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Transition-state inhibitors of purine salvage and other prospective enzyme targets in malaria

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Abstract

Malaria is a leading cause of human death within the tropics. The gradual generation of drug resistance imposes an urgent need for the development of new and selective antimalarial agents. Kinetic isotope effects coupled to computational chemistry have provided the relevant details on geometry and charge of enzymatic transition states to facilitate the design of transition-state analogs. These features have been reproduced into chemically stable mimics through synthetic chemistry, generating inhibitors with dissociation constants in the pico- to femto-molar range. Transition-state analogs are expected to contribute to the control of malaria.

Malaria is the most deadly of the human protozoan infections. This tropical disease can be caused by five different species of the *Plasmodium* genus: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium knowlesi* and *Plasmodium malariae* [1]. Its name arose from the Italian term for 'bad air' (*mala aria*), used to describe fevers associated with marsh air. The disease is endemic to warm and humid tropical areas worldwide, where mosquito larva can grow and multiply appropriately. Individuals infected with these species develop a common set of nonspecific clinical symptoms, which include multiple shaking chills, headaches, high fever, muscular aches, tachycardia, diarrhea, nausea and mild anemia, usually 2 weeks following infection [2,3]. Non-treated malaria caused by *P. falciparum*, the predominant species in Africa and the most severe or complicated malaria-causing species, progresses to a severe condition, where patients can no longer swallow medicines or food, and symptoms are characterized by severe anemia, kidney failure, hepatic failure, cerebral ischemia, altered consciousness and coma, ultimately leading to death.

Although the human disease can be triggered through infection by different species of the genus, all parasites display a common and complex life cycle (Figure 1) featuring three distinct stages: mosquito; human liver; and human blood [201]. The host infection cycle is initiated when a small number of *Plasmodium* sporozoites are injected into the human skin by an infected female *Anopheles* mosquito taking a blood meal. Once reaching the bloodstream, the parasites are transported throughout the body, gaining access to the liver, where they infect hepatocytes and undergo asymptomatic exoerythrocytic asexual

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multiplication (the liver stage). The infected hepatocytes rupture and several thousand merozoites are released into the circulatory system to infect red blood cells (RBCs), the site of major parasite expansion. The infecting parasites remodel the cell to support their intraerythrocytic asexual multiplication (the blood stage). The majority of antimalarial agents target the intraerythrocytic phase of the parasite, as pathology associated with RBC infection is responsible for the clinical disease. Although most merozoites released upon cell rupture re-infect RBCs and remain as merozoites, some divert from asexual replication and develop into gametocytes. The ingestion of gametocytes by a female *Anopheles* mosquito taking a blood meal re-establishes the *Plasmodium* life cycle into the mosquito stage [4].

Despite its complexity, the *Plasmodium* infection cycle is sufficiently robust to have survived for countless millennia. A robust infection cycle is supported by a genetic mechanism of *Plasmodium* causing rapid cell surface epitope switching to evade the human immune system. Decades of effort to develop vaccines have not yet led to a successful immunization program (see below). The complex life style of *Plasmodium* infections permits, in theory, therapeutic interruption at many key developmental steps, including: gametocyte development and multiplication in mosquitoes, transmission to humans during the blood meal, hepatocyte attachment, multiplication of merozoites in hepatocytes and attachment or multiplication of merozoites in erythrocytes. Public health eradication of mosquitoes with insecticides and bed nets has also had success (see below), but in cases of active disease, more direct intervention is essential.

Antimalarial agents & its limitations

Epidemiologists estimate that over 2 billion people are currently at risk of malaria infection worldwide within the tropics. Annual statistical data from the WHO have accounted for over 200 million new cases reported worldwide, 80% of which are in Africa, and almost 1 million deaths, 86% of which occur in children below the age of five [202]. These numbers have dire health and economic consequences for tropical nations.

The history on strategies to treat and control malaria infections is nearly as old as the disease itself. The first class of antimalarial drugs, the quinolines, include quinine, mefloquine, amidoquinine, chloroquine and halofantrine [5], which are among the earliest drugs discovered for malaria treatment. Quinine, was isolated from cinchona tree bark in the early 19th century, and was one of the first agents to be used on standardized dose treatments for malaria. Its use is still recommended, particularly on severe malaria treatment [6]. The mechanism of action of quinoline drugs has been thought to be on the inhibition of heme polymerization in the *Plasmodium* acidic food vacuole [7–9]. Resistance to quinolines has been attributed to mutations in membrane proteins involved in transport of antimalarial drugs into the *Plasmodium* acidic vacuole. In the early and mid-20th century, chloroquine was chemically synthesized and reported to be as effective as the natural product [6]. The *P. falciparum* chloroquine-resistant transporter is one putative protein at the parasite food vacuole membrane that has been reported to be responsible for *Plasmodium* resistance towards the quinolines. Critical mutations, such as K76T, confer resistance by reducing drug accumulation in the digestive vacuole [10,11].

The antifolate class of drugs targets enzymes found in the parasite cytosol involved in folate metabolism. This class includes sulfadoxine (inhibits DHPS; EC 2.5.1.15) and pyrimethamine (inhibits DHFR; EC 1.5.1.3), which in combination (Fansidar™) are more effective than chloroquine against *P. falciparum* [12]. Resistance towards antifolates has been attributed to mutations in DHPS and DHFR, which result in reduced binding affinity for the drugs, but with retention of catalytic function. Resistance to Fansidar is prevalent in east and central Africa, as well as some parts of Asia and South America.

The artemisinin combination therapies (ACTs) are now the first-line malaria treatment in endemic regions. The origin of the artemisinins comes from traditional medicine in China, where leaves of wormwood plants were used for thousands of years to treat fevers [13]. These sesquiterpene lactones contain a peroxide bridge that is thought to decompose into damaging free radicals. Its exact mechanism of action is uncertain, but is thought to be the inhibition of hemozoin production in the parasite vacuole [14,15]. In 2001, the WHO recommended the use of four ACTs to treat malaria: artemether-lamifantrine; artesunate-mefloquine; artesunate-amodiaquine; and artesunate-sulfadoxine/pyrimethamine. Although resistance towards ACTs has already begun to evolve, it still is the recommended and most effective class of antimalarial treatment [16].

