Purine nucleoside phosphorylase (PNP)\(^1\) is an important component of the nucleotide salvage pathway in Apicomplexan parasites, and a potential target for drug development. The intracellular pathogen *Toxoplasma gondii* was therefore tested for sensitivity to immucillins, transition state analogs that exhibit high potency against PNP in the malaria parasite *Plasmodium falciparum*. Growth of wild-type *T. gondii* is unaffected by up to 10 \(\mu\)M immucillin-H (ImmH), but mutants lacking the (redundant) purine salvage pathway enzyme adenosine kinase are susceptible to the drug, with an IC\(_{50}\) of 23 nM. This effect is rescued by the reaction product hypoxanthine, but not the substrate inosine, indicating that ImmH acts via inhibition of *T. gondii* PNP.

The primary amino acid sequence of *Tg* PNP is >40% identical to *Pf* PNP, and recombinant enzymes exhibit similar kinetic parameters for most substrates. Unlike the *Plasmodium* enzyme, however, *Tg* PNP cannot utilize 5'-methylthio-inosine (MTI). Moreover, *Tg* PNP is insensitive to methylthio-immucillin-H (MT-ImmH), which inhibits *Pf* PNP with a \(K_i\) of <1 nM. MTI arises through the deamination of methylthio-adenosine, a product of the polyamine biosynthetic pathway, and its further metabolism to hypoxanthine involves PNP in purine recycling (in addition to salvage). Remarkably, analysis of the recently-completed *T. gondii* genome indicates that polyamine biosynthetic machinery is completely lacking in this species, obviating the need for *Tg* PNP to metabolize MTI. Differences in purine and polyamine metabolic pathways among members of the phylum Apicomplexa, and these parasites and their human hosts, are likely to influence drug target selection strategies. Targeting *T. gondii* PNP alone is unlikely to be efficacious for treatment of toxoplasmosis.

The phylum Apicomplexa consists of >5000 species of obligate intracellular parasites, and is responsible for many important diseases in humans and other animals. Malaria (caused by *Plasmodium*) is a serious global problem with mortality rates in excess of 1 million per year (1). *Toxoplasma gondii* is a chronic infection estimated to affect ~30% of the world's population, and poses a significant threat to immunocompromised individuals and congenitally infected children (2). The emergence of drug-resistant malaria parasites, and complications associated with long-term treatment of chronic toxoplasmosis, underscores the need for new chemotherapeutic agents.

Focusing on differences between host and parasite metabolism provides an attractive strategy for identifying potential drug targets. One metabolic discrepancy between apicomplexan parasites and their mammalian hosts is the lack of...
de novo purine biosynthesis in the former, making them completely reliant on host cells for these essential nutrients (3). Apicomplexan purine salvage pathways have been explored using a combination of biochemical, genetic, and genomic studies (4-11), providing complete transport and metabolic maps for several species (8). Comparative analysis reveals two alternative, and functionally redundant, salvage routes for purine assimilation by *Eimeria* and *Toxoplasma*. Adenosine kinase (AK) converts the nucleoside adenosine into the nucleotide AMP, hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPT) converts guanylate nucleobases into nucleotides, including GMP (see Discussion). *Cryptosporidium* and *Theileria* rely on AK alone, while only HXGPRT is present in *Plasmodium*, but the ability to enzymatically interconvert AMP and IMP provides all of these parasites with a supply of all necessary purine nucleotides.

Purine nucleoside phosphorylase (PNP) converts inosine to hypoxanthine, and guanosine to guanine, providing an important source of nucleobases for HXGPRT (3). PNPs have been examined in a variety of species (12), and may be grouped into two main families: trimeric forms (such as the human enzyme) are typically ~31 kDa and prefer 6-oxopurines (e.g. inosine, guanosine); hexameric PNPs (such as the *E. coli* enzyme) are ~26 kDa and active against both 6-oxopurines and 6-aminopurines (e.g. adenosine). *P. falciparum* PNP (*PfPNP*) has been characterized in detail, and while its amino acid sequence is most similar to hexameric PNPs, its substrate is distinct from either family (10,13). In particular, *PfPNP* is able to utilize 5'-methothioinosine (MTI), produced by the action of adenosine deaminase (AdoDA) on methiothioadenosine (MTA), a byproduct of polyamine metabolism. The involvement of *PfPNP* in both purine and polyamine pathways makes this enzyme an attractive drug target, and rationally designed PNP inhibitors (immucillins) inhibit both parasite and host erythrocyte enzymes to produce purine-less death of *P. falciparum* parasites (11,14).

