Discovery of a novel inhibitor of human purine nucleoside phosphorylase by a simple hydrophilic interaction liquid chromatography (HILIC-UV) enzymatic assay

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Abstract: Purine nucleoside phosphorylase (PNP) belongs to the human purine salvage pathway. Genetic deficiency of this enzyme triggers apoptosis of activated T-cells due to the accumulation of deoxyguanosine triphosphate (dGTP). Therefore, potential chemotherapeutic applications of human PNP inhibitors includes the treatment of T-cell leukemia, autoimmune diseases and transplant tissue rejection. In this report, we present the discovery of novel human PNP (*HsPNP*) inhibitors by coupling experimental and computational tools. A simple, economic, direct and non-radioactive enzymatic assay coupled to hydrophilic interaction liquid chromatography and UV detection (HILIC-UV) was developed for experimental screening of *HsPNP* inhibitors. Enzymatic reactions were performed in batch and activity was assessed by monitoring the phosphorolysis of inosine (Ino) to hypoxanthine (Hpx) by HILIC-UV. A small library of 6- and 8-substituted nucleosides was synthesized and screened. The inhibition potency of the most promising compound, 8-aminoinosine (**4**), was quantified through K_i and IC_{50} determinations. The effect of *HsPNP* inhibition was also evaluated *in vitro* through the study of cytotoxicity on human T-cell leukemia cells (CCRF-CEM). We also carried out docking studies for the most potent compound allowing further insights into the inhibitor interaction of *HsPNP* active site. This study provides both new tools and a new lead for developing novel *HsPNP* inhibitors.

bond of purine (deoxy)ribonucleosides (guanine and hypoxanthine) in presence of inorganic orthophosphate (Pi) as a second substrate to generate the corresponding purine nucleobase and α -D-(deoxy)ribose-1-phosphate.^[1] In the absence of human PNP (*HsPNP*), 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) to 2'-deoxyguanosine triphosphate (dGTP) is responsible for T-cell cytotoxicity. dGTP allosterically inhibits the enzyme ribonucleoside-5'-diphosphate reductase, 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] Therefore, *HsPNP* is considered an attractive chemotherapeutic target for T-cell proliferative disorders, such as psoriasis, multiple sclerosis, rheumatoid arthritis, organ transplant rejection and inflammatory bowel disorders.^[5] Additionally, T-cell leukemias and lymphomas would be primary proliferative targets for PNP inhibitors.^[6] PNP inhibitors have been also explored due to their ability to potentiate the activity of nucleoside antiviral and anticancer drugs when tested in combination.^[7]

The first inhibitors of PNP were developed using structure-based inhibitor design focused on iterative group alignment established from the PNP crystal structure.^[8] The transition state structure of *HsPNP* has subsequently permitted the design of several transition state analogues with picomolar dissociation

Introduction

Purine Nucleoside Phosphorylases (PNPs) are homotrimeric enzymes that catalyze the reversible cleavage of the glycosidic

constants.^[6a, 9] Four structurally distinct generations of transition state analogues have been synthesized (Figure 1). The first-generation inhibitor, Immucillin-H (clinically Forodesine and Mundesine), designed to mimic the early dissociative transition state of bovine PNP,^[10] has been approved in Japan for the treatment of resistant and relapsed peripheral T-cell lymphoma. Immucillin-H [(1S)-1-(9-deazahypoxanthin-9-yl)-1,4-dideoxy-1,4-imino-D-ribose] binds with a K_d of 23 pM to the bovine enzyme and with a slightly higher K_d of 56 pM to HsPNP.^[6a, 10b] The fully dissociative (S_N1) transition state of HsPNP led to the second generation DADMe-Immucillins (4'-deaza-1'-aza-2'-deoxy-1'-(9-methylene)-Immucillins). These include a methylene bridge between the hydroxypyrrolidine mimic of the ribocation and the 9-deazapurine to mimic the fully dissociative transition state of HsPNP. DADMe-Immucillin-H (clinically Ulodesine) binds tightly to HsPNP, giving a dissociation constant of 8.5 pM.^[9b, 11] The third generation of Immucillins has the ribose ring replaced by an acyclic, chiral ribocation mimic (the acyclic chiral iminoalcohols). The tightest-binding inhibitor in this group is DATMe-Immucillin-H with an 8.6 pM dissociation constant for HsPNP.^[9b] The fourth generation transition state analogues for HsPNP is represented by SerMe-Immucillin-H, an acyclic, achiral mimic of the ribocationic transition state with a 5.2 pM dissociation constant for HsPNP.^[9d] Despite their potential therapeutic, the clinical use of these PNP inhibitors is limited by their complex production technology, high cost and adverse side effects such as neutropenia, hypocalcemia, peripheral edema and orthostasis^[12]. Thereby, the identification of new HsPNP inhibitors hold promise for the treatment of proliferative T-cell disorders.

