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Substrate and inhibitor specificity of the *Plasmodium berghei* Equilibrative Nucleoside Transporter Type 1 (PbENT1)

Avish Arora, Roman Deniskin, Yvett Sosa, Sita Nirupama Nishtala, Philipp P. Henrich, T. R. Santha Kumar, David A. Fidock and Myles H. Akabas

Department of Physiology& Biophysics (AA, RD, YS, SNN, MHA), Departments of Neuroscience and Medicine (MHA), Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461
Department of Medicine (DAF) and Department of Microbiology & Immunology (PPH, TRSK, DAF), Columbia University Medical Center, 630 West 168th Street, New York, NY, 10032

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Running title: Inhibitors of the murine malaria purine transporter PbENT1

Address correspondence to: Myles H. Akabas, Department of Physiology & Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY, 10461, USA, E-mail: myles.akabas@einstein.yu.edu

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Abbreviations: ACT, artemesinin-based combination therapies; ADQ, amodiaquine; CM, cerebral malaria; CPM, counts per minute; CQ, chloroquine; ECM, experimental cerebral malaria; ENT1, equilibrative nucleoside transporter type 1; EV, empty vector; HTS, high throughput screen; IC_{50}, concentration causing 50% of maximal inhibition; NBMPR, nitrobenzylmercaptopurineriboside; PbENT1, *P. berghei* ENT1; PfENT1, *P. falciparum* ENT1; PNP, purine nucleoside phosphorylase; uRBCs, uninfected RBCs; WT, wild type.

Abstract

Malaria is a critical public health issue in the tropical world causing extensive morbidity and mortality. Infection by unicellular, obligate intracellular *Plasmodium* parasites causes malaria. The emergence of resistance to current antimalarial drugs necessitates the development of novel therapeutics. A potential novel drug target is the purine import transporter. Because *Plasmodium* parasites are purine auxotrophic, to fulfill metabolic requirements they must import purines from their host. They import purines via Equilibrative Nucleoside Transporter 1 (ENT1) homologues. Recently, we used a yeast-based high throughput screen (HTS) to identify inhibitors of the *Plasmodium falciparum* ENT1 (PfENT1) that kill *P. falciparum* parasites in culture. *P. berghei* infection of mice is an animal model for human malaria. Because *P. berghei* ENT1 (PbENT1) shares only 60% amino acid sequence identity with PfENT1, we sought to characterize PbENT1 and its sensitivity to our PfENT1 inhibitors. We expressed PbENT1 in purine auxotrophic yeast and used radiolabeled substrate uptake to characterize its function. We showed that PbENT1 transports both purines and pyrimidines. It preferred nucleosides compared to nucleobases. Inosine (IC_{50}=3.7 μM) and guanosine (IC_{50}=21.3 μM) had the highest affinities. Our recently discovered PfENT1 inhibitors were equally effective against both PbENT1 and PfENT1 mediated purine uptake. The PfENT1 inhibitors are at least 10-fold more potent against PfENT1 than human hENT1. They kill *P. berghei* parasites in 24 hour *ex vivo* culture. Thus, the *P. berghei* murine malaria model may be useful to evaluate the efficacy of PfENT1 inhibitors *in vivo* and their therapeutic potential for treatment of malaria.

Introduction

Every year about 500,000 deaths, mostly children under the age of five, result from nearly 200 million cases of malaria (World Health Organization, 2014). In Southeast Asia, *P. falciparum* parasites have developed resistance to current first-line artemisinin-based combination therapies (ACT) (Uhlemann and Fidock, 2012; Ariey et al., 2014; Ashley et al., 2014; Burrows et al., 2014; Straimer et al., 2015). Therefore, it is essential to identify new drug targets to facilitate the development of novel antimalarial drugs.

The *Plasmodium* parasite purine import pathway is one potential target, because the parasites are purine auxotrophic and require imported purines to synthesize RNA and replicate DNA during proliferation in the human host. The parasites import purines via equilibrative nucleoside transporters (Baldwin et al., 2004; Downie et al., 2008; Riegelhaupt et al., 2010; Frame et al., 2015a). In *P. falciparum*, knockout of the primary purine transporter, PfENT1, is conditionally lethal at purine concentrations found in human blood, <10 μM (Traut, 1994; El Bissati et al., 2006; Frame et al., 2015b). Thus, we and others hypothesized that small molecule inhibitors of the parasite purine transporter would kill malaria parasites (El Bissati et al., 2006; Baldwin et al., 2007; El Bissati et al., 2008; Frame et al., 2015a). We developed a robust, yeast-based HTS to identify PfENT1 inhibitors (Frame et al., 2015b). The basis for the HTS was that 5-flurouridine (5-FUrd) is cytotoxic for PfENT1-expressing *fui1*Δ yeast due to 5-FUrd entry via PfENT1. With 5-FUrd in the growth media, PfENT1-expressing *fui1*Δ yeast will only grow if a PfENT1 inhibitor is present in the media. We screened 64,500 compounds, identified 171 hits, and characterized nine of the top compounds. The nine compounds, representing six distinct chemical scaffolds, inhibit tritiated adenosine uptake into red blood cell (RBC) free parasites with IC_{50} values in the 5-50 nM range (Frame et al., 2015b). They kill *P. falciparum* parasites in culture with 5-50 μM IC_{50} values. Furthermore, the compounds inhibit the *P. vivax* ENT1 transporter and PvENT1 non-synonymous single nucleotide polymorphic variants identified in field isolates (Deniskin et al., 2016). Collectively, these finding support the hypothesis that PfENT1 inhibitors may be developed into novel antimalarial drugs.

P. berghei causes malaria in mice. It is currently the best animal model to study the human disease, although significant differences have been noted between human *P. falciparum* malaria and the *P. berghei* mouse model (Chisholm et al., 2016). PbENT1 is 60% amino acid sequence identical with PfENT1 (Supplemental Fig. 1). Mice infected with PbENT1- knockout *P. berghei* ANKA parasites develop a non-fatal, high parasitemia without cerebral malaria symptoms (Niikura et al., 2013). However, knockouts of other *P. berghei* genes that reduce parasite viability also cause loss of the ability to induce cerebral malaria (Chisholm et al., 2016). Thus, the inability to induce cerebral malaria may not be related to the PbENT1 knockout, but rather could reflect the loss of parasite fitness. Loss of fitness after ENT1 knockout is also seen in mice infected with ENT1-disrupted *P. yoelii*, another murine malaria species. They do not develop obvious infection but develop sterilizing immunity against subsequent infection with wild-type (WT) *P. yoelii* (Aly et al., 2010). The limited viability of ENT1-knockout murine-infective *Plasmodia* indicates that they have a secondary purine import pathway. This pathway plus higher mouse blood purine concentrations, presumably allows the ENT1-knockout parasites to remain viable during *in vivo* infection, albeit with reduced pathogenicity.

Adenosine uptake into human RBCs is mediated by human ENT1 (hENT1) (Domin et al., 1988; Griffiths et al., 1997). hENT1 is 17-18% amino acid sequence identical to PbENT1 and PfENT1. It is inhibited by nitrobenzylmercaptopurineriboside (NBMPR) and the FDA approved drug, dipyridamole at nanomolar concentrations (Domin et al., 1988; Griffiths et al., 1997). Neither NBMPR nor dipyridamole inhibit PfENT1 at concentration up to 20 μM (Carter et al., 2000a; Parker et al., 2000; Riegelhaupt et al., 2010). Thus, it may be feasible to develop inhibitors with high specificity for malaria ENT1s. The existence of known nanomolar potency hENT1 inhibitors indicates that the ENTs are druggable targets.

