Intracellular rebinding of transition-state analogues provides extended in vivo inhibition lifetimes on human purine nucleoside phosphorylase

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Purine nucleoside phosphorylase (PNP) is part of the human purine salvage pathway. Its deficiency triggers apoptosis of activated T-cells, making it a target for T-cell proliferative disorders. Transition-state analogues of PNP bind with picomolar dissociation constants. Tight-binding PNP inhibitors show exceptionally long lifetimes on the target enzyme. We solve the mechanism of the target residence time by comparing functional off-rates in vitro and in vivo. We report in vitro PNP-inhibitor dissociation rates (t1/2) from 3 to 31 min for seven Immucillins with dissociation constants of 115 to 6 pm. Treatment of human erythrocytes with DADMe-Immucillin-H (DADMe-ImmH, 22 pm) causes complete inhibition of PNP. Loss of [14C]DADMe-ImmH from erythrocytes during multiple washes is slow and biphasic, resulting from inhibitor release and rebinding to PNP catalytic sites. The slow phase gave a t1/2 of 84 h. Loss of [14C]DADMe-ImmH from erythrocytes in the presence of excess unlabeled DADMe-ImmH increased to a t1/2 of 1.6 h by preventing rebinding. Thus, in human erythrocytes, rebinding of DADMe-ImmH is 50-fold more likely than diffusional loss of the inhibitor from the erythrocyte. Humans treated with a single oral dose of DADMe-ImmH in phase 1 clinical trials exhibit regain of PNP activity with a t1/2 of 59 days, corresponding to the erythropoiesis rate in humans. Thus, the PNP catalytic site recapture of DADMe-ImmH is highly favored in vivo. We conclude that transition-state analogues with picomolar dissociation constants exhibit long lifetimes on their targets in vivo because the probability of the target enzyme recapturing inhibitor molecules is greater than diffusional loss to the extracellular space.

Analyses of drug efficacy have considered the significance of the dissociation rate constant or off-rate (koff) and its correlation with residence time for drug candidates binding to their biological targets (1–3). Residence time is an important factor in determining biological efficacy and can be determined from half-life measurements obtained from the inhibitor off-rates (4). Off-rates are most commonly measured in vitro by incubating the enzyme and inhibitor, diluting the complex, and observing the resulting regain of enzymatic activity (5).

Transition-state analogues often exhibit physiological inhibitory effects extending beyond inhibitory expectations extrapolated from in vitro studies. For example, in mice, a single oral dose of the PNP inhibitor DADMe-ImmH (Fig. 1) gave inhibition in circulating erythrocytes for the life of the cells, whereas koff in solution predicted a much shorter efficacy (4). Extended inhibition on the target suggests a reduced koff rate under physiological conditions, cellular accumulation of inhibitor, covalent inhibition, or frequent rebinding events. Extended inhibitor-target efficacy will occur when the inhibitor has a higher probability of rebinding to the target enzyme than loss through cell membrane diffusion. Rebinding contributions to long-term inhibition have been mathematically treated and are known to contribute to cell receptor-ligand interactions (6–8).

Purine nucleoside phosphorylase (EC 2.4.2.1; PNP) is a homotrimeric enzyme containing three active sites (9). It is essential in human purine salvage by catalyzing the phosphorylation of 6-oxypurine nucleosides and 2’-deoxynucleosides to purine bases and α-D-ribose 1-phosphate in the production of purine bases for salvage and recycling or for urate production (10). The human genetic deficiency of PNP causes the accumulation of 2’-deoxyguanosine (dGuo) in the blood and induces T-cell immunodeficiency when activated T-cells convert dGuo to 2’-deoxyguanosine triphosphate (dGTP). The unbalanced dGTP pool causes T-cell specific apoptosis, making PNP a chemotherapeutic target for T-cell proliferative disorders (10–14). The catalytic sites of homotrimeric PNP bind transition-state analogues with negative cooperativity. Inhibitor binding at the first site has the highest affinity and causes complete inhibition of the enzyme. Inhibitor-binding exhibits negative cooperativity, with weaker binding at the second and third sites of the trimer, each by more than an order of magnitude relative to the first site (15).

The abbreviations used are: dGuo, 2’-deoxyguanosine; DADMe-ImmH, DADMe-Immucillin-H; PNP, purine nucleoside phosphorylase; ENT, equilibrative nucleoside transporter.

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This article contains supplemental Figs. S1 and S2.

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Immucillin-H (ImmH; Fig. 1) gains access to human leukemia CCRF-CEM cells via the equilibrative nucleoside transporters (ENT1 and ENT2), whereas dGuo uptake depends on ion-dependent concentrative nucleoside transporters (sodium-dependent transporters). Transporters ENT1 and ENT2 are also located in the plasma membranes of erythrocytes (16, 17). Here we demonstrate that the ENT1 and ENT2 transporters are involved in the transport of DADMe-ImmH by dipyridamole, a known inhibitor of these transporters.

The transition-state structure of PNP has provided design parameters for the development of the Immucillins; four generations of transition-state analogues with picomolar dissociation constants (Fig. 1; Refs. 11, 14, and 18–22). ImmH (clinically Forodesine and Mundesine®) has received approval for peripheral T-cell lymphoma in Japan. DADMe-ImmH (clinically Udodesine) has completed Phase 2 clinical trials for gout (23).

ImmH was designed from the transition-state structure of bovine PNP and is also a transition-state analogue of human PNP. The second generation analogues, DADMe-ImmH and DADMe-ImmG, were designed specifically from the transition-state structure of human PNP (24). Although bovine and human PNP’s are 87% identical, their transition states differ. ImmH binds more tightly to bovine PNP and is severalfold weaker for human PNP. DADMe-ImmH (clinically Udodesine) has entered clinical trials for gout (11, 14, and 18–22).