This report describes the cloning, recombinant expression, and characterization of *T. gondii* PNP (*TgPNP*), and comparison with *PfPNP*. Although, broadly similar, *TgPNP* lacks activity against methythio-purines. Surprisingly, examination of the *T. gondii* genome (15) indicates the absence of polyamine biosynthetic machinery in this parasite.

**MATERIALS AND METHODS**

**Parasites, Host Cells, Chemicals and Reagents** - RH strain *T. gondii* tachyzoites and adenosine kinase knockout (AK–) mutants (7) were maintained by serial passage in primary human foreskin fibroblasts (16). Immucillins were generously provided by Drs. Peter C. Tyler, Gary B. Evans, and Richard H. Furneaux (Industrial Research Ltd, Lower Hutt, New Zealand), and Dr. Vern L. Schramm (Albert Einstein College of Medicine, Bronx NY). [3H]-uracil (20 Ci mmol⁻¹) was purchased from Moravek Biochemicals (Brea, CA). Xanthine oxidase and all purine substrates were obtained from the Sigma (St. Louis, MO). DNA modifying enzymes were obtained from New England Biolabs (Ipswich, MA).

**Sensitivity of *T. gondii* to ImmH, and rescue by purine nucleobases** - The growth of intracellular *T. gondii* (wild-type and AK– mutants) was measured in 24-well plates containing confluent HFF cell monolayers, by the incorporation of [3H]-uracil into acid-precipitable material. Parasites were grown for 24 h in the presence of varying concentrations of ImmH (0-100 µM), after which the cultures were subjected to a 4 h pulse of [3H]-uracil (5 µCi; 20 Ci mmol⁻¹), and plates were processed as previously described (16). For metabolic rescue experiments, AK– parasites were inoculated into 96 well plates containing 10 µM ImmH plus various concentrations of hypoxanthine or xanthine (0-100 µM) for 4 days. The disruption of host cell monolayers (an indicator of parasite viability) was measured by crystal violet staining and optical density measurements at 650 nm (16).

**Genomic Analysis and Cloning of *T. gondii* Nucleoside Phosphorylases** - *T. gondii* genome sequence data (10-fold coverage) is available at http://ToxoDB.org (15), and TBLASTN (WU-BLAST 2.0) (17) was used to search predicted protein sequences for similarity to the PNP, uridine phosphorylase (UdP), methylthioadenosine phosphorylase (MTAP), methylthioadenosine nucleosidase (MTAN), and polyamine biosynthetic pathway enzymes from various...
organisms. Based on the most significant match in T. gondii genome obtained when PNP sequences were used as query, the following primers were constructed for 5’ and 3’ RACE (rapid amplification of cDNA ends) using the SMART™ RACE cDNA amplification kit (Clontech, Palo Alto CA): 5’-GCTGCCGGTGACTTGATGCC-3’ (sense primer for 3’ RACE); 5’-GGCAAGACCCAGGCCAGGAC-3’ (antisense primer for 5’ RACE). T. gondii tachyzoite cDNA was synthesized from total cellular RNA prepared using the RNeasy RNA extraction kit (Qiagen, Valencia CA), and the complete TgPNP open reading frame was amplified using sense primer 5’-acaGTCATGCAAAATGATAGGTACGCTC-3’ and antisense primer 5’-cgggatccGTACTGGCGACGCAGATTC-3’ (upper case indicates native coding sequence; restriction sites underlined). The PCR product was gel-purified (Qiagen Gel Extraction kit), digested with SphI and BglII, ligated into appropriately-digested pQE-70 plasmid (Qiagen), and its sequence verified. The resulting construct (pQE-TgPNP-His6) encodes TgPNP in frame with a C-terminal His6 tag under the control of an IPTG-inducible promoter. A second putative nucleoside phosphorylase was also identified and cloned from T. gondii (see Supplementary Materials), and has been shown to function as a Udp (not shown).

Nucleoside phosphorylase amino acid sequences obtained from Genbank were aligned with T. gondii nucleoside phosphorylases (TgPNP and TgUdp) using ClustalX (18). Unambiguously aligned sequences were used to construct phylogenetic trees using the neighbor joining method (19) and subjected to bootstrap analysis, with 1000 replicates (20).

Expression and Purification of TgPNP - E. coli strain M15[pREP4] (Qiagen) was transformed with the pQE-TgPNP-His6 plasmid and grown at 37°C in 100 µg/ml ampicillin and 25 µg/ml kanamycin. Expression of His-tagged protein was induced with 1 mM IPTG when the culture OD600 reached 0.6, and cells were harvested 5 h later by centrifugation at 4000 x g for 20 min.

