Structural Determinants of the 5′-Methylthioinosine Specificity of Plasmodium Purine Nucleoside Phosphorylase

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Abstract

Plasmodium parasites rely upon purine salvage for survival. Plasmodium purine nucleoside phosphorylase is part of the streamlined Plasmodium purine salvage pathway that leads to the phosphorylysis of both purines and 5′-methylthiopurines, byproducts of polyamine synthesis. We have explored structural features in Plasmodium falciparum purine nucleoside phosphorylase (PfPNP) that affect efficiency of catalysis as well as those that make it suitable for dual specificity. We used site directed mutagenesis to identify residues critical for PfPNP catalytic activity as well as critical residues within a hydrophobic pocket required for accommodation of the 5′-methylthio group. Kinetic analysis data shows that several mutants had disrupted binding of the 5′-methylthio group while retaining activity for inosine. A triple PfPNP mutant that mimics Toxoplasma gondii PNP had significant loss of 5′-methylthio activity with retention of inosine activity. Crystallographic investigation of the triple mutant PfPNP with Tyr160Phe, Val66Ile, and Val73Ile in complex with the transition state inhibitor immucillin H reveals fewer hydrogen bond interactions for the inhibitor in the hydrophobic pocket.

Introduction

Malaria, caused by Plasmodium spp, continues to be an important public health problem for which new interventions are needed. While much progress has been made in malaria control, in 2010 there were an estimated 219 million clinical cases estimated worldwide, with 660,000 deaths primarily in children in sub-Saharan Africa [1]. Because Plasmodium is unable to synthesize purines de novo, Plasmodium purine salvage enzymes have been investigated as potential chemotherapeutic targets. Unlike many other protozoa, Plasmodia have a streamlined purine salvage system consisting of adenosine deaminase (ADA)+purine nucleoside phosphorylase (PNP)+hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPR) [Figure 1] [2]. PNP catalyzes the phosphorolysis of purine nucleosides to ribose-1-phosphate and a purine base [3]. PADA converts adenosine to inosine. PfPNP converts inosine or guanosine to hypoxanthine or guanine that is then acted upon by HXGPR to generate IMP or GMP. Hypoxanthine is the major purine precursor utilized by Plasmodium.

Polypine synthesis pathways are also critical for Plasmodium viability [4–8] and generate 5′-methylthioadenosine (MTA) as a byproduct of polypine synthesis. Humans recycle purines from MTA via the action of methylthioadenosine phosphorylase (MTAP) but Plasmodium species recycle purines via the sequential activities of ADA and PNP, which are unique in their ability to utilize methylthiopurines [9]. In P. falciparum, PfADA converts MTA to 5′-methylthioinosine (MTI), which is then converted to hypoxanthine by PfPNP. Humans do not produce MTI, and human PNP does not catalyze the phosphorylysis reaction of methylthioinosine [10].

The unique dual specificity of P. falciparum PNP can be exploited for anti-malarial drug design. Immucillin-H (ImmH) and 5′-methylthioimmucillin-H (MT-ImmH) are transition state analogs of inosine and MTI, respectively (Figure 2). Immucillins are extremely potent with picomolar Ki for PNP [4,5,11,12]. In the purine-rich environment of cultured red blood cells, ImmH causes P. falciparum cell death by purine starvation [2]. MT-ImmH exhibits 100-fold greater specificity for PfPNP versus mammalian PNP [13]. Genetic studies have revealed that Plasmodium parasites lacking PNP are attenuated [14,15], demonstrating the impor-
tance of this enzyme for viability of malaria parasites. The genetic studies also validated PNP as the target of immucillins [14,15]. In addition, DADMe-Immucillin-G a picomolar transition state analogue of human and Plasmodium PNs is effective against P. falciparum in the Aotus model, illustrating that purine salvage is critical for P. falciparum survival [16].

PPNP, like Escherichia coli PNP, is hexameric and a member of the nucleoside phosphorylase family I [3]. Unexpectedly, the PPNP crystal structure revealed that the 5′-hydroxyl group of ImmH and 5′-methylthio group on the MT-ImmH are positioned differently in relationship to PPNP [17]. The 5′-methylthio of MT-ImmH is rotated ~135° when compared to the 5′-hydroxyl group of ImmH, and therefore the residues that surround the 5′- group are different [17]. If Plasmodium-specific PNP inhibitors are developed further, the capacity of the parasite to develop resistance to new agents must be explored. Thus the structural features of PNP responsible for inosine and MTI metabolism are of great interest.

The purine and polyamine pathways of the related apicomplexan Toxoplasma gondii have significant biologically relevant differences to those of Plasmodium [18,19]. While Plasmodium species must synthesize polyamines, T. gondii salvages polyamines from host cells and therefore does not require enzymes to metabolize MTAs [18]. Consistent with this, TgPNP does not catalyze MTI conversion to hypoxanthine [18].

We hypothesized that the differences between TgPNP and PPNP would enable us to determine the unique structural features responsible for 5′-methylthio purine activity. After comparison of the amino acid sequences of apicomplexan PNs (Figure 3) with the PPNP crystal structure [17], we identified conserved and nonconserved residues potentially critical for catalytic activity. We made a series of PPNP mutants and performed detailed kinetics and structural studies. In particular, PPNP mutants with activity for inosine but not MTI provided clues as to the malleability and conformation of the active site, providing insights that may be useful for future design of anti-malarial compounds.

Figure 1. Purine salvage in P. falciparum. The enzymes that comprise the purine salvage pathway in Plasmodium: ADA, adenosine deaminase; PNP, purine nucleoside phosphorylase; HXGPT, hypoxanthine-xanthine-guanine phosphoribosyltransferase. Substrates are: MTA, 5′-methylthioadenosine; Ado, adenosine; MTI, 5′-methylthiopurine; Ino, inosine; Hyp, hypoxanthine; IMP, inosine monophosphate; AMP, adenosine monophosphate; XMP, xanthine monophosphate; GMP, guanosine monophosphate; ATP, adenosine triphosphate; SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine.

