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Purine import into malaria parasites as a target for antimalarial drug development

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Abstract

Infection with *Plasmodium* species parasites causes malaria. *Plasmodium* parasites are purine auxotrophs. In all life cycle stages, they require purines for RNA and DNA synthesis and other cellular metabolic processes. Purines are imported from the host erythrocyte by equilibrative nucleoside transporters (ENTs). They are processed via purine salvage–pathway enzymes to form the required purine nucleotides. The *P. falciparum* genome encodes four putative ENTs (PfENT1–4). Genetic, biochemical, and physiologic evidence suggest that PfENT1 is the primary purine transporter supplying the purine-salvage pathway. Protein mass spectrometry shows that PfENT1 is expressed in all parasite stages. PfENT1 knockout parasites are not viable in culture at purine concentrations found in human blood (< 10 μM). Thus, PfENT1 is a potential target for novel antimalarial drugs, but no PfENT1 inhibitors have been identified to test the hypothesis. Identifying inhibitors of PfENT1 is an essential step to validate PfENT1 as a potential antimalarial drug target.

Keywords

purines; nucleoside transporter; malaria; drug development

Introduction

Plasmodium species parasites, like many other protozoan parasites, are purine auxotrophs, unable to perform *de novo* purine biosynthesis. They rely on the host to provide purines that they modify through enzymes of the purine-salvage pathway to generate the purine nucleotides necessary for cellular metabolic processes including RNA and DNA synthesis, cellular energetics (ATP), and the synthesis of purine-containing molecules such as NADH, NADPH, coenzyme A and S-adenosylmethionine, among others. Purine metabolic pathways

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Conflict of Interest

The authors declare a conflict of interest. The authors (MHA, IJF, RD) hold a patent on a high-throughput screening assay for inhibitors of protozoan parasite purine transporters. A second patent for 171 hits for inhibitors of PfENT1 from a high-throughput screen is pending (MHA, IJF, RD and others).

in *Plasmodium* parasites have been extensively reviewed and will not be discussed further in the present work.[1–8]

Purine monomers exist in three major forms, as nucleobases, nucleosides, and nucleotides. Two families of membrane transporters have been identified that transport purine nucleobases and nucleosides, the equilibrative nucleoside transporters (ENT, SLC29 family)[9,10] and the Concentrative Nucleoside Transporters (CNT, SLC28).[11] The ENTs and CNTs are distinct gene families with no apparent sequence or structural homology. While the gene family names suggest that the ENT family are facilitated transporters and the CNTs ion-coupled transporters, that distinction does not always hold, because some ENTs may be proton–purine symporters.[12,13] The *P. falciparum* genome contains four ENT homologues, PfENT1–4, and no CNT homologues.[14–17] Thus, as discussed in greater detail below, ENTs are likely to be the major purine import pathway into *Plasmodium* parasites. In the subsequent sections, we will review previous studies on the structure, function, and pharmacology of non-*Plasmodium* ENTs and then we will review the *Plasmodium* ENTs. We will then discuss other aspects of purine uptake and metabolism of relevance to ENTs as potential drug targets for novel antimalarial compounds.

Equilibrative nucleoside transporters: cloning, structure, and pharmacology

Four ENT homologues have been identified in the human genome. In humans, hENT1 and hENT2 are the major plasma membrane purine transporters.[18,19] They are 40% sequence identical. HENT3 is present in intracellular membranes and mutations in *hENT3* cause a variety of human disorders.[20–23] The fourth human ENT homologue was initially characterized as a plasma membrane monoamine transporter (PMAT), but at acidic pH it transports purines.[12,24,25] Both hENT1 and hENT2 transport both purines and pyrimidines. Both have a strong preference for nucleosides as substrates as compared to nucleobases.[9,26,27] The pharmacology of hENT1 and hENT2 is quite distinct. hENT1 is inhibited by low nanomolar concentrations of nitrobenzylthioinosine (NBMPR), dipyridamole, and dilazep.[18] In contrast, these compounds only inhibit hENT2 in the 10-micromolar concentration range.[19] Residues responsible for these differences have been identified through experiments involving chimeric constructs and site-directed mutagenesis.[28–38]

