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N-Phosphonocarbonylpyrrolidine Derivatives of Guanine: A New Class of Bi-Substrate Inhibitors of Human Purine Nucleoside Phosphorylase

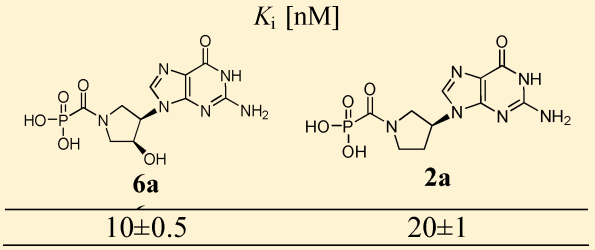
Dominik Rejman,*[,†] Natalya Panova,[†] Pavel Klener,*[,‡] Bokang Maswabi,[‡] Radek Pohl,[†] and Ivan Rosenberg*[,†]

[†]Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, v.v.i. Flemingovo nám. 2, 166 10 Prague 6, Czech Republic

[‡]Institute of Pathological Physiology, First Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 128 53 Prague 2, Czech Republic

Supporting Information

ABSTRACT: A complete series of pyrrolidine nucleotides, (3*R*)- and (3*S*)-3-(guanin-9-yl)pyrrolidin-1-*N*-ylcarbonylphosphonic acids and (3*S*,4*R*)-, (3*R*,4*S*)-, (3*S*,4*S*)-, and (3*R*,4*R*)-4-(guanin-9-yl)-3-hydroxypyrrolidin-1-*N*-ylcarbonylphosphonic acids, were synthesized and evaluated as potential inhibitors of purine nucleoside phosphorylase (PNP) isolated from peripheral blood mononuclear cells (PBMCs) and cell lines of myeloid and lymphoid origin. Two compounds, (*S*)-3-(guanin-9-yl)pyrrolidin-1-*N*-ylcarbonylphosphonic acid (**2a**) and (3*S*,4*R*)-4-(guanin-9-yl)-3-hydroxypyrrolidin-1-*N*-ylcarbonylphosphonic acid (**6a**), were recognized as nanomolar competitive inhibitors of PNP isolated from cell lines with K_i values within the ranges of 16−100 and 10−24 nM, respectively. The low $^{MESG}K_i$ and $^{Pi}K_i$ values of both compounds for PNP isolated from PBMCs suggest that these compounds could be bisubstrate inhibitors that occupy both the phosphate and nucleoside binding sites of the enzyme.



INTRODUCTION

In the purine salvage pathway, the enzyme purine nucleoside phosphorylase (PNP, EC 2.4.2.1) catalyzes the reversible phosphorolytic cleavage of guanine and hypoxanthine nucleosides in the presence of the second substrate, inorganic orthophosphate (P_i), releasing the corresponding purine nucleobase and sugar phosphate.[1] In the absence of PNP, nucleoside substrates, such as 2′-deoxyguanosine (dGuo), accumulate.[2] The accumulation of dGuo has been observed in children with an inherited PNP deficiency. These children exhibit severe T-cell immunodeficiency but retain normal B-cell function. Phosphorylation of dGuo (by 2′-deoxycytidine kinase, dCK, EC 2.7.1.74) to 2′-deoxyguanosine triphosphate (dGTP) is responsible for T-cell cytotoxicity. dGTP allosterically inhibits the enzyme ribonucleoside-5′-diphosphate reductase (EC 1.17.4.1), impairing DNA synthesis and initiating T-cell apoptosis.[3] Human T-cells are unique in that they display a combination of high dCK activity and relatively low nucleotidase activity, which allows these cells to accumulate dGTP.[4] PNP has been identified as a therapeutic target for the control of numerous T-cell disorders, including organ transplant rejection, T-cell malignancies, rheumatoid arthritis, psoriasis, and some other autoimmune diseases.[1,5−7] B-Cell chronic lymphocytic leukemia (CLL) cells also exhibit high dCK activity. Recently, CLL cells where shown to respond to PNP inhibition.[8]

PNP is widely expressed in human tissues, predominantly in the cytosol and mitochondria of cells. The highest activity of PNP was detected in both normal and malignant leukocytes, including peripheral blood mononuclear cells (PBMCs), neutrophil leukocytes (granulocytes), and lymphocytes. Increased levels of PNP in cancer cells and the relationship between PNP activity and the degree of malignancy have been reported.[9]

The first inhibitors of PNP were developed using structure-based inhibitor design focused on iterative group alignment established from the PNP crystal structure.[10] Following a novel approach based on the identification of the transition-state structures stabilized by the target enzyme, the potent inhibitors immucillin-H (IMH, 1-(9-deazahypoxanthin)-1,4-dideoxy-1,4-imino-D-ribitol) and immucillin-G (IMG, 1-(9-deazaguanin)-1,4-dideoxy-1,4-imino-D-ribitol) (Figure 1) of bovine, malarial, *Mycobacterium tuberculosis*, and human PNPs were identified.[11−14] IMH was reported to induce apoptosis and selectively suppress the growth of human T-cell leukemia lines with an IC_{50} of 5 nM.[15] Furthermore, IMH has completed phase IIb trials against cutaneous T-cell lymphoma (as BCX-1777 or Forodesine).[16,17] IMH was observed to selectively inhibit the in vitro growth of malignant T-cell lines in the presence of dGuo

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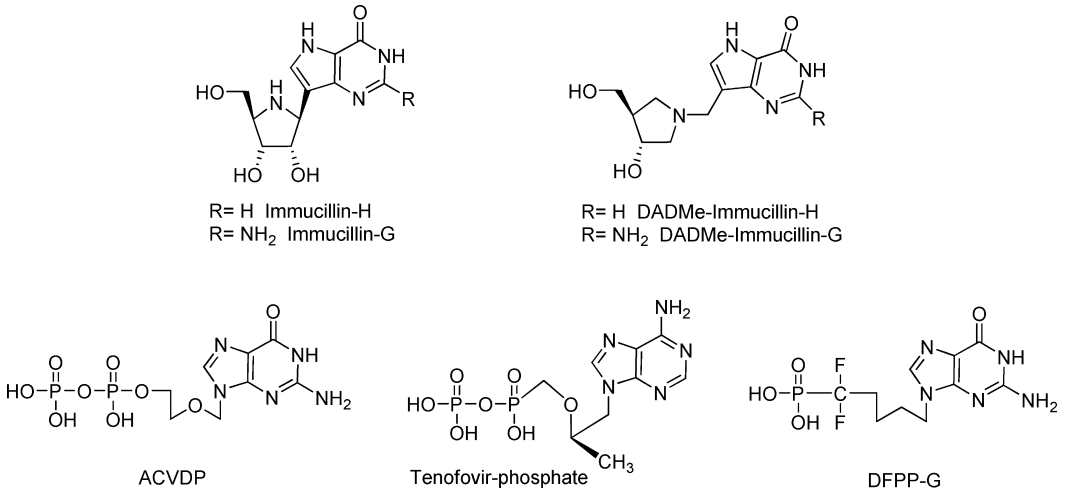


Figure 1. Inhibitors of PNP.

without affecting non-T-cell tumor lines. Activated human peripheral blood T-lymphocytes were also sensitive to inhibition by IMH.[15] Moreover, recent clinical and preclinical data showed activity of Forodesine in B-cell acute lymphoblastic leukemia (B-ALL), which supports the potential use of IMH for the treatment of selected B-cell malignancies.[8,18]

More recently, the second generation of the immucillins, the DADMe-immucillins (Figure 1), has been reported.[19] In contrast to IMHs mimicking a substrate-like transition state, DADMe-immucillin-H is more closely related to the product-like. Among these new compounds, the most potent DADMe-immucillin-G was eight times more active than IMH.