Figure 2. PPNP substrates and inhibitors. Structures of substrates (inosine and 5′-methylthiopurine) and immucillins transition state analogues (ImmH and MT-ImmH) of PPNP utilized for this study.
were sonicated in 10 mM imidazole lysis buffer (50 mM NaPO₄, 1 mM ß-mercaptoethanol, 300 mM NaCl, at pH 8) with protease inhibitor cocktail (Sigma) and 1 mg/ml lysozyme while on ice. After centrifugation at 12000 rpm for 30 minutes, cleared cell lysate was shaken at 4°C with Ni-NTA resin for 1 hr, and then packed into a column. The column was washed twice with 50 mM imidazole buffer (50 mM NaPO₄, 300 mM NaCl, at pH 8), and eluted with 250 mM imidazole buffer (50 mM NaPO₄, 300 mM NaCl, at pH 8). The purity of the proteins (>95%) was confirmed by SDS page gel electrophoresis (data not shown). Protein concentration was measured using protein assay reagent from BioRad (Hercules, CA).

Enzymatic Assays and Inhibition Studies

Kinetic assays were completed in 50 mM KH₂PO₄ at pH 7.4 measuring phosphorylisis of inosine or MTI by PNP in a coupled reaction with 60 milliunits/ml xanthine oxidase to convert hypoxanthine to uric acid. Formation of uric acid was measured at 293 nm wavelength (E 293 = 12.9 mM⁻¹ cm⁻¹) [2,20]. In cases where activity for MTI was low, increased protein concentration (10 mg/ml) was used to check enzyme function. Assays were performed with excess substrate in the presence of inhibitors. Inhibition studies measured both initial (K_i) and slow onset inhibition (K_i*) for inhibitors [2]. The initial onset inhibition was analyzed by using the following equation:

\[ \text{no} = \frac{k_{\text{cat}} [S]}{K_m (1 + I/K_i) + S} \]

no is the steady state rate, k_cat is the catalytic rate, S is substrate concentration, K_m is Michealis constant for inosine, I is inhibitor concentration, and K_i is the equilibrium dissociation constant.

Molecular Modeling

Model of site-directed mutants were comparatively designed based on the X-ray crystallography structure of P. falciparum purine nucleoside phosphorylase PIPNP [17] bound to MT-ImmH (Protein Data Bank entry 1Q1G). Point mutant structures were created utilizing MODELLER 8v2 program [21,22]. Structural visualization was performed using the PyMOL molecular graphics program [23].

Gel Filtration

Samples were concentrated to 1 mg/ml and 0.1 ml was loaded onto a Superose 12 gel filtration column for initial screening. The enzyme was eluted with 20 mM HEPES containing 50 mM KCl, 5m MK H₂PO₄, 0.1 mM DTT (pH 7.4) at a rate of 0.5 ml/min. Larger scale purification was performed on a Superdex 200 gel filtration column with a sample concentration of 10 mg/ml with 5 ml loaded. Both columns were run on an AKTA FPLC (GE Healthcare).

Circular Dichroism Analysis

Secondary structure measurements were taken from 300–185 nm on Aviv 215 CD spectrometer at Mount Holyoke College in the laboratory of Dr. Sean Decatur (Figure S1). The experiments were performed at 4°C in a Peltier temperature-controlled cell chamber. Samples were equilibrated for 10 minutes before measurements were taken. The average time for each wavelength was 10 seconds. Enzyme concentration for each run was 0.1 mg/ml (3.3 nM). Spectra were converted to molar ellipticity (Θ) after subtracting the baseline values of 10% HEPES buffer measured at 4°C. Molar ellipticity conversion = millidegrees/(pathlength × number of residues × molar protein concentration).

Crystallization and X-ray methods

Diffraction quality crystals of PIPNP V66I:V73I:Y160F-ImmH were grown by sitting drop vapor diffusion at 18°C by mixing 2 µl of 10 mg/ml P. falciparum PNP with ImmH (1:1.5 molar equivalents) and 1 µl of reservoir solution consisting of 0.2 M magnesium chloride hexahydrate, 0.1M HEPES at pH 7.5, and

Figure 3. Alignment of apicomplexan PNP s. ClustalW alignment of PNP protein sequences from T. gondii (TgPNP), P. yoelli (PyPNP), and P. falciparum PNP (PfPNP). Residues involved in substrate binding are highlighted [38]. Residues in blue font indicate those surrounding the catalytic domain that were mutated in this study. Amino acids marked: (*) are from the adjacent subunit, (') residues are associated with the hydrophobic cavity for accepting the 5'-Methylthio group of MTI.

doi:10.1371/journal.pone.0084384.g003
30% 2-propanol. The drop was equilibrated with 100 μl of reservoir solution. Crystals with a cubic habit appeared after one month. The crystals were cryo-protected in mother liquor supplemented with 15% glycerol and flash cooled in liquid nitrogen. Data were collected at Brookhaven National Lab National Synchrotron Light Source at beam line X29A with an ADSC Quantum 315 detector. Diffraction data were collected to a resolution of 2.8 Å, and integrated and scaled with HKL2000 [24]. Diffraction of the PfPNP V66I:V73I:Y160F -ImmH•PO4\(^{3-}\) crystals was consistent with the cubic space group 1432, with unit cell parameters a = b = c = 234.97 Å, α = β = γ = 90° and 2 molecules in the asymmetric unit. An initial structure of PfPNP V66I:V73I:Y160F -ImmH•PO4\(^{3-}\) complex was determined by molecular replacement with the program PHASER [25], using the PfPNP•ImmH•SO4\(^{2-}\) structure (1NW4) as the search model. The final model was refined with REFMACS [26]. PfPNP V66I:V73I:Y160F -ImmH•PO4\(^{3-}\) complex PDB ID is 3FOW.