IPTG-induced, TgPNP-transformed E. coli cells were resuspended and sonicated in lysis buffer (50 mM NaH2PO4, 300 mM NaCl, 10 mM imidazole, 1 mg ml⁻¹ lysozyme) containing a protease inhibitor cocktail (Sigma). The lysate was then cleared by centrifugation at 10,000 x g for 20 min. 1 ml of Ni-NTA agarose (Qiagen) was added to the cleared lysate, mixed gently for 1 h, packed into a column, and washed twice with 4 ml wash buffer (50 mM NaH2PO4, 300 mM NaCl, 30 mM imidazole). Tagged protein was then released with elution buffer (50 mM NaH2PO4, 300 mM NaCl, 250 mM imidazole), and estimated to be >95% pure based on denaturing polyacrylamide gel electrophoresis and staining with Coomassie Blue. Protein concentration was measured using a protein assay kit (BioRad, Hercules CA).

Enzyme assays - All PNP assays were performed with purified enzyme in 50 mM KPO4, pH 7.4. Phosphorylisis of inosine, 2-deoxyinosine and 5’-methylthio-inosine was measured in a coupled assay with 115 milliunits ml⁻¹ xanthine oxidase to convert hypoxanthine into uric acid. Uric acid formation was followed by spectrophotometric measurement at 293 nm (E293 = 12.9 mM⁻¹ cm⁻¹). Guanosine phosphorylisis was monitored by measuring the disappearance of guanosine at 258 nm (E258 = 5.2 mM⁻¹ cm⁻¹). Adenosine and 5’-methylthio-adenosine phosphorylase activities were measured by following the disappearance of the substrate at 274 nm (E256 = 1.9 mM⁻¹ cm⁻¹). Uridine phosphorylase activity was measured by following the conversion of uridine to uracil at 272 nm (E260 = 2.9 mM⁻¹ cm⁻¹).

For inhibition assays, excess substrate (0.5 mM) was used in combination with inhibitor ranging from 0 - 10 µM; the concentration of inhibitor was always at least ten-fold greater than the enzyme concentration. The rapidly reversible inhibition of PNP was analyzed by fitting to the equation

\[ v_0 = \frac{k_{cat} x S}{(K_m + I/K_i) + S} \]

where \( v_0 \) is the initial reaction rate, \( k_{cat} \) the maximal catalytic rate, \( S \) the substrate concentration, \( K_m \) the Michaelis constant, \( I \) the inhibitor concentration, and \( K_i \) the dissociation constant for the enzyme-inhibitor complex. Since transition state mimics typically exhibit a slow-onset tight-binding inhibition (21), reaction rates were measured continuously, to monitor for a second phase with a markedly different steady state rate \( v_s \), in which case, \( K_i* \) (the dissociation constant for steady state following slow-onset inhibition) was assessed by fitting to

\[ v_s = \frac{k_{cat} x S}{(K_m (1 + I/K_i*) + S)} \]
RESULTS

Immucillin-H inhibits the growth of AK- parasites but not wild-type T. gondii - Immucillins are transition state inhibitors that exhibit potent activity against mammalian and P. falciparum PNP (14,22). ImmH kills P. falciparum in culture with an IC_{50} of 35 nM, with the mode of action dependent on inhibition of both Plasmodium and erythrocyte PNP (14). In contrast, wild-type RH-strain T. gondii parasites (wt) are insensitive to 100 µM ImmH (Fig. 1A). Parasites lacking the purine salvage enzyme adenosine kinase (AK-) exhibit a dose-dependent inhibition of growth, however, with an IC_{50} value of 23 ± 9 nM. (Inhibition of host cell PNP is also expected at this concentration, as ImmH inhibits human PNP with a K_i of 72 pM.) The difference in sensitivities between wild-type and AK- parasites is consistent with previous observations on the redundancy of purine salvage pathways in T. gondii (8): this parasite can survive by salvaging either adenosine (via AK) or hypoxanthine (via HXGPRT). AK- parasites are dependent on HXGPRT, which may depend on PNP activity as a source of nucleobases.

In order to determine whether ImmH exerts its effect by targeting T. gondii PNP (as opposed to the host enzyme, or another target), we assayed the ability of hypoxanthine and xanthine to rescue drug-treated AK- parasites, as shown in Fig. 1B. Both of these nucleobases were able to rescue AK- parasites from the inhibitory effects of 10 µM ImmH. For example, 25 µM hypoxanthine restored parasite viability to 75% of the levels observed without ImmH treatment, and 25 µM xanthine restored parasite viability to 60% of control levels. In contrast, incubation with up to 100 µM concentrations of the nucleoside inosine was unable to rescue AK- parasites from the effects of ImmH (not shown). These data strongly suggest that ImmH acts by inhibiting T. gondii PNP directly.

