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Targeting the *Plasmodium vivax* equilibrative nucleoside transporter 1 (PvENT1) for antimalarial drug development

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ABSTRACT

Infection with *Plasmodium falciparum* and *vivax* cause most cases of malaria. Emerging resistance to current antimalarial medications makes new drug development imperative. Ideally a new antimalarial drug should treat both *falciparum* and *vivax* malaria. Because malaria parasites are purine auxotrophic, they rely on purines imported from the host erythrocyte via Equilibrative Nucleoside Transporters (ENTs). Thus, the purine import transporters represent a potential target for antimalarial drug development. For *falciparum* parasites the primary purine transporter is the *P. falciparum* Equilibrative Nucleoside Transporter Type 1 (PfENT1). Recently we identified potent PfENT1 inhibitors with nanomolar IC_{50} values using a robust, yeast-based high throughput screening assay. In the current work we characterized the *Plasmodium vivax* ENT1 (PvENT1) homologue and its sensitivity to the PfENT1 inhibitors. We expressed a yeast codon-optimized *PvENT1* gene in *Saccharomyces cerevisiae*. PvENT1-expressing yeast imported both purines ([^{3}H]adenosine) and pyrimidines ([^{3}H]uridine), whereas wild type (*fui1*Δ) yeast did not. Based on radiolabel substrate uptake inhibition experiments, inosine had the lowest IC_{50} (3.8 μM), compared to guanosine (14.9 μM) and adenosine (142 μM). For pyrimidines, thymidine had an IC_{50} of 183 μM (vs. cytidine and uridine; mM range). IC_{50} values were higher for nucleobases compared to the corresponding nucleosides; hypoxanthine had a 25-fold higher IC_{50} than inosine. The archetypal human ENT1 inhibitor 4-nitrobenzylthioinosine (NBMPR) had no effect on PvENT1, whereas dipyridamole inhibited PvENT1, albeit with a 40 μM IC_{50}, a 1000-fold less sensitive than human ENT1 (hENT1). The PfENT1 inhibitors blocked transport activity of PvENT1 and the five known naturally occurring non-synonymous single nucleotide polymorphisms (SNPs) with similar IC_{50} values. Thus, the PfENT1 inhibitors also target PvENT1. This implies that development of novel antimalarial drugs that target both *falciparum* and *vivax* ENT1 may be feasible.

1. Introduction

Malaria is a major global health problem and a socioeconomic burden in malaria endemic countries (Sachs and Malaney, 2002). According to the World Health Organization (WHO), in 2014 approximately 3.4 billion people were at risk for malaria infection (World Health Organization, 2014). Over 200 million clinical cases of malaria resulted in ~600,000 deaths. Most deaths occurred in sub-Saharan Africa in young children and pregnant women (Snow et al., 2005; World Health Organization, 2014). Malaria is caused by infection with single-cell protozoan parasites from the genus *Plasmodium*. Five *Plasmodium* species infect humans (*Plasmodium falciparum, vivax, malariae, ovale,* and *knowlesi*). Ninety percent of clinical cases are due to infection with either *P. falciparum* or *Plasmodium vivax* (World Health Organization, 2014). *P. falciparum* is associated with the highest mortality (~80% of all malaria-related deaths) but *P. vivax* infection is prevalent and associated with high morbidity (Rogerson and Carter, 2008; Anstey et al., 2009).

Abbreviations: ACT, Artemisinin-based Combination Therapies; CQ, chloroquine; EC_{50}, concentration causing 50% of maximal effect; ENT, equilibrative nucleoside transporter; EV, empty vector; hENT1, human ENT type 1; HTS, high throughput screen; IC_{50}, concentration causing 50% inhibition; NBMPR, 4-nitrobenzylthioinosine; PfENT1, *P. falciparum* ENT type 1; PvENT1, *P. vivax* ENT type 1; SDM, synthetic defined media; SNP, single nucleotide polymorphism; WHO, World Health Organization; WT, wild type.

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The geographic overlap between *P. falciparum* and *P. vivax* endemic areas is significant, especially in tropical regions. Thus, new antimalarial drugs should target both species.