Results

Based on the conservation of residues in the sequence alignment of PNP and the PfPNP crystal structure, we identified several residues that were predicted to be critical for catalytic activity. PfPNP and PyPNP activities are enzymatically indistinguishable with conservation of most catalytic residues [14], but, notably, there are residues within the hydrophobic pocket in contact with the 5'-methylthio group that are different in TgPNP (Figure 3). Of the five residues that surround the 5'-methylthio group in PfPNP, there are three substitutions in TgPNP. His7 and Met183 are conserved in human PNP, E. coli PNP as well as T. gondii and Plasmodium PNP. The PfPNP residues Val66, Val73, and Tyr160 correspond to residues in TgPNP Ile68, Ile75, and Phe162. We tested whether these three residues are determinants in the accommodation of the 5'-methylthio group in the active site of PfPNP [17].

Characterization and Purification of PfPNP

Using site-directed mutagenesis, recombinant PfPNP mutants were created, over-expressed and nickel affinity-purified from E. coli. Mutant and wild-type preparations were analyzed by SDS PAGE gel, and final protein purity was estimated to be >95% (data not shown). Both wild-type and mutant PfPNPs have a 6xHis and C-Myc tag at the C-terminus [20]. Mutants show similar migration on SDS-PAGE and prior studies showed that neither 6xHis nor C-Myc tag interfere with activity but enable rapid efficient purification [10,13,17].

Kinetic Activity of Wild-type PfPNP and Active Site Mutants

Conserved residues in contact with the 5’ group of the substrate and residues critical for catalysis were mutated to alanine (Table 1). His7 and Met183 are conserved residues involved in binding substrate, whereas Arg45 is a conserved residue involved in phosphate binding [27]. Asp206 is proposed to be the general acid/base for protonation of N7 of substrate in the transition state. The catalytic efficiency of wild-type PfPNP with inosine and 5’-methylthioinosine substrates was similar to previous reports [17] with \( k_{cat}/K_m \) values of 1.5×10\(^5\) M\(^{-1}\) s\(^{-1}\) and 9.4×10\(^4\) M\(^{-1}\) s\(^{-1}\), respectively (Table 1). Arg45Ala and Tyr47Ala PfPNP mutants have low activity for both substrates, Met183Ala has reduced activity for inosine (at 1.5×10\(^4\) M\(^{-1}\) s\(^{-1}\)) but no detectable activity for MTI, whereas Tyr160Ala has ~1000 fold reduced catalytic efficiency with both inosine and MTI when compared to wild-type PfPNP. Asp206Ala has low activity with both inosine and MTI.

Substrate Specificity of PfPNP Mutants Surrounding 5’-Methylthio Group

Since the 5’-hydroxyl and 5’-methylthio groups have different orientations when bound to PfPNP, we further tested how the inosine and 5’-methylthioinosine activities could be separated. We used the amino acid sequence of TgPNP, which has negligible 5’-methylthioinosine activity [18], as a guide (Figure 3 and Table 2). Val66 and Val73 line the 5’-methylthio pocket, but Val66le and Val73le mutants and the Val66le:Val73le double mutant, show no significant change in \( K_m \) or catalytic efficiency for MTI.

PfPNP Val66lle and Val73lle individual and combined mutations have no discernable effect upon PfPNP catalytic efficiency for inosine (Table 2). In contrast, the Tyr160Phe mutation alone and in combination with Val66lle or Val73lle renders PfPNP at least 10-fold less efficient with MTI as a substrate when compared with wild-type PfPNP. Tyr160Phe PfPNP \( K_a/K_m \) with MTI is 7.1×10\(^5\) M\(^{-1}\) s\(^{-1}\), whereas Val66lle:Val73lle:Ty160Phe \( K_a/K_m \) values are 4.7×10\(^5\) M\(^{-1}\) s\(^{-1}\) and 4.6×10\(^5\) M\(^{-1}\) s\(^{-1}\), respectively. Inosine remains an effective substrate for these of mutant PfPNPs with efficiency coefficients comparable to wild-type PfPNP.

V66I:V73I:Y160F PfPNP has a 83-fold decrease in \( K_a \) for MTI with 2-fold increase in \( K_m \) (Table 2). MTI is a poor substrate for the V66I:V73I:Y160F PfPNP with catalytic efficiency 160-fold lower than for inosine (9.0×10\(^4\) M\(^{-1}\) s\(^{-1}\) compared to 5.6×10\(^5\) M\(^{-1}\) s\(^{-1}\)). These kinetics are similar to those observed for TgPNP, which has <0.5% efficiency for MTI when compared to inosine [18]. Thus while Tyr160 appears to contribute significantly to the methylthio specificity of PfPNP, Val66lle and Val73lle mutations contribute to decrease PfPNP activity with MTI substrate.

Mutations were also made to more drastically alter the chemical properties of the residues that interact with the 5’-methylthio group (Table 1). His7, Val66 and Val73 were mutated to hydrophilic (serine) or bulky aromatic (phenylalanine) residues. These mutations resulted in greater \( K_m \) and/or lower \( K_a \) with lower overall catalytic efficiency. The Val73Ser mutation had a pronounced effect on MTI activity but not inosine activity with \( K_a \) of 5 μM for inosine and a \( K_m \) of 2100 μM for MTI. The catalytic efficiency was 4100 fold greater for inosine \( (k_{cat}/K_m \) with inosine is 4.6×10\(^4\) M\(^{-1}\) s\(^{-1}\) versus MTI \( 1.1×10^3 \) M\(^{-1}\) s\(^{-1}\)), suggesting that the OH side chain of Ser perturbs the pocket critical for MTI side chain binding. The bulky Val66Phe mutation in PfPNP results in \( K_a \) of 1500 μM for inosine, ~100 fold greater than for wildtype PfPNP, and this mutant has no detectable activity with MTI. Val73Phe has no activity for either inosine or MTI.

