Chapter

Purine and Pyrimidine Pathways as Antimalarial Targets

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Abstract

Malaria continues to plague the endemic regions of sub-Saharan Africa and Southeast Asia. With the current development of artemisinin resistance and a risk of failure of the current first line therapies, there is a growing need for novel antimalarials. Purine and pyrimidine metabolism in *Plasmodium* is distinctly different from the human host, making these pathways valid targets for the development of novel antimalarials. Targeting key enzymes in these pathways with transition state analogs has provided high affinity inhibitors. Transition state mimicry can also provide selectivity for the parasite enzymes over the homologous enzymes of the human host. Resistance of *Plasmodium* parasites to current antimalarials will be compared to resistance development induced by transition state analogs inhibitors, a feature that may contribute to decreased resistance development. Tight binding and specificity of transition state analog inhibitors provide important features for novel antimalaria therapy with low toxicity and prevention of antibiotic resistance.

Keywords: purine salvage, pyrimidines, antimalarials, transition state analog inhibitors, resistance

1. Introduction

Malaria remains the leading cause of mortality in the endemic regions of Sub-Saharan Africa and Southeast Asia. The WHO estimates that in 2020, there were 241 million malaria cases globally with 627,000 associated deaths, an increase from years prior [1, 2]. Additionally, although progress continues to be made in some regions, such as Southeast Asia reporting a 78% reduction in malaria cases, Sub-Saharan Africa accounts for more than 90% of the global malaria incidence. Children 5 years and younger are highly susceptible to malaria with a 77% mortality rate [1, 3, 4].

Malaria is caused by the apicomplexan parasite, *Plasmodium sp*. The five main species able to cause disease in humans are *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium knowlesi* and *Plasmodium malariae. Plasmodium falciparum (P. falciparum)* is the most prevalent and the most lethal of these [5]. Malaria infection is established when an infected female anopheles mosquito takes a blood meal from humans and introduces sporozoites into the bloodstream which invade human liver hepatocytes. The sporozoites replicate in the hepatocytes for 5–8 days and can generate thousands of merozoites which are released to infect the erythrocytes [6, 7]. Asexual replication occurs in the erythrocytes where parasites

undergo several nuclear divisions and generate between 6 and 30 daughter merozoites per infected erythrocyte [6]. Symptomatic malaria is characterized by the sustained parasitization and destruction of red blood cells as well as the host immune responses. Thus, drug development efforts are directed toward the intra-erythrocytic asexual stage of the parasite.

Over the centuries-long battle with malaria, treatment has been developed from both natural product and chemical synthetic sources. Quinine, originally isolated from the bark of the cinchona tree, gave way to its synthetic counterparts, chloroquine, amodiaquine, and mefloquine, among others [8, 9]. Likewise, artemisinin was isolated from the Chinese herb, *Artemisia annua* (Qinghao), and gave way to the synthetic artemisinin derivatives now used in the first line malaria treatment, artemisinin-based combination therapy (ACT) [10]. However, resistance to these antimalaria treatments, have slowed global efforts toward the eradication of malaria [11–15]. Resistance development has led to increased efforts to identify novel antimalarials, including the recent development of the RTS,S malaria vaccine, providing a potential positive outlook for malaria control [16, 17]. New drug development efforts for malaria must identify novel targets, their mechanisms of action, and must be well tolerated with minimal side effects. Short treatment periods are essential to promote compliance and to minimize resistance development.

Transition state mimicry is a promising approach to malaria drug discovery. The technique utilizes features of transition state structure of essential target enzymes to produce tight binding and highly selective analogs [18]. Transition state analogs (TSAs) against the purine and pyrimidine pathway enzymes of *P. falciparum* have resulted in inhibitor molecules with the potential for new antimalarial combination therapies.

Rapid nucleic acid synthesis during intra-erythrocytic parasitic growth makes purine and pyrimidine synthesis in *Plasmodium* parasites an important target for novel drug development. Although both host and parasite share some enzymes in these pathways, key differences allow for *Plasmodium*-specific and selective molecules.

2. Purine metabolism

Plasmodium parasites are purine auxotrophs as they do not express the enzymes necessary to perform *de novo* purine synthesis [19, 20]. Substantial new DNA and RNA synthesis occurs during the asexual cycle of *Plasmodium*, requiring large amounts of purine and pyrimidine nucleotide precursors and the energetic contribution from ATP. Parasites therefore rely completely on the host erythrocytes for the salvage of purine nucleosides and nucleobases for the synthesis of adenylate and guanylate compounds. Hence the parasite genome contains highly expressed genes corresponding to purine transporters and purine salvage enzymes [21]. Human erythrocytes contain high (millimolar) concentrations of adenylate nucleotides, but *Plasmodium* parasites have no kinase to phosphorylate adenosine molecules or ribosyltransferases to salvage adenine from the host. Therefore, purine salvage in *Plasmodium* requires ATP catabolism, through hypoxanthine formation, by essential enzymes making up the purine salvage pathway (**Figure 1**) [22]. The central concept in targeting purine salvage pathways for *Plasmodium* involves prevention of hypoxanthine and inosine formation in both erythrocytes and parasites and/or prevention of hypoxanthine or inosine conversion to purine nucleotides in the parasites.

Purine precursors formed in erythrocytes must gain entry to the parasite for conversion to nucleotides. Pathways proposed to facilitate the uptake of purine

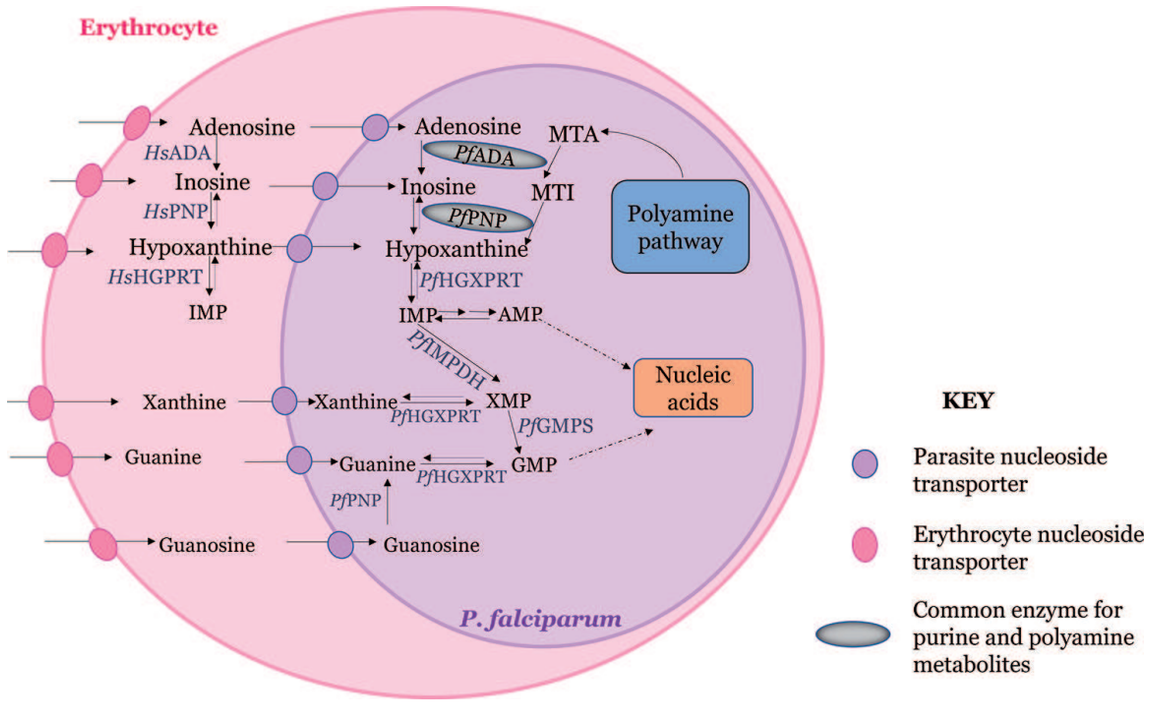

Figure 1.
Purine salvage pathway in P. falciparum and human erythrocytes. In P. falciparum, but not in humans, there is a link to the polyamine pathway via MTA.

nucleosides and nucleobases across the parasite membrane include; (1) saturable or facilitated transport of adenosine, (2) non-saturable, channel like transport and (3) the tubovesicular membranes induced by parasitization of the host erythrocytes [23, 24]. A major path for purine uptake is known to be the equilibrative nucleoside transporters (ENTs) that promote nucleoside and nucleobase transport into the parasite. Four classes of ENTs have been identified in *Plasmodium falciparum* and *Plasmodium vivax* (*Pf*ENT 1-4, *Pv*ENT 1-4) [24–28]. Inhibitors designed against *Pf*ENT1 prevent the uptake of purines and inhibit the growth of parasites in culture [29, 30]. The essentiality of the purine salvage pathway to the survival of *Plasmodium* parasites, makes targeting the component enzymes attractive for novel antimalarial therapies. These include adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT). Antimalarial validation of these targets has been confirmed by potent inhibitors that cause purine starvation and death of the parasites.