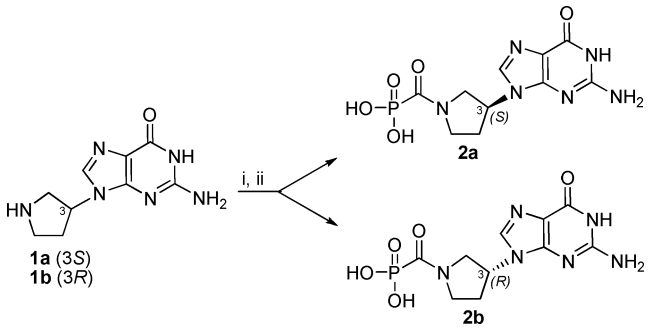
In addition to these nucleoside-based inhibitors, several acyclic nucleotide analogues were found to inhibit human PNP (Figure 1) in a nanomolar range. Acyclovir diphosphate (ACVDP)[20−22] and Tenofovir-phosphate[23] exhibited K_i values of 10 and 38 nM, respectively. However, neither ACVDP nor Tenofovir-phosphate are considered as drug candidates for PNP inhibition because of the instability of phosphate groups in vivo. High-resolution differentiation X-ray data obtained from a crystal of PNP with 9-(5′,5′-difluoro-5′-phosphonopentyl)guanine (DFPP-G, K_i of 10.8 nM) confirmed DFPP-G as a multi-substrate inhibitor. This crystal structure revealed that the nucleoside and phosphonate parts of one molecule of DFPP-G were bound simultaneously into the nucleoside and phosphate binding sites of PNP, respectively.[24] Recently, a variety of deazaguanine analogues of DFPP-G have also been recognized as potent PNP inhibitors.[25]

Our systematic search for nucleoside phosphonate-based inhibitors of enzymes involved in nucleoside/nucleotide catabolism has led to the synthesis of a series of pyrrolidine nucleoside phosphonic acids. A recent study on the inhibition of thymidine phosphorylase from rat T-cell lymphomas by these type of compounds revealed their nanomolar inhibitory effect, suggesting their possible role as bisubstrate inhibitors.[26] On the basis of these findings, we synthesized the corresponding guanine derivatives to examine their potential inhibitory effect on PNP partially purified from human lymphoma and leukemia cell lines of T- and B-cell origin as well as PNP from the lymphocytes of healthy donors.

RESULTS AND DISCUSSION

Chemistry. Enantiomeric pyrrolidine derivatives of guanine **1a** and **1b**, prepared as previously reported,[27] were treated with phenyl diisopropylphosphonoformate[28] in DMF at 90 °C overnight.[26] The diisopropyl ester groups were removed using trimethylsilyl bromide in DMF, and the crude **2a** and **2b** were purified by the preparative HPLC on C18 column. The following conversion into sodium salts via Dowex 50 × 8 (Na^+ form) and lyophilization from water afforded the final enantiomeric phosphonic acids **2a** and **2b** (Scheme 1).

Scheme 1. Synthesis of Enantiomeric Pyrrolidinyl Nucleotide Analogues 2a and 2b[a]



[a]Reagents: PhO(O)CP(O)$(OPr)_2$, DMF 90 °C; (i) Me_3SiBr, DMF.

The synthesis of diastereoisomeric hydroxypyrrolidine derivatives of guanine **5a−d** was initiated from fully protected 3,4-dihydroxypyrrolidines **3a−d**[29] (Scheme 2). The mesyl group of these compounds was converted into an azido group by treatment with sodium azide in DMF. The DMTr group of the obtained azido derivatives was selectively removed with 1.5% TFA in DCM.[30] The *N*-Boc group was not cleaved under these conditions. Subsequent hydrogenation over palladium catalyst afforded good yields of amino compounds **4a−d**, which were transformed into guanine derivatives in several steps. The reaction with 2,5-diamino-4,6-dichloropyrimidine followed by cyclization to form the five-membered imidazole ring using only 1.2 equiv of diethoxymethyl acetate in DMF, instead of using diethoxymethyl acetate as a solvent, provided the desired 2-amino-6-chloropurine derivative.[30] This modification eliminated the formylation of the purine exocyclic 2-amino group

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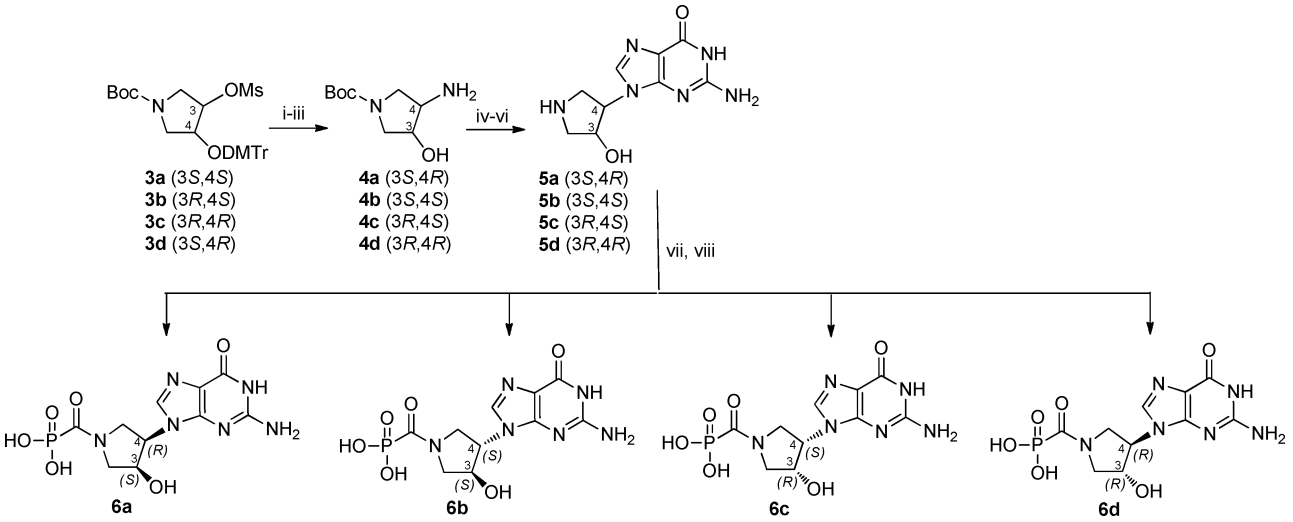
Scheme 2. Synthesis of Diastereoisomeric Hydroxypyrrolidinyl Nucleotide Analogues 6a–d[a]



[a]Reagents: (i) NaN_3, DMF, 110 °C; (ii) (i) 1.5% TFA/DCM; (ii) $NaHCO_3$; (iii) H_2, Pd/C, EtOH; (iv) 2,5-diamino-4,6-dichloropyrimidine, TEA, *n*BuOH, 130 °C; (v) $CH_3COOCH(OCH_2CH_3)_2$, DMF, from rt to 110 °C; (vi) 1.5M aq HCl, 80 °C; (vii) $PhO(O)CP(O)(OiPr)_2$, DMF, 90 °C; (viii) Me_3SiBr, MeCN.

that would otherwise render the isolation of the 2-amino-6-chloropurine intermediate difficult.[30] Final treatment of 6-chloro-2-aminopurine intermediates with 1.5 M aq HCl afforded guanine derivatives **5a–d** in moderate yields.

The resulting diastereoisomeric hydroxypyrrolidine derivatives of guanine **5a–d** were treated with phenyl diisopropylphosphonoformate[28] in DMF at 90 °C overnight.[26] The diisopropyl ester groups were removed using trimethylsilyl bromide in DMF, and the crude **6a–d** were purified by the preparative HPLC on C18 column. The following conversion into sodium salts via Dowex 50 × 8 (Na^+ form) and freeze-drying from water afforded the final diastereomeric phosphonic acids **6a–d**.

^1H and ^{13}C NMR spectra of each nucleoside phosphonate (**2a**, **2b**, and **6a–d**) showed the presence of two compounds, which were recognized as *cis* and *trans* rotamers around the C6′–N1′ linkage. These rotamers arise as a consequence of the conjugation of the carbonyl and pyrrolidine nitrogen electron pairs, which partially prevents unrestricted free rotation around the C6′–N1′ linkage (Figure 2).[31]

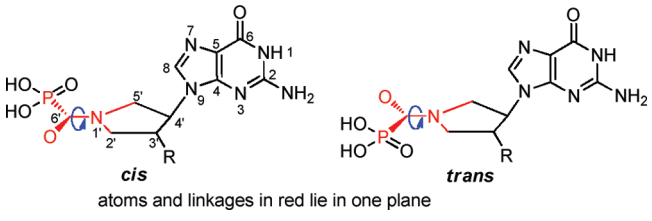


Figure 2. *Cis* and *trans* rotamers of carbonylphosphonates **2a**, **2b**, and **6a–d**.

Biochemistry. The main focus of this study was to assess the inhibitory potential of novel *N*-phosphonopyrrolidine derivatives of guanine toward PNP isolated from various cell lines. To achieve this, we completed the following: (a) optimized two step partial purification of PNP to remove a large excess of low molecular weight compounds and substantial part of balast proteins, (b) performed the inhibition assays to select potent inhibitors of PNP, and (c) performed inhibition study with selected inhibitors and PNPs isolated from various human lymphoma and leukemia cell lines of T- and B-cell origin to check potential different sensitivity of the enzymes to inhibition in comparison with PNP isolated from healthy donors.

