INHIBITION AND STRUCTURE OF *TOXOPLASMA GONDII* PURINE NUCLEOSIDE PHOSPHORYLASE

Running title: *Toxoplasma gondii* purine nucleoside phosphorylase

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The intracellular pathogen *Toxoplasma gondii* is a purine auxotroph that relies on purine salvage for proliferation. We have optimized *T. gondii* purine nucleoside phosphorylase (TgPNP) stability and crystallized TgPNP with phosphate and Immucillin-H, a transition-state analogue that has high affinity for the enzyme. Immucillin-H bound to TgPNP with a dissociation constant of 370 pM, the highest affinity of eleven immucillins selected to probe the catalytic site. The specificity for transition-state analogues indicated an early dissociative transition state for TgPNP. Compared with PfPNP, large substituents surrounding the 5′-hydroxyl group of inhibitors demonstrate reduced capacity for TgPNP inhibition. Catalytic discrimination against large 5′ groups is consistent with the inability of TgPNP to catalyze the phosphorylisis of 5′-methylthioinosine to hypoxanthine. In contrast to mammalian PNP, the 2′-hydroxyl group is crucial for inhibitor binding in the catalytic site of TgPNP. This first crystal structure of TgPNP describes the basis for discrimination against 5′-methylthioinosine and similarly 5′-hydroxy-substituted immucillins; structural differences reflect the unique adaptations of purine salvage pathways of Apicomplexa.
INTRODUCTION

Toxoplasma gondii, the etiologic agent of toxoplasmosis, is an opportunistic pathogen that is widespread among various warm-blooded animals, including domestic felines and humans (1). Approximately 1 billion people worldwide, including 22.5% of the population in the US, are seropositive for T. gondii (2). Toxoplasmosis affects immunocompromised individuals, such as AIDS patients, organ transplant recipients, and the fetuses of newly infected mothers (2). Although the incidence of congenital and AIDS-associated toxoplasmosis has been decreasing, recent water-borne outbreaks have shown that toxoplasmosis in immunocompetent individuals is more common than initially realized. Unfortunately, current therapeutic options for toxoplasmosis are limited. Antifolate drugs such as pyrimethamine are effective against the tachyzoite stage of T. gondii but do not affect the bradyzoite stage that causes chronic infection in the host. Lifelong maintenance with a combination of pyrimethamine/sulfadiazine for toxoplasmic encephalitis often leads to side effects, including severe allergic reactions and hematotoxicity (6). Alternative chemotherapeutic strategies are needed to prevent the onset of these adverse reactions (7, 8).

Toxoplasma gondii is a member of the phylum Apicomplexa, which also includes Plasmodium, the causative agent of malaria. Both parasites replicate rapidly and require large amounts of purines for the synthesis of their nucleic acids and other vital components. These obligate intracellular parasites cannot synthesize purines de novo and depend on purine salvage from the host. Toxoplasma gondii (Tg) nucleobase and nucleoside transporters have been identified and include TgNBT1, TgAT1, and TgAT2 (9-11). TgAT2 has an affinity for nucleosides, with a high affinity for adenosine (9, 12,
The rate of adenosine incorporation is higher than that of other purines, and adenosine kinase (AdK) has a high level of activity relative to other enzymes in the pathway, indicating that adenosine is the major purine utilized by T. gondii (12). In contrast, Plasmodium falciparum (Pf) has no AdK activity (14), and no AdK gene has been identified in the Plasmodium genome (15). However, in the presence of excess adenosine, P. falciparum can use AMP synthesized by human erythrocyte AdK, which is followed by parasite uptake of AMP from the erythrocyte cytosol (14).

Toxoplasma can replicate normally in vitro using adenosine kinase, or in the absence of adenosine kinase by using pathways that require hypoxanthine-xanthine-guanine phosphoribosyl transferase (HXGPRT) activity (16). Toxoplasma gondii with ∆AdK background are viable, but genetic ablation of AdK plus PNP inhibition kill the parasite (16). PNP converts inosine to hypoxanthine and guanosine to guanine. TgPNP, which is structurally similar to P. falciparum PNP (PfPNP), has a homohexameric structure in contrast to homotrimeric mammalian PNP (17, 18). The structure and function of PfPNP is unique because of its acceptance of 5´-methylthioinosine (MTI), a specific metabolite of many Plasmodium species but one that is not present in the human host or in T. gondii (18, 19). The TgPNP amino acid sequence is 41% identical to that of PfPNP, and a previous study showed that MTI is a poor substrate for TgPNP (16).