2.1 Adenosine deaminase (ADA, EC 3.5.4.4)

Plasmodium falciparum ADA (*Pf*ADA) catalyzes the zinc-dependent irreversible deamination of adenosine and deoxyadenosine to form inosine and deoxyinosine. *Pf*ADA can also catalyze the hydrolysis of 5′-methylthioadenosine (MTA) to 5′-methylthioinosine (MTI), a reaction that is distinctly absent from the mammalian ADA [31]. MTA is a product of the polyamine biosynthetic pathway, establishing that polyamine synthesis also plays a role in the purine salvage pathways for *Plasmodium* parasites. Parasites must metabolize MTA to prevent feedback inhibition of the polyamine biosynthetic pathway. Polyamine synthesis is also critical to the parasites since they cannot salvage polyamines from the host erythrocytes [see below]. Coformycin and 2′-deoxycoformycin are transition state or intermediate-like powerful inhibitors

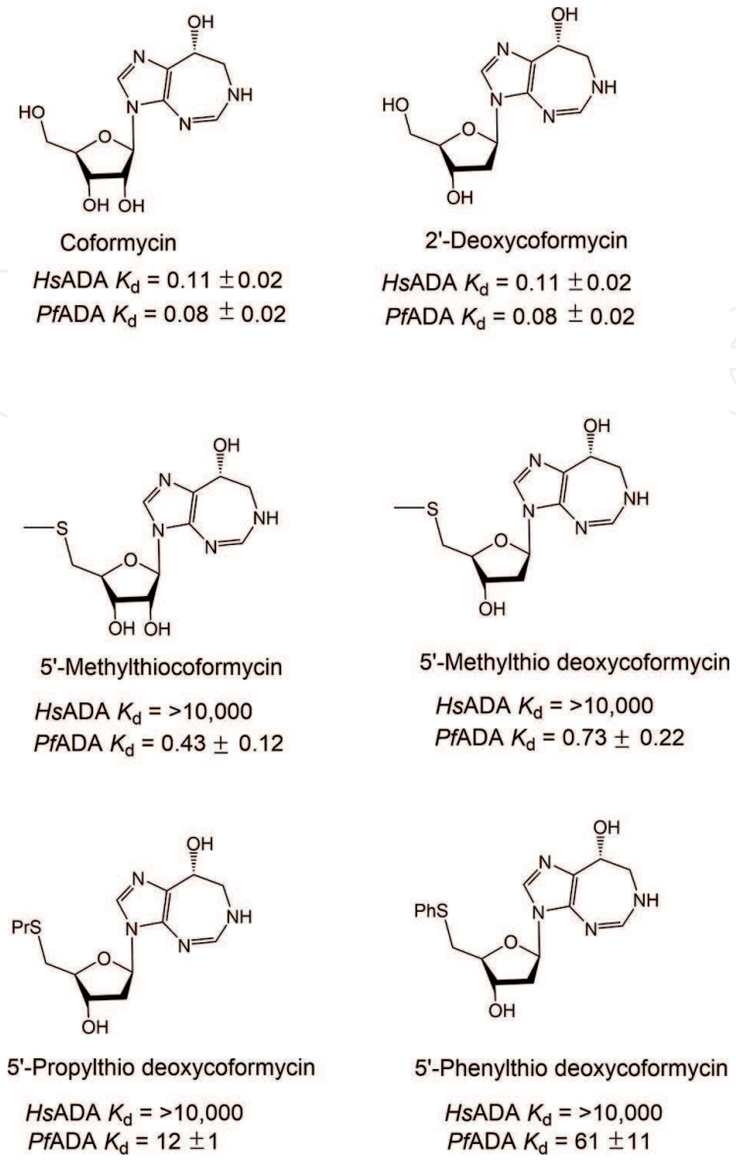

Figure 2.
Transition state analogs of HsADA and PfADA. The 5′-functionalized inhibitors provide improved selectivity for the parasite enzyme over the human enzyme. 5′-methylthio inhibitors are more potent thant the 5′-propylthio and 5′-phenylthio inhibitors.

of ADA. They were originally identified as natural products and inhibit both human and parasite enzymes with picomolar affinities (**Figure 2**) [32]. 2′-Deoxycoformycin (Pentostatin) is an FDA-approved treatment for hairy cell leukemia, where the accumulation of adenosine or 2′-deoxyadenosine, leads to an unbalanced nucleotide pool, initiating apoptosis in B and T cells [32]. Unfortunately, there is insignificant inhibition of *Plasmodium* parasite growth by these inhibitors.

The dual specificity of *Pf*ADAs serves to guide the synthesis of novel *Pf*ADA inhibitors with a high degree of selectivity for the parasite enzyme. In addition, the transition state structures of *Pf*ADA and human ADA (*Hs*ADA) are similar but distinct, with *Pf*ADA displaying an early transition state and a more intermediate-like transition state for *Hs*ADA, corresponding to their distinct catalytic turnover numbers (k_{cat}) (**Figure 3**). These differences are reflected by the state of protonation at N1 of the purine ring and the N1-H bond distances. The differences permit the design and synthesis of *Plasmodium*-specific transition state analogs [33]. Earlier work

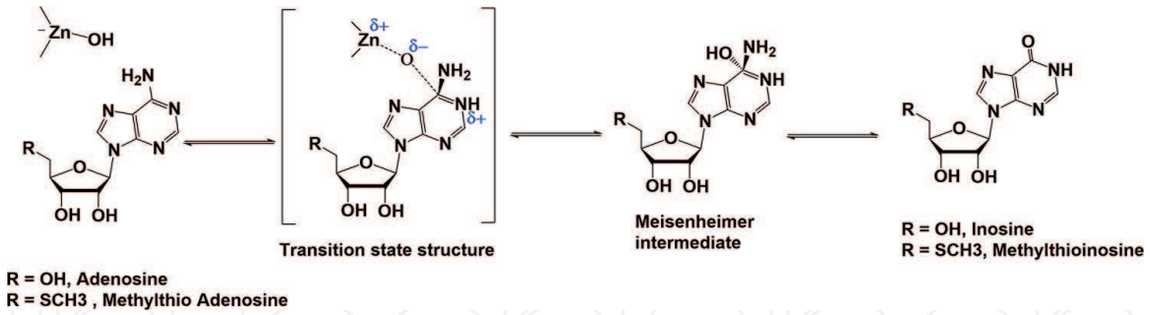

Figure 3.
Reaction scheme and transition state structure of ADA.

reported the synthesis of 5′-methythiolcoformycin (MT-coformycin), a sub nanomolar (K_i^* = 0.43 nM) transition state analog inhibitor of *plasmodium* ADA with over a 20,000-fold selectivity over the human ADA. Other functionalized 5′- and 2′-deoxy-coformycin molecules also retained selectivity for the *Plasmodium* enzyme (**Figure 2**) [31, 32]. MT-coformycin and coformycin inhibited the growth of *plasmodium* parasites in culture when MTA is used as the sole purine source, clearly demonstrating the deaminase as an essential step in conversion of MTA toward hypoxanthine [31, 34]. This effect is not observed when adenosine or MTI is used as a purine source, demonstrating that an intact polyamine pathway is not required for purine salvage in *Plasmodium* species. The high degree of selectivity of the 5′-functionalized coformycins demonstrates the importance of utilizing substrate specificity and transition state analysis to design target-selective inhibitors.

2.2 Purine nucleoside phosphorylase (PNP, EC 2.4.2.1)

The enzyme responsible for forming purine bases from nucleosides in the purine salvage pathway is PNP, catalyzing the reversible phosphorolysis of inosine to hypoxanthine and ribose 1-phosphate [35]. *Plasmodium* PNP substrate specificity includes MTI, to generate hypoxanthine and 5-methylthioribose 1-phosphate [36]. MTI appears to be a parasite specific metabolite and has not been identified in mammalian pathways [37]. Hypoxanthine serves as a key precursor to nucleotide synthesis; therefore, the parasite adopts multiple pathways to generate hypoxanthine. PNP also converts guanosine and 2′-deoxyguanosine to form guanine and (2-deoxy) ribose 1-phosphate (**Figure 1**). PNP displays specificity for 6-oxopurines with adenine containing purine rings having no effect as substrates or inhibitors [31].

Plasmodium falciparum PNP (*Pf*PNP) and human PNP (*Hs*PNP) have distinct structures and catalytic sites and share only ~20% sequence similarity [38]. Structurally, *Pf*PNP is more similar to PNP from bacterial sources and is homohexameric, displaying a trimer of dimer structure, while *Hs*PNP like other mammalian PNP is trimeric (**Figure 4**). Like *Pf*ADA, *Pf*PNP displays a broad substrate specificity that includes 6-oxopurine nucleosides and 5′-methylthio 6-oxopurine nucleosides as substrates. These differences form the basis for generation of species-specific inhibitors with discrimination for the *Plasmodium* enzyme.

The transition state structures of *Pf*PNP and *Hs*PNP, reveal similar catalytic mechanisms involving the formation of an oxocarbenium ion at the transition state (**Figure 5**). However, the two catalytic site interactions differ in the hydrogen bond and van der Waals interactions formed with the leaving group. The result is a more constrained active site environment at the transition state for *Pf*PNP than for *Hs*PNP [39]. The

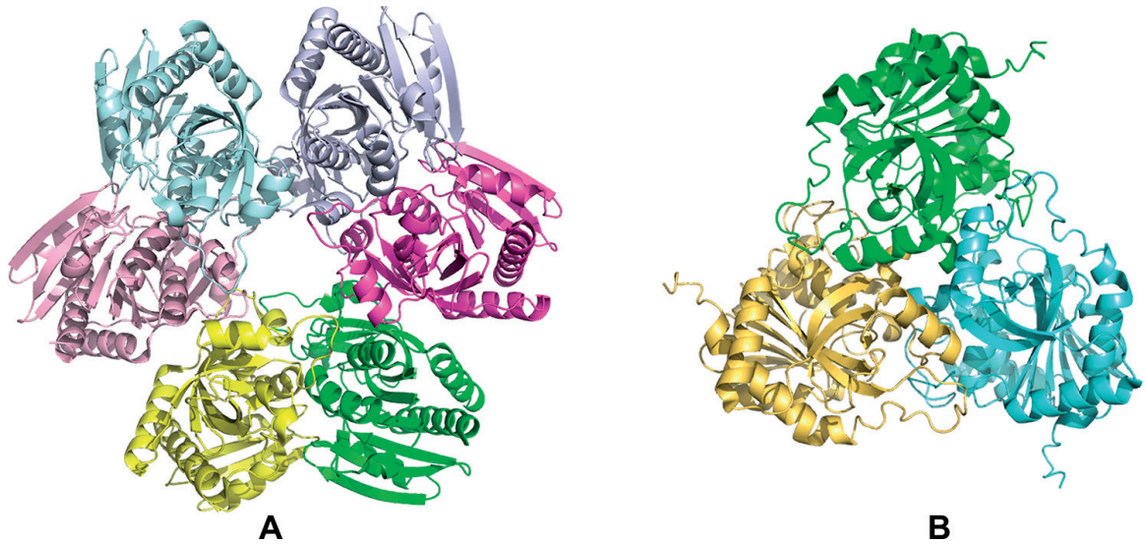

Figure 4.
A, Homohexameric structure of PfPNP displaying a trimer of dimer conformation (1NW4). B, trimeric structure of HsPNP (1PF7).

Immucillin-H (ImmH) transition state analog was designed to resemble the transition state structure of bovine PNP and inhibits that enzyme with a K_i^* of 23 pM. Although there are distinct differences between the transition state structures of *Hs*PNP, *Pf*PNP and bovine PNP, ImmH is a potent inhibitor of all three enzymes, with *Hs*PNP K_i^* = 56 pM and *Pf*PNP K_i^* = 600 pM [40, 41]. DADMe-ImmH and DADMe-ImmG were developed as second generation PNP inhibitors designed to mimic the fully dissociative transition state, and thereby more closely resemble the transition state structures of *Hs*PNP and *Pf*PNP [42]. The introduction of a methylene bridge increases the bond distance between the 9-deazahypoxanthine (DADMe-ImmH) or the 9-deazaguanine (DADMe-ImmG) and the riboxocarbenium mimic. Additionally, the 9-deazapurine scaffold increases the pK_a to permit protonation at N7 and the cation at N1′ mimics the cationic charge at this position of the transition state to more closely mimic the transition state. These features of the transition state translate to an improvement in the dissociation constant for *Hs*PNP from 56 pM for ImmH to 16 pM for DADMe-ImmH and to 2 pM for DADMe-ImmG. Transition state analogs bind tightly to their cognate enzymes by converting the catalytic potential into thermodynamic binding. The k_{cat} for *Hs*PNP is approximately 50 times greater than for *Pf*PNP, and this is reflected in the affinity of the inhibitors for the two enzyme species. Thus, the dissociation constant (K_i^*, the K_d after slow onset inhibition) for *Pf*PNP is 500 pM for DADMe-ImmH and is 670 pM for DADMe-ImmG, reflecting its lower catalytic potential [42–44].