Results

Inhibition studies

Inhibition constants (K_i and K_i^*) were determined for seven PNP transition-state analogues at 25 and 37 °C (Fig. 3). The data obtained from the assays were fit to Equation 1 (Fig. 4).

Inhibition constants were calculated from Equation 1 for each inhibitor (Table 1). All inhibitors show slow-onset tight-binding inhibition with dissociation constants in the picomolar range. At both temperatures, SerMe-ImmG was the tightest, whereas ImmH was the weakest inhibitor. The K_i values are more sensitive to temperature than K_i^* values, suggesting that the more open, Michaelis-like complexes governing K_i are more temperature-sensitive than the more constrained ensem-
and Ki were measured at 37 °C by diluting a stoichiometric
Inhibitor dissociation rates from PNP
analysis. Immucillins, significant here for comparing
direct temperature comparison for the four generations of
bles with bound transition-state analogues. Table 1 provides a
direct temperature comparison for the four generations of
Immucillins, significant here for comparing in vitro and in vivo
analysis.

Inhibitor dissociation rates from PNP
Initial off-rates and inhibitor half-lives for each PNP inhibi-
tor were measured at 37 °C by diluting a stoichiometric
enzyme-inhibitor mixture and observing the partial recovery of
PNP catalytic activity (Equation 2; Table 2; Fig. 5). DATMe-
I represents inhibitor, enzyme, substrate, and tightly bound
enzyme-inhibitor, respectively.

Temperature dependence for the K* dissociation constant is
contrary to the usual pattern for enzyme-inhibitor complexes,
where affinity increases at lower temperatures. For all of the
inhibitors, the values of K* indicate equal or tighter binding at
37 °C. This pattern has been observed before for 5’-methylth-
iodenosine phosphorylase and has been interpreted to indi-
cate catalytic site dynamic motion contributing to favorable
interaction with transition-state analogues (28).

Off-rate without added inhibitor
Excess [14C]DADMe-ImmH was incubated with RBCs in
nutrient media to permit binding to cellular PNP. Extensive
washing of the labeled cells before initiation of the experiment
removed excess unbound inhibitor. At each time point over
a period of 48 h, cells were subject to additional washes,
maintaining the external concentration of [14C]DADMe-
ImmH near zero. Under these conditions, the amount of
[14C]DADMe-ImmH retained in the cell is in equilibrium
with diffusive recapture or is non-exchangeable.

The amount of [14C]DADMe-ImmH decreased over the
early incubation period (0–2 h) with a t1/2 of 48 min for 2/3
of the inhibitor loss (Fig. 6). The initial concentration of
[14C]DADMe-ImmH in erythrocytes was estimated to be 3.8
μM based on the specific radioactivity of the inhibitor. After the
initial loss, a slower loss over the following 48 h gave a t1/2 of 84 h
for the last 1/3 of the [14C]DADMe-ImmH. The concentration of
[14C]DADMe-ImmH in the erythrocytes remained at ~1.2 μM
at 48 h. The initial loss rate constant was 8.7-fold slower than
the in vitro off-rate for DADMe-ImmH, and the final rate con-
tant was 916-fold slower than observed off-rate studies. Human
PNP is trimeric with the first site binding tightly to transition-state analogues and sites two and three with reduced
affinity. Loss of [14C]DADMe-ImmH in the initial phase is
interpreted to be from the two more weakly binding sites and
the slow phase loss from the tightly bound final, inhibitory cat-
alytic site. Quantitation of the bound [14C]DADMe-ImmH in

Table 2
Dissociation rates and half-times (t1/2) of transition-state analogues at
37 °C

| Inhibitor   | k  | t1/2 |
|-------------|----|------|
| ImmH        | 0.064 ± 0.002 | 11 ± 0.2 |
| DADMe-ImmH  | 0.084 ± 0.003 | 8.3 ± 0.3 |
| DADMe-ImmgG | 0.072 ± 0.003 | 9.7 ± 0.4 |
| DATMe-ImmH  | 0.26 ± 0.04   | 2.6 ± 0.4 |
| DATMe-ImmgG | 0.022 ± 0.004 | 31 ± 0.5 |
| SerMe-ImmH  | 0.030 ± 0.0005 | 23 ± 0.4 |
| SerMe-ImmgG | 0.031 ± 0.0005 | 23 ± 0.4 |

B. 3. Example of slow-onset, tight-binding inhibition of product for-

A. 3. Example of slow-onset, tight-binding inhibition of product for-

C. 3. Example of slow-onset, tight-binding inhibition of product for-

D. 3. Example of slow-onset, tight-binding inhibition of product for-

E. 3. Example of slow-onset, tight-binding inhibition of product for-

F. 3. Example of slow-onset, tight-binding inhibition of product for-

G. 3. Example of slow-onset, tight-binding inhibition of product for-

H. 3. Example of slow-onset, tight-binding inhibition of product for-

I. 3. Example of slow-onset, tight-binding inhibition of product for-

J. 3. Example of slow-onset, tight-binding inhibition of product for-

Ki were measured at 37 °C by diluting a stoichiometric
erythrocytes for this experimental approach indicates a trimeric PNP concentration of $1.5 \times 10^{-11}$ M (4.5 $\mu$M monomeric subunits). This result is in close agreement with quantitation of PNP in RBCs, eliminating the possibility of another cellular reservoir for [14C]DADMe-ImmH.