ENTs are polytopic membrane proteins. When hENT1 was initially cloned, hydrophobicity analysis predicted it to have 11 transmembrane segments.[18,19] Experimental data shows that the N-terminus is cytoplasmic and the C-terminus is extracellular, suggesting an odd number of membrane-spanning segments. Glycosylation site–insertion analysis is consistent with 11 membrane-spanning segments.[39–41] No X-ray crystal structures of ENTs have been solved to date, but using the Rosetta molecular modeling software, an *ab initio* model of the *Leishmania donovani* LdNT1.1 transporter, an ENT family member, has been constructed.[42] Experimental studies using disulfide cross- linking between engineered cysteine residues have verified some predictions of the model.[43,44] The water-surface accessibility of residues in transmembrane segments of several ENT family members have been analyzed by the

substituted cysteine accessibility method (SCAM).[45,46] SCAM experiments have identified residues that may line the ENT substrate permeation pathway in TM4, 5, 6, and 9–11.[47–52]

Purine transport and metabolism by red blood cells

Red blood cells (RBCs) provide the host environment for asexual-stage *Plasmodium* blood-stage parasites. Like *Plasmodium* parasites, RBCs are unable to synthesize purines by *de novo* biosynthesis. RBCs import purines and modify them via a subset of purine salvage–pathway enzymes (Fig. 1). Thus, purines in the plasma are the source for both the RBCs and the parasites.

Human plasma contains micromolar concentrations of purines. Early determinations of the plasma purine concentrations, particularly adenine/adenosine/ATP, were likely overestimates, owing to hemolysis and release from RBCs during sample acquisition and storage: With better techniques, more accurate measurements have been obtained that more likely reflect the composition of human plasma *in situ*.[53,54] The major purines found in human blood are hypoxanthine (1–5 μM) and inosine (~1 μM).[53,55–58] Adenosine is the other purine found in plasma. Adenosine acts as a hormone regulating local vasodilation and platelet aggregation. The plasma concentration of adenosine is regulated by local circumstances and ranges from nanomolar values up to 1–5 μM.[53,54,57,59] Thus, in humans, the total plasma purine concentration is below 10 μM.[57]

The human RBC plasma membrane has two major purine transport pathways. Nucleosides enter via hENT1 and nucleobases largely enter via an NBMPR-insensitive nucleobase facilitated transporter (hFNT1) whose molecular identity is uncertain.[60–66] In *P. falciparum*–infected RBCs, these two purine-import pathways seem to provide the primary pathway for purine entry into the RBC cytoplasm, but there is a component of adenosine uptake into infected RBCs that is insensitive to NBMPR and may be mediated by the new permeability pathway.[67–71]

The RBC cytoplasm contains a subset of purine salvage–pathway enzymes. They have two important roles: to remove adenosine from the blood and to provide the purine molecules necessary for RBC function. Adenosine is an important extracellular signaling molecule through its interactions with adenosine receptors, members of the G protein–coupled receptor (GPCR) superfamily.[72–74] The main pathway for removal of adenosine from plasma is uptake into RBCs. In the RBC, adenosine is either phosphorylated by adenosine kinase to convert it into AMP or deaminated by adenosine deaminase to inosine, which is converted to hypoxanthine by purine nucleoside phosphorylase (PNP).[8,64] Hypoxanthine can then be phosphoribosylated by HGPRT to form IMP, which can be further metabolized to guanylates.[4,75] Alternatively, hypoxanthine can be released into the plasma and either oxidized to xanthine or uric acid or cleared by metabolic processes in other cells. The composition of the purine pool inside RBCs maintained in supraphysiological (367 μM) hypoxanthine-containing media was recently reported.[76] The relationship to the purine composition in cells maintained in physiological purine-containing media (<10 μM) is uncertain because previous studies have shown changes in RBC purine composition and content as a function of media purine supplementation.[77] Inside the RBC, the main

constituent of the purine pool is ATP, which is present at a concentration of ~ 2 mM. Given the volume of an RBC, this would not even provide a sufficient amount of purine to allow eight-fold replication of the *Plasmodium* genome, the minimal amount of DNA replication that occurs during the 48-h intraerythrocytic life cycle. This implies that purines must be imported into the RBC in order to supply sufficient amounts of purines to the developing intracellular parasite.