Identification of T. gondii PNP - Similarity searches using sequences from other species to interrogate the T. gondii genome database identified a single significant match to the P. falciparum (10) and E. coli (23) PNP genes, but not to human PNP (24). Further analysis of protein ortholog groups (25) also identified another putative nucleoside phosphorylase in the T. gondii genome, based on significant similarity to E. coli uridine phosphorylase (26) (see Supplemental Fig. 1). Preliminary enzymatic characterization (not shown) indicates that this gene exhibits Udp activity.

Full-length cDNA sequence for TgPNP, was obtained by 5' and 3' RACE, and predicts a 247 amino acid protein of molecular mass 26,803 Da. Alignment of TgPNP with related enzymes shows 39% average similarity to the hexameric PNP (family 1, including bacterial PNP and UdpPs), vs. 21% similarity to trimeric PNP (family 2, including mammalian PNP and eukaryotic MTAPs). TgPNP exhibits 41% sequence identity to P/PNP and 27% identity to EcPNP (Fig. 2). Phylogenetic analysis also associates the Apicomplexan PNP with family 1, as TgPNP and Pf/PNP cluster more closely with UdpPs than with any of the PNP (Fig. 3). TgUdP is an outlier, not grouping strongly with other UdpPs or PNP.

Neither TgPNP and P/PNP (10) exhibit the complete consensus motif for either family of phosphorilases. Sequence alignment (Fig. 2) shows that eight of the sixteen residues known to be involved in substrate binding in the active site of EcPNP (23) are conserved, and five more are conservatively substituted. The remaining three sites are conserved within the apicomplexa, but differ from EcPNP. Asp206 in P/PNP and Asp207 in TgPNP probably correspond to Asp204 in EcPNP, which has been proposed to be the general acid/base for N7 protonation of the substrate purine ring (13).

Based on biochemical and structural studies, it has been established that P/PNP exhibits activity against MTI, a metabolite not found in humans (11,13). The crystal structure of P/PNP complexed with the transition state analogue MT-ImmH reveals that the methylthio group nestles within a hydrophobic pocket formed by Val66, Tyr160, and Met183 from one subunit, and His7 and Val73 from a neighboring subunit of the P/PNP hexamer. It is interesting to note that despite the overall similarity of apicomplexan PNP, three conservative substitutions (Ile68, Ile75, and Phe162) render the methylthio-binding pocket of TgPNP more similar to EcPNP, which does not possess activity against MTI (L.M. Ting and K. Kim, unpublished observations).
Expression, purification, and biochemical characterization of TgPNP - Recombinant TgPNP engineered to contain a C-terminal hexa-histidine tag was over-expressed and purified from bacteria using a Ni-NTA column under native conditions (Fig. 4). On a denaturing polyacrylamide gel, the protein ran as a single band close to its predicted molecular weight of 27 kDa. The protein was stored in elution buffer (see Materials and Methods) at 4°C with minimal loss of activity over a period of 3 months.

Among the various substrates tested against recombinant TgPNP, the highest catalytic efficiencies (kcat/Km) were observed for phosphorylysis of inosine (1.98 x 10^5 mole^-1 sec^-1) and guanosine (3.83 x 10^5 mole^-1 sec^-1), as shown in Table I. The kinetic parameters determined for these substrates were most similar to those observed in PfPNP, with a turnover rate ~10-fold lower than reported for HsPNP and EcPNP. Deoxynucleosides, which serve as substrates for mammalian PNP, showed very poor catalytic efficiency using either parasite PNP, and virtually no activity was detected against adenosine or uridine (substrates for EcPNP and EcUDP, respectively). While MTI is readily transformed by PfPNP, however, this purine is not an effective substrate for TgPNP, with a catalytic efficiency <0.5% that for inosine. MTA is not a substrate for any of these enzymes. Overall, the substrate specificity of TgPNP is distinct from both mammalian and bacterial PNP and also from the closely related P. falciparum ortholog.