Figure 1. Four generations of PNP transition state analogue inhibitors with dissociation constants in the pM range.

The rational design of enzyme inhibitors usually starts from a screening (through radiometric, spectrophotometric or chromatographic assays) of chemical libraries consisting of potential drug candidates. Usually an activity assay, specific for the target enzyme, is developed to initially compare the inhibition

percentage induced by different compounds, using a fixed concentration of enzyme, substrate and tested inhibitor. After this screening step, IC_{50} (inhibitor concentration that reduces the enzymatic activity to 50%) and K_i (enzyme-inhibitor dissociation constant) are determined for the most interesting molecules in order to quantify the inhibitor potency and the affinity for its target. The activity assay in this case is performed in presence of increasing amounts of inhibitors.^[13]

Methods reported for PNP inhibition studies comprise radiometric and spectrophotometric assays^[14] with specific drawbacks such as the use of radiolabelled compounds, or the need to couple a second enzyme (typically xanthine oxidase in the spectrophotometric assay) to achieve the required sensitivity. An innovative enzymatic method based on immobilized PNP in flow conditions has been reported for screening studies. However, the enzyme needs to be immobilized on the inner surface of silica capillaries and specific analytical skills are needed to run the experiments.^[15] More recently, a 96-well enzymatic assay based on LC-ESI-MS/MS quantification for the identification of selective inhibitors against *Mycobacterium tuberculosis* PNP (*Mtb*PNP) was reported.^[16] Nonetheless, the method requires the use of a sophisticated analytical platform amenable to high-throughput screening. HPLC-based enzymatic methods in combination with HPLC-UV detection are the simplest and most common approaches currently used to derive accurate and precise quantitative data.

In this paper, we report the discovery of a novel HsPNP inhibitor by integrated experimental and computational assays. For assessing the enzymatic activity of HsPNP in vitro, we first developed a simple, inexpensive, nonradioactive, and reliable chromatographic assay based on hydrophilic interaction liquid chromatography (HILIC) and UV detection. Enzymatic reactions were carried out in batch and activity was determined by monitoring the phosphorolysis of inosine (Ino, substrate) to hypoxanthine (Hpx, product). Secondly, we reported the design and synthesis of a small library of both 6- and 8-substituted purine ribonucleosides for screening as potential PNP inhibitors. The presented HILIC-UV assay was used for characterizing the kinetic of HsPNP enzyme, for screening the library of purine nucleoside analogues as potential PNP inhibitors and for quantitatively assessing the inhibitory activity of the most promising candidate, 8-aminoinosine (**4**). Quantum chemical approaches were applied to determine the most favorable tautomeric form of purine nucleoside analogue **4** in the gas phase and aqueous solution. Moreover, to provide insights into inhibitor binding at molecular level and facilitate the design of more potent ligands, we also performed docking studies of **4** into HsPNP catalytic site. Finally, **4** was tested under different conditions in CCRF-CEM lymphocytes cell culture which highly express HsPNP. Altogether, this paper describes a novel pipeline for screening PNP inhibitors in vitro, as well as the discovery of a new HsPNP inhibitor lead.