Other approaches to combat malaria include disease prevention methods, such as vector control using indoor insecticide spraying (dichlorodiphenyl-trichloroethane) and the use of long-lasting insecticidal nets. Both methods reduce infection incidence but have not been successful in eradicating malaria [17]. A preventative method for malaria currently in use provides chemoprophylaxis for travelers, as infections can be prevented by pre-exposure administration of chloroquine (for nonresistant areas), atovaquone-proguanil, doxycycline and mefloquine [18]. *P. falciparum* is the predominant malaria parasite in endemic African regions, and numerous epidemiology studies associate a prevalence of the sickle cell gene as a protective mutation [19–21]. Other protective human genetic mutations for malaria include thalassemia and G6PDH (EC 1.1.1.49) deficiency, adaptations commonly found in malaria-endemic populations.

Hope for the future is that a vaccination strategy will be developed to prevent and eradicate malaria. The need for a vaccine is clear; however, this field has been very challenging. Although there is no current US FDA-approved malaria vaccine, the RTS,S vaccine developed by GlaxoSmithKline and others has entered clinical trials [22,23]. Until then, chemotherapeutic treatment and vector control approaches must be improved to control current disease. Enduring problems are the development of resistance to drug management approaches and insecticides by the parasites and vectors. These public health problems emphasize the need for new drugs to become available for malaria treatment.

Transition-state theory & inhibitor design

Enzymatic transition-state analysis provides an excellent tool for developing powerful drugs. During enzymatic catalysis, reactants go through an unstable structure between the structures of substrates and products, called the transition state [24,25]. The lifetime of the transition state is estimated to be approximately 10^{-14} s, time for a single bond vibration [24,26]. The lifetime of enzymatic transition states prevents formation of tightly bound transition-state complexes, thus, making it difficult to develop methods that can be used to directly observe their structures. The best approach to determine the transition states of enzymatic reactions is by measuring intrinsic **kinetic isotope effects** (KIEs), which compare reaction rates for the chemical step within enzymatic reactions using isotopically labeled and unlabeled substrates. This information is provided when making or breaking covalent bonds are slow steps in the reaction, or when corrections can be made for interfering steps. Using quantum chemistry, a theoretical transition-state structure matching the intrinsic KIEs can be obtained. Chemically stable **transition-state analogs** developed from electrostatic maps have been shown to bind tighter than the actual substrates with dissociation constants into the femtomolar range (10^{-14} M). Upon binding of transition-state analogs to the enzyme, the dynamic motions involved in forming the enzymatic transition state convert to stable thermodynamic interactions and, thus, the enzyme undergoes conformational changes. As a result, the release of the chemical transition-state analog is very slow and energetically unfavored, with release rates ranging from minutes to days [27]. Transition-state analogs

have proven to be powerful and specific enzyme inhibitors with favorable biological properties based on their chemical similarity to natural metabolites [24,27].

Purine salvage enzymes & transition-state analogs

Extensive DNA replication occurs during blood and liver stages of the malarial parasite, when there is a high demand for purine nucleotides. *P. falciparum*, as with most parasitic protozoa, lacks a *de novo* purine nucleotide biosynthetic pathway [28], relying exclusively on the salvage of preformed purines from its human host to supply the requirement for purine nucleotides [29,30]. The component enzymes of purine salvage pathways have been targeted for the development of antimalarials since the discovery that *Plasmodium* parasites are purine auxotrophs [31]. Although the purine salvage pathway is present both in the parasite and its human host, most temporary interruptions of human purine salvage have no adverse health effects. A thorough understanding of the component enzymes and enzyme-catalyzed reactions from the pathogen can reveal features to be exploited in rational target-selective drug design to inhibit *Plasmodium* viability. Based on the transition-state analog approach, the new class of compounds should also be active against *Plasmodium* strains that display resistance to the antimalarial agents currently used to treat this disease.

Several homologs to enzymes of the purine salvage pathway have been identified in *P. falciparum* based on the published genome sequence from the human malarial parasite [32]. Within this pathway (Figure 2), purine bases, nucleosides and nucleotides can be interconverted through the activities of ADA (EC 3.5.4.4), PNP (EC 2.4.2.1), HGXPRT (EC 2.4.2.8), IMPDH (EC 1.1.1.205), AdSS (EC 6.3.4.4), AdSL (EC 4.3.2.2), and GMPS (EC 6.3.5.2) [33]. In this review, examples of transition-state analog inhibitors of enzymes involved in different pathways in *P. falciparum* are provided, highlighting transition-state analog design and lead compounds targeting enzymes involved in the purine salvage pathway.

PNP

PNP plays a fundamental role in the *P. falciparum* purine salvage pathway, catalyzing the reversible phosphorolysis of the N-glycosidic bond of β -purine (deoxy)ribonucleosides to generate β -(deoxy)ribose 1-phosphate and the corresponding purine bases (Figure 3) [34]. This enzyme is essential for the formation of hypoxanthine in humans and in the protozoan, and hypoxanthine is the primary purine needed as a purine precursor in the parasite.

The most powerful inhibitors known for PNP have all been developed from KIE-based transition-state analysis. Some of the first transition-state analogs were based on the transition-state structure of bovine PNP, and were derived from (1*S*)-1-(9-deazahypoxanthin-9-yl)-1,4-dideoxy-1,4-imino-D-ribitol (Immucillin H; ImmH; Figure 4), displaying IC₅₀ values within the sub-micromolar range in mammalian cells, and demonstrated to be effective for T-cell-selective immunosuppression [35]. These slow-onset tight-binding inhibitors were modeled to exhibit an elevated p*K*_a of N7 of the purine ring and the formation of an oxocarbenium ion on the ribosyl ring, and displayed equilibrium dissociation constants in the picomolar range [36–39]. As the KIEs and transition-state structures became available for human and malarial PNP [40], second-generation immucillins were synthesized, exemplified by 4-deaza-1-aza-2-deoxy-1-(9-methylene)-ImmH (DADMe-ImmH; Figure 4), with dissociation constants in the low picomolar range. Structural changes included a nitrogen atom replacing the anomeric carbon and the ribosyl ring oxygen replaced by a methylene group to mimic the ribocation moiety at the transition state. The 9-methylene bridge linking the 9-deazahypo-xanthine leaving group with the ribocation mimic served to place the cationic N1 group near the ribocationic C1 position state. In this transition-state mimic, the 2-hydroxyl group was removed to provide chemical

stability [41]. As an attempt to chemically simplify immucillin analogs with fewer chiral chemistry centers, a series of acyclic immucillin analogs were synthesized [42], such as the simplified analog of Immucillin G (SA-ImmG; Figure 4), which had dissociation constants comparable to the first generation of immucillins. These acyclic derivatives simplified chemical synthesis, an essential issue towards human applications. The series of acyclic immucillins was further expanded to the third generation based on these findings, the most active compound of which being 2 -deoxy-2 -amino-tetritol-*N*-(9-methylene)-ImmH (DATMe-ImmH; Figure 4), with dissociation constants within the low picomolar range [43]. A fourth generation of transition-state analogs has been presented [44], the most active compound being serinol-*N*-(9-methylene)-ImmH (SerMe-ImmH; Figure 4), with a low pico-molar dissociation constant. Isothermal titration calorimetry studies demonstrated that the conformationally flexible immucillins reduced the system entropic penalty [45]. The achiral structure of the inhibitor reduces eventual challenges for large scale synthesis.