Inhibition of PfPNP mutants by Immucillins

Immucillin H (ImmH) and 5’-methylthio-immucillin H (MT-ImmH) are potent transition state inhibitors of PfPNPs [15,17,18]. Immucillins bind wild-type PfPNP with 2 distinct binding stages—an initial binding, followed by slow onset tighter binding [20]. MT-ImmH was synthesized based on the transition structure of MTI [13], and inhibits PfPNP with slow onset kinetics and \( K_s = 1.8 \) nM. MT-ImmH is more than 160-fold less effective for TgPNP with \( K_s = 290 \) nM.

ImmH and MT-ImmH were tested against PfPNP mutants. Neither inhibitor exhibits slow onset inhibition of activity of the PfPNP mutants. In general, the inhibition constants correlate with the ability of mutants to utilize inosine or MTI. ImmH binds V66I:V73I:Y160F PfPNP more than 600 times better than MT-
Table 1. Kinetic constants for mutant and wild type PNP from P. falciparum and T. gondii.

| PNP      | Inosine  | 5'-Methylthioinosine | Ino/MTI |
|----------|----------|----------------------|---------|
|          | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $k_{cat}/K_m$ Ratio |
|          |          |                      |         |          |                      |         |                      |                      |
| PiPnP    | 11 ± 5   | 1.7 ± 0.7            | 1.5 x 10$^5$ | 8.8 ± 0.2 | 0.83 ± 0.03         | 9.4 x 10$^4$ | 1.6                  |
| Hi57Ala$^{a,b,c}$ | 45 ± 7   | 1.1 ± 0.5            | 2.5 x 10$^4$ | 2.6 ± 0.0  | 0.29 ± 0.01         | 1.1 x 10$^4$ | 2.2                  |
| Hi57Ser$^{a,b,c}$ | 14 ± 3   | 0.5 ± 0.3            | 3.8 x 10$^4$ | 3.2 ± 0.7  | 0.88 ± 0.30         | 2.7 x 10$^4$ | 0.1                  |
| Hi57Phe$^{a,b,c}$ | 353 ± 22 | 2.2 ± 0.7            | 6.5 x 10$^3$ | 9.6 ± 0.8  | 0.21 ± 0.03         | 2.2 x 10$^4$ | 0.3                  |
| Arg45Ala$^{a,b}$ | 470 ± 291| 0.04 ± 0.03         | 1.1 x 10$^5$ | (-)       | (-)                | (-)        | (-)                  |
| Tyr47 Ala | 200 ± 0  | 0.04 ± 0.00         | 1.9 x 10$^5$ | 960 ± 0.0  | 0.02 ± 0.0          | 2.1 x 10$^5$ | 9.3                  |
| Val66Ala$^{a}$ | 13 ± 10  | 2.4 ± 0.6            | 1.8 x 10$^5$ | 0.3 ± 0.4  | 0.12 ± 0.04         | 3.5 x 10$^5$ | 0.5                  |
| Val66Ser$^{a}$ | 78 ± 4   | 1.4 ± 1.7            | 1.8 x 10$^4$ | 0.7 ± 0.7  | 0.21 ± 0.05         | 2.8 x 10$^5$ | 0.06                 |
| Val66Phe$^{a}$ | 1500 ± 410 | 5.7 ± 3.4            | 3.8 x 10$^5$ | (-)       | (-)                | (-)        | (-)                  |
| Val73Ala$^{a,b,c}$ | 7 ± 4    | 1.1 ± 0.1            | 1.6 x 10$^5$ | 20 ± 10    | 1.9 ± 0.3           | 9.7 x 10$^4$ | 1.6                  |
| Val73Ser$^{a,b,c}$ | 5 ± 0    | 0.2 ± 0.0            | 4.6 x 10$^5$ | 2100 ± 0.0 | 0.02 ± 0.0          | 1.1 x 10$^5$ | 4100                 |
| Val73Phe$^{a,b,c}$ | (-)  | (-)                   | (-)         | (-)       | (-)                | (-)        | (-)                  |
| Tyr160 Ala$^{c}$ | 5400 ± 120 | 0.1 ± 0.0            | 2.2 x 10$^2$ | 200 ± 59   | 0.09 ± 0.01         | 4.5 x 10$^5$ | 0.5                  |
| Met183Ala$^{a}$ | 260 ± 64 | 0.4 ± 0.3            | 1.5 x 10$^3$ | (-)       | (-)                | (-)        | (-)                  |
| Asp206Ala  | 63 ± 6   | 0.04 ± 0.01          | 6.3 x 10$^2$ | (-)       | (-)                | (-)        | (-)                  |

The mean (± SD) is calculated under the reaction conditions as described in the Materials and Methods section.

(−) Activity not detected under the experimental conditions.

*Values reported in Chaudhary, PfPNP 11.

Structure Comparisons of Wild-type and V66I:V73I:Y160F PiPnP

Wild-type PiPnP has a hexameric quaternary configuration. Since any mutation has the potential to disrupt the structural conformation of the enzyme, the mutants were assessed by gel decreased binding for MT-ImM, but has similar binding to WT PiPnP for ImM.