PNP activity and inhibition assays were based on the phosphorolysis of 7-methyl-6-thioguanosine (MESG) to 7-methyl-6-thioguanine catalyzed by PNP.[32−34] This conversion resulted in a significant spectrophotometric shift from 330 nm (MESG) to 360 nm (7-methyl-6-thioguanine). MSEG, a substrate of PNP, was chosen with respect to the kinetics of cleavage, which are well described by the Michaelis–Menten equation.[32]

Partial Purification of PNP. Partially purified PNPs suitable for inhibition studies were obtained in two steps from a 105 × *g* supernatants of the cell homogenates including ammonium sulfate precipitation, dialysis, and subsequent fast anion-exchange chromatography on a HiTrap Q column (see Supporting Information page S3). The purification protocol is summarized in Table 1.

Determination of K_m and Enzyme Activity. The K_m and *V* values for MESG and inorganic phosphate were determined with PNP from different sources (Table 2). Using GraphPad Prism 4,[35] the measurement of the reaction rates of MSEG cleavage at various concentrations (up to 200 μM) at a constant potassium phosphate concentration (500 μM) provided the appropriate $^{MESG}K_m$. Similarly, we obtained the $^{Pi}K_m$ values from the reaction rate of the MESG cleavage (80 μM) and various potassium phosphate concentrations (up to 2500 μM). Simultaneously, *V* values were also obtained from the appropriate plots. All K_m and *V* values are averages of three measurements.

Inhibition Assay. The initial inhibition experiments with compounds **2a–b** and **6a–d** were carried out with PNP from PBMCs at two inhibitor concentrations (10 and 1 μM). The results summarized in Table 3 clearly show that only phosphonates **2a** and **6a** strongly inhibited the PNP even at an 80:1

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Table 1. Partial Purification of PNP and Its Distribution in Cancer Cell Lines

PNP source	no. of cells × 10^6	vol of $10^5 \times g$ supernatant (mL)	protein concentration (μg/mL)		PNP specific activity (μmol·h^{-1} per mg of protein)		purification factor [fold]/ recovery (%)
			$10^5 \times g$ supernatant	HiTrap Q column[b]	$10^5 \times g$ supernatant	HiTrap Q column[b]	
PBMC[a]	>1000	15	6000	250	2	27	14/4
JURKAT	300	4	4000	300	0.50	11	21/41
K-562	<300	3	nd	125	nd	10	nd/nd
DoHH2	600	3	3200	350	2.40	24	8.6/36
MINO	200	3	3000	250	2.32	35	16/42
MC-116	10	3	2500	200	3.50	54	14/49
SU-DHL-1	412	4	1300	100	1.59	23	14/28
RPMI 8226	235	2.5	8000	150	0.25	11	56/33
OPM-2	235	2.5	8000	50	0.30	26	87/22
HBL2 (culture)	300	2.5	2000	50	1.62	60	36/37
HBL2 (tumor)	nd	2.5	1500	125	1.09	24	10/73
CML[c]	5000	6	nd[e]	450	nd[e]	4	nd
CML[c]	5000	6.5	nd[e]	400	nd[e]	8	nd
AML[d]	2000	5	nd[e]	250	nd[e]	3.7	nd
AML[d]	2000	5	nd[e]	150	nd[e]	5.5	nd

[a]Peripheral blood mononuclear cells were obtained as buffy coats from healthy donors. [b]PNP activity was detected in only one 1 mL fraction. [c]Two different patients. [d]One patient. [e]Protein and PNP activity could not be measured due to the presence of a colored substance interfering with spectrophotometric assay.

Table 2. K_m and V Values for MESG and Inorganic Phosphate Determined for the Phosphorolysis Catalysed by Partially Purified PNPs

PNP source	$^{MESG}K_m$ (μM)	$^{Pi}K_m$ (μM)	^{MESG}V (nmol/min)	^{Pi}V (nmol/min)
PBMC[a]	80 ± 20	50 ± 10	2.5 ± 0.1	1.05 ± 0.05
hrPNP[b]	60 ± 10	100 ± 20	1.4 ± 0.09	1.4 ± 0.06
DoHH2	40 ± 5	nd	1.6 ± 0.04	nd
MINO	120 ± 20	nd	1.8 ± 0.4	nd
MC-116	70 ± 10	nd	1.5 ± 0.2	nd

[a]From healthy donors. [b]Human recombinant PNP.

ratio of substrate to inhibitor. Therefore, these compounds were assayed with hrPNP and PNP from PBMCs and cell lines derived from different human tumors to obtain K_i values with respect to MESG with the aim to find possible differences in the inhibitory potency. The results are summarized in Table 4. The initial reaction rates of the cleavage of MESG (at 40 and 80 μM) in the absence and presence of inhibitors **2a** and **6a** (up to 500 nM) and the constant inorganic phosphate concentration (500 μM) were measured. The appropriate $^{MESG}K_i$ values were calculated from Dixon plots using SigmaPlot software (see Experimental Section and the Supporting Information for plots).[36]

In the case of PNP from PBMCs, K_i values for **2a** and **6a** with respect to the inorganic phosphate were also determined. The measurements of the initial rates of MESG (80 μM) cleavage at three inorganic phosphate concentrations (250, 500, and 1000 μM) in the absence and presence of inhibitors (up to 500 nM) provided $^{Pi}K_i$ values of 50 and 20 nM for **2a** and **6a**, respectively. All K_i values in Table 4 represent the average values of three independent measurements. The patterns of Dixon plots (Supporting Informationo) suggest that **2a** and **6a** are competitive inhibitors of the PNPs studied with respect to MESG. In the case of PNPs from cancer cell lines, the K_i values of compound **2a** varied from 16 to 100 nM, whereas lower values (10−24 nM) were obtained for the hydroxy derivative **6a**. The low $^{MESG}K_i$ and $^{Pi}K_i$ values of **2a** and **6a** for PNP from PBMCs suggest that these compounds could be bisubstrate inhibitors, occupying both the phosphate and nucleoside binding sites of the enzyme.

The inhibitory activity of **2a**, in contrast to **6a**, varied widely between 16 and 100 nM (Table 4). Because the plausibility of these data is given by the arrangement of kinetic experiments (both **6a** and **2a** were measured simultaneously with the same batch of the enzyme), one can speculate that the obtained data for **2a** resulted from different affinity of **2a** toward PNP isoforms,[37] the ratio and type of which could vary in dependence on the type of cancer cell lines. On the other hand, it seems that the inhibitor **6a** providing a relatively narow window of inhibitory activity lost a selectivity due to the presence of hydroxy group allowing the formation of additional hydrogen bond(s) and thus a more tight binding to the enzyme catalytic site (regardless of the PNP isoforms).

Time-Dependent Inhibition. To quantify the possible time- and concentration-dependent inactivation of PNP during preincubation with the most potent inhibitor **6a**, we measured the remaining enzyme activity using MESG as a substrate at various inhibitor concentrations (0−250 nM) during 10 and 20 min periods of preincubation. The results demonstrated an increase in inhibition activity at each inhibitor concentration (Figure 3). In contrast, there was no difference between 10 and 20 min of preincubation. We observed that the IC_{50} values decreased from approximately 40 nM (no preincubation) to 10 nM within 10 or 20 min of preincubation. This time-dependent inhibition could be explained by a two-step mechanism where binding involves the rapid formation of an enzyme−inhibitor collision complex followed by slow conformational changes that

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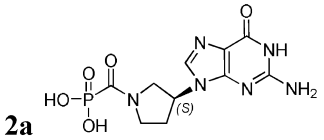
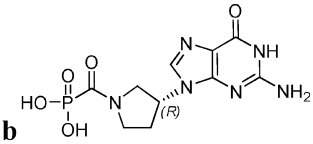
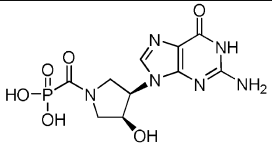
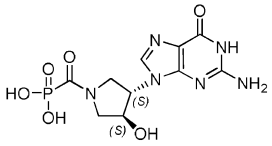
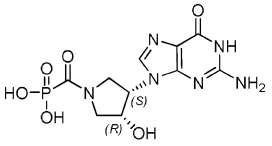
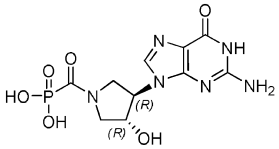
Table 3. Inhibition of PNP Isolated from PBMCs of Healthy Donors by Enantiomeric and Diastereomeric Guanine-Based Pyrrolidine Nucleoside Phosphonic Acids

Inhibitor	Remaining activity [a] [%]	
	10 μM	1 μM
 **2a**	0	10
 **2b**	0	34
 **6a**	0	0
 **6b**	100	100
 **6c**	30	100
 **6d**	100	100

[a]Measured at 80 μM of MSEG and 500 μM of inorganic phosphate.

