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PLASMODIUM PURINE METABOLISM AND ITS INHIBITION BY NUCLEOSIDE AND NUCLEOTIDE ANALOGUES

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ABSTRACT

Malaria still affects around 200 million people and is responsible for more than 400,000 deaths per year, mostly children in subequatorial areas. This disease is caused by parasites of the *Plasmodium* genus. Only a few WHO-recommended treatments are available to prevent or cure plasmodial infections, but genetic mutations in the causal parasites have led to onset of resistance against all commercial antimalarial drugs. New drugs and targets are being investigated to cope with this emerging problem, including enzymes belonging to the main metabolic pathways, while nucleoside and nucleotide analogues are also a promising class of potential drugs. This review highlights the main metabolic pathways targeted for the development of potential antiplasmodial therapies based on nucleos(t)ide analogues, as well as the different series of purine-containing nucleoside and nucleotide derivatives designed to inhibit *Plasmodium falciparum* purine metabolism.

INTRODUCTION

WHO, in 2018, reported more than 435,000 deaths associated with malaria, and at least 219 million people—mostly children—are still affected by this disease in 91 countries located in Africa and Asia.[1] The disease, which represents an endemic in tropical areas, is caused by protozoal parasites of the *Plasmodium* genus in the Apicomplexa phylum. Among the six human *Plasmodium* species (*P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri* [2,3] *and P. knowlesi* originally known to cause simian malaria), *P. falciparum* is the most widespread and lethal. The parasite is transmitted to humans by female mosquitos of the *Anopheles* genus (**Figure 1**). *Plasmodium* begins its development cycle in humans by multiplication in hepatocytes. After 10-12 days, the parasites are released in the blood and invade the erythrocytes (asexual multiplication). This intraerythrocytic phase constitutes the symptomatic phase of the disease, with massive destruction of erythrocytes and sometimes adherence to blood vessels of large organs like the brain, thus restricting the blood flow with serious consequences. A small proportion of the blood stages develop into sexual forms (so-called gametocytes) that are ingested by a feeding mosquito, therefore completing the cycle. Parasites of the *P. vivax* and *P. ovale* species can persist for years as dormant stages in the liver (so-called hypnozoites) and can cause relapses.

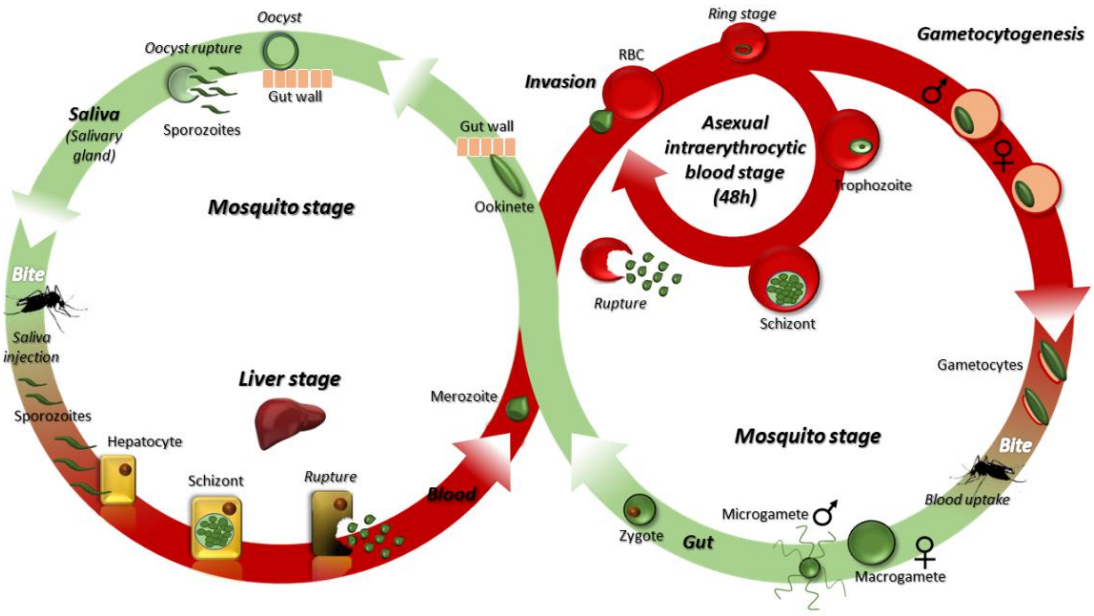


Figure 1 : *Plasmodium falciparum* life-cycle

Since the historical use of quinine 400 years ago, particularly as of the late 19th century, several treatments of synthetic or natural origins have been developed (**Figure 2**) and have been successfully used to cure malarial diseases in infected human hosts. The various classes of antimalarial drugs belong to a few families according to their chemical structures, such as: aryl amino alcohols (quinine, mefloquine, halofantrine, etc.); 4-aminoquinolines (chloroquine, amodiaquine, piperaquine, etc.); 8-aminoquinolines (primaquine, tafenoquine), antifolates (proguanyl, pyrimethamine, sulfadoxine, dapsone, etc.); naphthoquinones (atovaquone); peroxides and sesquiterpene derivatives (artemisinin, artesunate, artemether, etc.); antimicrobials (tetracycline, azithromycin, fluoroquinolones, etc.). In addition to chemotherapy, an efficient vaccine would be a desirable tool to control and eventually eradicate malaria, but such a vaccine has yet to be validated despite clinical assays.[4,5]

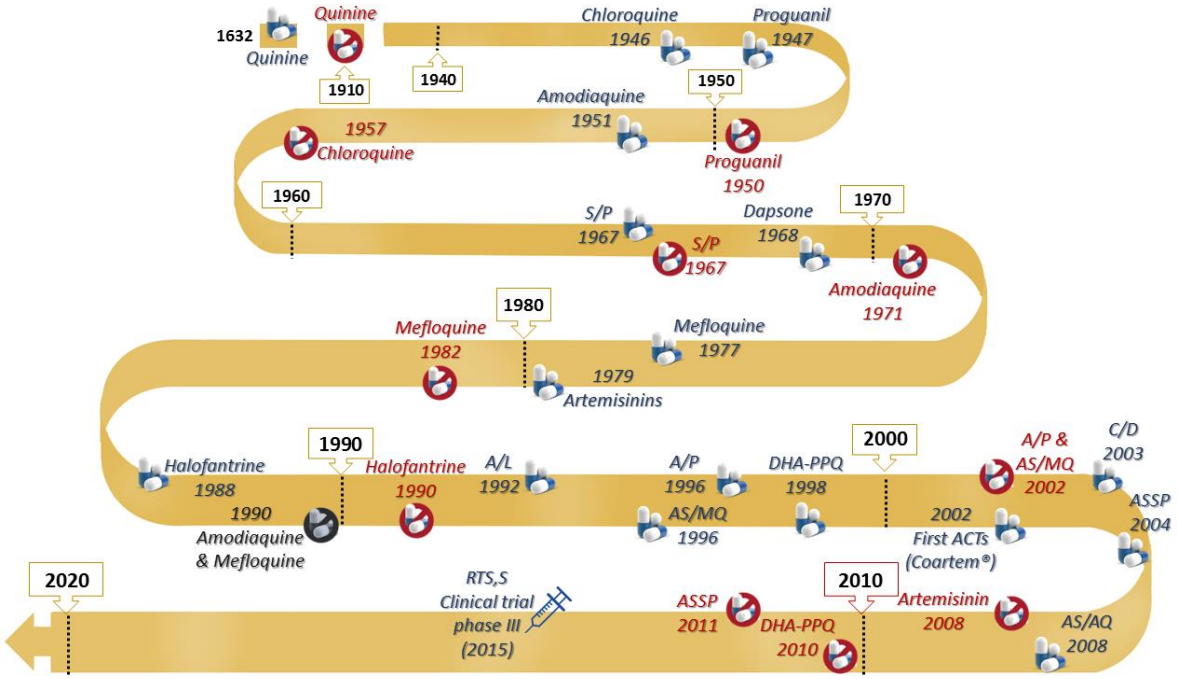


Figure 2: Timeline of main antimalarials and occurring resistance

Blue pills: first used as antimalarial; red circles: first reported resistance; black circles: no longer marketed AS/AQ, artesunate/amodiaquine; DHA-PPQ, dihydroartemisinin-piperaquine; A/P, atovaquone/proguanil; A/L, artemether/lumefantrine; ASSP, artesunate sufadoxine pyrimethamine; S/P, sulfadoxine/pyrimethamine; AS/MQ, arestunate/mefloquine; ACTs, artemisinin combination therapies; and C/D, chlorproguanil/dapsone.

Mutations in the parasite genome have appeared over the last century, inducing fast spreading resistance against all commercial drugs (**Figure 2**). To address these issues, and in the absence of an available efficient vaccine, new drugs with original modes of action are crucial as resistance to front-line antimalarial drugs (essentially artemisinin-based combination therapies, ACTs) is spreading in Southeast Asia. A number of collaborative drug discovery programs have thus been initiated worldwide and a few drug candidates are currently under clinical development or have recently been approved for clinical use. Among them, most of the new drugs correspond to compounds that are structurally related to previously used drugs or artemisinin-based combinations, and some molecules with a novel mechanism of action

have been proposed. To our knowledge, none of them belong to the nucleos(t)ide analogue family, targeting proteins involved in purine or pyrimidine metabolism, except DSM265 (currently in phase IIa clinical trials).[6] The latter is a selective inhibitor of *Plasmodium* dihydroorotate dehydrogenase (*Pf*DHODH), which plays a central role within the *de novo* pyrimidine pathway. However, target mutations were found in patient's parasites (already observed in earlier *in vitro* studies) and concerned a few patients with recurrence at day 28 after receiving a single oral dose of DSM265. Once again, this data highlights the need to use combination therapies.

Herein, we will focus on nucleobase, nucleoside and nucleotide analogues, which so far have been reported to target and inhibit proteins involved in the purine salvage pathway of *Plasmodium falciparum*. This family of derivatives is of special interest as they can mimic the endogenous substrates of these proteins, so the design of analogues and their study may result in a new class of antimalarial drugs. In addition, as *Plasmodium falciparum* relies solely on the *de novo* pathway for pyrimidine nucleotide biosynthesis and on the salvage pathway for purine nucleotides, both metabolic processes appear to be attractive chemotherapeutic targets. The development of these analogues as inhibitors of plasmodial purine metabolism is relatively recent and somehow associated with the discovery of the antiviral and antitumoral potential of such compounds. The main nucleic acid biosynthesis pathways of *Plasmodium* will be described as well as the wide collection of analogues targeting the corresponding proteins.

I/ NUCLEIC ACID METABOLISM IN *PLASMODIUM FALCIPARUM*

Mammalian cells are able to synthesize nucleic acids from small molecules through the *de novo* pathway but requiring 6 ATP equivalents for the synthesis of IMP from ribose-5-phosphate and a dozen steps. In contrast, the salvage pathway is based on the recycling of related metabolites, which is less

energy-demanding (a single step from hypoxanthine to IMP and 1 ATP equivalent is required for phosphoribosylpyrophosphate formation). The human host has both pathways to produce purine and pyrimidine nucleotides, whereas protozoan parasites such as *Plasmodium* only possess the salvage pathway for purine containing derivatives and *de novo* synthesis of pyrimidine derivatives. The latter are therefore essential for parasite growth, which requires a huge amount of purine and pyrimidine nucleoside triphosphates for its nucleic acid biosynthesis. A few reviews have provided detailed accounts of purine and pyrimidine pathways as chemotherapeutic targets.[7–9]

1) Pyrimidine Metabolism

Protozoan parasites rely solely on the *de novo* pathway to produce their pyrimidine derivatives, and they are not very capable of salvaging pyrimidines from erythrocytes. Briefly, *Pf* pyrimidine biosynthesis involves six enzymatic steps (**Figure 3**) as in the human host producing a central metabolite, *i.e.* uridine-5'-monophosphate (UMP), which is then used as precursor for all pyrimidine nucleotides (dUMP, dCTP, dTTP, UTP and CTP) required for DNA/RNA biosynthesis. The related enzymes can be divided in two groups of monofunctional and multifunctional proteins, i.e. the first and last three, respectively. Little attention has been focused on carbamoyl phosphate synthetase (CPS II) and aspartate transcarbamoylase (ATC), whereas *Pf* dihydroorotase (DHOase) has received much attention. The latter has been characterized and found to share many properties with mammalian proteins.[10] The three following enzymes, i.e. orotate phosphoribosyltransferase (OPRT), dihydroorotate hydrogenase (DHODH) and dihydroorotase (DHOase), are considered as key enzymes to target the human parasite, and DHODH is one of the most widely studied.[11,12] Their 3D crystal structures have been solved, their characteristics have been studied in detail and compared to their human counterpart.

This review is not intended to provide a detailed account of these proteins and their inhibitors, but these data may be gleaned from the literature[7,13]

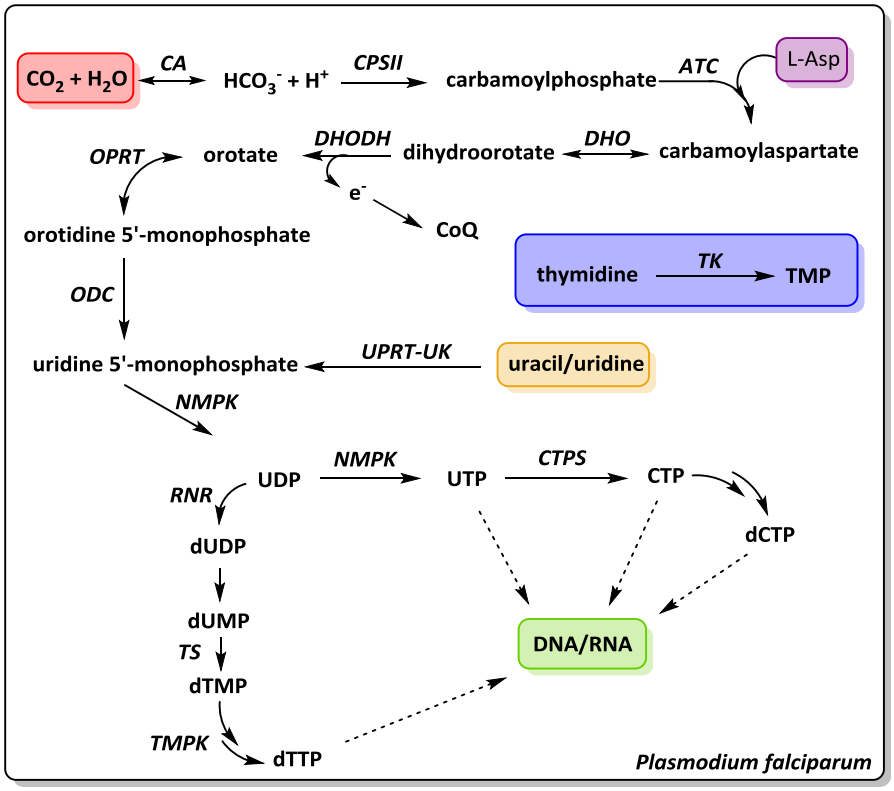


Figure 3 : *Pf* pyrimidine metabolism

Enzymes: CPSII, carbamoyl phosphate synthetase II; CA, carbonic anhydrase; ATC, aspartate transcarbamoylase; DHO, dihydroorotase; DHODH, dihydroorotate dehydrogenase; OPRTase, orotate phosphoribosyl transferase; ODC, orotidine 5'-monophosphate decarboxylase; NMPK, nucleoside monophosphate kinase; RNR, ribonucleotide reductase; TS, thymidylate synthase; TMPK, thymidylate monophosphate kinase; CTPS, cytosine 5'-triphosphate synthetase; TK, thymidine kinase; UPRT, uracile phosphoribosyl transferase; and UK, uridine kinase.

Metabolites: UDP, uridine-5'-diphosphate; UTP, uridine-5'-triphosphate; dUDP, 2'-deoxyuridine-5'-diphosphate; dUMP, 2'-deoxyuridine-5'-monophosphate; CTP, cytidine-5'-triphosphate; dTMP, 2'-deoxythymidine-5'-monophosphate; dTTP, 2'-deoxythymidine-5'-triphosphate; dCTP, 2'-deoxycytidine-5'-triphosphate; and CoQ, co-enzyme Q.

2/ Purine Metabolism

Plasmodium species are purine auxotroph parasites that depend exclusively on purine materials derived from erythrocytes. Furthermore, as the *Plasmodium* genome has an extremely high adenine content (over 80% AT content), the parasite requires a considerable amount of adenine nucleotides (dATP and ATP) to maintain its high replication rate. Erythrocytes are unable to perform *de novo* purine synthesis alone, so the corresponding nucleobases and nucleosides are taken up from the plasma by the erythrocytes and then by the parasites.[14,15] Purine import from the host cell is mediated by membrane transporters such as equilibrative nucleoside transporters (ENTs, **Figure 5**), *Pf* ENT1 is the main one from the four transporters (*Pf* ENT1-4) reported to date and supplying the purine salvage pathway.[15,16] Although the transport of purine and pyrimidine derivatives in mammalian cells is well-documented,[17,18] few studies have been performed on the parasite transporters in the last decade and mainly concerned *Pf* ENT1. Further investigations are thus required for rational design of purine transport inhibitors as antimalarial drugs.

A few of the enzymes involved in *Pf* purine metabolism have attracted special attention, namely purine phosphoribosyl transferases (PRT), adenosine deaminases (ADA) and purine nucleoside phosphorylases (PNP). Phosphoribosyl transferases induce the ribophosphorylation of purine bases in a single step and are present in many parasites, as well as in mammalian cells. In *Plasmodium*, hypoxanthine-guanine-xanthine-phosphoribosyl transferase (HGXPRT) converts hypoxanthine, guanine and xanthine into inosine-5'-monophosphate (IMP), guanosine-5'-monophosphate (GMP) and xanthosine-5'-monophosphate (XMP), respectively (**Figure 6**). Purine nucleoside phosphorylases degrade nucleosides into their corresponding nucleobases and sugar counterparts (**Figure 7**). Adenosine deaminases convert the exocyclic amino group of purine nucleos(t)ides into their oxo-derivatives (**Figure 8**). Moreover, HGXPRT, ADA and PNP enzymes are highly expressed and key players in purine

metabolism, but other enzymes are also involved, such as guanosine-5'-monophosphate synthase (GMPS), adenylosuccinate synthetase (AdSS), adenosine-5'-monophosphate deaminase (AMPDA) and inosine-5'-monophosphate dehydrogenase (IMPDH).

