Energetic Mapping of Transition State Analogue Interactions with Human and *Plasmodium falciparum* Purine Nucleoside Phosphorylases*

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Human purine nucleoside phosphorylase (huPNP) is essential for human T-cell division by removing deoxyguanosine and preventing dGTP imbalance. *Plasmodium falciparum* expresses a distinct PNP (PfPNP) with a unique substrate specificity that includes 5'-methylthioinosine. The P/PNP functions both in purine salvage and in recycling purine groups from the polyanine synthetic pathway. Immucillin-H is an inhibitor of both huPNP and PfPNPs. It kills activated human T-cells and induces purine-less death in *P. falciparum*. Immucillin-H is a transition state analogue designed to mimic the early transition state of bovine PNP. The DADMe-Immucillins are second generation transition state analogues designed to match the fully dissociated transition states of huPNP and PfPNP. Immucillin-H and DADMe-Immucillin-H are 860 and 500 pM inhibitors against huPNP and PfPNPs, respectively. These are extraordinary inhibitors of huPNP (K<sub>d</sub> values of 16 and 7 pM, respectively) and have both characterized by symmetric near fully dissociated ribooxacarbonium ion transition states (10). The second generation transition state analogue inhibitors were synthesized to mimic these dissociated transition states and include DADMe-Immucillin-H and DADMe-Immucillin-G. These are extraordinary inhibitors of huPNP with K<sub>d</sub> values of 6pM for huPNP and P/PNP. Immucillin-H (ImmH) is the first transition state analogue inhibitor developed in this class and shows a 56 pm K<sub>d</sub> for huPNP (6). It has recently entered phase II clinical trials against T- and B-cell cancers (7). ImmH was designed from the early ribooxacarbonium ion transition state structure of bovine PNP (8). More recently, the transition state structures have been solved for huPNP and P/PNP, which are both characterized by symmetric near fully dissociated ribooxacarbonium ion transition states (9).

Inhibition of human purine nucleoside phosphorylase (huPNP) by transition state analogue inhibitors shows promise for the control of T-cell cancers and autoimmune diseases (1–5). The combined inhibition of human and *P. falciparum* PNP kills parasites cultured in human erythrocytes by purine-less death (4, 5). Immucillin-H (ImmH) is the first transition state analogue inhibitor developed in this class and shows a 56 pm K<sub>d</sub> for huPNP (6). It has recently entered phase II clinical trials against T- and B-cell cancers (7). ImmH was designed from the early ribooxacarbonium ion transition state structure of bovine PNP (8). More recently, the transition state structures have been solved for huPNP and P/PNP, which are both characterized by symmetric near fully dissociated ribooxacarbonium ion transition states (9).

*Plasmodium falciparum* cultured in human erythrocytes without added hypoxanthine are killed by ImmH due to purine starvation in these purine auxotrophs (4, 5). The addition of exogenous hypoxanthine but not inosine rescues the parasites from killing by ImmH. This metabolic rescue pattern supports the Immucillin metabolic block at PNP (4). ImmH binds more tightly to huPNP than to P/PNP and inhibitors that bind selectively to P/PNP are important to establish if...
both huPNP and P.fPNP must be blocked to induce purine-less death.

Immcullin and DADMe-Immcullin inhibitor families have been expanded to include the 5’-methylthio functional group specific for the P. falciparum enzyme (11). DADMe-ImmmG and DADMe-ImmmH are the tightest binding inhibitors for both huPNP and P.fPNP. A methylene group is used in DADMe-ImmmH to increase the distance between the ribooxacarbenium cationic mimic and the 9-deazaahxopxanthine leaving group. These features closely resemble the transition states for both huPNP and P.fPNP. DADMe-ImmmH binds tighter to huPNP than ImmH; however, it binds less well to P.fPNP, despite its similarity in ribooxacarbenium character to the P.fPNP transition state. Differences in catalytic site properties can explain the difference in binding energy for transition state analogues of human and P. falciparum PNPs. The catalytic turnover number for huPNP (inosine) is 18-fold greater than for P.fPNP. Binding of transition state analogue inhibitors is proportional to the catalytic rate enhancement imposed by the enzyme (12). Inhibitors capturing similar features of the transition state for inosine phosphorylation are therefore expected to bind ~18-fold weaker to the malarial enzyme and this is observed in the inhibitor family. However, 5’-methylthioinosine (MTI) is a good substrate for P.fPNP and a weaker substrate for human PNP (11). Thus, transition state mimics of MTI that contain a 5’-methylthio group are bound more tightly to P.fPNP.

Human PNP achieves the transition state by a combination of ribooxacarbenium ion stabilization and purine leaving group activation. A mechanistic feature for ribooxacarbenium ion formation for huPNP involves crowding or overlap of the lone pair electrons from the 5’-hydroxyl to interact with the ribosyl 4’-ring oxygen. This facilitates the electron “push” into the leaving group (13). The action of P.fPNP on 5’-methylthioinosine precludes use of the 5’-oxygen lone pair mechanism. Immucillin-H binds to human PNP as the neutral molecule and in a second, slow onset tight binding step is protonated at 4’-N in the enzyme active site to form the cationic mimic of the transition state (14). This pattern is also involved in catalysis where neutral inosine binds and becomes the cationic ribooxacarbenium ion at the transition state. Recently, the x-ray crystal structure was solved for P.fPNP with ImmH and MT-ImmH at the catalytic sites (11). Together with the transition state analogue inhibitor specificity shown here, it is apparent that the leaving group interactions for P.fPNP are stronger than for huPNP as they must compensate for the loss of the 5’-hydroxyl lone pair interaction.