Table 2. Kinetic constants for P. falciparum PNP mutants simulating T. gondii PNP.

| PNP      | Inosine  | 5'-Methylthioinosine | Ino/MTI |
|----------|----------|----------------------|---------|
|          | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $K_m$ (μM) | $k_{cat}$ (s$^{-1}$) | $k_{cat}/K_m$ (M$^{-1}$ s$^{-1}$) | $k_{cat}/K_m$ Ratio |
|          |          |                      |         |          |                      |         |                      |                      |
| PiPnP    | 11 ± 6   | 1.7 ± 0.7            | 1.5 x 10$^5$ | 8.8 ± 0.2 | 0.83 ± 0.03         | 9.4 x 10$^4$ | 1.6                  |
| TgPNP$^a$ | 13 ± 1   | 2.6 ± 0.0            | 2.0 x 10$^5$ | 31 ± 3    | 0.03 ± 0.00         | 9.4 x 10$^2$ | 230                  |
| Val66Ile | 16 ± 4   | 2.0 ± 0.2            | 1.3 x 10$^3$ | 12 ± 5    | 1.2 ± 0.7           | 9.4 x 10$^4$ | 1.4                  |
| Val73lle | 13 ± 2   | 1.9 ± 0.7            | 1.4 x 10$^3$ | 11 ± 8    | 0.81 ± 0.03         | 7.1 x 10$^4$ | 2.0                  |
| Tyr160Phe | 2.5 ± 1.2 | 0.21 ± 0.02        | 8.3 x 10$^3$ | 44 ± 5    | 0.30 ± 0.04         | 6.8 x 10$^3$ | 12                   |
| Val66Ile/Val73lle | 28 ± 11  | 4.6 ± 0.6            | 1.7 x 10$^3$ | 15 ± 5    | 0.91 ± 0.20         | 6.2 x 10$^4$ | 2.7                  |
| Val66Ile/Thr160Phe | 6.2 ± 3.5 | 1.2 ± 0.16          | 1.9 x 10$^3$ | 11 ± 2    | 0.05 ± 0.00         | 4.7 x 10$^3$ | 39                   |
| Val73lle/Tyr160Phe | 3.6 ± 1.3 | 0.59 ± 0.13         | 1.6 x 10$^3$ | 60 ± 33   | 0.28 ± 0.04         | 4.6 x 10$^3$ | 35                   |
| V660/V730/Y160F | 4.0 ± 1.8 | 0.36 ± 0.23         | 9.0 x 10$^4$ | 18 ± 6    | 0.01 ± 0.00         | 5.6 x 10$^2$ | 160                  |

$^a$Values reported in Chaudhary, et al. [18].
The mean (± SD) is calculated under the reaction conditions as described in the Materials and Methods section.

(−) No activity detected under the experimental conditions.

doi:10.1371/journal.pone.0084384.t001

doi:10.1371/journal.pone.0084384.t002

doi:10.1371/journal.pone.0084384.t003
The electron density map clearly supported the presence of bound inhibitor and phosphate. Figure 4 shows the ligand, ImmH, in a 2Fo-Fc map contoured at 1.0σ. Figure S2 features the omitted Fo-Fc at 3.0σ. The omit Fo-Fc map was generated prior to building in the ligand. Similar to wild-type PIPNP, V66I/V73I/Y160F PIPNP active site residues are mainly contained in one monomer with His7 and Arg45 from the adjacent subunit binding 5'-hydroxyl of ImmH and the phosphate, respectively [17].

Cross-eyed stereo views showing residues that surround the transition state analogue inhibitor, ImmH, in V66I/V73I/Y160F PIPNP. The figure was created using MacPyMol [23]. The parental monomer surrounding the bound ImmH (green), while the yellow side chains indicate residues contributed from the adjacent subunit (Figure 5a). There is a reduction of hydrogen bonds in V66I/V73I/Y160F PIPNP to wildtype PIPNP (Figure 5b). Tyr160, Val181, Met183, Asp206, and Trp212 surround the 9-deazapurine base of ImmH. The Tyr160Phe mutant is unable to participate in hydrogen bonding with water and Asp206 due to the lack of the hydroxyl group on the Phe side chain (Figures 6 & 7).

Phosphate ion sits 4.5 Å under the O3' hydroxyl group of ImmH in the catalytic pocket. Each phosphate oxygen participates in 2 hydrogen bonds with Ser91, Arg88, Gly23, and Arg45 from the adjoining subunit. Phosphate oxygen is 3.4 Å from the Ser91 side chain of V66I/V73I/Y160F PIPNP. In wild type PIPNP the sulfate oxygen interacts with ImmH O3' [17], but in V66I/V73I/Y160F PIPNP, phosphate does not seem to interact with the 5'-hydroxyl of ImmH. The V66I/V73I/Y160F PIPNP pocket in the catalytic site near the 5'-hydroxyl group of ImmH is in closer proximity to CD1 of Val66Ile (4.7 Å), CD1 Val73Ile (5 Å), and CZ of Tyr160Phe (4.2 Å), than with wild-type PIPNP. Wild-type values as stated in Shi, et al, shows the distance of the ImmH 5'-hydroxyl group CD1 of Val66 (5.1 Å), CD1 Val73 (6.2 Å), and CZ of Tyr160 (4.6 Å) [17]. A water molecule is 3.8 Å from the 5'-hydroxyl group of ImmH in the PIPNP mutant active pocket. The O3' and O2' of the ImmH iminoribitol ring directly interact with a hydroxyl group located on the side chain of Glu184 (2.8 Å). His7 NE2 is 3 Å from the 5'-hydroxyl group of ImmH (Figure 6).