Results and Discussion

Design and synthesis of nucleosides 2-10

With the aim to identify new selective inhibitors of the HsPNP, we designed and synthesized a small library of both 6- and 8-substituted purine ribonucleosides (Figure 2) based on the

evidence that some related analogues had been reported to inhibit *HsPNP* (e.g. 8-aminoguanosine, $K_i = 17 \mu\text{M}$).^[17] Moreover, the inhibition data recently collected for some 8-halo-, 8-amino-, 8-*O*-alkyl-substituted purine ribonucleosides^[16] prompted us to select new congeners. Specifically, the new set of nucleosides includes four 8-aminoinosine derivatives bearing a differently sterically hindered substituent [8-methylaminoinosine (**2**), 8-*N,N*-dimethylaminoinosine (**3**), 8-aminoinosine (**4**), 8-*i*-propylaminoinosine (**5**)] and 8-bromoinosine (**6**). From the 6-substituted series, 6-*i*-propylaminopurine-9-ribose (**7**) and its isoster counterpart 6-*i*-propoxypurine-9-ribose (**8**), 6-dimethylaminopurine-9-ribose (**9**) and 6-chloropurine-9-ribose (**10**) were chosen. Compounds **2** and **3** were previously found to inhibit the activity of *HsPNP*⁶ and were thus used as reference controls together with acyclovir (Acv, **1**).

Figure 2. Structures of the nucleoside analogues tested as potential PNP inhibitors.

The 6-substituted purine ribonucleosides (**7-10**) were prepared according to Scheme 1, as previously reported¹⁷.

The synthesis of 8-substituted inosines was achieved by following an approach similar to that adopted for the functionalization of position 6 of the purine ring, that is halogenation either on the free nucleosides or on the protected ones followed by treatment with the appropriate nucleophile and successive manipulations, when necessary¹⁷. Specifically, 2',3',5'-tri-*O*-acetyl inosine (**11**) was brominated by reaction with Br₂ in a 10% aqueous Na₂HPO₄/1,4-dioxane mixture at moderately basic pH (Scheme 2). The resulting 8-bromo derivative (**13**) was then treated with primary or secondary amines in 1,4-dioxane at 80 °C to give 8-alkylamino- and 8-*N,N*-dialkylaminoinosines (**2**, **5**, **3**), respectively. Because of the slow reactivity of position 8 in purines, which is generally less prone to nucleophilic attack than C2 and C6, long reaction times as well as high reagent concentrations and temperatures were necessary. The low yield of compound **5** (25%) might be attributed to the steric hindrance of *i*-PrNH₂, in sharp contrast to disubstituted **3**⁸. Because of the low *R_f* in TLC on silica, all these products were purified by reverse phase semi-preparative HPLC (see Experimental Section for details).

Scheme 1. Synthesis of compounds **7-10**. Reagents and conditions: a) Ac₂O, Et₃N, DMAP (10 mol %), CH₃CN (95%); b) SOCl₂, CH₂Cl₂-DMF, reflux (98%); c) NH₃, MeOH (72%); d) NaH, *i*-PrOH, reflux (32%); e) *i*-PrNH₂ or Me₂NH, Et₃N, EtOH, reflux (94% for **7** and 69% for **9**).

8-Aminoinosine (**4**) was obtained by prolonged hydrogenolysis of the corresponding benzyl derivative (**14**)¹² with HCOONH₄ in MeOH in the presence of Pd-C (10 mol %) as the catalyst. Reflux temperature was necessary to both enhance decomposition of the ammonium salt to H₂ and speed up the reaction.

HILIC-UV method development

A new HILIC-UV method was developed to monitor the phosphorolysis of Ino to Hpx and consequently *HsPNP* activity under different conditions. The chromatographic conditions that allowed to properly separate and quantify Ino, Hpx and their internal standards [Acv and 2'-deoxyadenosine (dAdo), respectively] required the use of a carbamoyl TSKgel Amide-80 column and a mobile phase composed of 92/8 (v/v) acetonitrile (ACN)/15 mM ammonium formate buffer, pH 7.0, as described in the experimental procedures. Figure 3 shows the chromatographic trace obtained applying the developed method. The linearity of the HILIC method was established for both the enzymatic substrate (Ino) and product (Hpx) in the concentration range 0.5–50 μM . Calibration curves of $y = 0.1192x + 0.0601$ ($R^2 = 0.9987$) and $y = 0.1225x + 0.0379$ ($R^2 = 0.9984$) were obtained for Ino/Acv and Hpx/dAdo, respectively.

Figure 3. Representative chromatogram of Ino, Hpx and their internal standards.

