most clinically advanced malaria vaccine to date [47, 48]. Phase III clinical trials are underway [49]. Preliminary results from Phase III trials are disappointing and suggested that the vaccine has lowered efficacy than observed previously [50, 51]. The administration of the vaccine to infants between the ages of 6 and 12 weeks conferred 31% reduced risk of developing clinical malaria, and 26% reduced risk of developing severe malaria [51]. The reduced efficacy in infants suggested that early vaccination, which is logistically easier, may not be as feasible as hoped for [50].

Vaccines designed against parasite stages of the IDC could, apart from preventing and combating initial emergence, reduce the symptoms of these proliferative stages [3]. Efforts to design such vaccines are hampered due to highly effective parasite immune evasion mechanisms including the antigenic variation of the parasite surface proteins [33, 52]. Additionally transmission-blocking vaccines are also needed to target gametocyte forms of the parasite [53].

This, in combination with antimalarial therapeutics, could prevent transmission of parasites [53]. This would aid in ensuring effective epidemic management and subsequently aid in elimination.

1.2.2 Vector control

ITN offer both physical and chemical protection, and together with IRS has dramatically reduced malaria incidence in areas of high transmission [14, 54-56]. ITN's have largely been replaced with long lasting impregnated nets (LLIN) which contain an embedded insecticide, rather than residually applied insecticide as found on ITN's [57]. Commonly used insecticides include pyrethroids such as permethrin and deltamethrin that are incorporated into the polymer before extrusion, or is applied as a resin coating over the polymeric fibers of the nets [57].

The insecticide bis[4-chlorophenyl]-1,1,1-trichloroethane, also known as dichlorodiphenyltrichloroethane (DDT) was introduced and extensively used during the 1940's as it was inexpensive ($3/kg in 1990) and extremely effective [58-60]. DDT was classified as a persistent organic pollutants (POP) by the Stockholm convention in May 2004 [61]. It is carcinogenic in animals, resulting in liver tumors, however, there is no substantial evidence that DDT causes cancer in humans [62]. DDT is still used in Africa and was reintroduced in South Africa due to increased malaria incidence rates and the rapid appearance of pyrethroid-resistant *An. funestus* mosquitoes [63]. The safer alternative to DDT includes various pyrethroids that are also the active ingredients in ITN. Pyrethroids, as well as DDT, target the sodium channels of the mosquito's neurons, resulting in paralysis leading to death [64]. Mosquito resistance against pyrethroids arises through either polymorphisms in the sodium channels, or through altered cytochrome P450-mediated metabolism and detoxification of these compounds [54, 64]. The increased spread of DDT-resistant mosquitoes, as well as the widespread inefficacy of pyrethroids is alarming, establishing an urgent need for better control measures to stem the spread of malaria [54, 65].

1.2.3 Therapeutics

Historically the administration of chemotherapeutics has been the most successful in treating and preventing malaria [46, 66]. Various effective antimalarials are listed in Table 1.1 and can be divided into several groups; 4-aminoquinolines, sesquiterpene endoperoxides, antifolates, and 8-aminoquinolines. These therapeutics are extensively discussed elsewhere, and here only selected

aspects concerning efficacy and drug resistance are mentioned [66, 67]. Antimalarials that have been widely used between the 1950's and 1980's belong to the 4-aminoquinoline group (Table 1.1) including quinine, chloroquine (CQ), mefloquine and amodiaquine all of which contain the quinoline chemical scaffold. CQ is considered as one of the most successful antimalarial drugs due to the fact that it was safe and relatively inexpensive [68]. Broadly, the 4-aminoquinolines are capable of interfering with the parasite's leading to the accumulation of haeme [69, 70]. More specifically, free-haeme from Hb digestion is polymerised into haemozoin, which is deposited in the acidic food vacuoles of the parasites in crystalline form. CQ is capable of binding to free haeme, prevent haemozoin formation and leading to the accumulation of toxic complexes and eventual parasite death [68].

Widespread parasite resistance against the 4-aminoquinolines, especially against CQ, has limited their effectiveness in almost 90% of all endemic areas and led to discontinued use [70]. In the 1980's no affordable alternative was available and CQ resistance led to a 3-fold increase in malaria mortality [67]. Single nucleotide polymorphisms (SNP's) in the genes encoding *Pf*CRT (chloroquine resistance transporter) and *Pf*MDR (Multidrug resistance transporter 1) are linked to reduced parasite susceptibility to 4-aminoquinolines [69]. Both *Pf*CRT and *Pf*MDR are components of the parasite vacuolar membrane and reduce the accumulation of 4-aminoquinolines in the vacuole [70]. Related compounds to the 4-aminoquinolines include the 8-aminoquinoline, primaquine (PQ), is also an effective gametocidal capable of reducing mature gametocyte development and subsequently transmission of the parasites [71-73]. PQ is also effective against exoerythrocytic parasites [71, 72]. Other 8-aminoquinolines such as aablaquine (also known as bulaquine or elubaquine) and tafenoquine are currently in clinical trials [72, 74, 75].

Transmission-blocking therapeutics are essential to reduce the spread of resistant parasites and malaria by targeting gametocyte transmission [74, 76]. However, semi-immune populations carrying parasites are often asymptomatic, do not receive treatment, resulting in the continual transmission of parasites - and potentially resistant forms - for extended periods of time [74]. Several strategies aimed at targeting these asymptomatic carriers have been proposed, including mass drug administration, however these are not readily implemented [74]. Chemotherapeutics form part of the toolkit for eliminating malaria, but for this reason need to be used in combination with other control measures [76].Chemotherapeutics form part of the toolkit for eliminating malaria, but for this reason need to be used in combination with other control measures [76].

