Chapter 5
Concluding discussion

Malaria has plagued the world for many millennia. Efforts aimed at eradication have not been successful and at the present time more than a third of the human population still lives in malaria-stricken areas. Administration of antimalarial therapeutics and prophylaxis has been the most successful strategy, and is still the main-line defence we have against the parasites. Continual parasite drug-resistance has become a severe concern and drives the discovery and development of newer effective compounds. Previously successful antimalarials such as 4-aminoquinolines have set the precedent by exploiting endogenous parasite-specific processes such as haem detoxification. These compounds had good efficacy, were cheap and had limited host toxicity [299]. Amongst the successful antimalarials the antifolates have shown that endogenous parasite-only processes need not only be targeted in order to have a profound effect on treatment of malaria. However, in order to avoid targeting the host proteins additional pharmacokinetic optimisations are often required, and host-related metabolic perturbations may also require additional management complicating treatment. For these reasons the ideal candidates for target-based drug design remain parasite-specific processes. PLP biosynthesis is a parasite-specific process which is intricately regulated by two proteins – *Pf*Pdx1 and *Pf*Pdx2. The product of the PLP synthase is PLP - one of the most important cofactors in amino acid and polyamine metabolism as well as folate biosynthesis. The PLP synthase, or DXP-independent route of PLP biosynthesis was only recently discovered, and much regarding the regulation of this pathway within the parasite is not known. The absence of PLP biosynthetic pathways in humans gives a selective advantage for the exploitation of PLP biosynthesis in the malaria parasite.

PLP is involved in a myriad array of different metabolic processes and is a fascinating molecule. PLP can also facilitate non-enzymatic reactions [109]. Each chemical substituent on the PLP molecule is functionally essential; the phosphate group facilitates anchoring in PLP-dependent enzymes, whereas the pyridine ring facilitates delocalisation of negative charge at the C_α of reaction intermediates [300]. The principal component on PLP is the aldehyde group which forms the initial imine with embedded lysine residues in PLP-dependent proteins. Within the cellular milieu this group is relatively reactive and could be protected in order to avoid unnecessary conjugations. The Pdx1 and Pdx2 enzyme complex is therefore interesting within this regard, and questions arise as to how PLP is protected once synthesised. There is some evidence to suggest that PLP is tightly bound within Pdx1 [221]. In contrast, PLP is measured as a Tris-conjugate in Pdx1 reaction assays, and

this freely diffusible form suggests that PLP is not that tightly bound. How the PLP synthase complex sequesters and releases PLP remains to be discovered.

The objective of this study was to identify compounds that could interfere with *Pf*Pdx1 biosynthesis of PLP, and ultimately affect the proliferation of *P. falciparum* parasites. Computational approaches were performed to aid in the identification and selection of potential inhibitors of *Pf*Pdx1. A structure-based pharmacophore screening approach of *Pf*Pdx1 was followed. Initially several *Pf*Pdx1 homology models were created which shared homologous features compared to the *Pb*Pdx1 and *Tm*Pdx1 structures. The residues in the R5P-active site of *Pf*Pdx1 were shown to correspond to other structures, and were used to create structure-based *Pf*Pdx1 pharmacophores. Screening of these resulted in the identification of several ligands, as well as distinctive ligand scaffold groups. Docking was used to confirm predicted favourable binding in the R5P-active site of *Pf*Pdx1. The ligands that were identified had comparable or better predicted binding modes than R5P in *Pf*Pdx1. Some analogues of R5P, such as E4P and 4PEHz, were shown to have similar orientations in the active site of both *Pf*Pdx1 and *Pb*Pdx1.

The inhibitory capacity of *in silico*-identified ligands and other rationally selected compounds was determined on *Pf*Pdx1. Of the rationally selected compounds E4P showed some inhibitory activity. The E4P tetrose scaffold molecule had two specific chemical substituents which formed part of the molecule's pharmacophore; The C1 terminal aldehyde group, which was postulated to facilitate entry into the R5P-binding site (Figure 5.1). The exact mechanism is still speculative, and could involve initial imine formation with K83, thereby leading to disruption of R5P binding. The other pharmacophore on E4P was the phosphate moiety, which could have facilitated more favourable binding interactions within the R5P-binding site. Both these characteristics are shared by the substrates of *Pf*Pdx1 (Figure 5.1).