Off-rate with added inhibitor

Erythrocytes labeled with [14C]DADMe-ImmH and washed as above were incubated in the presence of excess (300 $\mu$M) unlabeled DADMe-ImmH (Fig. 7). Microscopic release of labeled inhibitor is prevented from rebinding by competition from the excess unlabeled inhibitor. The experiment determines if the tightly bound inhibitor (Fig. 6) is in dynamic exchange. An increased release rate for [14C]DADMe-ImmH would be expected if the PNP-bound inhibitor is exchanging and subject to diffusive recapture by PNP. The concentration of erythrocyte [14C]DADMe-ImmH decreased from an initial concentration of 3.5 $\mu$M to a concentration of 0.61 $\mu$M after 130 min of exchange (Fig. 7). Without excess DADMe-ImmH, the initial phase had a $t_{1/2}$ of 48 min and the slower phase had a $t_{1/2}$ of 84 h. With excess DADMe-ImmH, the initial phase gave a $t_{1/2}$ of 9.1 min, and the slower phase gave a $t_{1/2}$ of 1.65 h.

PNP catalytic activity studies

From the amount of bound [14C]DADMe-ImmH (Figs. 6 and 7) the PNP subunit concentration was estimated to be 3.8–4.5 $\mu$M in erythrocytes. A direct titration of PNP catalytic activity was made in extracts equivalent to 22.5% hematocrit (Fig. 8). Extracts were incubated with varying concentrations of DADMe-ImmH, and the residual PNP activity was analyzed. The concentration of PNP from a catalytic site titration was $0.40 \pm 0.01$ $\mu$M (Fig. 8). Extrapolation to 100% hematocrit indicates a 5.3 ± 0.1 $\mu$M subunits and 1.8 ± 0.1 $\mu$M trimer.

[14C]DADMe-ImmH uptake

[14C]DADMe-ImmH uptake experiments were performed to determine the role of ENT1 and ENT2 transporters in DADMe-ImmH uptake. Dipyridamole, a known inhibitor of these transporters (29), was tested for its effects at 10 $\mu$M (Fig. 9). In the absence of dipyridamole and with 2 $\mu$M [14C]DADMe-ImmH, the concentration of inhibitor inside the RBCs increased from 0.39 to 1.27 $\mu$M over a 20-min incubation at 37 °C. Dipyridamole blocked all but the time 0 level of [14C]DADMe-ImmH associated with the RBC pellet, supporting a role for ENT1 and/or ENT2 in [14C]DADMe-ImmH uptake.

DADMe-ImmH in phase 1 human trials

Analysis of DADMe-ImmH inhibitor rebinding together with the results from a human phase 1 dose-ranging study reveals a mechanism where the initial blood level of DADMe-ImmH rapidly inhibits PNP of RBCs (Fig. 10). In the following 72 h, the clearance of DADMe-ImmH causes blood levels to decline below 10 ng/ml. However, even at 504 h (21 days), the RBC PNP remains strongly inhibited. Slow regain of PNP activity in blood samples occurs with a $t_{1/2}$ of 59 days after single oral doses of 0.25 to 3 mg/kg. Human erythrocyte lifetime is 120 days. Regain of activity by hematopoiesis alone would, therefore, be expected to give a $t_{1/2}$ of 60 days. This PNP activity analysis demonstrates that the dominant factor in the regain of PNP catalytic activity is new cell regeneration rather than loss of DADMe-ImmH from the PNP target. To achieve this durable inhibition in vivo, DADMe-ImmH rebinding to PNP inside RBCs occurs with greater efficiency than in the isolated cell systems (e.g. Fig. 6). With isolated erythrocytes, inhibitor rebinding is 50-fold more likely than diffusional loss of DADMe-ImmH from cells. In human trials with DADMe-ImmH, rebinding of the inhibitor is significantly more efficient. With an intrinsic release rate of 1.65 h (Fig. 7) and no significant
loss of inhibitor over 504 h (21 days) in human trials, the ratio of rebinding to diffusional loss is >300-fold. The regain of PNP catalytic activity is equivalent to the rate of RBC replacement. Thus, near-stoichiometric DADMe-ImmH re-binding is required to give the extended PNP inhibition kinetics seen in humans (Fig. 10).

Discussion

Background

Oral administration of a single dose of DADMe-ImmH to mice caused rapid inhibition of blood PNP \((t_{1/2} = 10\) min) and an in vivo \(t_{1/2}\) of 11.5 days for regain of catalytic activity (4). As the lifespan of mouse erythrocytes is \(\sim 25\) days, regain of PNP activity can be attributed to erythropoiesis rather than diffusional loss of DADMe-ImmH. Thus, DADMe-ImmH causes apparently irreversible binding to PNP in vivo or is efficiently rebound over multiple release and recapture cycles. A similar pattern was found for inhibition of human blood PNP in phase 1 clinical trials (Fig. 10). Single oral doses of DADMe-ImmH (BCX-4208 in clinical trials) caused elevated blood levels peaking at 4 h and returning to near-baseline levels by 72 h (Fig. 10, left panel). However, inhibition of blood PNP extended well beyond 72 h, with an in vivo \(t_{1/2}\) of 59 days, consistent with the 120-day lifetime of human erythrocytes.

Here, we compare the microscopic dissociation rates for human PNP-Immucillin complexes in vitro and in vivo inside human erythrocytes. Comparison of release and exchange rates establishes that DADMe-ImmH is being released and rapidly rebound in human erythrocytes. The relative rates permit a mechanistic analysis of the frequency of release and re-binding. Comparison of seven Immucillins provides parameters for evaluating the relative efficiency of these compounds. ImmH has been approved for use against recurrent or resistant peripheral T cell lymphoma in Japan as Mundesine®. DADMe-ImmH has completed phase 2 clinical trials for treatment of gout (23), and comparative kinetics will be useful in considering other members of the Immucillin family for pharmaceutical potential. For example, DADMe-ImmG clears Plasmodium falciparum from an Aotus primate model (22). Autoimmune disorders based on auto-antigen T cell activity are also expected to respond to inhibitors of PNP activity (13).