Antifolates are blood schizontocides that selectively target asexual forms of the parasites [77]. These drugs have been very successful antimalarials and interfere with the formation of folates, leading to decreased pyrimidine synthesis as DNA nucleobase precursors and hence affecting DNA formation [77, 78]. Progaunil, a triazine chloroguanide, was discovered in the 1940s and used to prevent and treat malaria [79]. The active metabolite of proguanil is cycloguanil which targets the dihydrofolate reductase (*Pf*DHFR) enzyme of the parasite [79]. Some antifolates such as sulphadoxine, which is a sulphonamide, inhibit dihydropteroate synthase (*Pf*DHPS) by mimicking *p*-aminobenzoic acid (PABA), whereas other antifolates such as pyrimethamine also target *Pf*DHFR (Table 1.1) [78]. Extensive amino acid mutations in the respective target proteins have led to resistance development and use of these drugs have become restricted. The introduction of the sulphadoxine-pyrimethamine (SP) drug combination in 1967 was rapidly followed by parasite resistance and decreased clinical effectiveness in the same year, despite this SP was utilised to effectively treat CQ-resistant parasites until the early 1990s [77, 80]. SP is now only used to treat malaria during pregnancy [79]. Multiple factors determine the rate and spread of resistance; SP has a longer half-life than other antifolate combinations (such as LapDap – chloroproguanil with dapsone) which was believed to have contributed to rapid resistance development [80]. Additionally, poor drug compliance leads to sub-optimal therapeutic dosing and can result in resistance development [80]. Newer antifolates are being developed to overcome problems associated with resistance, one such compound - P218 – which inhibits both wildtype (WT) and mutated DHFR has shown promise as a potential therapeutic candidate [81]. Antifolates remain good drugs against *P. falciparum* and alternative strategies include using different antifolate combinations with other effective antimalarials. Malarone®, a fixed-dose combination of the antifolate proguanil with atovaquone, is used to prevent and treat multi-drug resistance *P. falciparum*, and has relatively mild side effects [82]. Reports of treatment failure in a patient treated with Malarone® have however also raised concerns whether resistant parasites might emerge [82].

Table 1.1: Most widely used antimalarial therapeutics and resistance markers.

Name	Structure	Trade name	Target	Benefits	Disadvantages	Resistance marker (SNP's)	Ref.
				4-aminoquinolines			
Quinine		Quininmax Aflukin	Haem detoxification mechanisms	Used for treatment of severe malaria. Can be used during pregnancy.	Short half-life (8h). Cannot be used as prophylactic.	*Pf*CRT (chloroquine resistance transporter) *Pf*MDR (Multidrug resistance transporter 1) *Pf*NHE1 (sodium proton exchanger 1)	[13, 67, 83-85]
Chloroquine		Resochin, Dawaquin, Daramal	haemozoin polymerisation Heme detoxification	Cheap, long half-life (60h). Suitable for children and pregnant woman.	Wide-spread resistance. Overdose risk.	*Pf*CRT, *Pf*MDR	
Mefloquine		Larium, Mephaquine	Haem detoxification or *Pf*MDR	Long half-life (14d) requiring weekly administration, ensures patient compliance.	Neuro-psychiatric side effects, not recommended during pregnancy. Vomiting in children.	*Pf*MDR	
Amodiaquine		Camoquin Flavoquine	Haem detoxification processes	-	Short half life	*Pf*CRT, *Pf*MDR	
				8-aminoquinolines			
Primaquine (PQ)		Primaquine	*Pf*CRT transporter inhibitor	Gametocytocidal. Targets *P. vivax* hypnozoites	Not compatible with G6PD deficiency. Not recommended during pregnancy.	-	[13, 67, 71, 83]
				Antifolates			
Sulphadoxine		Fansidar (combined with pyrimethamine)	Dihydropteroate synthase (*Pf*DHPS)	Inexpensive, long half-life (100 – 200h)	Extensive parasite resistance, severe allergic reactions.	SNP in *Pf*DHPS	[78]
Pyrimethamine		Daraprim	Dihydrofolate reductase (*Pf*DHFR)	Long half-life (80h)	Widespread resistance	SNP in *Pf*DHFR	[78, 84, 86]

Proguanil (cycloguanil *in vivo*)		Lapdap (combined with dapsone) Malarone® (combined with atovaquone)	*Pf*DHFR, may include additional targets.	-	Parasite resistance, short half-life	SNP in *Pf*DHFR	[67, 78, 86]
Sesquiterpene endoperoxides							
Artemisinin		Not used as monotherapy	Haemoglobin digestive processes (falcipain 4) or Mitochondrion electron transport processes.	Early ring and gametocidal activity	Short half-life.	No definitive resistance markers	[67, 87, 88]
Artemether		Coartem® (in combination with lumefantrine)		Effective against SP and chloroquine resistant parasites. Cheap.	Not recommend for pregnant woman.		
Artesunate		Larimal (in combination with amodiaquine)		More hydrophilic	Short half-life.		
Dihydro-artemisinin		Artenimol Eurartesim® (combination with piperaquine)		Active form	Short half-life.		
Other classes							
Atovaquone (hydroxynaptho quinone)		Malarone® (combination with proguanil) Mepron	Targets cytochrome bc_1 complex, arrests mitochondrial respiration	Well tolerated	Expensive	SNP in cytochrome b gene	[67, 86]
Doxycycline (tetracycline)		Vibramycin	Protein synthesis inhibitor, apicoplast targeting	Well tolerated, inexpensive.	Slow acting, used only as prophylactic. Cannot be used in children or during pregnancy	Increased copy numbers of *pfmdt* and *pftetQ*	[83, 89]