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In this paper, we expressed PbENT1 in *Saccharomyces cerevisiae* and characterized its substrate specificity profile. We investigated whether our PfENT1 inhibitors block PbENT1-mediated transport and their effects on the human RBC ENT1 homologue. We show that PbENT1 is a purine and pyrimidine transporter. We found that the PfENT1 inhibitors inhibit PbENT1 with similar nanomolar affinities, but with 10-1,000-fold lower affinity for hENT1. Thus, following the development of more potent PfENT1 inhibitors, the *in vivo* mouse malaria model may be useful to test their efficacy.

Materials and Methods

Yeast DNA Construct. We purchased a yeast codon-optimized gene of PbENT1 with a C-terminal HA epitope tag (*pbent1-HA-CO*) for expression in *S. cerevisiae* (DNA 2.0, Newark, CA) (Supplemental Fig. 2). The construct was cloned into a Gateway entry vector pENTR using pENTR/D-TOPO cloning kit (Life Technologies, Waltham, MA). From there, *pbent1-HA-CO* was cloned into a modified pYES2 destination vector using LR clonase enzyme (Life Technologies). The construct contains an upstream GAL1 promoter and a downstream CYC1 terminator. The construct also contains the *Ura3* gene, to allow for positive selection of yeast carrying the plasmid when using media lacking uracil.

Yeast growth media. Purine auxotrophic yeast were maintained on synthetic defined media (SDM) that contained 2% (w/v) galactose, 1% (w/v) raffinose, 0.5% (w/v) ammonium sulfate, 0.17% yeast nitrogen base (US Biologicals, Salem, MA), 0.02% (w/v) yeast dropout mix lacking uracil, adenine, histidine, and tryptophan (US Biologicals), 40 mg/l tryptophan, and 40 mg/l histidine. Media was supplemented with 300 µM adenine for *ade2*Δ-empty vector yeast or 1 mM adenosine for PbENT1-HA expressing yeast. Solid media plates contained 2% agar.

Yeast Strains and Transformation. DNA constructs were transformed into purine auxotrophic yeast as previously described (Frame et al., 2015b). Briefly, *S. cerevisiae* BY4741 with FUI1 and ADE2 gene deletions were used as WT (MATa; *his3*Δ1; *leu2*Δ0; *met15*Δ0; *ura3*Δ0; *fui1*Δ::KanMX4; *ade2*Δ::hphNT1). Hereafter, this yeast strain is referred to as purine auxotrophic yeast. Yeast were transformed using the lithium-acetate/DMSO method (Hill et al., 1991). WT yeast were grown in 10 ml YPD media to a cell density of 2 x 10^7 cells/ml. Cells were pelleted and washed with 20 ml cold lithium acetate (LiOAc: 100 mM LiOAc, 10 mM Tris pH 8). Pellet was resuspended in 100 µl LiOAc, and 10 µl of salmon-sperm DNA (2 mg/ml) and 1 µg of DNA construct was added. After incubation at room temperature for 5 min, 280 µl 50% PEG (polyethylene glycol 3350; %w/v) was added. Final solution was incubated at 30°C for 45 min.

DMSO was added (8% v/v) and sample was heat shocked at 42°C for 15 min. Cells were pelleted and resuspended in 5 ml YPD and allowed to recover for 3 h. Cells were pelleted and plated on SDM/Agar.

Yeast growth assays. Growth of PbENT1-HA expressing and empty vector transformed purine auxotrophic yeast was assessed in media where adenine or adenosine was the sole purine source. First, empty vector yeast and PbENT1-HA expressing yeast were grown overnight in SDM containing adenine to mid-log phase. Yeast cell density was determined by measuring OD_{600} (Biorad Benchmark Plus, Hercules, CA). Cells were pelleted and washed 3x with sterile water. Final cell pellet was diluted to 4 x 10^6 cells/ml in 2x SDM lacking purine. A 96-well plate was preloaded with 100 µl serially diluted adenosine in sterile water. 100 µl of cells were added and incubated for 30 h at 30°C. EC_{50} values were calculated using Prism 6 (GraphPad Software, La Jolla, CA). All experiments were repeated at least three times on different days.

Radiolabel uptake experiments with PbENT1-HA-expressing purine auxotrophic yeast. PbENT1-HA-expressing purine auxotrophic yeast were grown to mid-log phase in SDM containing 1 mM adenosine. Cells were pelleted and washed 3x in PBS supplemented with galactose (150 mM NaCl, 10 mM KH_2PO_4, 40 mM K_2HPO_4, 11 mM galactose, pH 7.4). Cells were resuspended in PBS to a concentration of 2 x 10^8 cells/ml. For uptake time course experiments, a 96 well plate was preloaded with 100 µl of 100 nM [^{3}H]adenosine ([2,8-^{3}H]adenosine, 35 Ci/mmol, Moravek Biochemicals, Brea, CA) or [^{3}H]uridine ([2,8-^{3}H]uridine, 22 Ci/mmol, Moravek Biochemicals). 100 µl of suspended yeast was added the at appropriate time points. For purine/pyrimidine uptake competition, a 96 well plate was preloaded with 50 µl serially diluted purines or pyrimidines. 50 µl of 200 nM [^{3}H]adenosine was added to pyrimidines and 50 µl of 1 mM [^{3}H]uridine was added to purines. 100 µl of resuspended yeast was added and uincubated for 15 min. For all experiments, uptake was terminated by harvesting cells onto glass fiber filtermats (Filtermat, GF/C; Perkin Elmer, Waltham, MA) using a TomTec 96 well cell harvester (#96-3-469, Hamden, CT). Filtermats were dried for > 1 h and sealed in plastic bags containing 5 ml Betaplate Scint LSC (Perkin Elmer). Filtermats were counted using 1450 Microbeta Trilux (Perkin Elmer). IC_{50} values were calculated using Prism 6 (GraphPad Software). All experiments were repeated at least three times on different days.

Inhibition of PbENT1 by PfENT1 inhibitors. PbENT1-HA-expressing purine auxotrophic yeast were grown to mid-log phase in SDM + 1 mM adenosine. Cells were washed and resuspended to a concentration of 2 x 10^8 cells/ml as above. A 96-well plate was preloaded with 100 µl of 100 nM [^{3}H]adenosine. 0.5 µl of compound, serially diluted in DMSO, was added to each well. 100 µl of cells were added and cells were harvested after 15 min, as above. Compounds were also tested on PfENT1-expressing purine auxotrophic yeast as described in (Frame et al., 2015b). PfENT1 inhibitors were purchased from Chembridge Corp. (San Diego, CA). Chembridge catalog numbers for the PfENT1 inhibitors are **1** (9001893), **2** (6718896), **3** (6946484), **4** (6081106), **5** (9039333), **6** (9011026), **7** (6736283), **13** (6517398), and **19** (9011680), structures are in Table 2. Compound names are listed in Frame et al., (2015b). Chemical structure and composition of PfENT1 inhibitors was validated previously by NMR and mass spectrometry (Frame et al., 2015b). All experiments were repeated at least three times on different days.