Immucllin affinity and release rates

Slow-onset, tight-binding inhibition is exhibited by the Immucillins described here, a characteristic of transition-state analogues (Figs. 3 and 4). Dissociation constants \((K_i^*)\) for the Immucillins vary from 115 to 6 pM at 37 °C under the conditions of our assays (Table 1). The unusual temperature dependence with equal or higher inhibitor affinity at elevated temperature has been seen before with transition-state analogues (28). One possible explanation is that increased dynamic motion at the catalytic site at increased temperature permits inhibitors to more closely mimic the dynamic interactions leading to the transition-state (27). Most in vitro inhibitor release rates \((t_{1/2})\) from PNP-Immucillin complexes scaled appropriately with the \(K_i^*\) values, with a fast value of \(t_{1/2} = 11\) min for ImmH (115 pM) and the slowest \(t_{1/2} = 31\) min for DATMe-ImmG (6 pM). A notable exception is provided by DATMe-ImmH. With a \(K_i^*\) value of 43 pM, a slow \(t_{1/2} = 157\) min. A possible explanation for this is increased dynamic motion at the binding site with increased temperature. The unusual temperature dependence seen in vitro is also expected, but it was near 3 min (Table 2). The more flexible chemical scaffolds of the DATMe- and SerMe-Immucillins are likely to promote rapid escape from the PNP catalytic sites. The slow release of DATMe-ImmG, SerMe-ImmH, and SerMe-ImmG suggest these three inhibitors will have the most powerful in vivo action as PNP inhibitors.

\(^{14}\text{C})\text{DADMe-ImmH efflux from erythrocytes}

The PNP activity of human erythrocytes is readily inhibited by \(^{14}\text{C})\text{DADMe-ImmH in vivo} (Fig. 10). The kinetics of \(^{14}\text{C})\text{DADMe-ImmH release from erythrocytes resolves tight binding from covalent interaction and provides rebound information. Human PNP is a homotrimer, where filling one of three catalytic sites causes complete inhibition. Binding at the first site gives the kinetic constants from steady-state and slow-onset analysis (Tables 1 and 2; Fig. 5). The second and third sites also fill with inhibitor but with negative cooperativity, each at least an order of magnitude weaker than the first (15). ITC titration of human PNP with DADMe-ImmH at 37 °C gave an enthalpy of \(-21.5\) kcal/mol for the first site and an average value of \(-12.0\) kcal/mol for the second and third sites (15). With a 4′-F analogue of DADMe-ImmH, binding at sites 1, 2, and 3 were 2, 64, and 667 nM (15). Incubation of erythrocytes with excess \(^{14}\text{C})\text{DADMe-ImmH loads all sites. Incubation
with multiple washing first removes inhibitor from the weaker sites (sites 2 and 3) for 2/3 loss of [14C]DADMe-ImmH with a t1/2 of 48 min (Fig. 6). This is slow compared with the 8.3 min t1/2 for activity recovery from [14C]DADMe-ImmH binding to the first tight site for PNP (Table 2). Even for PNP weak sites two and three, these are rebinding [14C]DADMe-ImmH approximately six times for each inhibitor molecule diffusing out of the erythrocytes.

The pattern of slow inhibitor loss from the first site for [14C]DADMe-ImmH wash-out experiments from RBCs gave a t1/2 of 84 h (Fig. 6). The addition of excess unlabeled DADMe-ImmH prevented [14C]DADMe-ImmH rebinding and increased the t1/2 of 84 h to a t1/2 of 1.65 h, a 51-fold increase in inhibitor loss rate (Fig. 7). Thus, the inhibitor is in rapid microscopic exchange inside the erythrocytes and rebinds at least 50 times more frequently in the absence of excess DADMe-ImmH than in its presence.

\[ ^{14}\text{C}]\text{DADMe-ImmH transport} \]

Transport of ImmH into human CCRF-CEM cells occurs on ENT1 and ENT2 equilibrative nucleoside transporters, also present in erythrocytes and sensitive to dipyridamole inhibition (29). Human RBCs transported \[^{14}\text{C}]\text{DADMe-ImmH} in a dipyridamole-sensitive mechanism, consistent with the same uptake mechanism.

\[ \text{Extended PNP inhibition: Extrapolation to human trials} \]

The long-term biological efficacy of DADMe-ImmH was first seen in mouse experiments, where the inhibitor time on the PNP target approximated the lifetime of mouse erythrocytes. This inhibition characteristic was termed “the ultimate goal” in inhibitor design. We define this interaction as an orally available drug that inhibits the target enzyme for the lifetime of the cell without the complications of covalent attachment. Work here extends the inhibitor “ultimate goal” status to the DADMe-ImmH inhibition of PNP in human blood. The rate of PNP activity regain following DADMe-ImmH treatment depends on the rate of cell replacement. Therefore, tissues with more rapid cell replacement rates are expected to recover PNP activity more rapidly than RBCs. The human clinical trials demonstrated that once a day oral dosing provides adequate whole body inhibition of PNP.

\[ \text{Conclusions} \]

Transition-state analogue inhibitors of human PNP bind with picomolar dissociation constants and cause slow-onset tight-binding inhibition. Residence times of these inhibitors on the PNP target are on the timescale of minutes with \textit{in vitro} enzyme-inhibitor complexes. The \textit{in vivo} efficacy in isolated RBCs is much greater than predicted by target residence times. Labeled inhibitor-exchange experiments in isolated RBCs demonstrate that the long inhibitor residence time on the PNP target is caused by inhibitor rebinding to the target more frequently than inhibitor loss from the cell. Inhibitor efficacy in human phase 1 clinical trials indicate that inhibitor rebinding is more effective \textit{in vivo}, with no significant PNP activity regain from circulating RBCs over their 120-day lifetime in humans.