The development of resistance to antimalarial drugs has been a recurring problem. Chloroquine (CQ) was the mainstay of antimalarial chemotherapy until CQ resistance developed worldwide (Wellems and Plowe, 2001). In 2006, the WHO recommended Artemisinin-based Combination Therapies (ACT) as first-line treatment for *P. falciparum* infection. Unfortunately, resistance to current ACT regimens is expanding in Southeast Asia (Dondorp et al., 2011; Ariey et al., 2014; Hastings et al., 2015; Straimer et al., 2015). The fact that resistance to a three day ACT treatment course emerged in as little as a decade after the large scale introduction of ACTs as first line therapy underscores the importance of identifying new drug targets that take advantage of weaknesses in *Plasmodium* biology.

One potential target for the development of novel antimalarial drugs is the purine salvage pathway (Downie et al., 2008; Cassera et al., 2011; Frame et al., 2015a). Similar to other protozoa, *Plasmodium* species can perform *de novo* pyrimidine synthesis but are incapable of *de novo* purine synthesis (Manandhar and Van Dyke, 1975; Gero and O'Sullivan, 1990; Downie et al., 2008; Frame et al., 2015a). Therefore, *Plasmodium* parasites must import purines from the host cytoplasm. Imported purines are processed via the purine salvage pathway enzymes to form the purines required for RNA synthesis, DNA replication, and metabolism. Hence, the purine import and processing pathways are potential targets for antimalarial drug development (Downie et al., 2008; Ducati et al., 2013; Frame et al., 2015a).

Plasmodium parasites use equilibrative nucleoside transporters (ENT) to import purines (Landfear et al., 2004; Downie et al., 2008). Genomic sequence analysis of *P. falciparum* (3D7) and *P. vivax* (Sal I) (www.PlasmoDB.org) shows that both species possess four putative ENT homologues: PfENT1-4 and PvENT1-4 (Martin et al., 2005; Kirk and Lehane, 2014). *P. falciparum* ENTs have been studied more extensively. Multiple genetic, biochemical, and functional experiments show that PfENT1 is the principle route for purine uptake into the *P. falciparum* parasites. PfENT1 is localized to the parasite plasma membrane and transports both purine and pyrimidine substrates (Carter et al., 2000a; Parker et al., 2000; Rager et al., 2001; Riegelhaupt et al., 2010a). Genetic knockout of the PfENT1 gene (*pfent1*Δ) is lethal if the parasites are grown in concentrations of purines present in human blood, <10 μM (Traut, 1994; El Bissati et al., 2006; El Bissati et al., 2008; Frame et al., 2015b). However, PfENT1-knockout parasites survive when grown in culture with supra-physiologic purine concentrations (>100 μM) (El Bissati et al., 2006; El Bissati et al., 2008; Frame et al., 2015b). Thus, a secondary low affinity and/or low capacity purine transport pathway must be present, at least in the *pfent1*Δ parasites. The molecular basis for this secondary purine uptake pathway is unknown but may involve the AMP uptake pathway (Cassera et al., 2008) or possibly, PfENT4 (Frame et al., 2012).

To test whether chemical inhibition of PfENT1 would be lethal to *P. falciparum* parasites, we identified PfENT1 inhibitors using a yeast-based, high-throughput screen (HTS) (Frame et al., 2015b). We screened 64,500 compounds and identified 171 hits. Nine of the highest activity compounds that represent six distinct chemical scaffolds were characterized in depth. They blocked [^{3}H]adenosine uptake into PfENT1-expressing yeast and into erythrocyte-free trophozoite stage parasites with 5–50 nM IC_{50} values and killed chloroquine-sensitive and -resistant *P. falciparum* parasites with 5–50 μM IC_{50} values (Frame et al., 2015b). These results provide strong support for the hypothesis that inhibition of purine uptake is a potential target for the development of novel antimalarial drugs.