The limited efficacy of most of the *in silico*-identified compounds could have been related to the nature of the *Pf*Pdx1 protein. The initial requirement of Schiff base formation between R5P and K83 suggests that the aldehyde reactivity of R5P is an important requirement to enter the R5P active site [157]. The protein may therefore allow only molecules which conform to this prerequisite. Such features were also absent from the identified ligands. These are drug design considerations for future inhibitors which could be used to exploit this initial imine formation feature. There are numerous imine formation steps within the Pdx1 reaction mechanism, also recently proposed to occur during a C1 to C5 imine migration of R5P with K83 [160]. Compounds that contain chemical groups liable to imine formation such as ketones in place of hydroxyl substituents on the basic R5P scaffold may be promising inhibitors of *Pf*Pdx1. Sulphonate groups

were proposed to mimic phosphate groups of R5P, however whether these might do so *in vitro* is still questionable, as results from this study were inconclusive. A sulphonated compound with terminal aldehyde arrangement, plus the potential to interfere with imine formation, could have potential as a *Pf*Pdx1 inhibitor.

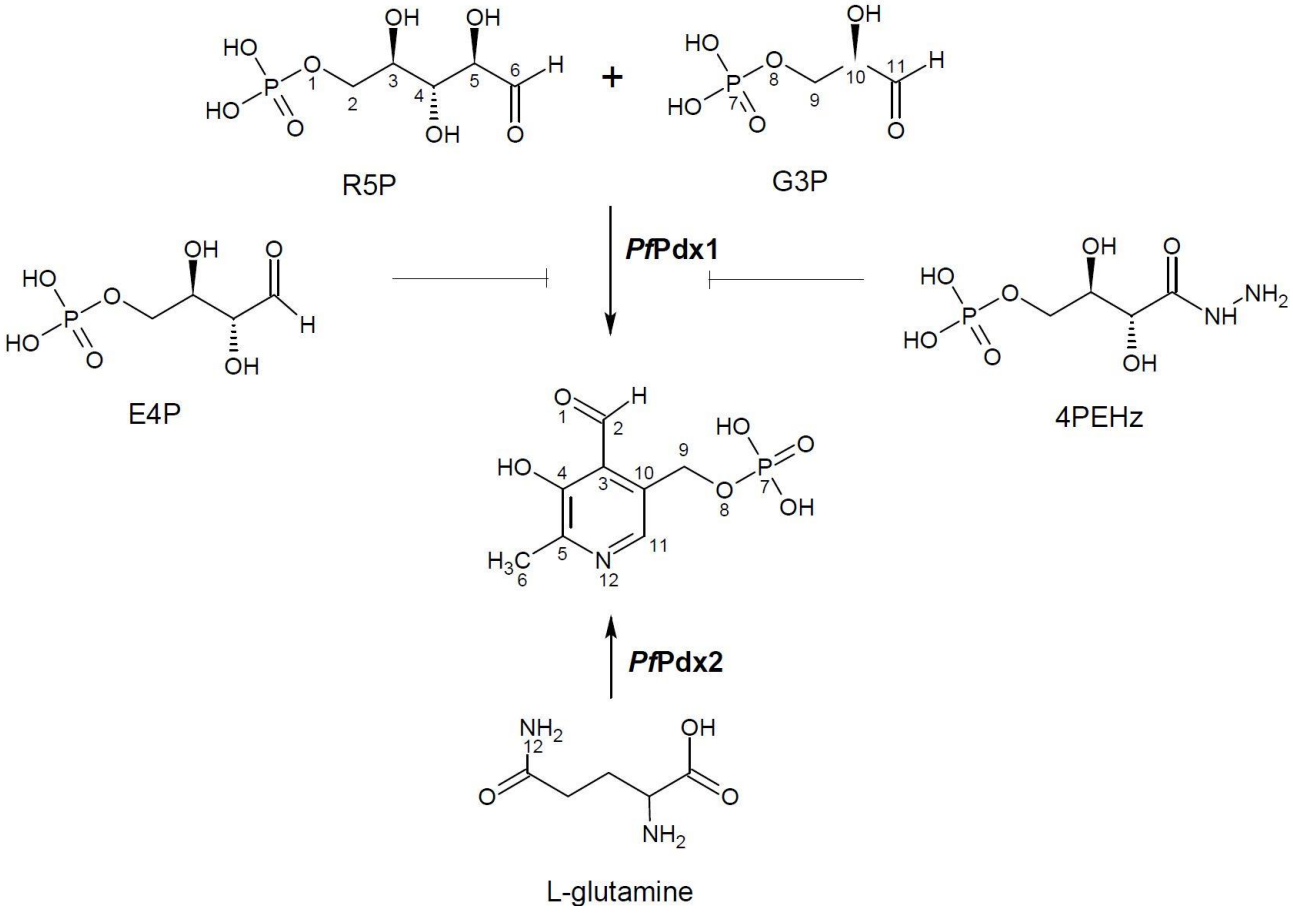

Figure 5.1: Summary of the formation of PLP by *Pf*Pdx1 and *Pf*Pdx2 with inhibitors. The PLP molecule is numbered according to the contribution of atoms from the R5P, G3P and L-glutamine substrates. E4P, R5P and G3P share chemical features such as terminal aldehyde groups and phosphate moieties. The 4PEHz inhibitor is based on the E4P scaffold with an additional hydrazide terminal group.

Analogues of E4P were selected and tested against *Pf*Pdx1. The 4PEHz compound showed appreciably inhibition of *Pf*Pdx1. Molecular docking supported the view that both E4P and 4PEHz could bind in the R5P-active site of *Pf*Pdx1. As part of the SAR the phosphate moiety of 4PEHz was established as part of the molecule's pharmacophore. This correlated with observations for E4P suggesting that this was an essential requirement for *Pf*Pdx1 inhibitors, and supported the prediction that 4PEHz occupies the same site as E4P. The increased inhibitory activity of 4PEHz when incubated with both *Pf*Pdx1 and *Pf*Pdx2 was linked to the cooperative binding of R5P in other PLP synthases and supported that 4PEHz could bind in the R5P-acitve site.