Immucillin-H is a transition state analogue inhibitor of PNP that causes purine starvation in cultured P. falciparum (20, 21) and inhibits both TgPNP and host cell PNP activity (16). To understand the structural basis of substrate and inhibitor recognition, TgPNP was co-crystallized with Immucillin-H. The structure of TgPNP in complex with Immucillin-H and phosphate revealed a reduced capacity to bind 5´-substituted
nucleosides. Two representative 4′-deaza-1′-aza-2′-deoxy-1′-(9-methylene) (DADMe)-immucillins (22) were also tested. These analogues have similar affinity to Immucillin-H for PfPNP and a higher affinity for human PNP (Figure 1). Surprisingly, these analogues show low affinity for TgPNP. Several PNP family transition-state analogues were used to explore catalytic site interactions of TgPNP (Figure 1). The results indicate an early dissociative transition-state for TgPNP.

MATERIALS AND METHODS

Reagents. Xanthine oxidase, inosine, ampicillin, isopropyl β-D-1-thiogalactopyranoside (IPTG), and protease inhibitor cocktail were purchased from Sigma (St. Louis, MO). One Shot Top 10 chemically competent Escherichia coli cells, DNase I, Superscript III reverse transcriptase, Platinum Taq High-Fidelity master mix, and PtrcHis 2 Topo vectors were purchased from Invitrogen (Carlsbad, CA). BL21-codon plus (DE3)-RIPL E. coli competent cells were purchased from Stratagene (Santa Clara, CA). RNase mini kits and Ni-NTA agarose were purchased from Qiagen (Valencia, CA). Imm-H, 5′-d-Imm-H, 5′-F-Imm-H, 5′-COOH-Imm-H, 2′-d-Imm-H, DADMe-Imm-H, DADMe-Imm-G, 5′-MT-Imm-H, 5′-CONH2-Imm-H, 5′-thio-Imm-H, and 1′,9-Me-Imm-H were synthesized as described previously (18, 23, 24). Crystallography reagents and plates were purchased from Hampton Research (Aliso Viejo, CA).

cDNA synthesis and polymerase chain reaction (PCR) analysis of TgPNP.

Toxoplasma gondii RH tachyzoite cDNA was synthesized from total cellular RNA, which was prepared using chloroform:TRIzol (1:5 vol:vol). RNA was quantified using a
Nanodrop spectrophotometer and then treated with DNase I (RNase-free) at 37°C for 15 min prior to cDNA synthesis. RNA was purified using a QIAGEN RNeasy extraction kit according to the manufacturer’s protocol. Aliquots containing 3.5 μg of RNA were stored at −80°C until needed. First-strand cDNA was generated using Invitrogen Superscript III reverse transcriptase and oligo dT20 as described by the manufacturer (25). PCR products from cDNA and gDNA were assessed on an agarose gel and analyzed via automated DNA sequencing (Albert Einstein College of Medicine DNA Sequencing Facility, Bronx, NY).

**Development of TgPNP constructs.** The coding region of TgPNP, without the stop codon, was amplified by PCR using High-Fidelity Taq with the sense primer 5′-AGGGCATGGAAGTTCAGCCTC-3′ and antisense primer 5′-GTACTGGCGACGCAGATT-3′. The coding region was then cloned into the pTrcHis2-TOPO vector (Invitrogen) with a C-terminal hexahistidine-tag and an ampicillin selection cassette. Each plasmid was transformed into E. coli strain BL21-codon plus (DE3)-RIPL (Stratagene). The TgPNP clone used in this study contains a Val233Ile substitution compared with the TgPNP amino acid sequences reported earlier (16). This conservative substitution is remote from the catalytic site and is not expected to cause significant differences in enzymatic activity. It should be noted that the genes for strains GT1 TGGT1_307030 and ME49 TGME49_307030 found at the Toxoplasma Genomics Resource website, toxoDb.org, are incorrectly predicted to encode a 330-amino acid protein, in contrast to the 247-amino acid protein previously characterized (16) and predicted for the *T. gondii* VEG strain TGVEG_050700.
Expression and purification of TgPNP for kinetic studies. Fresh enzyme was expressed and purified before each experiment. The recombinant enzyme was expressed by inducing a 100-mL bacterial culture with 1 mM IPTG at 37°C for 18 h. Cells were ruptured by resuspension in BugBuster™ (Novagen, Darmstadt, Germany) according to the manufacturer’s instructions, and the cell debris was removed by centrifugation 16,000 x g for 20 min at 4°C. Recombinant TgPNP was purified using a nickel-nitrilotriacetic acid affinity chromatography spin column (Ni-NTA, Qiagen) according to the manufacturer’s instructions. Purified recombinant protein was buffer-exchanged against 50 mM Na₂HPO₄/KH₂PO₄ (pH 5.0), 1 mM DL-dithiothreitol (DTT), and 50 mM NaCl. This buffer condition was selected after screening (described below). The enzyme concentration was determined from the extinction coefficient (24,005 M⁻¹ cm⁻¹) at 280 nm.