Inhibition of TgPNP by immucillins - Immucillins mimic the transition state structure for PNP, and strongly inhibit both mammalian and Plasmodium PNP (10,22). These compounds usually exhibit a two-step mechanism, with modest inhibition of the initial reaction rates followed by the slow onset of a tighter binding, powerful inhibition phase (22). ImmH and ImmG (analogues of inosine and guanosine, respectively; see Fig. 5) show significant inhibition of TgPNP, with equilibrium dissociation constants (K*) of 2.03 and 1.89 nM (Table II). This corresponds to K_m/K* ratios of 6450 for ImmH and 4970 for ImmG, indicating that these inhibitors bind to the enzyme considerably more tightly than do the substrates. 5'-deoxy-ImmH inhibits parasite PNP somewhat less strongly, with a K* of 10 nM, corresponding to the relatively high K_m for deoxy-inosine (Table I). All three of these inhibitors exhibit biphasic inhibition, with inhibition at equilibrium 68-195-fold stronger than the initial inhibition. All three also bind even more strongly to human PNP (for which they were designed), with K* for ImmH and ImmG in the picomolar range.

Taking advantage of the Plasmodium enzyme's unusual substrate specificity, MT-ImmH was found to bind PfPNP more than 100-fold more strongly than human PNP (13). MT-ImmH is a poor inhibitor of TgPNP, however, with K* of 290 nM, ~142 times the inhibition constant for ImmH (Table II). This observation is consistent with the observation that MTI is not a substrate for TgPNP (Table I).

Comparative genomics of purine and polyamine metabolism in T. gondii and P. falciparum - We recently carried out a comparative analysis of purine salvage pathways in five apicomplexan species and the ciliate Tetrahymena thermophila (8). Purine auxotrophy is common in intracellular pathogens, and T. gondii possesses redundant pathways, including both AK and HXGPRT (Fig. 6). Other organisms (such as T. thermophila) are also capable of salvaging adenine via phosphoribosylation to produce AMP. P. falciparum maintains a more stripped-down version of these pathways, retaining HXGPRT but not AK; in contrast, Theileria parasites have retained only AK.

Most organisms are also capable of recycling purines via the action of MTAP or MTAN on MTA (a byproduct of polyamine metabolism and a dead-end metabolite) producing adenine. These enzymes are lacking in apicomplexan parasites, but the ability of P. falciparum PNP to utilize MTI as a substrate provides an alternative route (11). The T. gondii genome encodes neither MTAP nor MTAN, raising the question of how methyliopurines are recycled or detoxified, given that TgPNP does not act on MTI. Remarkably, in contrast to Plasmodium, which possesses a robust polyamine synthetic pathway, including S-adenosylmethionine decarboxylase (SAMDC), ornithine decarboxylase (ODC), and spermidine synthase (SpdS) activities (27), T. gondii genome lacks all of these genes. Consistent with this observation, T. gondii is insensitive to the ODC
inhibitor difluoromethylornithine (DFMO), at concentrations as high as 10 mM (see Supplementary Fig. 2). The *T. gondii* genome also lacks arginine decarboxylase or agmatinase, which could provide an alternate route for polyamine biosynthesis (28). In sum, *Toxoplasma* appears to be the first eukaryote known to have completely dispensed with *de novo* polyamine biosynthesis, presumably in favor of salvaging these metabolites from the host cell.

**DISCUSSION**

Purine nucleoside phosphorylase is an important component of the purine salvage pathway in several apicomplexan parasites. This report describes the identification and biochemical characterization of *T. gondii* PNP, and analysis of its contribution to the parasite's purine economy. AK and HXGPRT provide redundant routes of purine salvage in *T. gondii* (8). Adenosine is the major source of purine utilized by *T. gondii* (4), and is normally phosphorylated by AK to produce AMP. Parasites lacking AK activity are viable, but completely dependent on HXGPRT (8). The flux of inosine is second only to adenosine in *T. gondii* (4) serving as the major source of hypoxanthine for HXGPRT (8). Parasites lacking AK activity are therefore strongly inhibited by the PNP inhibitor ImmH (Fig. 1). This is analogous to the situation in *P. falciparum*, which naturally lacks AK activity, and is killed by similar concentrations of ImmH (14). The rescue of ImmH treated *T. gondii* with hypoxanthine and xanthine (but not inosine) confirms that *Tg*PNP is the relevant drug target.