Inhibition of both *Hs*PNP and *Pf*PNP is required to cause a purine-less death of *Plasmodium falciparum* parasites in culture, as formation of hypoxanthine in erythrocytes or in the parasite will meet the needs for purine base salvage. In cell cultures, *Hs*PNP inhibition occurs at low concentrations of PNP inhibitors with inhibition of *Pf*PNP occurring at higher concentrations. Inhibition of both PNPs is thus required for the antiparasitic effects of the immucillins, with the IC_{50} values corresponding to the molar concentration of PNP present [45, 46]. The purine-less death induced by PNP inhibitors targets hypoxanthine production. Confirmation of hypoxanthine starvation as the mechanism of action, and the lack of other targets from this therapy, comes from the full restoration of parasite growth when hypoxanthine is added to culture media [45].

The genetic deficiency of *Hs*PNP is known to cause a T cell immune deficiency by causing accumulation of 2′-deoxyguanosine (dGuo), a metabolite completely dependent on *Hs*PNP for its recycling. Without *Hs*PNP, activated T cells transport dGuo and convert it to dGTP which can reach toxic levels for DNA polymerase. In humans, activated T cells are therefore sensitive to the loss of *Hs*PNP function with no effect observed in naive T cells, B cells, and other mammalian cells [47, 48]. The selective toxic effects of *Hs*PNP inhibition in human T cells is slow to develop, taking a year or more to be symptomatic in newborns. The more rapid effect in *Plasmodium* suggests that inhibiting both human and parasite PNPs for antimalaria therapy will have minimal side effects. Furthermore, oral administration of DADMe-ImmG clears *Plasmodium falciparum* parasites from infected *Aotus* monkeys [49]. Although not yet tested in clinical trials for malaria, the companion compound, DADMe-ImmH, is also a powerful inhibitor of both *Hs*- and *Pf*PNPs and has been in extensive phase 2 clinical trials for gout [50]. PNP inhibitors as an antimalarial therapy block hypoxanthine formation in both erythrocytes and parasites. Human blood hypoxanthine is present at approximately 1 μM, while *Aotus* blood was found to have approximately 40 μM hypoxanthine [49]. Therefore, PNP inhibition as an antimalarial therapy is expected to be a more effective in humans than in the *Aotus* test animal where several days of oral drug therapy were required to clear the parasites [49]. This hypothesis remains to be tested.

Other selective inhibitors remain an attractive option when developing novel antimalarial therapeutics. Therefore, using the dual specificity of *Pf*PNP for MTI, the transition state analog, 5′-methylthio-immucillin-H (MT-ImmH) was synthesized [46]. MT-ImmH has a 100-fold specificity for *Pf*PNP over *Hs*PNP, underscoring the importance of exploring transition state chemistry and substrate specificity to develop selective inhibitors (**Figure 5**) [46].

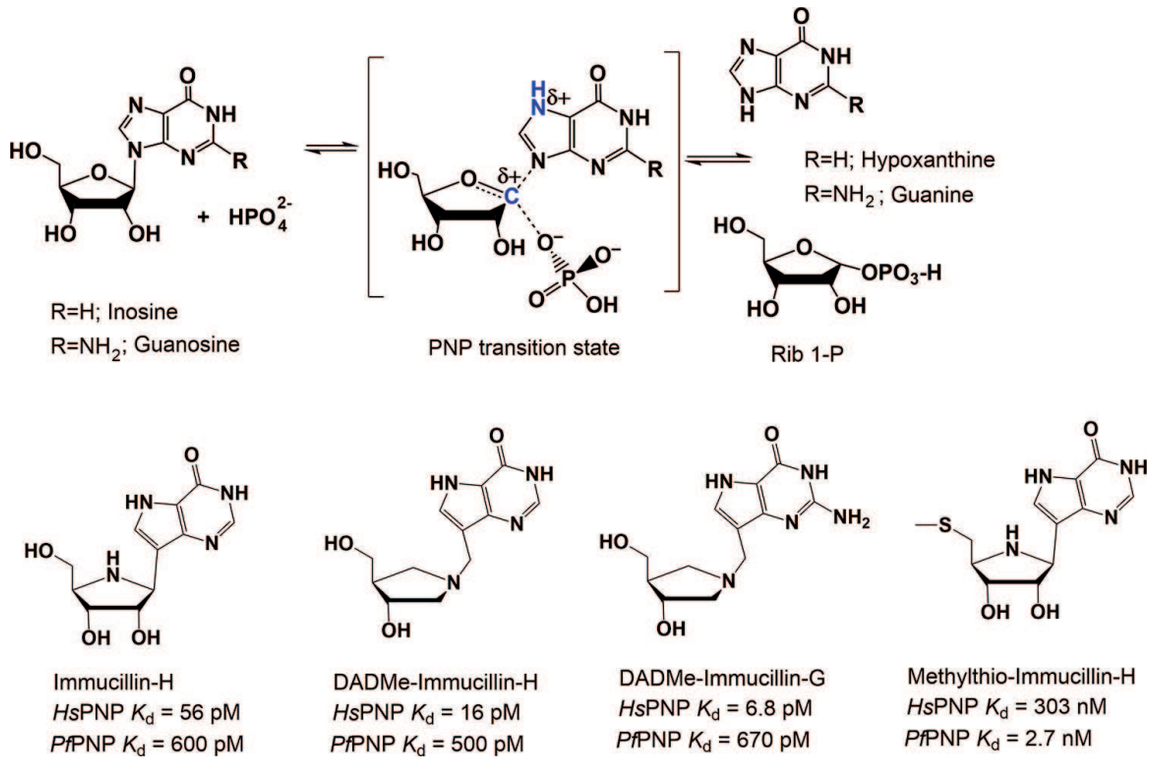

Figure 5.
Reaction scheme and transition state structure of PNP (upper panel). Lower panel; transition state analogs of PNP with dissociation constants for HsPNP and PfPNP. Methylthio-Immucillin-H provides over 100-fold selectivity for the parasite enyzyme.

2.3 Hypoxanthine-guanine-xanthine phosphoribosyltransferase (HGXPRT, EC 2.4.2.8, EC 2.4.2.22)

Hypoxanthine is the essential purine precursor for *Plasmodium* and HGXPRT is an essential enzyme in the *Plasmodium* purine salvage pathway, as the only enzyme to incorporate hypoxanthine into the parasite nucleotide pool. HGXPRT catalyzes the phosphoribosylation of hypoxanthine, guanine, and xanthine using 5-phosphoribosyl-α-D-1-pyrophosphate (PRPP) to generate inosine 5′-monophosphate (IMP), guanosine 5′-monophosphate (GMP) and xanthine 5′-monophosphate (XMP) respectively [51]. Pyrophosphate (PPi) is generated as the second product of the reaction (**Figure 6**). IMP serves as the precursor for the synthesis of both adenine and guanine-based nucleotides. The single distinction in substrate specificity between the *Plasmodium* HGXPRT (*Pf*HGPRT) and the human HGPRT (*Hs*HGPRT) is the inability of the human homolog to use xanthine as a substrate [52]. Xanthine in humans is converted to uric acid by xanthine oxidase and is the terminal end of purine metabolism, followed by its excretion into the urine [53, 54].

*Pf*HGXPRT has been identified as the most highly expressed purine salvage enzyme in *P. falciparum* and has been reported to have the highest activity compared to other enzymes of purine salvage identified by Reyes and coworkers [55]. Parasites may have evolved to use hypoxanthine as a key nucleotide precursor because it is the end-product of purine catabolism in human erythrocytes, with subsequent oxidation to uric acid occurring in other tissues. The high amounts of intracellular phosphates in erythrocytes favors the activity of PNP to produce hypoxanthine [55, 56]. Since its identification, *Pf*HGXPRT has been the most targeted enzyme for purine salvage in *P. falciparum*. Detailed kinetic and structural analyses have led to the design of many inhibitors of *Pf*HGXPRT activity. Challenges in targeting *Pf*HGXPRT include its similarities to *Hs*HGPRT and the need for the anionic phosphate group, making cell access a problem [57–60].

*Pf*HGXPRT and *Hs*HGPRT share 76% sequence similarity and 44% sequence identity [61]. Active site residues in contact with substrates and inhibitors are conserved (**Figure 7**). Purine nucleoside analogs and transition state analogs have been investigated and many prove to be good inhibitors of both *Pf*HGXPRT and *Hs*HGPRT.

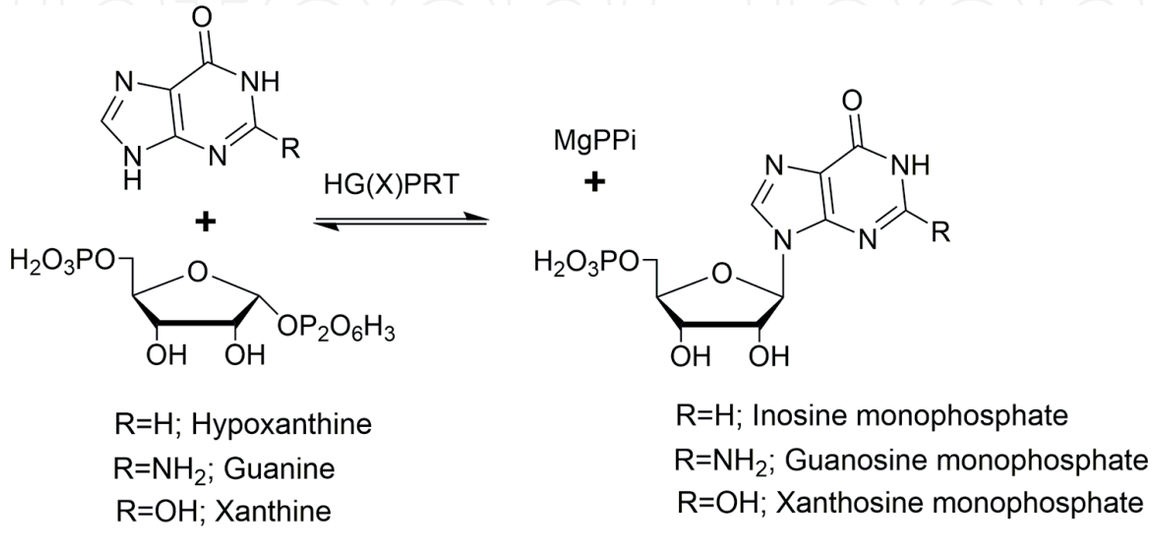

Figure 6.
Reaction scheme for HG(X)PRT. PfHGXPRT has a unique substrate specificity for xanthine. HsHGPRT does not.