Buffer optimization for protein stability. Recombinant TgPNP was expressed and purified as described above. Immediately after purification, the recombinant protein was diluted 100-fold into 50 mM test buffer containing 50 mM NaCl and 1 mM DTT (Table S1, Supplemental Material), and an aliquot from each condition was tested for PNP activity as described below. The remaining protein was incubated for 6 hours at 4°C and then retested for PNP activity.

Enzymatic assays and inhibition studies. Recombinant proteins were used for enzymatic assays directly following purification. Kinetic assays were carried out in 50
mM KH$_2$PO$_4$ at pH 7.4, measuring phosphorylysis of inosine by PNP in a coupled reaction with 60 mU/mL of xanthine oxidase to convert hypoxanthine to uric acid. Formation of uric acid was measured at a wavelength of 293 nm (ε$_{293}$ = 12.9 mM$^{-1}$ cm$^{-1}$) (20, 21). Assays were performed with excess substrate in the presence of inhibitors. Inhibition assays for determining the $K_i$ value for initial- and slow-onset inhibition constants ($K_i^*$) were performed using varied concentrations of Imm-H, 5’-d-Imm-H, 5’-F-Imm-H, 5’-COOH-Imm-H, 2’-d-Imm-H, DADMe-Imm-H, DADMe-Imm-G, 5’-MT-Imm-H, 5’-CONH$_2$-Imm-H, 5’-thio-Imm-H, and 1’,9-Me-Imm-H along with 500 μM inosine. Inhibition constants were determined as described previously (26). Initial-onset inhibition was analyzed using the following equation: $v_0 = (k_{cat} \times [S])/(K_m(1+[I]/K_i)+[S])$, where $v_0$ represents the steady state rate, $k_{cat}$ represents the catalytic rate, $[S]$ represents the substrate concentration, $K_m$ represents the Michaelis constant for inosine, $[I]$ represents the inhibitor concentration, and $K_i$ represents the equilibrium dissociation constant for the inhibitor. Human and P. falciparum PNP were used as controls and were expressed and purified as described elsewhere (18, 19).

**Protein crystallization and data collection.** Bacterial cultures for expressing TgPNP were grown in Luria Bertani-ampicillin broth at 37°C to an optical density at 595 nm of 0.6, induced with IPTG at a final concentration of 1 mM, and grown at 25°C for an additional 18 h. Cells were harvested by centrifugation (4,000 x g for 30 min) and then ruptured by passage through a French press. The resulting cell debris was pelleted by centrifugation (16,000 x g for 30 min), and the remaining supernatant was purified over a 3-mL Ni-NTA affinity column (Qiagen) with elution by a step gradient of 50, 75, 100,
200, 300, and 500 mM imidazole in 50 mM HEPES (pH 8.0), 300 mM NaCl, and 1 mM DTT. The purified recombinant protein was dialyzed overnight against two different conditions: ammonium acetate buffer (50 mM ammonium acetate [pH 5.0], 50 mM NaCl, and 1 mM DTT) and phosphate buffer (25 mM Na$_2$HPO$_4$/KH$_2$PO$_4$ [pH 5.0], 50 mM NaCl, and 1 mM DTT). The final concentration of TgPNP for crystallization was 10 mg/mL in the presence of 3 mM Imm-H and 10 mM phosphate. The crystallization condition of 25% polyethylene glycol monomethyl ether 2000, 100 mM Tris (pH 8.5), and 0.2 M trimethylamine N-oxide dihydrate was determined using Hampton Research Index HT screening by sitting-drop vapor diffusion. Crystals were transferred into a fresh drop of the crystallization solution containing 25% glycerol and rapidly frozen in liquid nitrogen. X-ray diffraction data for Imm-H bound to TgPNP was collected using a Beamline X29A at the Brookhaven National Laboratory. All data were processed using the HKL2000 program suite, and the data processing statistics are provided in Table 1 (27). The coordinates and structure factors for Imm-H bound TgPNP were deposited in the Protein Data Bank (PDB) under accession code 3MB8.