Compounds with a sesquiterpene endoperoxide functionality include artemisinin (Table 1.1) and its more hydrophilic derivatives artemether, artesunate and dihydroartemisinin (DHA) [88]. These have become the current mainstay antimalarial therapeutics. Artemisinin and derivatives are converted into to the clinically active DHA form [88]. Early studies showed that treatment of malaria-infected individuals with artesunate and artemether had a significant gametocidal effect, and are therefore capable of reducing transmission of malaria [90]. The compounds do not target mature gametocytes, rather the early gametocytes (stages I – III) as well as IDC-ring forms of the parasite [88, 90, 91]. Artemisinin inhibits chloroplast electron transport and is phytotoxic leading to impaired plant growth [92]. In the malaria parasite the endoperoxide ring of the compounds create reactive oxygen species (ROS), and are believed to affect mitochondrial electron transport functions [93]. Increased parasite recrudescence rates against artemisinin combinational therapeutics (ACT) was first reported in 2004, later in 2006, and most recently in 2009 [94]. A study in western Cambodia showed reduced clearance times in artesunate-treated patients compared to individuals in western Thailand [94]. Postulated polymorphisms in *Pf*ATPases or *Pf*MDR were not linked to ACT resistance development and there are still no definitive resistance markers for ACT-resistant parasites [87, 94].

The Medicines for Malaria Venture (MMV) is a non-profit organization jointly established by the Government of Switzerland, the WHO (RBM partnership), and private philanthropists in 1999. Through collaborating with industrial and academic partners the MMV aims to discover and develop newer and effective antimalarial drugs which would aid in reducing the burden of malaria (http://www.mmv.org). The MMV has developed antimalarials of which Eurartesim® (DHA and piperaquine combination) [95] and Coartem® *Dispersible* are successful commercially available products. The MMV is involved in lead generation and optimisation, and has several compounds in preclinical developmental stages. The MMV also boasts several compounds in Phase I, IIa, IIb and III clinical trials of which tafenoquine is one. A recent venture of the MMV in collaboration with, amongst others the University of Cape Town, has resulted in a compound which was identified from phenotypic whole cell high-throughput screens (HTS) [2]. The compound was highly effective against two drug resistant malaria strains and boasts a single dose cure capability against *Plasmodium berghei*-infected mice [2].

The discovery of new antimalarials is intensive and expensive. Typically from the original hit (molecule) to approved medicine can take up to 12 years and cost more than $1 billion [96, 97]. There are several phases of a typical drug discovery scheme program; generally a target is selected, followed by identification of hits (usually chemical antagonists or agonists – modulators of target function) [98]. The hits are generally confirmed using dose-response curves, and tested in

functional cellular assays to evaluate efficacy and membrane permeability. Hit-to-lead transition further establishes the hit cytotoxicity and biocompatibility. Lead optimisation aims to improve lead efficacy and pharmacokinetic properties. Large phenotypic or whole cell HTS are used to identify hits – its major drawback is high cost and time expenditure. Some strategies involve rigorous target validation, which is then followed by target-based HTS, which results in the identification of more specific molecules compared to whole cell HTS approaches. Attrition rates of target-based HTS hits during the hit-to-lead transition are expected to be less (considering that these are more specific), but these hits often have poorer permeability characteristics, requiring additional lead optimisation. An overview of these methodologies, as well as the benefits of computational screening, is given in Chapter 2.

Over the last 30 years only a few antimalarial therapeutics with unique chemical scaffolds have been identified or developed, and strategies have largely focussed on the modification of effective chemotypes [99]. From the collection of antimalarial therapeutics, it is estimated that there are only around 30 different chemical scaffolds, of which only around 10 are clinically useful [99]. More alarming is the fact that parasite resistance to almost all of these scaffolds have been reported, necessitating the identification and expansion of newer chemotypes. Different drug discovery approaches, such as targeting of validated proteins, optimisation of known effective chemotypes, and chemical screening, have resulted in the successful identification of antimalarials.

In drug discovery chemotypes can be expanded by modifications to known scaffolds, or by designing and identifying newer classes of compounds against cellular target [99]. Validated chemotypes can be considered as classes of compounds which have known efficacy, of which the mode of action (MOA) is not necessarily always known, and often of interest. However, conservation and optimisation of a chemotype does not render newer chemical scaffolds. Furthermore the occurrence of drug-resistance can render whole chemotypes useless. In the case of malaria this has occurred numerous times, as demonstrated for aminoquinolines [67]. This severely negates highly effective treatment and possible elimination of the parasites.

A question that arises in target-based drug discovery is whether a single protein is truly tractable or "druggable" *in vivo* and whether chemical attenuation does achieve the desired effect? For this reason target-based drug discovery essentially focuses on validated protein targets. A validated protein target can be considered as a target which has been successfully attenuated using chemotherapeutics and kills the parasite. Target validation includes demonstrating that chemical attenuation is achievable in an *in vitro* as well as *in vivo* setting [100]. Chemical knock-down as a tool for validation of the target is not always possible due to target-specific compounds having poor

bioavailability, low efficacy *in vivo*, high toxicity and off-target effects [99, 100]. Moreover, protein target validation using forward genetic approaches such as antisense- or RNAi-knockdown is based on the premise that the implementation of chemical compounds will have the same effect [100]. This is not always the case. The RNA product could have numerous biological functions apart from providing the ribosomes with a protein assembly sequence, and could obscure the interpretation of the knock-down [101]. Gene deletions or knock-outs and loss–of-function mutations are also used to validate proteins for their druggability. The increasing appreciation of the involvement of coding as well as non-coding regions of DNA in cellular homeostasis questions whether this approach is truly comparable to chemical attenuation of protein targets [101]. In the human genome it has become apparent that a large number of genes are interlaced and transcripts can be synthesized from both DNA strands [101]. Therefore these approaches can complement the validation of protein targets but should not be relied on for absolute validation of druggability.