Table 4. K_m and K_i Values of Partially Purified PNP from PBMCs and Cancer Cell Lines

Source of PNP	$^{MESG}K_m$ [μM]	$^{Pi}K_m$ [μM]	$^{MESG}K_i$ [nM] **6a**	$^{MESG}K_i$ [nM] **2a**
PBMC	80±20	50±10	10±0.5/20[a]	20±1.0/50[a]
hrPNP[b]	60±10	100±20	7±0.35	25±5.0
JURKAT	nd	nd	10±0.5	16±0.8
K-562	nd	nd	10±0.5	60±3.0
DoHH2	40±5	nd	6±0.3	50±2.5
MINO	120±20	nd	10±0.5	30±1.5
MC-116	70±10	nd	11±0.5	50±2.5
SU-DHL-1	nd	nd	11±0.5	38±2.0
RPMI 8226	nd	nd	20±1.0	50±2.5
OPM-2	nd	nd	16±0.8	78±4.0
HBL2 (culture)	Nd	nd	16±0.8	98±10
HBL2 (tumor)	Nd	nd	24±1.2	100±10
CML[c]	Nd	nd	15±0.75	nd
CML[c]	Nd	nd	14±0.7	77±4.0
AML[d]	Nd	nd	12±0.6	60±3.0
AML[d]	Nd	nd	17±1.0	90±10

[a]$^{Pi}K_i$ values. [b]Human recombinant enzyme. [c]Two different patients. [d]One patient.

lead to a more stable enzyme−inhibitor complex, as observed for immucillin H[13] or DFPP-DG.[25]

CONCLUSION

A series of enantiomeric and diastereomeric *N*-phosphonocarbonylpyrrolidine (**2a**,**b**) and *N*-phosphonocarbonyl-3-hydroxypyrrolidine (**6a**−**d**) derivatives of guanine, respectively, were synthesized and assayed with native human PNPs partially purified from PBMCs of healthy donors and various cancer cell lines. From the series of potential inhibitors, compounds **2a** and **6a** were recognized as potent, potential bisubstrate inhibitors of PNP from PBMCs with nanomolar K_i values. The patterns of the Dixon plots obtained suggested that these compounds are competitive inhibitors of PNPs from various sources with respect to the MESG substrate. The K_i values of compound **2a** for PNPs from cancer cell lines varied from 16 to 100 nM, whereas lower values (10−24 nM) were obtained for the hydroxy derivative **6a**, suggesting a slightly higher selectivity of **2a** toward several PNPs. The low $^{MESG}K_i$ and $^{Pi}K_i$ values for **2a** and **6a** may suggest that these compounds are bisubstrate inhibitors of PNP from PBMCs. Regarding the stereochemistry of inhibitors **2a** and **6a**, these compounds undergo the *cis* ↔ *trans* isomerism around the N−C(O) linkage and therefore exist as *cis* and *trans* conformers as confirmed by ^{1}H and ^{13}C NMR spectroscopy (Figure 2). One can conclude that only one of these conformers is capable of binding efficiently to the PNP phosphorus binding site. The availability of X-ray coordinates of the enzyme with **6a** and **2a** would not only be an instructive tool for determining the conformational and binding properties of these nucleotide analogues but also a starting point for the rational design of potent bisubstrate inhibitors of human PNP based on pyrrolidine nucleotide analogues. Such a study is underway and the results will be published in due course.

EXPERIMENTAL SECTION

Chemistry. Unless otherwise stated, all solvents used were anhydrous. Pyrrolidine derivatives of guanine (**1a**, **1b**) and mesyl derivatives (**3a**−**d**) were synthesized according to previously described procedures.[27,28] TLC was performed on TLC plates precoated with silica gel (silica gel/TLC-cards, UV 254, Merck). Compounds were detected using UV light (254 nm), heating (for the detection of dimethoxytrityl group; orange color), spraying with 1% ethanolic solution of ninhydrine to visualize amines, or by spraying with a 1% solution of 4-(4-nitrobenzyl)pyridine in ethanol, followed by heating and treating with gaseous ammonia (for the detection of alkylating agents, such as mesyl derivatives; blue color). The purity of the final compounds was greater than 95%. The purity of the prepared compounds was determined by LC-MS using a Waters AutoPurification System with 2545 quarternary gradient module and 3100 single quadrupole mass detector using Luna C18 column (100 mm × 4.6 mm, 3 μm, Phenomenex) at a flow rate of 1 mL/min. The following mobile phase was used, where A, B, and C represent 50 mM NH_4HCO_3 and 50 mM NH_4HCO_3 in 50% aq CH_3CN and CH_3CN, respectively: A → B over 10 min, B → C over 10 min, and C for 5 min. Preparative RP HPLC was performed on an LC5000 liquid chromatograph (INGOS-PIKRON, CR) using a Luna C18 (2) column (250 mm × 21.2 mm, 5 μm) at a flow rate of 10 mL/min. A gradient elution of methanol in pH 7.5 0.1 M TEAB (A, 0.1 M TEAB; B, 0.1 M TEAB in 50% aq methanol; C, methanol) was used. All final compounds were lyophilized from water. The optical rotation values were measured on an AUTOPOL IV (Rudolph Research Analytical, USA) polarimeter for the sodium D line at 20 °C. Mass spectra were collected on an LTQ Orbitrap XL (Thermo Fisher Scientific) using ESI ionization. The phosphorus content in the compounds was determined using a simultaneous energy-dispersive X-ray fluorescence spectrometer SPECTRO iQ II. NMR spectra were collected in DMSO-d_6 or D_2O on a Bruker AVANCE 400 (^{1}H at 400.0 MHz, ^{13}C at 100.6 MHz, and ^{31}P at 162.0 MHz) and/or Bruker

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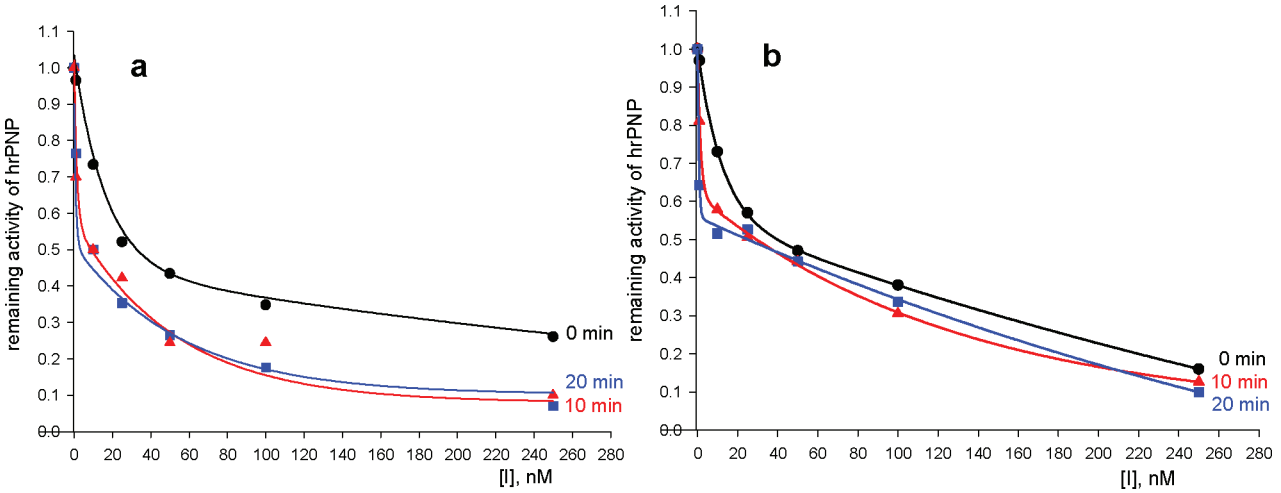

Figure 3. The effects of 0, 10, and 20 min preincubation of hrPNP with inhibitor **6a** on the remaining PNP activity measured by the phosphorolysis of MESG ((a) remaining activity measured after 2 min; (b) remaining activity measured after 5 min).