**Structure determination and refinement.** The crystal structure of TgPNP bound to Immucillin-H was determined by molecular replacement in MolRep using the published structure of *Pf*PNP bound to Immucillin-H (PDB ID: 1NW4) as the search model, followed by model building using Phenix (28, 29). The model without Immucillin-H was first rebuilt using the Crystallographic Object-Oriented Toolkit and refined in Refmac5 (30, 31). Immucillin-H was added last using $F_o$-$F_c$ map and refined in Refmac5 (31). The refinement statistics are summarized in Table 1.
RESULTS

Buffer optimization for protein stability. Recombinant TgPNP enzyme activity was unstable, thus precluding previous attempts at crystallization. Buffer composition and pH were optimized to stabilize the enzyme activity (Table S1, Supplemental Material). With inosine as the substrate and Na₂HPO₄/KH₂PO₄ buffer at pH 5 and pH 6 or ammonium acetate buffer at pH 7, PNP activity was retained (Figure S1, Supplemental Material).

Kinetic analysis of TgPNP. TgPNP accepts multiple substrates, including guanosine and inosine (16). Inosine is a purine salvage metabolite and was used to establish kinetic parameters. The catalytic efficiency (kcat/Km) with inosine was 5.5 x 10⁴ for TgPNP and 1.5 x 10⁵ for PfPNP, similar to the values reported previously for these enzymes (Table 2) (16), (18).

Transition-state analogue screening. Transition-state analogues that mimic the specific geometry and electrostatic features of a molecule can be used to predict the position of the transition state in the reaction coordinate (32). The dissociation constants of an array of transition-state analogue inhibitors were measured to explore the specificity of TgPNP. Immucillin-H bound TgPNP tightly, with a Kᵢ of 0.37 nM, similar to the Kᵢ of 0.86 nM for PfPNP (Figure 1 and Table S2) (16). DADMe-Imm-H and DADMe-Imm-G are second-generation immucillins and are powerful inhibitors of human PNP, with Kᵢ values of 0.0085 and 0.007 nM, respectively. These analogues inhibited PfPNP with an affinity similar to Imm-H (Figure 1 and Table S2) (18). Surprisingly, these transition-
state analogues bound poorly to TgPNP, with $K_i$ values of 3,600 nM for DADMe-Imm-H and 1,500 nM for DADMe-Imm-G. Both Imm-H ($K_i = 0.37$ nM) and Imm-G ($K_i^* = 1.9$ nM) are powerful inhibitors of TgPNP (16). Imm-H and Imm-G also bound PfPNP tightly, with $K_i^*$ values of 0.6 and 0.9 nM, respectively (18). DADMe-Imm-H and DADMe-Imm-G mimic a fully-dissociated $S_N1$ ribocationic transition state.

High-affinity transition state analogues for human PNP (HsPNP) were designed with a methylene bridge between the purine and ribocation group, and these analogues lacked the 2'-hydroxy group (Figure 1) (33). The linear distance between the deazapurine ring and the C1' of Imm-H was found to be 1.5 Å, characteristic of an early transition state, whereas the distance to the N1' of DADMe-Imm-H was determined as 2.5 Å, which was characteristic of a late transition state (33). To evaluate if the presence of the methylene bridge is responsible for the loss of affinity, 1',9-Me-Imm-H was tested with TgPNP. No inhibition was observed at 50 μM with 1',9-Me-Imm-H; although, 1',9-Me-Imm-H inhibited HsPNP with a $K_i$ of 250 nM. These results indicate an early dissociative transition state for TgPNP. In addition, 2'-d-Imm-H displayed a 522-fold decrease in affinity for TgPNP as compared to Imm-H, indicating that the 2'-hydroxyl group is necessary for tight binding (Figure 1 and Table S2). This specificity suggests a physiological role for TgPNP in nucleoside salvage. TgPNP did not show slow-onset inhibition with any inhibitor.