\[ \text{Experimental procedures} \]

Immmillins (Fig. 1) were synthesized at the Ferrier Research Institute at the Victoria University of Wellington in New Zealand and are the generous gifts of Drs. Peter C. Tyler and Gary B. Evans. \[^{14}\text{C}]\text{Formaldehyde} was obtained from Moravek. Isolated RBCs were obtained from human volunteers under informed consent with IRB# 2000-031, approved under 45 CFR 46.110 and 21 CFR 56.110. Culture medium 1640 RPMI was purchased from Invitrogen. Xanthine oxidase, inosine, and hydrochloric acid in diethyl ether, sodium acetate, 30% formaldehyde, dichloromethane, methanol, and ammonium hydroxide were purchased from Sigma. Hexanes and ethyl acetate were purchased from Fisher.
**Protein expression and purification**

Human PNP was expressed and purified as previously described with slight variations (4). A starter culture containing ampicillin (15 ml) was added to 1 liter of LB broth along with ampicillin stock to 100 μg/ml. Expression was induced using isopropyl β-D-1-thiogalactopyranoside (IPTG, 1 mM). Freshly harvested cells (~20 g) were treated with a few mg of DNase (powdered form), and the cells were disrupted by sonication. Protein was purified by AKTA FPLC with a nickel-nitrilotriacetic acid column of 5 ml. The column was loaded with 42 ml of extract. It was washed with 10 mM imidazole, 300 mM NaCl, and 50 mM potassium phosphate (pH 8.0). Elution was effected by a gradient (1 ml/min) from wash buffer to 150 mM imidazole in the same buffer over 30 min followed by a second gradient from 150 to 500 mM imidazole over 10 min. Human PNP was eluted at 150–250 mM imidazole. The protein was dialyzed against 100 mM NaCl and 50 mM potassium phosphate (pH 8.0). Concentrated protein was stored frozen at ~80 °C. Hypoxanthine co-purifying with the PNP was removed by dialysis against the same buffer containing 0.5% (w/v) powdered charcoal.

**Synthesis of radiolabeled [14C]DADMe-ImmH-HCl**

[Methylene-14C]DADMe-ImmH-HCl was synthesized from 3-hydroxy-4-(hydroxymethyl)pyrrolidin-1-ium as described for the synthesis of DADMe-ImmH (supplemental Figs. S1 and S2) with slight variations. The reagents NaOAc, 4-(hydroxymethyl)-3-pyrrolidinol hydrochloride, and [14C]formaldehyde were added in a 1.1 equivalent ratio to 9-deazahypoxanthine. [14C]Formaldehyde was carrier-free with a specific activity of 58 μCi/μmol. The [14C]formaldehyde (250 μCi) in H2O contributed 100 μl to the reaction mixture. The total volume (~156 μl) was larger than used in the unlabeled reaction (~65 μl).

[Methylene-14C]DADMe-ImmH was purified by HPLC using a C18 preparatory column (10 × 250 mm). Elution of DADMe-ImmH used a gradient of 0.1% TFA in H2O (A) and 0.1% TFA in acetonitrile (B): solvent A, 0−5 min; 100% A to 7.5% B, 5−20 min; 7.5% to 15% B, 20−27 min; 15% B, 27−29 min; 100% A, 30−40 min.

**Kinetic inhibition studies**

Kinetic parameters for PNP inhibition by Immucilinins were measured in a mixture of 1 ml containing 60 milliunits of xanthine oxidase, 1 nM enzyme, 50 mM phosphate buffer (pH 7.5), and inosine (1 mM or 10 mM depending on the inhibitor), with concentrations of inhibitor from 0.1 nM to 316 nM. The reaction was monitored using a CARY 300 Bio UV spectrophotometer running the reactions at 25 or 37 °C and monitoring the progression at 293 nm for 1 h. To determine \( K_i \) and \( K_i^* \), the competitive inhibition equation was used (Equation 1),

\[
\frac{v_0'}{v_0} = \frac{[A] + K_m}{K_m \left( 1 + \frac{[I]}{K_i} \right) + [A]}
\]

(Eq. 1)

where \( v_0' \) is the initial reaction rate in the presence of inhibitor, \( v_0 \) is the initial reaction rate in the absence of inhibitor, \([I]\) is the inhibitor concentration, and \([A]\) is the substrate concentration.

**Biological efficacy of transition-state analogues**

In addition to this equation, Equation 2 below was used to calculate the amount of free inhibitor concentration (for use in Equation 1),

\[
[I]' = [I] - \left(1 - \frac{v_0'}{v_0}\right)[E]
\]

(Eq. 2)

where \([I]\) is the total inhibitor concentration, \([E]\) is the total enzyme concentration, and \([I]'\) is the amount of free inhibitor. For each inhibitor, dissociation constants were determined for the initial reaction rates (\( K_i \)) and from reaction rates after the system had equilibrated (\( K_i^* \)). Experimental data were fit to the equations by the use of GraphPad Prism 7.