The comparison between transition states and substrate specificities from different PNP sources indicates a possible species-specific inhibitor design to be used as therapeutic agents [46]. It has already been demonstrated that even within mammalian homologs, such as bovine and human PNPs, which share 87% sequence identity and have totally conserved active site residues, inhibitors with differential specificity can be designed [47]. The appropriate inhibition for PNP requires the use of compounds with extraordinary affinity, since this enzyme is highly expressed in the host erythrocytes and in *P. falciparum* [48]. Individuals with genetic defects on PNP are normal at birth, however, in cases of complete deficiency in PNP-catalytic activity, develop severe immunodeficiency after several years [49] due to the accumulation of 2 -deoxyguanosine in the blood and dGTP in proliferating T-cells, resulting in inhibition of RNR (EC 1.17.4.2) [50]. The selective increase in the levels of dGTP leads to T-cell deficiency, although B-cell function is maintained. Such selective toxicity has made human PNP a target for T-cell cancer and autoimmune drug development. Immucillins have been demonstrated to be powerful picomolar transition-state analog inhibitors of both human and malarial PNPs [46]. Recent studies on *Aotus lemurinus lemurinus* monkeys infected with *P. falciparum* demonstrated that *in vivo* inhibition of PNP from the non-human primate model and the parasite (*Pf*PNP) by a transition-state analog inhibitor (DADMe-ImmG, also known as BCX4945) causes blood hypoxanthine depletion, blocking the purine salvage pathway, inducing purine starvation, thus inhibiting parasite proliferation, and clearing the infection [51]. In theory, as humans have ten-times less hypoxanthine in the blood than *Aotus* monkeys [52], the limiting metabolite for purine salvage in the parasite, PNP inhibition with this class of transition-state analog may be even more effective on antimalarial therapy in humans. Although metabolic studies also indicate the inhibition of the polyamine biosynthetic pathway in *P. falciparum*, as *Pf*PNP also recycles 5 -methylthioinosine (MTI; not found in human metabolism [53]), a product of polyamine metabolism in parasites (Figure 2), polyamines are reduced, not depleted, supporting purine metabolism as the primary target of DADMe-ImmG [54]. The PNP transition-state analog inhibitors' oral availability, chemical stability, singular mechanism of action, and low toxicity to humans [55] enable them as potential candidates to be introduced in combination therapies for the treatment of malaria.

ADA

ADA plays an important role in the purine salvage pathway, catalyzing the irreversible hydrolytic deamination of (deoxy)adenosine to generate (deoxy)inosine and ammonia (Figure 5). It belongs to the superfamily of zinc-dependent hydrolases, a large group of proteins that display conservation in their 3D fold and in details of the active site. Most members of this group have a conserved metal-binding site involving three histidines and one aspartic acid. In the common reaction mechanism, the metal ion(s) deprotonates a water

molecule for a nucleophilic attack on the substrate [56,57]. Besides its role in the purine salvage pathway, *P. falciparum* ADA (*Pf*ADA), as with *Pf*PNP, is also part of the polyamine biosynthetic pathway (Figure 2), displaying broader substrate specificity than its human homolog as *Pf*ADA also accepts 5 -methylthioadenosine (MTA) to generate MTI. Human ADA does not deaminate MTA, which is recycled through the activity of a specific MTA phosphorylase (EC 2.4.2.28) [54]. The dual specificity of *Pf*ADA increases its attraction as an antimalarial target due to the possible disruption of both necessary pathways by the use of a single compound.

Coformycin, a microbial product, was identified as a potent inhibitor of ADA, capable of inhibiting *in vitro* lymphocyte maturation [58,59]. 2 -deoxycoformycin, a coformycin analog, is used to treat hairy cell leukemia and refractory chronic lymphocytic leukemia [60]. However, its use is limited to such life-threatening conditions due to the severe CNS, bone marrow and renal toxicity [61]. Both compounds are powerful pico-molar inhibitors of human and malarial ADAs [62]. Humans deficient on this enzyme activity have been associated to a form of severe combined immunodeficiency disease caused by a dysfunction of B and T lymphocytes with impaired cellular immunity and decreased production of immunoglobulins. Although invariably fatal at an early age, this condition can be treated through compatible bone marrow transplantation, gene therapy and/or enzyme replacement infusion [63,64]. A series of *Pf*ADA inhibitors were synthesized based on the expanded substrate specificity of the malarial enzyme, which permits the design of parasite-specific transition-state analogs. The 5 -methylthio derivatives of coformycin and 2 -deoxycoformycin bind specifically to the parasite's enzyme, with sub-nanomolar dissociation constants [62,65]. 5 -methylthio-coformycin (MT-coformycin), a specific malarial ADA transition-state analog inhibitor demonstrates over 20,000-fold selectivity for *Pf*ADA relative to the human homolog [62]. Such specificity has been supported by structural studies, which revealed an unprecedented binding geometry for the 5 -methylthioribosyl groups of MT-coformycin in the malarial ADA [65]. Although host and parasite ADA activities might not be essential for parasite growth in cultured erythrocytes [66], the relatively large expression of this enzyme in malarial parasites suggests a metabolic importance in the essential purine salvage pathway. Furthermore, even though coformycin and 2 -deoxycoformycin have a weak effect on parasite growth in cultured erythrocytes [66,67], the latter was shown to decrease the parasitaemia in *P. knowlesi*-infected primates [68]. Further studies on the effect of MT-coformycin in *Plasmodium*-infected primates will give light on the hypothesis that the parasite ADA is important on establishing a viable infection. It is worth noting that the use of MT-coformycin as a potential antimalarial avoids the neurotoxicity of ADA inhibitors, such as 2 -deoxycoformycin, in humans [69].