***Ex vivo* parasite drug susceptibility assay.** Donor outbred CD1 mice (female, 6–8 weeks of age; Charles River Laboratories, Stone Ridge, NY), were infected with 1 ×10^7 *P. berghei* parasites (strain ANKA 676m1cl1). This strain was obtained from the BEI Resources Repository, NIAID, NIH, as item MRA-868, contributed by Chris J. Janse and Andrew P. Waters. Once the parasitemia reached 5–7%, the parasites were harvested by cardiac puncture. 1 ml of the harvested blood was incubated in 50 ml RPMI1640 malaria culture medium (supplemented with 25% fetal bovine serum) with gentle shaking and incubated at 36.8°C for 23 hr (Janse et al., 2006). The parasites were then pelleted, washed in physiological saline solution, and reinjected (in saline solution, total volume 0.5 ml) into another mouse by tail vein injection. After one day (the duration of one intra-erythrocytic developmental cycle), we harvested ring-stage parasites by cardiac puncture and diluted these in 10 ml of malaria culture medium per 100 µl of packed blood cell pellet. 0.5 ml of this parasite suspension (at ~3–4% parasitemia) was mixed with 0.5 ml of compound across a range of ten 2-fold dilutions in 24-well plates. Plates were incubated for 24 hr at 36.8°C. Parasitemias were determined by microscopic analysis of Giemsa-stained thin blood smears, and IC_{50} values extrapolated by non-linear regression analysis. Experiments were performed on two separate occasions in duplicate. As a positive control, we included amodiaquine, which yielded IC_{50} values of 3.9 ± 0.3 nM, consistent with earlier reports of its activity against *P. berghei* parasites assayed *ex vivo* (Orjuela-Sanchez et al., 2012). All animal experiments were conducted under a protocol approved by the Columbia University Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals.

PfENT1 inhibitor effects on the human RBC hENT1 transporter. The PfENT1 inhibitors were evaluated for their ability to inhibit uptake of 50 nM [^{3}H]adenosine ([2,8-^{3}H]adenosine; 35 Ci/mmol Moravek Biochemicals) into human RBCs. Uninfected RBCs (uRBCs) were washed with a Ringer solution (122.5 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 11 mM D-glucose, 25 mM HEPES, 1 mM Na_2HPO_4, pH 7.4) and resuspended at 3% hematocrit in pre-warmed (37°C) Ringer solution. The PfENT1 inhibitors were serially diluted 1:4 in DMSO and mixed with radiolabeled purine in Ringer solution (final DMSO concentration was 0.5%). 100 µl of uRBCs were mixed with 100 µl of radioisotope/PfENT1-inhibitor solution and incubated at room temperature for 15 min (final Hct was 1.5% ~ 18 million cells). Discs punched from fiberglass filters (Filtermat A) were inserted into 1 ml filter tips (Cat. 1182-1830, USA Scientific, Ocala, FL) and mounted on a vacuum suction manifold. At the end of the incubation, the samples were transferred to the filter tips, vacuum-filtered onto the filtermats and each sample was washed with 4 ml of ice-cold Ringer solution. Filtermats were transferred to scintillation vials and allowed to dry for 1 h. RBCs were solubilized with 200 µl 5% SDS for 30 min and then mixed with 3 ml of scintillation fluid (UltimaGold, PerkinElmer). Samples were counted for 1 min each using a Wallac TriCarb Liquid Scintillation Counter. Experiments were repeated at least three times on different days.

We also used a second method to measure the concentration dependence of compound inhibition of [^{3}H]adenosine uptake into uRBCs, which gave similar IC_{50} values to the method described above. Values from the two methods were pooled in calculating the average values. 96-well plates (Flat Bottom 96 well plate, clear, PS, Non-Sterile, Fisher Scientific, #12565501) were preloaded with 0.75 µl of compound (serially diluted 4-fold in DMSO from a 25mM stock) and 100 µl of 100 nM [^{3}H]adenosine ([2,8-^{3}H]adenosine;23 Ci/mmol Moravek Biochemicals) in PBS solution (137 mM NaCl, 2.7 mM KCl, 10 mM KH_2PO_4,10mM Na_2HPO_4, pH 7.4). RBCs acquired from healthy blood donors were washed five times with PBS solution and resuspended at 4% hematocrit (hct) in PBS. 100 µl of RBCs (4% hct) were added to each well containing compound/radiolabel, resuspended, and incubated at room temperature for 15 min. At the end of the time course, cells were harvested onto glass fiber filter mats (Filtermat A, GF/C; Perkin Elmer) using a TomTec 96-well cell harvester system (#96-3-469). Filtermats were dried and sealed in plastic bags with Betaplate Scint (Perkin Elmer) scintillation fluid. Counts were measured using a microplate scintillation counter (1450 Microbeta Trilux, Perkin Elmer).

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Results

PbENT1 shares 60% amino acid identity with PfENT1 (Supplemental Fig. 1), so we hypothesized that PbENT1 should have a similar substrate transport profile. Because *Plasmodium* genes are often A-T rich, heterologous expression is often difficult. To eliminate effects of codon-usage bias on expression, we used a yeast codon-optimized version of PbENT1 (Supplemental Fig. 2). The expression vector was transformed into yeast lacking the endogenous uridine transporter, *FUI1* and one of the enzymes in the *de novo* purine synthesis pathway, *ADE2*. Growth of these purine auxotrophic yeast can be rescued in media containing adenine, which can enter via the endogenous yeast FCY2 transporter (Weber et al., 1990). However, yeast lack an endogenous adenosine transporter. Thus, growth in media containing adenosine as the sole purine source can only be rescued if there is heterologous expression of an adenosine transporter. As expected, PbENT1-expressing yeast grew in the presence of adenosine (EC_{50} 542 ± 59 µM, mean $\pm$ s.d.), but yeast transformed with empty vector (EV) did not (Fig. 1).

Next, we examined the uptake time course of radiolabeled substrate into PbENT1-expressing yeast. PbENT1-HA expressing yeast take up [^{3}H]adenosine and [^{3}H]uridine in a time-dependent, linear fashion over a one hour period (Fig. 2). Uptake was significantly greater for PbENT1-HA-expressing yeast than for EV-transformed yeast. Subsequent uptake experiments were done at 15 min, within the linear uptake range.

To determine the substrate specificity of PbENT1, we measured the ability of unlabeled purines and pyrimidines to inhibit the uptake of radiolabeled tracer into PbENT1-expressing yeast. To ensure that the radiolabel and test substrate were competing at the transporter, and not at a downstream metabolic enzyme, we used [^{3}H]adenosine to test pyrimidine substrates and [^{3}H]uridine to test purine substrates. Thus, the radiolabel and test substrate would not share metabolic enzymes and competition could only occur at the transporter. We determined the IC_{50} values for four purine nucleosides and their equivalent nucleobases (Fig. 3). All of the tested purines inhibited [^{3}H]uridine uptake. Inosine and guanosine showed the highest affinity with IC_{50} values of 3.7 µM and 21.3 µM, respectively (Table 1). IC_{50} values for nucleobases were 1.6 to 50 fold higher than for the corresponding nucleosides (Table 1).

The uptake of [^{3}H]uridine showed that PbENT1 was able to transport a pyrimidine (Fig. 2). We tested the ability of six pyrimidine nucleosides and their equivalent nucleobases to inhibit [^{3}H]adenosine uptake (Fig. 4). The nucleosides thymidine and uridine showed the highest affinity with IC_{50} values of 91.3 µM and 400 µM, respectively) (Table 1). The nucleobase cytosine and nucleoside cytidine were unable to completely inhibit radiolabel uptake, even at the maximum concentration tested, 12.5 mM (Fig. 4).

Recently we identified and characterized nine small molecule inhibitors of PfENT1 (Table 2) (Frame et al., 2015b). We showed that these compounds inhibit [^{3}H]adenosine uptake into erythrocyte-free *P. falciparum* parasites and kill parasites in culture (Frame et al., 2015b). In the present study, we tested their ability to inhibit [^{3}H]adenosine uptake into PbENT1-HA-expressing yeast (Fig. 5). All nine compounds inhibited PbENT1 with IC_{50} values in the 3 to 60 nM range (Table 2). The ratios of PbENT1 IC_{50} to PfENT1 IC_{50} for the different compounds were all within a factor of 2 (Table 2). Thus, the compounds have similar efficacy against both transporters despite the 40% amino acid sequence differences.

