Achieving the Ultimate Physiological Goal in Transition State Analogue Inhibitors

for Purine Nucleoside Phosphorylase*

Andrzej Lewandowicz‡, Peter C. Tyler§, Gary B. Evans§, Richard H. Furneaux§ and Vern L. Schramm‡

‡Department of Biochemistry, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY 10461 and §Carbohydrate Chemistry Team, Industrial Research Limited, Lower Hutt, New Zealand.

Address correspondence to V. L. Schramm. Telephone: 718-430-2813. Fax: 718-430-8565. Email: vern@aecom.yu.edu.

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Summary: Genetic deficiency of human purine nucleoside phosphorylase (PNP) causes T-cell immunodeficiency. The enzyme is therefore a target for autoimmunity disorders, tissue transplant rejection and T-cell malignancies. Transition state analysis of bovine PNP led to the development of Immucillin-H, a powerful inhibitor of bovine PNP but less effective for human PNP. The transition state of human PNP differs from the bovine enzyme and transition state analogues specific for the human enzyme were synthesized. Three first-generation transition state analogues; Immucillin-G ($K_d = 42$ pM), Immucillin-H ($K_d = 56$ pM) and 8-aza-Immucillin-H ($K_d = 180$ pM) are compared with three second-generation DADMe-compounds [4’-Deaza-1’-Aza-2’-Deoxy-1’-(9-Methylene)-Immucillins] tailored to the transition state of human PNP. The second-generation compounds DADMe-Immucillin-G ($K_d = 7$ pM), DADMe-Immucillin-H ($K_d = 16$ pM) and 8-aza-DADMe-Immucillin-H ($K_d = 2.8$ nM) are superior for inhibition of human PNP by binding up to 6-fold tighter. The DADMe-Immucillins are the most powerful PNP inhibitors yet described, with $K_m/K_d$ ratios up to 5,400,000. Immucillin-H and DADMe-Immucillin-H are orally available in mice and DADMe-Immucillin-H is more efficient than Immucillin-H. DADMe-Immucillin-H achieves the ultimate goal in transition state inhibitor design in mice. A single oral dose causes inhibition of the target enzyme for the approximate lifetime of circulating erythrocytes.
**Introduction:** In 1975 Eloise Giblett discovered a child with a specific T-cell immunodeficiency and traced the biochemical defect to a genetic deficiency of PNP \(^1\) (1). Subsequent investigations demonstrated that the biochemical link between PNP and T-cell deficiency was the failure to degrade deoxyguanosine and its conversion to dGTP in activated T-cells (2, 3). Stimulated T-cells require DNA synthesis for cell division, and excess dGTP acts to allosterically inhibit ribonucleotide diphosphate reductase causing unbalanced deoxynucleotide pools and the induction of apoptosis (4, 5). Dividing T-cells induce deoxycytidine kinase activity relative to resting T-cells and this enzyme also accepts deoxyguanosine when blood levels are elevated (6). Under normal conditions the blood level of deoxyguanosine is less than 0.1 µM because of high levels of PNP in human erythrocytes and in liver, spleen and intestine (7). In PNP-deficient patients the deoxyguanosine level in blood can reach 10 µM, causing its accumulation as dGTP in stimulated T-cells.

Structure-based inhibitor design using early X-ray crystal structures of human PNP led to a family of inhibitors with dissociation constants to 15 nM, but these were insufficient to cause the sustained elevation of blood deoxyguanosine needed to suppress division of activated T-cell populations (9). Transition state analogue inhibitor design based on the transition state structure of bovine PNP led to the design and synthesis of ImmH \(^1\), a 23 pM inhibitor for the bovine enzyme but a 2 - 3 fold weaker inhibitor for the human enzyme (10, 11). In the presence of deoxyguanosine, ImmH induces apoptosis in dividing human T-cells but not other cell types. Normal human T-cells that are not stimulated to divide are not affected by Immucillin-H and deoxyguanosine (12). In a mouse model of host vs graft disease, Immucillin-H is as effective as cyclosporin in prolonging host survival (13). These favorable features of PNP inhibition have
recently gained Immucillin-H approval to enter phase I/II clinical trials against T-cell leukemia (14).