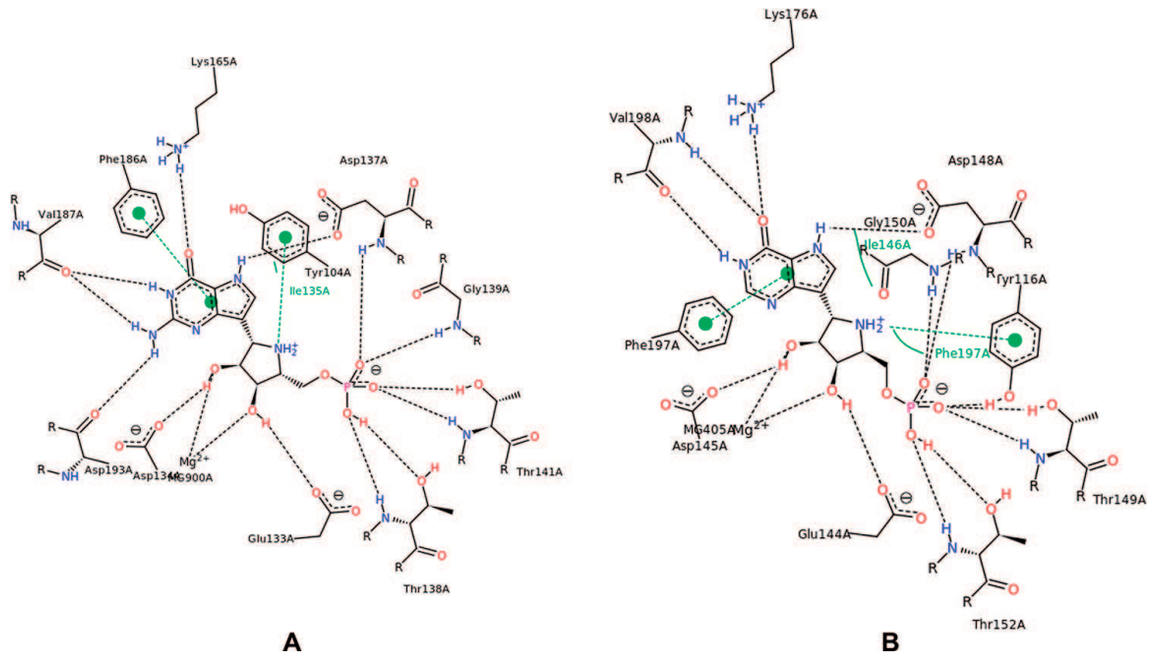

Figure 7.
Two-dimensional ligand interaction map showing A, Immucillin-G phosphate bound to HsHGPRT (1BZY) and B, Immucillin-H phosphate bound PfHGXPRT (1CJB). Active side residues that make contact with inhibitors are similar in both enzymes.

Acyclic nucleoside phosphonates (ANPs) however provide the greatest selectivity for the *Plasmodium* enzyme compared to phosphate-based compounds (**Figure 8**) [62, 63]. Crystal structures of *Hs*HGPRT in complex with ANP and free *Pf*HGXPRT suggest that the increased in selectivity when the phosphate groups are replaced with phosphonates is attributed to tighter hydrogen bonding around the phosphonate group and flexibility of the 6-oxo binding pocket [60].

TSAs are the tightest binding HG(X)PRT inhibitors reported to date. Although the first-generation transition state analogs, immucillin-G phosphate (ImmGP) and immucillin-H phosphate (ImmHP) were designed based on the proposed similarity of the transition state structure to other phosphoribosyltransferases, they bind potently but without discrimination for parasite or human HG(X)PRT (**Figure 8**) [41] and are subject to host enzymatic degradation by phosphomonoesterases. Structural analysis of TSAs bound to both *Hs*HGPRT and *Pf*HGXPRT as well as downfield proton chemical shift differences observed by NMR of bound inhibitors suggested that selectivity for *Pf*HGXPRT may be achieved by substituting TSAs with electron-withdrawing or electron-donating groups to alter hydrogen-bonding distances [41]. Recently, the transition state structure of *Pf*HGXPRT has been solved and provides detailed information on bond distances in the transition state [61]. With this knowledge, serinol-based mimics of the riboxocarbenium with a methylene bridge linking the oxocarbenium mimic to the 9-deazapurine ring were designed to mimic the bond distances in the transition state structure and synthesized. These compounds have proven to be the tightest and most selective inhibitors of *Pf*HGXPRT, with K_i^* values as low as 650 pM and selectivity indices for *Pf*HGXPRT greater than 500-fold relative to *Hs*HGXPRT [64, 65].

HGXPRT TSAs are phosphate or phosphonate-based compounds with a net negative charge at physiological pH, making them impermeable to cell membranes. Prodrug approaches are therefore required for their biological activity. Prodrugs of *Pf*HGXPRT inhibitors involve ANPs with intramolecular esters in the form of lysophospholipid prodrugs, designed to be activated by intracellular phospholipase C,

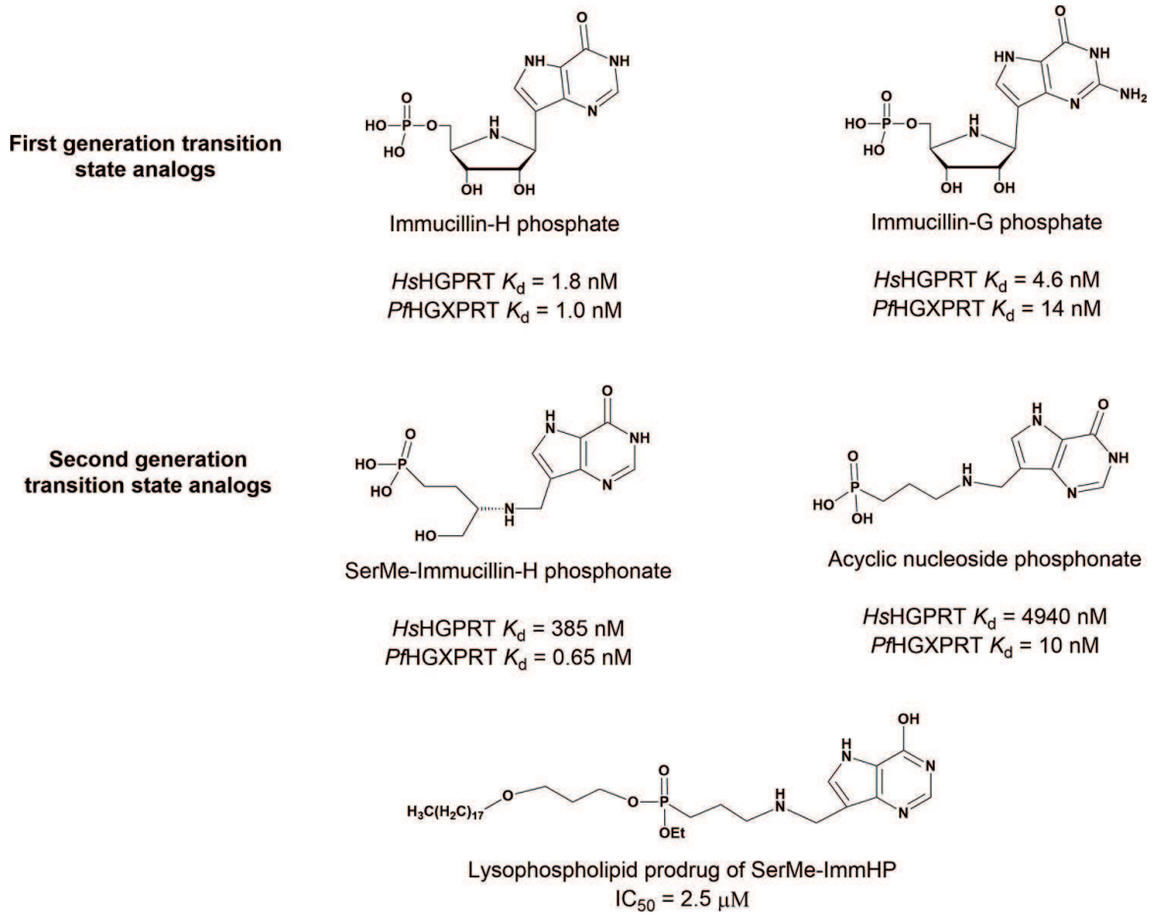

Figure 8.
First generation and second generation transtion state analogs for HG(X)PRT. Dissociation constants for HsHGPRT and PfHGXPRT are shown for each inhibitor. The second generation analogs provide greater selectivity for the PfHGXPRT. Lysophospholipid prodrug with biological activity against P. falciparum parasites in culture shown in lower panel.

or phosphoramidate prodrugs with a multistep activation mechanism [66]. Prodrug approaches have been validated in several FDA-approved nucleoside antiviral drugs [67]. The prodrug approaches improve the biological activities of TSAs of *Pf*HGXPRT to give anti-parasite IC_{50} values in the micromolar range (2–7 μM) against *P. falciparum* parasites cultured in human blood (**Figure 8**) [65]. With a K_i^* of 650 pM, disparity between the K_i^* for the enzyme and the micromolar IC_{50} values of lysophospholipid prodrugs suggests that more efficient prodrug approaches are needed to fully capture the tight-binding capabilities of TSAs intracellularly. *Pf*HGXPRT is a challenging target because of the multiple cellular and membrane barriers between an oral drug and the intracellular parasites. However, similar challenges have been overcome for antivirals and they provide a lesson for prodrug approaches. In summary, *Pf*HGXPRT is a challenging but valid target for the development of novel antimalarials and TSAs currently provide the most promising approach.