This metabolism is remarkable because of the few enzymes involved even though it has a crucial role in parasite survival. In addition, these enzymes are also present in the metabolic pathway of other plasmodial parasites and may have a high degree of similarity. As example *Pv*ENT1 shares ~75% amino acid sequence identity with *Pf*ENT1. However, differences between *P. vivax* and *P. falciparum* were reported and resulted in modifications of the 3D protein structure and/or conformation.[19] As example, structural differences between *Pv* and *Pf* ADA have been highlighted by computational studies and crystal structure analysis, and mainly concern the ligand recognition domain.[20]

The parasite salvages adenosine from the erythrocytes and the combined action of ADA and PNP successively leads to the formation of inosine and hypoxanthine (**Figure 4**). As very little adenosine is available from the erythrocyte (due to the activity of erythrocytic adenosine kinases), the parasite essentially relies on hypoxanthine, which is also salvaged from the host. The key purine metabolism precursor is consequently hypoxanthine, and therefore malarial cultures are often enriched with this nucleobase, which serves as a nutrient to promote parasite growth. Hypoxanthine is then converted into IMP by HGXPRT (**Figure 4**). IMP is thus the nucleotidic precursor of all other purine nucleotides required for nucleic acid biosynthesis, especially adenosine 5'-monophosphate (AMP). As *Pf* does not have the genes coding for nucleoside kinases[21,22] (AMP synthesis directly from adenosine), there is no other alternative to obtain AMP, and IMP is the sole adenosine 5'-monophosphate metabolite available. To a lesser extent, it has been shown that by using high concentrations of adenosine in *Pf* cultures, AMP synthesized by RBC could induce erythrocytic AMP uptake into the parasite.[22] IMP is also transformed into XMP via IMPDH and then into GMP via GMPS. GMP is also produced via a second pathway

involving guanosine and guanine uptake, with the latter being PNP and HGXPRT substrates, respectively. Similarly, XMP is produced through HGXPRT activity following xanthine uptake.[7]

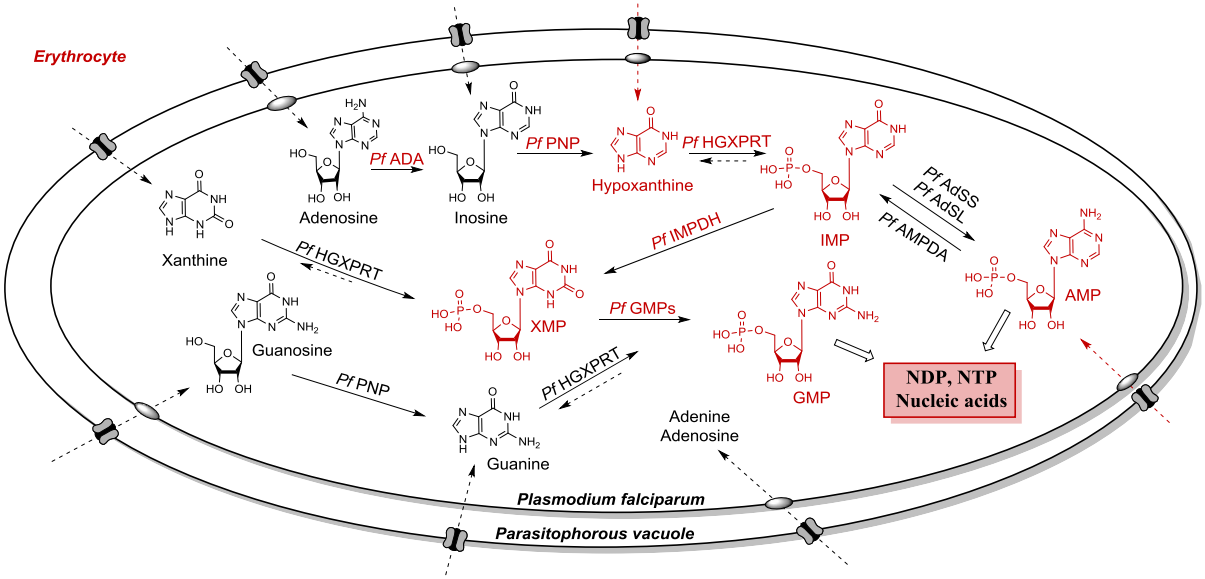


Figure 4: Representative scheme of *Pf* purine metabolism

In red: main path or nucleic acids synthesis in *Pf*

Metabolites: IMP, inosine-5'-monophosphate; XMP, xanthosine-5'-monophosphate; AMP, adenosine-5'-monophosphate; NDP, nucleoside diphosphate; and NTP, nucleoside triphosphate.

Enzymes: HGXPRT, hypoxanthine-guanine-xanthine-phosphoribosyl transferase; ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; GMPs, guanosine-5'-mono-phosphate synthase; IMPDH, inosine-5'-monophosphate dehydrogenase; AMPDA, adenosine-5'-monophosphate deaminase; AdSS, adenylosuccinate synthase; and AdSL, adenylosuccinate lyase.

Transporters: ENT, equilibrative nucleoside transporter, notably *Pf* ENT1-4. PVM channel are represented at the parasitophorus vacuole membrane.

To date, the main strategy developed to inhibit *Plasmodium falciparum* growth involved targeting PNP and ADA *Pf* enzymes, because of their importance in the hypoxanthine pathway in the parasite.

1. *Uptake of purine bases and nucleosides into the parasite*

a. Erythrocyte transport pathways

As for many human cells, mature erythrocytes have a profusion of transport systems (transporters and channels) located at the plasma membrane and responsible for the uptake of ions, hexoses, amino acids and other nutrients **(Figure 5).** Human nucleoside transporters (hNTs) thus include various transport system families, such as hENT1 and hENT2 (both equilibrative nucleoside transporters and responsible for purine and pyrimidine uptake, with nucleosides being the preferred substrates)[18], hFNT1 (facilitated nucleobase transporter, which transports hypoxanthine and adenine) and hCNTs (concentrative ion-coupled nucleoside transporters).[16,14] It has been demonstrated that hENT1 and hFNT1 are the most important source of purine-containing derivatives in *Pf*-infected RBC, while parasite-induced new permeability pathways (NPP, an alteration in membrane permeability) has also been mentioned.[23,14] NPP are designed to facilitate the uptake of low molecular weight metabolites required for the parasite growth, such as sugar, sugar-alcohols, polyamine, nucleosides, nucleobases, ions, amino-acids [24], etc.[25,26]

b. Parasite transporters

Four equilibrative nucleoside transporters *(Pf* ENT1-4) have been identified to date **(Figure 5)**—but they have yet to be fully characterized—and are expressed during the intraerythrocytic stage of the *Plasmodium* life cycle. [202] According to genetic studies, *Pf* ENT1 has only 17% identical sequence homology with hENT1 and only shares 15 to 22% identical amino acid sequence with other parasite ENT transporters, and all four share only 2% similarity between them.[16]

Among them, *Pf* ENT1 (also called *Pf* NT1) is mainly responsible for the import of most purine nucleosides and nucleobases inside the parasite. Its characteristics (affinity and substrate specificity) have been the focus of some debate with regard to the model used for transport studies and the effects of purine metabolism upon import.[27] In 2008, Quashie *et al.*[15] suggested that hypoxanthine and purine nucleoside

uptake was mediated by a high-affinity and broad-specificity transporter, i.e. *Pf* NT1 (located at the parasite plasma membrane[28]), and the existence of another high-affinity transporter and two low-affinity systems. Downie and co-workers proposed an alternative model, taking into account the fact that high-affinity proteins of purine metabolism readily transform *Pf* ENT substrates into nucleotides.[27] They thus reported a fast, equilibrative and low-affinity mechanism for *Pf* NT1.[29] *Pf* ENT1 is a proton-dependent transporter[30] accepting inosine, hypoxanthine, guanine, adenosine and guanosine as substrates, but with higher affinities for hypoxanthine and adenosine (K_i 300-700μM range).[31] They are reported to be 11 transmembrane helix segment proteins, with intracellular N-ter and extracellular C-ter, which is the common structure for all protozoan and mammalian ENT1. Helices are mostly linked by short hydrophobic connectors, except for two large loops (one extracellular and one intracellular).[32] As its human homologue, *Pf* ENT1 may also accept pyrimidine nucleosides as substrate.[33]

Pf ENT2 and ENT3 have received much less attention. [16] *Pf* ENT2 was shown to be located at the parasitic endoplasmic reticulum membrane and thus may not be involved in purine uptake.[34] *Pf* ENT3 has yet to be localized or shown to be involved in purine or pyrimidine transport.

Frame *et al.* have shown that *Pf* ENT4 is a low-affinity (in the millimolar range) purine transporter able to transport both nucleosides and bases such as adenine, adenosine and 2'-deoxyadenosine with low affinity.[35] *Pf* ENT4 does not transport AMP or hypoxanthine (the main metabolite in the purine pathway), thus presenting a different substrate profile from that of *Pf* ENT1.

Like *Pf*, *Pv* parasites also present four equilibrative nucleoside transporters. *Pv* ENT1 shares an almost identical amino acid sequence with *Pf* ENT1, although small differences have been observed with variable affinities of both transporters for the same substrate.[36] The substrate and inhibitor specificity of *P. berghei* ENT1 has been reported recently.[37]

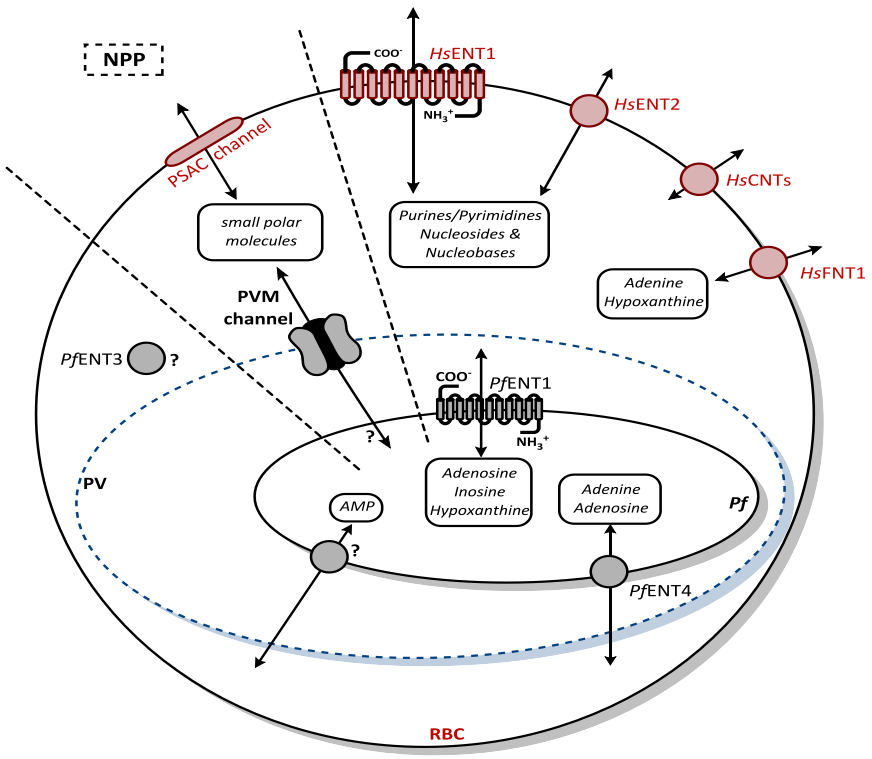


Figure 5: Main transporters involved in the uptake of purines and derivatives into RBC and the parasite.

*Pf*ENT3 is not yet localized; "?" sign, unknown transporters.

2. *HGXPRT*

HGXPRT is the most targeted purine metabolism enzyme in *P. falciparum* due to its major and multiple implications in the metabolism of this parasite (**Figure 4**). The crucial role of HGXPRT in purine metabolism has also been observed in *P. vivax*.[38] Indeed, it provides the sole PRT activity for three substrates, i.e. converting hypoxanthine, xanthine and guanine into the corresponding nucleoside 5'-monophosphates (IMP, GMP and XMP). Briefly, HG(X)PRT facilitates transfer of the phosphoribosyl moiety from *P*Rib-*PP* (alpha-D-5-phopshoribosyl-1-pyrophosphate) to 6-oxopurines in the presence of divalent metal ions, i.e. usually Mg^{2+} *in vivo* (**Figure 6**). Reyes *et al*. reported a detailed enzymatic

characterization of *Pf* HGXPRT, demonstrating that phosphoribosyl transferases are the most important enzymes in purine metabolism.[39]

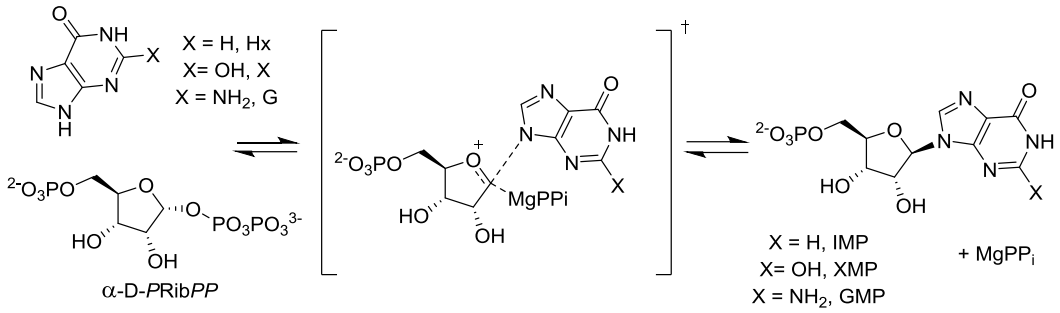


Figure 6: Mechanism and transition state of the 6-oxopurinephosphoribosyltransferase catalyzed reaction

Hx, hypoxanthine; X, xanthine; G, guanine; IMP, inosine 5'-monophosphate; XMP, xanthosine 5'-monophosphate; GMP, guanosine 5'-monophosphate; α-D-*P*Rib-*PP*, α-D-ribofuranose-5-phosphate-1-diphosphate; and PP_i, pyrophosphate.

Few differences between the human and *Pf* HGXPRT were revealed by Keough *et al.*[40] when comparing the specific activity (greater for human enzymes), kinetic parameters and substrate selectivity (the human enzyme does not accept xanthine as substrate, contrary to the parasite protein). Both enzymes are active as a homotetrameric form and share 44% sequence identity and 76% similarity. Superposition of their structures (in the presence of substrates of the catalytic reaction) generates a good root-mean-square deviation (rmsd) value (nearly 1,7 Å).[41] Considering these structural similarities between the two enzymes, the challenge is to selectively target and inhibit the parasite phosphoribosyl transferases so as to avoid any toxicity for human cells.

The crystal structures of the human 6-oxopurinephosphoribosyltransferase (*Hs* HGPRT) in the presence of nucleoside analogues have been often described. The human enzyme has three important binding sites

hosting the nucleobase (through hydrogen bonds with residues), the 5'-phosphate (binding pocket is located between residues 137-141) and the pyrophosphate moiety (of *P*rib-PP or only PP_i).

In humans, *P*Rib-*PP* first binds to the metal ion and then the required nucleobase associates with the complex to form the intermediate. Then the pyrophosphate dissociates from the ribooxocarbenium complex and nucleoside 5'-monophosphate is released from the enzyme catalytic site.[42] The initial proposed mechanism for *Pf*HGXPRT was slightly different and consisted of a ping-pong exchange, where *P*Rib-*PP* first binds to the enzyme, followed by PP_i release and then nucleobase entry[43]. These results have been contested by the reported crystal structure of *Pf*HGXPRT complexed with hypoxanthine.PP_i.Mg^{2+} and a few immucillin transition state analogues.[175] It has been shown that nucleobase binding is conditional to *P*Rib-*PP* entry, suggesting that *Pf*HGXPRT proceeds through a steady-state-ordered mechanism. [176]

3. *PNP*

PNP are also important enzymes present in both mammals and protozoan parasites. Like HGXPRT, PNP inhibition leads to death of the parasite by blocking the formation of hypoxanthine, a key metabolite of the *Pf* purine metabolism **(Figure 4)**.[46,47] *Pf* PNP is structurally different from the human protein (hexamer versus homotrimer and low sequence homology, ~20% identity of the primary amino acid sequence) and exhibits different catalytic rates with inosine as substrate (56 s^{-1} for hPNP and 0.34s^{-1} for *Pf* PNP).[48–50]

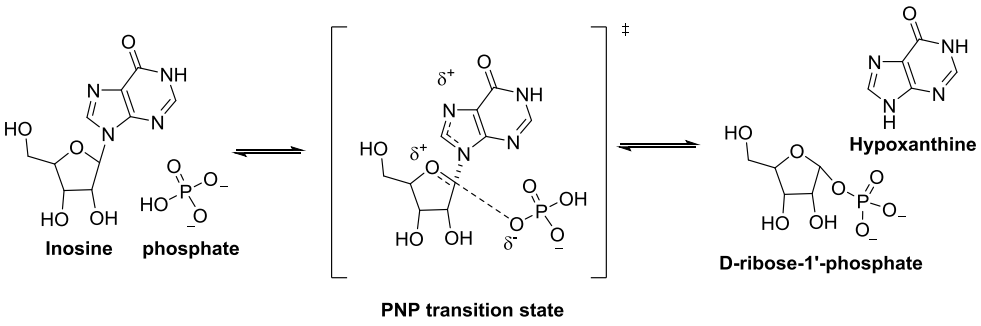


Figure 7: Mechanism and transition state of the *Pf* purine nucleoside phosphorylase catalyzed reaction

According to the mechanism proposed by Schramm *et al.* for both human and *Pf* PNPs,[51,52] the nucleoside and phosphate (as nucleophilic participant) first bind to the active site of the enzyme and the reaction transforms the inosine/enzyme complex (located on a single active site of the homotrimer), into a hypoxanthine/ribose/enzyme complex (**Figure 7**). Then the enzyme catalyzes cleavage of the glycosidic bond to release the ribose-1'-phosphorylated moiety, giving rise to a tight binding hypoxanthine/enzyme complex. The latter finally dissociates within the corresponding nucleobase and the D-ribose phosphorylated moiety. Both enzymes have similar transition states, with the active site environment being more constrained in the parasite enzyme than in the human one (on the basis of different interactions between the active site and substrate).