Pdx1 proteins share high degree of structural conservation, and a similar inhibitory MOA is therefore expected for E4P and 4PEHz against Pdx1 homologues in other organism. Both components of the PLP synthase are good drug targets. Inhibition of the *Pf*Pdx2 activity or inhibition of dimerization could be additional strategies to target the PLP synthase. The compounds identified here can also be utilised to manage other parasitic or bacterial infections. The causative agent of toxoplasmosis - *Toxoplasma gondii* – possess a functional *de novo* vitamin B_6 pathway which could similarly be exploited using E4P and 4PEHz [187]. Moreover *Mycobacterium tuberculosis* is solely reliant on the DXP-independent pathway for survival and knockout experiments have already shown that attenuation of the Pdx1 is a viable strategy to combat tuberculosis [301]. The importance of vitamin B_6 biosynthesis in plants could be exploited by using Pdx1 inhibitors as alternative herbicides, and considering the antioxidant properties of PLP which aids during abiotic stress, these in conjunction with other herbicides are expected to have a synergistic effect in management of unwanted crops [302].

The proliferation of *P. falciparum* parasites was analysed during treatment with the *Pf*Pdx1 inhibitor 4PEHz. The compound was detrimental to the proliferation of *P. falciparum* parasites *in vitro*. In order to establish whether inhibition was attributed to targeting of *Pf*Pdx1 and subsequently PLP biosynthesis, a cell line overexpressing *Pf*Pdx1 and *Pf*Pdx2 was tested with 4PEHz. These cells were significantly less susceptible to 4PEHz, and the parasite growth was not significantly perturbed compared to cells lacking additional copies of *Pf*Pdx1 and *Pf*Pdx2. These results suggested that 4PEHz had some specificity and was capable of targeting *Pf*Pdx1. To determine the global functional consequences of inhibiting *Pf*Pdx1 the transcriptome and proteome of the parasites treated with 4PEHz was analysed. Transcriptomic responses suggested that 4PEHz caused PLP-specific perturbations, however unrelated functional pathways were also affected. Attenuation of PLP biosynthesis could have caused major disruptions attributed to the ubiquitous involvement of PLP in cellular metabolism. Disruption of folate metabolism, which results in DNA lesion, has previously been linked to PLP depletion [274]. Corroborating with this, transcripts of enzymes involved in folate metabolism and recycling, such as SHMT, were found to be systematically affected during treatment of parasites with 4PEHz. This suggested that PLP biosynthesis was disrupted and also resulted in damage of DNA. In particular the *Pf*Pdx2 transcript expression was increased in 4PEHz-treated parasites. *Pf*Pdx2 has no other function apart from forming an obligate heterodimer with *Pf*Pdx1. Through guilt by association PLP biosynthesis was directly affected by 4PEHz suggesting that the compound had specificity for *Pf*Pdx1. Results from proteomic perturbations supported that L-glutamine catabolism was favoured, possibly due to decreased utilisation of this substrate by *Pf*Pdx2.