Preferential binding of transition state analogue inhibitors to P.fPNP was observed only in the case of MT-Immcullin-H (Fig. 1). Interaction of other analogues with huPNP and P.fPNP provides a geometric and electrostatic map for interactions at their catalytic sites. The specificity studies support two essential features for tight binding to both PNPs, elevated $pK_a$ of the leaving group, and the ability to form a cation to mimic the ribooxacarbenium ion transition state.

MATERIALS AND METHODS

Human PNP—Human PNP was expressed in a His$_6$-N-terminal construct to facilitate purification and purified to homogeneity for these kinetic studies (9). The enzyme has also been expressed without N-terminal extension and the kinetic properties are unchanged by the N-terminal modification.

P. falciparum PNP—Purine nucleoside phosphorylase from P. falciparum was expressed as described earlier, except that a His$_6$-N-terminal extension was added to facilitate purification (11). P.fPNP has been expressed in Escherichia coli with and without the His-tag, and the extension does not change the kinetic properties.

Inhibitor Synthesis—Chemical synthesis, purification, and structural characterization of most of the inhibitors described here have been described previously (15–20). 5’-Methylthio- and 5’-phenylthio-Immcullin-H (MT-ImmmH and Pht-ImmmH) were prepared from 1,4-dideoxy-1,4-imino-D-ribitol in a similar manner to that described for the preparation of 5’-aza-2’-deoxy-1’(9-methylene) modification of MT-DADMe-ImmmH, 5’-PfT-DADMe-ImmmH, and 5’-5’-Me-DADMe-ImmmH were synthesized by application of the Mannich reaction on the parent amines with formaldehyde and 9-deazaahxopxanthine as described for the 9-deazaadenine analogues (22). These compounds were prepared from their amines by a Mannich reaction with formaldehyde and 9-deazaahxopxanthine (17). 4’-OH-HDADMe-ImmmH was synthesized by the same Mannich reaction applied to (3R,3S,4,4-dihydroxy-3-methylthiopropyldimethylcarboxylate (23). This amine was also converted into the corresponding 3-methylthiopropylcarboxylate from which 5’-MT-4’-OH-HDADMe-ImmmH was obtained. Treatment of 7-benzyloxymethyl-9-deaza-9-formyl-6-O-methylhyxopxanthine (19) with triphenylphosphorane and hydorboration of the product followed by oxidation gave 7-benzoyloxymethyl-9-deaza-6-O-methyl-9(2-oxoethyl)-hyxopxanthine which on reductive amination with (3R,4R)-3-hydroxy-4-hydroxymethylcarboxylate and degradation (19) gave DADET-ImmmH. Treatment of 7-benzyloxymethyl-9-deaza-6-O-methylhyxopxanthine with butyl lithium and then DMP gave 7-benzyloxymethyl-9-deaza-8-formyl-6-O-methylhyxopxanthine, which on reductive amination with (3R,4R)-3-hydroxy-4-hydroxymethylcarboxylate and degradation (19) gave 1,8-DADMe-ImmmH. Desilylation of (1S,2S,3S)-1-(6-O-benzyl-7-benzyloxymethyl-9-yl)N-tert-butoxycarbonyl-5-O-tert-butylidemethylsilyls-1,4-dideoxy-1,4-imino-2,3-O-isopropylidene-n-ribitol (16) and oxidation (PDC in DMP) gave the 5’-carboxylic acid, which was deprotected to 5’-COOH-ImmmH. Methylation of the protected carboxylic acid afforded the methyl ester that was treated with ammonia in methanol and then deprotected to give 5’-CONHOH-ImmmH (Fig. 1).

Inhibition Studies—Inhibitor dissociation constants for the phosphorylation of inosine were based on initial and equilibrium reaction rate measurements with varied inhibitor concentrations (6, 24). Reactions were started by adding huPNP (1.4 nM) or 1.0 µg of P.fPNP (32 nM) to reaction mixtures (25 °C) containing 1 mM inosine in 50 mM KHPO$_4$ pH 7.4, with xanthine oxidase at 60 milliunits/ml. Hyxopxanthine formed by phosphorylation of inosine was oxidized to uric acid and monitored spectrophotometrically at 295 nm (extinction coefficients for uric acid in 0.1 M phosphate buffer, pH 7.4, $e_{295} = 12.9$ mmol$^{-1}$ cm$^{-1}$). The Michaelis constants for inosine were measured at the same phosphate concentration for huPNP and P.fPNP. Enzyme concentration was adjusted to give absorbance changes not exceeding 1.0 during the time required to characterize initial and final slow onset inhibition equilibria. The large excess of substrate and continuous product depletion provided extended initial rate conditions. In most cases the inhibitor concentration was $>10$-fold greater than the enzyme concentration as required for simple analysis of two-state slow onset tight binding inhibition (24). In the cases of the most tightly bound inhibitors it was not possible to maintain this condition and corrections were made to compensate for the concentration of bound inhibitor (10). The inhibition constant $K_i$ describes the reversible equilibrium between enzyme and inhibitor for the initial inhibitor binding step, $K_i$ was determined by fitting the initial rates at different inhibitor concentrations to the equation for competitive inhibition: $v = (k_{cat} \times S) / (K_i (1 + I / K_i) + S)$, where $v$ is initial reaction rate, $k_{cat}$ is the catalytic turnover number, $K_i$ is the Michaelis constant, $K_i$ is the dissociation constant of enzyme-inhibitor complex ($EI$), $I$ is inhibitor concentration, and $S$ is substrate concentration. The dissociation constant for the complex formed after slow onset equilibrium ($K_{eq}$) was determined by $v = (k_{cat} \times S) / (K_{eq} (1 + I / K_{eq}) + S)$, where $v$ is the steady state reaction rate and the other variables are the same as above.