AVANCE 500 (^{1}H at 500.0 MHz, ^{13}C at 125.7 MHz, and ^{31}P at 202.3 MHz) NMR spectrometers. Chemical shifts (in ppm, δ scale) were referenced to the residual DMSO-d_6 signal (2.5 ppm for ^{1}H and 39.7 ppm for ^{13}C) or to the 1,4-dioxane signal (3.75 ppm for ^{1}H and 69.3 ppm for ^{13}C) as an internal standard in D_2O. ^{31}P NMR spectra were referenced to H_3PO_4 (0 ppm) as an external standard. Coupling constants (J) are given in Hz. The complete assignment of ^{1}H and ^{13}C signals was performed by analysis of the correlated homonuclear H,H−COSY and heteronuclear H,C-HSQC and H,C-HMBC spectra. The relative configuration of compounds was checked using DPFGSE-NOE and 2D-ROESY techniques. The numbering for signal assignment is shown in Figure 2.

*General Method A: Phosphonocarbonylation and Preparation of Phosphonic Acids **2a**, **2b**, and **6a**−**d**.* Phenyl diisopropylphosphonoformate (2 mmol) was added to a DMF solution (10 mL) of pyrrolidine derivative of nucleobase (**1a**, **1b**, and **5a**−**d**; 1 mmol) previously (dried by coevaporation with DMF) at 90 °C. The course of the reaction was followed by LC-MS or TLC (10% EtOH/$CHCl_3$). After 2 h, the solvent was removed in vacuo, and the respective diisopropyl esters of compounds **2a**, **2b**, and **6a**−**d** were recovered by silica gel chromatography using a linear gradient of ethanol in chloroform (0 → 10%). The obtained diesters were coevaporated with DMF and dissolved in the same solvent (10 mL/mmol). Then bromotrimethylsilane (1.32 mL/mmol) was added, and the reaction mixture was stirred at 70 °C. The reaction was monitored by LC-MS. Subsequently, the reaction mixture was concentrated in vacuo, the residue was dissolved in a mixture of 2 M TEAB (1 mL) and ethanol (5 mL), and the solution was concentrated in vacuo.

The preparative RP HPLC afforded desired products **2a**, **2b**, and **6a**−**d** as triethylammonium salts, which were converted into the sodium salts using Dowex 50 (Na^+ form). The triethylammonium salts of phosphonic acids were subjected to HR ESI measurements, whereas the sodium salts was checked, after lyophilization from water, for phoshorus content, which was used for the calculation of molecular weights.

*General Method B: Nucleosidation, Preparation of Compounds **5a**−**d**.* A mixture of the appropriate amino derivative (**4a**−**d**) (1 mmol), 2,5-diamino-4,6-dichloropyrimidine (0.27 g, 1.5 mmol), and triethylamine (0.56 mL, 4 mmol) in *n*-butanol (10 mL) was stirred in a pressure vessel at 130 °C for two days. The course of the reaction was monitored by LC-MS or TLC (10% EtOH/$CHCl_3$). The chloropyrimidine intermediate, obtained by chromatography on silica gel using a linear gradient of ethanol in chloroform, was treated with diethoxymethyl acetate (1.2 equiv) in DMF (10 mL/mmol) at rt for 2 h and then at 110 °C for 2 d. The reaction mixture was concentrated in vacuo, and the resulting residue was treated with 3 M HCl (10 mL/mmol) at 70 °C overnight. The solution was diluted with water (20 mL/mmol) and applied onto a Dowex 50 column in H^+ (30 mL/mmol). The resin was washed with water, and the product was eluted with 3% aq ammonia. The final product was obtained by preparative RP-HPLC using a linear gradient of methanol in water.

*(S)-3-(Guanin-9-yl)pyrrolidin-N-ylcarbonylphosphonic Acid (**2a**).* Compound **2a** was prepared from the pyrrolidine derivative **1a** (60 mg, 0.27 mmol) in a 46% yield (45.9 mg, 0.123 mmol) as a white amorphous solid using general method A. ^{1}H NMR (500.0 MHz, D_2O, 25 °C): 2.28−2.56 (m, 4H, H-4′-*cis*+*trans*), 3.55−3.70 (m, 2H, H-5′-*cis*), 3.83 (ddd, 1H, J_{gem} = 13.4, $J_{2'b,3'}$ = 5.5, J_{HP} = 1.6, H-2′b-*trans*), 3.99 (ddd, 1H, J_{gem} = 13.4, $J_{2'a,3'}$ = 7.2, J_{HP} = 1.8, H-2′a-*trans*), 4.08 (dt, 1H, J_{gem} = 12.3, $J_{5'b,4'}$ = 6.7, H-5′b-*trans*), 4.11 (dt, 1H, J_{gem} = 12.3, $J_{5'a,4'}$ = 7.7, H-5′a-*trans*), 4.37 (d, 2H, $J_{2',3'}$ = 5.4, H-2′-*cis*), 5.01 (m, 1H, H-3′-*cis*), 5.03 (m, 1H, H-3′-*trans*), 7.85 and 7.86 (2 × s, 2 × 1H, H-8-*cis*+*trans*). ^{13}C NMR (125.7 MHz, D_2O, 25 °C): 31.64 (CH_2-4′-*cis*), 34.13 (CH_2-4′-*trans*), 46.34 (d, $J_{C,P}$ = 4.2, CH_2-5′-*cis*), 48.54 (CH_2-5′-*trans*), 52.79 (d, $J_{C,P}$ = 4.7, CH_2-2′-*trans*), 54.38 (CH_2-2′-*cis*), 54.80 (CH-3′-*trans*), 56.76 (CH-3′-*cis*), 118.93 (C-5-*cis*+*trans*), 140.15, 140.25 (CH-8-*cis*+*trans*), 154.15, 154.19 (C-4-*cis*+C-4-*trans*), 156.74, 156.79 (C-2-*cis*+*trans*), 162.14, 162.17 (C-6-*cis*+*trans*), 181.08, 181.18 (d, $J_{C,P}$ = 189.4, C═O-*cis*+*trans*). ^{31}P{^{1}H} NMR (162.0 MHz, D_2O, 25 °C): −1.65. IR ν_{max}(KBr) 3125 (m, br), 2960 (m, sh), 1694 (vs), 1652 (m, br), 1601 (s, sh), 1581 (s), 1536 (m), 1179 (m), 1085 (m, br), 574 (m, br) cm^{-1}. HR-ESI $C_{10}H_{12}O_5N_6P$ $[M - H]^-$ calcd 327.0608, found 327.0612. $[\alpha]^{20}$= −15.8 (*c* 0.253, H_2O). Elem. Anal.: P, found 7.18% (MW 431.4).

*(R)-3-(Guanin-9-yl)pyrrolidin-N-ylcarbonylphosphonic Acid (**2b**).* Compound **2b** was prepared from pyrrolidine derivative **1b** (0.41 g, 1.86 mmol) in a 33% yield (228.4 mg, 0.614 mmol) as a white amorphous solid using general method A. ^{1}H NMR, ^{13}C NMR, and ^{31}P{^{1}H} NMR spectra are identical to **2a**. HR-ESI $C_{10}H_{12}O_5N_6P$ $[M - H]^-$ calcd 327.0608, found 327.0607. $[\alpha]^{20}$ = +19.4 (*c* 0.237, H_2O). Elem. Anal.: P, found 7.66% (MW 404.4).

*(3S,4R)-4-Amino-1-N-tert-butyloxycarbonyl-3-hydroxypyrrolidine (**4a**).* A mixture of the mesyl derivative **3a** (19.8 g, 34 mmol) and sodium azide (11 g, 170 mmol) in DMF (340 mL) was stirred at 100 °C overnight. The solvent was removed in vacuo. The azido derivative, obtained by silica gel chromatography using a linear gradient of ethyl acetate in toluene (0 → 100%), was treated with 1.5% TFA in DCM (350 mL) for 30 min (TLC in 10% EtOH/$CHCl_3$). Solid $NaHCO_3$ (20 g) and methanol (150 mL) were then added, and the suspension was stirred until a neutral pH value was reached. The suspension was filtered, and the filtrate was concentrated in vacuo. The azido derivative obtained by silica gel chromatography using a linear gradient of ethanol in chloroform was dissolved in methanol and subjected to hydrogenation in the presence of Pd/C catalyst (1.5 g) overnight. After filtration over Celite and evaporation of the solvent, compound **4a** was obtained in a 55% yield (3.78 g, 18.7 mmol) as a colorless, viscous oil. NMR of trifluoroacetate salt: ^{1}H NMR (500.0 MHz,