4. *ADA*

ADA is a zinc-dependent hydrolase that catalyzes the irreversible hydrolysis of adenosine into inosine, its corresponding 6-oxopurine derivative. The reaction is triggered by the deprotonation of a water molecule which then reacts with the substrate, giving rise to 6-amino-6-hydroxypurine as intermediate, which provides the corresponding oxopurine by releasing one ammonia molecule (**Figure 8**).[53]

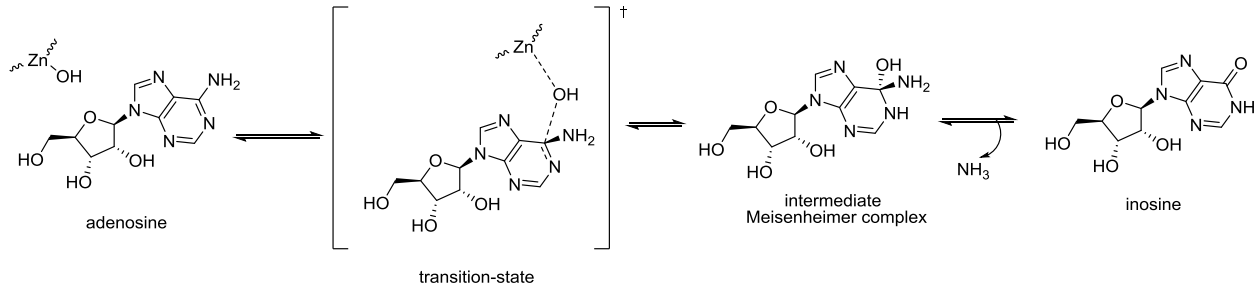


Figure 8: Mechanism and transition state of the *Pf* adenosine deaminase catalyzed reaction

Human and *Pf* ADAs are functionally similar and have high sequence homology, but they differ slightly in terms of substrate specificity and catalytic turnover. *Pf* ADA thus accepts both adenosine and 5'-methylthioadenosine (MTA, a polyamine biosynthesis metabolite) as substrate, which is not the case for its human homologue.[54]

Pv ADA has been crystallized in a complex with three different ligands (adenosine, guanosine and 2'-deoxycoformycin, i.e. a known human ADA inhibitor)[55]. A parasite-specific conformation was observed, which closed the substrate binding site within a solvent-less cavity, whereas the human enzyme does not close its active site. This difference may explain the recognition of MTA, as the solvent-less cavity hosts the hydrophobic thiomethyl group. Amino-acid changes in the active sites of *Pv* and *Pf* ADAs have been highlighted via computational studies and comparison with a *Pf* ADA homology model.[20]

In addition, *P. knowlesi* growth (in infected primates) was found to be inhibited by 2'-deoxycoformycin, indicating that parasite survival was dependent on adenosine deaminase.[56]

In 2017, the *Pf* proteome was studied to identify commercial drugs which could target and inhibit *Pf* enzymes. Dipyridamole (a nitrogen heterocycle derivative used as blood vessel dilatator) was thus reported to be a *Pf* ADA inhibitor with an IC_{50} of 30 nM.[57]

5. *IMPDH*

Inosine 5'-monophosphate dehydrogenase catalyzes XMP formation from IMP through a covalent mechanism (**Figure 9**) and serves as an alternative/complementary pathway for providing GMP. As GMP synthesis mediated by HGXPRT is not substantial enough to provide the overall amount of GMP required for nucleic acid biosynthesis in the parasite, IMPDH inhibition could indirectly and partially block GMP formation from XMP.[58,59] IMPDH is a homotetramer protein. The active site is located at the C-terminal chain, whereas the cofactor (NAD^+) binds at another site.[60] Consequently, two classes of IMPDH

inhibitors can be developed, i.e. one targeting the substrate binding site and the other targeting the cofactor binding loops via an uncompetitive mechanism (e.g. mycophenolic acid and phenyl-oxazole urea, respectively).[61]

Human and *Pf* IMPDH genome sequences shown only 48% of similar amino acid sequences and exhibit differences in the structure of the active site.[60] That could possibly help to design selective inhibitors of the plasmodial enzyme.

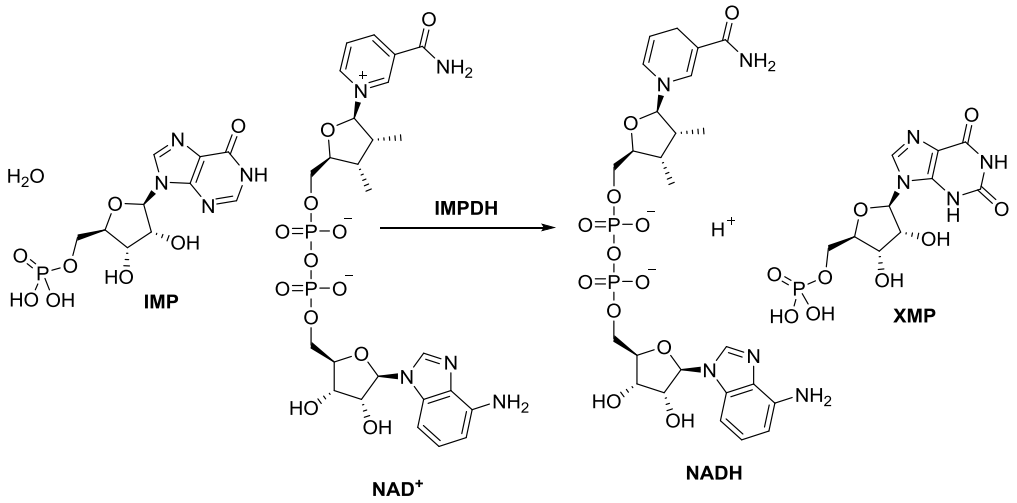


Figure 9: *Pf* inosine 5'-monophosphate deshydrogenase catalyzed reaction

6. *GMPS*

Pf GMP synthetase (belonging to the glutamine amidotransferase family) is a dimeric protein catalyzing the irreversible formation of GMP through XMP amination. GMP synthesis via HGXPRT is not enough to maintain optimal GMP levels in the parasite,[62] thus blocking this enzyme may be a valuable approach to inhibit nucleic acid biosynthesis. *Pf* and human GMPS share 20-30% common chain sequence and both contain two domains, i.e. one catalyzing formation of the XMP-adenyl complex (ATPPase), and the other providing the required ammonia from glutamine hydrolysis (GATase, glutamine amidotransferase). The ammonia molecule is channeled to the other domain in order to generate the GMP product [63] (**Figure 10**),

which is a common amidotransferase reaction step (ping-pong mechanism).[62] Both ATPPase and GATase sites can be targeted in competitive and uncompetitive ways, respectively. For instance, glutamine analogues, such as acivicin and 6-diazo-5-oxo-norleucine (use as antibiotics and known hGMPS inhibitors), are able to inactivate the enzyme by covalent modification of the GAT domain.[62] *Pf* GMPS also differs with regard to other plasmodial species associated with a single 20 amino-acid long sequence located in its GAT domain.[63]

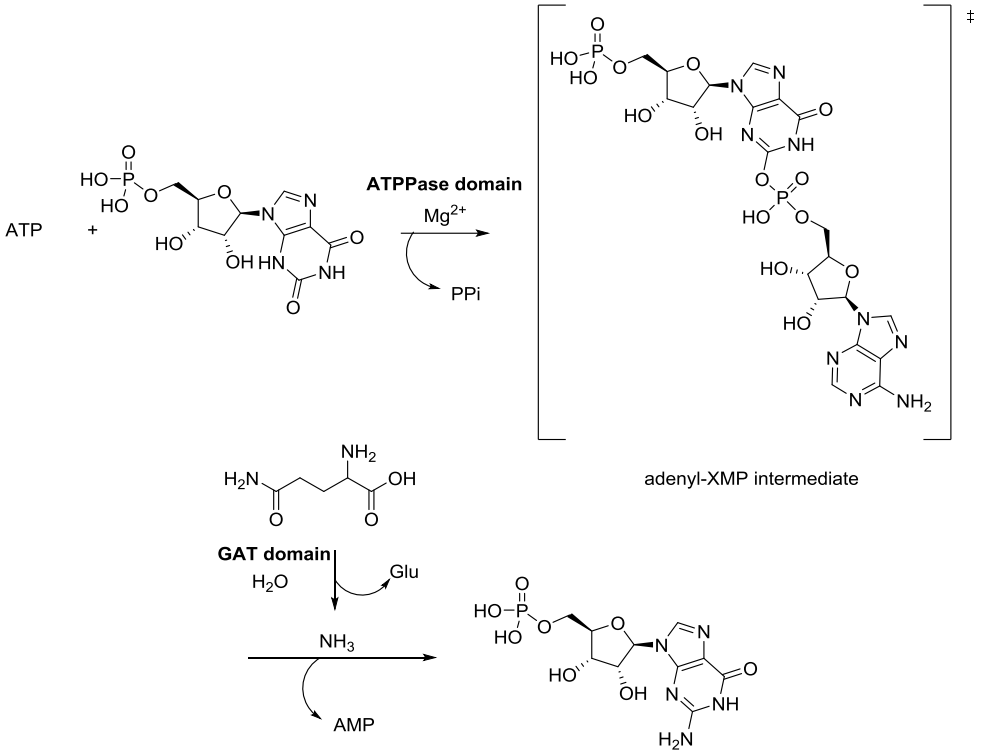


Figure 10: Formation of GMP from XMP, catalyzed by *Pf* GMPS from its GAT and ATPPase domains

II/ TARGETING PURINE METABOLISM WITH NUCLEOS(T)IDE ANALOGUES

Because of the importance of purine metabolism for plasmodial parasites, the inhibition of related enzymes has been considered since 1984.[64] Nucleoside analogues are well-known inhibitors of the nucleic acids biosynthesis, so they may also be viewed as potential candidates for targeting of purine metabolism

enzymes due to their structural similarities with their substrate. Series of purine-based heterocycles, nucleoside and nucleotide analogues have been studied with the aim of targeting these enzymes and related transporters. A few of them were designed on the basis of transition state studies. This part of the review concerns such compounds as inhibitors of transporters and/or enzymes involved in plasmodial purine metabolism.

1. *Purine-based Analogues*

In 2006, Keough *et al.* studied the effects of purine nucleobase skeleton modifications and the ability of these derivatives to bind to human and *Pf* HGXPRT, and the hypothetical discrimination between these two enzymes.[65] By analogy with 6-mercaptopurines used in cancer chemotherapy[66], the proposed mechanism of action consists of their conversion—mediated by 6-oxopurinePRTases—into their corresponding nucleoside 5'-monophosphate derivatives, thereafter incorporated into DNA or RNA and thus interfering with cell replication. Determination of the K_m for these derivatives revealed that the presence of an exocyclic oxygen-containing group at position-2, and/or a chlorine atom at position-6, and/or the replacement of the carbon atom at position-8 by a nitrogen allowed strong discrimination between human and *Pf* HGXPRT. In addition, these nucleobases are able to inhibit *P. falciparum in vitro* growth. The best IC_{50} values were 1.3 μM and 6.6 μM for 6-mercaptopurine and 8-azaguanine, respectively.

2. *Nucleoside analogues*

Since natural substrates of the targeted enzymes are mostly nucleosides and derivatives, the easiest and most promising way to selectively inhibit them is to design analogues. In the field of antiviral and antitumoral chemotherapy, the search for nucleoside analogues has led to the discovery of the active

compounds which are nowadays used as drugs. Some of these derivatives were tested against the *Pf* purine salvage pathway, leading to the identification of lead compounds which served as a starting point for the synthesis of novel derivatives.

a. Cordycepin

Cordycepin (**Table 1**), an antibiotic and anti-tumoral nucleoside extracted from *Cordyceps militari*, was tested *in vitro*, by Trigg *et al*. in 1971 against *P. knowlesi* and *in vivo* in *P. berghei*-infected mice.[67] Effects were noted on *in vitro* parasite growth at micromolar concentrations, but a full cure was not obtained in mice even at high concentration. The fact that nucleic acid biosynthesis in the parasite was affected suggested that the purine metabolism of the parasite was targeted.

b. Mizoribine and ribavirin derivatives

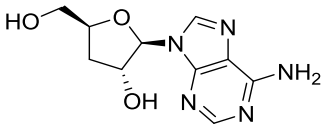
Mizoribine **(Table 1)** is an immunosuppressive drug that was isolated from *Eupenicillium brefeldianum* in Japan in 1971. It is clinically used for renal transplantation[68] as well as an inhibitor of *Hs* IMPDH in leukemia and viral diseases. Its proposed mechanism of action involves IMPDH inhibition by the mizoribine-5'-monophosphate, thus limiting both XMP formation and GMP synthesis mediated by GMPS.[69] When tested as an antimalarial by Webster and Whaun in 1982, mizoribine appeared to be active against drug-resistant *Pf* strains at 50 μM.[70] Variations in the concentration of several purine-containing nucleosides and nucleotides in infected RBCs have been studied and a significant decrease was observed in the presence of mizoribine.

Recently, Raza *et al.* performed virtual screening of a library of ribavirin analogues (**Table 1**, a base modified nucleoside analogue known as a competitive inhibitor of human IMPDH[71,72]) using a 3D-homology model of *Pf* IMPDH.[60] Five promising ribavirin derivatives were identified as potential ligands, which should be able to selectively target the parasite enzyme by interacting with the active site (hydrogen

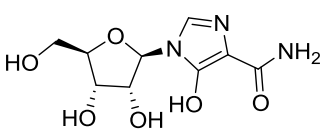
bonds and hydrophobic pockets). To date, these molecules have not yet been tested *in vitro* against *P. falciparum* growth.

Table 1. Representative nucleoside analogues as potential purine metabolism inhibitors

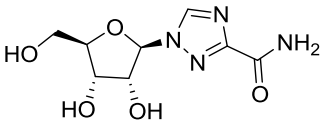
Cordycepin (3-deoxy-adenosine)[67]



Inhibitors of *Pf* IMPDH[60,70]

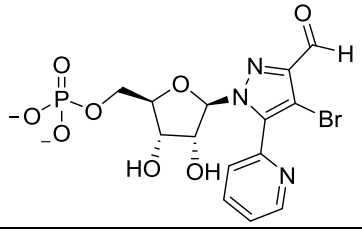


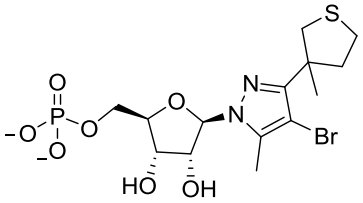
Mizoribine (Bredinin)



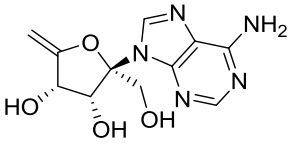
Ribavirin (Virazole)

Ribavirin derivatives screened by Raza *et al.*[60]

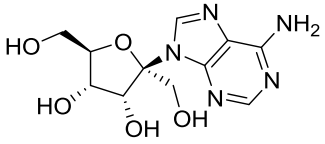




Adenosine analogues tested against *Pf* GMPS by Bhat *et al.*[62]

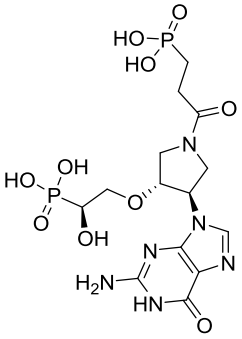


Decoyinine



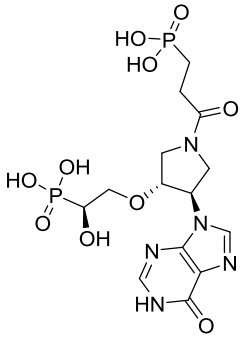
Psicofuranine

Pyrrolidine nucleoside mono- and bisphosphonates[73]



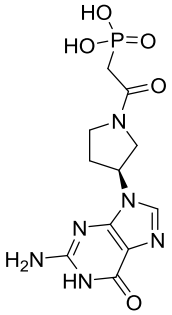
1

K_i 8 nM on *Pv* HGPRT
K_i 7 nM on *Pf* HGXPRT
K_i 3 nM on *Hs* HGPRT



2

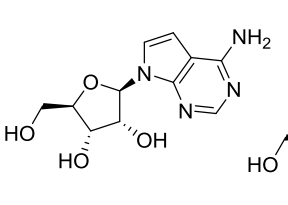
K_i 6 nM on *Pv* HGPRT
K_i 2 nM on *Pf* HGXPRT
K_i 1.3 nM on *Hs* HGPRT



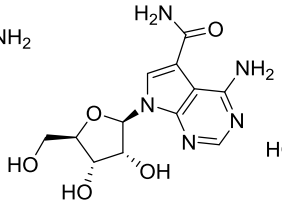
3

K_i 2,6 mM on *Pv* HGPRT
K_i 0.4 mM on *Pf* HGXPRT
K_i 73 mM on *Hs* HGPRT

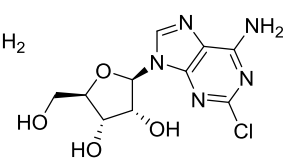
Tubercidin and derivatives[74]



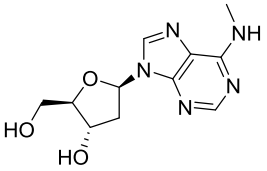
Tubercidin
IC_{37} 0.7 µM on *Pf*



Sangivamycin
IC_{37} 0.3 µM on *Pf*

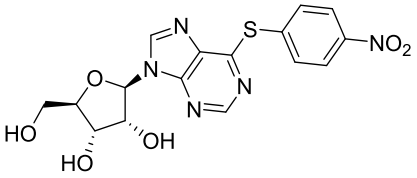


2-chloroadenosine
IC_{37} 11 µM on *Pf*

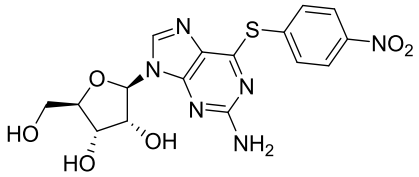


6-methylamino-deoxyadenosine
IC_{37} 10 µM on *Pf*

Nitrobenzylthionucleosides[75]



Nitrobenzylthioinosine (NBMPR)
ID_{50} 4.2 µM on K-1 *Pf* strains



Nitrobenzylthioguanosine (NBTGR)
ID_{50} 4.5 µM on K-1 *Pf* strains

c. Psicofuranine and decoyinine

As psicofuranine and decoyinine (**Table 1**) were reported to inhibit hGMPS (with 17.3 and 46.5 µM IC_{50} values),[76] Baht *et al.* evaluated them on purified *Pf* GMPS. They reported a weak inhibitory effect of psicofuranine (25% inhibition at 0.5 mM) and no effect of decoynine.[62] Only psicofuranine had an IC_{50} of 0.3 mM when tested against *Pf* cultures (3D7 strain).[77]

d. PNBPs – Pyrrolidine nucleoside bisphosphonates

Initially synthesized and tested for their ability to inhibit *E. coli*, 6-oxopurine phosphoribosyltransferases,[78] pyrrolidine nucleoside phosphonates (**Table 1**) were subsequently assessed as potential inhibitors of *Pv*HGPRT and *Pf*HGXPRT. The design of such compounds was based on a central 5-membered ring skeleton, bearing three substituents anchored through free rotation bonds, thus allowing to adopt the best conformation at the active site of the enzyme.[73]

In comparison with compound **3**, the addition of a second phosphonate tail to the pyrrolidine ring markedly decreased the K_i values (from millimolar to nanomolar range) for all three enzymes but to the detriment of selectivity. It should be noted that: (i) the nature of the nucleobase did not seem to have a real impact

on the activity, (ii) the addition of the hydroxyl group on the second phosphonate tail enhanced the inhibitory activity of these derivatives and the stereochemistry had an influence on the affinity of the studied enzyme. For instance, the (*R*)-isomer of compound **1** exhibited a 7.5-fold higher K_i value than that of its (*S*)-isomer on *Pv* HGPRT. Studies of the crystal structures of the *Pv* HGPRT-compound **1** complex revealed that the oxygen-linked phosphonate tail located within the 5'-phosphate binding pocket could explain the difference in K_i observed between the two stereoisomers. Additional interactions also occurred between water molecules, the hydroxyl group and the active site for the (*S*)-isomer of compound **1**. Structural data regarding the *Pf* protein are currently not available.

e. Tubercidins

The successful use of adenosine analogues in cancer chemotherapy led Coomber *et al.* to test some of them against *Plasmodium falciparum. In vitro*, the most efficient ones were the 7-deaza-adenosine derivatives tubercidin and sangivamycin (**Table 1**), with IC_{37} of 0.7 and 0.3 µM (IC_{37} values are quite unusual compared to IC_{50}, but it was used by the authors to compare their results with previously reported ones). Their mechanism of action was suggested to be related to purine metabolism, especially with a decrease of IMP content in treated *Pf*-infected erythrocytes.[74] In addition, 5-iodotubercidin (a known human adenosine kinase inhibitor) has been shown to inhibit *Pf* growth *in vitro* (IC_{50} of 2 µM)[22] and several tubercidin derivatives appeared to be *Pf* ENT1 substrates.[31] However, no further studies have been recently reported in the literature concerning this family of compounds.

f. NBMPR – nitrobenzylthioinosine

Nitrobenzylthioinosine (NBMPR) and its guanine analogue (NBTGR) were first assayed as antimalarial drugs in 1989 **(Table 1)**.[75] In addition to their micromolar IC_{50} values in *Pf*-K1 strains (multi-drug resistant)-infected erythrocytes, they were successfully used in an original approach based on the alteration of infected erythrocyte membrane permeability. Briefly, the two derivatives were used to

facilitate the uptake of cytotoxic nucleosides (presumably through permeability pathways induced by the parasite) into infected erythrocytes, while their ability to selectively inhibit the human transporters (hENT1)[79] prevented the uptake and further toxic effects in uninfected cells. Later on, it was also proven that *Pf* ENT1 and *Pf* ENT4 were insensitive to NBMPR.[80]

3. *Transition state analogues as Pf purine metabolism inhibitors*

In theory, a perfect and chemically stable transition state analogue will bind tighter to the enzyme active site than the substrate. The protein will enclose the compound with a more thermodynamically stable and static conformation, thus preventing natural substrate binding until release of the transition state analogue. Moreover, an ideal transition state analogue will be highly selective of the targeted enzyme, avoiding toxicity and unwanted side-effects.