From validated targets additional compounds can be generated based on chemical analogy or designed from structure-based mining approaches. However, currently there are only a few validated antimalarial drug targets. Moreover, the identification of newer chemical scaffolds to these targets depends on the finite availability of ligands that may have complementary fit with the target, and such approaches often do not yield more effective compounds than what is currently available [99, 102]. For this reasons there is an urgent necessity to exploit non-validated protein targets using chemotherapeutic intervention. This would aid in expanding the antimalarial armamentarium. Vitamin B_6 biosynthesis in the parasite, and the proteins involved in the pathway, are attractive candidates for drug design and identification.

1.3 The significance of vitamin B_6

Vitamin B_6 is one of the most versatile co-factors found in nature. The B_6 family is composed of several vitamers and their respective phosphorylated forms; pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) as shown in Figure 1.3 [103-105]. The term vitamin B_6 is given to all of the vitamers [106]. Pyridoxal 5'-phosphate (PLP) is the most prominent vitamer acting as the active cofactor in PLP-dependent enzymes, and is also involved in non-enzymatic reactions [107-109]. PLP is involved in more than 140 different biochemical reactions ranging from transamination, deamination, racemisation and decarboxylation, and is most notably utilised as co-factor during amino acid metabolism [103, 110-112].

pyridoxine (PN) pyridoxal (PL) pyridoxamine (PM)

pyridoxine 5'-phosphate (PNP) pyridoxal 5'-phosphate (PLP) pyridoxamine 5'-phosphate (PMP)

Figure 1.3: Different physiological forms of vitamin B_6. The basic pyridine scaffold of B_6 vitamers containing various functional chemical moieties attached to the 4' position of the pyridine ring [111]. Both pyridoxal 5'-phosphate and pyridoxal 5-phosphate are accepted nomenclature according to the International Union of Pure and Applied Chemistry (IUPAC) [113, 114].

The physiological significance of vitamin B_6 is further highlighted due to its anticancer properties. Recently, it has been demonstrated that a dietary supplement of vitamin B_6 effectively decreased colon tumorigenesis in mice, partly attributed to the antioxidative activity of the molecule [115]. Vitamin B_6, particularly PLP, can effectively scavenge ROS by quenching singlet molecular oxygen ($^1O_2$) [116, 117].

1.3.1 Vitamin B_6 metabolism

Vitamin B_6 biosynthesis is limited to most *Archaea*, bacteria, fungi and plants, whereas humans and mammals acquire this essential metabolite exclusively from their diet [106]. Two major routes of vitamin B_6 biosynthesis have been established, the deoxy-D-xylulose 5-phosphate (DXP) dependent and independent routes. The DXP-dependent pathway is found in *Escherichia coli* and the γ-division of proteobacteria. The pathway is composed of enzymes PdxA (E.C. 1.1.1.262) and PdxJ (E.C. 2.6.99.2), and produces PNP from DXP and 4-phosphohydroxy-L-threonine (Figure 1.4) [106]. The final PNP is oxidized to PLP by PdxH to complete the biosynthesis of the cofactor [106]. The DXP-independent PLP synthesis is more prevalent in bacteria (other than *E. coli*), archaea and eukaryotes [107]. The DXP-independent pathway consists of a PLP synthase which uses D-ribose 5-phosphate (R5P), DL-glyceraldehyde 3-phosphate (G3P), and L-glutamine as substrates (Figure 1.4) [106]. The PLP synthase is composed of two proteins Pdx1 and Pdx2. These proteins are present in organisms lacking proteins from the DXP-dependent pathway, and a clear divergence is evident

from the DXP-dependent route of PLP synthesis [116, 118]. Pdx1 and Pdx2 proteins are named YaaD and YaaE in *Bacillus subtilis*, SNZ and SNO in *Sacchromyces cerevisiae*, and PdxS and PdxT in *Geobacillus stearothermophilus*, respectively [107, 119]. Other names of Pdx1 proteins are PyroA in *Aspergillus nidulans* and SOR1 in the filamentous fungi *Cercospora nicotianae* [116, 120].