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DMSO-d_6, 80 °C): 1.41 (s, 9H, $(CH_3)_3C$)), 3.25 (dd, 1H, J_{gem} = 10.7, $J_{5b,4}$ = 7.0, H-5b), 3.27 (dd, 1H, J_{gem} = 11.6, $J_{2b,3}$ = 3.1, H-2b), 3.44 (dd, 1H, J_{gem} = 11.6, $J_{2a,3}$ = 5.0, H-2a), 3.57 (dd, 1H, J_{gem} = 10.7, $J_{5a,4}$ = 7.4, H-5a), 3.62 (ddd, 1H, $J_{4,5}$ = 7.4, 7.0, $J_{4,3}$ = 4.7, H-4), 4.28 (ddd, 1H, $J_{3,2}$ = 5.0, 3.1, $J_{3,4}$ = 4.7, H-3). ^{13}C NMR (125.7 MHz, DMSO-d_6, 80 °C): 28.06 (($\mathbf{C}H_3)_3C$), 46.96 (CH_2-5), 51.50 (CH-4), 51.86 (CH_2-2), 67.80 (CH-3), 78.66 ($\mathbf{C}(CH_3)_3$), 117.25 (q, JC,F = 299.7, $\mathbf{C}F_3COO$), 153.41 (CO), 158.33 (q, JC,F = 31.1, $CF_3\mathbf{C}OO$). HR-ESI $C_9H_{18}O_3N_2Na$ $[M + Na]^+$ calcd 225.1210, found 225.1209.

*(3S,4S)-4-Amino-1-N-tert-butyloxycarbonyl-3-hydroxypyrrolidine (**4b**).* Compound **4b** was prepared from mesyl derivative **3b** (15.5 g, 26.55 mmol) following the same procedure used to obtain the amino derivative **4a** in a 64% yield (3.44 g, 19.38 mmol). Compound **4b** was obtained as a colorless, viscous oil that solidified to an amorphous solid upon standing in the refrigerator. ^{1}H NMR (500.0 MHz, DMSO-d_6, 80 °C): 1.41 (s, 9H, $(CH_3)_3C$)), 2.95 (dd, 1H, J_{gem} = 10.7, $J_{5b,4}$ = 3.5, H-5b), 3.06 (dd, 1H, J_{gem} = 11.2, $J_{2b,3}$ = 3.1, H-2b), 3.12 (dt, 1H, $J_{4,5}$ = 5.9, 3.5, $J_{4,3}$ = 3.5, H-4), 3.42 (dd, 1H, J_{gem} = 10.7, $J_{5a,4}$ = 5.9, H-5a), 3.47 (dd, 1H, J_{gem} = 11.2, $J_{2a,3}$ = 5.1, H-2a), 3.75 (ddd, 1H, $J_{3,2}$ = 5.1, 3.1, $J_{3,4}$ = 3.5, H-3), 4.74 (bs, 1H, OH). ^{13}C NMR (125.7 MHz, DMSO-d_6, 80 °C): 28.10 (($\mathbf{C}H_3)_3C$), 51.55 (CH_2-2), 51.70 (CH_2-5), 56.90 (CH-4), 75.30 (CH-3), 77.80 ($\mathbf{C}(CH_3)_3$), 153.79 (CO). IR ν_{max} (KBr) 2981 (s), 2942 (m), 2887 (m), 1687 (vs), 1678 (vs, sh), 1478 (m), 1455 (m), 1415 (s), 1394 (s), 1368 (s), 1251 (m), 1162 (s), 1078 (m) cm^{-1}. HR-ESI $C_9H_{18}O_3N_2Na$ $[M + Na]^+$ calcd 225.1210, found 225.1209.

*(3R,4S)-4-Amino-1-N-tert-butyloxycarbonyl-3-hydroxypyrrolidine (**4c**).* Compound **4c** was prepared from mesyl derivative **3c** (13.9 g, 23.81 mmol) in an 81% yield (3.92 g, 19.38 mmol) following the same procedure used to obtain the amino derivative **4a**. Compound **4c** was obtained as colorless, viscous oil that solidified to an amorphous solid on standing in refrigerator. ^{1}H NMR and ^{13}C NMR spectra are identical to **4a**. HR-ESI $C_9H_{18}O_3N_2Na$ $[M + Na]^+$ calcd 225.1210, found 225.1209.

*(3R,4R)-4-Amino-1-N-Boc-3-hydroxypyrrolidine (**4d**).* Compound **4d** was prepared from mesyl derivative **3d** (31.22 g, 53.49 mmol) in a 48% yield (5.24 g, 25.91 mmol) following the same procedure as used to obtain the amino derivative **4a**. Compound **4d** was obtained as colorless, viscous oil that solidified to an amorphous solid upon standing in the refrigerator. ^{1}H NMR and ^{13}C NMR spectra are identical to **4b**. HR-ESI $C_9H_{18}O_3N_2Na$ $[M + Na]^+$ calcd 225.1210, found 225.1209.

*(3S,4R)-4-(Guanin-9-yl)-3-hydroxypyrrolidine (**5a**).* Compound **5a** was prepared in an overall yield (134 mg, 0.57 mmol) of 15% as an amorphous solid according to general method B from compound **4a** (0.83 g, 3.77 mmol). ^{1}H NMR (400.0 MHz, DMSO-d_6, 25 °C): 2.79 (dd, 1H, J_{gem} = 11.9, $J_{2'b,3'}$ = 2.9, H-2′b), 3.10 (dd, 1H, J_{gem} = 11.0, $J_{5'b,4'}$ = 8.9, H-5′b), 3.15−3.22 (m, 2H, H-2′a,5′a), 4.14 (td, 1H, $J_{3',4'}$ = $J_{3',2'a}$ = 5.6, $J_{3',2'b}$ = 2.9, H-3′), 4.56 (td, 1H, $J_{4',5'}$ = 8.9, $J_{4',3'}$ = 5.6, H-4′), 5.12 (bs, 1H, OH), 6.43 (bs, 2H, NH_2), 7.69 (s, 1H, H-8). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C): 48.94 (CH_2-5′), 54.19 (CH_2-2′), 56.74 (CH-4′), 69.61 (CH-3′), 116.17 (C-5), 137.34 (CH-8), 151.65 (C-4), 153.54 (C-2), 157.06 (C-6). IR ν_{max}(KBr) 3373 (m, vbr), 3125 (m, vbr), 1694 (vs), 1680 (vs), 1639 (s, sh), 1612 (m), 1584 (m, sh), 1568 (m, sh), 1538 (m), 1477 (m), 1433 (m), 1420 (m, sh), 1384 (m), 1365 (m) cm^{-1}. HR-ESI $C_9H_{11}O_2N_6$ $[M - H]^-$ calcd 235.09490, found 235.09491. $[\alpha]^{20}$= +69.2 (*c* 0.416, DMSO).

*(3S,4S)-4-(Guanin-9-yl)-3-hydroxypyrrolidine (**5b**).* Compound **5b** was prepared from the amino derivative **4b** (0.8 g, 3.96 mmol) in a 25% overall yield (237 mg, 1 mmol) as an amorphous solid according to general method B. ^{1}H NMR (400.0 MHz, DMSO-d_6, 25 °C): 2.72 (dd, 1H, J_{gem} = 11.6, $J_{2'b,3'}$ = 4.4, H-2′b), 2.96 (dd, 1H, J_{gem} = 11.8, $J_{5'b,4'}$ = 4.9, H-5′b), 3.24 (dd, 1H, J_{gem} = 11.6, $J_{2'a,3'}$ = 6.1, H-2′a), 3.31 (dd, 1H, J_{gem} = 11.8, $J_{5'a,4'}$ = 7.3, H-5′a), 4.32 (ddd, 1H, $J_{3',2'}$ = 6.1, 4.4, $J_{3',4'}$ = 3.5, H-3′), 4.52 (ddd, 1H, $J_{4',5'}$ = 7.3, 4.9, $J_{4',3'}$ = 3.5, H-4′), 5.84 (bs, 2H, NH_2), 7.75 (s, 1H, H-8). ^{13}C NMR (100.6 MHz, DMSO-d_6, 25 °C): 50.93 (CH_2-5′), 53.92 (CH_2-2′), 62.55 (CH-4′), 76.57 (CH-3′), 114.01 (C-5), 135.24 (CH-8), 152.75 (C-4), 154.96 (C-6), 159.46 (C-2). HR-ESI $C_9H_{13}O_2N_6$ $[M + H]^+$ calcd 237.10945, found 237.10942. $[\alpha]^{20}$= +29.1 (*c* 0.326, H_2O).