Recently, we have examined the transition state structure of human PNP\textsuperscript{2}, since it and the bovine PNP are known to have different binding affinities with the Immucillin transition state analogues (11, 15). When a transition state analogue binds with different strengths to isozymes such as the human and bovine PNPs, a difference in their transition state structures is likely to be the cause. Despite an 87% amino acid identity between human and bovine PNPs, differences at the homotrimeric subunit interfaces cause differences in catalytic site cooperativity and apparently, differences in transition state properties (11). Kinetic isotope effect analysis\textsuperscript{2} established that the transition state is more dissociated for the human enzyme, with greater separation between the ribosyl group and the departing purine ring. While Immucillin-H is well-suited to the transition state of bovine PNP, DADMe-Immucillin-H is better matched to the more dissociated transition state (Figure 1). Here we demonstrate that the DADMe-Immucillins are more powerful inhibitors of human PNP than the first-generation Immucillins designed to match the transition state of bovine PNP. The oral availability of DADMe-Immucillin-H is established in a mouse model and its biological efficiency is compared to Immucillin-H. DADMe-Immucillin-H is a sufficiently powerful inhibitor of PNP in circulating mouse erythrocytes that regain of blood PNP activity occurs primarily by synthesis of new erythrocytes.

**Experimental Procedures**

*Human PNP.* The plasmid (pEPD) containing human PNP sequence was the generous gift of Scott McIvor, M.D. (16). Polymerase chain reaction with designed primers purchased from Invitrogen was performed to amplify PNP DNA which was cloned to T7/NT TOPO vector and transformed in BL21(DE3) *E.Coli* strain.
Inhibitor synthesis. Immucillin-H was synthesized from D-gulonolactone to introduce appropriate stereochemistry in the iminoribitol ring as described earlier (Figure 1; 17-19). DADMe-Immucillins were synthesized via the reductive amination of 5-N-benzyloxyethyl-4-methoxy-3H,5H-pyrrolo[3,2-d]pyrimidine-7-carbaldehyde, derived from the lithiation and subsequent formylation of 5-N-benzyloxyethyl-7-bromo-4-methoxy-3H,5H-pyrrolo[3,2-d]pyrimidine with (3R,4R)-3-hydroxy-4-hydroxymethylpyrrolidine. Acid deprotection and chromatography provided DADMe-ImmH1 as the HCl-salt and was characterized by NMR, mass spectroscopy and elemental analysis as outlined earlier (Figure 1; 20). A full description of the chemical synthetic routes for DADMe-Immucillins will be reported elsewhere3.

Inhibition studies. Inhibitor dissociation constants for phosphorolysis of inosine by PNP were based on reaction rates measurements with different inhibitor concentrations. Reactions were started by addition of 0.05 µg of human PNP (final concentration 1.4 nM) to 1 mM inosine in 50 mM KPO4, pH = 7.5 buffer with xanthine oxidase added to final concentration 60 mU/mL at 25 °C. In the coupled assay hypoxanthine formed by phosphorolysis of inosine was oxidized to uric acid and followed spectrophotometrically at 293 nm (extinction coefficient for uric acid ε293 = 12.9 mM⁻¹) (21). The dissociation constant for slow-onset tight-binding inhibitors was determined from reaction rates after slow onset inhibition had occurred according to the equation v = (kcat x S)/(Km(1+I/Kd) + S), where v is the steady state reaction rate after the slow-onset inhibition period has reached equilibrium, kcat is the rate at substrate saturation, S is substrate concentration, Km is the Michaelis constant for inosine (38 µM under these conditions), I is inhibitor concentration and Kd is the equilibrium dissociation constant for the tightly-inhibited PNP-inhibitor complex (22). For inhibitors without slow-onset properties, fits to the same equation were made, but using initial reaction rates.
Rate constants for dissociation of PNP•Immucillin•PO₄—Inhibition of PNP by the Immucillins is a two step process to form the tightly-bound complex of PNP (11). The rate of inhibitor release was estimated by the regain of enzyme activity following dilution of stoichiometric complexes formed by prolonged incubation similar to experiments described earlier for Immucillin-H and Immucillin-G with bovine PNP (11). Briefly, human PNP (96 µM, inhibitors (105 to 108 µM and PO₄ (50 mM pH 7.5) were incubated at 25 °C for 5.5 hr to assure equilibrium. Mixtures were diluted 1:500,000 by a 1:500 dilution into 20 mM Tris-HCl pH 7.5 followed immediately by a 1:1000 dilution into assay mixtures containing 1 mM inosine as described above. The rate constant for dissociation of inhibitors was calculated from fits to the initial regain of catalytic activity as described earlier (11). These experiments also demonstrate the reversible inhibition caused by Immucillins.