Based on the ability of the PfENT1 inhibitors to block PbENT1, we sought to test the hypothesis that they would inhibit proliferation of *P. berghei* parasites. It should be noted that the compounds are not cytotoxic to yeast at concentrations up to 125 µM, the highest concentration tested (Frame et al., 2015b). We tested the effect of three of the inhibitors, **3**, **4**, and **13**, on *P. berghei* parasite proliferation in 24 hour *ex vivo* culture. The compounds inhibited parasite proliferation with IC_{50} values between 5 to 25 µM (Table 3). Amodiaquine, 4-aminoquinoline compound similar to chloroquine, was included as a positive control (Table 3). Similar IC_{50} values were obtained for inhibition of *P. falciparum* parasite proliferation in culture (Table 3).

We also tested the specificity of the PfENT1 inhibitors relative to the human erythrocyte hENT1 purine transporter. We assessed their effect using uninfected human RBCs where hENT1 function was assayed by [^{3}H]adenosine uptake (Fig. 6A). Compound **7** displayed the lowest selectivity - it had 27 times higher affinity for PfENT1 than for hENT1 (Fig. 6B). Compound **1** had the highest selectivity, it inhibited hENT1 at a concentration 1200 times higher than the concentration at which it inhibited PfENT1 (Fig. 6B). These results confirmed that the compounds displayed significant specificity for PfENT1 over the human erythrocyte hENT1 transporter.

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Discussion

The long term goal of this project is to develop antimalarial drugs against a novel target, the primary purine import transporter. As a step towards that goal, in the current work we sought to determine the feasibility of using the mouse malaria model. To establish the feasibility of using the mouse model, we characterized the functional properties of PbENT1 and determined whether the best hits from our HTS for PfENT1 inhibitors would also work on the *P. berghei* homologue, PbENT1. PbENT1, like its homologues in *P. falciparum* and *P. vivax* (Riegelhaupt et al., 2010; Deniskin et al., 2016), transports both purines and pyrimidines (Fig. **2**, Table 1). PbENT1 has higher affinity for purines compared to pyrimidines and higher affinity for nucleosides compared to nucleobases (Table 1). Inosine and guanosine showed the highest affinities (Table 1), much like the *Leishmania* LdNT2 nucleoside transporter (Carter et al., 2000b). For *P. falciparum*, adenosine and hypoxanthine are the preferred substrates for purine import, as these purines are present at the highest concentration in human plasma and are also present in human erythrocytes (Moser et al., 1989; Traut, 1994). The inhibition constants (K_i) for PfENT1 transport of adenosine and hypoxanthine are around 650 µM and 300 µM, respectively (Riegelhaupt et al., 2010). In contrast, for PbENT1 the IC_{50} values were 4-to-6 fold lower for these substrates (Table 1). The different affinities for various purines are presumably due to amino acid differences between PfENT1 and PbENT1, as they are only 60% sequence identical. Whether these differences in substrate affinity are physiologically significant is unknown at present. Of note, differences exist in the purine import pathways of *Plasmodium* species that infect primates and rodents. Genome sequencing reveals that *P. vivax* and *P. falciparum* encode 4 ENT homologues (ENT 1-4), while the species that infect rodents, *P. berghei and P. yoelii*, lack an ENT3 orthologue (Frame et al., 2012; Frame et al., 2015a). The substrate specificity and functional role of PfENT3 remains to be determined. However, its presence in *Plasmodium* species that infect humans and not in those that infect rodents suggests that there may be differences in purine transport and metabolism between rodents and humans that may be important for proliferation of the respective *Plasmodium* species.

Efforts to combat malaria have been hampered by the development of resistance to antimalarial drugs (Sa et al., 2011). Thus, it is important to have a robust pipeline of new therapeutics that target novel aspects of *Plasmodium* parasite biology to replace current drugs as they become less effective (Burrows et al., 2014). One potential target is the purine import and salvage pathway, which is essential for parasite survival (Ducati et al., 2013; Frame et al., 2015a). Using a yeast-based HTS, we previously identified compounds that inhibit PfENT1 at concentrations in the nanomolar range (Frame et al., 2015b). We characterized nine of the hits in a series of secondary assays and showed that they inhibit *P. falciparum* parasite proliferation in culture. In this paper, we show that despite the 40% amino acid sequence differences between PbENT1 and PfENT1, these nine inhibitors also block PbENT1-mediated purine transport with IC_{50} values comparable to those for PfENT1 (Table 2).

Ideally, an antimalarial drug would not inhibit hENT1. However, dipyridamole, an FDA approved drug, that inhibits hENT1 has been safely used in patients (Griffiths et al., 1997). Thus, avoiding interactions with hENT1 may not be essential for a viable antimalarial drug targeting PfENT1. None the less, because hENT1 is only 17% amino acid sequence identical with PfENT1 and dipyridamole does not block PfENT1 (Riegelhaupt et al., 2010), it may be feasible to identify inhibitors that display specificity for PfENT1 over hENT1. In fact, the PfENT1 inhibitors that we have identified have lower potency against the human RBC purine transporters, hENT1 (Fig. 6). They are 27 to 1,200-fold more potent against the *Plasmodium* ENT1 homologues relative to the human hENT1 (Fig. 6B). Thus, it may be possible to maintain selectivity for the *Plasmodium* ENTs during the hit-to-lead medicinal chemistry process that will be necessary to develop our current compounds into antimalarial drugs.

Based on the assumption that PbENT1-mediated purine import is essential for *P. berghei* parasite proliferation, we expected that the PfENT1 inhibitors would kill *P. berghei* parasites. Because these compounds are HTS hits that need to be optimized through medicinal chemistry, we did not think it worthwhile to determine the mouse pharmacokinetics for these compounds at this time. Thus, we did not test the efficacy of the inhibitors in *P. berghei* infected mice. To assess their efficacy on parasite proliferation, we tested the effect of three of the inhibitors on *P. berghei* parasite proliferation in *ex vivo* culture (Table 3). The inhibitors blocked parasite proliferation with IC_{50} values similar to their efficacy against *P. falciparum* parasites in culture (Frame et al., 2015b). This suggests that with improved potency through medicinal chemistry efforts, *Plasmodium* ENT1 inhibitors will display efficacy in the *in vivo* mouse malaria model.

One strategy to reduce the development of resistance to antimalarial drugs has been to pair drugs with different targets. This was the rationale behind pairing artemisinin derivatives with other drugs in ACTs. Unfortunately, because the partner drugs for the artemisinins already had been widely used, resistance to the partner drugs was already present in the *P. falciparum* parasite population. This has contributed to the development of resistance to the artemisinin component of the ACTs (Uhlemann and Fidock, 2012; Ariey et al., 2014; Ashley et al., 2014; Burrows et al., 2014; Straimer et al., 2015). With our PfENT1 inhibitors, we were surprised that PfENT1-knockout parasites grown in high purine concentrations were also killed by the PfENT1 inhibitors with 2 to 4 fold higher IC_{50} values (Frame et al., 2015b). Killing of PfENT1-knockout parasites showed a delayed-death phenotype not observed in the killing of the WT parasites (Frame et al., 2015b). This indicates that the secondary target causing death in the PfENT1-knockout parasites is distinct from the primary target in WT parasites. The existence of two targets with similar affinities may reduce the likelihood of parasites developing resistance to these compounds. Whether the affinity for both targets can be improved simultaneously during medicinal chemistry optimization is uncertain. An alternative strategy might be to pair a PfENT1 inhibitor with another drug that acts elsewhere in the purine salvage pathway. Schramm and coworkers have shown that transition state analogue inhibitors of the purine salvage pathway enzyme, purine nucleoside phosphorylase (PNP), kill malaria parasites (Cassera et al., 2011; Ducati et al., 2013). Targeting two points in the purine metabolic pathway might lead to synergistic effects. Future experiments will be necessary to test this hypothesis. In summary, we showed that the PfENT1 inhibitors were active against PbENT1 and that they were able to inhibit the proliferation of *P. berghei* parasites in *ex vivo* culture. This indicates that we will be able to utilize the mouse malaria model to test the utility of inhibition of purine uptake as a strategy for development of novel antimalarial drugs.