*(3R,4S)-4-(Guanin-9-yl)-3-hydroxypyrrolidine (**5c**).* Compound **5c** was prepared according to general method B from **4c** (0.95 g, 4.32 mmol) in a 14% overall yield (142.2 mg, 0.61 mmol) as an amorphous solid. ^{1}H NMR and ^{13}C NMR spectra are identical to **5a**. HR-ESI $C_9H_{13}O_2N_6$ $[M + H]^+$ calcd 237.10945, found 237.10940. $[\alpha]^{20}$= −64.7 (*c* 0.306, DMSO).

*(3R,4R)-4-(Guanin-9-yl)-3-hydroxypyrrolidine (**5d**).* Compound **5d** was prepared in a 21% overall yield (142 mg, 0.6 mmol) as an amorphous solid according to general method B from **4d** (0.52 g, 2.57 mmol). ^{1}H NMR and ^{13}C NMR spectra are identical to **5b**. HR-ESI $C_9H_{13}O_2N_6$ $[M + H]^+$ calcd 237.10945, found 237.10941. $[\alpha]^{20}$= −31.1 (*c* 0.228, H_2O).

*(3S,4R)-4-(Guanin-9-yl)-3-hydroxypyrrolidin-1-N-ylcarbonylphosphonic Acid (**6a**).* Compound **6a** was prepared from pyrrolidine derivative **5a** (58 mg, 0.25 mmol) in a 15% yield (14.5 mg, 0.037 mmol), using general method A, as a white amorphous solid. ^{1}H NMR (400.0 MHz, D_2O, 25 °C): 3.60 (dt, 1H, J_{gem} = 13.6, $J_{2'b,3'}$ = $J_{H,P}$ = 2.1, H-2′b-*cis*), 3.84 (ddd, 1H, J_{gem} = 13.6, $J_{2'a,3'}$ = 5.2, $J_{H,P}$ = 1.8, H-2′a-*cis*), 3.97 (ddd, 1H, J_{gem} = 12.8, $J_{5'b,4'}$ = 8.4, $J_{H,P}$ = 1.9, H-5′b-*trans*), 4.09 (ddd, 1H, J_{gem} = 12.8, $J_{5'a,4'}$ = 8.2, $J_{H,P}$ = 1.7, H-5′a-*trans*), 4.17 (d, 2H, $J_{2',3'}$ = 3.9, H-2′-*trans*), 4.31 (dd, 1H, J_{gem} = 11.5, $J_{5'b,4'}$ = 9.5, H-5′b-*cis*), 4.60−4.69 (m, 3H, H-3′-*cis*+*trans*, H-5′a-*cis*), 5.02 (ddd, 1H, $J_{4',5'}$ = 9.5, 7.7, $J_{4',3'}$ = 4.3, H-4′-*cis*), 5.07 (ddd, 1H, $J_{4',5'}$ = 8.4, 8.2, $J_{4',3'}$ = 4.3, H-4′-*trans*), 7.96, 7.97 (2 × s, 2 × 1H, H-8-*cis*+*trans*). ^{13}C NMR (125.7 MHz, D_2O, 25 °C): 49.00 (d, $J_{C,P}$ = 4, CH_2-5′-*trans*), 50.34 (CH_2-5′-*cis*), 54.15 (d, $J_{C,P}$ = 4, CH_2-2′-*cis*), 55.75 (CH_2-2′-*trans*), 57.18 (CH-4′-*trans*), 58.69 (CH-4′-*cis*), 70.53 (CH-3′-*cis*), 72.54 (CH-3′-*trans*), 118.44, 118.47 (C-5-*cis*+*trans*), 141.40, 141.47 (CH-8-*cis*+*trans*), 154.78 (C-4-*cis*+*trans*), 154.89 (C-2-*cis*+*trans*), 156.56 (C-6-*cis*+*trans*), 181.54 (d, $J_{C,P}$ = 189, CO-*cis*+*trans*). ^{31}P{^{1}H} NMR (162.0 MHz, D_2O, 25 °C): −1.63, −1.75. HR-ESI $C_{10}H_{12}O_6N_6P$ $[M - H]^-$ calcd 343.0561, found 343.0565. $[\alpha]^{20}$= +47.4 (*c* 0.177, H_2O). Elem. Anal.: P, found 7.64% (MW 405.4).

*(3S,4S)-4-(Guanin-9-yl)-3-hydroxypyrrolidin-1-N-ylcarbonylphosphonic Acid (**6b**).* Compound **6b** was prepared from pyrrolidine derivative **5b** (100 mg, 0.42 mmol) in an 18% yield (30 mg, 0.077 mmol), using general method A, as a white amorphous solid. ^{1}H NMR (500.0 MHz, D_2O, 25 °C): 3.50 (ddd, 1H, J_{gem} = 13.3, $J_{2'b,3'}$ = 4.2, $J_{H,P}$ = 1.1, H-2′b-*cis*), 3.82 (ddd, 1H, J_{gem} = 13.3, $J_{2'a,3'}$ = 6.2, $J_{H,P}$ = 1.2, H-2′a-*cis*), 3.92 (m, 2H, H-2′b,5′b-*trans*), 4.12 (m, 1H, H-5′a-*trans*), 4.36 (dd, 1H, J_{gem} = 12.5, $J_{2'a,3'}$ = 5.9, H-2′-*trans*), 4.46 (dd, 1H, J_{gem} = 12.9, $J_{5'b,4'}$ = 5.4, H-5′b-*cis*), 4.55 (dd, 1H, J_{gem} = 12.9, $J_{5'a,4'}$ = 6.9, H-5′a-*cis*), 4.69 (m, 2H, H-3′-*cis*+trans), 4.85 (m, 2H, H-4′-*cis*+*trans*), 7.85, 7.86 (2 × s, 2 × 1H, H-8-*cis*+*trans*). ^{13}C NMR (125.7 MHz, D_2O, 25 °C): 50.13 (d, $J_{C,P}$ = 4.6, CH_2-5′-*trans*), 51.72 (CH_2-5′-*cis*), 52.90 (d, $J_{C,P}$ = 4.1, CH_2-2′-*cis*), 54.73 (CH_2-2′-*trans*), 60.66 (CH-4′-*trans*), 62.52 (CH-4′-*cis*), 73.97 (CH-3′-*cis*), 75.69 (CH-3′-*trans*), 118.89, 118.91 (C-5-*cis*+*trans*), 140.15, 140.40 (CH-8-*cis*+*trans*), 154.53, 154.58 (C-4-*cis*+*trans*), 156.43 (C-6-*cis*+*trans*), 161.71, 161.74 (C-2-*cis*+*trans*), 181.07, 181.20 (d, $J_{C,P}$ = 190.0, CO-*cis*+*trans*). ^{31}P{^{1}H} NMR (202.3 MHz, D_2O, 25 °C): −1.84, −1.82. IR ν_{max}(KBr) 3414 (m, vbr), 3126 (m, vbr), 1696 (vs), 1587 (s, br), 1537 (m), 1184 (m), 1085 (m) cm^{-1}. HR-ESI $C_{10}H_{12}O_6N_6P$ $[M - H]^-$ calcd 343.05614, found 343.05606. $[\alpha]^{20}$= −25.5 (*c* 0.247, H_2O). Elem. Anal.: P, found 7.61% (MW 407.0).

*(3R,4S)-4-(Guanin-9-yl)-3-hydroxypyrrolidin-1-N-ylcarbonylphosphonic Acid (**6c**).* Compound **6c** was prepared from pyrrolidine derivative **5c** (40 mg, 0.17 mmol) in a 28% yield (18 mg, 0.05 mmol) as a white amorphous solid using general method A. ^{1}H NMR and ^{13}C NMR are identical to **6a** HR-ESI $C_{10}H_{12}O_6N_6P$ $[M - H]^-$ calcd 343.05614, found 343.05617. $[\alpha]^{20}$= −46.5 (*c* 0.256, H_2O). Elem. Anal.: P, found 7.69% (MW 402.8).

*(3R,4R)- 4-(Guanin-9-yl)-3-hydroxypyrrolidin-1-N-ylcarbonylphosphonic Acid (**6d**).* Compound **6d** was prepared from pyrrolidine derivative **5d** (150 mg, 0.635 mmol) in an 11% yield (25.7 mg, 0.07 mmol) using general method A, as a white amorphous hygroscopic solid. ^{1}H NMR and ^{13}C NMR spectra are identical to **6b**. HR-ESI $C_{10}H_{12}O_6N_6P$ $[M - H]^-$ calcd 343.05614, found 343.05624. $[\alpha]^{20}$= +21.7 (*c* 0.240, H_2O). Elem. Anal.: P, found 6.77% (MW 457.515).