Oral availability of ImmH and DADMe-ImmH—A solution containing 10⁻⁷ mole (27 µg) of DADMe-ImmH and 3 mg glucose was pipetted into the mouth of a 32 g Balb-c mouse that had been fasted overnight. The oral uptake of ImmH was measured by pipetting 27 µg in 10 µL solution onto solid food and fed to mice under observation. Small samples (5 µL) of blood were collected from the tail and added to 6 µL of PBS (140 mM NaCl, 3 mM KCl, 10 mM KHPO₄, pH = 7.4) containing 1 U heparin and 0.3% Triton X-100. Control blood samples were collected before administration of Immucillins to the mice. The mixture was kept on ice for 25 min and frozen in dry ice/ethanol for storage at –70 °C. For catalytic activity of whole blood samples, 10 µL of the lysate was added to 735 µL complete reaction mixture containing 1 mM inosine, 50 mM phosphate, pH = 7.4 and 60 mU/ml xanthine oxidase. After mixing, the reaction progress was followed spectrophotometrically at 293 nm. For determination of the equilibrium dissociation constant ($K_d$) the slope was taken after the slow-onset steady-state inhibition had
occurred (typically 40-50 min under conditions of Figure 3). In separate experiments, samples of tail blood (5 µL) were pipetted into 1 mL ice-cold PBS, centrifuged in the cold, resuspended in a second 1 mL sample of fresh PBS, and the pellet of washed erythrocytes finally lysed in 5 µL of PBS containing Triton X-100 and assayed as above.

RESULTS AND DISCUSSION

Inhibition of PNP by Immucillins—Human PNP was inhibited by ImmG, ImmH and 8-aza-ImmH to give equilibrium dissociation constants ($K_d$) of 42, 56 and 180 pM (see Figure 1). The kinetic properties exhibited slow-onset, tight binding inhibition typical of inhibition by compounds that resemble transition state analogues (22). The Immucillins were designed to match the transition state for bovine PNP, which has a C1’-N9 ribosidic bond of 1.77 Å at the transition state (Figure 2; 10). The Immucillin transition state analogues are found to be better inhibitors of the bovine enzyme than the human PNP (to 23 pM for the bovine enzyme; 11), establishing that the transition states of the two enzymes differ, despite 87% amino acid sequence identity and the observation that every amino acid at the catalytic site is conserved (23). Tight-binding of the Immucillins to bovine PNP depends on two essential features (24). The 9-deazahypoxanthine structure increases the $pK_a$ at N7 to above 10, and the N7 of substrate at the transition state is known to be a proton acceptor. The 4’-N in the iminoribitol has a $pK_a$ of 6.9 in ImmH and is protonated at the catalytic site to resemble the cationic feature of substrates at the transition state (25). Loss of either of these features destroys the tight binding to PNP (24).

Design principles of the DADMe-Immucillins—Recent kinetic isotope effect studies with human PNP have established that the transition state structure differs from that of the bovine enzyme, to give a C1’-N9 ribosidic bond length of greater than 2.5 Å and weak participation of the anionic nucleophile (>3.0 Å for arsenate). These features cause increased cation character at
C1’ compared to the transition state for bovine PNP (Figure 2). The Immucillins have a covalent C-C bond between the iminoribitol and the 9-deazapurine group and this bond length is near 1.5 Å, making it at least 1.0 Å shorter than the corresponding atoms at the transition state. In more dissociated transition states like that of the human PNP, the cationic charge on the riboxacarbenium ion becomes more highly developed and centered at the C1’ carbocation, rather than being distributed over O4’, C1’ and N9 when the transition state has significant residual bond order remaining between C1’ and N9 (26). We proposed that spacing the iminoribitol and the 9-deazapurine rings closer to the actual transition state, and placing the carbocation charge of the transition state closer to its location in the transition state may increase the affinity specifically for the human PNP. The DADMe-Immucillins (Figure 1) were synthesized as outlined in the Methods. The methylene bridge between the deazapurine and ribosyl-analogue groups of DADMe-Immucillins adds approximately 1 Å linear distance between these groups but does not alter the favorable pKₐ of the 9-deazahypoxanthine. Moving the nitrogen to the position corresponding to C1’ retains the cationic nature to more closely mimic the riboxacarbenium ion transition state since N-substituted hydroxymethylpyrrolidines have pKₐ values near 10, and will be fully protonated at physiological pH values near 7.5 (27).