Scheme 2. Synthesis of compounds 2-6. Reagents and conditions: a) Br₂, 10% Na₂HPO₄, 1,4-dioxane (83%); b) NaOMe, MeOH (90%); c) MeNH₂, Me₂NH, *i*-PrNH₂ or BnNH₂, 1,4-dioxane, 80°C (81% for **2**, 87% for **3**, 25% for **5** and 83% for **14**); d) HCOONH₄, Pd/C (10 mol %), MeOH, reflux (96%).

Enzymatic activity assay

Kinetic and inhibition enzymatic assays were performed following the procedures and the experimental details reported in the experimental procedures. The time for enzymatic reactions was fixed at 5 minutes, after which the enzyme was inactivated by dilution of the reaction mixture in ACN containing internal standards. Before the quantification of the produced Hpx by HILIC-UV, the denatured enzyme was separated from the supernatant through centrifugation.

Kinetic studies

HsPNP was incubated with increasing amounts of Ino (0.01-1 mM, three replicates for each concentration) to investigate the enzyme activity. Since the reaction catalyzed by PNPs requires the presence of inorganic phosphate (P_i) as a second substrate, the assay was performed in 10 mM ammonium phosphate buffer,

pH 7.5, used as a source of P_i in saturating concentration. The resulting kinetic parameters are reported in Table 1, while Michaelis-Menten kinetic is shown in Figure 4. The obtained values are consistent and of the same order of magnitude compared to those reported by Cattaneo *et al.*^[16]

Table 1. HsPNP kinetic parameters (K_M, V_{max} and K_{cat}).

Enzyme	K _M (mM)	V _{max} (μmol/min)	K _{cat} (1/sec)
HsPNP	0.1267 ± 0.0213 ^[a] [0.15 ± 0.02] ^[b]	0.0078 ± 0.0004 ^[a] [0.0022 ± 0.0003] ^[b]	52.52 ± 3.00 ^[a] [14 ± 2] ^[b]

[a] Values were calculated using Ino as a substrate. [b] Values reported in Ref. 8 are indicated in square brackets.

HsPNP kinetic profile

Figure 4. Kinetic profile of HsPNP. The enzyme was incubated with increasing concentrations of Ino. Each point is the mean of three replicates, with error bars indicating ± standard deviation.

Inhibition studies

After demonstrating the reliability of the enzymatic assay in the determination of HsPNP activity, the synthesized 6- and 8-substituted purine ribonucleosides (Figure 2) were tested as potential inhibitors. The inhibition induced by the different potential lead candidates was initially determined by performing a screening assay. Thus, the developed enzymatic activity assay was carried out at fixed concentrations of the HsPNP

substrate (close to the K_M for Ino, saturating for P_i) and of the tested compounds (0.5 mM). The enzyme activity was calculated in presence and in absence of each compound and inhibition percentages (expressed as enzyme residual activity) were compared.

The majority of the tested nucleosides showed *HsPNP* inhibition under 30% (Figure 5). Compound **4** was the only exception, considered a strong candidate for additional studies since it exhibited a very high inhibitory activity, with $29.26 \pm 1.75\%$ *HsPNP* residual activity. Nucleoside **4** was therefore selected for further investigations on inhibitor potency and affinity for its target through K_i and IC_{50} determination.

Figure 5. Residual activity of *HsPNP* upon incubation with 0.5 mM of each nucleoside. Results derive from the mean of three independent determinations, with standard deviation indicated by error bars. A residual activity of 100% corresponds to the produced Hpx in absence of the tested compound.

K_i measures the enzyme-inhibitor dissociation constant, while IC_{50} expresses the inhibitor concentration required to obtain a 50% reduction in enzyme activity. Both K_i and IC_{50} quantify inhibitor potency, but usually K_i is preferred because, unlike IC_{50} , it is independent of substrate identity and concentration^[13]. For K_i and IC_{50} determination, the activity assay was performed under the same conditions used for the screening assays, but with increasing inhibitor concentrations (0.01–1 mM, three replicates). Residual activity of *HsPNP* was calculated by comparing the results obtained in presence and in absence of each nucleoside. The two inhibition parameters were calculated by fitting the curve of inhibitor concentrations (Log(M)) vs residual activities (%) through Prism® software (see experimental procedures).