**Off-rates from purified human PNP**

Purified human PNP (0.5 μM) and the inhibitors (0.5 μM) in 50 mM phosphate buffer (pH 7.5) (total volume of 50 μl) were incubated at 37 °C for 1 h. The mixture was diluted 1:1000 using 50 mM phosphate buffer and assayed in the presence of inosine (2 mM) and 60 milliunits of xanthine oxidase. Product formation was observed at 293 nm for 3.5−5 h. Controls were included where enzyme or inhibitor were absent. The data were fit to Equation 3,

\[
p = v_t + \frac{(v_0 - v_s)(1 - e^{-kt})}{k}
\]

(Eq. 3)

where \( P \) is product formation, \( k \) is rate constant for inhibitor release, and \( v_0 \) and \( v_s \) are the initial and steady-state rates, respectively. The half-life for the PNP-inhibitor complex was calculated from Equation 4,

\[
t_{1/2} = \frac{ln 2}{k}
\]

(Eq. 4)

**Inhibitor release from isolated red blood cells**

Freshly collected blood was washed 3 times by dilution into equal volumes of PBS and centrifugation for 3 min at 400 × g. Pelleted erythrocytes (RBCs) were suspended in 1640 RPMI media to the desired hematocrit before use. Inhibitor [14C]DADMe-ImmH (4 μM) and washed RBCs (100 μl, 50% hematocrit) were incubated (15 min at 37 °C) and centrifuged at 3000 × g for 2 min, and the cells were washed with medium twice to eliminate any excess inhibitor. The RBCs were suspended in 450 μl of medium (to give 500 μl) and incubated for 5 min. The supernatant along with 200 μl of a second wash was collected for analysis. Cells were suspended in 450 μl of medium. A sample (10 μl) was compared with the collected supernatant. This analysis gave the amount of inhibitor in the supernatant and RBCs at time 0. The sample was incubated, and the analysis was repeated for time points 0.5, 1, 1.5, 2, 4, 6, 8, 16, 24, 32, 40, and 48 h. The washed RBC pellets were lysed by the addition of 90 μl of water, and samples of the RBCs and supernatants were transferred to scintillation vials where 10-ml scintillation fluid was added. The radioactivity in each sample was counted in a liquid scintillation analyzer Tri-Carb 2910 TR. The data were analyzed using Equation 3, where CPM replaced \( P \) in the formulation. In this formulation, \( v_0 \) and \( v_s \) are the initial and steady-state release rates (CPM/min), and \( k \) is the rate constant.
**Biological efficacy of transition-state analogues**

**PNP inhibitor off-rates from isolated human RBCs**

Erythrocyte cellular release of $[^{14}C]$DADMe-ImmH was measured by multiple washes of erythrocytes previously equilibrated with the inhibitor. Cell labeling with radiolabeled $[^{14}C]$DADMe-ImmH permitted monitoring inhibitor content in RBCs and release to the extracellular media.

$[^{14}C]$DADMe-ImmH release with excess unlabeled inhibitor

$[^{14}C]$DADMe-ImmH release from human RBCs was measured in the presence of excess unlabeled inhibitor. The procedure was the same as described above except the resuspension of the RBCs was in RPMI medium containing 300 or 3000 μM unlabeled DADMe-ImmH followed by incubation at 37 °C. Immediately after resuspension, a 50-μl sample was collected and centrifuged at 3000 × g for 0.5 min, and the supernatant was removed. This procedure was repeated for: 1, 4, 8, 16, 32, 48, 64, 96, and 128 min. Those samples were then set aside. The RBC pellets for each time point were lysed with 90 μl of water. The RBC lysates and supernatant samples were transferred to scintillation vials, and the radioactivity was measured and analyzed (Equation 3).

**PNP catalytic activity studies**

Erythrocytes were washed with PBS and centrifuged at 400 × g for 2 min. Washed, packed RBCs (600 μl, 100% hematocrit) were lysed in 100 mM phosphate (pH 7.4) containing 0.1% Triton X-100 (final volume 2.0 ml, 30% hematocrit). Incubation mixtures containing lysate and varying concentrations of DADMe-ImmH (0–0.4 μM) (22.5% hematocrit) were incubated for 30 min at 25 °C and assayed for PNP activity. PNP assay mixtures contained 60 milliunits of xanthine oxidase, 1 mM inosine, and 100 mM phosphate (pH 7.5). Reactions (triplicate) were initiated with 8 μl of the incubation mixtures and monitored at 293 nm at 25 °C using a CARY 100 Bio UV spectrophotometer. Control experiments used purified PNP (1 μM) as a concentration standard.

**$[^{14}C]$DADMe-ImmH Uptake**

Whole human blood (150 μl) was washed with PBS (850 μl) and centrifuged at 400 × g for 2 min, removing the supernatant. The isolated RBCs (75 μl) were suspended in 675 μl of 1640 RPMI media (total volume of 750 μl) and divided into two 300-μl samples to examine the uptake of $[^{14}C]$DADMe-ImmH with and without 10 μM dipyridamole. The mixtures (343 μl) were incubated at 37 °C for 10 min with or without dipyridamole, after which $[^{14}C]$DADMe-ImmH (100 μM, 7 μl) was added to each tube (2 μM final). Samples of 7 μl were taken to determine the total counts in each mixture. Subsequent samples (35 μl) were taken at the desired times and added to 1 ml of ice-cold PBS to quench transport. The cold samples were centrifuged (2000 × g for 2 min), discarding the supernatant. Water (90 μl) was added to lyse the cells, and samples were counted as described above (Fig. 9).

**Human studies**

BCX4208 (DADMe-ImmH) was evaluated in a phase I clinical study. The study was conducted as a single-center, randomized, double-blind, placebo controlled, sequential, dose-escalating study consisting of seven dose groups. Doses were escalated from 0.25, 0.5, 1, 1.5, 2, 2.5, and 3 mg/kg, and subjects were randomized to receive either BCX4208 (8 subjects) or placebo (4 subjects) in each group. Safety, tolerability, pharmacodynamics, and pharmacokinetics were evaluated after single oral doses of BCX4208. Blood samples were collected in heparin Vacutainer tubes for pharmacokinetics analysis. The target time for collection of the blood was predose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, 60, and 72 h after a single oral dose. Blood samples were also collected for PNP activity determination at predose, 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 36, 48, and 72 h after a single oral dose and 7, 14, and 21 days post dosing.