Inhibition of human PNP by DADMe-Immucillins—Human PNP binds Immucillin-G more tightly than other members of the Immucillin family to give a Kₐ of 42 pM (Figure 1). Likewise, DADMe-ImmG is the most tightly bound member of the DADMe family with a Kₐ of 7 pM, improving the affinity by a factor of 6. In parallel with the ImmG and DADMe-ImmG affinities, DADMe-ImmH binds a factor of 4 more tightly than ImmH and like DADMe-ImmG, gives slow-onset, tight-binding inhibition (Figure 3). This pattern does not continue with the 8-aza-DADMe-ImmH. Thus, 8-aza-ImmH binds with a Kₐ of 0.18 nM while 8-aza-DADMe-
ImmH binds with a dissociation constant of 2.0 nM, and does not exhibit slow-onset inhibition. The affinity of 8-substituted ImmH analogues with the bovine PNP have shown that the pK\textsubscript{a} at N7 is an important parameter for Immucillin binding (24). The carbonyl oxygen of Asn243 forms a short H-bond to the N7 proton of ImmH (28). Loss of binding energy in 8-aza-DADMe-ImmH relative to 8-aza-ImmH reveals that either the pK\textsubscript{a} or the contact geometry between N7 and Asn243 is not as favorable as it is in 8-aza-DADMe-ImmH.

The kinetics of DADMe-ImmH inhibition of human PNP demonstrate two distinct inhibitory phases (Figure 3). In the first few minutes, the initial rate slopes change rapidly to reach a second steady-state plateau of increased inhibition. This is characteristic of transition state analogue inhibitors and reflects a slow enzymatic conformational change to form the tightly bound complex: E + I \rightarrow EI \rightarrow E*I, where E is free enzyme, I is inhibitor, EI is the rapidly-reversible complex of enzyme and inhibitor and E*I is the tightly-bound slowly releasing form of the inhibited complex. The K\textsubscript{d} values reported here are calculated from the slopes of the curves at 40-50 min, where the slow onset-phase of inhibition has been completed. This part of the curve provides the overall dissociation constant for E*I (22).

*In vivo inhibition of PNP by the DADMe-Immucillins*—The goal of the PNP inhibitor design program is to use transition state design principles to develop more effective inhibitors against mammalian PNPs in vivo. In vitro measurements suggest that the DADMe-Immucillins should be the more effective inhibitors in vivo, provided that both inhibitor families have similar bioavailability. We elected to compare ImmH and DADMe-ImmH for bioavailability in mice, since the in vivo behavior of ImmH is becoming well-characterized from studies in mice and primates (13, 14, 29). Oral administration of 0.1 µmol (27 µg, 0.8 mg/kg) of ImmH or DADMe-ImmH caused rapid loss of the PNP activity in mouse blood with loss of 50% of total catalytic
activity in blood occurring in 10 and 14 min for DADMe-ImmH and ImmH respectively (Figure 4). The catalytic activity protocol of whole-blood lysis does not establish if the Immucillins penetrate the erythrocyte compartment, since serum ImmH could inhibit erythrocyte PNP following lysis. In parallel experiments, blood samples were washed in cold buffer to remove extracellular Immucillins followed by lysis and assay. The inhibition of erythrocyte PNP was complete for both Immucillins in this assay, demonstrating the cellular permeability of these inhibitors.

Thus, both ImmH and DADMe-ImmH are readily available from the gastrointestinal system and equilibrate with whole blood PNP more rapidly than they can be removed by hepatic or renal systems. Continued monitoring for the regain of enzymatic activity demonstrated that 50% of PNP in the blood regained activity at 100 hr (4.2 days) with ImmH (Figure 4). In contrast, blood PNP regained activity more slowly with DADMe-ImmH, for a 50% activity regain at 275 hr, or 11.5 days.