Because of the extensive geographic overlap between *vivax* and *falciparum* malaria, an effective antimalarial drug should treat infection by both parasites. In the current work, we sought to characterize *P. vivax* ENT1 (PvENT1) functionally and determine whether the PfENT1 inhibitors also inhibit PvENT1. Based on its genomic sequence, PvENT1 is a 47.3 kDa, 416 amino acid protein. PvENT1 shares ~75% amino acid sequence identity with PfENT1. However, unlike its *P. falciparum* homologue, the *pvent1* gene is only moderately AT rich (57%; vs. 72% *pfent1*). Although no crystal structures are available for any known ENTs, glycosylation scanning and modeling algorithms support an 11-transmembrane segment topology with a cytoplasmic N-terminus and an extracellular C-terminus (Sundaram et al., 2001; Valdes et al., 2009). Hydropathy plots suggest that PvENT1 has a similar transmembrane topology to PfENT1. In the current work, we expressed PvENT1 in *Saccharomyces cerevisiae*. We showed that a similar group of purine and pyrimidine nucleobases and nucleosides competed with radioactive uridine or adenosine uptake into PvENT1-expressing yeast, but the measured IC_{50}s were distinct from those previously reported for PfENT1-expressing yeast (Frame et al., 2015b). We determined the sensitivity of PvENT1 to the recently identified PfENT1 inhibitors. All nine PfENT1 inhibitors block PvENT1 with similar efficacy. Furthermore, the five known PvENT1 non-synonymous single nucleotide polymorphisms (SNP) have minimal impact on inhibitor potency.

2. Materials and methods

2.1. Purine auxotrophic yeast (*ade2Δ*)

All genetically modified yeast were created using the *S. cerevisiae* BY4741strain that also had a deletion of the *FUI1* gene (MATa; *his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; fui1Δ::KanMX4*) (Winzeler et al., 1999). To disrupt *de novo* purine synthesis in *S. cerevisiae*, we replaced the yeast *ADE2* gene (Chr. 15; phosphoribosyl-aminoimidazole carboxylase) with the *hphNT1* (hygromycin B phosphotransferase) selectable marker using homologous recombination. Briefly, using two-step PCR, we created 45 nt 5′ and 3′ *ade2* homology arms flanking the *hphNT1* gene (which was amplified from the pFA6a-hphNT1 plasmid; gift from Dr. Ian Willis). The following sets of primers were used: 1st-stepF: 5′⁻GACAAAACAATCAAGTATGCGTACGCTGCAGGTCGACGGATCCCC-G-3′, 1st stepR: 5′-GTATATCAATAAACTTATA-TATTAATCGATGAATTCGAGCTCG-3′; 2nd-stepF: 5′-AACAATCAAGAAAAACAAGAAAATCGGACAAAACAATCAAGTATG-3′, 2nd stepR: 5′-TTATAATTATTTGCTGTACAAGTATATCAATAAACTTA-TATATTA-3′. The underlined portion corresponds to the sequence of yeast chromosomal DNA flanking the *ADE2* gene. Each 100 μL PCR reaction contained 50 ng DNA, 1x *Pfu*UltraII Reaction Buffer, 250 μM of each dNTP (dATP, dCTP, dGTP, dTTP), 0.2 μM of each primer (F/R), 2% (v/v) *Pfu*UltraII Fusion HS DNA Polymerase (Agilent). The PCR conditions were: 1 cycle—95 °C (2 min); 26 cycles—95 °C (30 s), 60 °C (30 s), 72 °C (30 s); 1 cycle—72 °C (2 min), 4 °C (hold). The PCR product was verified using ethidium bromide agarose gel electrophoresis, excised, and column purified. The amplicon was transformed into yeast (see below) and plated on YPD + 500 μM hygromycin B selection plates at 30 °C. The resulting yeast strain was MATa; *his3Δ 1; leu2Δ 0; met15Δ 0; ura3Δ 0; fui1Δ::KanMX4, ade2Δ::hphNT1*. Disruption of the *ade2* gene was confirmed by PCR. The purine auxotrophic yeast was pigmented red and displayed a retarded growth phenotype under purine starved conditions (Kokina et al., 2014). Single colonies of the purine auxotrophic yeast were picked and expanded for transformation with the pCM189 constructs.

The purine auxotrophic yeast strain with the *ADE2* gene deletion (*ade2Δ::hphNT1*) was used in all experiments in this paper and was