Data reported in Table 2 confirmed the information deduced by the screening assay and provided a quantitative assessment of the inhibitory activity of **4**, which showed a high potency, with K_i and IC_{50} in the micromolar range.

Table 2. Inhibition results of 8-aminoinosine (**4**) towards *HsPNP*.

Inhibitor	Enzyme	K_i (mM)	Log(K_i) Log(M)	IC_{50} (mM)	Log(IC_{50}) Log(M)
4	<i>HsPNP</i>	0.0481	-4.32 ± 0.0539	0.0860	-4.06 ± 0.0539

Structural basis of *HsPNP* inhibition by 8-aminoinosine

In an effort to elucidate the possible binding mode of compound **4** within *HsPNP* active site, we carried out docking experiments using the crystal structure of *HsPNP* in complex with the transition-state analogue I-DADMe-ImmH (PDB codes 3BGS^[18]). Purine derivatives can exist in different keto-enol and amino-imino tautomeric forms. The potential structures which may be adopted by **4** are schematically presented in Figure 6. Thus, before docking into the PNP active site, we carried out separate quantum chemical calculations on the possible tautomeric forms of **4** and their relative stability (A–F in Figure 6). The ground-state molecular geometries of the tautomers were optimized at the MP2/6-311G** level of theory.^[19]

Figure 6. Schematic structures of the six possible tautomeric forms of 8-aminoinosine (**4**, A–F).

Geometries of the selected tautomers were also optimized in water solution using the integral-equation-formalism polarizable continuum model (IEF-PCM). The resulting energies were combined with the thermochemical corrections computed using the same approach to yield the Gibbs free energies and the Boltzmann population ratios (BPRs) at 298.15 K. The results are summarized in Table 3. Both the MP2 and IEF-PCM methods predicted that the most stable and dominant tautomer of **4** corresponds to the keto form **A** with BPRs at 298 K of 81.76 and 99.64 %, respectively. The second stable structure was the tautomer **B**, which is characterized by the presence of an enol group connected to the C6 carbon atom. The Gibbs free energy difference is less than 1 kcal/mol in gas phase and more than 3 kcal/mol in solution. However, the enol tautomer **B** was predicted to be much less abundant with BPRs at standard temperature of 18.24 % in gas phase and 0.36 % in solution. This agrees with experimental evidence, which clearly indicates that Ino exists as the N1H keto species in gas phase^[20] and aqueous solutions^[21] and this is also the structure determined crystallographically.^[22]

Docking simulations into the *HsPNP* active site showed that the keto form of compound **4** adopted a binding mode very similar to that previously reported for I-DADMe-ImmH (Figure 7B) in terms of both orientation within the active site and interactions with the enzymes. As depicted in Figure 7A the ligand forms three H-bonds between the purine O6, N7H and 8-NH₂ groups and N243 side chain. A further H-bond between the 8-NH₂ group and T242

Table 3. Relative values of the total energies (ΔE , kcal/mol) and the Gibbs (ΔG , kcal/mol) free energies of the different tautomers of **4** calculated at the MP2/6-311G** level of theory in the gas phase and in the solution by using the IEF-PCM model along with the results of the Boltzmann population weighted analysis (BPR, %).

Molecule	Tautomer	Gas phase			Solution		
		MP2			IEF-PCM		
		ΔE	ΔG	BPR	ΔE	ΔG	BPR
8-aminoinosine (4)	A	0.0	0.0	81.76	0.0	0.0	99.64
	B	0.78	0.89	18.24	3.96	3.33	0.36
	C	15.01	13.82	0	15.41	13.48	0
	D	14.31	13.36	0	17.78	15.12	0
	E	22.98	21.69	0	15.26	14.25	0
	F	23.93	23.14	0	15.45	15.18	0

Figure 7. *In silico* docking of compound **4** into HsPNP active site. (A) Binding mode of **4** (limegreen sticks) into the HsPNP active site located near the interface of two subunits (marine and slate ribbons for HsPNP) within the trimer. Only amino acids located within 4 Å of the bound ligand are displayed and labeled. H-bonds discussed in the text are depicted as dashed gray lines. Phosphate is shown in orange sticks, whereas structural water molecules are displayed as red spheres. (B) Ca superposition of the complex of HsPNP with 8-aminoinosine (**4**) and I-DADMe-ImmH (sand sticks), a 2 pM transition state analogue inhibitor.

residue within the catalytic site is also observed, thus contributing to the complex stabilization. The purine N1 and O6 (via a water molecule) atoms are also in H-bond distance to E201. The 5'-OH group makes a H-bond to the imidazole ring of H257. The 3'-OH group establishes a H-bond with the OH group of Y88. Finally, the 2'-OH group interacts with the amide backbone of M219 and a structurally observed water molecule.