Whether PLP biosynthesis in *P. falciparum* is regulated transcriptionally, translationally, post-translationally is not known. Several investigations have shown that the transcripts of *Pf*Pdx1 and *Pf*Pdx2 are transcriptionally modulated, however were not always associated with general stress responses. This study supported the transcriptional regulation of *Pf*Pdx2, which was linked to inhibition of *Pf*Pdx1. Evidence from this study implicated that the PLP synthase activity could be regulated through inhibition by endogenous E4P. Both E4P, as well as D-xylulose 5-phosphate, is formed during the condensation of F6P and G3P by transketolase which are present in the parasites [216]. Transketolases play an essential role in the non-oxidative PPP branch by generating R5P from G3P and F6P via D-xylulose 5-phosphate [216]. D-xylulose 5-phosphate can be reversibly converted to Ru5P [215, 303], and in *P. falciparum* a putative Ru5P 3-epimerase (PFL0960w) has been implicated in this process [304]. The Ru5P is then isomerised to R5P by putative R5P epimerase (PFE0730c) [215]. Interestingly, the non-oxidative PPP pathway accounts for more than 80% of the carbon flux derived from [1-^{14}C]glucose which incorporated into nucleic acids [305, 306]. Moreover, the transcripts of enzymes involved in the non-oxidative PPP branch are co-ordinately expressed during the IDC of the parasites, highlighting the essential functional activity of this pathway [215]. Considering that E4P is the indirect by-product of the formation of R5P, appreciable levels of E4P are expected in the parasites, additionally also diverted into the shikimate pathway for the production of chorismate [216]. E4P metabolite levels in *P. falciparum* have not yet been established, nor have detectable levels been found in erythrocytes, and could be of interest for future investigations [307]. E4P could have a weak regulatory role on PLP biosynthesis, however other molecules or stereoisomers of E4P such as D-threose could also affect *Pf*Pdx1, and remains to be determined. Other stereoisomers of R5P which possess similar reactive groups, such as the product of the non-oxidative PPP arm - D-xylulose 5-phosphate - could also inhibit *Pf*Pdx1. Other means of regulating PLP biosynthesis includes the possibility of allosteric sites on the PLP synthase.

Based on results presented 4PEHz is a good therapeutic candidate, however additional chemical optimisation will be required to increase the potency of the compound before this compound is suited for clinical or *in vivo* investigations. Additional analogues of the lead E4P compound could also be created to further explore variations in the basic scaffold. The chemical reactivity of both the lead compound E4P and 4PEHz are undesirable properties, however the nature of the interaction with *Pf*Pdx1 was shown to require these functional groups. Both the substrates of *Pf*Pdx1 are also considered to be chemically reactive, and additional considerations should be made during lead optimisations.

Contributions of this study showed that the PLP biosynthetic pathway of the parasite is chemically tractable or druggable. Some questions remains as the influence of B_6 vitamer salvage which could diminish the effects of *Pf*Pdx1 inhibitors. Currently salvage is believed to minimally contribute towards PLP metabolism; the proliferation of parasites in PN-free culture medium is not affected [108]. Additionally, the efficacy of 4PEHz was tested in conditions which should promote B_6 vitamer salvage (5 µM PN in the culture media), and the erythrocyte is considered a rich source of other B_6 vitamers [125, 127]. Unphosphorylated B_6 vitamers can enter the parasite by diffusion, however the final phosphorylation reaction catalysed by PdxK requires ATP. The *de novo* biosynthesis of PLP utilises substrates from glycolysis and the PPP pathways, moreover is ATP-independent. This supports the notion that B_6 vitamer salvage is not the predominant route in order to obtain PLP. This also underscores the importance of the *de novo* PLP biosynthetic route in the parasites. Inhibition of this key pathway, in combination with other antimalarials, could be a feasible clinical combination to treat and eliminate *P. falciparum* infections. Overall results from this thesis support the hypothesis that inhibition of *Pf*Pdx1 in malaria parasites is detrimental and leads to perturbations in PLP-dependent processes. This study has contributed new knowledge regarding inhibitors that target *Pf*Pdx1 and the effects of inhibiting PLP biosynthesis in *P. falciparum* parasites.