RESULTS AND DISCUSSION

Inhibition of huPNP by Immucillins—ImmmH, ImmG, and their 2’-deoxy analogues (2’-d-ImmmH and 2’-d-ImmG) are the four most potent Immucillin inhibitors for huPNP with dissociation constants of 42 to 180 nM (Table I, Fig. 1). Two 8-sub-
stututated Immucillins, 8-aza-ImmH and 8-F-ImmH, are also pm inhibitors with dissociation constants of 180 and 390 pm, respectively, but the 8-Me group increases the dissociation constant to 20 nm, similar to N7 methylation, which gives a dissociation constant of 100 nm for 7-Me-ImmH. Immucillin-H molecules with 2'-alterations (2',2'-difluoro, 2'-ara, 2'-methoxy) are still good inhibitors with dissociation constants of 1.4–5.9 nm. Thus, the catalytic site of huPNP is tolerant at the 2'-position, as expected for its substrate specificity to accept both inosine and guanosine and their 2'-deoxy analogues with approximately equal catalytic efficiency. 3'-o-ImmH gave a dissociation constant of 7.5 nm indicating that 3'-hydroxyl contacts are more important than those at the 2'-hydroxyl. Alterations in the exocyclic 6-carbonyl oxygen to give 6-MeO-ImmH or 6-S-ImmH increased the dissociation constants to 4.7 and 25 nm. Changes in the 5'-hydroxyl group causes large losses in affinity for huPNP, consistent with the catalytic mechanism involving the neighboring group participation of the lone pair of the 5'-hydroxy in stabilizing the riboxoacarbenium ion (13). Thus, the 5'-F-ImmH, 5'-o-ImmH, 5'-PhT-ImmH, 5'-MT-ImmH, 5'-CONH2-ImmH, and 5'-COOH-ImmH increase K_i values to 6.8 nm, 25 nm, 250 nm, 300 nm, >120 μm, and >190 μm, respectively. Binding of 1'-aza-inosine and 4'-N'-ImmH to huPNP was not detected at 38–240 μm (Fig. 1). Addition of a propyl group to N4', or switching the ring N3 and C4 atoms decreased affinity to give dissociation constants of 410 nm and 1.9 μm, respectively. The changes in ImmH chemical structure summarized in Fig. 1 cause up to a 5,700,000-fold (9.4 kcal/mol) change in binding affinity for huPNP.

An important feature of the specificity result (Fig. 1) is that ImmH and ImmG, the transition state analogues originally designed for bovine PNP, are the tightest binding Immucillin inhibitors for both huPNP and bovine PNP (26). Second, there are six Immucillins with dissociation constants in the pm range. The ps inhibitors are modified only at the C8 or by being 2'-deoxy.

The pK_a of N7 is an important determinant for transition state formation for mammalian PNPs. Modest perturbations in pK_a values with little alteration in steric volume cause only modest changes in K_i. Thus, 8-F-ImmH and 8-aza-ImmH have K_i values of 390 and 180 pm, while the bulkier 8-Me-ImmH has K_i increased to 20 nm. Likewise, 7-Me-ImmH has a K_i of 100 nm. The ability of N7 substituted purine nucleosides to bind and to act as substrates of PNPs has been used as a rationale to distinguish the group in H-bond contact to O4, and N7 at the transition state is not important to catalysis. In contrast, N7 methylation generates a cationic site on the purine and serves to chemically activate the purine leaving group, making the H-bond at N7 unnecessary for leaving group departure. Crystallographic studies with huPNP have demonstrated that Asn243, the group in H-bond contact with N7, is mobile and can move to accommodate N7 substituents, a finding consistent with the modest perturbation in Immucillin binding by the N7-methyl substitution and the observation that 7-Me-inosine is a substrate for huPNP (27).

Purine nucleoside phosphorylases have no protein contacts to O4' of purine nucleosides or to N4' of Immucillins, permitting space in the catalytic site for the ribosyl group to migrate between the purine and the phosphate (13, 28). Even the relatively bulky 5'-Pr-ImmH (K_i = 410 nm) binds better than substrate (K_m ~ 30 μm) confirming the ability of huPNP to accept this volume, even though binding 7,300-fold more weakly than ImmH.