In Vitro Effect of 8-aminoinosine (**4**) on CCRF-CEM

The effects of **4** on T cell leukemia cell line CCRF-CEM were evaluated by treating cell cultures with increasing concentrations of **4** for 96 hours in the presence or absence of dGuo (0 or 10

μM), which mimics the extracellular accumulation of this metabolite in HsPNP deficiency.^[23] Figure 8 shows the CCRF-CEM viability after treatment with **4**. The significant differences between cell viabilities in the presence or absence of dGuo strongly indicated that **4** could inhibit HsPNP and lead to intracellular dGuo metabolism into dGTP, which is an inhibitor of DNA synthesis, as discussed by other authors who evaluated Immucillin-H under conditions very similar to those of this work.^[24] Derivative **4** was able to reduce CCRF-CEM viability in the absence of dGuo (0 μM) only at 250 μM , showing its intrinsic cytotoxic effect only at high concentrations. From 10 to 100 μM , derivative **4** significantly reduced cell viability in the presence of 10 μM dGuo, demonstrating that the CCRF-CEM cell viability was decreased exclusively by PNP inhibition effect in this range.

CCRF-CEM T-lymphocytes present low dGMP catabolism and high rates of dGuo phosphorylation, leading to even more pronounced toxic effects during *HsPNP* inhibition^[6a].

Figure 8. Cytotoxic effects of 8-aminoinosine (**4**) in presence/absence of dGuo on T cell leukemia cell line CCRF-CEM. Cell-growing conditions and method of measurement are described in Experimental Section. Control values for dGuo absence were obtained by cell incubation without dGuo and **4**. Control values in the presence of dGuo were obtained by cell incubation with 10 μ M dGuo and 0 μ M **4**. Each data point represents a mean across 4 assays.

Conclusion

Driven by the evidence that human PNP present a significant role in peripheral T-cell lymphoma, we designed and synthesized a small library of 6- and 8-substituted purine ribonucleosides as potential drug candidates (Figure 2). Compounds were designed according to previous reports about the inhibitory action of some related nucleoside analogues on *HsPNP*.^[16–17] Starting from the conditions described in our previous work,^[16] we developed an enzymatic assay by HILIC-UV based on the in batch phosphorolysis of Ino to Hpx followed by the identification and quantification of substrate and product in order to evaluate the inhibitory activity of nucleoside analogues incubated with the enzyme substrate (Ino). This method is characterized by a suitable sensitivity and specificity, it does not need the use of radiolabeled compounds and it requires an user-friendly instrumentation virtually available in any drug discovery lab.

The linearity of the HILIC-UV method was assessed by using the internal standard method and no matrix effect due to the presence of denatured enzyme was observed, thus confirming that the centrifugation step after the enzymatic reaction was sufficient to separate PNP from Ino, Hpx and their internal standards. In addition, since nucleoside solubilization required the use of dimethylsulfoxide (DMSO), the enzymatic assay was performed both in presence and in absence of DMSO and no significant differences on the enzymatic activity were observed. Before starting with the investigation of potential enzyme inhibitors, it is necessary to characterize the target enzyme by defining its activity in absence of inhibitor. Usually, enzymatic activity assays quantify the product formation or the substrate consumption over time to define reaction rate. The kinetic properties of most enzymes can be described by the Michaelis-Menten equation which allows to derive the maximum reaction rate V_{max} and K_M which is the substrate concentration that allows to reach half of the V_{max} . Another important kinetic parameter to

assess enzyme activity is the turnover number K_{cat} , which expresses the number of substrate molecules converted into product per catalytic site per unit of time and is given by the ratio between enzyme concentration and V_{max} .