Natural transition state species have a very short longevity and consequently are not directly observable, but they can be predicted, or hypothesized, by studying the reaction process mechanisms and determining the intrinsic kinetic effects of isotopic compounds. A chemical structure could then be proposed on the basis of i*n silico* calculations. Transition state analogues of three parasite enzymes have thus been reported to date, i.e. *Pf* ADA, *Pf* PNP and *Pf* HGXPRT.[81]

a. <u>*Pf* ADA inhibition</u>

- *Coformycin and derivatives*

Coformycin and 2'-deoxycoformycin (namely pentostatin) were both known as mammalian ADA inhibitors. In 2007, Tyler *et al.* studied the affinity of these derivatives toward human, bovine and *Pf* ADAs (**Table 2**) and inhibition constants in the low nanomolar range were observed, but with no specific selectivity.[82] On the basis of previous results showing that 5'methylthioadenosine or inosine[54] were *Pf* ADA substrates, 5'-alkylthio and 5'-phenylthio analogues of coformycin and 2'-deoxycoformycin were

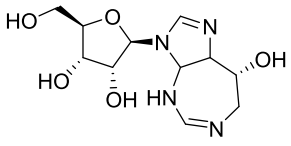
synthesized.[82] Among them, 5'-methylthio derivatives appeared to be highly selective of the parasite enzyme, with K_i of less than 1 nM (**Table 2**), especially 5'-methylthiocoformycin, which showed >20,000-fold selectivity for *Pf* versus *Hs* ADA. However, no further studies have been reported.

- *L-nucleoside analogues*

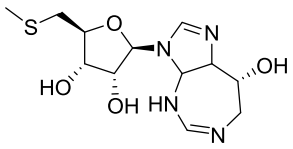
In 1995, Upston *et al.* showed that L-adenosine was significantly transported (via a unique parasite-induced pathway) and selectively metabolized (through ADA activity) within parasites infected-erythrocytes.[83] These preliminary results led the same team to investigate series of L-nucleoside analogues (**Table 2**).[84,85] Among them, L-coformycin and L-isocoformycin were shown to specifically inhibit *Pf* ADA activity in the picomolar range (contrary to their D-isomer, especially 2'-deoxycoformycin, which interacted with both human and parasite enzymes).[85] L-coformycin, L-isocoformycin and L-sangivamycin inhibited the *in vitro* growth of *Pf* with an ID_{50} in the micromolar range.[84]

Table 2. Nucleoside analogues as *Pf* ADA inhibitors

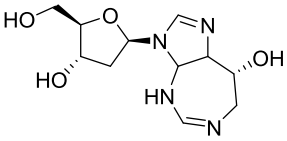
Coformycin and derivatives as inhibitors of human and plasmodic adenosine deaminases[82]



Coformycin
K_i 13.9 nM / Ki* 0.11 nM on *Hs* ADA
K_i 0.68 nM / K_i* 0.08 nM on *Pf* ADA



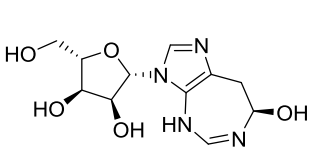
5'-Methylthiocoformycin
K_i >10 µM / K_i* ND on *Hs* ADA
K_i 2.66 µM / **K_i* 0.43 nM on *Pf* ADA**



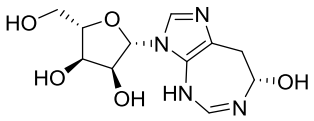
2'-deoxycoformycin
K_i 0.5 nM / K_i* 0.026 nM on *Hs* ADA
K_i 8.2 nM / K_i* 0.038 nM on *Pf* ADA

K_i* values correspond to the equilibrium dissociation constant upon completion, after a slow onset, for tight binding inhibition of the enzyme (consequent upon a time-dependent conformational change in the enzyme that improve the binding after a moment, thus K_i* is lower than K_i) reported for transition state-analogues. Compounds with K_i* values are called slow-onset tight binding inhibitors. K_i values of >10,000 indicate that assays with 10 µM concentration of the compound showed no inhibition under the assay conditions

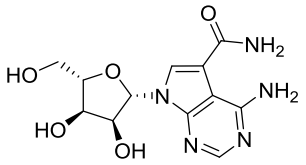
L-nucleoside derivatives as *Pf*ADA inhibitors[84,85]



L-isocoformycin
K_i 7 pM on *Pf* ADA



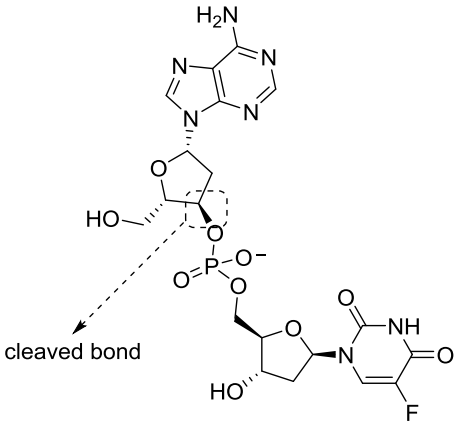
L-coformycin
K_i 250 pM on *Pf* ADA



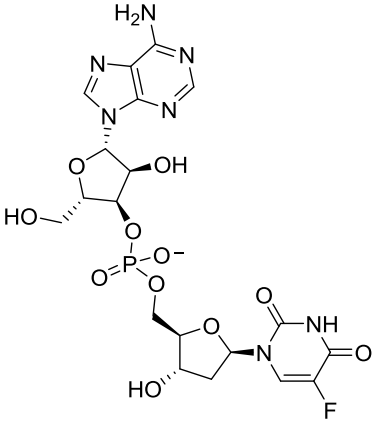
L-sangivamycin
ID_{50} 25 µM on *Pf* strains

no inhibition of *Hs* ADA up to 200µM

Dimers with L-nucleosides as carrier for cytotoxic D-nucleosides[25]



β-L-dA and β-D-FUdR dimer
ID_{50} 5 µM on *Pf*



β-L-A and β-D-FUdR dimer
ID_{50} 4 µM on *Pf*

Gero *et al.* took advantage of the ability of some L-nucleosides to be selectively transported into infected erythrocytes and proposed dinucleoside monophosphate prodrugs (**Table 2**) that combined a L-nucleoside as carrier and a cytotoxic nucleoside as antimalarial agent.[25] The release mechanism involved the cleavage of the 3'-5' phosphodiester linkage, delivering the free 3'-OH L-nucleoside and the 5'-monophosphate D-nucleoside (the position of the phosphate moiety, 3'- or 5'- inside the targeted cell, was shown to be crucial for the activity. The best results were obtained for dinucleotides containing 5-fluoro-2'-D-deoxyuridine (FUdR) as cytotoxic nucleoside and 2'-deoxy-L-adenosine or L-adenosine as carriers, with *in vitro* antimalarial activities in the low micromolar range. No further information is currently available.

b. *Pf* PNP Inhibition

- *First generation: immucillins*

In 1966, Reist *et al.* first synthesized an adenosine analogue in which the sugar ring oxygen was replaced by a nitrogen.[86] In 1998, studies on bovine PNP transition states led Miles *et al.* to design immucillin-H (Imm-H) and immucillin-G (Imm-G), as analogues of the proposed riboxocarbenium ion transition state.[87] These compounds are iminoribitol *C*-glycoside derivatives incorporating an iminoribitol sugar scaffold and 9-deazapurines. Replacement of the labile glycosidic bond by a C-N link led to a stable analogue with increased pKa values for the nucleobase N^7 atom (**Figure 11**). Tight binding constants, in the picomolar range, were determined for these two immucillins on bovine and human PNP.

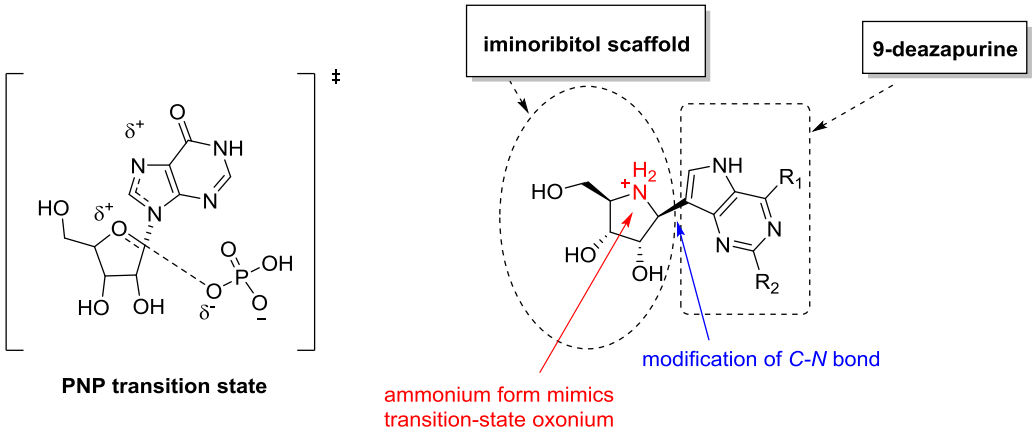


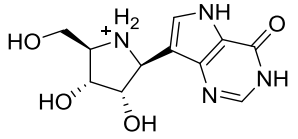
Figure 11: Common structure of first-generation immucillins

In 2002, Imm-H, Imm-G and analogues were evaluated as *Pf*PNP inhibitors **(Table 3)**[48] and Imm-H was also shown to be able to kill parasites in culture experiments, with an IC_{50} of 63 nM (3D7 strain).[54] But these immucillin derivatives were first designed as substrates of human PNP and exhibited low to modest selectivity for parasite PNPs, which is a crucial point for the development of antimalarial immucillins. As the structures of the *Pf* PNP-ImmH complex revealed that the enzyme could accept 5'-substitued nucleosides,[49] 5'-methylthioimmucillin (MT-ImmH) was designed to selectively target the parasite

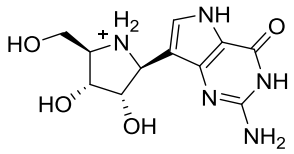
enzyme. 5'-MT-ImmH inhibited both human and *Pf* PNP in infected erythrocyte cultures and selectively bound to the *Pf* enzyme according to the K_i values **(Table 3).** Later, it was also demonstrated that MT-ImmH was able to kill *Pf* in cultures, with an IC_{50} of 50 nM (3D7 strain).[54] Other immucillin analogues (e.g. 2'-deoxy-Imm, 8-aza or fluorinated purine, 6-methoxy or thiopurine, 5'-acid or amide-Imm, ara-Imm, etc.) were reported by Lewandowicz *et al.*, but they all exhibited higher binding affinity for human protein.[88] Overall, these studies highlighted that the 5'-methylthiosubstitution resulted in a unique *Pf* PNP substrate specificity, as observed for MT-ImmH.

Table 3. Transition state analogues as *Pf* PNP inhibitors

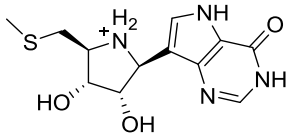
Immucillins and methylthioimmucillin (ND: not determined) [48,49]



Mundesine
Imm-H
K_i 29 nM K_i* 0.6 nM on *Pf* PNP
K_i ND K_i* 72 nM on *Hs* PNP
IC_{50} 63 nM on 3D7

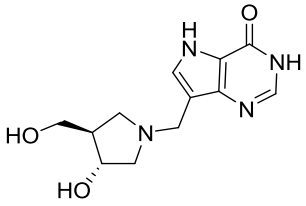


Imm-G
K_i ND K_i* 0.9 nM on *Pf* PNP
K_i ND K_i* 29 nM on *Hs* PNP

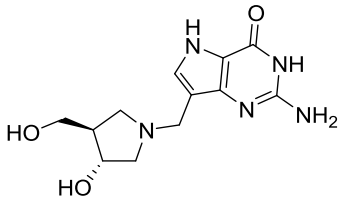


MT-Imm-H
K_i* 2.7 nM on *Pf* PNP
K_i 303 nM on *Hs* PNP
IC_{50} 50 nM on 3D7

Second-generation immucillin derivatives[88]



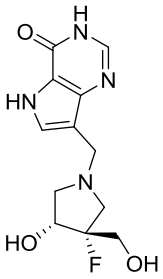
Ulodesine
DADMe-Immucillin-H
K_i 1.1 nM / K_i* 16 pM on *Hs* PNP
K_i 0.5 nM on *Pf* PNP



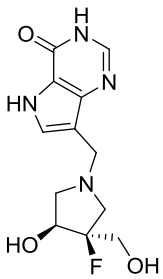
DADMe-Immucillin-G
K_i 0.16 nM / K_i* 7 pM on *Hs* PNP
K_i 0.89 nM on *Pf* PNP

These compounds are represented in neutral form, but the nitrogen is protonated at physiological pH thus mimicking the cation of the transition state. No slow-onset tight binding inhibition was observed for *Pf* PNP

Fluorinated second-generation immucillins[89]



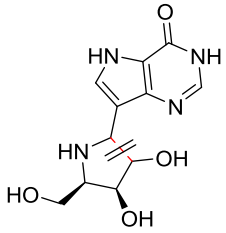
(3R,4R)-F-DADMe-ImmH
K_i 18 nM K_i*1.82 nM on *Hs* PNP
K_i 2070 nM K_i* 260 nM on *Pf* PNP



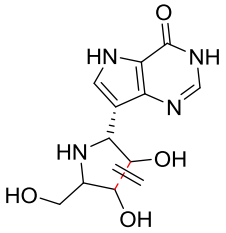
(3S,4S)-F-DADMe-ImmH
K_i 0.33 nM K_i* 0.032 nM on *Hs* PNP
K_i 19 nM K_i*2.63 nM on *Pf* PNP

Racemic
K_i 0.50 nM K_i* 0.066 nM on *Hs* PNP
K_i 23.3 nM K_i*3.6 nM on *Pf* PNP

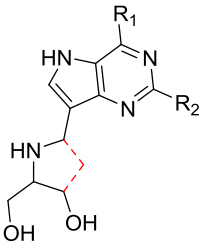
Third-generation immucillin derivatives[90,91]



DATMe-Imm-H
K_d 8.6 pM on *Hs* PNP
K_d 55 nM on *Pf* PNP
The complete iminoribitol ring shown in red

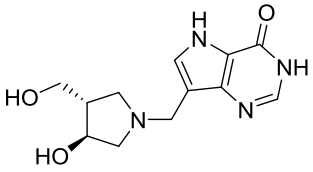


K_d 0.21 nM on *Hs* PNP
K_d 297 nM on *Pf* PNP

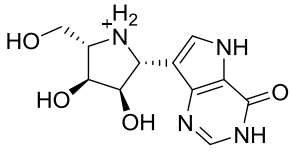


R_1 = OH, R_2 = H, SerMe-ImmH
K_d 5.2 pM on *Hs* PNP
R_1 = OH, R_2 = NH_2, SerMe-ImmG
K_d 2.1 pM on *Hs* PNP
The intact iminoribitol ring is shown in red

L-enantiomers of potent immucillins[92]



L-DADMe-ImmH
K_i 1.5 nM / K_i*0.68 nM on *Hs* PNP
K_i 1700 nM / K_i* 80 nM on *Pf* PNP



L-ImmH
K_i 18 nM / K_i*0.9 nM on *Hs* PNP
K_i 32 nM / K_i* 9 nM on *Pf* PNP

- *Second generation immucillins: N1-branched analogues*

To overcome the expensive and complex synthesis of these immucillins, the development of a second generation of Imm derivatives was considered while focusing on easier synthetic pathways. The latter initiatives generally involved the displacement nitrogen close to the C1' position on the iminoribitol ring

to mimic the PNP transition state. This modification nevertheless requires removal of the 2'-hydroxy group to avoid the formation of an unstable hemiaminal scaffold and to eliminate one asymmetric carbon. On the basis of a structural analysis and theoretical calculations, Schramm's group proposed DADMe-immucillin-H (4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene)-immucillin-H, derivatives **(Table 3)**, where the introduction of a methylene bridge between the sugar residue and 9-deazahypoxanthine allows the leaving group to be more distant from the oxacarbenium ion mimic than in Imm-H.[52]

DADMe-immucillin-H (BCX-3408) and DADMe-immucillin-G (4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene)-immucillin-G, BCX4945) **(Table 3)** have been identified as potent human PNP inhibitors (with picomolar K_i^* values)[93] since 2003 while exhibiting lower affinities for *Pf* PNP. [88] Despite this, DADMe-immucillin-G has been shown to completely inhibit *Pf* PNP and suppress *P. falciparum* development in *Aotus* primates,[94] but no further development has been reported to date.

Many immucillin derivatives have been proposed and studied (fluorinated, *N*-alkylated, 5'H, 5'-thiophenol, 5'-carboxylic acids, etc.) to revert the selectivity of these derivatives toward the *Pf* enzyme, but they preferentially bound to *Hs* PNP rather than *Pf* PNP **(Table 3)**.[88,89]

Second and first generation immucillins and transition state studies on bovine, human and malarial PNPs were reviewed by Schramm in 2005.[95]

In 2010, Cui *et al.* proposed novel *N*-branched immucillin derivatives where the purine base was replaced by uracil, as uridine was reported to be a PNP substrate, including variations in the linker length between the base and the pyrrolidine ring.[96] Modest affinities for *Pf* PNP were determined for uracil-based transition state analogues (K_i in the micromolar range), and no significant antiparasitic activity was observed *in vitro*.

- *Third generation immucillins: Acyclic analogues*

As a continuation of their research program on transition state analogues as PNP inhibitors, Schramm's group designed third generation acyclic immucillin derivatives by cleaving the iminoribitol ring, which led to iminoalcohol chains linked to 9-deazapurine [90], or by replacing the pyrrolidine ring by a serinol chain.[91] The flexibility of the acyclic scaffold allowed the chain to rotate freely inside the active site, possibly enhancing the positioning of the compound. In addition, these compounds were easier to synthesized while using cheaper precursors. Only the acyclic iminoalcohol series was assessed against both human and *Pf* PNPs, and all compounds exhibited better selectivity for human protein. The best K_d value for the human enzyme was reported for the imino-tri-alcohol DATMe-Imm-H **(Table 3)**. *Pf* PNP thus seemed to be more selective for the ribose ring than its human homologue, and consequently DADMe-immucillins and immucillins are still the best compounds in this series to date.