5'-MT-Imm-H was specifically designed to examine the substrate specificity of PfPNP for MTI. MTI is a poor substrate for TgPNP, and thus 5'-methylthio-purines are unlikely to be formed during T. gondii metabolism (16). Consistent with this, 5'-MT-ImmH was found to be a poor inhibitor of TgPNP, with a $K_i$ of 5,600 nM (Figure 1 and...
The inhibitory activity of 5′-CONH₂-Imm-H was similar to that of 5′-MT-ImmH, with a \( K_i \) of 8,900 nM, and no inhibition was observed with 5′-thio-Imm-H at 50 μM. Other 5′-modifications revealed a high selectivity for a hydroxyl group in the 5′-position (Figure 1 and Table S2).

**Crystal structure of TgPNP-bound ImmH.** The hexameric quaternary structure was similar to that of the Apicomplexa phylum member *P. falciparum* PNP and to *E. coli* PNP (Figure 2A) but differed from that of mammalian trimeric family 1 PNP (18, 34). TgPNP co-crystallized in the presence of Imm-H showed a monomer structure consisting of a core containing 11 β sheets (Figure 2B). TgPNP consists of a trimer of dimers, with six catalytic sites formed by residues at the interface of the monomeric pairing (Figure 2A). Each subunit primarily houses one inhibitor molecule, with residues His9 and Arg47 from the adjacent subunit binding to the 5′-hydroxyl of Imm-H and the phosphate ion, respectively (Figure 3).

Clear electron density at 1.8-Å resolution was observed for the transition state analogue and phosphate in the active site of the enzyme. The deazahypoxanthine base was bound in a pocket formed by nonpolar residues Phe162, Pro210, Ile182, and Trp213 and by polar residue Asp207. The N1′ proton of the deazapurine hydrogen bonded with structurally conserved water at 2.8 Å, which was stabilized in a water lattice with another water molecule and the backbone oxygen of Tyr161 (Figure 4). Asp207 was observed to interact with the N7 proton of Imm-H at 3.0 Å, and formed water-mediated hydrogen bonds with both O6 and O5′ of the inhibitor and is favored to be the general acid/base for protonation of N7 with formation of the transition state (18, 35, 36).
Tyr218 formed a water-mediated interaction with the 5’-hydroxyl of the iminoribitol group (2.7 Å) and formed hydrogen bonds with the O2 of a glycerol molecule (2.6 Å) from the crystallization buffer in the active site. His9 NE2 from the adjacent subunit was within hydrogen bonding distance to the 5’-hydroxyl (2.7 Å). Glu185 was bound to both the 2’- and 3’-hydroxyls of the iminoribitol group. The phosphate ion was stabilized in an anionic pocket consisting of Arg90, Arg47b, Arg29, and the α-polar residue Thr93 beneath the inhibitor. The OG1 of Thr93 was within hydrogen bonding distance to the N1’ of the ribitol group (3.2 Å). Ion pairing to the phosphate ion occurred with residues Arg90 NH1 and NH2 (3.1–2.8 Å), Arg47b NH1 and NH2 (2.9–3.0 Å), and Arg29, with distances of 2.7 Å for both NH1 and NH2.

Comparison of the active sites of TgPNP and PfPNP. In the structures depicted in Figure 5, residues His9, Ile68, Ile75, Phe162, and Tyr218 are shown to illustrate the similarities found in the active sites of TgPNP to PfPNP bound to Imm-H (PDB 1NW4).