Molecular Modeling of V66I/V73I/Y160F PIPNP with bound inhibitors

The V66I/V73I/Y160F PIPNP complex with MT-ImmH ligand was modeled based on the crystal structures of PIPNP with MT-ImmH using the Modeller 8v2 comparative modeling program. The model allowed for prediction of the accommodation of MT-ImmH in V66I/V73I/Y160F PIPNP catalytic pocket (Figure 7). The overlap of the ImmH and MT-ImmH shows the difference in the 5' group jutting into the active site with representative residues. According to the comparative model with MT-ImmH, Val66Ile (CD1) is calculated to be 3.4 Å from the 5'methylthio group, whereas Val66Ile (CD1) is 4.7 Å away from 5' hydroxyl group of ImmH (Table 4). Val73Ile(CD1) is calculated to be about 4.7 Å from the 5'methylthio group, compared to Val73Ile (CD1), which is 5 Å away from 5' hydroxyl group of ImmH. The proximity of Tyr160Phe (CE2) is 4.1 Å to the MT-ImmH 5' group, compared to (CE2) 5.2 Å for 5' hydroxyl of ImmH. This model predicts that the 5' group of MT-ImmH is more crowded in the active site relative to 5' group of ImmH.

Discussion

Malaria parasite survival depends upon access to hypoxanthine obtained from direct uptake from the host environment or through the action of the Plasmodium purine salvage pathway. Since erythrocytes do not synthesize the polyamines that are essential for Plasmodium, malaria parasites are dependent on their own

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Table 3. Inhibition constants for Immucillins.

| PNP          | ImmH  | MT-ImmH |
|--------------|-------|---------|
|              | Kᵢ*  | Kᵢ*    |
|              | nM   | nM     |
| PIPNP WT     | 15   | 0.69    |
|              | 10   | 1.8     |
| TrpPNPWT*    | 450  | 2.0     |
|              | 7100 | 290     |
| Pf Val66Ile  | 15   | (-)*    |
|              | 31   | (-)     |
| Pf Val73Ile  | 280  | (-)     |
|              | 31   | (-)     |
| Pf Tyr160Phe | 0.93 | (-)     |
|              | 1200 | (-)     |
| Pf V66I/V73I | 270  | (-)     |
|              | 180  | (-)     |
| Pf V66I/V73I/Y160F | 3.6 | (-)     |
|              | 2200 | (-)     |

*Inhibition constants for Immucillins. Assays were performed with excess substrate in the presence of inhibitors. Inhibition studies measured both initial and slow onset to ascertain the initial dissociation (Kᵢ) and to determine if a steady state exists (Kᵢ*) [2].

**Values reported in Chaudhary, et al. [18].

(*) No slow onset phase was detected.

doi:10.1371/journal.pone.0084384.t003

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The electron density map clearly supported the presence of bound inhibitor and phosphate. Figure 4 shows the ligand, ImmH, in a 2Fo-Fc map contoured at 1.0σ. Figure S2 features the omitted Fo-Fc at 3.0σ. The omit Fo-Fc map was generated prior to building in the ligand. Similar to wild-type PIPNP, V66I/V73I/Y160F PIPNP active site residues are mainly contained in one monomer with His7 and Arg45 from the adjacent subunit binding 5'-hydroxyl of ImmH and the phosphate, respectively [17].

Cross-eyed stereo views showing residues that surround the transition state analogue inhibitor, ImmH, in V66I/V73I/Y160F PIPNP. The figure was created using MacPyMol [23]. The parental monomer surrounding the bound ImmH (green), while the yellow side chains indicate residues contributed from the adjacent subunit (Figure 5a). There is a reduction of hydrogen bonds in V66I/V73I/Y160F PIPNP to wildtype PIPNP (Figure 5b). Tyr160, Val181, Met183, Asp206, and Trp212 surround the 9-deazapurine base of ImmH. The Tyr160Phe mutant is unable to participate in hydrogen bonding with water and Asp206 due to the lack of the hydroxyl group on the Phe side chain (Figures 6 & 7).

Phosphate ion sits 4.5 Å under the O3' hydroxyl group of ImmH in the catalytic pocket. Each phosphate oxygen participates in 2 hydrogen bonds with Ser91, Arg88, Gly23, and Arg45 from the adjoining subunit. Phosphate oxygen is 3.4 Å from the Ser91 side chain of V66I/V73I/Y160F PIPNP. In wild type PIPNP the sulfate oxygen interacts with ImmH O3' [17], but in V66I/V73I/Y160F PIPNP, phosphate does not seem to interact with the 5'-hydroxyl of ImmH. The V66I/V73I/Y160F PIPNP pocket in the catalytic site near the 5'-hydroxyl group of ImmH is in closer proximity to CD1 of Val66Ile (4.7 Å), CD1 Val73Ile (5 Å), and CZ of Tyr160Phe (4.2 Å), than with wild-type PIPNP. Wild-type values as stated in Shi, et al, shows the distance of the ImmH 5'-hydroxyl group CD1 of Val66 (5.1 Å), CD1 Val73 (6.2 Å), and CZ of Tyr160 (4.6 Å) [17]. A water molecule is 3.8 Å from the 5'-hydroxyl group of ImmH in the PIPNP mutant active pocket. The O3' and O2' of the ImmH iminoribitol ring directly interact with a hydroxyl group located on the side chain of Glu184 (2.8 Å). His7 NE2 is 3 Å from the 5'-hydroxyl group of ImmH (Figure 6).