Plasmodium equilibrative nucleoside transporters

Sequence analysis of the *P. falciparum* genome identified four putative ENT homologues (PfENT1–4).[14,16] Extensive information about the four genes and their expression patterns in parasite life cycle stages is available on the PlasmoDB website (http://plasmodb.org/plasmo/). The gene identification numbers are *PfENT1*, PF3D7_1347200; *PfENT2*, PF3D7_0824400; *PfENT3*, PF3D7_1469400; and *PfENT4*, PF3D7_0103200. Curiously, *PfENT3* is only found in *Plasmodium* species that infect primates and humans.[78] Homologues of PfENT1, 2, and 4 are found in all *Plasmodium* species sequenced to date.

The four PfENT homologues have diverged significantly both from hENT1 and from each other. PfENT1 is 17% identical to hENT1 in amino acid sequence. Individually, PfENT1 is 15–22% identical to PfENT2–4. Only 2% of residues are identical between the four homologues.

PfENT1

The primary import pathway for purines to supply the parasite purine-salvage pathway is via PfENT1. PfENT1 mRNA is found in all parasite life-cycle stages. The mRNA level has a small peak in the early trophozoite stage and then decreases in late schizonts.[79,80] By mass spectrometry, PfENT1 peptides have been identified in all asexual and sexual blood stages and in sporozoites.[81–88] PfENT1 was cloned and heterologously expressed in *Xenopus laevis* oocytes.[89,90] Interestingly, the native *pfent1* nucleotide sequence can be functionally expressed in oocytes and did not require codon optimization for expression. There were significant differences in the substrate and inhibitor profiles and affinities reported in the initial pair of papers.[89,90] Some of these differences arose from technical issues related to the effects of substrate metabolism on the radioactive substrate–uptake experiments in oocytes that formed the basis of the papers.[91,92] PfENT1 transports both purine and pyrimidine nucleobases and nucleosides with affinities in the hundreds-of-micromolar to millimolar range.[90–93] The characteristics of purine nucleobase uptake by RBC-free parasites is similar to the characteristics determined for heterologously expressed PfENT1.[93] Uptake experiments in isolated parasites and in *Xenopus* oocytes must be done with care so as to distinguish the kinetic properties of the transporter from those of metabolic enzymes that may metabolize the transported substrates.[92,94] Difficulties separating transport and metabolism have adversely affected some studies in isolated parasites.[94,95] Human hENT1 inhibitors including NBMPR, dipyridamole, and dilazep do not inhibit PfENT1 expressed in oocytes at concentrations up to 10 μM.[50,90,91] These characteristics are similar to those reported for intact, RBC-free parasites.[91,93] Immunoelectron microscopy with anti-PfENT1 antibodies show that it is mainly localized in the parasite plasma membrane.[96]

Knockout of *pfent1* is conditionally lethal.[97,98] At purine concentrations found in human blood (<10 μM), *pfent1*Δ parasites are not viable. Growth can be rescued by addition of purines, hypoxanthine, adenosine, or inosine to the growth media at concentrations above 50 μM. Maximal growth is not observed until a purine concentration of 500 μM.[97] The growth rescue at high purine concentrations suggests that the *pfent1*Δ parasites have a secondary, low-affinity, low-capacity purine transport pathway besides PfENT1.

The results of purine-uptake experiments into *pfent1*Δ parasites released from the host RBCs differs between two papers from the same group.[97,98] The initial paper reported that uptake rates of adenosine and inosine were about 50% of uptake amounts in wild-type parasites, but no hypoxanthine uptake was observed.[97] The subsequent paper reported no uptake of adenosine, inosine, or hypoxanthine by *pfent1*Δ parasites, but restoration of uptake in *pfent1*Δ parasites expressing PfENT1 through complementation from a plasmid.[98] Parasites lacking *pfent1* did not import xanthine, guanine, or guanosine, implying that PfENT1 is the only import pathway for these purines.[98] Although there are discrepancies in the results as to whether the *pfent1*Δ parasites can transport adenosine and inosine, the results in the second paper by the same authors presumably represent their view of the transport properties of the *pfent1*Δ parasites. This emphasizes the importance of PfENT1 in the uptake of purine nucleobases and nucleosides. Even though the *pfent1*Δ parasites cannot transport physiologically relevant nucleosides and nucleobases, they can grow on hypoxanthine-, adenosine-, inosine-, or adenine-supplemented media because the RBC purine-salvage pathway likely converts these purines into a form that can be transported and used by the parasites, possibly as nucleotides.[99] The identity of the specific chemical forms of the purines imported by *pfent1*Δ parasites remains unknown.