HGXPRT

The malarial reliance on purine salvage creates a critical role for HGXPRT in the parasite's purine metabolism. Human erythrocytes can salvage adenosine by the AK (EC 2.7.1.20) reaction and APRT (EC 2.4.2.7) reaction. However, neither of these enzymes is encoded in the *Plasmodium* genome, making hypoxanthine the key precursor salvaged for purine nucleotide synthesis in *P. falciparum* [70]. This is the only enzyme that incorporates hypoxanthine into the purine nucleotide pool, making *P. falciparum* HGX-PRT (*Pf* HGXPRT) an essential enzyme [70,71]. It catalyzes the Mg^{2+} -dependent reversible conversion of 6-oxopurine bases to their respective nucleotides and inorganic pyrophosphate (PP_i), with the phosphoribosyl group being derived from phosphoribosyl pyrophosphate (PRPP) (Figure 6) [72,73]. In humans, the reaction strongly favors nucleotide formation ($K_{eq} \approx 1.6 \times 10^5$) [73]. Its kinetic mechanism has been elucidated for the human [74], schistosomal [75], *Tritrichomonas foetus* [76], and *Trypanosoma cruzi* [77] enzymes as ordered sequential bi bi, in which PRPP binds first to the enzyme, followed by nucleobase

binding. For product release, PP_i dissociates first from the enzyme, followed by nucleotide release. Although a detailed kinetic mechanism has not been described so far for *Pf* HGXPRT, it may follow the same mechanism.

The human and malarial enzymes are homotetrameric, and share 44% sequence identity and 76% similarity. Both enzyme structures have been solved in complex with transition-state analogs, PP_i and two Mg²⁺ ions [78,79]. The enzymes consist of core and hood subdomains, and the active site is formed by a cleft between these subdomains. The residues that make contact with catalytic site ligands are identical. Although there is a high degree of similarity among these proteins on sequence and structure, they differ significantly in substrate specificity and catalytic activity. In addition to hypoxanthine and guanine, malarial HGXPRT is also capable of catalyzing the phosphoribosylation of xanthine [80]. While values in the literature vary considerably regarding its specific catalytic activity, the *Pf*H-GXPRT *K_M* values reported for hypoxanthine are one order of magnitude (or more) lower than those reported for the human homolog [72,80–82]. Tight binding of hypoxanthine is indicative of its metabolic role for sequestering even low concentrations of free purine bases and preventing their diffusional release until the enzyme is able to convert them into the respective nucleotide mono-phosphates, thereby directing valuable purine bases into salvage pathways for the parasite.

Since the genetic deficiency of the human enzyme is related to Lesch–Nyhan syndrome [83–85], a debilitating neurological disorder, or gouty arthritis [86], potential inhibitors must also be characterized against the human enzyme. Inhibitor design approaches based on nucleoside analog searches, structure-based design and catalytic site-based screening programs identified lead compounds with binding constants on the same range to substrate *K_M* values. Enzymes related to HGXPRT indicate dissociative reaction mechanisms with ribooxocarbenium ion transition states [87,88]. Other *N*-ribosyltransferases also have ribooxocarbenium ions and protonated leaving groups at the transition state [89]; thus, it was hypothesized that transition-state features of HGXPRT could be added to immucillins phosphorylated at the O5' position, corresponding to the respective nucleotide monophosphate substrate. Indeed, ImmH and ImmG 5'-phosphates displayed low nanomolar inhibition affinities, binding at least three orders of magnitude tighter than previously reported inhibitors [90]. Unfortunately, the phosphate monoesters are not compatible with biological activity, since their negative charges make immucillin nucleotides impermeable to cell membranes and/or the phosphate group is susceptible to phosphomonoesterase activity [91]. However, these phosphorylated immucillins provided leads for the development of species-specific tight-binding inhibitors against *Pf*H-GXPRT. The acyclic immucillin phosphonates (AIPs), a new, biologically stable and selective class of HGXPRT inhibitors that mimic the transition state, were synthesized in the form of cell permeable lysophospholipid prodrug analogs that are delivered and activated inside the parasite. These inhibitors blocked proliferation of *P. falciparum* in culture. Metabolic and labeling studies demonstrated that the block was due to the inhibition of purine incorporation into the parasite nucleotide pool at the level of *Pf*HGXPRT [91]. These lysophospholipid prodrugs of the AIPs have extended the work on neutral 6-oxopurine acyclic nucleoside phosphonates [92–94]. A significant feature of these analogs is their greater specificity for the malarial enzyme than for the human homolog. Further optimization of the prodrug scaffold is still needed [91]. It should be noted that *Pf*HGXPRT is a more attractive target than *Pf*PNP, as the latter can be compensated by exogenous hypoxanthine rescue, but blockade at *Pf* HGXPRT cannot [95].

Downstream purine salvage enzymes

P. falciparum expression patterns suggest that PNP, ADA and HGXPRT form the major path for purine salvage in the parasite [54,96,97]. The nutrient-rich environment of human

blood allowed *Plasmodium* parasites to evolve enzymes with substrate specificity for two pathways. Thus, the parasite PNP and ADA function in both the purine salvage and polyamine biosynthesis pathways, a unique strategy for providing genomic economy for efficient purine metabolism with fewer expressed proteins. However, this strategy also exposes an Achilles heel for parasite metabolism. Targeting either enzyme may disrupt two essential pathways. All downstream enzymes that comprise the purine salvage pathway are also potential targets. Efforts to understand *Plasmodium* purine metabolism have been complicated by the limited understanding of **purine salvage enzymes** in the parasite. Although some of these enzymes are still poorly understood, below are the downstream enzymes required in *P. falciparum*.

AdSS

Inosine monophosphate (IMP) formed by HGXPRT is a precursor for the synthesis of both AMP and GMP. AdSS catalyzes the first committed step in the synthesis of AMP, more specifically the Mg^{2+} -dependent reversible formation of adenylosuccinate (also named succinyl-adenosine monophosphate; *SAMP*) from IMP and aspartate, in a two-step reaction driven by the hydrolysis of GTP to GDP and inorganic phosphate (Figure 7). Initially, the γ -phosphate of GTP is transferred to O6 of IMP, yielding 6-phosphoryl IMP. The nucleophilic attack by the α -amino group of aspartate then displaces the phosphate group to form the final product, *SAMP* [98]. AdSS is present in a broad range of organisms and is involved in the maintenance of cellular ATP/GTP ratios. Regulation can be expected to differ between *P. falciparum* and its human host, as the extremely A+T-rich genome of the parasite [99] demands a considerably higher production of adenine compared with guanine nucleotides. Given the primary importance of this enzyme in both *de novo* and salvage pathways for AMP biosynthesis in humans, both of which branch from IMP formation, species-specific inhibition of *P. falciparum* AdSS (*PfAdSS*) becomes essential. Even though *PfAdSS* is active as a homodimer [100], as with its homologs from most other species, its kinetic mechanism is different from their general random sequential one [101–103]. The malarial enzyme displays an ordered sequential mechanism of substrate binding [104]. This unique kinetic mechanism within AdSSs might have an important role in the regulation of enzyme activity *in vivo*. The ordered binding of GTP ensures its binding to a productive complex (enzyme–IMP), an essential factor in *P. falciparum*, which maintains relatively low levels of guanine nucleotides. The subsequent binding of aspartate to a productive complex (enzyme–IMP–GTP) might offer an additional advantage to *P. falciparum*, given its inability to synthesize amino acids [105]. Although a more thorough understanding of *PfAdSS* is still needed, these singular features indicate possible means to be explored for species-specific drug design.