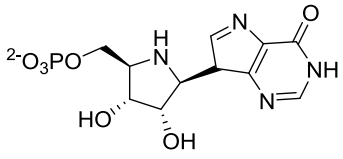
- *L-nucleosides*

Several studies carried out on L-enantiomers of known biologically active nucleoside analogues have highlighted the importance of isometry and the potential of L-nucleosides.[97–99] Based on these observations, Clinch *et al.* synthesized and compared D-immucillins (**Table 3)** to their L- enantiomers against human and *Pf* PNPs (**Table 3**).[92] All L-analogues were less potent than their D-enantiomers against malarial and human protein, while showing nanomolar range inhibition. No selectivity towards *Pf* enzymes was reported.

c. <u>*Pf* HGXPRT Inhibition</u>

In 1999, Li *et al.* hypothesized that known transition state inhibitors of PNPs could also mimic HGPRT (human enzyme) and HGXPRT ribooxocarbenium species after phosphorylation of the 5'-hydroxyl of the nucleoside analogue.[100] The nitrogen atom replacing the intracyclic oxygen of ribose in immucillins can provide a cation which may serve as the ribocation transition state of the enzyme. 5'-

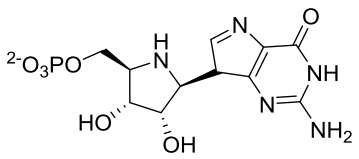
monophosphate derivatives of immucillinH and immucillinG (**Figure 12: Comparative affinities of 5'-phosphate immucillin derivatives for Hs HGPRT and Pf HGXPRT**) were thus synthesized and evaluated towards *Pf* and human HG(X)PRT, and the two compounds were found to exhibit similar affinities to both proteins in the low nanomolar range. Immucillin 5'-monophosphate derivatives were not further investigated due to their susceptibility to phosphates present in biological media.



immucillinHP

K_i ND / K_i* 1 nM on *Pf* HGXPRT

K_i 250 nM / K_i * 1.8 nM on *Hs* HGPRT



immucillinGP

K_i ND / K_i* 14 nM on *Pf* HGXPRT

K_i 56 nM / K_i*4.6 nM on *Hs* HGPRT

Figure 12: Comparative affinities of 5'-phosphate immucillin derivatives for *Hs* HGPRT and *Pf* HGXPRT

In 2012, Hazleton *et al.* reported the synthesis and biological assessment findings of a second generation of immucillin-5'-monophosphate analogues belonging to the acyclic phosphonate family. In this review, these derivatives are part of the aza-ANPs section.[101] Briefly, acyclic immucillin phosphonates (AIPs) were the first compounds to show tight binding inhibition of *Pf* (with K_i in the nanomolar range) and human (with K_i in the micromolar range) phosphoribosyl transferases, thus good selectivity for the parasite protein was observed.

d. <u>Conclusion on immucillins and transition state analogues</u>

The use of transition state analogues has been successful in inhibiting PNP, ADA and HGXPRT. A wide variety of structures, mainly based on the immucillin skeleton (four generations developed through the years), have been synthesized along with studies on the purified enzymes. One of their limitations is their lack of selectivity regarding human and parasite enzymes, and their entry into the parasite may also

be problematic due to their high polarity. To our knowledge, the mechanism of their uptake into parasites does not involve *Pf* ENT1 transporters.[31] In 2012, Frame *et al.* demonstrated that immucillin and DADMe-immucillins were recognized by *Pf* ENT4,[35] suggesting the possible role of this transporter in the uptake of these compounds into the parasite

The fourth generation of immucillins designed as human PNP transition state analogues were reviewed by Ho *et al.* in 2007.[102] DADMe-immucillin-G was under preclinical development for malaria treatment in 2011,[94] but the data are currently unavailable. Finally, as for all known antimalarial treatments, *Plasmodium falciparum* has developed genetic drug resistance (gene amplification and drug-binding mutations of *Pf* PNP gene) to PNP inhibition by DADMe-Imm-G, as recently demonstrated by Ducati *et al.*[103]

4. *Nucleotide analogues: the acyclic nucleoside phosphonate family*

   a. Introduction to acyclic nucleoside phosphonate derivatives

Since the 1960s, nucleoside analogues have been proposed as antiproliferative agents based on their ability to interfere (as their 5'-triphosphate derivatives) with the last steps of nucleic acid biosynthesis. Extensive modification of the nucleobase and/or sugar moiety was carried out to enhance their efficacy. In 1978, A. Holy and E. De Clercq described the broad spectrum antiviral activity of a new family of nucleoside analogues consisting of a simple aliphatic chain linked to a nucleobase, i.e. (S)-DHPA ((S)-9-(2,3-dihydroxypropyl)adenine, also known as Duviragel®, (**Figure 13: First acyclic nucleoside and nucleoside phosphonate analogues designed**).[104] A few months earlier, G. Elion had reported a new antiherpetic agent, another acyclic nucleoside analogue,[105] *i.e.* 9-(2-hydroxyethoxymethyl)guanine, currently known as acyclovir (**Figure 13: First acyclic nucleoside and nucleoside phosphonate analogues designed**) and in 1988 she shared the Nobel Prize with G. H. Hitchings and Sir J. Black for

this discovery. Studies on the antiviral potential of phosphonoacetic acid, with the aim of developing isopolar and catabolically stable nucleotide analogues, gave rise to the design of the first acyclic nucleoside phosphonate (ANP) in 1986 by Holy *et al*, i.e. (S)-HPMPA or ((S)-9-(3-hydroxy-2-phosphonomethoxy-propyl)adenine) (**Figure 13)**. The latter was described as a nucleotide analogue resistant to phosphoesterase hydrolysis due to the presence of the phosphonate moiety (P-C bond) and an acyclic chain replacing the sugar ring, and it was found to be active against anti-DNA viruses.[106] Meanwhile, the antiviral activity of 2-(phosphonomethoxy)ethyl-adenine (PMEA) was also reported.[107] (S)-HPMPC (cidofovir) was the first ANP marketed (in 1996) for cytomegalovirus retinitis treatment. Over the last three decades, many ANPs have been designed, synthesized and evaluated against viruses (cytomegalovirus, hepatitis B and HIV), and they still represent a promising and efficient family of drugs.[108,109]

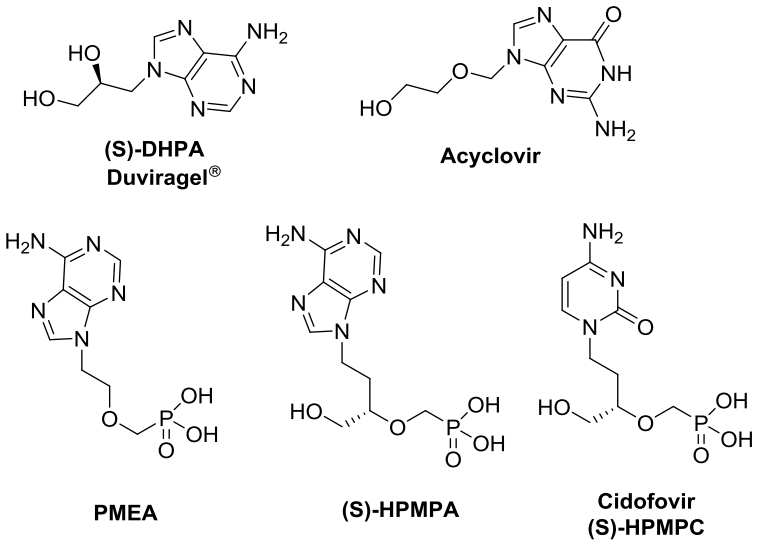


Figure 13: First acyclic nucleoside and nucleoside phosphonate analogues designed

Due to their negative charge and relatively small structure, phosphonate derivatives are too polar to easily cross cell membranes, except by endocytosis. Infected erythrocytes, like normal RBCs, are not able to undergo endocytosis.[110] The drug therefore must reach the intracellular parasite by successively crossing

erythrocyte, parasitophorous vacuole and parasite membranes. Consequently the use of prodrugs to enhance the lipophilicity of the drug by hiding charges and/or adding long aliphatic tails is essential to increase cell uptake and the overall bioavailability of these derivatives.[111,112] These temporary phosphonate moiety protecting groups, once within the parasite or RBC, are cleaved to release the parent drug and eventually limit possible side-effects before delivery.

Prodrug approaches have been successfully applied to ANPs in antiviral chemotherapy, including adefovir dipivoxil (Hepsera®, approved in 2002 for chronic HBV infection treatment) and tenofovir disoproxil fumarate (Viread®, approved in 2001 for HIV infection treatment).[113,114]

b. Standard acyclic nucleoside phosphonates

Based on the success of ANPs in chemotherapeutic treatment of DNA virus infections, several groups have assessed their potential for treating other diseases, namely in protozoan infections like malaria. In this respect, (*S*)-HPMPA, (*S*)-3-deaza HPMPA and (*S*)-HPMPADAP (2,6-diaminopurine) were first tested in 1991 against *Pf* and *P. berghei* infected RBCs (**Figure 14**). S-HPMPA is active *in vitro* against *Pf* at nanomolar concentration, which is more than 1,000-fold less than the cytotoxic dose for human cells. [115] When evaluated in *P. berghei* infected mice, four injections of 20 mg/kg HPMPA at 3-day intervals decreases parasitemia to low detectable levels but does not lead to a complete cure.[115] Using extracted plasmodial DNA polymerases, the inhibitory activity of these compounds was also observed and interference with the replication process was hypothesized. Unfortunately, the effects of (*S*)-HPMPA in *Pf* infected mice were hampered by nephrotoxicity and hepatotoxicity as well as the limited half-life in plasma.[116]

In 1999, Smeijsters *et al.* conducted an *in vitro* study of 48 ANPs belonging to five main structural groups with the aim of inhibiting plasmodial DNA polymerase activity **(Figure 14).**[117] All HPMPA derivatives (except (S)-HPMPA and (S)-3-deaza-HPMPA) exhibited antiplasmodial activity in the micromolar range.

(S)-HPMPA was found to be less potent (0.18 μM) than reported by de Vries *et al.* due to the use of a different IC_{50} determination method. One of the most interesting results was the difference in IC_{50} between the two isomers of HPMPA and 3-deaza-HPMPA. Indeed, (*R*)-enantiomers of both compounds were less potent than the (*S*)-enantiomers. This was attributed to the fact that kinases may not be able to recognize (*R*)-enantiomers of phosphorylated substrates.

Conversely, PMEA derivatives had no significant activity, presumably due to the lack of the hydroxyl group present in the HPMPA derivatives. Studies on hydrogenated and fluorinated ((*S*)-FPMPA) derivatives revealed no inhibition, thus confirming the previous hypothesis. However, the difference in antimalarial activity observed between PMEA (IC_{50}> 250 μM) and its prodrugs bis(POM)PMEA (IC_{50} = 37.3 μM) was significant. As the prodrug is more lipophilic and neutral than PMEA, the improved activity could be explained by the efficient prodrug uptake through the host cell and parasite membranes.

Modification of the nucleobase counterpart with similar phosphonate tail was then envisaged. (*S*)-HPMPG exhibited a similar antimalarial activity of about 4 μM, as well as 2-chloroadenine, 2,6-diaminopurine, 7-deazaadenine HPMP compounds, whereas 2-aminomethyladenine, 2-aminomethylhypoxanthine or 2-methylthioadenine derivatives did not exhibit any activity at 100 μM.[117] The nature of the nucleobase and the presence of the phosphonate moiety thus seemed to be crucial as non-phosphorylated compounds (acyclic nucleoside) were inactive. For the latter, either the lack of appropriate kinases in *Plasmodium* limits their transformation into active form and/or their polarity prevents their passive passage through the membranes.

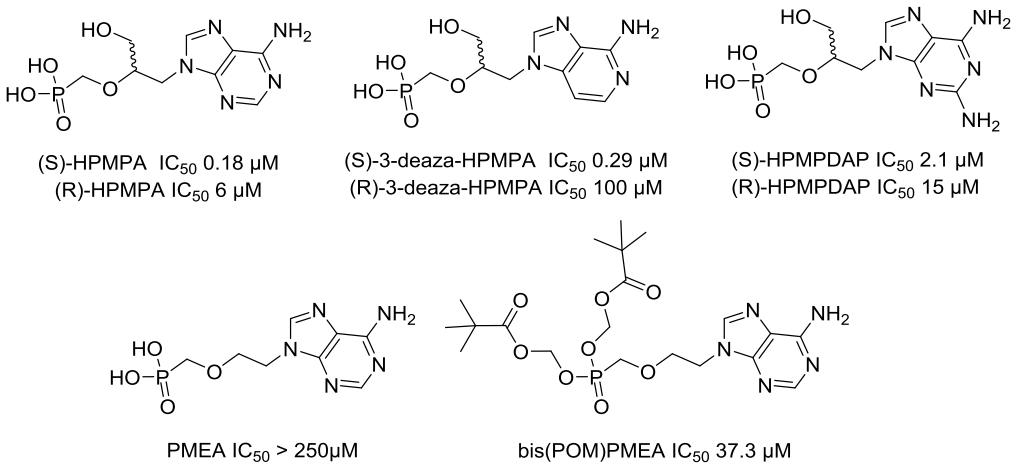


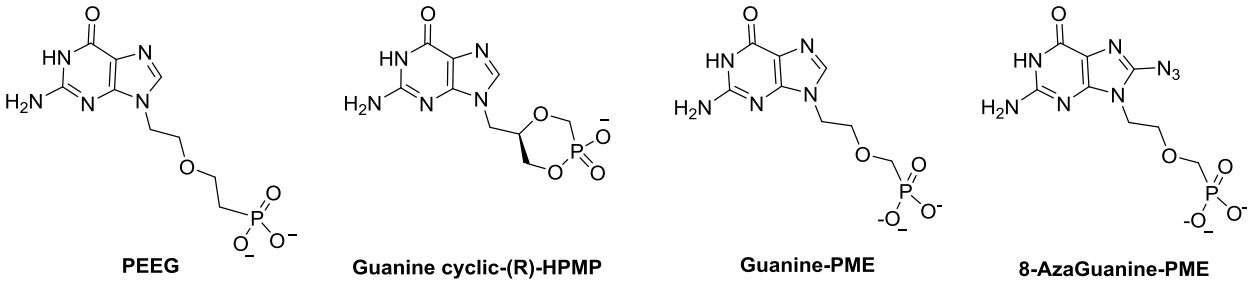
Figure 14: IC_{50} of several ether ANPs in *Pf*-infected RBCs (from Smeijsters *et al.*)

Obviously these compounds could target any enzymes involved in nucleic acid metabolism pathways, but Smeijsters *et al*. focused on the potency of ANPs to inhibit DNA replication (because (*S*)-HPMPA has been described as a viral DNA polymerase inhibitor) but did not envisage any other possibilities. Note also that pyrimidine-containing ANPs, such as (S)-HPMPU, (S)-HPMPT and (S)-HPMPC, did not show any parasite growth inhibition.[117]

In conclusion, this pioneer work highlighted the antimalarial potency of ANPs and the importance of the nature of the nucleobase and the stereochemistry for *in vitro* antiplasmodial activity, as well as the possible passive uptake of these drugs when the corresponding prodrugs were used.

In 2009, Keough *et al*. reported, for the first time, novel series of ANPs to target *Pf* HGXPRT.[118] These authors performed an SAR study to compare the length and position of the oxygen atom within the phosphonate tail (e.g. cyclic-3-hydroxy-2-(phosphonomethoxy)-propyl (cyclic-HPMP), 2-(phosphonoethoxy)ethyl (PEE), 2-(phosphonomethoxy)ethyl (PME), 3-fluoro-2-(phosphono-methoxy)propyl (FPMP), 2-(phosphonomethoxy)propyl (PMP), etc., (**Figure 15: Inhibition constants of two series (C4 and C5) of ether ANPs on parasite and human 6-oxopurine PRTs**) and the nature of the nucleobase on the affinity for *Pf* and human enzymes, and selectivity toward the parasite protein. A comparison of several PME analogues revealed that the nature of the nucleobase had a crucial effect on

binding to the human HGPR catalytic site. The best tradeoff in terms of affinity (K_i in the low micromolar range) and selectivity was found to be the combination of a PEE tail and a 8-substituted guanine as nucleobase (i.e. 8-azaguanine, etc.). Selected derivatives were tested *in vitro* and were found to be able to inhibit the *Pf* growth, with IC_{50} values in the micromolar range (**Figure 15**).[118]



PEEG
K_i 0.1 µM on *Pf* HGXPRT
K_i 1 µM on *Hs* HGPRT

Guanine cyclic-(R)-HPMP
K_i 1 µM on *Pf* HGXPRT
K_i 19 µM on *Hs* HGPRT
IC_{50} 46 µM (*Pf*)
IC_{50} > 1 mM (human A549 cells)

Guanine-PME
K_i 1.6 µM on *Pf* HGXPRT
K_i 29 µM on *Hs* HGPRT
IC_{50} 14 µM (*Pf*)
IC_{50} 17 µM (human A549 cells)

8-AzaGuanine-PME
K_i 3 µM on *Pf* HGXPRT
K_i 175 µM on *Hs* HGPRT

Figure 15: Inhibition constants of two series (C4 and C5) of ether ANPs on parasite and human 6-oxopurine PRTs

In the absence of any crystallized *Pf* HGXPRT with the studied ANPs, human HGPRT complexes were used as models in structural studies and highlighted that the loop hosting the phosphonate or phosphate group seemed to be identical for both enzymes.[118] The three oxygen atoms of the phosphonate group makes hydrogen bonds with the protein, whereas the natural substrate (GMP) has fewer hydrogen bonds, indicating that the phosphonate group is essential for tight binding at the active site while not being involved in any selectivity contribution. According to docking studies, the purine ring of ANPs binds at the nucleobase binding site of the enzyme. These results pave the way for the development of ANPs targeting HGXPRT, based on the fact that the potency of this family was found to inhibit *Pf* growth *in vitro* and on findings regarding their interaction within the active site of the enzyme.

Meanwhile, these authors also reported the synthesis of 43 α- and β-substituted PEE derivatives bearing guanine and hypoxanthine (**Figure 16: Structure of α- and β- branched 2-(phosphonoethoxy)ethyl ANPs**) and their inhibitory activity against *Pf* and human HG(X)PRT was determined.[119] Overall, α-substituted (R_1=methyl or ethyl) phosphonate derivatives were either non-active or weakly active against both enzymes and it was suggested that these results were due to steric hindrance.