The lethal impact of *pfent1* knockout for parasites grown in purine concentrations found in human blood suggests that it might be a target for the development of novel antimalarial drugs.[89,90,92,97,100] However, no inhibitors of PfENT1 have been identified to date. Experiments are in progress in our lab using a high-throughput screen that we have developed to identify PfENT1 inhibitors and to characterize their effects on the proliferation of malaria parasites in culture.

Rodent malaria parasites lacking the PfENT1 homologue

Knockout of the *P. yoelli* homologue of *pfent1*, *pynt1*, resulted in parasites that survived within the mouse. The *pynt1*Δ parasites displayed a growth defect compared to wild type. Peak parasitemia was only 2% for mice infected with 5000 *pynt1*Δ parasites but reached 30% with the same number of wild-type parasites. Infection with 50 *pynt1*Δ parasites did not yield observable parasitemia, but conferred immunity to challenge with wild-type *P. yoelli* and *P. berghei* strains. The authors noted that *pynt1*Δ parasites were unable to complete ookinete development in the mosquito.[101]

A *P. berghei pbnt1* knockout in the ANKA strain used as a model for cerebral malaria was also reported.[102] Mice infected with the *pbnt1*Δ parasites did not develop cerebral malaria symptoms or pathology.[102] The observations from both rodent malaria parasite models is clear: removing the ENT1 protein is clearly detrimental to parasite growth and virulence.

The latter report supports the idea that blocking ENT1 with a small molecule might prevent the development of cerebral malaria.

PfENT2

Like PfENT1, PfENT2 is expressed in all parasite blood stages but was not detected in sporozoites.[81–88] PfENT2 mRNA levels peak significantly in the early-to-late trophozoite period.[79,80] Attempts to heterologously express either native or codon-optimized PfENT2 in either *Xenopus* oocytes or in *Saccharomyces cerevisiae* (yeast) did not provide evidence for functional purine transport.[91,103] PfENT2-expressing yeast were somewhat more sensitive to the pyrimidine analog 5-fluorouridine, but there was no other evidence of pyrimidine transport.[103] Immunoelectron microscopy showed localization in internal parasite membranes, likely to be predominantly the endoplasmic reticulum.[103]

PfENT3

PfENT3 is the least studied of the *Plasmodium* ENTs. Curiously, *pfent3* homologues are not found in the genomes of murine or avian *Plasmodium* species that have been sequenced to date.[78] However, homologues are found in all species that infect primates or humans. PfENT3 mRNA expression is fairly constant through the 48-h intraerythrocytic life cycle.[79,80] PfENT3 transcript levels appear to increase in response to antimycin A exposure.[104] No evidence for PfENT3 expression has been reported in proteomic studies.[81–88] This may be due to low levels of protein expression or to specific expression in only a limited set of life-cycle stages. Attempts to express either native or yeast codon-optimized PfENT3 in either *Xenopus* oocytes or in *S. cerevisiae* did not result in evidence of functional purine or pyrimidine transport, although protein expression was documented using an epitope-tagged construct (Arora and Akabas, unpublished results). Its membrane localization both *in situ* and in these heterologous systems is unknown.