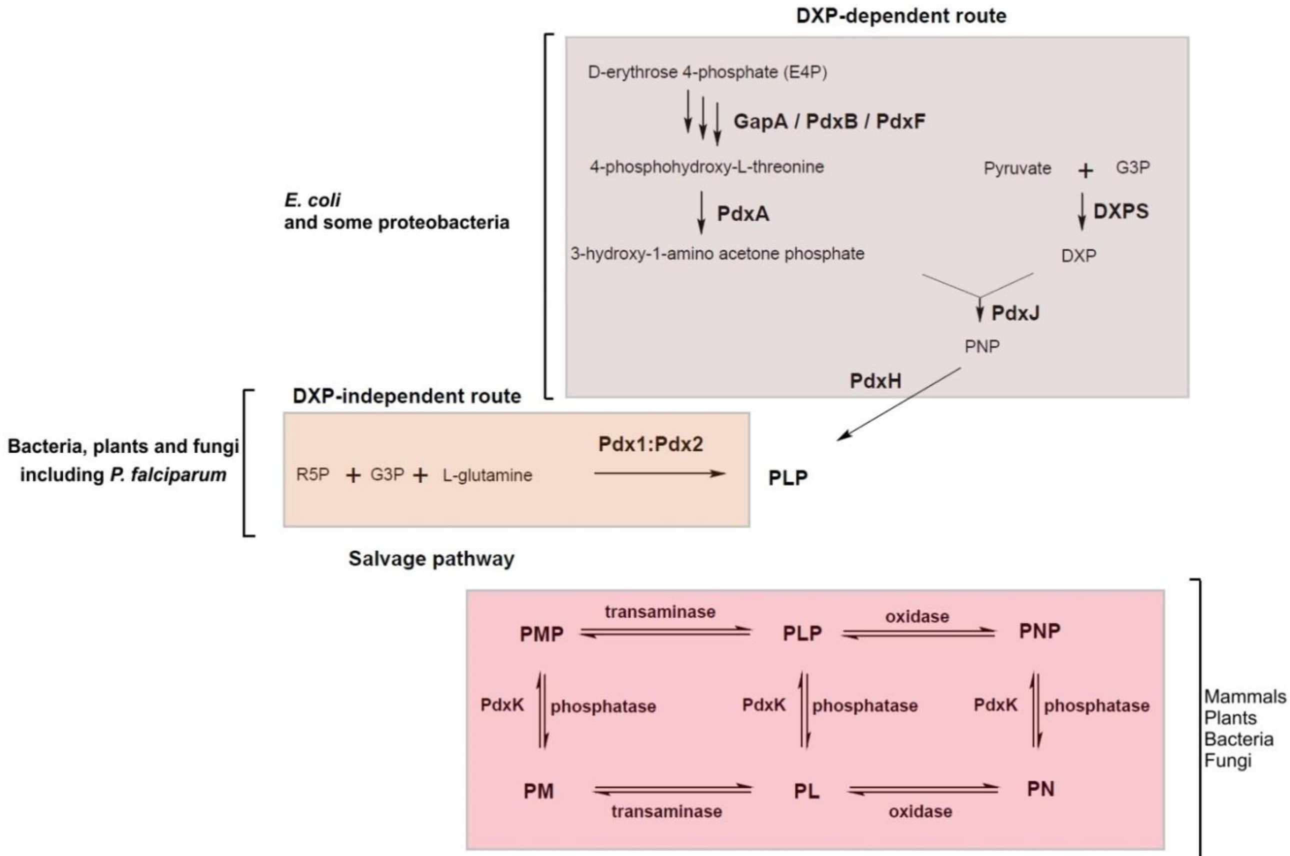

Figure 1.4: Summary of vitamin B_6 biosynthesis and salvage. There are two biosynthetic pathways for the formation of PLP. The DXP-dependent route utilises E4P, which undergoes enzymatic conversion,together with DXP to form PNP. This reaction is catalysed by PdxJ. DXP is derived from pyruvate and G3P by DXP synthase. The final PNP molecule is oxidised to PLP by PdxH. The DXP-independent route involves a single PLP synthase, composed of proteins Pdx1 and Pdx2. These proteins condense R5P, G3P and L-glutamine to form PLP. Salvage of the B_6 vitamers also constitute towards the PLP pool. Pyridoxine kinase (PdxK) are responsible for phosphorylation of PM, PL and PN. Phosphatases are involved in the dephosphorylated of PLP, PNP and PMP. For a comprehensive summary of biochemical entities in the pathways see Mukherjee *et al.* [121].

Mammals do not have the required biochemical machinery for *de novo* PLP synthesis, and instead salvage B_6 vitamers from their diet (Figure 1.4) [104]. Unphosphorylated vitamers are absorbed through diffusion from the intestine and pyridoxine kinases (PdxK, E.C. 2.71.35) are involved in phosphorylation to form PLP, PMP and PNP [106]. This effectively traps the different B_6 vitamers. Flavin mononucleotide (FMN)-dependent PNP oxidases are responsible for conversion of PNP and PMP to PLP (Figure 1.4) [122]. Pyridoxal phosphatases (PdxP, E.C. 3.1.3.74) can dephosphorylate

PLP, PNP and PMP in order to allow transport across cellular membranes [122]. Transaminases facilitate interconversion of PMP and PM to PLP and PL, respectively (Figure 1.4) whereas pyridoxine 5-phosphate oxidases (E.C. 1.4.3.5) are able to convert PNP and PN to PLP and PL respectively [106, 121, 123, 124].

1.3.2 Vitamin B_6 in the erythrocyte

Erythrocytes transport B_6 vitamers within the body. Dietary acquired PN, PL and PM enters the erythrocytes through diffusion, and are converted to their phosphorylated forms by PdxK [123-126]. Molecular trapping through phosphorylation, rather than active transport, drives the accumulation of B_6 within the erythrocytes (Figure 1.5) [127]. The phosphorylated vitamers PNP and PMP are converted to PLP by pyridoxine 5-phosphate oxidase (E.C. 1.4.3.5) and [123, 124] or via transamination reactions [121]. Within the erythrocyte, phosphorylated B_6 vitamers can be de-phosphorylated by PdxP whereas tissue non-specific alkaline phosphatases (E.C. 3.1.3.1) and liver acid phosphatases (E.C. 3.1.3.2) with broad substrate specificity performs this function in other tissues [123, 128]. In the erythrocyte a large proportion of PLP is bound to Hb and this both protects free PLP from hydrolysis and aids in establishing the concentration gradient to facilitate accumulation [127, 129]. PLP forms a Schiff base with a N-terminal valine of the β chains and reduces the oxygen affinity of Hb [130]. Hb constitutes 95% of the total erythrocyte protein content and is present at a intracellular concentration of 5mM [46]. The average erythrocyte PLP concentration is 410 pmol/g Hb, equivalent to 0.247 ng PLP/g Hb, and this low physiological concentration compared to Hb suggests that a very small proportion of Hb is PLP-bound (PLP binding to Hb is therefore not saturated at physiological conditions) [131]. Human erythrocytes possess the full complement enzymes required to interconvert B_6, contain greater oxidase activity compared to other tissues, and high levels of Hb, therefore are believed to be the main courier of B_6 within the body [128].