Transition state analogues require faithful representation of both geometric and electrostatic properties of the transition state. 4'-N'-ImmH violates this principle by linking the purine to the 4'-imino nitrogen, a geometric change. No inhibition is detected at 38 μm, indicating a million-fold loss in affinity. Similar consequences accompany electrostatic changes. Thus, DAD'-1',9-hypoxanthine and 4-aza-3-deaza-ImmH have low pK_a values at N7, while ImmH and related analogues with 9-deazaahypoxanthine rings have a pK_a of above 10 for N7 (14). Without the increased basicity at N7, tight binding inhibition is not detected, resulting in up to 4.3 million-fold loss in binding affinity, despite the geometric similarity of these molecules to both substrate and transition state.

Inhibition of P/PNP by Immucillins—The k_cat values for phosphorolysis of inosine by huPNP and P/PNP are 31 s^-1 and 1.7 s^-1, respectively, making huPNP 18-fold more efficient than P/PNP. Catalytic efficiency is only an approximation of transition state stabilization for these PNPs, since chemistry is not completely rate-limiting for either enzyme (9). On the basis of transition state principles, it would be expected that transition state analogues of inosine would bind more weakly to P/PNP by the rate enhancement factor. This relationship is confirmed for the better Immucillin inhibitors (Fig. 1). Thus, ImmH, ImmG, 2'-d-ImmH 2'-d-ImmG, 8-F-ImmH, 8-aza-ImmH, and 3'-d-ImmH are relatively faithful mimics of an early riboxoacarbenium ion transition state and contain the essential recognition elements of the elevated pK_a at N7, the imino cation at N4', and the 5'-hydroxyl. These inhibitors bind better to the huPNP by factors of 15-, 21-, 16-, 47-, 23-, 72-, and 6-fold, respectively, in good agreement with the approximate 18-fold difference in transition state efficiency. Deviations from this relationship are seen in the analogues where the iminoboritol character, features of leaving group activation, or the 5'-hydroxyl modifications are made. Thus, 5'-MT-ImmH and 5'-PhT-ImmH bind 112- and 2-fold better to P/PNP than to human PNP. This reversal reflects the P/PNP substrate specificity for 5'-methylthioinosine, which is equal to inosine as a substrate for P/PNP (11). The methylthio group is found in a hydrophobic region of the catalytic site for P/PNP and this site also favors interaction with the larger phenyl group.

The ability of P/PNP to use 5'-methylthioinosine as a substrate precludes the lone pair interaction between the 5'-hydroxyl and the 4'-ring oxygen, an interaction proposed to be important for phosphorolysis of inosine and guanosine (29). Loss of this transition state stabilizing interaction may be associated with the decreased k_cat for P/PNP. However, the P/PNP compensates for this loss with other interactions to increase catalytic efficiency. Strengthened interactions to N7, O6, and O4', at the transition state are all possibilities. Interactions at the 2'-hydroxyl are more important for the malarial enzyme, since 2'-MeO-ImmH and 2',2'-diF-ImmH bind huPNP 140 and >3,300 times more tightly than P/PNP. Increased H-bond donation from the enzyme to the 2'-oxygen would decrease electronic contribution into the carbocation and facilitate transition state formation.

Volume near the 4'-imino cation is more restricted at the catalytic site of P/PNP than for huPNP, since N4'-Pr-ImmH binding was not detected at 300 μm with P/PNP but binds with a dissociation constant of 410 nm to huPNP. The confined geometry around the iminoboritol cation in P/PNP is also apparent with DAD'-1', 9-hypoxanthine and 4'-N'-ImmH, since neither bind to P/PNP at concentrations of 240–870 μm. However, these inhibitors also fail to bind to huPNP.

Transition states of mammalian PNPs involve leaving group activation by N7 protonation or H-bonding at N7 via a carbonyl oxygen hydrogen bond to Asn243, in addition to interactions at
**Fig. 1. Immucillin inhibitors of huPNP and PfPNP.** Each inhibitor is shown with two individual $K_i$ or $K_i^*$ values. The *upper line* (in blue) is the equilibrium dissociation constant for huPNP, and the *middle line* (in red) is the equilibrium dissociation constant for PfPNP. Where the value is a $K_i^*$, the dissociation constant occurs following slow onset tight binding inhibition as described under “Materials and Methods.” Where the value is a $K_i$ value, there is no slow onset, and a single dissociation constant is observed. Both $K_i^*$ and $K_i$ values listed here are thermodynamic dissociation constants and can be directly compared. The *bottom line* is in blue to indicate tighter binding to huPNP and in red to indicate tighter binding to PfPNP. Note that only two inhibitors bind more tightly to PfPNP than to huPNP and are shown in bold. The value given in the *bottom line* is the ratio of dissociation constants. Atomic numbering used for all inhibitors is given for ImmH.
Immucillin Inhibitors of Human and Malarial PNPs