AdSL

AdSL is the only known enzyme to catalyze two distinct, although biochemically similar, reactions in purine biosynthesis. One reaction, which is exclusive to the *de novo* purine biosynthetic pathway and, therefore, absent in *P. falciparum*, involves the cleavage of 5-aminoimidazole-4-(*N*-succinylcarboxamide) ribonucleotide (SAICAR) into 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) and fumarate. The other reaction, which does function in *P. falciparum*, involves the reversible cleavage of *SAMP* to AMP and fumarate through β -elimination, being common to both purine *de novo* and salvage pathways, and the final step in AMP synthesis (Figure 8) [106,107]. AdSL belongs to the fumarylase superfamily of enzymes, where high conservation exists in the tertiary structure despite their low amino acid sequence identity [108,109].

Humans defective in AdSL activity accumulate its substrates in body fluids and are affected at both biochemical and behavioral levels. Although variable, the symptoms include autism,

psychomotor retardation and, in some cases, growth retardation associated with muscle wasting and epilepsy [108]. The human enzyme shows significant sequence divergence from *P. falciparum* AdSL (*PfAdSL*) [110]. The cleavage of *SAMP* to AMP and fumarate catalyzed by *PfAdSL* follows a rapid equilibrium uni bi ordered mechanism. Catalysis may proceed through a carbanion transition state, as the overall rate is dictated by the substrate C-N bond cleavage. Interestingly, *PfAdSL* was shown to be capable of acting on both the *de novo* cleavage of SAICAR with similar thermodynamic and kinetic constants to those for *SAMP* and binding to AICAR with similar affinity to that of AMP [106]. Although these have no known function within the *P. falciparum* metabolism, such residual memory of its evolutionary history may be explored for selective drug development.

GMPS

GMP production through the activity of *PfH-GXPRT* using host-salvaged guanine would be insufficient for the rapidly multiplying parasite in the intraerythrocytic stage due to the considerably low levels of such free base in the human blood [111]. In *P. falciparum*, GMP is mainly formed from IMP by the sequential activities of IMPDH (yet to be characterized) and GMPS. Therefore, the final step in GMP synthesis in the purine salvage pathway of *P. falciparum* is accomplished by the activity of GMPS (*PfGMPS*). This Mg^{2+} -dependent enzyme catalyzes the irreversible biosynthesis of GMP from XMP and glutamine in the presence of ATP, producing GMP, PP_i , AMP and glutamate (Figure 9). The reaction involves the irreversible transfer of an amino group from glutamine to the C2 atom of XMP via an adenylyl-XMP intermediate, a characteristic of the G-type family of GAT. These enzymes use glutamine as the source of ammonia for amination of their substrates. The general GMPS structure in prokaryotes and eukaryotes consists of two different domains associated with distinct functions. An N-terminal GAT domain hydrolyzes glutamine to glutamate and ammonia. The C-terminal domain is an ATP pyrophosphatase domain, where the adenylyl-XMP intermediate is formed [112]. The substrate-protective environment created for the channeling of ammonia between the two separate although connected GMPS domains ensures an efficient catalysis and classifies this enzyme as a Class I amidotransferase [113–115]. The strict requirement for selective toxicity for inhibitors directed towards enzymes in the purine salvage pathway evident in cases like HGXPRT, where co-inhibition of the human homolog has pathologic consequences [83–85], may not apply to GMPS. Thus, human GMPS is considered to be a target for immunosuppressive and anticancer therapies [116,117]. A thorough understanding of the mechanism of GMPS from humans or other sources is still needed.

Putative enzyme targets in *Plasmodium falciparum* for transition-state structure studies

OPRT

OPRT (EC 2.4.2.10) is the fifth enzyme of the *de novo* pyrimidine biosynthetic pathway. It catalyzes the Mg^{2+} -dependent formation of OMP and PP_i from PRPP and orotate. OMP is subsequently decarboxylated to UMP by OMPDC (EC 4.1.1.23). In most multicellular organisms, OPRT is encoded into a single gene with OMPDC, making the resulting protein bifunctional, while in prokaryotes the enzymes exist as separate monofunctional units [118,119]. *P. falciparum* OPRT (*PfOPRT*) was shown to exist as a bifunctional multienzyme complex with a molecular weight of approximately 140 kDa [120,121].

The essential nature of pyrimidine synthesis in *P. falciparum* makes OPRT a good target for the development of antimalarial drugs. Several compounds including the C-nucleoside, pyrazofurin, have been studied as potential inhibitors of the enzyme [122]. Originally isolated from *Streptomyces candidus*, pyrazofurin was demonstrated to moderately inhibit *P.*

falciparum growth with an IC_{50} of 20 μ M. Parasitaemia was reduced in several parasite strains, including FCQ-27, FCI-1 and the chloroquine-resistant strain K-1 with IC_{50} values ranging from 6 to 10 μ M for pyrazofurin. This agent works by halting parasite progression to schizonts from the trophozoite intraerythrocytic asexual stage. The structural analog of orotate, 5-fluoroorotic acid, and the pyrimidine analog, 5-aminouracil, both inhibit *in vitro* growth of FCB *P. falciparum* strain with IC_{50} values of 42 nM and 8.2 μ M, respectively [123].

Transition-state structures of both human and malarial OPRT have been analyzed by KIEs, using phosphonoacetic acid as the slow substrate analog of PP_i , and quantum chemistry (Figure 10) [88]. Although these homolog enzymes have only 26% sequence homology, they display similar transition-state structures. The transition state is developed following ribooxocarbenium ion formation, characterized by fully dissociated orotate and partially bonded attacking nucleophiles. The substrate analog *p*-nitrophenyl -D-ribose 5 -phosphate was used to test the hypothesis that catalysis in *Pf*OPRT originates from enzymatic formation of a ribocation. If so, *p*-nitrophenyl -D-ribose 5 -phosphate would be expected to be an excellent substrate, as the leaving group (*p*-nitrophenol anion) needs no assistance from the enzyme. Poor catalysis displayed by *Pf*O-PRT on *p*-nitrophenyl -D-ribose 5 -phosphate supports leaving group activation as the driving force of the *Pf*OPRT-catalyzed reaction. To date, no transition-state analog inhibitor for *Pf*OPRT has been reported as an antimalarial agent; however, transition-state structures provide a blueprint for developing transition-state analog antimalarials.