PfENT4

Evidence of PfENT4 protein expression was detected in all blood stages and in sporozoites.[81–88] PfENT4 mRNA levels peak during the trophozoite stage.[79,80] While the native nucleotide sequence did not result in functional expression of PfENT4 in *Xenopus* oocytes, a *Xenopus* codon-optimized gene did exhibit functional purine transport.[105] PfENT4 has a distinct substrate specificity profile from PfENT1. In marked contrast to PfENT1, we found no evidence of hypoxanthine transport by PfENT4-expressing oocytes either by uptake of [^{3}H]hypoxanthine or by competition with uptake of [^{3}H]adenine.[105] PfENT4 displayed millimolar affinities for most transported purines, both nucleosides and nucleobases. Strikingly, PfENT4 displayed the highest affinities for isoprenylated adenine derivatives, including isopentenyladenine (3 μM), trans-zeatin (20 μM), and cis-zeatin (190 μM).[105] These compounds are plant hormones called cytokinins.[106–108] In plants, cytokinin receptors are transmembrane proteins with cytoplasmic histidine kinase domains.[109] There are no histidine kinase domain homologues in the *Plasmodium* genome, so the physiological significance of the high affinity of PfENT4 for these modified purines is uncertain.

Other purine transport pathways in *Plasmodium*

RBC-free *P. falciparum* parasites can also transport the purine nucleotide AMP.[99] The gene encoding the AMP transporter has not been identified, but neither PfENT1 nor PfENT4 transports AMP.[99,105] Further studies will be necessary to characterize the AMP-transport pathway and define its substrate selectivity. RBCs have potent adenosine kinase activity,[110,111] but the parasites have none.[99] At physiological concentrations, adenosine entering the RBC will mostly be converted to AMP, not to hypoxanthine.[64] The ability to transport AMP may allow the parasite to take advantage of this potential purine source; however, the concentration of AMP in RBCs is in the low micromolar range.[57]

Plasmodium parasites also express a mitochondrial ADP/ATP exchanger.[112–115] While it appears to be localized to the mitochondrial membrane, some early studies suggested that it might also be present in the parasite plasma membrane,[112,113] although subsequent studies questioned that localization.[114] Inhibitors of the ADP/ATP exchanger such as bongkrekic acid and atractyloside kill malaria parasites in culture.[112,113,116] Although the mitochondria do not appear to have an ATP-generating role, they do perform other important metabolic functions.[117]

Another potential purine source for the parasites might be host-cell ATP taken up during endocytosis of host-cell cytoplasm. How the ATP might be transported from the digestive vacuole into the parasite cytoplasm remains unknown, but ATP is present in the RBC cytoplasm at a concentration of about 2 mM.

Summary

Purine import is an essential function for *Plasmodium* parasites. The PfENT1 and PfENT4 nucleoside/nucleobase transporters have been characterized. PfENT1 is the primary purine-import pathway for the parasite purine-salvage pathway. The functional role of PfENT4 in parasite physiology is uncertain. Other purine-transport pathways exist, but their molecular bases remain to be identified. Identification of these purine-transport pathways may provide new potential targets for antimalarial drug development.