At long times for regain of erythrocyte PNP activity, factors other than inhibitor release and metabolism must be considered. The average life-span for mouse erythrocytes is 25 days (30), therefore after 12.5 days, one-half of the erythrocytes present at the start of the experiment will have been replaced by new erythrocytes. When the red cell replacement rate is considered, approximately 90% of the PNP activity regain that occurs over 11.5 days is due to synthesis of new erythrocytes. The in vivo inhibition of mouse blood PNP by a single dose of DADMe-ImmH therefore approximates the life-span of mouse erythrocytes. The enhanced inhibition seen for DADMe-ImmH in enzyme inhibition experiments with human PNP is confirmed in the whole animal mouse model. The DADMe-Immucillins represent the culmination of transition state inhibitor design in efficient inhibition of the target enzyme following a single oral dose.
Comparison of off-rates and biological dissociation rates—The biological half-life of DADMe-ImmH in the mouse results from the competition between binding to erythrocyte PNP and elimination from the animal. In the simplest case, the biological half-life would be directly related to the $t_{1/2}$ for the E*I complex. That is not the case for the Immucillins, with binding half-lives of 8 to 120 min for ImmH, DADMe-ImmH and DADMe-ImmG respectively (Table 1). These are similar to the rates reported for other tight-binding inhibitors of enzymes (Table 1). The concentration of PNP inside mouse erythrocytes is approximately $10^{-6}$ M and the $K_d$ for DADMe-ImmH is $1.6 \times 10^{-11}$ M. Release of DADMe-ImmH from an intracellular catalytic site makes recapture more likely than release by diffusion. Therefore, with a $t_{1/2}$ of 20 min for DADMe-ImmH release at near infinite dilution, we propose that once trapped inside mouse erythrocytes, each DADMe-ImmH molecule binds sequentially to hundreds of PNP molecules before loss to the plasma. Rebinding of DADMe-ImmH is sufficient to cause nearly full inhibition of target PNP for the 25 day lifetime of mouse erythrocytes. As indicated above, approximately 90% of PNP activity regain corresponds to synthesis of new erythrocytes. We hypothesize that rebinding traps the inhibitor into individual cells and at the death of these cells, the inhibitor is excreted or metabolized more efficiently than transport or diffusion into developing cells.

CONCLUSIONS

Transition state theory teaches that the most powerful noncovalent enzyme inhibitors can be obtained with chemically stable mimics of the transition state structures of the cognate enzymes. Perfect analogues of enzymatic transition states can never be obtained because of the non-equilibrium bond lengths and charges that exist at the transition state. However only a fraction of the $10^{12}$-fold increased binding potential theoretically available for PNP analogues is
required for biological efficiency. In the case of mammalian PNP inhibitors, we have achieved $5 \times 10^6$-fold tighter binding, sufficient to reach the ultimate goal in transition state analogue design. DADMe-ImmH binds sufficiently tightly to the target enzyme in an in vivo mouse model to be released only on the time scale of cellular replacement.
REFERENCES


**FOOTNOTES AND ACKNOWLEDGEMENTS**

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1Abbreviations are: PNP, purine nucleoside phosphorylase; ImmH, Immucillin-H; DADMe-ImmH, 4’-Deaza-1’-Aza-2’-Deoxy-1’-(9-Methylene)-Immucillin-H. The IUPAC nomenclature for DADMe-ImmH is (3R,4R)-N-[(9-deazahypoxanthin-9-yl)methyl]-4-hydroxymethyl-pyrrolidin-3-ol.

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Table 1. Examples of enzyme-inhibitor release rates and dissociation constants compared to Immucillins with human PNP.\(^a\)