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Biochemistry. The instruments used to perform enzyme purification were the following: (a) a Beckman Coulter Allegra X-22R centrifuge, (b) a Beckman Coulter Optima L-100 XP ultracentrifuge, and (c) a Cell ultrasound desintegrator Soniprep 150 (Sanyo). Spectrophotometric enzyme assays were performed on an Infinite F500 Tecan reader. Protease inhibitor cocktail was obtained from Sigma, and 7-methyl-6-thioguanosine (MESG) was purchased from Berry & Associates. HiTrap Q ion exchange columns (1 mL volume) were obtained from GE Healthcare Life Sciences.

Normal peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats of healthy donors who signed informed consent forms. Primary chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) cells were obtained from patients who underwent cytapheresis therapy, all of whom signed an informed consent form. The cells were provided by the Institute of Hematology and Blood Transfusion in Prague. Cell lines and cell line xenografts were provided by the Institute of Pathological Physiology, First Faculty of Medicine, Charles University, in Prague. The following cell lines were used in the study: K562 (erythroleukemia progressed from chronic myeloid leukemia), MC-116 (B-cell lymphoma), JURKAT (acute T-cell lymphoblastic leukemia), DoHH2 (diffuse large B-cell lymphoma progressed from follicular lymphoma), MINO (mantle cell lymphoma), HBL2 (mantle cell lymphoma), SU-DHL-1 (anaplastic large T-cell lymphoma), OPM-2 (multiple myeloma), and RPMI-8226 (multiple myeloma). Dry pellets were obtained either from in vitro cultures of the aforementioned cell lines or ex vivo from murine xenografts. The xenografts were derived using the following procedure: 10^6 cells were subcutaneously injected into the right flank of 6−8 week old immunodeficient mice NOD-SCID; tumors were allowed to grow subcutaneously until they reached 2−3 cm in any diameter; the mice were sacrificed; tumors were excised and passed through 45 μM nylon mesh; and the cells were counted and frozen in the form of dry pellets. Human recombinant purine nucleoside phosphorylase was kindly provided by Dr. Larissa Balakireva (NovoCIB, Lyon, France).

Partial Purification of Purine Nucleoside Phosphorylase (PNP). All steps were carried out at 4 °C. A suspension of human PBMCs from healthy donors was centrifuged at 4000*g*, the sedimented cells were resuspended in buffer A (pH 7.5 20 mM TRIS-citric acid, 135 mM sodium chloride, 2.5 mM EDTA, 5.5 mM glucose, 100 mL) and washed twice with the same buffer. The washed cells were stored at −80 °C. Cells (10^9) were suspended in 50 mM Tris-HCl buffer pH 7.5 (15 mL) containing a protease inhibitor cocktail (0.1 mL), disrupted by freezing and thawing, and the suspension was subsequently sonicated three times for 10 s. The crude lysate was centrifuged at 10^4g for 30 min to remove cell debris and then at 10^5g for 1 h. The resulting supernatant (15 mL) was brought to 60% saturation by adding solid ammonium sulfate (5.5 g). After 1 h, the precipitate was collected by centrifugation at 10^4g for 40 min and dissolved in pH 7.5 50 mM Tris-HCl buffer (10 mL), and the solution was dialyzed against the same buffer (2 L) overnight. The desalted solution was loaded on a HiTrap Q column equilibrated with 50 mM Tris-HCl buffer (pH 7.5; 20 mL). The column was eluted with a linear gradient (0−1 M) of NaCl in the same buffer at a flow rate of 1 mL/min. Fractions (1 mL, 20 fractions totally) were collected and analyzed for PNP activity. PNP activity was detected only in one fraction (at approximately 200 mM NaCl), which was used immediately for assays. Protein concentration was determined by the method of Bradford[38] using bovine serum albumin as a standard.

PNPs from cancer cell lines and blood cells of patients suffering from chronic myeloid leukemia (CML) and acute myeloid leukemia (AML) were purified using the same method described above (Table 1).

Determination of PNP Activity. To determine PNP activity, we employed a spectrophotometric method described by Webb et al[34] that uses 7-*N*-methyl-6-thiopurineriboside (MESG) as a substrate. The incubation mixture (200 μL) contained 50 mM Tris-HCl buffer (pH 7.5), 500 μM potassium phosphate, 20−100 μM MSEG, and a partially purified PNP. Enzyme phosphorolytic activity was calculated from changes in absorbance at 360 nm using $\varepsilon_{360\ nm}$ = 11000 M^{-1} cm^{-1}. Assays were performed in 96-well plates and monitored using the Infinite F500 Tecan reader. Initial steady-state rates were calculated from a linear part of the time-dependent absorbance changes over a wide range of MESG concentrations at a constant concentration of inorganic phosphate. The data obtained for partially purified PNP from various sources fit the Michaelis−Menten equation. The GraphPad Prism4[33] software was used to calculate a K_m and V_{max}.

Inhibition Study. Compounds **2a**, **2b**, and **6a−d** were tested for their inhibition activity against PNP with respect to MESG and inorganic phosphate substrates. The reaction mixture (200 μL) for each assay contained 50 mM Tris buffer (pH 7.5), MESG (40 or 80 μM), potassium phosphate (500 or 250 μM), and the inhibitor (0−500 nM). Phosphorolysis was initiated by the addition of the appropriate amount of enzyme sample (2−5 uL; approximately 3 μg) and monitored for 5−7 min at 360 nm. The type of inhibition and K_i values were determined from Dixon plots ($1/v$ versus [I]) using SigmaPlot software. K_i values were calculated from three independent measurements.

Time-Dependent Inhibition. Human recombinant enzyme (0.002 units, 2 μL of stock solution in 20 mM Tris buffer in 50% aqueous glycerol, pH 7.5) was preincubated with various concentrations of inhibitor **6a** (0, 0.1, 10, 25, 50, 100, and 250 nM) in 50 mM Tris buffer (pH 7.5, 445 μL) at 20 °C for 0, 10, and 20 min. Then 50 mM potassium dihydrogenphosphate (5 μL) followed by 110 μM MSEG (100 μL) in Tris buffer were added to final concentrations of 500 and 20 μM, respectively, (total volume 550 μL).

After 2 and 5 min of incubation at 25 °C, aliquots (100 μL) of each mixture were removed and quenched with 10 μL of 20% HCl. The extent of MESG phosphorolysis was subsequently analyzed using HPLC (Luna C18 3 μm, 100 mm × 4.6 mm).

The enzyme activity is expressed as the remaining activity related to assays without inhibitor. See Figure 3 and Figure S13 in Supporting Information for these results.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Graphical data used to determine the K_i and K_m values. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*For I.R.: phone, +420 220 183 381; E-mail, rosenberg@uochb.cas.cz. For D.R.: phone, +420 220 183 371; E-mail, rejman@uochb.cas.cz. For P.K.: phone, +420 608 177 782; E-mail, pklener@yahoo.com.

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■ ABBREVIATIONS USED

ACVDP, acyclovir diphosphate; AML, acute myeloid leukemia; B-ALL, B-cell acute lymphoblastic leukemia; B-CLL, B-cell chronic lymphocytic leukemia; CML, chronic myeloid leukemia; dCK, deoxycytidine kinase; DFPP-G, 9-(5′,5′-difluoro-5′-phosphonopentyl)guanine; dGuo, 2′-deoxyguanosine; DoHH2, diffuse large B-cell lymphoma progressed from follicular lymphoma; HBL2 , mantle cell lymphoma (culture = in vitro cultured cells, tumor = ex vivo obtained cells grown in

form of subcutaneous tumors in immunodeficient mice); hrPNP, human recombinant purine nucleoside phosphorylase; IMG, immucillin-G; IMH, immucillin-H; JURKAT, T-cell leukemia; K-562, erythroleukemia progressed from chronic myeloid leukemia; k_{cat}, catalytic constant; K_i, inhibition constant; $^{Pi}K_i$, inhibition constant related to inorganic phosphate; K_m, Michaelis constant; $^{MESG}K_i$, inhibition constant related to MESG; $^{MESG}K_m$, Michaelis constant for MESG; $^{Pi}K_m$, Michaelis constant for P_i; MC-116, B-cell lymphoma; MESG, 7-methyl-6-thioguanosine; MINO, mantle cell lymphoma; OPM-2, multiple myeloma; PBMCs, peripheral blood mononuclear cells; P_i, inorganic phosphate; PNP, purine nucleoside phosphorylase; RPMI 8226, multiple myeloma; SU-DHL-1, anaplastic large cell lymphoma; *V*, reaction velocity

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