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Authorship Contributions

Participated in research design: Arora, Fidock and Akabas.

Conducted experiments: Arora, Deniskin, Sosa, Nishtala, Henrich, Kumar.

Contributed new reagents or analytic tools: Arora.

Performed data analysis: Arora, Deniskin, Sosa, Nishtala, Henrich, Kumar, Fidock and Akabas.

Wrote or contributed to the writing of the manuscript: Arora, Deniskin, Sosa, Nishtala, Henrich, Kumar, Fidock and Akabas.

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Footnotes

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Figure Legends

Fig. 1. PbENT1-HA-CO expression in purine auxotrophic yeast. Concentration dependent growth of yeast containing PbENT1-HA construct or empty vector in the presence of adenosine, after 30 hours. Adenosine growth EC_{50} of PbENT1-expressing yeast is 542 ± 59 µM. Data shown is the average of three independent experiments. OD_{600}, optical density at 600 nm.

Fig. 2. Time course of uptake of [^{3}H]adenosine and [^{3}H]uridine into PbENT1-HA expressing purine auxotrophic yeast. Uptake of (A) 50 nM of [^{3}H]adenosine and (B) 250 nM [^{3}H]uridine into PbENT1-HA expressing yeast. Open boxes represent yeast transformed with empty vector. Filled boxes represent yeast expressing PbENT1-HA. Uptake is measured in counts per minute (CPM) per million cells. Note differing y-axis scales in the two panels. Data shown is the average of three independent experiments.

Fig. 3. Inhibition of [^{3}H]uridine uptake into PbENT1-HA expressing yeast by various purine nucleosides and nucleobases. Uptake inhibition of 250 nM [^{3}H]uridine into PbENT1-HA expressing yeast by (A) adenosine, adenine; (B) inosine, hypoxanthine; (C) guanosine, guanine; (D) xanthosine, xanthine. Uptake is normalized to remove background and is shown as % of maximum uptake. All tested substrates fully inhibited [^{3}H]uridine uptake, with the exception of the nucleobases xanthine and guanine, and the nucleoside xanthosine. IC_{50} values are shown in Table 1. Data from a representative experiment is shown.

Fig. 4. Inhibition of [^{3}H]adenosine uptake into PbENT1-HA expressing yeast by various pyrimidine nucleobases and nucleosides. Uptake inhibition of 50 nM [^{3}H]adenosine into PbENT1-HA-CO expressing yeast by pyrimidine (A) nucleobases and (B) nucleosides. Uptake is normalized to remove background and is shown as % of maximum uptake. Of the tested

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substrates, only uridine and thymidine showed complete inhibition. IC_{50} values are shown in Table 1. Data from a representative experiment is shown.

Fig. 5. Concentration dependent inhibition of PbENT1 function by PfENT1 inhibitors. Concentration dependent inhibition of 50 nM [^{3}H]adenosine uptake in the presence of nine PfENT1 inhibitor compounds. Uptake is normalized to remove background and is shown as % of maximum uptake. Single experiment inhibition curves are shown, representative of n=3 independent trials. IC_{50} values are shown in Table 2.

Fig. 6. Specificity of the PfENT1 inhibitors for the malaria transporter compared to the human RBC hENT1 transporter. (A) Inhibition of 50 nM [^{3}H]adenosine uptake into RBC-free parasites (solid symbols) and uninfected human RBCs (open symbols) by compounds **5** and **7**. Representative experiments are shown. (B) Ratio of IC_{50} values for inhibition of radiolabeled purine uptake via hENT1 compared to the IC_{50} values for inhibition of uptake into RBC-free 3D7 strain *P. falciparum* parasites. IC_{50} values used to calculate the ratios were the average of at least three separate experiments. Activity of hENT1 measured using [^{3}H]adenosine uptake into uninfected RBC. IC_{50} values for inhibition of [^{3}H]adenosine uptake into RBC-free *P. falciparum* parasites are from Frame *et al*. (2015b). Note that the y-axis is a log scale.

Table 1. IC_{50} values for inhibition of radiolabel substrate uptake by purine and pyrimidine substrates.

IC_{50} values are mean ± SD in µM for n≥3 experiments. Pyrimidines were competed with 50 nM [^{3}H]adenosine; purines were competed with 250 nM [^{3}H]uridine.

Uptake Inhibition IC_{50} values (Mean ± SD, µM)			
Pyrimidine	IC_{50} vs [^{3}H]Adenosine	Purine	IC_{50} vs [^{3}H]Uridine
Uracil	2833 ± 243	Adenine	306 ± 19
Uridine	400 ± 152	Adenosine	190 ± 2.5
Thymine	1142 ± 185	Hypoxanthine	53 ± 1.65
Thymidine	91.3 ± 38	Inosine	3.7 ± 0.1
Cytosine	9697 ± 455	Guanine	1142 ± 44
Cytidine	26726 ± 4116	Guanosine	21.3 ± 0.6
		Xanthine	979 ± 13
		Xanthosine	626 ± 17

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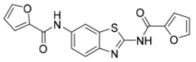
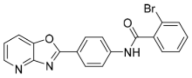
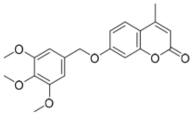
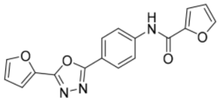
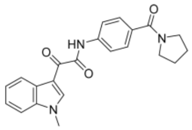
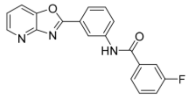
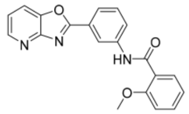
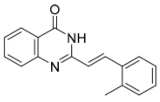
Table 2. IC_{50} values for inhibition of uptake of 50 nM [^{3}H]adenosine into PbENT1-expressing and PfENT1-expressing yeast by the PfENT1 inhibitors identified by Frame et al. (2015b). IC_{50} values are mean ± SD in nM from n=3 experiments. Compounds are numbered as in Frame et al. (2015b), with rank order number from the high throughput screen. Chembridge catalog numbers of the compounds are in the Materials and Methods section. The compound names are in Frame et al., (2015b).

Compound	Structure	PbENT1 IC_{50} nM	PfENT1 IC_{50} nM	Pb:Pf
1		3.4 ± 0.7	4.8 ± 0.5	0.7
2		16 ± 7.2	33.5 ± 6.5	0.47
3		5.4 ± 2.3	7 ± 0.4	0.77
4		21.6 ± 2.3	23.1 ± 3.5	0.93
5		56 ± 14	54.5 ± 7.2	1.02
6		27 ± 7	14 ± 2.7	1.93
7		12.3 ± 3.2	15 ± 2.3	0.82
13		11.6 ± 3.1	10.3 ± 2.4	1.12

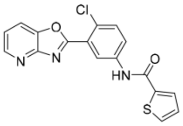
19		17 ± 3.6	42.5 ± 2.5	0.4

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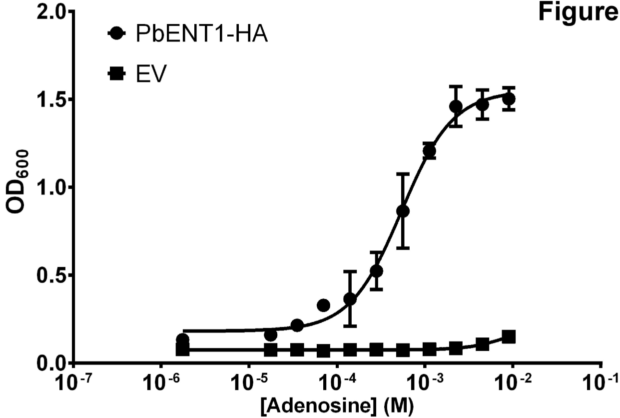
Table 3. IC_{50} values for inhibition of *P. berghei* parasite proliferation in 24 h *ex vivo* culture.