### Table I

| Inhibitor | HuPNP $K_i$ (nm) | HuPNP $K_i^*$ (nm) | PfPNP $K_i$ (nm) | PfPNP $K_i^*$ (nm) |
|-----------|------------------|--------------------|------------------|--------------------|
| ImmH$^*$  | 3.3 ± 0.2        | 0.056 ± 0.015      | 0.86 ± 0.08      | ND$^b$             |
| ImmG$^*$  | 0.54 ± 0.1       | 0.042 ± 0.006      | 0.9 ± 0.2        | ND$^a$             |
| 2'-n-ImmH$^*$ | 0.14 ± 0.01    | ND                 | 2.2 ± 0.1        | ND$^a$             |
| 5'-MT-ImmH | 300 ± 80        | ND                 | 22 ± 3           | 2.7 ± 0.4          |
| 2'-n-ImmG$^*$ | 0.18 ± 0.01    | ND                 | 8.5 ± 0.9        | ND$^a$             |
| Ara-ImmH$^*$ | 3.5 ± 0.3       | ND                 | 9.1 ± 2.4        | ND$^a$             |
| 8-F-ImmH$^*$ | 5.6 ± 0.7       | 0.39 ± 0.02        | 9.1 ± 0.5        | ND$^a$             |
| 8-Aza-ImmH | 1.4 ± 0.2        | 0.18 ± 0.02        | 110 ± 10         | 13 ± 2             |
| 6-MeO-ImmH | 4.7 ± 0.5        | ND                 | 450 ± 30         | 26 ± 7             |
| 5'-n-ImmH$^*$ | 7.5 ± 0.8       | ND                 | 42 ± 2           | ND$^a$             |
| 5'-n-ImmH  | 25 ± 1           | ND                 | 280 ± 20         | 53 ± 6             |
| 6-T-ImmH   | 25 ± 1           | ND                 | 58 ± 6           | ND$^a$             |
| 5'-F-ImmH  | 81 ± 5           | 6.8 ± 1.9          | 240 ± 20         | 60 ± 11            |
| 5'-PhT-ImmH | 250 ± 5         | ND                 | 150 ± 8          | ND$^a$             |
| 2'-MeO-ImmH | 6 ± 1           | ND                 | 840 ± 70         | ND$^a$             |
| 4'-N-Pr-ImmH | 410 ± 90        | ND                 | >300,000$^a$     | ND$^a$             |
| 2', 2',6-dIf-ImmH | 1.4 ± 0.2 | ND                  | >15,000          | ND$^a$             |
| 8-Me-ImmH$^*$ | 20 ± 1          | ND                 | >100,000         | ND$^a$             |
| 5'-CONH$_2$-ImmH | >120,000     | ND                 | >120,000         | ND$^a$             |
| 5'-COOH-ImmH | >190,000       | ND                 | >190,000         | ND$^a$             |
| 4'-Aza-3-deaza-ImmH | 1,900 ± 400  | ND                 | >150,000         | ND$^a$             |
| 7-Me-ImmH   | 100 ± 30        | ND                 | 900 ± 160        | ND$^a$             |
| DAD-D'-9'-hypoxanthine | >240,000     | ND                 | >240,000         | ND$^a$             |
| 4'-N'-ImmH  | >38,000         | ND                 | >870,000         | ND$^a$             |

$a$ Inhibition constants were originally reported in Kiesca et al. (25). Some values reported here differ from the earlier report and reflect use of overexpressed PNPs and more extensive kinetic analysis with both huPNP and PfPNP.

$b$ ND, there is no slow onset observed, thus, only $K_i$ is measured from inhibition studies.

When no inhibition was observed at a given concentration, the $K_i$ limit was set at three times the inhibitor concentration that gave no inhibition.

### Table II

| Inhibitor | HuPNP $K_i$ (nm) | HuPNP $K_i^*$ (nm) | PfPNP $K_i$ (nm) | PfPNP $K_i^*$ (nm) |
|-----------|------------------|--------------------|------------------|--------------------|
| DADMe-ImmH$^*$ | 1.1 ± 0.1    | 0.016 ± 0.001      | 0.50 ± 0.04      | ND$^b$             |
| DADMe-ImmG$^*$ | 0.16 ± 0.03  | 0.007 ± 0.001      | 0.89 ± 0.06      | ND$^a$             |
| 5'-d-5'-Me-DADMe1ImmH | 1.48 ± 0.3 | 0.36 ± 0.03        | 11.13 ± 1.7      | 1.6 ± 0.2          |
| 8-Aza-DADMe-ImmH | 2.0 ± 0.05   | ND                 | 5.5 ± 0.2        | ND$^a$             |
| 5'-MT-DADMe-ImmH  | 0.30 ± 0.01  | 0.07 ± 0.01        | 11 ± 4           | 0.9 ± 0.1          |
| 5'-P't-DADMe-ImmH  | 0.39 ± 0.04  | 0.041 ± 0.005      | 160 ± 40         | ND$^a$             |
| 4'-OH-5'n-or-3'-4'Seco-DADMe-ImmH | 1.3 ± 0.1 | ND                  | 170 ± 10         | ND$^a$             |
| 4',9-N'-Me-DADMe-ImmH | 2.8 ± 0.1   | ND                 | 530 ± 70         | ND$^a$             |
| DADMe1-ImmH | 1.6 ± 0.3    | 0.46 ± 0.05        | 90,000 ± 23,000  | 610 ± 150         |
| 3',4'-Seco-DADMe-ImmH | 120 ± 8     | ND                 | >3,000$^a$       | ND$^a$             |
| 4'-OH-DADMe1-ImmH | 430 ± 10   | ND                 | 45,000 ± 3,000   | ND$^a$             |
| 5'-MT-4'-OH-DADMe1-ImmH | 430 ± 10 | ND                  | 45,000 ± 3,000   | ND$^a$             |
| 1',9'-Me-ImmH | 250 ± 7     | ND                 | >200,000         | ND$^a$             |
| 1',8'-DADMe1-ImmH | 6,600 ± 500 | ND                 | 130,000 ± 20,000 | ND$^a$             |
| 1',9'-Me-Amma | 440 ± 90    | ND                 | >150,000         | ND$^a$             |