Plasma BCX4208 was determined by a validated LC/MS/MS Method. BCX4208 was extracted from plasma using a Waters Oasis® MCX solid phase extraction cartridge on a Zymark RapidTrace® Work station. Chromatography was performed isocratically using a Zorbax® SB C-3 column with isocratic elution using a mobile phase of 5% methanol in 0.1% acetic acid and methanol (97:3). An Agilent 1100 HPLC system was used for the chromatography. Column effluent was analyzed by positive ion multiple reaction monitoring 265 m/z and 147.9 m/z using a PE Sciex® API 2000 MS/MS equipped with Turbo Ion Spray® in positive ion mode. The concentration of BCX-4208 was then determined by weighted (1/x) quadratic regression analysis of peak areas produced from the standard curve spanning 5 to 2000 ng/ml.

Oral dosing with BCX4208 resulted in a dose-dependent increase of $C_{\text{max}}$ for the doses up to 2.5 mg/kg, cohort 6, with possible plateau for $C_{\text{max}}$ at the 2.5 mg/kg dose. $C_{\text{max}}$ was achieved 3–7 h after oral administration of BCX4208. Overall, $C_{\text{max}}$ and the area under the curve (AUC) increased in a dose-proportional manner.

**Erythrocyte PNP inhibition in vivo**

Inhibition of PNP enzyme activity was measured ex vivo in erythrocytes taken from subjects in all dose groups (0.25–3.0 mg/kg) in the phase I study. PNP enzyme activity in erythrocyte extracts was measured by the conversion of inosine to hypoxanthine using a spectrophotometric assay. A Cary 3 Spectrophotometer, Varian Model 1001206, equipped with Cary WinUV software was used for these studies.

Oral administration of BCX4208 resulted in rapid inhibition of erythrocyte PNP activity. Maximum mean PNP inhibitory activity was >80% in all dose groups. Inhibition was rapid and maintained at the elevated levels for at least 21 days after the last dose. The data were used to show a dose-dependent decrease in the time to maximum PNP inhibition and the rates of recovery with the slopes almost identical across the dose groups.

**Author contributions**—S. T. G. accomplished the experimental work shown in the figures and drafted the manuscript. S. A. C. designed and directed the synthesis of labeled inhibitors. L. L. completed preliminary feasibility experiments and an early draft of the manuscript. Y. S. B. provided the data of Fig. 10. V. L. S. conceived the experimental plan, designed the experiments, and edited the final drafts of the manuscript.
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References

1. Copeland, R. A., Pompliano, D. L., and Meek, T. D. (2006) Drug-target residence time and its implications for lead optimization. Nat. Rev. Drug Discov. 5, 730–739

2. Spagnuolo, L. A., Eltschkner, S., Yu, W., Daryaeef, F., Davoodi, S., Knudson, S. E., Allen, E. K., Merino, J., Pschibil, A., Moree, B., Thivalapill, N., Truglio, J. I., Salafsky, J., Slayden, R. A., Kisker, C., and Tonge, P. J. (2017) Evaluating the contribution of transition-state destabilization to changes in the residence time of triazole-based InhA inhibitors. J. Am. Chem. Soc. 139, 3417–3429

3. Cramer, J., Krimmer, S. G., Frith, V., Wulsendorf, T., Karlsson, R., Heine, A., and Klebe, G. (2017) Elucidating the origin of long residence time binding for inhibitors of the metalloproteinase thermolysin. ACS Chem. Biol. 12, 225–233

4. Lewandowicz, A., Tyler, P. C., Evans, G. B., Furneaux, R. H., and Schramm, V. L. (2003) Achieving the ultimate physiological goal in transition state analogue inhibitors for purine nucleoside phosphorylase. J. Biol. Chem. 278, 31465–31468

5. Morrison, J. F., and Walsh, C. T. (1988) The behavior and significance of slow-binding enzyme inhibitors. Adv. Enzymol. Relat. Areas Mol. Biol. 61, 201–301

6. Vauquelin, G. (2015) On the “micro”-pharmacodynamic and pharmacokinetic mechanisms that contribute to long-lasting drug action. Expert Opin. Drug Discov. 10, 1085–1098

7. Hothersall, D. J., Brown, A. J., Dale, I., and Rawlins, P. (2016) Can residence time offer a useful strategy to target agonist drugs for sustained GPCR responses? Drug Discov. Today 21, 90–96

8. Schaf, C. T., Fay, J. F., Janz, J. M., and Farrans, D. L. (2016) Decay of an active GPCR: Conformational dynamics govern agonist rebinding and persistence of an active, yet empty, receptor state. Proc. Natl. Acad. Sci. U.S.A. 113, 11961–11966

9. Pugmire, M. J., and Ealick, S. E. (2002) Structural analyses reveal two distinct families of nucleoside phosphorylases. J. Biol. Chem. 361, 1–25

10. Murkin, A. S., and Schramm, V. L. (2010) Purine nucleoside phosphorylases as targets for transition-state analog design. In Drug Design: Structure- and Ligand-based Approaches (Merz, K. M., Jr., Ringo, D., Reynolds, C. H., eds) pp. 215–247, Cambridge University Press: Cambridge, UK