2.4 Downstream enzymes of purine salvage

Given how essential purine salvage is to the survival of *Plasmodium* parasites, it is remarkable that the pathway evolved to rely on only three major enzymes, ADA, PNP and HGXPRT, based on the genome interpretation and protein expression levels

in the parasites [55]. As concentration of free guanine in human blood is very low, it is not feasible for *Plasmodium* spp. to synthesize guanosine monophosphate (GMP) using HGXPRT. [51] Two enzymes downstream of HGXPRT, inosine 5′-monophosphate dehydrogenase (IMPDH) and GMP synthetase (GMPS) work sequentially to synthesize GMP (**Figure 1**) [68]. These two enzymes control GMP synthesis in most organisms [69]. IMPDH catalyzes the NAD^+ dependent reaction that coverts IMP to xanthine monophosphate (XMP) and NADH [70]. GMPS is composed of two domains, an ATP pyrophosphatase domain and a glutamine amidotransferase (GAT) domain [68, 71]. By this mechanism, GMPS catalyzes the ATP-dependent irreversible amination of XMP on carbon 2 to form GMP. Inhibitors of both IMPDH and GMPS have been shown to have antimalaria properties and have been the subject of recent drug development [21, 68].

Mycophenolic acid is an IMPDH inhibitor and inhibits *P. falciparum* parasites in culture with an IC_{50} of about 5 μM [21, 72, 73]. Bredinin is an inhibitor that has been shown to inhibit both IMPDH and GMPS [68, 74] and suggest that both IMPDH and GMPS are targets to explore for novel antimalarials. Although the IMP to GMP pathway involves phosphorylated intermediates, both mycophenolic acid and bredinin are non-phosphorylated natural products (or derivatives) that provide orally available access to tissues. Both mycophenolic acid and bredinin are used in immune suppression, and they have not been useful as antimalarials.

2.5 Purine salvage and polyamine synthesis

Polyamines are synthesized by the transfer of propyl amino groups from decarboxylated S-adenosylmethionine to putrescine to form spermidine (one transfer) and spermine (two transfers) [75]. MTA is the product of each transfer in the polyamine biosynthetic pathway. MTA is also a substrate for *Pf*ADA, whereby it is deaminated to methylthioinosine. These steps provide a path for this abundant product of the polyamine pathway to be channeled into the into the purine salvage pathway [76]. Polyamines are present at high amounts in the intraerythrocytic stages of *Plasmodium* spp., which can perform both *de novo* synthesis and salvage of polyamines [76, 77]. Given that parasites can only perform salvage of purines, the valuable purines used in the polyamine pathway are economically recycled. MTA is converted by the action of *Pf*ADA to produce MTI. MTI is a substrate for *Pf*PNP to form hypoxanthine and methylthioribose 1-phosphate. MTI is not found in human metabolism and recent studies have suggested that MTI production by *Plasmodium falciparum* in infected humans leads to activation of human Toll-like receptor 8 (TLR8), a signal for the stimulation of host innate immunity [37]. This connection provides an interesting link to the potential use of *Pf*PNP inhibitors as a potential therapeutic in human malaria. MTI is removed from *P. falciparum* metabolism exclusively by *Pf*PNP as *Hs*PNP does not use MTI as substrate. Therefore, inhibition of purine salvage via *Pf*PNP will increase MTI, increase signaling from TLR8 and have an immune stimulatory effect for the clearance of infected erythrocytes. Crosstalk between the polyamine pathway and purine salvage in *Plasmodium* parasites, together with the potential immunostimulatory effect of polyamine metabolites, underscores the importance of the polyamine pathway to purine salvage in *Plasmodium* parasites [37]. Inhibition of either the polyamine or the purine salvage pathways in *Plasmodium* parasites will be detrimental to parasites by; (1) purine starvation and death and (2) immune stimulation to promote clearance by host immune cells.

3. Pyrimidine metabolism

Opposite to the purine requirements of *Plasmodium falciparum*, the parasites are incapable of pyrimidine salvage from host erythrocytes; and pyrimidine synthesis requires the *de novo* pathway. This requirement contrasts with host cells with pathways for *de novo* synthesis and salvage of pyrimidine nucleotides [78]. Pyrimidine synthesis occurs by the action of six sequential enzymes to produce uridine monophosphate (UMP) as the primary product and a precursor to the other pyrimidine nucleotides (**Figure 9**) [79]. The enzymatic steps following UMP synthesis in *P. falciparum* are not well characterized, however, pyrimidine biosynthesis is linked to folate metabolism via the action of thymidylate synthetase (TS) [80]. Differences in the pathway between the human host and *Plasmodium* are important for selective targeting of *Plasmodium* parasites. In humans, the first three enzymes of the pyrimidine biosynthetic pathway, carbamoylphosphate synthetase II (CPS II), aspartate transcarbamoylase (ATC) and dihydroorotase (DHO) are a single protein, multiple domains, multifunctional enzyme complex, the CAD complex. In *Plasmodium*, CPS II, ATC and DHO are encoded as distinct genes, produce independent proteins and are organized on different locations on chromosomes, Ch. 13 for CPS II and ATC, and Ch. 14 for DHO [78, 81].

CPS II and ATC in *P. falciparum* are poorly characterized despite the role of CPS II as a key regulator of the pathway [20, 82]. The most thoroughly characterized enzymes of *Plasmodium* pyrimidine biosynthesis are dihydroorotate dehydrogenase (DHODH) and orotate phosphoribosyltransferase (OPRT) [83]. The transition state

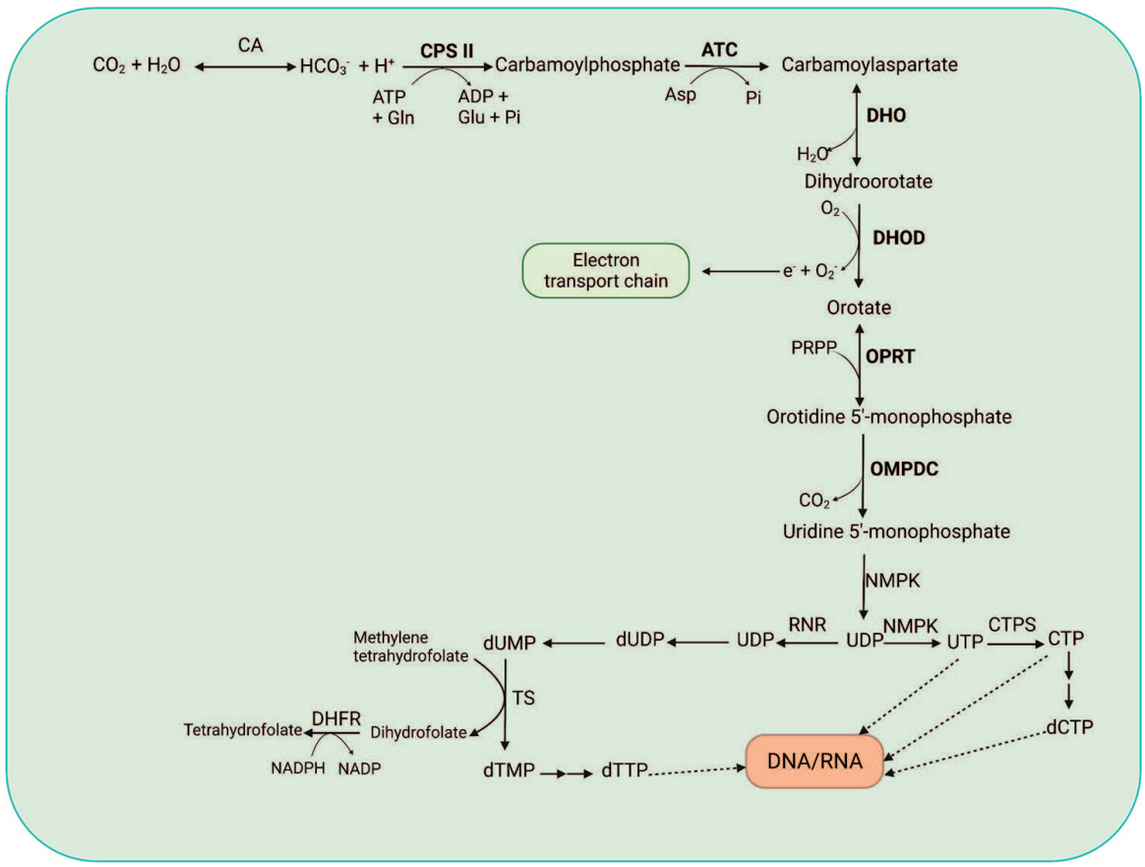

Figure 9.
De novo pyrimidine biosynthetic pathway. The pyrimidine biosynthetic pathway is conserved in Plasmodium and humans.

structures of human (*Hs*OPRT) and *Plasmodium falciparum* OPRT (*Pf*OPRT) have previously been solved and transition state analog inhibitors of both *Hs*OPRT and *Pf*OPRT have been designed (detailed below).

3.1 DHODH (EC 1.3.5.2)

DHODH is the fourth enzyme in the pyrimidine biosynthetic pathway and is expressed as two isozymes. The isozyme expressed from chromosome 7 codes for a mitochondria-associated DHODH and chromosome 9 expresses a cytosolic DHODH, where the mitochondrial form is essential for *Plasmodium* growth [84]. DHODH catalyzes the formation of orotate from dihydroorotate, a rate-limiting step for pyrimidine biosynthesis. *Plasmodium* spp., like the human host, express a type II DHODH, localized in the inner membrane of the mitochondrion [84, 85]. The electron transport chain provides quinone for the redox activity of DHODH. Recent studies have highlighted *Pf*DHODH as a potential antimalaria target, showing that inhibiting *Pf*DHODH leads to parasite death in culture and animal models [85–87].

Selective inhibition of *Pf*DHODH has been achieved with DSM265, a triazolopyrimidine with a 4000-fold selectivity over mammalian DHODH. DSM265 has resulted in phase II clinical trials for the treatment of malaria, a first for any inhibitor of the parasite pyrimidine biosynthetic pathway. DSM265 inhibits both blood and liver stages of the malaria parasite with a biological efficacy similar to chloroquine in murine malaria models [87–89]. The pharmacokinetic analysis of DSM265 supported single dose therapy for use as a once weekly prophylactic [87]. Single dose administrations are considered important for patient compliance. Since the discovery of DSM265, DSM421, an improvement on DSM265 in terms of compound solubility, plasma stability and equal targeting of *P. falciparum* and *P. vivax* has also been reported in preclinical development [90]. Other studies have followed suit, using pharmacophore screening and structure-guided virtual studies to identify *plasmodium* specific DHODH inhibitors with submicromolar IC_{50} values [91, 92].

Pyrimidine biosynthesis is coupled to the mitochondrial electron transport chain (ETC) by DHODH and its requirements of quinones for catalytic activity (**Figure 9**). This coupling results in indirect inhibition of DHODH by ETC inhibitors such as atovaquone, an approved antimalarial often used in combination with proguanil. [93] Although DHODH and the linked mitochondrial ETC were promising targets with extensive discovery and development programs providing powerful inhibitors, the rapid development of resistance in clinical trials has hindered their development toward approved drugs [94, 95].