$a$ Inhibition constants were originally reported in Lewandowicz et al. (10).

$b$ ND, there is no slow onset observed; thus, only $K_i$ is measured from inhibition studies.

When no inhibition was observed at a given concentration, the $K_i$ limit was set at three times the inhibitor concentration that gave no inhibition.

O6 and N1 (13, 21). PfPNP has Asp$^{296}$ as a general acid at this position and acts as a more favorable H-bond partner to N7, promoting leaving group activation (11). Interaction between Asp$^{296}$ and N7 is explored with N7 methylation of ImmH in 7-Me-ImmH (Fig. 1). While 7-Me-ImmH is a 4.7 ns inhibitor of huPNP, no inhibition of PfPNP was detected at 240 μs; thus, the discrimination factor is >51,000 against PfPNP. The N7-methyl effect supports the proposal that more of the transition state energy in PfPNP arises from the leaving group interaction at N7 than in huPNP.

### Inhibition of huPNP by DADMe (methylene-bridged)-Immucillins

The recent determination that both huPNP and PfPNP have highly dissociated riboxacarbenuim transition states led to the development of the DADMe-Immucillins (9, 10, 17). DADMe-ImmH and DADMe-ImmG are the two tightest binding inhibitors known for huPNP with $K_i^*$ values of 16 and 7 ps, respectively (Table II and Fig. 2). Disruption of the 5'-hydroxyl interaction in 5'-d-5'-Me-DADMe-ImmH, 5'-MT-DADMe-ImmH, and 5'-P't-DADMe-ImmH show small decreases in affinity of 1.3–23-fold relative to DADMe-ImmH and DADMe-ImmG. DADMe-Immucillins do not permit the 5'-hydroxyl lone pair interaction with the 4'-amino cation found in the Immucillins; thus, loss of the 5'-hydroxyl interaction has a smaller energetic consequence in huPNP binding.

Chemical stability of the DADMe-Immucillins requires them to be 2'-deoxy. The physiological substrate for huPNP is 2'-deoxyguanosine; thus, the 2'-deoxy feature of DADMe-ImmG increases its structural mimicry of the transition state with
dGuo. Placement of the cation-generating pyrrolidine nitrogen at the 1'-position more accurately mimics the carboxocation electrostatics of this dissociated S$_2$1-like transition state (9). Accordingly, DADMe-ImmG is the most tightly bound transition state analogue yet known for huPNP. The leaving group $pK_a$ value at N7 is also important to capture transition state binding energy. 8-Aza-DADMe-ImmH has a decreased $pK_a$ at N7 (9.6) compared with that of DADMe-ImmH, which is >10, based on the $pK_a$ for Immucillin-H (14). This difference causes a 125-fold decrease in binding affinity.

The hydroxypyrrolidine ring-opened analogues (4'-OH-5'-nor-3',4'-secodADMe-ImmH and 3',4'-secodADMe-ImmH) retain the 9-deaza-hypoxanthine feature of DADMe-ImmH and bind with 1.3–120 nM affinity, a decrease in binding energy reflecting the increased entropic cost of freely rotating groups (Fig. 2). Additional volume and a potential for new H-bonds in the hydroxypyrrolidine are provided by the 4'-hydroxyl substituent in 4'-OH-DADMe-ImmH. This change decreases binding by 875-fold relative to DADMe-ImmH to 14 nM. The decrease in binding due to the 4'-hydroxyl substituent between 5'-MT-DADMe-ImmH and its 4'-hydroxy derivative is similar at a 840-fold decrease, emphasizing the importance of geometry around the ribooxacarbenium ion mimic (Fig. 2).

The methylene bridge that characterizes the DADMe-Immucillins adds both distance and angular geometric relationships between the deazapurine and the ribocation mimic. This geometry was explored with an ethyl bridge in DADMe-ImmH, a methylene bridge to N4' of iminoribitol in 4',9-Me-ImmH, a link to C8 of the deazapurine in 1',8-DADMe-ImmH, and a methylene spacer inserted into ImmH in 1',9-Me-ImmH. These alterations decreased binding affinity relative to DADMe-ImmH, by factors of 29, 170, 410,000, and 16,000 respectively. 1',9-Me-ImmA retains the high $pK_a$ at N7 and the iminoribitol group as the carbocation mimic, but the 6-amino group prevents interaction at the catalytic site and the $K_i$ value is >150 μM.