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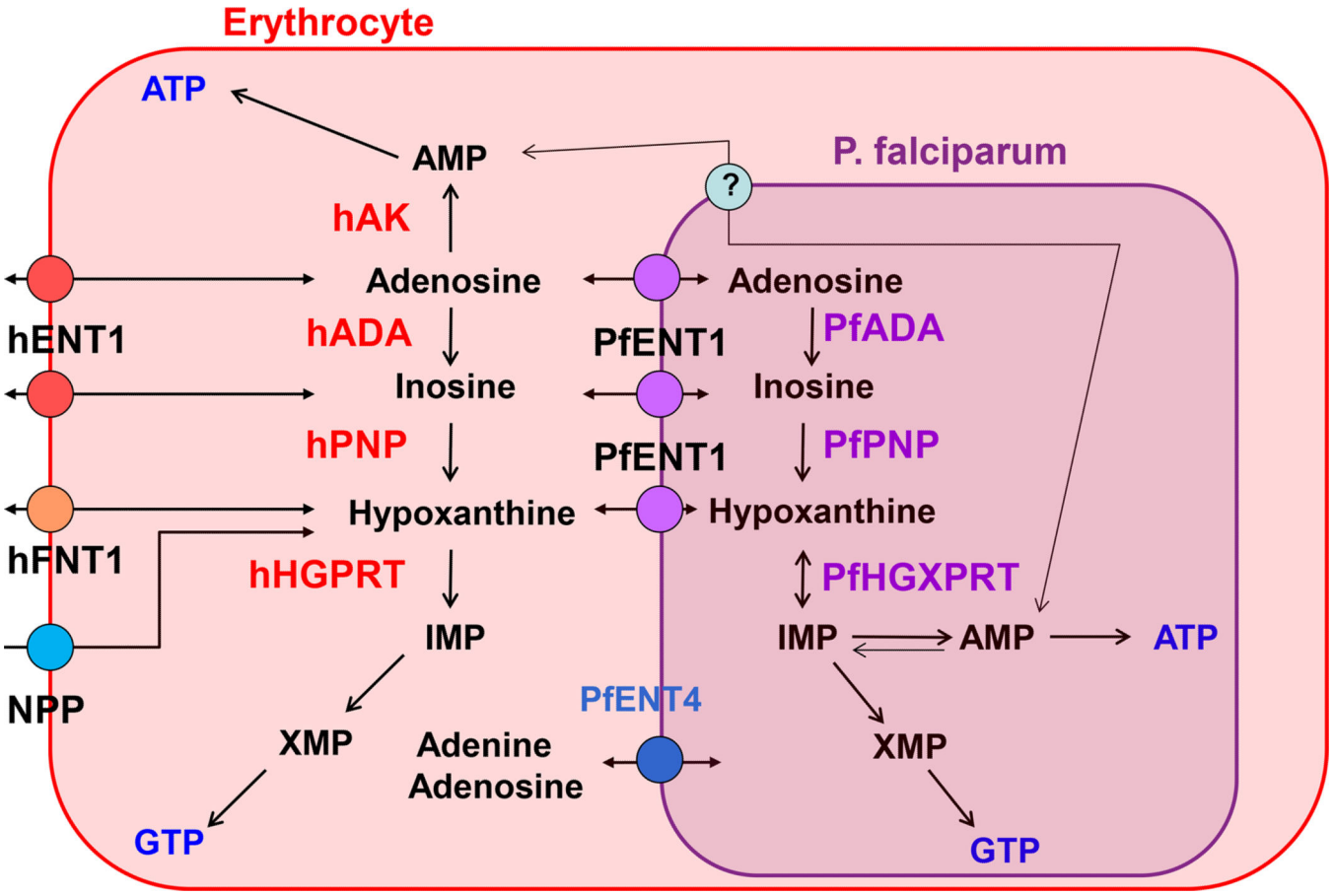

Figure 1.

Simplified illustration of the purine transport and metabolism pathways in a *P. falciparum*–infected RBC. More detailed metabolic pathways are available in other reviews.[7,8] Colored circles represent transporters and channels for the given substrates. The number of circles for a given transporter does not indicate relative abundance or transport capacity; they simply indicate the pathway a given substrate may take to cross a particular membrane. The light blue circle with the question mark represents the AMP-transport pathway that has been shown to exist functionally but whose molecular identity is unknown.[99] In the interest of simplifying the figure, the parasitophorus vacuole membrane that surrounds the parasite in not shown because it is non-selectively permeable to small molecules such as purines.[17] Purine transport pathways into various subcellular organelles that contain DNA that must be replicated during the parasite life cycle, such as the mitochondrion and apicoplast, are not shown.[118,119] Nor is PfENT2, whose substrate specificity is unknown but is localized in the parasite endoplasmic reticulum.[103] PfENT3, whose cellular localization and substrate specificity is unknown, is also not shown. PfENT4 is shown in the parasite plasma membrane, but localization experiments have not been performed. hENT1, human ENT1; hFNT1, human facilitated nucleobase transporter 1; NPP, new permeability pathway; hAK, human adenosine kinase; hADA, human adenosine deaminase; hPNP, human purine nucleoside phosphorylase; hHGPRT, human hypoxanthine guanine phosphoribosyl transferase; IMP, inosine monophosphate; PfADA, *P. falciparum* adenosine deaminase;

PfPNP, *P. falciparum* purine nucleoside phosphorylase; PfHGXPRT, *P. falciparum* hypoxanthine guanine xanthine phosphoribosyl transferase; XMP, xanthine monophosphate.