Compound[a]	IC_{50} *P. berghei* parasites *ex vivo* culture	IC_{50} *P. falciparum* 3D7 parasites[b]
3	6.5 ± 0.6 μM	19.2 ± 4.3 μM
4	23.4 ± 1.0 μM	15.0 ± 1.5 μM
13	5.6 ± 0.4 μM	6.9 ± 0.4 μM
ADQ	3.9 ± 0.4 nM	

[a] Compound numbers refer to PfENT1 inhibitors as per Table 2 and from Frame et al. (2015b). Data show mean ± SEM from two independent experiments. ADQ, amodiaquine

[b] From Frame et al. (2015b).

Figure 1



● PbENT1-HA
■ EV
2.0
1.5
1.0
0.5
0.0
OD_{600}
10^{-7}
10^{-6}
10^{-5}
10^{-4}
10^{-3}
10^{-2}
10^{-1}
[Adenosine] (M)

Figure 2

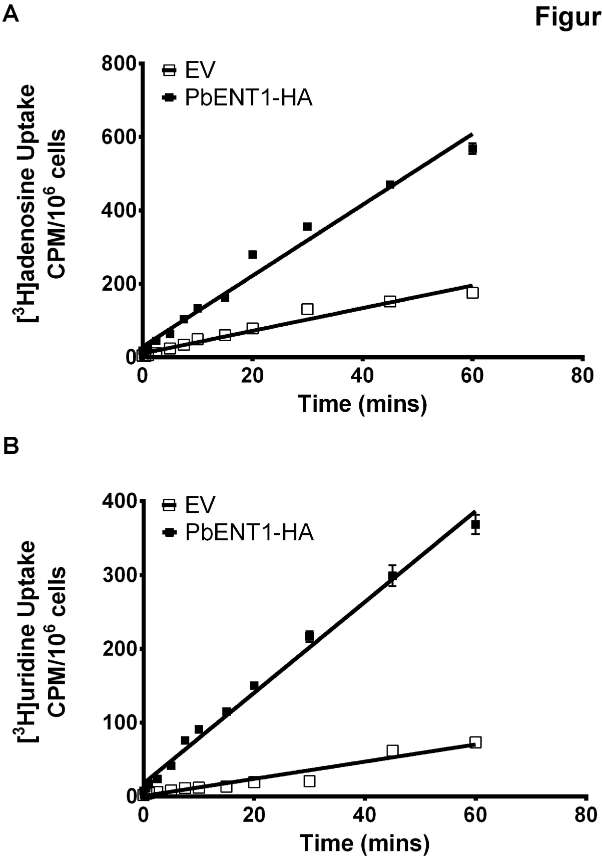

A
800
600
400
200
0
[^{3}H]adenosine Uptake CPM/10^6 cells
EV
PbENT1-HA
0
20
40
60
80
Time (mins)
B
400
300
200
100
0
[^{3}H]uridine Uptake CPM/10^6 cells
EV
PbENT1-HA
0
20
40
60
80
Time (mins)

Figure 3

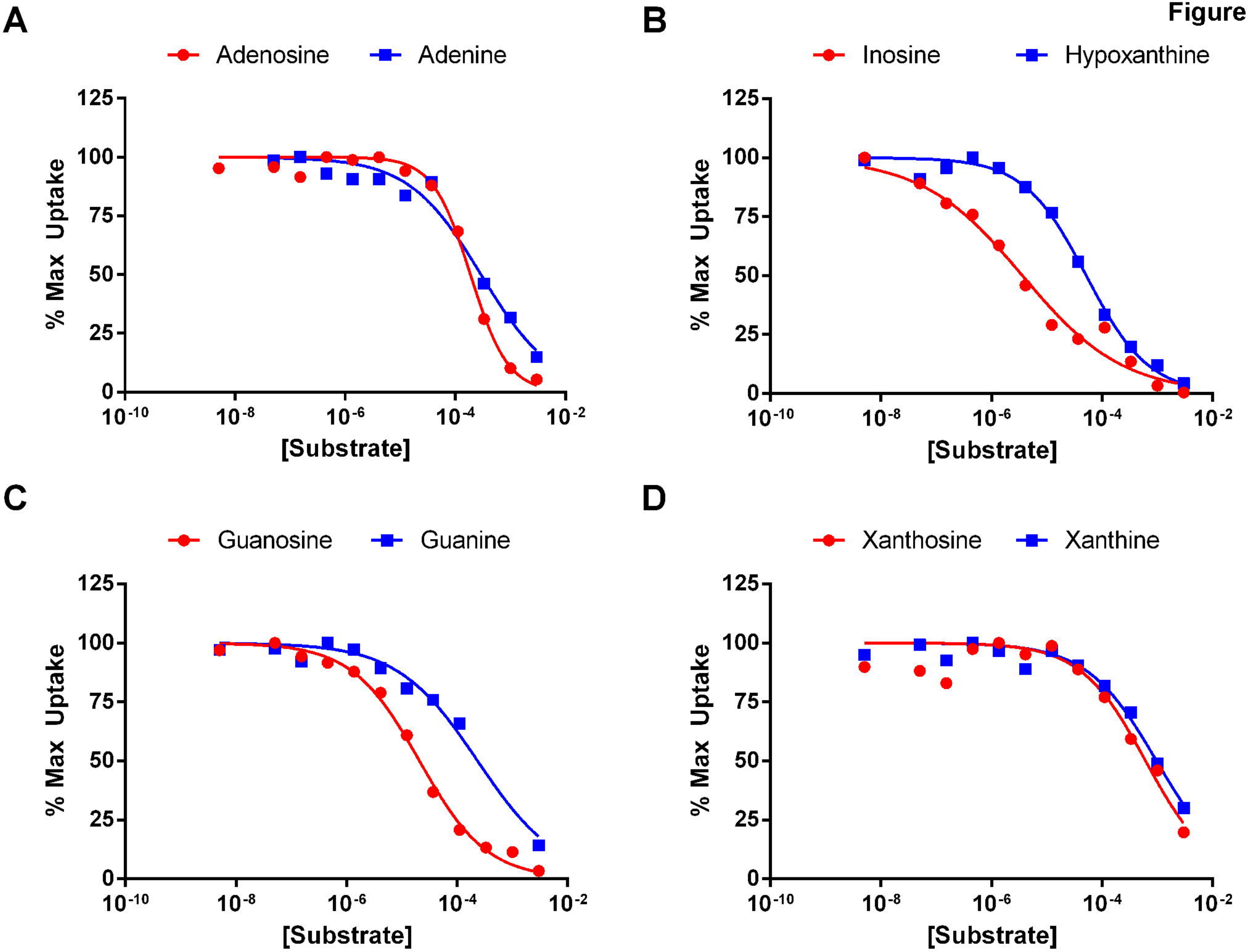

A
Adenosine
Adenine
125
100
75
50
25
0
% Max Uptake
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[Substrate]
B
Inosine
Hypoxanthine
125
100
75
50
25
0
% Max Uptake
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[Substrate]
C
Guanosine
Guanine
125
100
75
50
25
0
% Max Uptake
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[Substrate]
D
Xanthosine
Xanthine
125
100
75
50
25
0
% Max Uptake
10^{-10}
10^{-8}
10^{-6}
10^{-4}
10^{-2}
[Substrate]