11. Kicska, G. A., Long, L., Hörg, H., Fairchild, C., Tyler, P. C., Furneaux, R. H., Schramm, V. L., and Kaufman, H. L. (2001) Immucillin H, a powerful transition-state analog inhibitor of purine nucleoside phosphorylase, selectively inhibits human T lymphocytes. Proc. Natl. Acad. Sci. U.S.A. 98, 4595–4598

12. Clinch, K., Evans, G. B., Fröhlich, R. F., Furneaux, R. H., Kelly, P. M., Legentil, L., Murkin, A. S., Li, L., Schramm, V. L., Tyler, P. C., and Woolhouse, A. D. (2009) Third-generation Immucillins: Syntheses and bioactivities of acyclic Immucillin inhibitors of human purine nucleoside phosphorylase. J. Med. Chem. 52, 1126–1143

13. Bantia, S., Miller, P. J., Parker, C. D., Ananth, S. L., Horn, L. L., Kilpatrick, J. M., Morris, P. E., Hutchison, T. L., Montgomery, J. A., and Sandhu, J. S. (2001) Purine nucleoside phosphorylase inhibitor BCX-1777 (immucillin-H): a novel potent and orally active immunosuppressive agent. Int. Immunopharmacol. 1, 1199–1210

14. Evans, G. B., Furneaux, R. H., Lewandowicz, A., Schramm, V. L., and Tyler, P. C. (2003) Synthesis of second-generation transition state analogues of human purine nucleoside phosphorylase. J. Med. Chem. 46, 5271–5276

15. Edwards, A. A., Mason, J. M., Clinic, K., Tyler, P. C., Evans, G. B., and Schramm, V. L. (2009) Altered enthalpy-entropy compensation in pimocolar transition state analogues of human purine nucleoside phosphorylase. Biochemistry 48, 5226–5238

16. Huang, M., Wang, Y., Gu, J., Yang, J., Noel, K., Mitchell, B. S., Schramm, V. L., and Graves, L. M. (2008) Determinants of sensitivity of human T-cell leukemia CCRF-CEM cells to immucillin-H. Leuk. Res. 32, 1268–1278

17. Löffler, M., Morote-Garcia, J. C., Eltzschig, S. A., Roei, I. E., and Eltzschig, H. K. (2007) Physiological roles of vascular nucleoside transporters. Arterioscler. Thromb. Vasc. Biol. 27, 1004–1013

18. Miles, R. W., Tyler, P. C., Furneaux, R. H., Bagdassarian, C. K., and Schramm, V. L. (1998) One-third-the-sites transition-state inhibitors for purine nucleoside phosphorylase. Biochemistry 37, 8615–8621

19. Evans, G. B., Furneaux, R. H., Hutchison, T. L., Kezar, H. S., Morris, P. E., Jr., Schramm, V. L., and Tyler, P. C. (2001) Addition of lithiated 9-deaza-purine derivatives to a carbohydrate cyclic imine: Convergent synthesis of the aza-C-nucleoside immucillins. J. Org. Chem. 66, 5723–5730

20. Evans, G. B., Furneaux, R. H., Tyler, P. C., and Schramm, V. L. (2003) Synthesis of a transition state analogue inhibitor of purine nucleoside phosphorylase via the Mannich reaction. Org. Lett. 5, 3639–3640

21. Taylor, E. A., Clinic, K., Kelly, P. M., Li, L., Evans, G. B., Tyler, P. C., and Schramm, V. L. (2007) Acrylic ribooxacarbonium immonium mimics as transition state analogues of human and malarial purine nucleoside phosphorylases. J. Am. Chem. Soc. 129, 6984–6985

22. Cassera, M. B., Hazleton, K. Z., Merino, E. F., Obaldia, N., Ho, M. C., Murkin, A. S., DePinto, R., Gutierrez, J. A., Almo, S. C., Evans, G. B., Babu, Y. S., and Schramm, V. L. (2011) P. falciparum parasites are killed by a transition state analogue of purine nucleoside phosphorylase in a primate animal model. PLoS ONE 6, e26916

23. Sattui, S. E., and Gaffo, A. L. (2016) Treatment of hyperuricemia in gout: recent therapeutic options, latest developments and clinical implications. Ther. Adv. Musculoskel. Dis. 8, 145–159

24. Lewandowicz, A., and Schramm, V. L. (2004) Transition state analysis for human and Plasmodium falciparum purine nucleoside phosphorylases. Biochemistry 43, 1458–1468

25. Edwards, A. A., Tipton, J. D., Brenowitz, M. D., Emmett, M. R., Marshall, A. G., Evans, G. B., Tyler, P. C., and Schramm, V. L. (2010) Conformational dynamics of human purine nucleoside phosphorylase at rest, at work, and with transition state analogues. Biochemistry 49, 2058–2067

26. Ho, M. C., Shi, W., Rinaldo-Matthis, A., Tyler, P. C., Evans, G. B., Clinic, K., Almo, S. C., and Schramm, V. L. (2010) Four generations of transition-state analogues for human purine nucleoside phosphorylase. Proc. Natl. Acad. Sci. U.S.A. 107, 4805–4812

27. Hirschi, J. S., Arora, K., Brooks, C. L., 3rd, Schramm, V. L. (2010) Conformational dynamics in human purine nucleoside phosphorylase with reactants and transition-state analogues. J. Phys. Chem. B. 114, 16263–16272

28. Firestone, R. S., Cameron, S. A., Karp, J. M., Arcus, V. L., and Schramm, V. L. (2017) Heat capacity changes for transition-state analogue binding and catalysis with human 5’-methylthioadenosine phosphorylation. ACS Chem. Biol. 12, 464–473

29. Molina-Arcas, M., Casado, F. J., and Pastor-Anglada, M. (2009) Nucleoside transporter proteins. Curr. Vasc. Pharmacol. 7, 426–434

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