3.2 OPRT (EC 2.4.2.10)

Reduction of dihydroorotate by DHODH produces orotic acid and the following step, catalyzed by *Pf*OPRT, catalyzes the production of the nucleotide orotidine monophosphate (OMP) from orotate and PRPP. OMP is subsequently converted to UMP by OMP decarboxylase (OMPDC). *Plasmodium* and human OPRTs differ in the early steps of the pyrimidine biosynthetic pathway. Thus, human OPRT is fused to OMPDC to form the single protein, bifunctional enzyme called UMP synthase [96]. In *Plasmodium*, OPRT and OPMDC have been purified as monofunctional enzymes however, there are recent reports suggesting that OPRT and OPMDC may exist as a heterotetrameric enzyme in *Plasmodium* with the potential for hydrolysis into the single enzymes during purification [83, 97, 98]. Detailed kinetic characterizations of

both human and *P. falciparum* OPRTs (*Hs*OPRT and *Pf*OPRT respectively) have been reported and active site differences between the homologs have been characterized [83, 98].

The essential function of OPRT in pyrimidine biosynthesis and therefore for parasite proliferation has been demonstrated by the selective killing of cultured *P. falciparum* parasites by pyrazofurin, an inhibitor of OPRT with anti-parasite IC_{50} values of 6–20 μM [21, 99]. Additionally, 5-fluororotate and 5-aminouracil have inhibitory activity against *Pf*OPRT with IC_{50} values of 42 nM and 8 μM respectively [21]. 5-Fluororotate selectively targets *P. falciparum* parasites in culture with an IC_{50} of 6 nM [100]. The mechanism of action is proposed to arise from an indirect effect of the toxic 5-fluorodeoxyuridylate metabolite on TS [101]. *Plasmodium* express TS as a single protein, multi-domain bifunctional enzyme with dihydrofolate reductase (DHFR) whereas TS exists as a monofunctional enzyme in mammals [21, 99, 100].

Transition state analysis of *Pf*OPRT and *Hs*OPRT reported an S_N1 transition state with a partial dissociative orotate and riboxocarbenium ion character for both enzymes [102]. Despite differences in the enzyme organization, *Pf*OPRT and *Hs*OPRT possess similar transition state structures, where the ribocation is fully developed, the dianionic orotate is fully dissociated and there is weak participation of the pyrophosphate nucleophile (**Figure 10**) [102]. The transition state structures provided information for the design of several TSAs with nanomolar affinities for both *Hs*OPRT and *Pf*OPRT (**Figure 10**) [103]. Inhibition of *Hs*OPRT might have potential utility for the treatment of autoimmune diseases and some malignant neoplastic diseases. However, no anti-parasitic activity was observed for any of the TSAs in cultured *P. falciparum*. The lack of activity was attributed to permeability barriers and target

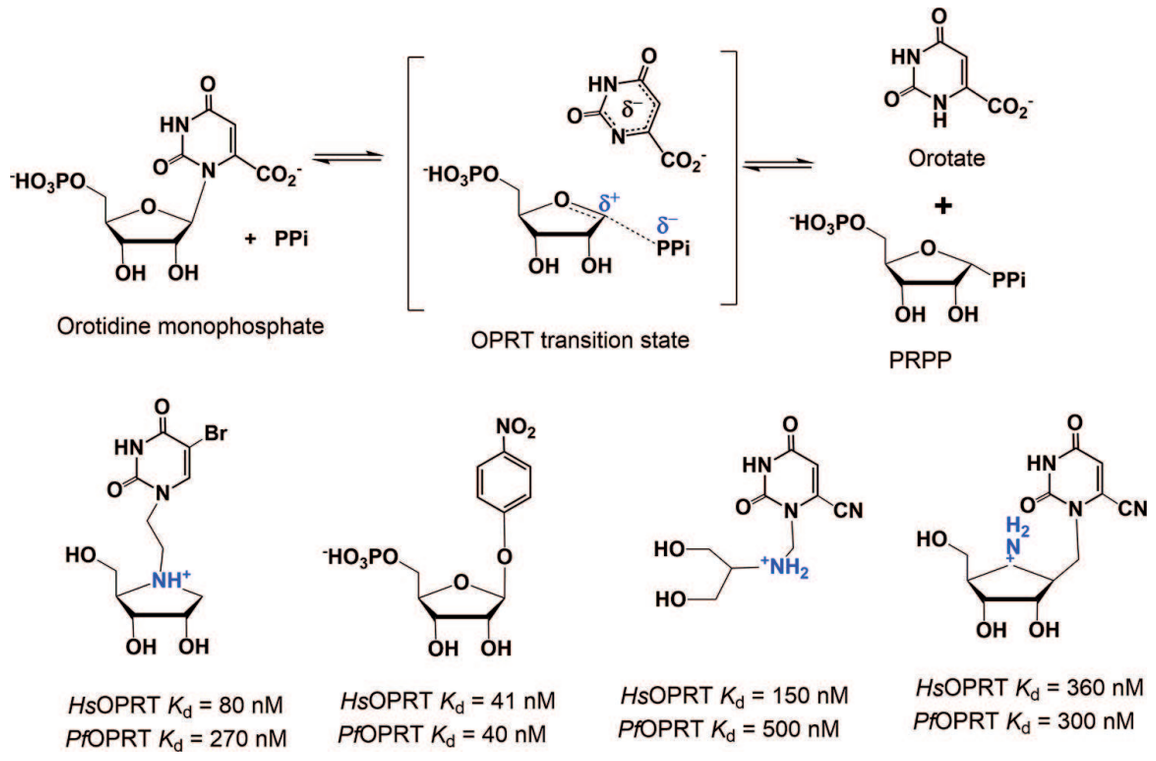

Figure 10.
Upper panel: OPRT reaction scheme and transition state structure. The transition state structure is similar for PfOPRT and HsOPRT. It features a fully developed ribocation and a fully dissociated dianionic orotate with weak participation of the pyrophosphate. Lower panel: Transition state analogs synthesized and tested on PfOPRT and HsOPRT.

access, but no permeability studies have been reported [103]. *Pf*OPRT remains a viable antimalarial target requiring further studies to elucidate and develop species-specific antimalarials.

3.3 OMPDC (EC 4.1.1.23)

The decarboxylation of OMP to form UMP is catalyzed by OMPDC, the sixth enzyme in the *de novo* pyrimidine biosynthetic pathway [78]. OMPDC has achieved catalytic notoriety as it has been considered the most proficient pure protein catalyst, giving a catalytic reaction rate enhancement of approximately 10^{17} [104, 105]. Unlike other decarboxylases, OMPDC requires no metal ions or cofactors for its catalytic activity [105]. Recent studies indicate that *Plasmodium falciparum* OMPDC may form a tight heterotetrameric complex with OPRT (*Pf*$OMPDC_2$-*Pf*$OPRT_2$) with properties distinct from the bifunctional human OPRT [106, 107]. Although both are expressed and encoded by two separate genes, unique amino acid insertions in both proteins not present in the homologous proteins of other organisms allow the formation of the heterotetrameric complex [98, 106]. Additional support comes from the kinetic characterization of fused *P. falciparum* OPMDC and OPRT, expressed in *Escherichia coli*, where the catalytic efficiency of the fused enzymes was enhanced several orders of magnitude relative to either enzyme acting as monofunctional proteins, leading the authors to call the fusion a 'super' enzyme [97].

Nucleoside 5′-monophosphate analogs, 6-azauridine 5′-monophosphate (AzaUMP), allopurinol-3-riboside 5′-monophosphate (allopurinol MP), pyrazofurin 5′-monophosphate (pyrazofuin MP), and xanthosine 5′-monophosphate (XMP) are potent inhibitors of both human and parasite OMPDCs (**Figure 11**) [108]. These display a strong preference for *Pf*OMPDC, with selectivity ranging from 11 to 150-fold preference for the parasite enzyme [108]. The differential binding of XMP has been investigated by comparing X-ray crystal structures of both enzymes. Few differences in active site residues were observed and specificity may be as a result in hydrogen bonding differences between ligand and active site residues (**Figure 12**). Another reported difference is the βα5-loop present at the dimerization interface, which displays different conformations and amino acid substitutions in *Pf*OMPDC compared to *Hs*OMPDC and is postulated to cause different active site rearrangements around the pyrimidine binding region [108]. The phosphate binding loop is also larger in *Hs*OMPDC than in *Pf*OMPDC [108]. Repositioning of the loop is a proposed requirement to bind XMP as observed for XMP-bound *Pyrococcus horikoshii* OMPDC [108]. Interestingly, the phosphate loop is the same size and shape in *P. horikoshii* OMPDC and *Pf*OMPDC, therefore, these enzymes may bind XMP similarly. XMP is a 150-fold more potent inhibitor of *Pf*OMPDC than of *Hs*OMPDC [108].

No crystal structures of inhibitor bound *Pf*OMPDC are available to understand the basis for inhibitor selectivity. However, the high preference of inhibitors for parasite OMPDC compared to the human enzyme indicates the possibility of developing potent and selective antimalarials. Inhibition of OMPDC has focused on nucleotide analogs, where the 5′-phosphate is a significant contributor to binding. A substantial barrier to drug development for nucleotide inhibitors is the anionic charge, as phosphoryl anions are membrane impermeable and phosphomonoesters are susceptible to phosphomonoesterases. Prodrug approaches, now being used in antiviral therapy for the delivery of phosphate nucleosides may be useful in development of OMPDC inhibitors.