DHFR

P. falciparum can produce folate *de novo*, which is required for parasite survival. The classical folate biosynthetic pathway begins with the rearrangement of GTP by GCH1 (EC 3.5.4.16) to dihydroneopterin triphosphate. This is followed by the cleavage of two of the carbons from dihydroneopterin catalyzed by a phosphatase and subsequently DHNA (EC 4.1.2.25) to provide 7,8-dihydroneopterin. However, DHNA is absent in the *P. falciparum* genome [32,124] and new biochemical evidence shows the presence of PTPS (EC 4.2.3.12), which may replace the phosphatase and DHNA activities (Figure 11) [124]. Following formation of 7,8-dihydrone-opterin is the pyrophosphorylation catalyzed by HPPK (EC 2.7.6.3). The subsequent steps involve *p*-amino benzoic acid (*p*ABA) and glutamate linkage to pterine, catalyzed by DHPS and DHFS (EC 6.3.2.12). The final reduction is catalyzed by DHFR. The inhibition of any of these enzymes in the folate biosynthetic pathway leads to reduced folate production, ultimately inhibiting DNA synthesis and the parasite life cycle.

DHFR is the best known target in *P. falciparum* (*Pf*DHFR). This enzyme catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate (Figure 12) during the *de novo* folate biosynthetic pathway. In *P. falciparum*, *Pf*DHFR exists as a bifunctional species with TS (EC 2.1.1.45) [125,126]. Currently, the most widely used regimen for malaria prophylaxis and treatment relies on the drug combination of the DHPS inhibitor sulfadoxine and DHFR inhibitor pyrimethamine (Fansidar). Pyrimethamine is a compound containing a 2,4-diaminoheterocyclic scaffold (Figure 13), which binds tightly to DHFR by providing key interactions between the 4-amino group in the scaffold and the enzyme. This interaction has been studied in the *E. coli* DHFR x-ray structure with methotrexate (anticancer agent), which resembles folate (Figure 13) except for the substitution of the 4-oxo group with a 4-amino group, which exhibits tighter binding to DHFR than folate by 10^3 -fold [127]. Pyrimethamine inhibits wild-type *Pf*DHFR with a K_i of 0.19 nM and an IC_{50} of 0.53 nM in the 3D7 pyrimethamine-sensitive strain [128]. The emergence of mutations in DHFR, however, results into reduced pyrimethamine sensitivity, as a single mutation reduces drug sensitivity 100-fold [129]. The accumulation of more mutations leads to even higher resistance to pyrimethamine, with a reported K_i of 385 nM for a *Pf*DHFR quadruple mutant

[130]. The four main *Pf*DHFR mutations reported in clinical isolates of pyrimethamine-resistant strains include codons at: Cys50 to Arg (C50R), Asn51 to Ile (N51I), Cys59 to Arg (C59R) and Ser108 to Asn (S108N) [131]. Crystal structures and binding studies of *Pf* DHFR wild-type and the highly pyrimethamine-resistant quadruple mutant revealed a probable reduction in binding affinity of the antifolate drugs pyrimethamine and cycloguanil to mutant DHFR [132,133].

WR99210 also contains the 2,4-diaminoheterocyclic scaffold and exhibits antifolate activity against both wild-type and pyrimethamine-resistant *Pf*DHFR. x-ray crystallography revealed the flexibility of a (2,4,5-trichlorophenoxy) propyloxy side chain within WR99210 that limits steric hindrance between the compound and Asn108-containing mutants. On the other hand, the presence of the chlorophenyl group of pyrimethamine causes a steric clash with Asn108-containing mutants, ensuing low binding affinity of pyrimethamine. WR99210 is approximately 200-fold more efficacious than pyrimethamine against *Pf*DHFR quadruple mutant (N51I/C59R/S108N/I164L) with a K_i value of 1.9 nM [132]. Due to its low bioavailability and toxicity, WR99210 was not approved as an antimalarial drug. Nevertheless, the important insight of flexibility with 2,4-diaminopyridines led to the design of P65 and P218 (Figure 13), which inhibit the quadruple mutant strain with an IC_{50} of 3495 nM and 56 nM for P65 and P218, respectively. P218 is the most potent compound in the series of WR99210 analogs and contains properties of slow-on, slow-off inhibition of both *Pf*DHFR wild-type and quadruple mutant enzymes [130]. Its flexible linker favors interactions with conserved amino acid residues at the opposite ends of the substrate binding site and has good oral bioavailability and enzyme selectivity compared with WR99210. For these reasons, P218 is undergoing preclinical development.

DHPS

P. falciparum DHPS (*Pf*DHPS) catalyzes the reversible condensation of *p*ABA and 6-hydroxy-methyl-7,8-dihydropterin pyrophosphate to yield 7,8-dihydropteroate (Figure 14). The mechanism of catalysis elucidated from *Yersinia peptis* DHPS revealed that the Mg^{2+} -dependent reaction is likely to undergo an S_N1 mechanism, where a cationic intermediate is formed as a result of loss of PP_i from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate [134]. *Pf*DHPS is a component enzyme of the folate biosynthetic pathway (Figure 11), and exists as a bifunctional species with its preceding enzyme in *P. falciparum* HPPK (*Pf*HPPK) [135].

*Pf*DHPS is a validated target for the treatment of malaria, as inhibition of this enzyme leads to decreased folate, an essential cofactor for DNA synthesis. The FDA-approved sulfa-based drugs, such as sulfadoxine (substrate analog of *p*ABA), in combination with the antifolate drug pyrimethamine have been used for malaria prophylaxis and treatment [136]. However, the emergence of resistance due to mutations in *Pf*DHPS has rendered these drugs less effective [131]. Evaluation of the activity of *Pf*HPPK–DHPS enzymes from sulfadoxine-sensitive and -resistant isolates demonstrates that mutations in codons S436A/F, A437G, K540G/E, A581G, and A613S/T affect inhibition of *Pf*DHPS by sulfadoxine [137]. *P. falciparum* D10 strain containing wild-type DHPS was reported to exhibit a low K_M of 16.4 nM for *p*ABA, suggesting efficient catalysis at low substrate concentration. Sulfadoxine showed good inhibition in the D10 strain, with a K_i of 0.14 μ M. A single amino acid mutation in *Pf*DHPS such as that of the 3D7 strain (A437G) results in an increase on the apparent K_M of *p*ABA and sulfadoxine K_i (to 1.39 μ M). Double mutations in the enzyme as in the case of the Tak9/96-C strain (S436A/A437G) and the K1 strain (A437G/A581G) and triple mutations in the W2mef strain (S436F/A437G/A613S) result into an even more dramatic increase in sulfadoxine K_i . In these cases, the amino acid mutations are close to the drug binding site in *Pf*DHPS, which result in decreased binding affinity of sulfadoxine to *Pf*

DHPS. In all cases, the enzyme retains sufficient catalytic activity to satisfy the biological need for folate production.