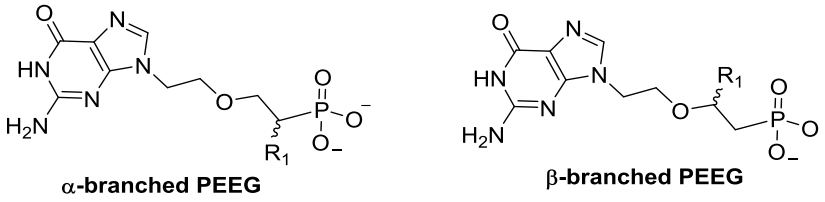


Figure 16: Structure of α- and β- branched 2-(phosphonoethoxy)ethyl ANPs

The study of β-branched ANPs revealed inhibition of both enzymes (**Figure 17: Inhibitory activities of β- branched PEE ANPs on human HGPRT and Pf HGXPRT**) in the micromolar range, but with low to reverse selectivity, with the natural nucleobases being preferred. The smallest substituents better accommodated the active site, and the absence of binding of a β-benzyl-substituted ANP was confirmed. It was hypothesized that the presence of substituents hampers proper closure of the loop in *Pf* HGXPRT.

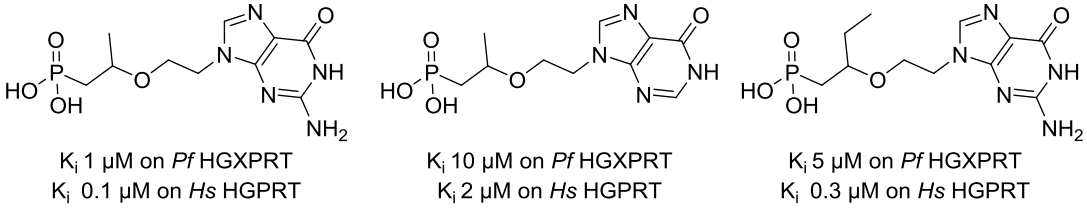


Figure 17: Inhibitory activities of β- branched PEE ANPs on human HGPRT and *Pf* HGXPRT

Similarly, Cesnek *et al.* synthesized series of 9-phosphonoalkyl and 9-phosphonoalkoxyalkyl purines and tested them on human and *Pv* HGPRT, and on *Pf* HGXPRT (**Figure 18: Studies of the impact of the chain length of ANPs on their inhibitory activities against Pf, Pv and human 6-oxopurinePRT**).[120]

The optimal number of atoms within the linker thus seemed to be four or five, compounds with six atoms or more being highly selective for human HGPRT. This latter enzyme appears to be able to host longer derivatives than the parasite enzyme. The presence of an oxygen atom within the four-atom linker was mandatory for interfering with parasite proteins, indicating that the oxygen in the ribose ring may be responsible for important binding site interactions. As previously observed, guanine is apparently the preferred nucleobase and all modified nucleobases (2-amino-6-chloropurine, 6-bromopurine, etc.) lead to derivatives with weaker inhibitory activities (K_i values above 100 μM). This indicates that efficient binding of both the phosphonate group and the nucleobase is essential at the catalytic site to obtain optimal K_i values. In conclusion, at least a four carbon atom chain length and a guanine as nucleobase were required for significant inhibition of these proteins, but with low or no selectivity towards parasite proteins, while the presence of an oxygen atom in the linker was somewhat favorable.

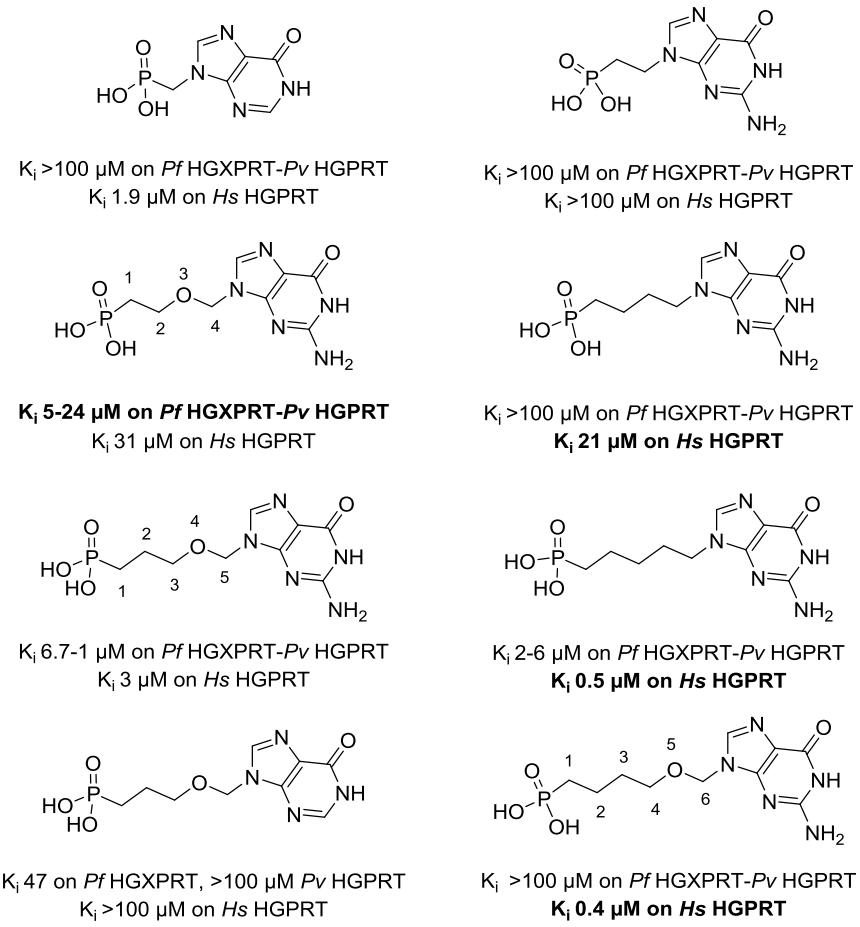


Figure 18: Studies of the impact of the chain length of ANPs on their inhibitory activities against *Pf*, *Pv* and human 6-oxopurinePRTs

Based on results on PEE derivatives, Krecmerova *et al.* proposed to study the effect of the insertion into the phosphonomethoxypropyl chain of a polar group (an hydroxyl or a carboxyl) at the β-position related to the nucleobase (**Figure 19**).[121] These new derivatives, which incorporated a small polar or anionic side chain, were less efficient but presented some selectivity for *Pf* and *Pv* enzymes. These derivatives were all tested as racemic mixtures, so the impacts of stereoisomery could not be determined.

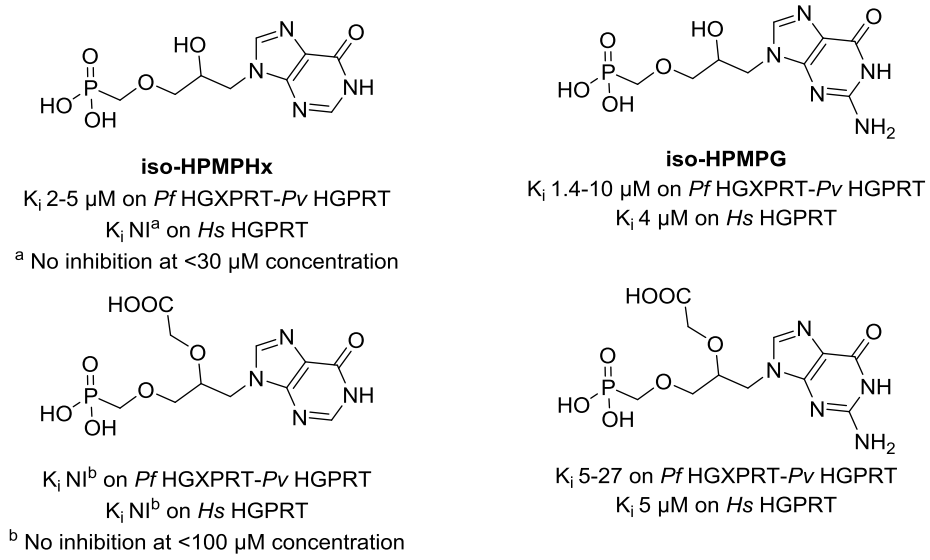


Figure 19: Representatives of PMP ANPs

In 2015, on the basis of the results obtained for the HPMP series (**Figure 14**) and computational studies, novel analogues bearing side chains in the β-position related to the nucleobase were designed (**Figure 20**), including (S)-2-(phosphonoethoxy)propanoic acid (CPEE), (S)-3-hydroxy-2-(phosphonoethoxy)propyl (HPEP) and (S)-2-(phosphonomethoxy)propanoic acid (CPME).[122] Biological studies on *Pf* and human PRT enzymes revealed that CPME analogues were inactive, in agreement with the fact that a five-atom chain is optimal for tight interactions at the active site.[123,124] Concerning HPEP derivatives, again the nature of the nucleobase was found to be crucial as only the 8-bromoguanine, guanine and hypoxanthine analogues showed some affinity (with K_i in the low micromolar range), but only the guanine HPEP ANP presented a 6-fold better selectivity for the parasite enzyme. CPEE derivatives exhibited similar activities against both enzymes, and in this case only the hypoxanthine derivatives appeared to be more selective for *Pf* HGXPRT than for human HGPRT. Finally, the presence of an acidic, ester or hydroxyl group as side chain did not lead to a significant difference between these three series of compounds. The crystal structures of HPEPG and HPEPHx revealed that residues located

in the vicinity of the active site were flexible and mobile, and the activity enhancement was hypothesized to be associated with the 'locking' of these residues (tighter binding).

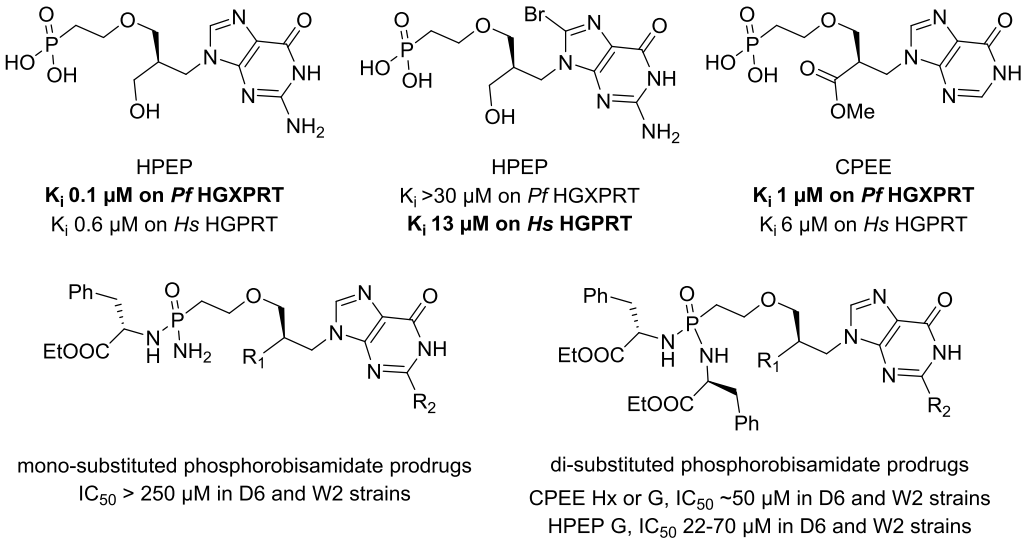


Figure 20: HPEP and CPEE ANPs and their corresponding prodrugs

R_1, HPEP or CPEE side-chain; R_2, H or NH_2.

In order to facilitate cell uptake, phosphorobisamidate prodrugs incorporating L-phenylalanyl moieties (**Figure 20: HPEP and CPEE ANPs and their corresponding prodrugs**) were prepared and assessed *in vitro* in *Pf* strains W2 and D6.[122] Mono-substituted phosphorobisamidates did not exhibit significant antimalarial activity, probably due to the fact that the free amino group may not be easily hydrolyzed in cells. IC_{50} values of di-substituted phosphoramidate prodrugs ranged from 22 to 103 µM.

c. Bisphosphonates ANPs - ANbPs

On the basis of an analysis of the crystal structure of human HGPRT complexed with PEEG analogues, new series of ANPs containing a second oxymethylphosphonate group and increased flexibility of the two phosphonate tails were designed **(Figure 21)**.[125] Most of the compounds had K_i values in the sub-micromolar range, but little selectivity towards *Pf* HG(X)PRT was observed. Inhibitory activity against the parasite enzyme was noted when the oxygen atom of the phosphonate tail was moved from

position 4 to 3. The position of the oxygen atom may thus modify the chain conformation while enhancing its binding at the active site. Studies on the crystal structure of human HGPRT complexed with the first derivative of **Figure 21** revealed that the second phosphonate group interacted with amino acid residues via Mg^{2+} ion, thus highlighting the contribution of this group in comparison to PEEG analogues. Docking studies also revealed that the second phosphonate group was located near the PP_i binding site but did not share interactions inside this pocket.

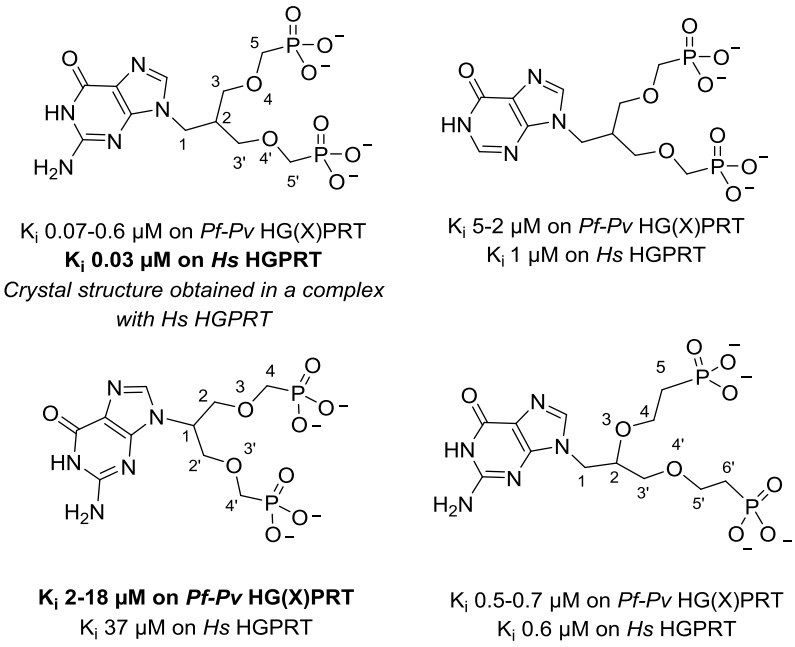


Figure 21: Bisphosphonate ANPs tested on human and malarial 6-oxopurinePRTs

Two types of prodrug were proposed to increase the cell permeability of parent ANPs. The negative charges of PEEG **(Figure 15)** were either masked by one or two lipophilic chains (**Figure 22**), or as a bis phosphoramidate including an alanine ethyl ester. In comparison to the parent drug (PEEG, IC_{50} 242 µM on *Pf* strain D6), the presence of two substituents decreased the biological activity (IC_{50} 56 µM) and the best result was obtained for the prodrug that included a single hexadecyloxypropyl chain (7.6 µM)[125], a behavior that had already been noted with antiviral compounds.[126] For ANbPs **(Figure 22)**, ethyl L-

phenylalanyl (instead of alanyl) moieties were envisaged for phosphorobisamidate prodrugs with the aim of further increasing the lipophilicity of the final compounds. *In vitro* evaluation of these three prodrugs on *Pf* strains D6 and W2 led to similar results, with IC_{50} values in the low micromolar range.[125] Note that some cytotoxicity on mammalian cells was observed for the lipophilic monoester PEEG prodrug (~36-61 µM CC_{50} on all three human cell lines tested) as well as for bisphosphonate prodrugs (~41-130 µM CC_{50}).[125]

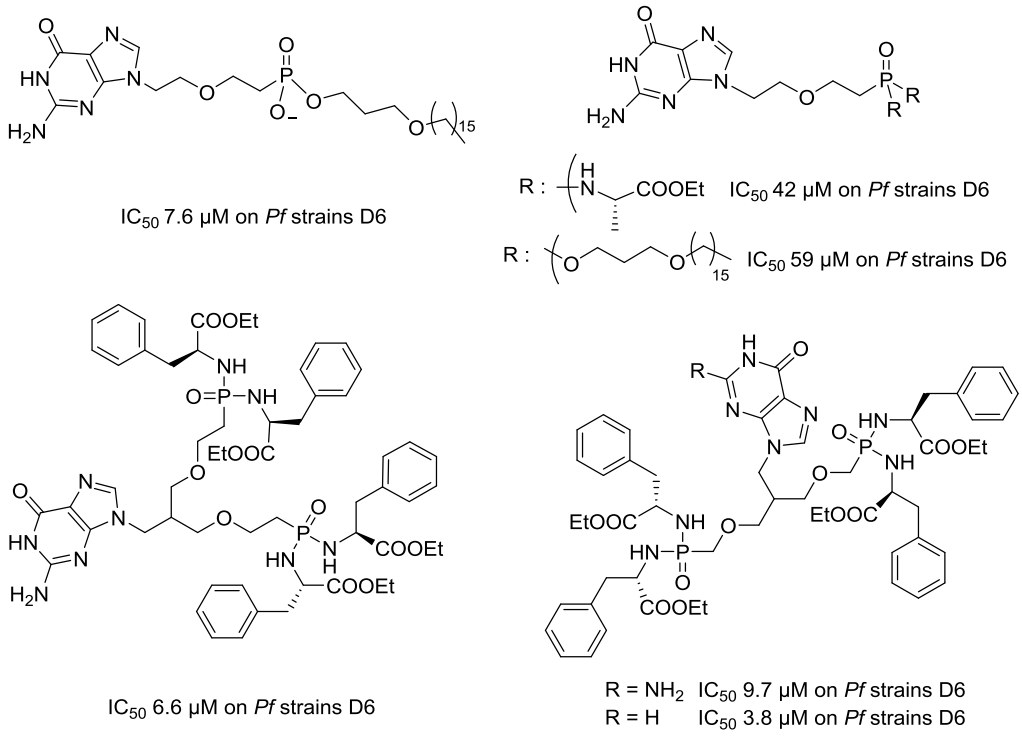


Figure 22: Biological assessment of ANbP prodrugs on *Pf* strains

In 2017, Spacek *et al.* proposed novel series of ANbPs derivatives (**Figure 23**) rationally designed on the basis of the crystal structure of the previous ANbP lead compound (**Figure 21**) complexed with human HGPRT and based on molecular docking studies, suggesting that increasing the length of the phosphonate tail by one atom could allow its location in the PP_i binding pocket.[127] Various nucleobase modifications showed that hypoxanthine (compound **7**), 8-bromoguanine (compounds **5** and **8**) and 7-deazapurine

(compound **6**) derivatives were equally or less active than the guanine derivative (compound **4**, **Figure 23**). Adding one carbon atom into the phosphonate chain (**Figure 23,** compound **4**) led to a 10-fold decrease in the K_i values on the human enzyme (0.006 µM to 0.03 µM) but had no effect on *Pf* HGXPRT (K_i = 0.07 µM). For this latter derivative, a crystal structure was obtained in a complex with human HGPRT and the affinity gain was associated with the location of the second phosphonate chain within the PP_i binding site, whereas the phosphonate tail of the previous derivative was too short to reach this site and share interactions. They also showed that this new compound bonded strongly in the active site through a network of hydrogen bonds between protein residues, phosphonyl oxygen atoms and magnesium ions. Finally, phenylalanine phosphoramidate prodrugs were synthesized and their antimalarial and cytotoxicity activities were determined. Most of them exhibited IC_{50} values in the micromolar range against D6 and W2 *Pf* strains, and prodrugs of the best ANbPs in this series exhibited low cytotoxicity compared to the others (CC_{50} 212 µM on human lung carcinoma cells).