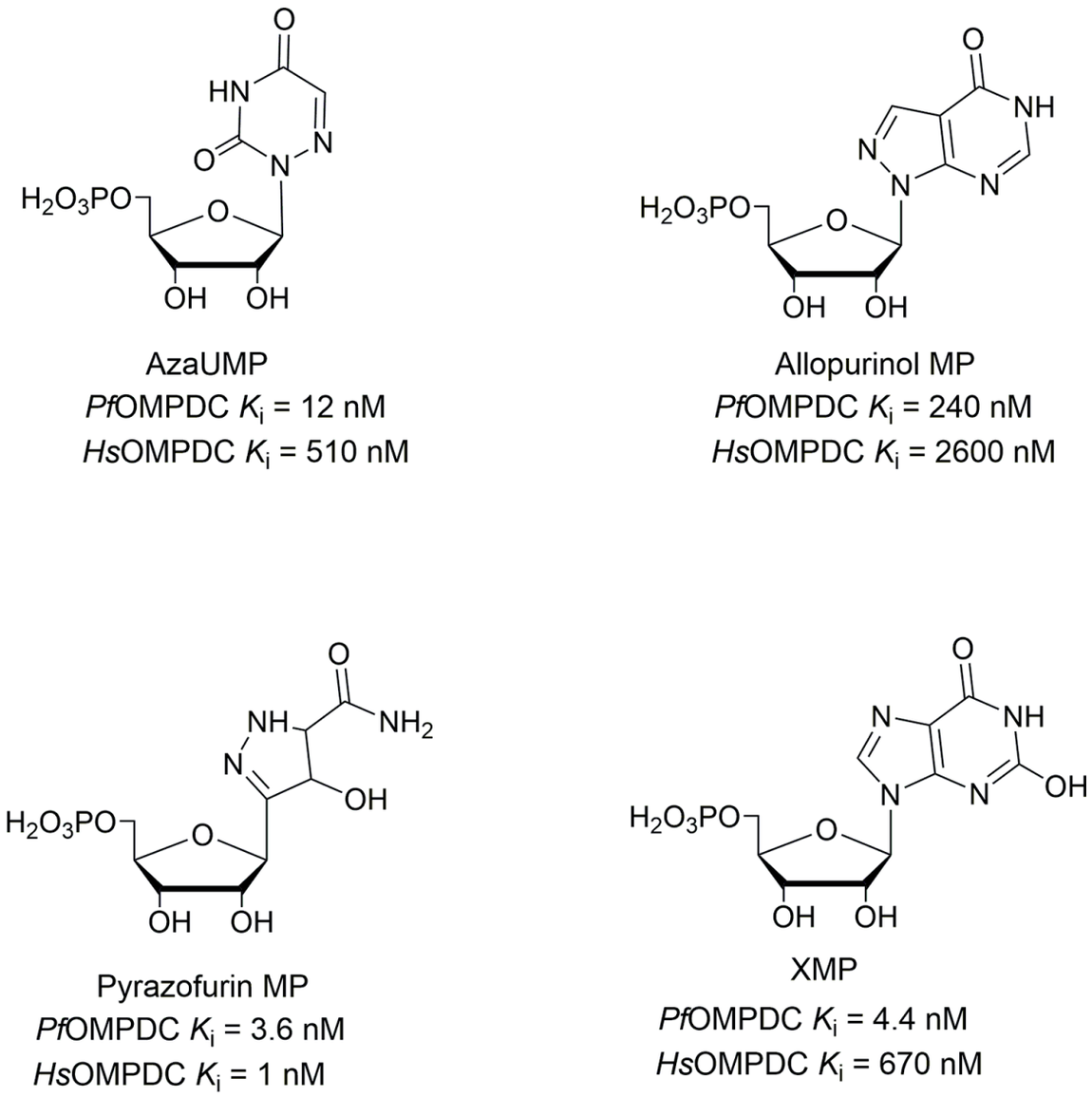

Figure 11.
Nucleoside 5′-analogs of OPMDC showing inhibitory constants for both PfOMPDC and HsOMPDC. Inhibitors are highly selective, 11–150-fold for PfOMPDC over HsOMPDC. Values are as reported in [108].

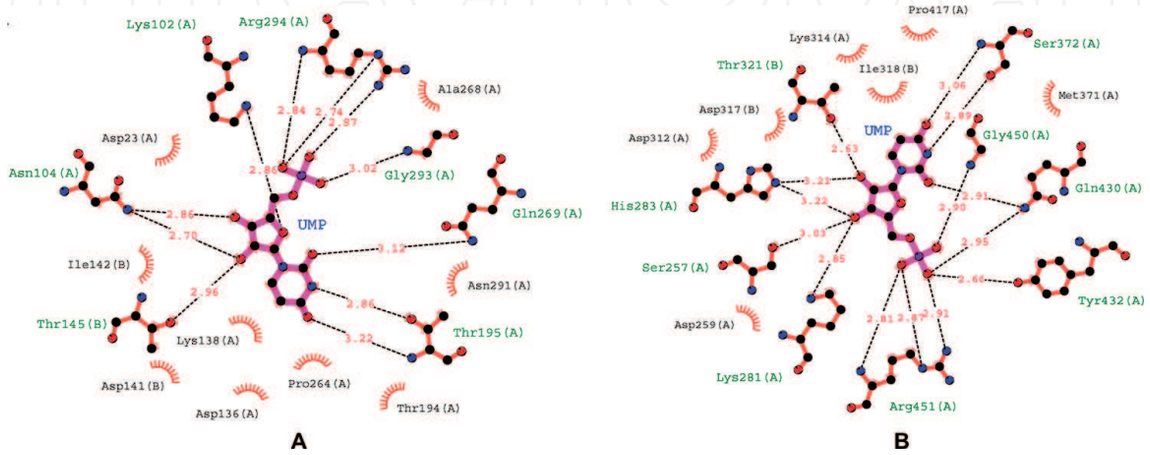

Figure 12.
2-dimensional ligand interaction map of UMP bound A, PfOPMDC (6DSR) and B, HsOMPDC (2V30). Similar active site residues make contact with UMP.

3.4 DHFR (EC 1.5.1.3) and TS (EC 2.1.1.45)

De novo purine synthesis in the host and pyrimidine synthesis in both host and parasites are dependent on folate metabolites for the donation of carbon units. The pyrimidine biosynthetic pathway is linked to folate metabolism by TS, which requires 5,10-methylene tetrahydrofolate as a methyl donor for its catalytic activity [109]. DHFR and TS are both targets for human cancers [110]. In *Plasmodium falciparum*, DHFR and TS are expressed as a single protein, two-domain bifunctional DHFR-TS enzyme system, catalyzing the synthesis of deoxythymidylate monophosphate (dTMP) from dUMP and the NADPH-dependent reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) [80]. The bifunctional coupling of adjacent steps in the pathway is proposed to cause "substrate channeling", an efficient utilization of folate production for the synthesis of purines, pyrimidines, and amino acids [111, 112]. Since malaria parasites are unable to salvage pyrimidines, if metabolites or cofactors required by the folate pathway are cut off, thymidine biosynthesis is blocked, and parasites die from pyrimidine starvation [20, 80]. Thus, DHFR has been a frequently-drugged target for antimalaria therapy.

Pyrimethamine (PYR) and proguanil (PG) are approved antimalaria therapeutics which selectively target *Pf*DHFR by several hundred-fold compared to the human counterparts [20, 113]. Mutations in the *DHFR* gene conferring resistance to both PYR and PG have led to diminished clinical effectiveness as single therapeutics for malaria. However, the combination of pyrimethamine with sulfadoxine (SP), which blocks dihydropteroate synthase, a folate precursor, act synergistically in the treatment of severe malaria [114]. Despite the prevalence of molecular mutations causing resistance to SP, WHO still recommends SP for intermittent preventative treatment for pregnant women and, often, in combination with amodiaquine, an inhibitor of *Plasmodium* heme polymerase, for seasonal malaria prevention [115]. PG is also used in combination with atovaquone, an inhibitor of the mitochondrion electron transfer chain, which also directly impacts pyrimidine biosynthesis because of its coupling to DHODH [116, 117].

Recent searches for additional inhibitors of DHFR-TS identified the antimicrobial triclosan to inhibit the proliferation of intraerythrocytic *P. falciparum* and is a 775 nM inhibitor of *P. vivax* DHFR. Triclosan displays a 20-fold selectivity for *Plasmodium* enzymes relative to human DHFR [118]. To preempt the development of resistance, Tarnchompoo and coworkers have developed hybrid inhibitors of DHFR with both flexible and rigid side chains that target both wild-type and multiple resistance *P. falciparum* [119]. Hybrid inhibitors include sub-nanomolar inhibitors of *Pf*DHFR with 10-fold selectivity over *Hs*DHFR [119]. Flexible cycloguanil analogs have been characterized that are low nanomolar inhibitors of multiple mutant *Pf*DHFR [120]. Fragment-based screening approaches to inhibitor design have identified non-pyrimidine scaffolds that inhibit *Pf*DHFR-TS with IC_{50} in the range of 28–695 μM and are highly selective over *Hs*DHFR [121]. This approach offers a new avenue to tackle antifolate resistance and develop new antifolate antimalarials, but remains in development [121].

DHFR-TS is a validated target for antimalaria combination therapy by blocking synthesis of dTMP. The functional characteristics of DHFR-TS including its crystal structure and the structures of drug-resistant DHFR-TS allows for the identification and development of next-generation selective inhibitors of either catalytic domain that will address resistance to current antifolate drugs for malaria. New approaches

are being explored, including peptide-based antagonists of *Pf*DHFR-TS in the quest for novel and potent antifolate/anti-pyrimidine drugs [122].

4. Transition state analogs and drug development

Transition state theory postulates that chemically stable mimics of the enzymatic transition state will bind tightly to the target [123, 124]. All chemical, including enzymatic reactions, proceed via a transition state, a transient high energy species that lies along the reaction coordinate between reactants and products [125, 126]. Transition states are the balance point of a catalytic reaction where bonds are partially broken or formed and the probability for product formation and return to reactants is equal. Enzymes have evolved to highly favor the transition state geometry, hence their very large rate enhancement factors, typically of 10^{10} to 10^{15}, a very large decrease in the activation energy of the reaction [18]. Chemically stable mimics of the transition state bind to and stabilize this favored enzymatic transition state geometry, capture part of the transition state energy and bind very tightly [18, 127–129]. Therefore, transition state analysis provides a powerful tool to develop molecules with high inhibitory potential for their target enzymes. Kinetic isotope effects (KIE) remain the best approach to study enzymatic transition states and together with quantum computational chemistry, allows the construction of electrostatic potential maps that provide information on the transition state structure [18]. This information enables the design of transition state analogs that bind with high fidelity to their cognate enzymes, millions of times tighter than substrates. Transition state analogs are some of the tightest binding enzymatic inhibitors and have the propensity to bind their target enzymes with dissociation constants in the nanomolar to femtomolar range (10^{-9} to 10^{-15} M) [123, 125, 126]. A feature of transition state analogs is the ability to convert the enzyme potential for catalysis into binding energy, therefore the more catalytically efficient the enzyme, the tighter the potential for transition state analog binding [127].