The two homologous enzymes shared a 41% sequence identity, and their Cα backbone superimposed well, with root-mean-square deviations of 0.7 Å out of 1,208 atoms for 1NW4 and 0.7 Å out of 1,220 atoms for 1Q1G. Even though the sequence identity was only 41%, with the exception of Ile68, Ile75, Phe162, and Thr93, there was a high degree of identity with respect to the active pocket residues between the two isozymes. The corresponding residues for PfPNP were: Val66, Val73, Tyr160, and Ser91. The first three residues are proposed to play a key role in binding the 5’-methylthio group in the pocket (18). A water molecule was found to be conserved between TgPNP-bound Imm-H and PfPNP-bound Imm-H (1NW4). Both Ile68 and Ile75 were found to carry an additional
methyl group in comparison with Val66 and Val73. These bulky groups protrude an additional 1.5 Å into the active site, a distance of 4.1 Å from C5’ and 5.0 Å from O5’ of the Imm-H in the crystal structure of 1NW4. When 5’-MT-Imm-H-bound PfPNP (PDB ID: 1Q1G) was overlaid with Imm-H-bound TgPNP, Val66 and Val73 were found to be within Van der Waals distances of 2.5 and 1.8 Å from Ile68 and Ile75, creating a steric clash (Figure 5B). The 5’-hydroxyl and 5’-methylthio groups of Imm-H and 5’-MT-Imm-H were in different orientations. In TgPNP-bound Imm-H, the 5’-hydroxyl was in a position that is surrounded by hydrophilic residues in a solvent-filled cavity, whereas PfPNP-bound 5’-MT-Imm-H (1Q1G) was observed to have the 5’-methylthio group rotated 135° away, towards a closed hydrophobic cavity encompassed by Val66, Val73, and Tyr160 (18).

DISCUSSION

Improvement of TgPNP stability after purification. The stability of TgPNP was improved by buffer screening. The $k_{cat}/K_m$ was similar to earlier reports, but the stabilized protein had a lower $k_{cat}$ (Table 2) (16). Buffer screening enabled the synthesis of viable TgPNP crystals for crystallographic structural determination and inhibition studies.

Transition-state analogue screening. Transition-state structures can be predicted based upon affinity to related transition-state analogues. The transition-state characteristics are compared by analyzing dissociation constants ($K_i$) with an array of representative transition-state analogues (32). These inhibitors mimic known early or late dissociative transition states. Transition-state structures of $N$-ribosyl phosphorylases that metabolize...
nucleosides can be either early or late dissociative and are distinguished by the distance between the ribosyl anomic carbon and the N9 of the purine base leaving group (26).

The first-generation PNP transition-state analogue Immucillin-H was designed from the transition state of bovine PNP, which revealed an early transition state with ribocation characteristics but a relatively close 1.8-Å distance between the leaving-group nitrogen and the anomic carbon (37). In contrast, HsPNP has a fully dissociated purine leaving group with a fully developed ribocation, with a distance greater than 2.6 Å between the leaving-group nitrogen and the anomic carbon (33). A second-generation HsPNP transition-state analogue inhibitor, DADMe-Imm-H, was designed to match this characteristic by the addition of a methylene bridge between the purine and the sugar moiety (22) (Figure 1). TgPNP was strongly inhibited by Immucillin-H, with a $K_i$ of 0.37 nM. Surprisingly, TgPNP was weakly inhibited by DADMe-Imm-H and DADMe-Imm-G, with $K_i$ values of 3,600 and 1,500 nM, respectively, indicating an early dissociative transition state for TgPNP. As shown in Figure 1, no inhibition was observed with 1´,9-Me-Imm-H at 50 μM, which also supports the formation of an early transition state. Despite the high degree of residue conservation found in the active sites of HsPNP and TgPNP, inhibitor specificity studies support distinct transition states. Moreover, different affinities of PfPNP and TgPNP for the analogues tested indicate divergence of the transition states among Apicomplexa PNPs.

**TgPNP substrate specificity.** Consistent with the observation that TgPNP exhibits substrate specificity for inosine but not for MTI, TgPNP inhibition assays with Immucillin-H and 5´-MT-Imm-H yielded $K_i$ values of 0.37 and 5,600 nM, respectively.
Because the 5'-CONH$_2$ group is able to hydrogen bond with His9, 5'-CONH$_2$-Imm-H was tested as an alternative transition-state analogue to the 5'-methylthio group of 5'-MT-Imm-H. However, 5'-CONH$_2$-Imm-H showed weak binding, with a $K_i$ of 8,900 nM, suggesting that this 5'-group is too bulky. For both 5'-MT-Imm-H and 5'-CONH$_2$-Imm-H, the weak binding reflects space constraints in the 5'-group binding cavity. This restriction at the 5'-position is supported by the results of tests involving several distinct inhibitors with specific 5'-modifications (Figure 1 and Table S2). TgPNP exhibits a high degree of selectivity for a hydroxyl group in the 5'-position, as evidenced by results showing that all 5'-modifications resulted in a decrease in binding affinity compared with Imm-H (Imm-H < 5'-d-Imm-H < 5'-F-Imm-H < 5'-COOH-Imm-H < 5'-MT-Imm-H < 5'-CONH$_2$-Imm-H < 5'-thio-Imm-H).