**Inhibition of PfPNP by DADMe-Immucillins**—The altered catalytic capacity of PfPNP relative to huPNP predicts ~18-fold decreased binding affinity of transition state analogues to the Pf enzyme (see above). The DADMe-Immucillins, in every example, bind more tightly to huPNP than PfPNP (Table II and Fig. 2). DADMe-ImmH, DADMe-ImmG, and MT-DADMe-ImmH are the most tightly bound inhibitors for PfPNP at 500, 860, and 900 pM, respectively, and are the only pM inhibitors for PfPNP in the DADMe-Immucillin family. The relative affinity for huPNP and PfPNP is close to the values expected from...
transition state stabilization with DADMe-ImmH giving a 31-fold preference for huPNP. However, DADMe-ImmG shows a 130-fold preference for huPNP, reflecting the catalytic role of huPNP for dGuo phosphorolysis as compared with the physiological roles of PfPNP of forming hypoxanthine from inosine and 5’-methylthioinosine (5, 11). For both physiological functions of PfPNP the substrate molecules contain a 2’-hydroxyl group. Other analogues, including 5’-4’-Me-DADMe-ImmH, 8-aza-DADMe-ImmH, and 1’-8-DADMe-ImmH differ only by 1.3–14-fold in affinity between huPNP and PfPNP.

Other DADMe-Immucillins that bind more tightly to huPNP than PfPNP include 5’-MT-DADMe-ImmH (13-fold), 5’-PrT-DADMe-ImmH (3,900-fold), 4’-9-N-Me-ImmH (190-fold), DADEt-ImmH (1,300-fold), 4’-OH-DADMe-ImmH (290-fold), 5’-MT-4’-OH-DADMe-ImmH (105-fold), and 1’-9-Me-ImmH (2,400-fold). This strong preference for huPNP relative to PfPNP reflects a structurally adaptable catalytic site for huPNP, enabling it to accept inosine, deoxyinosine, guanosine, and deoxyguanosine as good substrates. The catalytic efficiency (kcat/Km) for huPNP is 106 M−1 s−1 for these four substrates, while the PfPNP has efficiencies of 103 to 104 for the same compounds (25). The physiologic role of PfPNP is hypoxanthine salvage from inosine and methylthioinosine, and deoxynucleoside salvage is unlikely to play a role. Thus, PfPNP has evolved for efficient catalysis of nucleosides rather than deoxynucleosides. In contrast, the human enzyme requires a catalytic site to accommodate both deoxynucleosides (deoxyguanosine and deoxyinosine) and nucleosides. In PNPD deficiency, purine nucleosides and deoxynucleosides appear in the blood and urine and little uric acid is formed, demonstrating the role of PNPD in the catabolism of both groups of purines (2).

Methylthio-Immucillin-H and Methylthio-DADMe-ImmH—
A surprise in the inhibition pattern of DADMe-Immucillins is 5’-MT-DADMe-ImmH with its 13-fold preference for huPNP. In the Immucillin inhibitors, 5’-MT-ImmH binds 112-fold better to PfPNP than to huPNP and this is attributed to the 5’-methylthioinosine specificity for the PfPNP, a metabolite not found in human metabolism (5, 11). Since DADMe-Immucillins are closer mimics of the highly dissociated transition states of huPNP and PfPNP, it was hypothesized that the 5’-methylthio group would also convey specificity for PfPNP in the DADMe-Immucillin series. Its failure to do so suggests that the 2’-hydroxyl group is in a cooperative binding interaction with the 5’-methylthio group and/or other determinants of the transition state. Cooperative binding interactions between individual groups of transition state analogues have been documented with bovine PNPD (26). Transition state analysis for the arsenolysis reaction catalyzed by bovine PNPD indicated significant bond order (0.38) to the leaving group without participation of the arsanate nucleophile (8). Loss of any feature critical to the transition state interaction weakens direct interactions with the catalytic site and also weakens nearby interactions related to the transition state (26). In the case of PfPNP, the 5’-methylthioinosine substrate and MT-ImmH have a 2’-hydroxyl group that is not present in 5’-MT-DADMe-ImmH. Thus, the loss of the 2’-hydroxyl for PfPNP is more significant than the replacement of the 5’-hydroxyl with 5’-thiomethyl for huPNP.