Molecular Modeling of V66I/V73I/Y160F PIPNP with bound inhibitors

The V66I/V73I/Y160F PIPNP complex with MT-ImmH ligand was modeled based on the crystal structures of PIPNP with MT-ImmH using the Modeller 8v2 comparative modeling program. The model allowed for prediction of the accommodation of MT-ImmH in V66I/V73I/Y160F PIPNP catalytic pocket (Figure 7). The overlap of the ImmH and MT-ImmH shows the difference in the 5' group jutting into the active site with representative residues. According to the comparative model with MT-ImmH, Val66Ile (CD1) is calculated to be 3.4 Å from the 5'methylthio group, whereas Val66Ile (CD1) is 4.7 Å away from 5' hydroxyl group of ImmH (Table 4). Val73Ile(CD1) is calculated to be about 4.7 Å from the 5'methylthio group, compared to Val73Ile (CD1), which is 5 Å away from 5' hydroxyl group of ImmH. The proximity of Tyr160Phe (CE2) is 4.1 Å to the MT-ImmH 5' group, compared to (CE2) 5.2 Å for 5' hydroxyl of ImmH. This model predicts that the 5' group of MT-ImmH is more crowded in the active site relative to 5' group of ImmH.

Discussion

Malaria parasite survival depends upon access to hypoxanthine obtained from direct uptake from the host environment or through the action of the Plasmodium purine salvage pathway. Since erythrocytes do not synthesize the polyamines that are essential for Plasmodium, malaria parasites are dependent on their own
**Figure 4. Catalytic Site of the V66I:V73I:Y160F PfPNP mutant.** Cross-eyed stereo view of catalytic site of the triple mutant (V66I:V73I:Y160F) PfPNP showing bound ligand, ImmH, in a 2Fo-Fc map (blue) contoured at 1.0σ. The resolution for this map is 2.8 Å. The figure was prepared with MacPyMol [23].
doi:10.1371/journal.pone.0084384.g004

**Figure 5. Structure of the V66I:V73I:Y160F PfPNP mutant with transition state inhibitor ImmH.** A) Cross-eyed stereo views of the catalytic site contacts in V66I:V73I:Y160F PfPNP with the transition state analogue inhibitor ImmH and PO$_4^{3-}$. The figure was created using MacPyMol [23]. **Light blue** side chains show the parental monomer surrounding the bound ImmH (green), while the **yellow** side chains indicate residues contributed from the adjacent subunit. The highlighted imino nitrogen is in **blue** and the 5'-hydroxyl oxygen is in **red**. **B** Side by side images of the decreased convalent interactions located in the enzymatic pocket of V66I:V73I:Y160F PfPNP mutant (3FOW) on the right with WT PfPNP (1NW4) as a comparison.
doi:10.1371/journal.pone.0084384.g005
Polyamine pathway for metabolic needs [31]. *Plasmodium* species need to metabolize MTA, the product of polyamine synthesis. MTA accumulation leads to feedback inhibition of polyamine synthesis, and has been shown to lead to antiproliferative effects [6,32,33]. MTA also represents an additional source of purines for *Plasmodium*.

PfPNP compensates for the streamlining of its non-redundant purine pathway by having multi-substrate activities. *Plasmodium* purine salvage enzymes, ADA and PNP, are unique in their ability to take either purines or 5'-methylthio purines. MTI is exclusively a *Plasmodium* metabolite that is neither produced nor metabolized in other Apicomplexa including *Toxoplasma* [18].

We investigated the residues in the catalytic pocket of PfPNP that interact with the 5'-methylthio group of MTI and transition state analogue MT-ImmH. We also created substrate binding site mutants of PfPNP with altered activity for inosine and MTI. V66I:V73I:Y160F PfPNP, a *P. falciparum* PNP mutant designed to mimic TgPNP, retains wild-type PfPNP efficiency with the inosine substrate, but has significantly reduced phosphorylysis of MTI. Residues that line the active site, such as Arg45 and Tyr47, are critical for enzyme function. Arg45 is conserved in family I PNPs including *Plasmodium*, *Trichomonas vaginalis*, *T. gondii*, and *E. coli* PNPs [17,18,29,34], whereas Tyr47 is conserved in *Plasmodium* species and *T. gondii*. Asp206 has been implicated in hydrogen bonding with inosine and essential for purine base binding [2,35].

*Plasmodium yoelii* PNP has Ile at position 75 that corresponds to PfPNP Val73, yet PyPNP has similar activity for both inosine and methylthioinosine as *P. falciparum* PNP [14]. As confirmed in mutagenesis studies, the Val73Ile substitution is not critical in reducing PfPNP activity for MTI, although it has a synergistic effect with the other mutations. Surprisingly, the Val73Ile PfPNP mutant binds less tightly to ImmH. The decreased sensitivity of the Val73Ile to ImmH and MT-ImmH is unexpected, since PyPNP is catalytically indistinguishable from PfPNP with similar Kd for ImmH and MT-ImmH as PfPNP ([14] and unpublished).

For many of the PfPNP mutations that affect PfPNP catalytic activity, only a modest change in Km was observed (Tables 1 and 2). Km is the concentration of substrate at which half of the enzyme catalytic sites are filled by substrate in the steady-state. Binding of transition state inhibitors will reflect the energy of binding to the transition state. Assuming the chemical step is rate limiting, the effect of mutations that alter transition state binding would be expected to correlate with kcat. Thus, the data suggest that Tyr160 of PfPNP is particularly important for binding to the transition state. Comparison of kcat/Km illustrates the changes in catalytic efficiency of the mutant enzymes compared to the wild-type for MTI and inosine.