**TgPNP crystal structure.** The residues in the active sites of TgPNP and PfPNP are highly conserved, with the exception of Ile68, Ile75, Phe162, and Thr93 in TgPNP, which correspond to Val66, Val73, Tyr160, and Ser91 in PfPNP. The first three residues are proposed to play a key role in binding the 5'-methylthio group in the pocket (18). It was found previously that PfPNP Tyr160 was able to form two water-mediated hydrogen bonds with O5' and Asp206, but TgPNP Phe162 does not participate in hydrogen bonding to water in the pocket according to our experiments. Residue Tyr218, which is located within the TgPNP active site, makes conserved water-mediated contacts that substitute for Tyr160 in PfPNP.

In the structure of PfPNP bound to 5'-MT-ImmH, the 5'-methylthio group is turned 135º relative to the 5'-hydroxyl group of Immucillin-H in TgPNP-Imm-H,
abutting residues in a densely packed hydrophobic area in the catalytic site. The 5´-hydroxyl group of Immucillin-H is surrounded by largely hydrophilic residues within a solvent-filled cavity in PfPNP. These findings agree with reported positions of both Immucillin-H and 5´-MT-Imm-H bound to PfPNP (18).

**T. gondii and polyamine salvage.** Similar to other members of the phylum Apicomplexa, such as Plasmodium and Cryptosporidium, *T. gondii* is a purine auxotroph that relies on purine salvage for its metabolic requirements (38, 39). The difference in the specificities of TgPNP and PfPNP relates to distinct metabolic functions within the respective parasites. *Toxoplasma gondii* lives in nucleated mammalian cells, as polyamines are abundant in these cells, it is believed that polyamine salvage replaces *de novo* polyamine synthesis (40, 41). Under conditions of polyamine abundance, no 5´-methylthioadenosine, a byproduct of polyamine synthesis, is formed in *T. gondii*. In contrast, *P. falciparum* must synthesize polyamines because erythrocytes are polyamine deficient. The resulting 5´-methylthioadenosine formed is metabolized by unique dual-specificity N-(2-acetamido)iminodiacetic acid and PNP enzymes in most *Plasmodium* species, hence the specificity for 5´-methylthioinosine in PfPNP.

**Conclusion.** Due to a lack of forward-directed polyamine biosynthetic enzymes, *T. gondii* is unable to generate 5´-methylthiopurines (40, 41). Examination of the structure of TgPNP indicates that the residues in the active site cannot accommodate 5´-methylthioinosine, but the solvent-filled pocket surrounding the 5´-OH group of Immucillin-H suggests that the active site may be able to accept a larger 5´ group.
Inhibitor screening of 5’-analogues revealed a high selectivity for a hydroxyl group in the 5’-position. Any modification resulted in a decrease in binding affinity compared with binding to Immucillin-H. Moreover, the results presented here show that TgPNP is strongly inhibited by Immucillin-H but weakly inhibited by DADMe-Imm-H and DADMe-Imm-G. In addition, no inhibition was detected with 1’,9-Me-Imm-H, indicating an early dissociative transition state for this enzyme. This information reveals a surprising degree of catalytic divergence among Apicomplexa PNP.

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Figure 1: Structure of transition-state analogues used in this study to reveal differences in the \( Tg \)PNP transition state and substrate specificity. The dissociation constants for \( Tg \)PNP (Tg), \( Pf \)PNP (Pf), and \( Hs \)PNP (Hs) were obtained using inosine as the substrate. Dissociation constant is defined as the tightest inhibition constant, either \( K_i \) or \( K_i^* \). (*) Indicates \( K_i^* \).