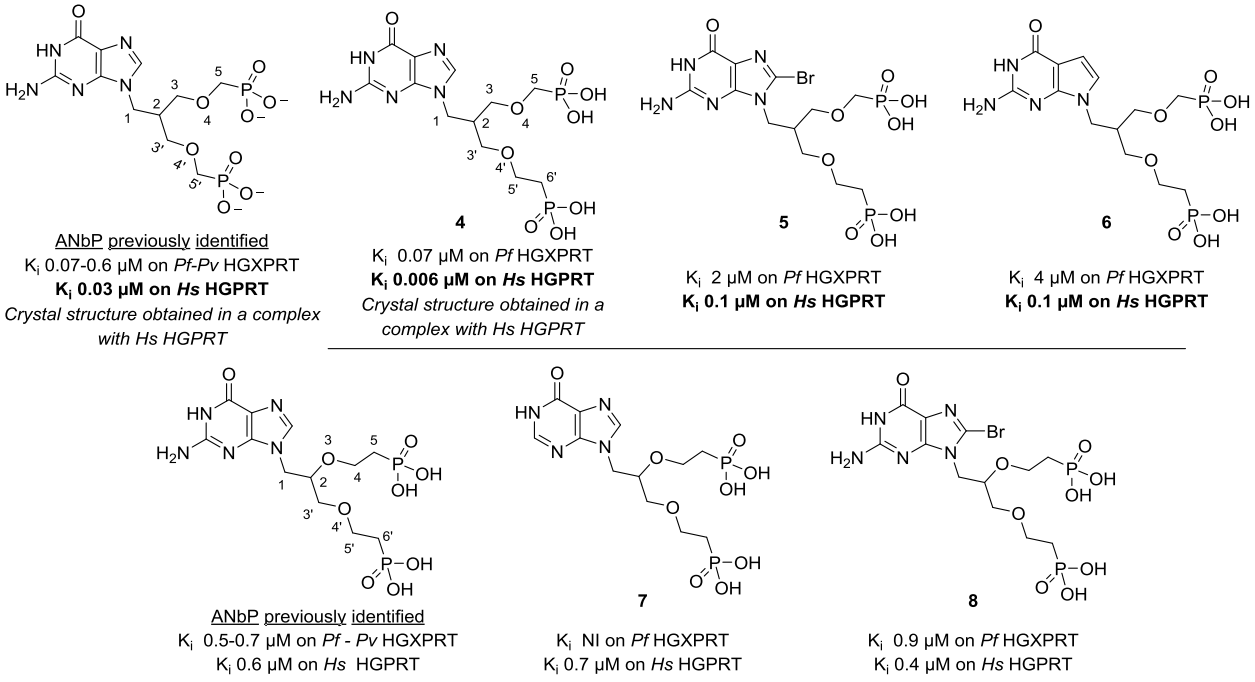
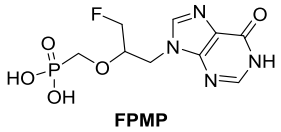


Figure 23: Novel ANbPs designed by Spacek *et al.* to study the impact of the phosphonate chain and nucleobase modifications on PRTase inhibition

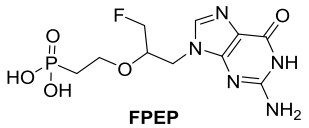
d. Fluorinated ANPs

In addition to the previously reported ANPs,[149,119] a fluoromethyl group was incorporated as substituent at the beta-position related to the nucleobase on the basis of the PEE and PME scaffolds, giving rise to 3-fluoro-(2-phosphonoethoxy)propyl (FPEP) and 3-fluoro-(2-phosphonomethoxy) propyl (FPMP) derivatives, which were obtained as pure isomers (**Figure 24**).[124]



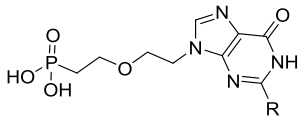
FPMP

(R) = K_i 63 µM on *Pf* HGXPRT, >200 µM on *Pv* HGPRT
K_i >200 µM on *Hs* HGPRT
(S) = K_i 13 µM on *Pf* HGXPRT, >250 µM on *Pv* HGPRT
K_i >250 µM on *Hs* HGPRT



FPEP

(R) = K_i 1-29 µM on *Pf* HGXPRT-*Pv* HGPRT
K_i 4.7 µM on *Hs* HGPRT
(S) = K_i 1.2-34 µM on *Pf* HGXPRT-*Pv* HGPRT
K_i 0.1 µM on *Hs* HGPRT



PEEHx, R = H, K_i 0.3 µM on *Pf* HGXPRT
K_i 3.6 µM on *Hs* HGPRT
PEEG, R = NH_2, K_i 0.1 µM on *Pf* HGXPRT
K_i 1 µM on *Hs* HGPRT

Figure 24: Fluorinated ANPs and their biological activities

Briefly, FPMP derivatives were found to be very weak inhibitors (K_i >50 µM) of the three enzymes, except for the (S)-isomer incorporating hypoxanthine which exhibited a K_i of 13 µM toward *Pf* HGXPRT. Among the FPEP series, the guanine analogues exhibited inhibition constants in the low micromolar range for the three enzymes, with no significant selectivity for the parasite enzymes (**Figure 24**). No FPEP and FPMP derivatives were more active than PEEHx and PEEG ANPs.

Molecular docking demonstrated that the fluoromethyl group was too small to interact significantly with protein residues at the active site, thus not really reflecting any effect of the fluorinated group on the activity. One interesting finding was the effect of stereoisomery, (*S*)- and (*R*)-isomers providing different profiles, as previously noted by other authors. Docking studies revealed different positioning of the nucleobase for each isomer, with one being closest to the aromatic ring of residue F186. The stereochemistry also influenced the position of the phosphonate group within the 5'-phosphate group loop, thus affecting the activity. Indeed, the binding of this group is one of the most crucial for the affinity.[124]

e. Acyclic immucillin phosphonates (AIP)

In 2012, Schramm and co-workers concluded from their preliminary studies on immucillin 5'-monophosphate derivatives that they were unable to act efficiently as antimalarials *in vitro* due to hydrolysis of the 5'-phosphate group by cellular phosphatases.[101] As an alternative and inspired by the work done on ANPs, they designed acyclic immucillin phosphonate derivatives (AIPs, **Figure 25**), by cleaving the sugar moiety of immucillin derivatives and replacing the 5'-phosphate group by a hydrolysis-resistant phosphonate bond and investigated them as potential PRTase inhibitors.[101,123]

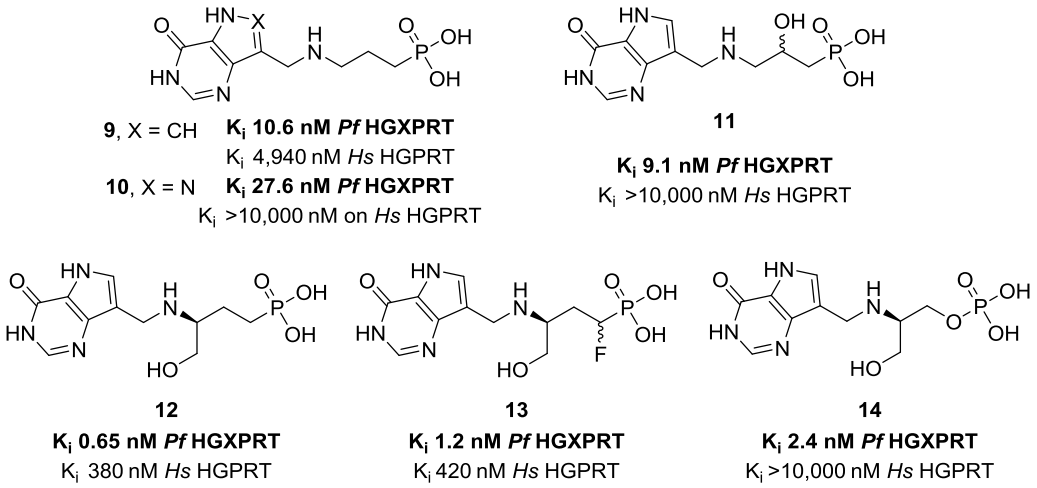
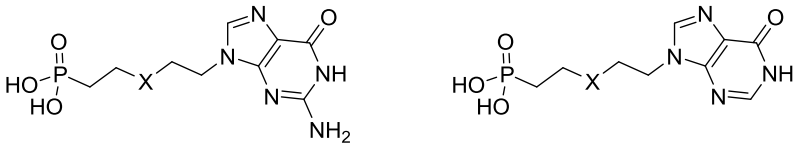


Figure 25: First AIPs tested to inhibit *Pf* HGXPRT and *Hs* HGPRT activities

Pf enzymes were found to be remarkably selective for all derivatives (SI >350). The most potent compounds included a hydroxymethyl group as side chain, with sub-nanomolar K_i values (**Figure 25**, compounds **12**-**14**). The most surprising result was the increased selectivity observed when a phosphate group linked to a serinol-like chain replaced the phosphonate tail (compound 14). The introduction of fluorine atom(s) in the α-position related to the phosphorus atom (compound 13) was envisaged so that phosphonate would mimic the pKa of a phosphate group, but this modification did not improve K_i values towards *Pf* and human PRTases.[123]

f. Aza-ANPs

In 2012, novel ANP analogues, where a nitrogen atom replaced the oxygen atom within the parent PEE scaffold, were compared for their ability to inhibit *Pf* HGXPRT, with the aim of introducing a novel class of nitrogen-containing ANPs (**Figure 26**)[128] This modification led to less active derivatives and increased the affinity for the human enzyme (reversed selectivity). These negative effects on the activity and selectivity may be attributed to the ability of the secondary amino group to protonate at physiological pH, thus modifying the chain conformation (confirmed by docking studies).[128]



X = O **K_i 0.1 µM on *Pf HGXPRT***
K_i 1 µM on *Hs HGPRT*
X = NH K_i 16 µM on *Pf* HGXPRT
K_i 3 µM on *Hs* HGPRT

X = O **K_i 0.3 µM on *Pf HGXPRT***
K_i 3.6 µM on Hs HGPRT
X = NH K_i >200 µM on *Pf* HGXPRT
K_i 40 µM on *Hs* HGPRT

Figure 26: Comparison the inhibitory activity of oxygen- and nitrogen-containing ANPs derivatives

Then, *N*-branched ANPs including a second chain linked to the nitrogen atom were synthesized, thus leading to a tertiary amine at the core of the compounds (**Figure 27: Evaluation of N-branched ANP potent inhibitors of malarial and human 6-oxopurinePRTs**).[128] The side chains were found to have various functionalities such as cyano- (compounds **15-16**), hydroxy- (compound **17**), methyl ester- (compounds **18-19**) and carboxylic acids (compounds **20-21**), as shown in **Figure 27**. In all cases, guanosine derivatives exhibited better affinity for the human enzyme than for the parasite enzyme, whereas some hypoxanthine derivatives exhibited sub-micromolar affinity and significant selectivity for the *Pf* protein. Cyano- and hydroxy-substituted aza-ANPs showed better affinity for the human enzyme. All compounds showed weak potency towards parasite enzymes. Finally, promising results were obtained

when the second tail had a length of three carbon atoms and included a carboxylic acid or a methyl ester group (compounds **19-20**).

Docking studies on these compounds were also performed with human HGPRT to support the biological results. The functional group is supposed to be located in a hydrophobic pocket differing from the PP_i binding site. In the case of the hydroxyl compound, the -OH group is supposedly bound through hydrogen bonds to the internal phosphonate, thus hampering it from occupying the 5'-phosphate bonding pocket. The nature of the linker has a marked impact on the location of the second group within the active site, e.g. in the case of the two cyano compounds **15** and **16**, thus lowering the affinity.

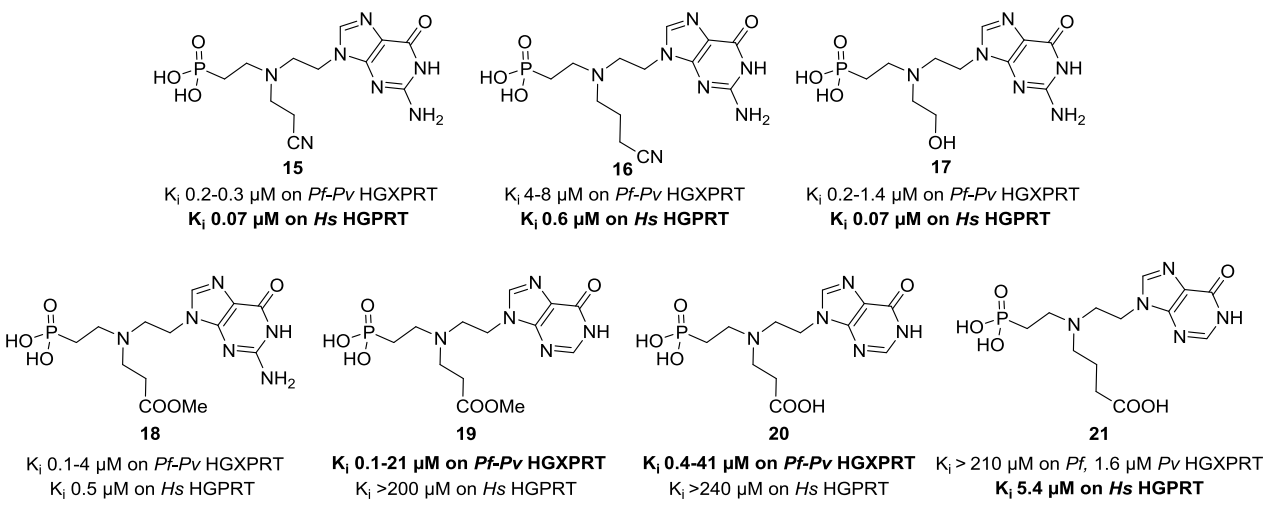


Figure 27: Evaluation of N-branched ANP potent inhibitors of malarial and human 6-oxopurinePRTs

Prodrugs (**Figure 28**) of the previously described aza-cyano ANPs were subsequently synthesized in order to perform cell culture experiments against *Pf* strains.[129] As expected, the use of bisamidate and diester prodrugs led to a potency gain in comparison to the parent compounds. However, some cytotoxicity was observed for the guanine derivatives, presumably due to the higher affinity of this ANP for the human protein, contrary to the hypoxanthine-containing prodrug.

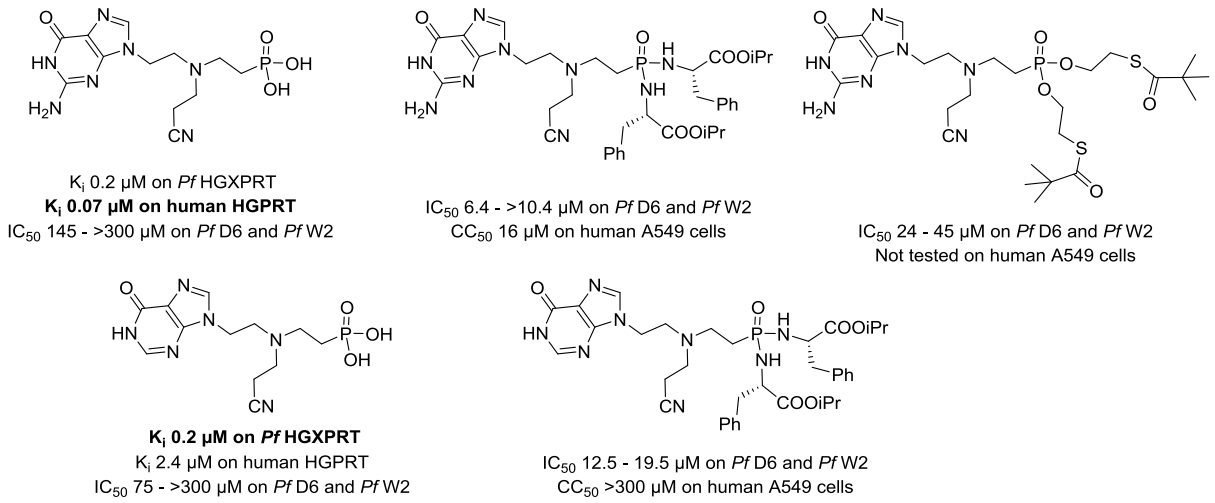


Figure 28: Cyano-aza-ANPs and prodrugs against *Pf* HGXPRT and human HGPRT

g. Bisphosphonate aza-ANPs

By merging the scaffold of *N*-branched ANPs (AIP and aza-ANPs) and bisphosphonate ANPs (ANbPs) described previously, Hockova *et al.* proposed bisphosphonate aza-acyclic nucleosides (**Figure 29:**). [128] In theory, these compounds should form a tight drug-enzyme complex. Indeed, the presence of the nitrogen atom allows triple anchoring of the compound through the nucleobase and the formation of two phosphonate chains of different lengths. One phosphonate tail was designed to fill the *Pf* HGXPRT pyrophosphate binding site and the other one the phosphate binding pocket. The main chain included five atoms, which was found to be the optimum length for this target. Studies on their affinity towards human, *Pf* and *Pv* PRTases showed that the addition of a second phosphonate group seemed to create new interactions at the active site with the human enzyme, which may have been responsible for the improvement in all K_i values compared to their monophosphonate analogues (**Figure 29**). These compounds however remained poorly selective for the parasite enzymes.

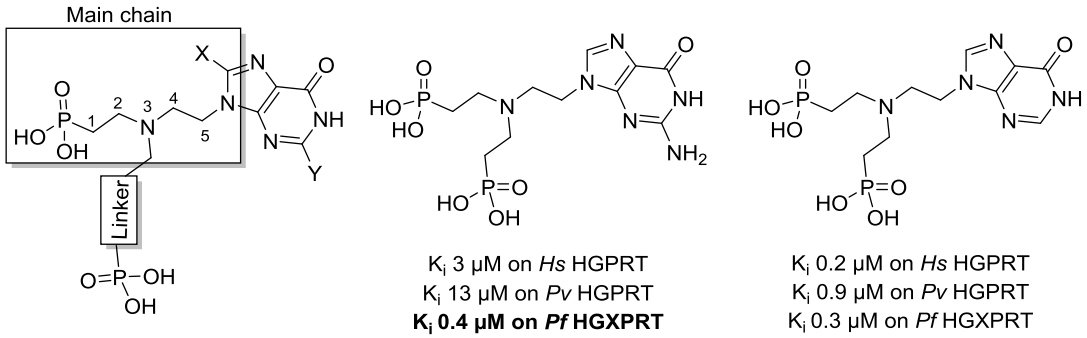


Figure 29: Generic structure of the aza-ANPs bisphosphonates and first derivatives studied

Modification of the length of the side chain and incorporation of an oxygen atom were then envisaged **(Figure 30).**[129] Regardless of the nature of the second phosphonate tail, guanine derivatives showed similar or better affinity for human HGPRT, with K_i values in the sub-micromolar range. The increased length of the side chain to eight atoms and the presence of oxygen atoms led in all cases to more potent inhibitors for all three enzymes. In this case, selectivity was in favor of the parasite enzymes for hypoxanthine and 8-bromoguanine derivatives.