<table>
<thead>
<tr>
<th>enzyme</th>
<th>inhibitor</th>
<th>(t_{1/2})(^b)</th>
<th>(K_d)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartate transcarbamylase</td>
<td>PALA</td>
<td>1 min</td>
<td>5 nM</td>
</tr>
<tr>
<td>\textit{E.coli} DHF reductase</td>
<td>trimethoprim</td>
<td>8 min</td>
<td>20 pM</td>
</tr>
<tr>
<td>HMGCoA reductase</td>
<td>compactin</td>
<td>15 min</td>
<td>240 pM</td>
</tr>
<tr>
<td>Chicken DHF reductase</td>
<td>methotrexate</td>
<td>35 min</td>
<td>9 pM</td>
</tr>
<tr>
<td>Xanthine oxidase</td>
<td>allopurinol</td>
<td>300 min</td>
<td>630 pM</td>
</tr>
<tr>
<td>Adenosine deaminase</td>
<td>deoxycoformycin</td>
<td>40 hr</td>
<td>2 pM</td>
</tr>
<tr>
<td>Human PNP</td>
<td>Immucillin-H</td>
<td>8 min</td>
<td>56 pM</td>
</tr>
<tr>
<td>Human PNP</td>
<td>DADMe-ImmH</td>
<td>20 min</td>
<td>16 pM</td>
</tr>
<tr>
<td>Human PNP</td>
<td>DADMe-ImmG</td>
<td>120 min</td>
<td>7 pM</td>
</tr>
</tbody>
</table>

\(^a\)Enzyme inhibition examples are summarized in reference 22 and the PNP values are from the present work. \(^b\)The value of \(t_{1/2}\) is the time to regain 50% of uninhibited enzyme activity. \(^c\)The \(K_d\) value for slow-onset inhibitors is governed by the expression \(K_d = (K_i k_6)/(k_5 + k_6)\) where \(K_i\) is the initial dissociation constant ([E][I]/[EI]) and \(k_5\) and \(k_6\) are rates of formation and relaxation of the tightly-bound complex (E*I) from (EI). \(K_d\) is the equilibrium dissociation constant = [E][I]/[E*I] where [E] is free enzyme concentration [I] is inhibitor concentration and [E*I]. \(K_i\) reflects capture of the inhibitor and \(K_d\) reflects overall equilibrium binding energy (22).
LEGENDS TO FIGURES

Figure 1. First and second generation inhibitors for human purine nucleoside phosphorylase. The values given are dissociation constants for the tightly inhibited complex after slow-onset is complete. This value is often called $K_i$ (11, 22). All compounds except 8-aza-DADMe-ImmH are slow-onset inhibitors. The numbering of atoms is shown for inosine and is also used for convenience here for the Immucillins and DADMe-Immcullins although it does not conform to IUPAC rules.

Figure 2. Transition state structures for bovine and human PNP. The transition states have been determined from kinetic isotope effect studies and computational matching of the isotope effects. The distances for the transition states have been reported for bovine PNP (10) and lower limits have been set for the transition state geometry for human PNP$^2$. The distances of 1.5 Å for Immucillin-H and 2.5 Å for DADMe-Immcillin-H refer to the linear distance between the deazapurine ring and C1’ or N1’ of the ribosyl analogues (see Figure 1 for numbering).

Figure 3. Slow-onset, tight binding inhibition of human PNP by DADMe-ImmH. The production of hypoxanthine from inosine is monitored by conversion to uric acid in a coupled assay as indicated in the Methods. The concentrations of DADMe-ImmH are indicated. The inset is a replot of the initial rate (no inhibitor) compared to rates at 40-50 min with inhibitor. This replot is used to calculate the $K_d$ value from the equation for competitive inhibition.

Figure 4. Oral availability for ImmH and DADMe-ImmH in mice. The $t_{1/2}$ for onset is the time after oral administration that 50% of PNP activity in blood is inhibited. The $t_{1/2}$ for DADMe-ImmH is 10 min and that for ImmH is 14 min as indicated in the text. The $t_{1/2}$ for PNP activity recovery following the single oral dose is 100 hr for ImmH (open circles) and 275 hr for
DADMe-ImmH (closed circles). The ordinate scale is nmoles of uric acid per min using the assay described in the methods.
first generation PNP transition state analogues

Immucillin-G 42 ± 6 pM
Immucillin-H 56 ± 15 pM
8-aza-Immucillin-H 180 ± 20 pM

second generation PNP transition state analogues

DADMe-ImmG 7 ± 1 pM
DADMe-ImmH 16 ± 1 pM
8-aza-DADMe-ImmH 2.0 ± 0.1 nM

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Figure 1
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Figure 2
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Figure 3
$t_{1/2} = 275$ h (11.5 days)

$t_{1/2}$ onset = 10 min

mouse blood activity

time [h]

dotted line: ImmH

circle: DADMelmmH

black circle: DADMelmmH

open circle: ImmH
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