Figure 2: A) X-ray crystal structure of the \( Tg \)PNP hexamer with Immucillin-H and \( \text{PO}_4^{3-} \) bound in the active site. The hexamer structure was generated by applying crystallographic symmetry to the dimers composed of the light blue and dark blue subunits on the bottom left, yielding the purple, green, yellow, and orange monomers. B) \( Tg \)PNP monomer with Immucillin-H and \( \text{PO}_4^{3-} \) bound in the active site (in yellow). The monomer consists of eight \( \alpha \) helices in light blue and surrounds 11 \( \beta \) sheets in the red subunit. Loop regions are shaded in purple. The figure was prepared with MacPyMol.

Figure 3: Stereo views of the catalytic site contacts in \( Tg \)PNP with the transition state analogue inhibitor Immucillin-H and \( \text{PO}_4^{3-} \). Structure shows light blue side chains of the parental monomer surrounding the bound Immucillin-H (yellow); the green subunits on the bottom right indicate residues contributed from the adjacent subunit. Residues participating in binding Immucillin-H and \( \text{PO}_4^{3-} \) are labeled in the active site of \( Tg \)PNP. Figure was prepared with MacPyMol.

Figure 4: Schematic diagram of catalytic site contacts for Immucillin-H and \( \text{PO}_4^{3-} \) at the active site of \( Tg \)PNP. Amino acids are from the parent subunit unless labeled with a \( \beta \), which marks residues from the adjacent subunit. Dashed lines indicate hydrogen bonding. Distances are shown in angstroms.

Figure 5: A) Cross-eyed stereo view superposition of \( Tg \)PNP:Imm-H:PO\(_4^{3-}\) (purple; PDB ID: 3MB8) and \( Pf \)PNP:Imm-H:SO\(_4\) (light blue; 1NW4) to show differences in amino acids surrounding the inhibitor. B) Cross-eyed stereo view superposition of \( Tg \)PNP:Imm-H:PO\(_4^{3-}\) (purple; PDB ID: 3MB8) and \( Pf \)PNP:5´-MT-Imm-H:SO\(_4\) (yellow; 1Q1G) to show the relative angular shifting of residues in the active site when 5´-MT-Immucillin-H is bound.
### TABLE 1. Data collection and refinement statistics

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<tr>
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<th>Imm-H-bound TgPNP</th>
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<td>PDB codes</td>
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<tr>
<td><strong>Data collection</strong></td>
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<td>Space group</td>
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<tr>
<td>Cell dimension</td>
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<tr>
<td>a, b, c (Å)</td>
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<tr>
<td>A, β, γ (°)</td>
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<tr>
<td>Resolution (Å)</td>
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<tr>
<td>(1.97-1.90)</td>
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<tr>
<td>R_{sym} (%)</td>
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<td>1 / σI</td>
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<tr>
<td>Completeness (%)</td>
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<td>Redundancy</td>
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<td>R_{work} / R_{free} (%)</td>
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<td><strong>B-factors (Å²)</strong></td>
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<td>(side chain)</td>
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<td>Ligand</td>
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<td><strong>No. of Atoms</strong></td>
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<td>Bond angles (°)</td>
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<tr>
<td>Disallowed region</td>
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*Numbers in parentheses are for the highest-resolution shell. One crystal was used for each data set.
TABLE 2. Kinetic constants for *T. gondii*, *P. falciparum*, and human PNPs with inosine as the substrate

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<tr>
<th></th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (µM)</th>
<th>$k_{cat}/K_m$ (M$^{-1}$s$^{-1}$)</th>
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<td><em>T. gondii</em></td>
<td>0.23 ± 0.06</td>
<td>4 ± 2</td>
<td>$5.5 \times 10^4$</td>
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<td><em>T. gondii</em> a</td>
<td>2.6 ± 0.0</td>
<td>13 ± 1</td>
<td>$2.0 \times 10^5$</td>
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<tr>
<td><em>P. falciparum</em></td>
<td>1.7 ± 0.7</td>
<td>11 ± 5</td>
<td>$1.5 \times 10^5$</td>
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<td><em>H. sapiens</em> b</td>
<td>12 ± 3</td>
<td>31 ± 5</td>
<td>$8.0 \times 10^5$</td>
</tr>
</tbody>
</table>

Values originally reported in "Chaudhary *et al.* (16) and "Lewandowicz *et al.* (33).