Due to the polyanionic nature of the parent compounds, only the corresponding phosphoramidate prodrugs were tested in cell experiments (*Pf* D6 and W2 strains), and cytotoxicity was assessed on human lung carcinoma A549 cells. The prodrugs exhibited similar IC_{50} values, in the micromolar range, against both *Pf* W2 and D6 strains, with weak cytotoxicity against mammalian cells (SI for prodrugs ≥ 15).

Extensive structural studies revealed that the main phosphonate chain was located in the 5'-phosphate binding pocket, whereas the other chain was hosted by the PP_i binding site. The importance of coordination of the magnesium ion with the second phosphonate moiety through a water molecule for tight binding was highlighted. It was also found that 8-bromoguanine induced inversion of the common positioning of the two phosphonate chains and led to occupation of the 5'-phosphate binding site by the second phosphonate group, not by the main one. The longer chain can enter deeply into the binding site,

and forming strong interactions. Finally, the hydrogen bond interactions of amino acid residues in the active site with the purine ring have been described and the most important ones were identified (hydrogen bond between V187 and the N^1 of the purine ring is present in every case). The authors also observed a hydrogen bond between the N^3 of the purine ring and water.[129]

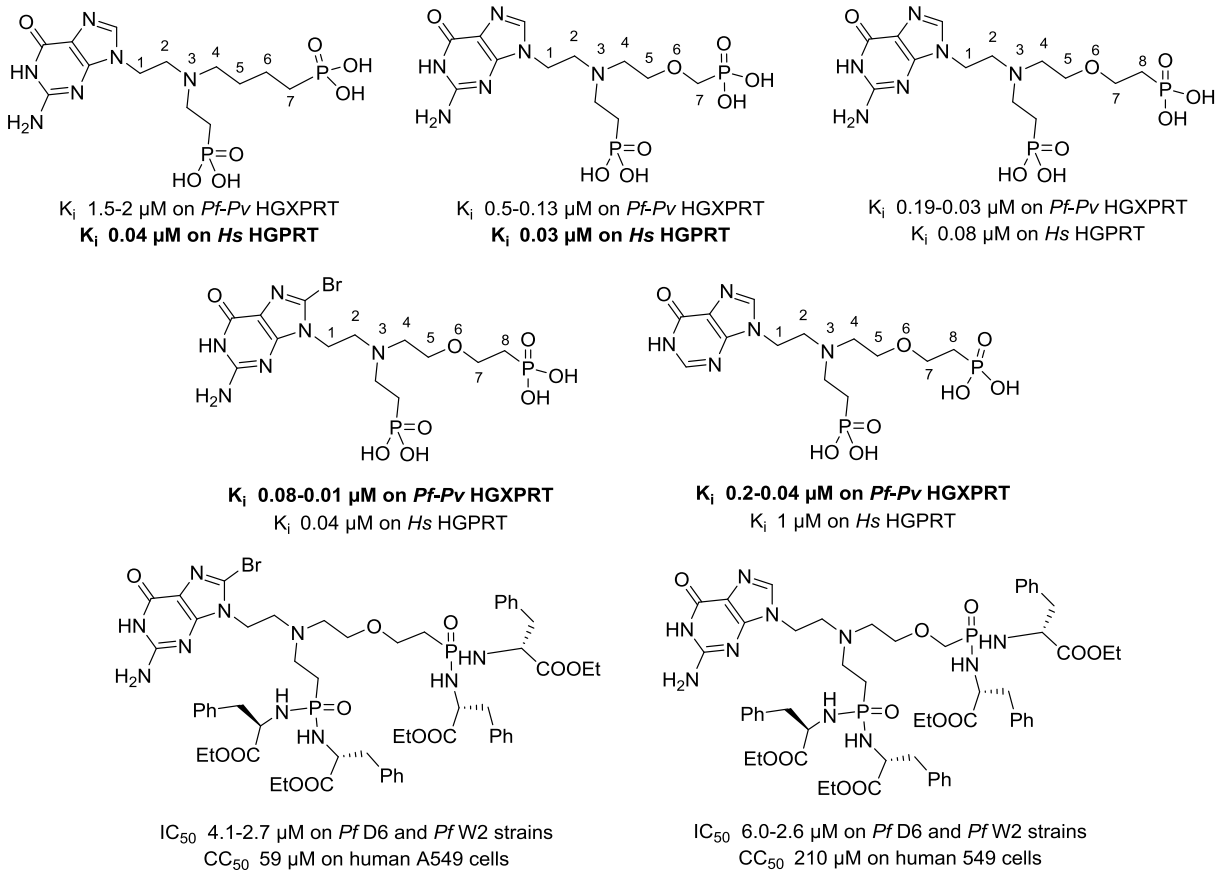
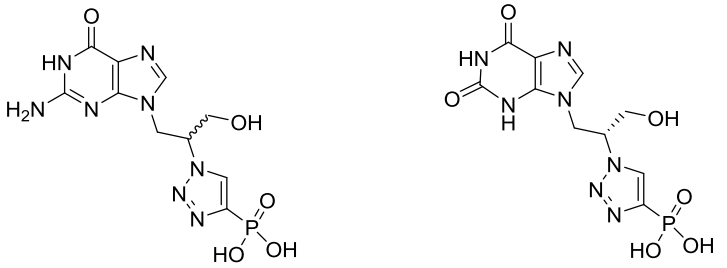


Figure 30: Biological activities of asymmetric aza-bisphosphonate-ANPs and their prodrugs

h. Triazolo-ANPs

Recently, ANPs containing a triazole ring instead of a ribose ring and a phosphonic acid group at position 4 were reported **(Figure 31).**[130] Both guanine enantiomers exhibited the same K_i on purified *Pf* and human HG(X)PRT, which surprisingly indicated that stereoisomery had no effect on affinity. Also surprisingly, the xanthine compound showed the best selectivity for *Pf* enzymes among others (SI > 250).

Hypoxanthine compounds were found to be modest inhibitors of P*f* and human HG(X)PRT ((R)-isomer K_i values of 3 and 8 µM were observed, respectively) and poorly active against *Pv* HGXPRT (K_i >100 µM).



K_i (R) 1-6 µM, (S) 2 µM on *Pf-Pv* HG(X)PRT
K_i (R) 0.1, (S) 0.4 µM on *Hs* HGPRT

K_i (R) 2 µM on *Pf* HGXPRT
K_i (R) >500 µM on *Hs* HGPRT

Figure 31: Triazolo-ANPs as potent inhibitor of *Pf* HGXPRT and *Hs* HGPRT

CONCLUSION

As the resistance of *Plasmodium* species to all antimalarial medicines is spreading in Asia and Africa, the search for new treatments is crucial. The main recommendations of WHO to address the drug-resistance problem include the use of a single-dose treatment to cure uncomplicated malaria, safety for vulnerable persons such as pregnant women and young children, combining drugs with different targets, activity against several stages of the parasite life cycle and low cost (~ US$1 per adult and US$0.60 for an average child's treatment). Consequently, the scientific community, especially medicinal chemists, biochemists and biologists working in the field of parasitology, is still facing a challenge to discover original drugs and identify novel targets.

Here we have discussed nucleos(t)ide analogues as a class of potential antimalarials and the related metabolic pathway, i.e. enzymes belonging to the purine salvage pathway which have been investigated for chemotherapy. Indeed, *Plasmodium* parasites behave as purine auxotrophs and inhibition of the corresponding enzymes would lead to death of the parasites through purine starvation. Among the possible

targets, *Pf* PNP and *Pf* HGXPRT inhibition has been intensively investigated, along with *Pf* ADA and *Pf* IMPDH to a lesser extent. Molecular modelling approaches have been used to improve potential inhibitors. However, the weak (or the lack) of specificity of many compounds towards human and *Pf* species has somewhat limited their development as drug candidates. The antimalarial activity of immucillin derivatives and analogues has been demonstrated *in vitro* and *in vivo*. Within this class, DADMe-immucillin-G (BCX4945) was identified as one of the most potent *Pf* PNP inhibitors (with subnanomolar affinity) while acting as a transition state analogue. This compound reached the preclinical phase in 2012, but no further information has been reported since then. The use of ANPs as inhibitors of the plasmodial purine metabolism was proposed in the late 1990s, and investigations are still ongoing. It could represent one of the most promising families of derivatives. Various series of ANPs have been reported to inhibit *Pf* HGXPRT and *Pv* HGPRT, leading to studies on the impact of the nucleobase nature, linker length, the presence of side-chains and their functionalities on the activity and selectivity. Docking studies were also performed to enhance their affinity and/or selectivity. However, the unknown structure of *Pf* HGXPRT is a major limitation which could hamper rational design of these derivatives based on docking studies. All computational studies have been performed with human and/or *Pv* HGPRT, which is not entirely comparable to the *Pf* HGPRT. In addition, the use of prodrug approaches is mandatory for such compounds since phosphonic acids do not readily cross biological membranes—an aspect that could complicate matters (i.e. synthesis and cost).

The overall findings of all of these studies show us that nucleos(t)ide analogues are far from revealing their full potential. Two decades have been enough to develop a broad range of compounds effectively and selectively targeting different enzymatic steps of the *Plasmodium* purine salvage pathway, and it is hoped that the future of this class of compounds will meet with the success it deserves.

ABBREVIATION USED

6-oxoPRTases or 6-oxopurinePRTs, 6-oxopurinephosphoribosyltransferases; A, adenine; ABC, ATP-binding cassette; ABS, asexual blood stage; ACTs, artemisinin combination therapies; ADA, adenosine deaminase; ADC, aminodeoxychorismate; ADCL, aminodeoxychorismate lyase; ADCS, amino-deoxychorismate synthase; ADP, adenosine 5'-diphosphate; AdSL, adenylosuccinate lyase; AdSS, adenylosuccinate synthase; AIP, acyclic immucillin phosphonate; AMPDA, adenosine-5'-monophosphate deaminase; ANbPs, acyclic nucleoside bisphosphonates; ANPs, acyclic nucleoside phosphonate; ATC, aspartate transcarbamoylase; ATP, adenosine 5'-triphosphate; ATPPase, adenosine 5'-triphosphate pyrophosphatase; BCX-1777, immucillin-H; BT1 family, high affinity folate-biopterin transporters C; cytosine CA, carbonic anhydrase; CC_{50}, 50% cytotoxic concentration; CH_2.THF, 5,10-methylene-tetrahydrofolate; CH.THF, 5,10-methenyltetrahydrofolate; CoQ, co-enzyme; QCPEE, (*S*)-2-(phosphonoethoxy)propanoic acid; CPME, (*S*)-2-(phosphonomethoxy)propanoic acid; CPSII, carbamoyl phosphate synthetase; IICS, chorismate synthase; CTP, cytidine-5'-triphosphate; CTPS, cytosine 5'-triphosphate synthetase; CVPS, 5-O-(1-carboxyvinyl)-3-phosphoshikimatecyclic; HPMP, cyclic-3-hydroxy-2-(phosphonomethoxy)-propyl; DADMe, 4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene); DAP, 2,6-diaminopurine; DATMe : , 4'-deaza-1'-aza-triol-1'-(9-methylene); dCTP, 2'-deoxycytidine-5'-triphosphated; GMP, 2'-deoxyguanosine-5'-monophosphate; DHF, 7,8-dihydrofolate; DHFR, dihydrofolate reductase; DHNTP, 7,8-dihydroneopterin triphosphate; DHO, dihydroorotase; DHODH, dihydroorotate dehydrogenase; DHP, 7,8-dihydropteroate; DHPA, 9-(2,3-dihydroxypropyl)adenine; DNA, desoxyribonucleic acid; DON, 6-diazo-5-oxo-norleucine; DOXP, 1'-deoxy-D-xylulose-5'-phosphate

pathway / non-mevalonate pathway; dTMP, 2'-deoxythymidine-5'-monophosphate; dTTP, 2'-deoxythymidine-5'-triphosphate; dUDP, 2'-deoxyuridine-5'-diphosphate; dUMP, 2'-deoxyuridine-5'-monophosphate; dUTP, 2'-deoxyuridine-5'-triphosphate; dUTPase, deoxyuridine 5'-triphosphate nucleotidohydrolase; EC_{50}, 50% effective concentration ; EMP1, erythrocyte membrane protein 1 ; ENT1/2/3/4, equilibrative nucleoside transporter 1/2/3/4; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase ; FUdR, 5-fluoro-D-2'-deoxyuridine ; FMet-tRNA, formylmethionyl-tRNA ; FPEP, 3-fluoro-(2-phosphonoethoxy)propyl ; FPGS, folylpolyglutamate synthase ; FPMP, 3-fluoro-(2-phosphono-methoxy)propyl; FOCM, folate-mediated one-carbon metabolism; FT1/2, folate/biopterin transporter 1/2; G, guanine; GAT, domain of GMPS responsible of glutamine hydrolyzation; GMP, guanosine monophosphate; GMPs, guanosine monophosphate synthetase; GTP, guanosine 5'-triphosphate; GTPC, GTP cyclohydrolase I; bis(POM)PMEA, bis(pivaloyloxymethyl)-9-[2-(phosphonomethoxy)ethyl]-adenine, brand name Adefovir; dipivoxil HBV, hepatitis B virus; HGPRT, hypoxanthine-guanine phosphoribosyltransferase (human); HGXPRT, hypoxanthine-guanine-xanthine phosphoribosyl-transferase (malarial); HG(X)PRT, both human HGPRT and malarial HGXPRT; *Hs*, Homo sapiens; HIV, human immunodefiency virus; HMDP, 6-hydroxymethyl-7,8-dihydropterin; HMDP-PP, 6-hydroxymethyl-7,8-dihydropterin pyrophosphate; HomoCys, homocysteine; HPEP, (S)-3-hydroxy-2-(phosphonoethoxy)propyl; HPMP, 9-(3-hydroxy-2-phosphonomethoxypropyl); IC_{37}, molar concentration of an antagonist that inhibits 37% of the activity of a targeted function *in vitro*; IC_{50}, 50% inhibitory concentration; ID_{50}, 50% infectious dose; Imm, immucillin ; IMP, inosine monophosphate ; IMPDH, inosine monophosphate dehydrogenase ; K_d, dissociation constant, measure of the propensity of a complex (such as the substrate-enzyme complex) to separate into each of its components; K_i, dissociation constant for the enzyme-drug complex, measured from initial rates; K_i*, equilibrium dissociation constant; Me.THF, 5-methyltetrahydrofolate; Met-tRNA, methionyl-tRNA; MTA, 5'-methylthioadenosine; MTHFD,

methylenehydrofolate dehydrogenase; MTHFR, methylenetetrahydrofolate reductase; MTI, 5'-methylthioinosine; MT-Imm, 5'-methylthioimucillin; NAD+, oxidized nicotinamide adenine dinucleotide (coenzyme); NADH, reduced nicotinamide adenine dinucleotide (coenzyme); NBMPR, nitrobenzylthioinosine; NDP, nucleoside diphosphate; NMPK, nucleoside monophosphate kinase; NPP, new permeability pathway; NTP, nucleoside triphosphate; ODC, orotidine 5'-monophosphate decarboxylase; OPRTase, orotate phosphoribosyltransferase; pABA, para-aminobenzoic acid; *Pb*, *Plasmodium berguei*; PDB, protein data bank; PEE, 2-(phosphonoethoxy)ethyl; *Pf*, *Plasmodium falciparum; Pf*3D7, *Plasmodium falciparum* strain used in lab assays; *Pf*crt, *Pf* chloroquine-resistant transporter gene; *Pf*CRT, *Plasmodium falciparum* digestive vacuole chloroquine-resistant transmembrane transporter; *Pf*MDR, *Plasmodium falciparum* multidrug resistance protein; *Pf*mdr1, gene of *Plasmodium falciparum* multidrug resistance protein; *Pk*, *Plasmodium knowlesi*; pKa, logarithm of acid dissociation constant Ka; PlasmoDB, the online plasmodic genomic resources http://PlasmoDB.org*Pm*: *Plasmodium malariae;* PME, 2-(phosphonomethoxy)ethyl; PMP, 2-(phosphonomethoxy)propyl; PNP, purine nucleoside phosphorylase; *Po*, *Plasmodium ovale;* PP_i : inorganic pyrophosphate; PRib-PP, phosphoribosyl pyrophosphate; PSAC, plasmodial surface anion channel; PTPS, 6-pyruvoyl-tetrahydropterin synthase III; *Pv*, *Plasmodium vivax;* PVM, parasitophorous vacuole membrane; RBC, red blood cell; RNA, ribonucleic acid; RNR, ribonucleotide reductase; ROS, reactive oxygen species; SHMT, serine hydroxymethyltransferase; SK, shikimate kinase; T, thymine; THF, 5,6,7,8-tetrahydrofolate; TK, thymidine kinase; TMP, thymidine-5'-monophosphate; TMPK, thymidylate monophosphate kinase; tRNA, transfer ARN; TS, thymidylate synthase; UDP, uridine-5'-diphosphate; UK, uridine kinase; UPRT, uracile phosphoribosyltransferase; UTP, uridine-5'-triphosphate; WHO, world health organization; XMP, xanthosine monophosphate.

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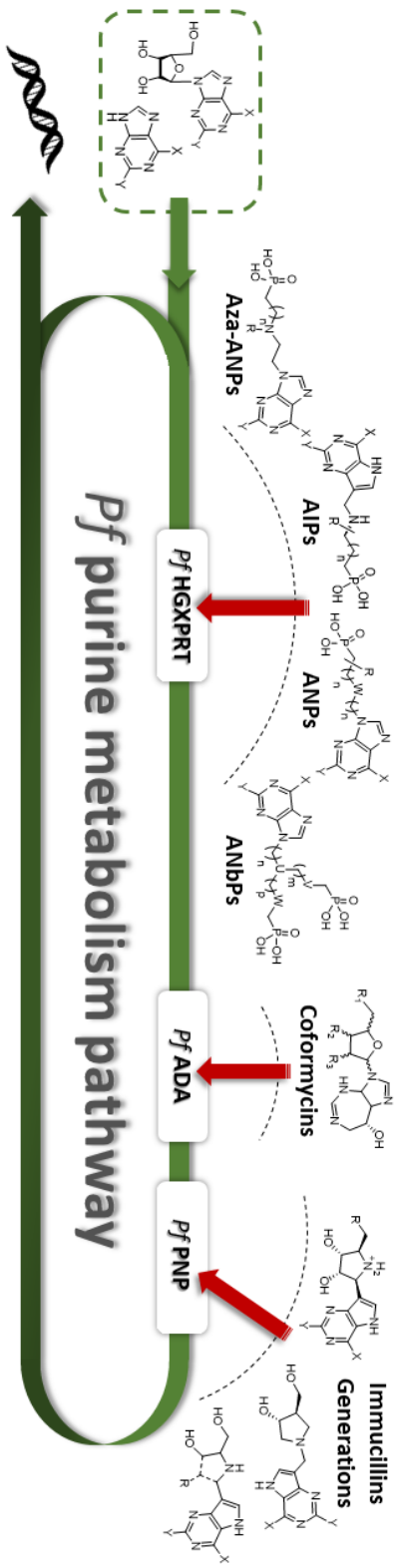

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