*Tg*PNP shows high sequence similarity to *P.f*PNP and family 1 hexameric PNPs (bacterial PNPs and UdPs), but lower similarity to family 2 trimeric PNPs (including mammalian enzymes) (Figs. 2 & 3). Inosine and guanosine are major substrates for *Tg*PNP, but the protein does not possess activity against the *P.f*PNP substrate MTI, 2'-deoxy nucleosides (substrates for mammalian PNPs), or adenosine or uridine (substrates for bacterial PNPs and UdPs) (Table 1). This substrate specificity is also reflected in unique aspects of *Tg*PNP sequence. Five of the ten residues known to be important for base binding in *Ec*PNP (22) are not conserved in *Tg*PNP (Fig. 2), providing a possible explanation for the lack of *Tg*PNP activity against adenosine. Furthermore, the differences observed in the residues known to be important for the formation of a hydrophobic pocket that accommodates the methylthio group in *P.f*PNP (13) may explain why MTI is a poor substrate for *Tg*PNP.

Several immucillins inhibit *Tg*PNP strongly (Table II), exhibiting a slow onset mechanism with equilibrium inhibition constants in the nM range. Measured inhibition profiles are consistent with the observed substrate specificity: the inosine and guanosine analogs ImmH and ImmG are far more effective than MT-ImmH against *Tg*PNP.

In *P. falciparum*, which lacks MTAP, the polyamine biosynthesis byproduct MTA is converted to MTI by AdoDA, which is further broken down to hypoxanthine and 5'-methylthio-ribose-1-phosphate by PNP, underlining the importance of these enzymes in both polyamine and purine metabolism (Fig. 6). The absence of either an MTAP gene in *T. gondii* or the ability of *Tg*PNP to utilize MTI poses an interesting conundrum: how is MTA recycled in this parasite?

Examination of the *T. gondii* genome indicates that this parasite is entirely lacking in polyamine biosynthetic machinery, in distinct contrast to *P. falciparum*, which possesses genes encoding a bifunctional ODC-SAMDC and SpdS. Lack of ODC activity in *T. gondii* cell extracts (29) and insensitivity of the parasite to DFMO (supplemental data) provides further support for this observation. An alternative route for polyamine biosynthesis via ADC and agmatinase is present in plants and bacteria, and has also been proposed for the apicomplexan parasite, *Cryptosporidium parvum* (28), but these genes are also missing from the *T. gondii* genome. The absence of polyamine pathway enzymes, and by extension, their MTA and MTI products, obviates the need for *Tg*PNP activity against MTI. In aggregate, the available data suggests that *T. gondii* is incapable of *de novo* polyamine biosynthesis, and must depend on the transport of these crucial metabolites from the host cell and/or extracellular media, making polyamine transport a potential target for anti-parasitic chemotherapy.

It is interesting to observe that orthologous enzymes may exhibit significantly divergent biochemical characteristics, even when operating in the same metabolic pathway, and that these differences can affect inhibitor specificity and
efficacy. Thus, while TgPNP and PfPNP are similar enzymes, performing similar functions in related parasites, PfPNP is a more promising drug target. The striking differences in the composition of purine and polyamine metabolic pathways between P. falciparum and T. gondii probably reflect the adaptation of parasite metabolic machinery to specific environmental conditions. These studies serve to further validate comparative genomic analysis of metabolic pathways.

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FOOTNOTES

1 Abbreviations used in the study: AdoDA, adenosine deaminase; AK, adenosine kinase; AK–, AK knockout T. gondii parasites; AMPDA, AMP deaminase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase; DFMO, difluormethylornithine; GMPS, GMP synthase; HXGPRT, hypoxanthine-xanthine-guanine phosphoribosyl transferase; Imm, Immucillin; IMPDH, IMP dehydrogenase; MTA, 5′-methylthio-adenosine; MTAN, MTA nucleosidase; MTAP, MTA phosphorylase; MTI, 5′-methylthio-inosine; MT-ImmH, 5′-methylthio-immucillin-H; ODC, ornithine decarboxylase; PNP, purine nucleoside phosphorylase; RACE (rapid amplification of cDNA ends); SAMDC, S-adenosylmethionine decarboxylase; SpdS, spermidine synthase; UdP, uridine phosphorylase; wt, wild-type.

2 The T. gondii PNP and UdP nucleotide sequences reported in this paper have been submitted to GenBank with accession numbers DQ385445 and DQ385446 respectively.

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FIGURE LEGENDS

Fig. 1. Susceptibility of T. gondii to ImmH. A, The growth of ImmH-treated, wild-type RH-strain T. gondii tachyzoites (squares) and AK– mutants (diamonds) was assayed by [3H]-uracil incorporation. Only AK– parasites showed sensitivity to the drug, with an IC50 value of 23 ± 9 nM (mean ± standard deviation). B, AK– parasites were cultured in the presence of 10 µM ImmH plus 0 - 100 µM hypoxanthine (triangles) or xanthine (circles). Growth was measured by monolayer disruption assay, and is represented as the percentage of untreated controls.