1.3.3 Vitamin B_6 metabolism in the malaria parasite

The malaria parasite occupies erythrocytes during asexual proliferation in the human, consuming host cell nutrients and proteolytically digests host proteins like Hb. This is complemented in part by *de novo* production of other essential metabolites, vitamins and amino acids. PLP in the parasite could be derived from two different routes – *de novo* biosynthesis through the action of PLP synthases, or through vitamin B_6 salvage pathways. In *P. falciparum* salvage of B_6 vitamers

minimally contributes toward the total vitamin B_6 pool, with recent isotopic tracer experimentation suggesting that *de novo* production through PLP synthase is the major route in which parasites obtain this essential cofactor (unpublished experiments, C. Wrenger). The necessary enzymes are present, as discussed below, however it has not yet definitively been established whether these promote salvage of B_6 vitamers or are involved in interconversion of different B_6 vitamers within the parasite.

Two potential salvage routes are possible - the salvaging unphosphorylated B_6 vitamers through diffusion from the erythrocyte or alternatively as Hb-bound forms (Figure 1.5). Pyridoxal kinases (PdxK) have been identified in *P. falciparum* and was shown to be involved in the phosphorylation of the various inactive B_6 vitamers, utilising adenosine triphosphate (ATP) as a substrate [132]. Through phosphorylation, PdxK effectively traps PLP within the parasites [133]. Prodrugs, consisting of PN piggybacked onto tryptophan methyl esters, were shown to be phosphorylated by parasitic PdxK resulting in trapping of toxic adducts capable of inhibiting other PLP-dependent enzymes [105]. Uptake of these was presumed to be through an amino acid transporter, which may recognise the tryptophan moiety.

Alternative sources of PLP which form part of the salvage pathway could include release of PLP during proteolysis of Hb in parasite acidic digestive food vacuoles (Figure 1.5). The recent discovery of acid phosphatases suggests that unphosphorylated B_6 vitamers could potentially be salvaged via this route [134]. The acid phosphatases are secreted via the plasma membrane to the parasite food vacuoles and have broad substrate acceptance, including the ability to accept PLP. This implies that PLP from Hb in the food vacuoles could be dephosphorylated, and capable of entering the parasites through diffusion. PdxK could re-activate PL into the active cofactor.

Enzymes involved in interconversion of PNP, PMP and PLP such putative pyridoxine oxidases (PF14_0570) have not experimentally been verified, and raises the question whether the parasites are capable of, or have need for, interconversion between PNP, PMP and PLP [135]. Other components of the salvage pathway include PLP phosphatase (PdxPP, PF07_0059) also termed 4-nitrophenyl phosphatase (PNPase), which dephosphorylate PLP to produce PL [133, 136]. *P. falciparum* parasites have a cytosolic expressed PNPase protein with broad substrate acceptance [136]. The exact contribution of these cytosolic PNPases and secreted acid phosphatases in B_6 metabolism remains to be determined.