Figure 4

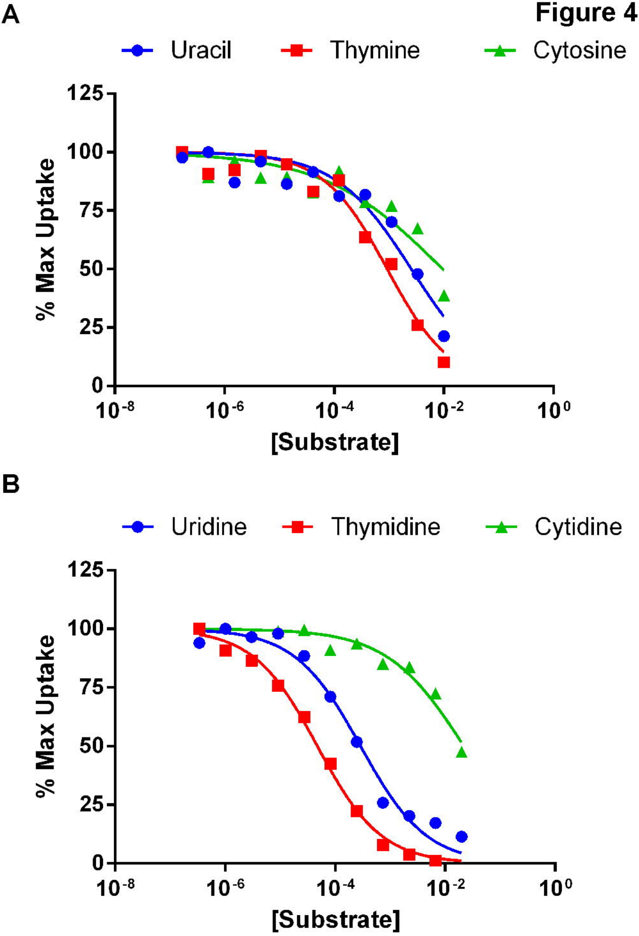

A
Uracil
Thymine
Cytosine
125
100
75
50
25
0
% Max Uptake
10^{-8}
10^{-6}
10^{-4}
10^{-2}
10^{0}
[Substrate]
B
Uridine
Thymidine
Cytidine
125
100
75
50
25
0
% Max Uptake
10^{-8}
10^{-6}
10^{-4}
10^{-2}
10^{0}
[Substrate]

Figure 5

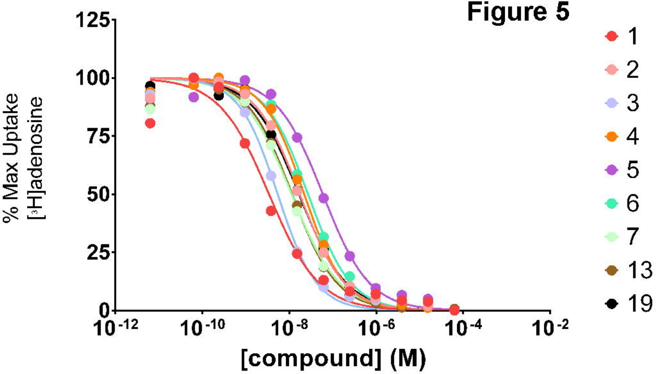
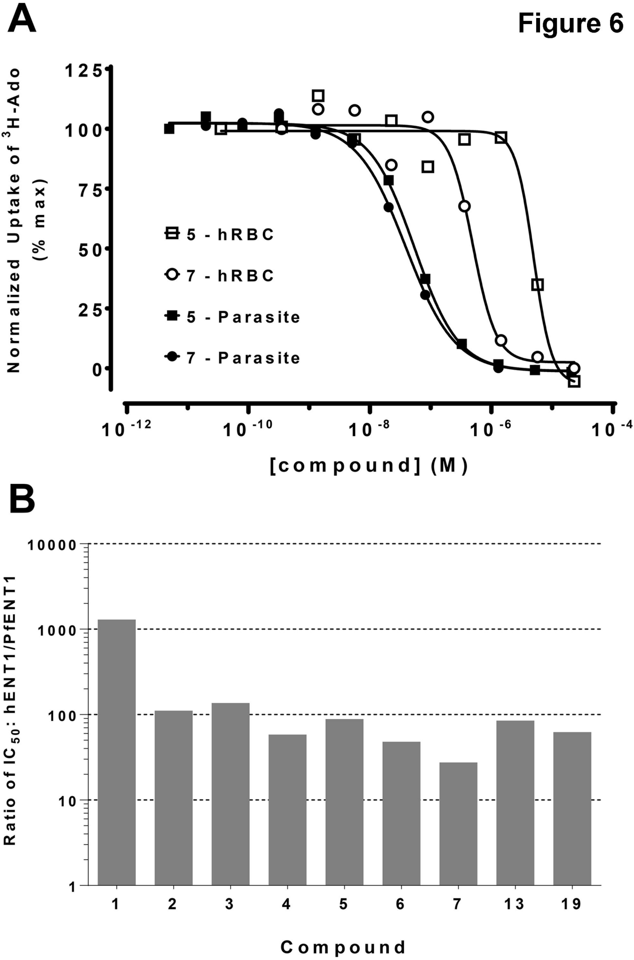

% Max Uptake
[3H]adenosine
125
100
75
50
25
0
10-12
10-10
10-8
10-6
10-4
10-2
[compound] (M)
● 1
● 2
● 3
● 4
● 5
● 6
● 7
● 13
● 19

Figure 6


A
Normalized Uptake of ^{3}H-Ado (% max)
125
100
75
50
25
0
5 - hRBC
7 - hRBC
5 - Parasite
7 - Parasite
10^{-12}
10^{-10}
10^{-8}
10^{-6}
10^{-4}
[compound] (M)
B
Ratio of IC_{50}: hENT1/PfENT1
10000
1000
100
10
1
1
2
3
4
5
6
7
13
19
Compound

Molecular Pharmacology

Supplemental Information for:

MOL# 101386

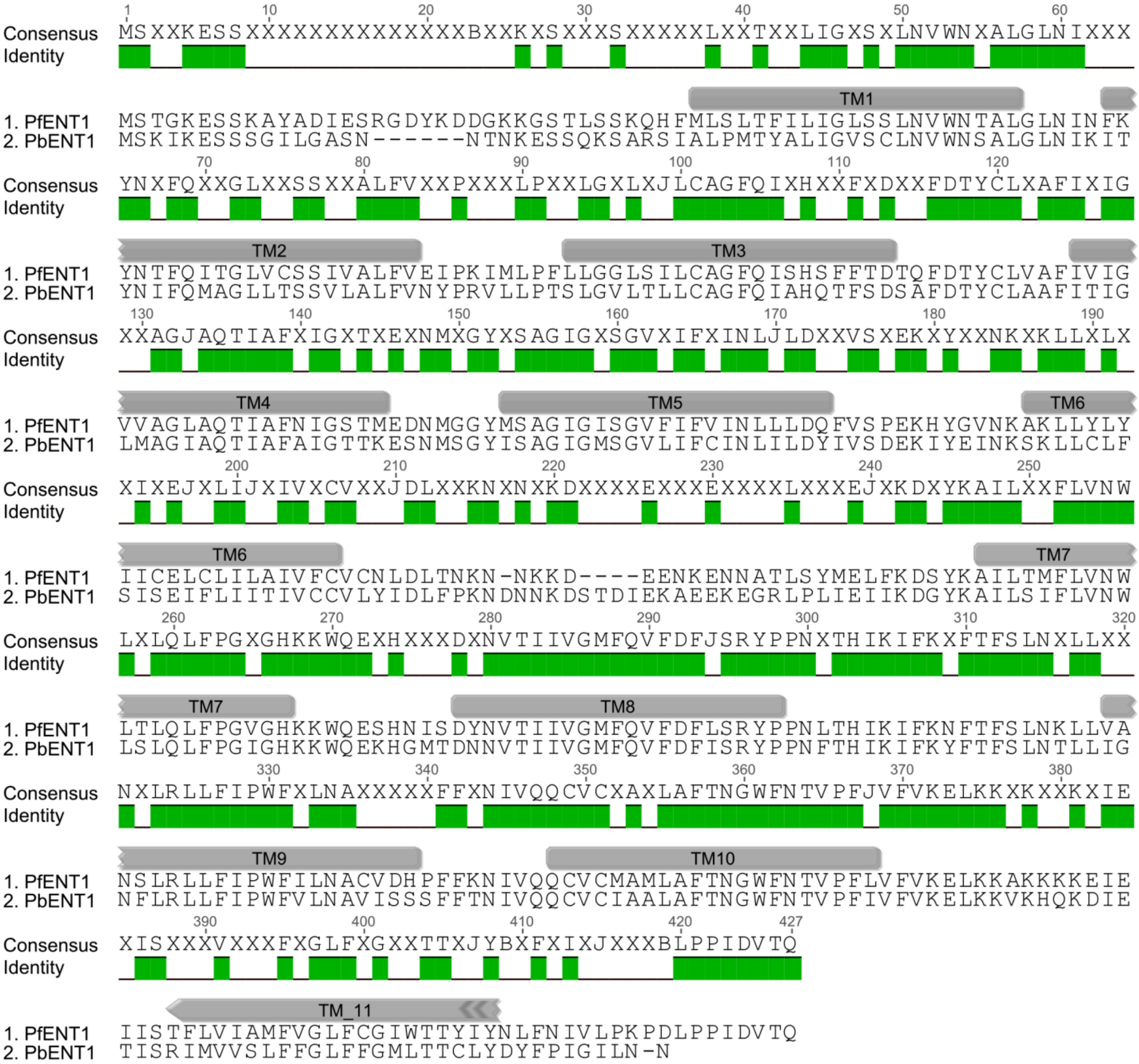
Substrate and inhibitor specificity of the *Plasmodium berghei* Equilibrative Nucleoside Transporter Type 1 (PbENT1)

Avish Arora, Roman Deniskin, Yvett Sosa, Sita Nirupama Nishtala, Philipp P. Henrich, T. R. Santha Kumar, David A. Fidock and Myles H. Akabas

Department of Physiology& Biophysics (AA, RD, YS, NN, MHA), Departments of Neuroscience and Medicine (MHA), Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461

Department of Medicine (DAF) and Department of Microbiology & Immunology (PPH,TRSK, DAF), Columbia University Medical Center, 630 West 168th Street, New York, NY, 10032

Address correspondence to: Myles H. Akabas, Department of Physiology & Biophysics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY, 10461, USA, Tel.: (718) 430-3360; Fax: (718) 430-8819; E-mail: myles.akabas@einstein.yu.edu



Supplemental Fig. 1. Alignment of *P. falciparum* and *P. berghei* ENT1 protein sequences. *P. berghei* ENT1 (PBANKA_1360100) shares 60% sequence homology with *P. falciparum* ENT1 (PF3D7_1347200). Green bars indicate regions of amino acid identity. Transmembrane domains of PfENT1 are indicated, as determined by Carter et al. (2000). Amino acid sequences obtained from PlasmoDB http://www.plasmodb.org/plasmo/

```
1       10        20        30        40        50        60
ATGAGTAAGATTAAGGAATCATCTTCTGGAATTCTAGGCGCAAGTAATAACACAAACAAA
        70        80        90       100       110       120
GAGTCCAGCCAGAAATCTGCCAGATCAATTGCTTTACCAATGACCTACGCTTTAATCGGT
       130       140       150       160       170       180
GTTAGCTGCCTGAATGTATGGAACTCAGCACTTGGTTTGAATATCAAGATCACTTACAAC
       190       200       210       220       230       240
ATCTTTCAAATGGCTGGTCTGTTAACATCTTCTGTTCTAGCACTATTTGTTAATTACCCT
       250       260       270       280       290       300
CGTGTGTTGTTGCCTACATCCCTTGGTGTCCTTACCTTGTTATGTGCTGGATTTCAAATT
       310       320       330       340       350       360
GCGCATCAGACATTCTCCGATTCTGCATTCGATACTTATTGTTTGGCTGCCTTTATAACA
       370       380       390       400       410       420
ATCGGTCTGATGGCTGGAATTGCCCAAACAATTGCCTTTGCCATTGGTACTACTAAAGAG
       430       440       450       460       470       480
TCTAATATGTCTGGGTATATTTCCGCTGGTATCGGCATGTCTGGAGTTCTAATCTTCTGC
       490       500       510       520       530       540
ATAAACCTCATACTAGACTACATTGTTTCTGATGAGAAGATCTACGAAATCAATAAGTCT
       550       560       570       580       590       600
AAACTATTGTGCTTGTTCTCAATCAGCGAAATCTTTCTAATCATTACAATCGTCTGTTGC
       610       620       630       640       650       660
GTCTTATACATTGATCTCTTTCCAAAGAATGACAACAACAAAGACTCAACAGATATAGAG
       670       680       690       700       710       720
AAAGCTGAAGAGAAGGAAGGAAGATTGCCATTGATTGAAATTATCAAAGATGGTTACAAA
       730       740       750       760       770       780
GCAATACTTTCTATCTTTCTCGTAAACTGGTTGTCATTACAACTCTTTCCTGGAATAGGC
       790       800       810       820       830       840
CATAAGAAATGGCAAGAGAAACACGGTATGACAGACAATAATGTTACTATTATAGTAGGC
       850       860       870       880       890       900
ATGTTTCAAGTATTCGACTTTATCTCAAGATACCCACCAAATTTCACCCACATCAAGATC
       910       920       930       940       950       960
TTTAAGTACTTTACTTTCAGTTTGAATACCTTACTGATCGGTAACTTTCTAAGGCTGTTA
       970       980       990     1,000     1,010     1,020
TTCATCCCATGGTTTGTACTGAATGCAGTGATTAGTTCCTCATTCTTCACGAATATAGTG
     1,030     1,040     1,050     1,060     1,070     1,080
CAACAATGTGTCTGTATTGCTGCACTCGCTTTCACTAATGGGTGGTTTAATACTGTACCA
     1,090     1,100     1,110     1,120     1,130     1,140
TTCATTGTCTTTGTTAAGGAACTCAAGAAAGTGAAACATCAGAAAGATATTGAAACTATA
     1,150     1,160     1,170     1,180     1,190     1,200
TCTAGAATTATGGTCGTTTCATTGTTCTTTGGCTTATTCTTTGGCATGTTGACAACATGT
     1,210     1,220     1,230     1,240     1,250     1,260
TTATATGATTACTTTCCAATTGGGATCTTGAATAACTACCTTATGATGTTCCTGATTATT
    1,269
GCGTAATGA
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Supplemental Fig. 2. Nucleotide sequence for *pbent1-HA-CO*, codon-optimized for *S. cerevisiae***.** Nucleotide sequence designed by DNA2.0 (Newark, CA) software https://www.dna20.com/