The crystal structure of V66I:V73I:Y160F PfPNP:ImmH:PO4<sup>3-</sup> supports the results from the enzymology studies of the mutants. The crystal structures of wild-type PfPNP bound to ImmH and MT-ImmH differ in the position of the 5'-
Table 4. V66I:V73I:Y160F PfPNP Mutant Residues R-Group Atom Distances to Immucillins.

| Site directed mutant PfPNP | Wild type PfPNP | Mutant PfPNP ImmH -3'-methylthio | Mutant PfPNP MT-ImmH -3'-methylthio |
|---------------------------|----------------|----------------------------------|------------------------------------|
| Val66 | CD1 | C5 | CS | Val66 | CD1 | C5 | CS | Val66 | CD1 | C5 | CS | Val66 | CD1 | C5 | CS |
| Val73 | CD2 | C5 | CS | Val73 | CD2 | C5 | CS | Val73 | CD2 | C5 | CS | Val73 | CD2 | C5 | CS |
| Tyr160 | CD1 | C5 | CS | Tyr160 | CD1 | C5 | CS | Tyr160 | CD1 | C5 | CS | Tyr160 | CD1 | C5 | CS |

R-group carbons of comparatively designed V66I:V73I:Y160F PfPNP:MT-ImmH and V66I:V73I:Y160F PfPNP:ImmH crystal structure distances from 5'-methylthio group of the inhibitor [17]. The 5'-methylthio group of MT-ImmH is rotated approximately 135° when compared to the 5'-hydroxyl of ImmH, allowing the 5'-methylthio group to occupy a hydrophobic pocket in the active site that is different from the hydrophilic pocket that faces the 5'-hydroxyl group [17]. When ImmH is bound to the wild-type PfPNP active site, Tyr160, through a water-mediated contact, interacts with the 5'-hydroxyl group of the inhibitor. The Tyr160Phe in V66I-V73I-Y160F PfPNP cannot participate in the water-mediated interaction with the 5'-hydroxyl of ImmH due to the hydrophobic properties of the Phe160 side chain. Tyr160Phe mutation in PfPNP reduces the water-mediated hydrogen bonds with Asp206 and water molecules in the pocket. The Tyr160Phe mutation coupled with either Val66Ile or Val73Ile significantly reduces the efficiency of PfPNP for MTI.

Although the V66I-V73I-Y160F PfPNP:ImmH-PO4 structure is limited by its 2.8 Å resolution, there appear to be fewer water molecules found in the hydrophobic region of the catalytic site that coincides with the increased number of hydrophobic residues in the V66I-V73I-Y160F PfPNP hydrophobic pocket of the active site. The V66I-V73I-Y160F PfPNP triple mutations reduce the docking of the 5'-methylthio side pocket in the catalytic domain. In the wild-type PfPNP (1NW4) structure, Val66 (5.1 Å CD1 and Val73 (5.4 Å CG2), and Val73 (6.4 Å CG1, 6.6 Å CG2) methyl groups sit further away from the 5'-hydroxyl group of the iminoribitol region of ImmH when compared to the structure of V66I-V73I-Y160F PfPNP with ImmH (3FOW) [Val66Ile - 4.7 Å CD1 and Val73Ile - 5.0 Å CD1].

This work shows that it is possible to selectively modify MTI activity while retaining inosine activity of PfPNP. Tyr160 is a conservative substitution for the Phe residue present in TgPNP, allowing the Tyr160Phe activity while retaining inosine activity of PfPNP, the Tyr160 is critical, and a Tyr160Phe substitution in PfPNP significantly diminishes PfPNP sensitivity to MT-ImmH. Similarly, the Val73Ser mutation has a dramatic effect upon MTI activity without loss of inosine activity, presumably because the hydrophobic pocket that accommodates the MT sidechain is disrupted by the hydrophobic OH of Serine.

New antimalarials are needed to subdue the increase of drug resistant parasites, which is part of a concerted global effort to control the spread of the disease [36]. Ideally, new chemotherapeutic agents will specifically inhibit parasite targets without significantly affecting host metabolic pathways. Since there is a lack of redundancy in the Plasmodium purine pathway, and purine salvage is essential, a variety of compounds that target the purine salvage pathway are being investigated as potential antimalarials [37]. Our studies elucidate the molecular basis of differences in substrate affinities and differential susceptibility to imincumilins between PfPNP and TgPNP. Since Plasmodium parasites develop resistance to antimalarials readily, our studies suggest that resistance could develop to antimalarials based on targeting the unique 5'-methylthio activity of PNPN without compromising activity against inosine. These studies should aid in the future rational design of PfPNP inhibitors as potential antimalarials.

Supporting Information

Figure S1 Secondary structure of wild-type and mutant PNPs. Circular dichroism of wild-type and mutant PfPNPs to compare secondary structure. Enzyme concentration for each run was 0.1 mg/ml (3.3 nM). Spectra was converted to molar ellipticity (θ) after subtracting the solution of 10% HEPES buffer in water for baseline measured at 4°C. Molar ellipticity conversion =

P. falciparum PNP Methylthio Specificity
Acknowledgments

Immucillins were provided by Drs. Peter C. Tyler, Gary B. Evans of the Industrial Research Ltd. (now named Callaghan Innovation, Lower Hutt, New Zealand), and Dr. Vern Schramm of the Department of Biochemistry of the Albert Einstein College of Medicine (Bronx, NY). We gratefully acknowledge Dr. Sean Decatur for technical advice and allowing us to use the Avizo 215 CD spectrometer at Mount Holyoke College for circular dichroism experiments and thank Dr. Maria Belen Cassera for technical advice throughout this project. We also thank Dr. Schramm for his advice and stimulating discussions throughout the course of this work. Most of this work was submitted in partial fulfillment of the requirements for a Doctor of Philosophy in the Graduate Program in Biomedical Sciences of the Albert Einstein College of Medicine (TMD). Data of X-ray diffraction for this study were measured at beamline X29A of the National Synchrotron Light Source and Beamline 24-ID-E at the Northeastern Collaborative Access Team beamlines of the Advanced Photon Source.

Author Contributions

Conceived and designed the experiments: TMD LMT WS RZ SCA KK. Performed the experiments: TMD LMT CZ WS RZ. Analyzed the data: TMD LMT CZ WS RZ SCA KK. Wrote the paper: TMD KK.

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