The immucillins are chemically stable transition state analogs that mimic the ribocation transition state of N-ribosyltransferases, enzymes that include *Pf*PNP and *Pf*HGXPRT, both important in purine salvage in *P. falciparum* [50]. Immucillin-H (also known as: BCX1777, Forodesine and Mundesine) (**Figure 5**) is an inhibitor of PNPs and has been approved in Japan for the treatment of resistant or relapsed peripheral T cell lymphoma (PTCL). Immucillin-A, as Galidesivir (also known as BCX4430) continues in phase I - II clinical trials for antiviral therapy. It is converted to the triphosphate form where it blocks RNA chain elongation in RNA viruses, including Yellow Fever and SARS-CoV2 [130]. DADMe-Immucillin-H (also known as: BCX4208, Ulodesine) has completed phase II clinical trials for gout, by virtue of its powerful inhibition of human PNP, an essential step in formation of uric acid in humans [50]. No immucillins or related transition state analogs for purine and pyrimidine pathways have yet entered clinical trials for antimalaria therapy though several are in preclinical testing [128].

The immucillins differ from traditional antibiotic discovery. Antibacterials are often discovered by cell wall screening or genomic targeting to inhibit cell wall synthesis [131]. Immucillins and related transition state analogs are designed to mimic the geometry of the transition state of their specific enzymes [129]. The resulting transition state analogs can be powerful tight-binding inhibitors with exquisite specificity for their targeted enzymes. Transition state inhibitor design is adding new candidates for drug development.

Immucillins in preclinical development as antimalaria drugs include the PNP-targeting DADMe-ImmG, a powerful inhibitor that is a picomolar inhibitor for both human and *Pf*PNPs. Administration at nanomolar concentrations to parasites cultured in human erythrocytes caused purine-less death of *P. falciparum*. Testing in *Aotus* primates infected with *P. falciparum* at an oral dose of 50 mg/kg and dosed for 7 days gave robust parasite clearance and no parasitemia was detected by day 6 of treatment, along with complete inhibition of host and parasite PNP [49]. Recrudescent parasites appeared several days after treatment stopped. [49] *Aotus* monkeys, however, have approximately 40 μM circulating hypoxanthine, compared to approximately 1 μM in humans [49]. Therefore, clearing *P. falciparum* from *Aotus* is a more stringent test for the antimalaria efficacy of DADMe-ImmG than anticipated in human infections. DADMe-ImmH, the compound tested extensively in phase I and phase II clinical trials for gout, has a good safety profile in humans. DADMe-ImmH has an inhibitory potential for both *Hs*PNP and *Pf*PNPs similar to DADMe-ImmG and is a strong candidate to enter human clinical trials [50]. The efficacy of DADMe-ImmH has been established in clinical trials. A single oral dose of 0.5 mg/kg completely inhibits erythrocyte PNP with inhibition lasting for 120 days, the lifetime of the erythrocyte, making it a candidate for single-dose therapy of *P. falciparum* [132]. As inhibition of both human PNP and *Pf*PNP is required for antimalarial properties, a single dose therapy has potential as a novel antimalarial, and as described below, has a reduced potential for the induction of resistance mutations.

4.1 Antimalaria resistance to transition state analogs

P. falciparum has developed resistance to most approved antimalarials, contributing to the difficulty of disease-eradication efforts worldwide. Rapid development of resistance has led to the clinical failure of several potent antimalaria drugs early in clinical trials and has resulted in World Health Organization recommendations for combination drug therapy for malaria treatment. Chloroquine which was once the gold standard treatment for malaria was widely used until the 1950s, when widespread resistance prompted its removal from the list of approved malaria therapeutics [133, 134]. Resistance soon followed for drugs such as atovaquone, pyrimethamine, proguanil, cycloguanil, sulfadoxine and sulfadoxine-pyrimethamine (SP) [133, 134]. Recently, resistance to artemisinin, one partner of the current first line treatment has been reported in the endemic regions of Sub-Saharan Africa and the Greater Mekong region of Southeast Asia [12, 14, 135, 136].

In the field, drug resistance is characterized by delayed-clearance of parasites from the blood of infected individuals [11]. Resistant parasites often have point mutations and gene amplification which can result in decreased drug uptake, increased efflux of drugs, target overexpression, target modification that reduces drug binding, and inactivation of a drug by modification (**Figure 13**) [134]. Knowledge of these mechanisms to resistance have prompted the search for novel and unique antimalaria compounds followed by investigation of the rates and mechanisms of resistance by subjecting cultured *P. falciparum* parasites to constant pressure of the novel compounds.

One such study involving DSM265, the *Pf*DHODH inhibitor. Using a drug pressure of 3 × EC_{50} revealed a minimum inoculum for resistance (MIR) of 2 × 10^6 compared to 2 × 10^7 for atovaquone [87]. Selection of resistant parasites to DSM265 required 8 x EC_{50} of DSM265 compared to 33 × EC_{50} required to suppress atovaquone resistant parasites. The resistance mechanism in Dd2 clones (resistant to chloroquine,

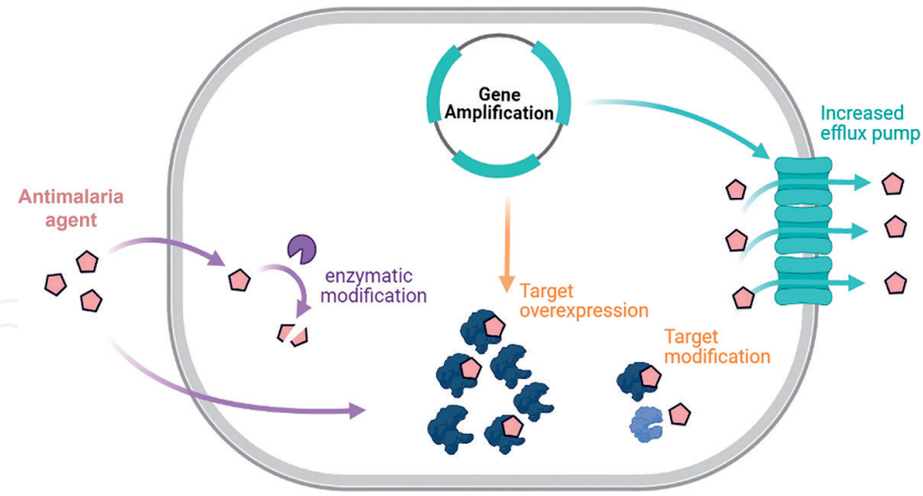

Figure 13.
Antimalarial resistance mechanism frequently employed by Plasmodium parasites to overcome drug pressure.

pyrimethamine and mefloquine) revealed both gene amplification of the DHODH gene and the G181C mutation that confers a 13-fold shift in IC_{50} of the drug compared to wild-type enzyme and 26-fold reduction of the EC_{50} in parasite growth assay. Kinetic characterization of the recombinant mutant enzyme revealed a 2-fold increase in the $k_{cat,}$ and K_m was unchanged [87]. Resistance to DSM265 in Dd2 parasites developed rapidly with a profile similar to resistance development for atovaquone. However, resistance was not as easily generated at higher concentrations of DSM265 in other *P. falciparum* strains, questioning if resistance would develop in the field [90]. DSM265 in phase II clinical trials for malaria led to the appearance of resistance during the clinical trial phase [94].

In contrast to DSM265, resistance to transition state analogs is not easily generated. Loss of transition state features in the protein also generates severe catalytic penalties predicted to induce a fitness cost to the parasites. For example, treatment of cultured *P. falciparum* with DADMe-ImmG for 1 year in culture resulted in a 6-fold resistance (6-fold increase in IC_{50}) [44]. The resistance was caused by a 6-fold amplification of wild-type *PfPNP* gene and protein levels. After 3 years of drug pressure (over 2^{136} clonal selections) in cultured cells, increased resistance was marked by a 12-fold amplification in the target *PfPNP* gene [44]. The 12-fold gene amplification was accompanied by two point mutations in PNP occurring in separate clones (M183L and V181D) to give rise to a 500-fold increase in the IC_{50}. Interestingly, point mutations were only present in 50% of the transcripts and the remainders were wild type [44]. Recombinant expression of mutant PNP revealed that the kinetic properties were incompatible with the purine salvage function of PNP [44]. Particularly, the M183L mutation resulted in a 17,000-fold decrease in the catalytic efficiency (30-fold decrease in k_{cat}) of the enzyme and a 39,000-fold decrease in affinity for DADMe-ImmG [44]. Therefore, the mutation reduced the catalytic efficiency and DADMe-ImmG efficacy by approximately the same amount, highlighting a key feature of transition state analogs. Mutations that prevent binding of the analog are expected to decrease catalytic activity to the same degree.

Resistance to DADMe-ImmG in *P. falciparum* arises by a unique mechanism. Hybrid PNP expression of 50% mutant M183L subunits and 50% wild-type subunits is proposed to generate hybrid hexameric PNPs demonstrating a 6-fold decrease in

catalytic efficiency, as three of the six subunits are native. This hybrid hexameric construct of three native and three mutant subunits displays negative cooperativity in binding to DADMe-ImmG, always leaving a fraction of the native subunits free to catalyze the formation of hypoxanthine [137]. Therefore, in addition to 12-fold target overexpression and point mutations, *P. falciparum* also employs hybrid multimeric PNP to achieve robust resistance [137]. This example emphasizes the severe catalytic costs and threat to biological function that resistance to transition state analogs produces in the parasites. This slow development of resistance to DADMe-ImmG suggests that resistance may be slow to develop in the field.

Conditions for generating laboratory DADMe-ImmG-resistant *P. falciparum* clones are stringent, requiring up to 3 years of drug pressure *on the same culture* before robust resistance appeared. In the field, effective anti-parasitics clear parasites in a matter of days, and it is unlikely that parasites would encounter conditions of continuous drug pressure. Notwithstanding, these *in vitro* resistance assays help identify mechanisms and molecular markers of resistance and resistance phenotypes that will aid in recognizing resistance development in the field. Like most antimalarials, the proposed use of DADMe-ImmG or related transition state analogs would be in drug combinations to provide an extra layer of protection against development of resistance.

5. Summary and conclusion

During the intraerythrocytic stages, *Plasmodium* parasites proliferate rapidly and require extensive nucleic acid synthesis. The building blocks are either salvaged from the human host in the form of purines or synthesized de novo (pyrimidines). There are distinct differences between the purine and pyrimidine synthesis pathways of humans and the malaria parasites. Therefore, targeting these pathways can aid in the development of novel chemotherapeutic agents to combat emerging resistance to the current first line antimalarials. Transition state analogs against various purine and pyrimidine pathway enzymes are emerging as promising candidates for antimalarial therapy. The slow development of resistance and the unusual resistance mechanism employed by the parasites to drug pressure against transition state analogs may lead to slow resistance development in the field. Finally, the recent advances made with the RTS,S vaccine in endemic African countries is promising and point in the right direction for population control of malaria. However, until an effective vaccine is approved for widespread use, chemotherapeutic agents managing the disease and vector control measures are still needed.

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Conflict of interest

The authors declare no conflict of interest.

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