Energetics of Immucillin Binding to huPNP and PfPNP—The binding affinity for Immucillin transition state analogues of huPNP and PfPNP is compared with the affinity for ImmH in Fig. 3 (upper panel). Atomic substitutions at all positions except the 2-aminoguanosine decrease binding affinity and therefore have a positive ΔΔG relative to ImmH binding with huPNP and PfPNP. Interesting atomic substitutions are those with large differentials in binding for the PNPD isozymes. One of these is 8-Me-ImmH, which decreases the binding energy by 3.5 kcal/mol compared with ImmH for huPNP but only 0.7 kcal/mol for PfPNP. This change is possible either through perturbation of the pKs at N7 or by a steric clash in the PfPNP that is not present in huPNP. The 8-F-ImmH answers this question, since the fluorine substitution also alters the pKs at N7 but does not alter the steric volume. The 8-F-ImmH decreases binding to both huPNP and PfPNP by the same amount. Therefore the large discrimination seen for the 8-methyl group indicates that the catalytic site of the PfPNP is more highly constrained.
around this position than huPNP. Another substitution of interest is in the 5' -methylthio group of 5'-MT-ImmH. The PfPNP uses 5'-MT-insine as a physiological substrate and has a hydrophobic pocket to accommodate the methylthio group (11). Placing a methylthio group in the 5'-position for huPNP decreases affinity relative to ImmH by 5.2 kcal/mol but for P/PNP the binding affinity changes only by 0.7 kcal/mol. This substitution confers high specificity for 5'-MT-ImmH on the P/PNP (11). Recently, this feature has been used to probe the relative contributions of erythrocyte PNP and P/PNP to purine salvage in P. falciparum cultured in human erythrocytes (4). The third feature to give isozyme discrimination is substitution of the difluoro at the 2' position of ImmH (2'2-diF-ImmH). Human PNP experiences a decrease of 1.9 kcal/mol for this substitution relative to a hydroxyl at this position with ImmH, but P/PNP experiences a loss in binding energy of > 5.9 kcal/mol. In contrast, huPNP and P/PNP are both tolerant of 2'-deoxy, 2'-ara, and 2'-OCH3 substitutions, since all of these changes affect both enzymes with similar changes in ΔDG.

Thus, the electron-rich difluoro substitution reflects an unfavorable electrostatic interaction or induces an unfavorable iminobitol ring pucker for P/PNP relative to huPNP. Although huPNP and P/PNP bind some Immucillins with large differences, for many other Immucillins, binding differences are unexpectedly small. For example, a catalytic site feature that most distinguishes huPNP from P/PNP is the hydrophobic cavity that accommodates the 5'-methylthio group, specifically for P/PNP. Substitution of a phenylthio group (5'-PhT-ImmH) at this position alters binding of both huPNP and P/PNP by a similar energy of ~5 kcal/mol.

**Energetics of DADMe-Immucillin Binding to huPNP and P/PNP**—The binding of DADMe-ImmH to huPNP and P/PNP exhibits dissociation constants of 16 and 500 pM, respectively. DADMe-ImmH is the most powerful inhibitor known for P/PNP and the second most powerful for huPNP. The rationale for this tight binding is the close mimicry of DADMe-ImmH to the fully dissociated transition states for both enzymes. Transition states for both huPNP and P/PNP have N-ribosidic bond (N9 to C1') distances of ~3.0 Å compared with 1.5 Å in the substrate molecules (9). Introduction of the methylene bridge in the DADMe-Immucillins increases this distance to 2.5 Å (C9 to N1') compared with a distance of 1.4 Å in the Immucillins (C9 to C1'). The deazapurine leaving group in the DADMe-Immucillins retains the high pK_a at N7, and the pyrrole nitrogen cation at the 1'-position closely mimics the electrostatics of the transition state. DADMe-ImmG binds ~0.5 kcal/mol more tightly than DADMe-ImmH to huPNP but binds 0.3 kcal/mol less well to P/PNP. While these are relatively small differences, they reflect the physiological roles of the enzymes. In humans PNP catalyzes dGuo and in P. falciparum PNP forms hypoxanthine from inosine and 5'-methylthioinosine. The 2.5-Å distance separating leaving group and oxacarbenium ion mimics in DADMe-ImmH does not exactly match the distance or the geometry of these transition states. Insertion of an ethylene bridge increases the distance to 3.5 Å, greater than the 3.0 Å of the transition state, and also introduces additional conformational flexibility. DAEt-ImmH binding is decreased by 2.0 kcal/mol for huPNP but only 0.1 kcal/mol for the P/PNP relative to DADMe-ImmH. These changes suggest that the transition state for huPNP is more closely matched by the 2.5 Å in DADMe-ImmH than the 3.5-Å bond separation in DAEt-ImmH and that the P/PNP transition state has bond separation between 3.0 and 3.5 Å. These bond distances are consistent with kinetic isotope effect measurements, since leaving group distances >3.0 Å are not accurately predicted by KIE. Surprisingly, the 5'-methylthio and 5'-propylthio substitutions in the DADMe-immucillins do not show strong preferences for the P/PNP. Transition state interactions at the catalytic sites of PNP are known to be cooperative. Since the DADMe-Immucillins are required to be 2'-deoxy for chemical stability, it is possible that cooperative binding occurs between the 2'-hydroxyl and the 5'-methylthio groups, and without the 2'-interaction, the methylthio group does not interact optimally with its hydrophobic site. This interpretation remains speculative pending structural analysis of these complexes.

**Summary and Conclusions**—The Immucillins transition state analogues of human and P. falciparum PNP give K_{pK_a} values up to 5,400,000 (10). Systematic atomic substitutions of these designed inhibitors only weaken the binding, supporting the proposal that these molecules are highly optimized to capture transition state binding energy. Although only a fraction of the transition state binding energy is captured, imperfect mimicry of transition state structure is a necessary feature of attempting to mimic unstable transition states with chemically stable analogues.

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