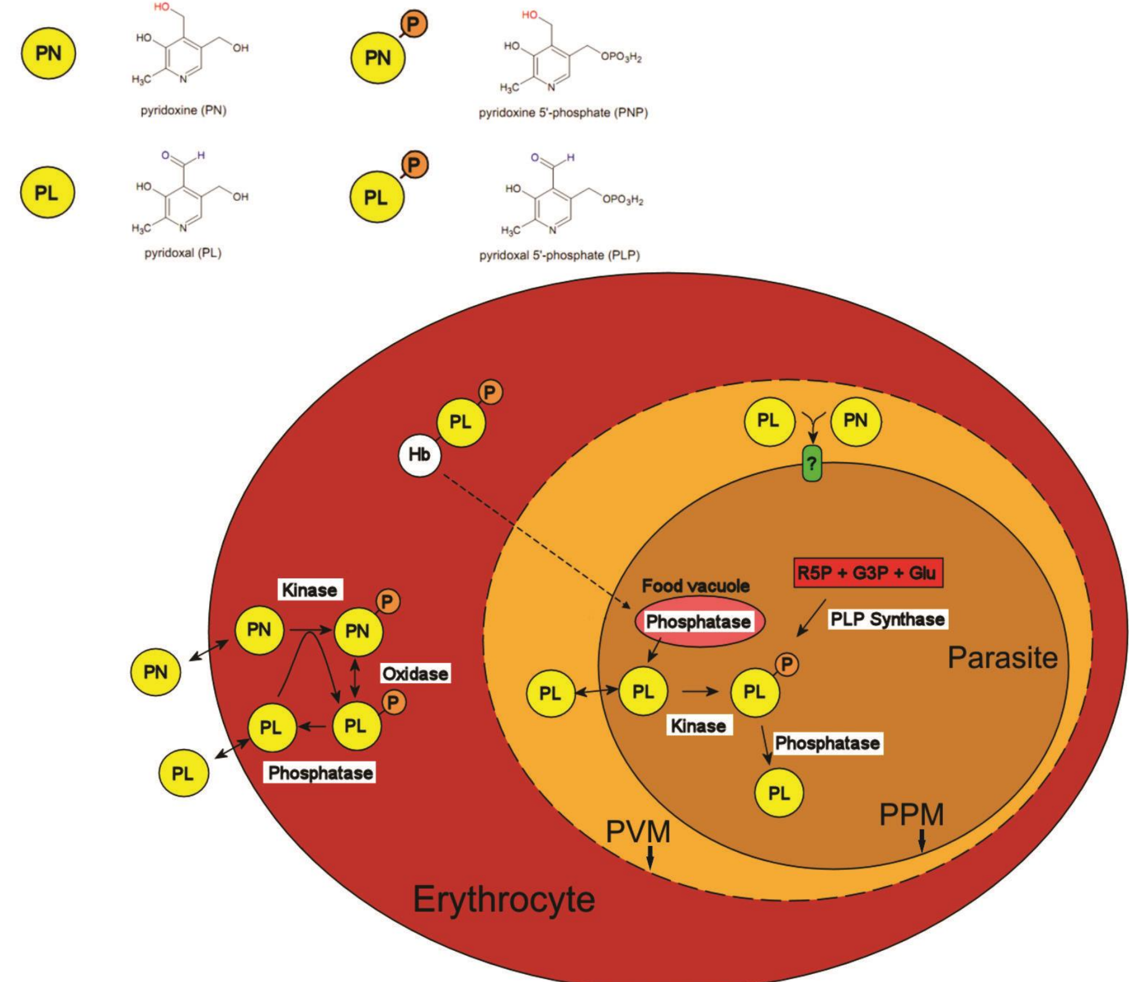

Figure 1.5: Erythrocyte and *P. falciparum* B_6 homeostasis. Unphosphorylated PN, PM and PL can diffuse into the erythrocyte, and are trapped within via phosphorylation by PdxK. The resulting PNP and PMP can be converted into PLP by oxidases and transaminases. PdxP phosphatases catalyse dephosphorylation of PLP and PNP to allow diffusion from the erythrocyte back to peripheral tissues. Unphosphorylated B_6 vitamers can diffuse into the malaria parasite. PLP can enter when attached to Hb, and is released in the acid food vacuoles in which phosphatases are involved. The parasite PdxK can convert salvaged PL to PLP. The parasites possess PLP synthases which can produce PLP from R5P, G3P and L-glutamine. PVM - parasitophorous vacuolar membrane and PPM - parasite plasma membrane.

In *P. falciparum* parasites, PLP is produced *de novo* by PLP synthase. The *P. falciparum* PLP synthase consists of *Pf*Pdx1 and *Pf*Pdx2 which assembles into a dimer, which arranges into a large dodecameric complex [133]. *Pf*Pdx1 and *Pf*Pdx2 form an obligate heterodimeric complex, and one cannot function without the other in the cellular environment (*in vitro Pf*Pdx1 can function without *Pf*Pdx2 when supplied with ammonia). *Pf*Pdx2 are glutaminases (or glutamine amidotransferases) which produce ammonia through the hydrolysis of L-glutamine which is channelled to Pdx1 subunits [104]. The *P. falciparum* Pdx2 protein was first annotated as the SNO-type pyridoxine biosynthesis protein in a proteomic study by Lasonder *et al*. [137]. Wrenger *et al.* confirmed the single gene copies of *Pf*Pdx1 and *Pf*Pdx2 were present in *P. falciparum* parasites, [132]. The

*Pf*Pdx1 and *Pf*Pdx2 proteins are translated from single gene copy mRNA's, with maximal mRNA transcript expression at 26 hours post invasion (HPI) corresponding to trophozoite parasite stages [132]. Gengenbacher *et al.* later revealed that corresponding protein levels only peaked at the later 34 HPI schizont parasite stages and the proteins are localized to the cytosol of the parasite [104]. Compared to the energy demanding PdxK ATP-catalysed reactions for salvage of PLP, *de novo* biosynthesis of PLP is a low cellular-energy cost route for maintaining the PLP levels.

1.3.4 Vitamin B_6 biosynthesis in *P. falciparum* as drug target

Free haem iron and haem moiety release during Hb digestion is associated with oxidative stress and generates superoxide anions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH·) [46]. The parasites combat oxidative damage through release of superoxide dismutases and catalases, and also inactivate free haem through polymerisation to form inert hemozoin [46]. PLP and other B_6 vitamers are potent antioxidants capable of quenching $^1O_2$ [117, 138, 139]. PLP can reduce superoxide anions (O_2^-), and is a potent antioxidant comparable to vitamin C [116, 117]. During the IDC of the malaria parasite $^1O_2$ was shown to peak during the trophozoite developmental stage and has been correlated with Hb digestion [140]. The expression of *Pf*Pdx1 and *Pf*Pdx2 during trophozoite and schizont parasite stages coincides with degradation of large amounts of Hb [132]. Moreover, *Pf*Pdx1 and *Pf*Pdx2 transcripts were overexpressed during methylene blue and cercosporin stress-treatment [132, 141]. This implied that *Pf*Pdx1 and *Pf*Pdx2 could aid during oxidate stress caused by ROS. Episomal over-expression of both *Pf*Pdx1 and *Pf*Pdx2 in the parasites resulted in increased levels of PLP and was shown to protect parasites from a sub-lethal dose of cercosporin, a singlet oxygen producing toxin from the pathogenic fungus *Cercospora nicotinae* [141]. Moreover, parasites over-expressing *Pf*Pdx1 and *Pf*Pdx2 proteins were less sensitive to cercosporin, emphasising the role PLP plays in neutralization of ROS [141]. The PLP synthase is therefore an important and indispensable protein that produces PLP which limits the cellular stress caused by ROS. These studies support *Pf*Pdx1 and *Pf*Pdx2 as good candidates for drug design; however there are no known inhibitors that target the PLP synthase. This therefore establishes the incentive for drug discovery on the PLP synthase.

Table 1.2: PLP-dependent enzymes in *P. falciparum*. Table was adapted from Müller *et al.* [108].

Enzyme name	EC number	PlasmoDB ID	Involvement in metabolism
Serine hydroxymethyltransferase (SHMT)	2.1.2.1	PFL1720w PF14_0534 (putative)	Folate metabolism
p-aminobenzoic acid synthetase	4.1.3.38	PFI1100w	Folate biosynthesis
Cysteine desulfurase	2.8.1.7	MAL7P1.150 (putative) PF07_0068 (putative)	Iron-sulphur cluster synthesis
Aspartate aminotransferase, aspartate transaminase (AspAT)	2.6.1.1 or 2.6.1.57	PFB0200c	Amino acid metabolism
Lysine decarboxylase	4.1.1.18	PFD0285c (putative) PFD0670c (putative)	Polyamine metabolism
Ornithine aminotransferase (OAT)	2.6.1.13	PFF0435w	Arginine and proline metabolism
Ornithine decarboxylase (ODC) in bifunctional S-adenosylmethionine decarboxylase-ODC	4.1.1.17	PF10_0322	Polyamine metabolism
Delta-aminolevulinic acid synthetase	2.3.1.37	PFL2210w	Porphyrin metabolism, amino acid metabolism
Serine C-palmitoyltransferase (SPT)	2.3.1.50	PF14_0155 (putative)	Sphingolipid metabolism
Phosphatidylserine decarboxylase (PSDC)	4.1.1.65	PFI1370c	Glycerophospholipid metabolism
Branched-chain amino acid aminotransferase	2.6.1.42	PF14_0557 (putative)	Pantothenate and CoA biosynthesis; amino acid metabolism