Parasite proteases

P. falciparum digests host cell hemoglobin in the acidic food vacuole during the intraerythrocytic life cycle. This process is mediated by proteolytic enzymes, the plasmepsins (aspartic proteases) and falcipains (serine proteases) [138], which are promising targets since hemoglobin digestion is essential for the survival of parasites. Ten plasmepsins have been identified in the *P. falciparum* genome [139], four of which are located in the digestive vacuole. They vary in sequence specificity cleavage sites and are involved in heme digestion (plasmepsin I, II, IV and histoaspartic protease). Individual knockout of each of the four vacuolar plasmepsins and the serine protease falcipain-2 demonstrate no effect on parasite growth, while accumulative knockout of the plasmepsins and falcipain-2, as well as triple knockout of plasmepsin I/IV and falcipains-2 result in attenuated growth of *P. falciparum*, implying that collective inhibition of both plasmepsins and falcipains is lethal for *P. falciparum*. Thus, new inhibitors of the malarial proteases may serve as drug cocktails for malaria treatment [140,141].

The HIV protease inhibitors saquinavir and ritonavir have been reported to kill *P. falciparum* *in vitro* and *in vivo* [142–144]. It has been suggested that these HIV-1 aspartic protease (EC 3.4.23.16) inhibitors may also target *P. falciparum* aspartic proteases (plasmepsins). Multiple sequence alignments revealed conserved sequences within the catalytic site of HIV protease and plasmepsin II, supporting the possibility of HIV protease inhibitors blockade of plasmepsins [145]. HIV-1 protease processes the gag proteins, generating mature gag pol, structural and viral enzymes, allowing for the maturation of virion and facilitating spread of virus [146]. The homodimeric HIV-1 protease consists of 99 amino acid subunits containing a flap at each subunit that covers the active site (Figure 15A). Each subunit contributes one aspartate that cleaves the substrate at the active site. The structure of the rate-limiting transition state of HIV-1 protease reaction has been resolved using KIEs and computational chemistry [147]. The transition-state structure reveals a species that has three protons directly associated with substrate, with proton transfer from the active site Asp25, partial hydrogen bonding to the accepting nitrogen and partial bond loss from the carbonyl carbon to the amide leaving group. Electrostatic potential maps of the HIV protease inhibitor, indinavir, further demonstrated that all the FDA approved transition-state analogs against HIV protease maintain the *gem-diol* mimic, but lack the charge character that results from the proton transfer revealed in the transition-state structure. Interestingly, the highly drug-resistant HIV pro-tease mutant (I84V) showed a very similar transition state, indicating that drug resistance arising in the mutants develop from mutations distant from the transition-state interactions and that a stable analog of the transition state developed from KIEs would likely inhibit both wild-type and resistant HIV protease strains [147].

In contrast to the HIV-1 protease structure, the *P. falciparum* aspartase plasmepsin II (EC 3.4.23.39) is a single chain of 329 amino acids folded into two N and C-terminal domains which contact at the bottom binding cavity containing the catalytic diad Asp34 and Asp214 and a flap around the substrate binding site (Figure 15B). The aspartase digests hemoglobin between Phe33 and Leu34 [148]. Most inhibitors developed for plasmepsin II mimic the tetrahedral intermediate formed during peptide bond cleavage of aspartases. C₂-symmetric inhibitors containing the 1,2-dihydroxyethylene scaffold were reported to inhibit plasmepsin II with nanomolar activity. Using binding affinity calculations, it was determined that an *RRRR* configuration of the 1,2-dihydroxyethylene core of the inhibitors is important for binding the enzyme [149,150]. A different class of inhibitors was developed by screening cathepsin D inhibitors containing a hydroxyethylamine scaffold against plasmepsin II, and

the lead was optimized using structure-based design with plasmepsin II crystal structure [151].

Individual inhibition of vacuolar plasmepsins and falcipains has been reported not to be a promising strategy, but rather the collective inhibition of the plasmepsins and falcipains [141]. Furthermore, the vacuolar plasmepsins have been reported to share common inhibitors [152]. Altogether, these reports highlight the importance of transition-state analysis of the *P. falciparum* vacuolar aspartases, as such would open doors to the development of stable mimics. An interesting possibility would be a shared transition-state structure among the plasmepsins resulting in a single stable inhibitor that would collectively inhibit the *P. falciparum* aspartases.

Conclusion

The ongoing public health concerns caused by malaria have fueled extensive efforts to be directed on developing efficacious prophylactic vaccines, making new insecticides with extended action against resistant mosquito strains, as well as discovering, developing and delivering chemotherapeutics capable of overcoming *P. falciparum* resistance to current drug regimens [153]. During the intraerythrocytic stage of *P. falciparum* life cycle, parasites exhibit rapid nucleic acid synthesis, demanding high levels of purine nucleotides, which are salvaged from the host. The *P. falciparum* purine salvage pathway is distinct from its host and, thus, parasite enzymes in this pathway are promising targets for selective drug development. By utilizing KIEs coupled to quantum chemistry, the transition state of enzymatic reactions in the pathway can be determined. These transition-state structures provide a blueprint for the development of powerful transition-state inhibitors for malaria treatment.

Future perspective

Hope for the future is that a vaccination strategy will be developed to prevent and eradicate malaria. However, the challenging effectiveness on a malarial vaccine has clearly indicated that such accomplishment still is a long-term goal. Until then, chemotherapeutic treatment and vector control approaches must be improved to control current disease. Enduring problems are the development of resistance to drug management approaches and insecticides by the parasites and vectors. These public health problems emphasize the need for new drugs to become available for malaria treatment within the next decade. Enzymes essential for *P. falciparum* stand as promising targets for the development of selective drugs to treat malaria. Solving their transition states will provide blueprints to design potential drugs or therapeutic agents to soon be evaluated on clinical trials and, hopefully, administered for this human disease in the near future.