Fig. 2. Sequence comparison of T. gondii PNP with P. falciparum and E. coli PPNPs. PNP protein sequences from P. falciparum (Pf), T. gondii (Tg), and E. coli (Ec) were aligned using ClustalX (17). Black shading indicates identity (and gray shading similarity) of two or more sequences. Amino acids known to be associated with the EcPNP active site are marked: squares indicate binding to the ribose sugar, circles bind to the phosphate group, and triangles are involved in binding to the nucleobase. Open and closed symbols represent amino acids contributed by different subunits in the PNP hexamer. Stars
indicate amino acids known to be associated with a hydrophobic cavity in PfPNP that can accommodate the 5'-methylthio group of MTI.

Fig. 3. Phylogeny reconstruction for PNP, UdP and MTAPs. Unambiguously alignable amino acids sequences from several organisms were used to construct a neighbor-joining tree, with horizontal branch lengths proportional to distance in the unrooted tree (bar indicates scale in substitutions per site). Solid dots define clades with 100% bootstrap support (shaded dot, 92% support) based on 1000 replicates. HsMTAP, Homo sapiens MTAP (GenBank accession no Q13126); BstPNP, Bacillus stearothermophilus PNP (JT0873); BsuPNP, B. subtilis PNP (D69614); MmPNP, Mus musculus PNP (NP_038660); HsPNP, H. sapiens PNP (PHHUPN); TgUDP, Toxoplasma gondii UdP (ABC94783); VcUDP, Vibrio cholerae UdP (B82249); EcUDP, Escherichia coli UdP (BAB38184); TgPNP, T. gondii PNP (ABC94782); PfPNP, Plasmodium falciparum PNP (CAD51497); StPNP, Salmonella typhimurium PNP (NP_463426); EcPNP, E. coli PNP (BAB38766); BstPNPII, B. stearothermophilus PNPII (JT0874); BsuPNPII, B. subtilis PNPII (O34925).

Fig. 4. Expression and purification of recombinant TgPNP. Lane 1, molecular weight markers (sizes indicated in kDa); lanes 2 and 3, total E.coli protein extract before and after induction with IPTG (respectively); lane 4, His6-tagged TgPNP after purification.

Fig. 5. Structures of PNP substrates and immucillin inhibitors used in this study.

Fig. 6. Purine and polyamine metabolism in T. gondii and P. falciparum. The presence or absence of polyamine biosynthesis and purine salvage enzymes involved in the conversion of nucleosides (ns) and nucleobases (nb) to nucleotides (nt) was determined based on an analysis of available genome sequences (see "Materials and Methods"), motif searches, and manual curation (25). Blue arrows indicate pathways present only in P. falciparum, and red arrows indicate pathways present only in T. gondii. Pathways lacking in both apicomplexans but found in the human host are indicated by dotted arrows. Polyamine biosynthetic enzymes: ODC, ornithine decarboxylase; SAMDC, S-adenosylmethionine decarboxylase; SpdS, spermidine synthase. Purine salvage enzymes: ADA, adenine deaminase; AdoDA, adenosine deaminase; AK, adenosine kinase; AMPDA, AMP deaminase; ASL, adenylosuccinate lyase; ASS, adenylosuccinate synthetase; GMPS, GMP synthase; HXGPRT, hypoxanthine-xanthine-guanine phosphoribosyl transferase; IMPDH, IMP dehydrogenase; PNP, purine nucleoside phosphorylase.
Table I: Kinetic constants for PNPs from *T. gondii*, *P. falciparum*, Human erythrocytes and *E. coli*