The malaria parasite possesses several PLP-dependent enzymes, and these have diverse involvement in amino acid, folate and polyamine-metabolism (Table 1.2). Several PLP-dependent enzymes are established targets for commercially available drugs; such as L-ornithine decarboxylase (ODC) for the treatment of African sleeping sickness caused by *Trypanosoma brucei gambiense* with α-difluoromethylornithine (Eflornithine) [142]), γ-aminobutyric acid (GABA)-aminotransferases for treatment of epilepsy using Vigabatrin [143], and L-3,4-dihydroxyphenylalanine (DOPA) decarboxylase for the treatment of Parkinson's disease and hypertension using carbidopa or Lodosyn [105, 144]. Some of these PLP-dependent enzymes have not been identified in *P. falciparum*, however the select few which are present, as listed in Table 1.2, are considered as good drug targets [145]. By targeting PLP biosynthesis and disrupting PLP homeostasis the multitude of PLP-dependent processes could be affected which should be lethal to the parasites. The diverse metabolic involvement of the PLP cofactor in these processes underscores the importance of the *de novo* PLP biosynthetic pathway, and makes *Pf*Pdx1 and *Pf*Pdx2 promising drug targets.

1.4 Hypothesis, research objective and aims

Hypothesis: Inhibition of *de novo* PLP biosynthesis in the malaria parasite *P. falciparum* is detrimental to parasite growth *in vitro* and results in compensatory responses functionally linked to PLP metabolism.

The objective of this study was the *in silico* identification of *Pf*Pdx1 inhibitors, analyses of their inhibitory capacity on parasite proliferation and the functional consequences thereof.

The aims of this study were to:

a) Use *in silico* / computational approaches to aid in the identification of potential inhibitory compounds against *Pf*Pdx1.
b) Determine the inhibitory capacity of *in silico*-identified and rationally selected compounds on *Pf*Pdx1 activity.
c) Analyse parasite proliferation when treated with *Pf*Pdx1 inhibitors.
d) Determine the global functional consequences of inhibiting *Pf*Pdx1 by analysis of its transcriptome and proteome of the parasites.

Chapter 2, describes homology modelling and structure-based virtual screening of *Pf*Pdx1 pharmacophores, of which hits were evaluated based on their chemical profiles and ability to dock into *Pf*Pdx1 homology models.

Subsequently, in Chapter 3, we asked if the *in silico* identified compounds were able to elicit inhibition of recombinantly expressed *Pf*Pdx1. Additionally, we also evaluated the inhibitory activity of rationally identified compounds on *Pf*Pdx1. Moreover, growth assays were used to show that an successful *Pf*Pdx1 inhibitor affected the proliferation of the parasites. Mutagenesis studies also helped identify essential residues required for a R5P reaction intermediate formation, contributing to the biochemical characterisation of this enzymatic activity.

Lastly, we asked if the identified *Pf*Pdx1 inhibitors were able to elicit inhibition of parasite proliferation and if so, what the functional consequences thereof were on the parasite. Chapter 4 describes an investigation of the inhibitory effect by monitoring global transcriptional and proteome-wide changes.

In a concluding discussion, Chapter 5 evaluates the scientific contributions made in this study and attempts to evaluate targeting of *de novo* PLP biosynthesis in the parasite as a viable strategy for novel antimalarial drug development.

1.5 Outputs

Research findings originating from this thesis has been presented at the following instances:

Conference proceedings:

1. Reeksting, S. B., Müller, I. B., Salmon, L., Louw, A. I., Birkholtz, L. M. and Wrenger, C. Identification of structure-based leads from the *Plasmodium falciparum* PLP synthase enzyme Pdx1. Polyamines in Parasites 2010, 6th Biennial Symposium, Phalaborwa, South Africa. (**Oral presentation**).
2. Reeksting, S. B., Müller, I. B., Salmon, L., Louw, A. I., Birkholtz, L. M. and Wrenger, C. Identification of inhibitors against the *Plasmodium falciparum* PLP synthase enzyme Pdx1. SASBMB 2012, Champagne castle, South Africa. (**Oral presentation**).
3. Reeksting, S. B., Müller, I. B., Salmon, L., Louw, A. I., Birkholtz, L. M. and Wrenger, C. Identification of inhibitors against the *Plasmodium falciparum* PLP synthase enzyme Pdx1. Molecular approaches to malaria (MAM) 2012. Melbourne, Australia. (**Poster presentation**).

Manuscripts:

1. Reeksting, S. B., Müller, I. B., Burger, P. B., Burgos, E. S., Salmon, L., Louw, A. I., Birkholtz, L. M. and Wrenger, C. (2013), Exploring inhibition of Pdx1, a component of the PLP synthase complex of the human malaria parasite *Plasmodium falciparum*. *Biochemical Journal*. **449**, p. 175-187.

2. Reeksting, S. B., Olivier, N., Burgos, E. S., Salmon, L., Louw, A. I., Birkholtz, L. M. and Wrenger, C. Functional consequences of targeting PLP synthesis as novel drug target in malaria parasites. S.B. Reeksting, J. Reader, N Olivier, C Wrenger and L Birkholtz. Manuscript in preparation.