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Key Term

Plasmodium falciparum

The predominant *Plasmodium* species in Africa and the most severe or complicated malaria parasite causing disease in humans

Enzymatic transition state

When chemical reactants participate in an enzymatic reaction, an activated complex or a transition state is formed. In this chemical

	reaction, the transition state exists momentarily and is less stable than both reactants or products
Kinetic isotope effect	The ratio of reaction rates of labeled (K_{labeled}) and unlabeled ($K_{\text{unlabeled}}$) reactants
Transition-state analog	Chemically stable compound that mimics the unstable transition-state structure
Purine salvage enzymes	Purine nucleotides may be derived from preformed purine bases and nucleosides by enzymes of the salvage pathway

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Executive summary

Background

- Malaria, caused by *Plasmodium falciparum*, is the most deadly human protozoan infection.
- The current classes of antimalarial drugs target the intraerythrocytic stages of *P. falciparum* life cycle; unfortunately, emergence of drug-resistant strains complicates treatment.

Transition-state theory & inhibitor design

- Transition-state analysis of enzymes and how it translates to the design of stable transition-state analogs has been discussed.
- After measuring kinetic isotope effects, a theoretical transition-state structure matching the intrinsic kinetic isotope effects can be obtained, which is used as a guide to design and synthesize stable transition-state analogs.

Purine salvage enzymes & transition-state analogs

- Upstream enzymes of the purine salvage pathway (PNP, ADA and HGXPRT) are described as good targets for developing transition-state inhibitors.
- The family of 'Immucillins' derived from transition-state analysis using kinetic isotope effect measurements are powerful inhibitors of some of the enzymes in the purine salvage pathway.

Downstream purine salvage enzymes

- Though not well understood, the downstream enzymes of the purine salvage pathway are possible drug targets.
- The enzymes AdSS, AdSL and GMPS are described as potential targets for transition-state analysis.

Putative enzyme targets in *P. falciparum* for transition-state structure studies

- Enzymes in essential pathways in *P. falciparum* can be inhibited to act as agents in fighting malaria. These include the *de novo* pyrimidine pathway, folate biosynthetic pathway and the proteases.
- DHFR and DHPS are enzymes of the folate biosynthetic pathway that are targets for US FDA-approved antimalarials. Mutations have caused resistance to the existing drugs. Transition-state analysis of these enzymes would be important for developing a new generation of inhibitors.

Figure 2. Purine salvage and polyamine pathways in an erythrocyte infected by *Plasmodium falciparum*

Bold arrows on reversible steps indicate the metabolically favored direction.

MTA: 5-methylthioadenosine; MTI: 5-methylthioinosine; SAMP: Adenylosuccinate.

Adapted from [33].

Figure 3. One of the reactions catalyzed by *Plasmodium falciparum* PNP in the purine salvage pathway

PfPNP catalyzes the reversible phosphorolysis of the N-glycosidic bond of β -purine (deoxy)ribonucleosides to generate β -(deoxy) ribose 1-phosphate and the corresponding purine bases.

PfPNP: *Plasmodium falciparum* PNP.

Figure 4. Immucillins, transition-state analogs for PNP

DADMe-ImmH: 4 -deaza-1 -aza-2 -deoxy-1 -(9-methylene) -ImmH; DATMe-ImmH: 2 -deoxy-2 -amino-tetritol-*N*-(9-methylene) -ImmH; ImmH: Immucillin H; SA-ImmG: Simplified analog of Immucillin G; SerMe-ImmH: Serinol-*N*-(9-methylene) -ImmH.

Figure 5. One of the reactions catalyzed by *Plasmodium falciparum* ADA in the purine salvage pathway

*Pf*ADA catalyzes the Zn²⁺-dependent irreversible hydrolytic deamination of (deoxy)adenosine to generate (deoxy) inosine and ammonia.

*Pf*ADA: *Plasmodium falciparum* ADA.

Figure 6. One of the reactions catalyzed by *Plasmodium falciparum* HGXPRT

*Pf*HGXPRT catalyzes the Mg²⁺-dependent reversible conversion of 6-oxopurine bases to their respective nucleotides and PP_i, with the phosphoribosyl group being derived from phosphoribosyl pyrophosphate.

*Pf*HGXPRT: *Plasmodium falciparum* HGXPRT; PP_i: Inorganic pyrophosphate.

Figure 7. Reaction catalyzed by *Plasmodium falciparum* AdSS

PfAdSS catalyzes the first committed step in the synthesis of AMP, more specifically the Mg^{2+} -dependent reversible formation of adenylosuccinate from IMP and aspartate, in a two-step reaction driven by the hydrolysis of GTP to GDP and inorganic phosphate.

IMP: Inosine monophosphate; *PfAdSS*: *Plasmodium falciparum* AdSS.

Figure 8. Reaction catalyzed by *Plasmodium falciparum* AdSL

PfAdSL catalyzes the final step in AMP synthesis, more specifically the reversible cleavage of adenylosuccinate to AMP and fumarate.

PfAdSL: *Plasmodium falciparum* AdSL.

Figure 9. Reaction catalyzed by *Plasmodium falciparum* GMPS

*Pf*GMPS catalyzes the Mg²⁺-dependent irreversible biosynthesis of GMP from XMP and glutamine in the presence of ATP, producing GMP, inorganic pyrophosphate, AMP and glutamate.

*Pf*GMPS: *Plasmodium falciparum* GMPS.

Figure 10.

Reaction catalyzed by *Plasmodium falciparum* OPRT with phosphonoacetic acid as the substrate, revealing the ribooxocarbenium ion formation, a fully dissociated orotate, and a partially bonded nucleophile transition-state structure.

Figure 11.
Folate biosynthetic pathway in *Plasmodium falciparum*.

Figure 12. Reaction catalyzed by *Plasmodium falciparum* DHFR

P. falciparum DHFR catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate during the *de novo* folate biosynthetic pathway.

PfDHFR: *Plasmodium falciparum* DHFR.

Figure 13.
DHFR inhibitors.

Figure 14. Reaction catalyzed by *Plasmodium falciparum* DHPS

P. falciparum DHPS catalyzes the Mg^{2+} -dependent reversible condensation of *p*-amino benzoic acid and 6-hydroxymethyl-7,8-dihydropterin pyrophosphate to yield 7,8-dihydropteroate during the *de novo* folate biosynthetic pathway. The reaction is likely to undergo an S_N1 mechanism, where a cationic intermediate is formed as a result of loss of PP_i from 6-hydroxymethyl-7,8-dihydropterin pyrophosphate.

PfDHPS: *Plasmodium falciparum* DHPS; PP_i : Inorganic pyrophosphate.

Figure 15. Protein database structures of aspartic proteases
(A) Homodimeric HIV-1 protease (3HVP). (B) Single chain polypeptide plasmepsin II (1LF4).