| Substrate       | T. gondii | P. falciparum | H. sapiens<sup>a</sup> | E. coli<sup>a</sup> |
|-----------------|-----------|---------------|------------------------|---------------------|
|                 | K<sub>m</sub> (µM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (M<sup>-1</sup>s<sup>-1</sup>) | K<sub>m</sub> (µM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (M<sup>-1</sup>s<sup>-1</sup>) | K<sub>m</sub> (µM) | k<sub>cat</sub> (s<sup>-1</sup>) | k<sub>cat</sub>/K<sub>m</sub> (M<sup>-1</sup>s<sup>-1</sup>) |
| Inosine         | 13.1 ± 1.2 | 2.60 ± 0.02 | 1.98 x 10<sup>5</sup> | 4.7 | 1.1 | 2.3 x 10<sup>5</sup> | 40 | 56 | 1.4 x 10<sup>6</sup> | 70 | 88 | 1.3 x 10<sup>6</sup> |
| deoxy-Inosine   | 259 ± 61  | 0.48 ± 0.01 | 1.85 x 10<sup>3</sup> | 91 | 0.89 | 9.8 x 10<sup>3</sup> | 66 | 180 | 231 | 1.3 x 10<sup>6</sup> |
| Guanosine       | 9.4 ± 1.6 | 3.60 ± 0.02 | 3.83 x 10<sup>5</sup> | 9.4 | 2.6 | 2.8 x 10<sup>5</sup> | 12 | 26 | 2.3 x 10<sup>6</sup> | 20 | 59 | 3.9 x 10<sup>6</sup> |
| MT-Inosine      | 31.9 ± 2.7 | 0.027 | 8.46 x 10<sup>2</sup> | 10.6 | 2.6 | 2.4 x 10<sup>d</sup> | 12 | 0.2 | 1.6 x 10<sup>d</sup> | – | ND<sup>b</sup> | – |
| Adenosine       | – | ND | – | – | ND | – |
| MT-Adenosine    | – | ND | – | – | ND | – |
| Uridine         | – | ND | – | 115 | 0.09 | 7.8 x 10<sup>2</sup> |

<sup>a</sup> Values from refs (10,13)
<sup>b</sup> ND, not detectable under reaction conditions

Table II: Inhibition constants for Immucillins (nM)

| Inhibitor       | T. gondii | P. falciparum<sup>a</sup> | H. sapiens<sup>a</sup> |
|-----------------|-----------|------------------------|------------------------|
|                 | K<sub>i</sub><sup>b</sup> | K<sub>3</sub><sup>c</sup> | K<sub>i</sub><sup>b</sup> | K<sub>3</sub><sup>c</sup> | K<sub>i</sub><sup>b</sup> | K<sub>3</sub><sup>c</sup> |
| ImmH            | 450       | 2.03                   | 29                     | 0.6                  | ND<sup>d</sup>   | 0.07 |
| MT-ImmH         | 7110      | 290                    | –                      | 2.7                  | –                 | 0.3  |
| ImmG            | 346       | 1.89                   | ND                     | 0.9                  | ND                | 0.03 |
| deoxy-ImmH      | 682       | 10                     | 130                    | 7.4                  | ND                | 9.4  |

<sup>a</sup> Values from refs (10,13)
<sup>b</sup> Inhibition constant during slow onset phase (nM)
<sup>c</sup> Equilibrium inhibition constant (nM)
<sup>d</sup> ND indicates that no slow-onset phase was observed
Chaudhary et al, Fig. 1 (top)

A. 

\[ \text{3H-uracil incorporation (x 10^3 cpm)} \]

\[ \text{ImmH concentration (log molarity)} \]

\[ \text{wild type} \]
\[ \text{AK^−} \]

B. 

\[ \text{Monolayer disruption (% control)} \]

\[ \text{Nucleoside concentration (µM)} \]

\[ \text{Hypoxanthine} \]
\[ \text{xanthine} \]
Chaudhary et al, Fig. 3 (top)
Chaudhary et al, Fig. 4 (top)
Chaudhary et al, Fig. 5 (top)
Chaudhary et al, Fig. 6 (top)

red = Toxoplasma only / blue = Plasmodium only

Polyamine Synthesis

Ado Met → dAdo Met

Orn → Put → Spd → Spm

ODC

Samdc

Purine Salvage

Guo → Gua → Xan → Hyp

PNP

HXGPRT

MTI

MTA

AdoDA

Ino → Ado → Ade

ADA

AMP

AMPDA

GMPS

IMPDH

ASL

ASS

red = Toxoplasma only / blue = Plasmodium only
### Multiple sequence alignment of uridine phosphorylases from various species.

ClustalX alignment for Udp amino acid sequences from *T. gondii* (Tg; obtained in the course of this work), *Dictyostelium discoideum* (Dd; from GenBank), *Escherichia coli* (Ec; from GenBank), and *Haemophilus influenzae* (Hin; from GenBank). Black shading indicates amino acid identity across two or more species.
Susceptibility of *T. gondii* to DFMO. The growth of wild-type RH strain parasites in various concentrations of DFMO was assayed by $[^3H]$-uracil incorporation. Parasite growth was not affected by up to 10 mM drug.
Toxoplasma gondii purine nucleoside phosphorylase: Biochemical characterization, inhibitor profiles and comparison with the plasmodium falciparum ortholog
Kshitiz Chaudhary, Li Min Ting, Kami Kim and David S. Roos

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