The enzymatic assay was therefore applied for the kinetic characterization of *HsPNP* by the definition of enzyme activity parameters (V_{max} , K_M and K_{cat}), which were found comparable to the data reported by Cattaneo *et al.*^[16] For the preliminary screening step, only one fixed concentration of substrates (Ino and P_i), inhibitors and enzyme was tested. The concentration of Ino was set close to the K_M level, the reaction buffer was used as a source of P_i in saturating concentration, the inhibitors were tested at the highest concentration dictated by the solubility of the compounds under the assay conditions and the same amount of enzyme for the kinetic studies was added. *HsPNP* residual activity was calculated by comparing the produced Hpx in presence and in absence of each compound. As shown in Figure 5, in addition to the control inhibitor Acv, one compound was found to be able to strongly inhibit *HsPNP*: compound **4**, which exhibited $29.26 \pm 1.75\%$ of *HsPNP* residual activity. The inhibition potency of this nucleoside was assessed by performing the enzymatic assay in presence of increasing amounts of inhibitors, while all the other conditions were the same as for the screening. K_i and IC_{50} values confirmed the significant inhibitory activity of **4**.

Quantum chemical calculations on the possible tautomeric forms (**A–F** in Figure 6) of **4** clearly demonstrated that this compound exists predominantly as keto form **A** both in gas phase and aqueous solution, which is in agreement with previous theoretical and experimental findings.^[20–22, 25] To elucidate the possible binding mode of compounds **4** within *HsPNP* active site and to shed light onto the structural determinants underlying its inhibitor activity, a docking study was also performed. In addition, compound **4** significantly reduced cell viability of T cell leukemia cell line CCRF-CEM in the presence of 10 μ M dGuo. In contrast, in the absence of dGuo the reduction of cell viability was irrelevant, suggesting that this effect is related to PNP inhibition, which results in intracellular dGuo metabolization into dGTP, an inhibitor of DNA synthesis and cellular proliferation.

In conclusion, we developed a simple and widely applicable assay for evaluating the activity of *HsPNP* and we showed that this assay was both qualitatively and quantitatively reliable in evaluating and screening *HsPNP* inhibitors. One leading compound was identified and validated with the experimental and computational protocols presented in this study. Due to the homologous feature of different PNPs, we suppose that our assay should be readily used for PNPs from other sources, accelerating the study and drug discovery of molecules with a selective PNP inhibitory activity.

Experimental Section

Reagents and chemicals

The *HsPNP* recombinant enzyme was kindly provided by Prof. Diógenes Santiago Santos (PUC-RS, in memoriam). *HsPNP* activity was 0.57 IU/mg (stock solution: 0.4 mg/mL), as previously described.^[16] Water was obtained from a Milli-Q® Integral system (Merck KGaA, Darmstadt, Germany). Acetonitrile HPLC grade, ammonium phosphate monobasic

(≥99%) and ammonium formate (≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA), while dimethylsulfoxide was from Carlo Erba Reagents (Cornaredo, Italy). Hypoxanthine (Hpx) was from Alfa Aesar (Haverhill, MA, USA). Inosine (Ino), acyclovir (Acv, **1**) and 2'-deoxyadenosine (dAdo) were purchased from Sigma-Aldrich (Milano, Italy). 8-Methylaminoinosine (**2**), 8-*N,N*-dimethylaminoinosine (**3**), 8-bromoinosine (**6**), 6-*i*-propylaminopurine-9-ribose (**7**), 6-*i*-propoxypurine-9-ribose (**8**), 6-dimethylaminopurine-9-ribose (**9**) and 6-chloropurine-9-ribose (**10**) were synthesized according to literature methods^[16, 26] (see Schemes 1 and 2). Their analytical data were consistent with those previously reported.^[16, 26]

8-Aminoinosine (4): 8-Benzylamino inosine^[27] (**14**, 60 mg, 0.16 mmol) and 10% Pd-C (90 mg) were suspended in MeOH (2.50 mL) under inert atmosphere and treated with HCOONH₄ (202 mg, 3.20 mmol); the resulting mixture was refluxed and monitored by TLC (CH₂Cl₂-MeOH, 4:1). Further portions of HCOONH₄ were progressively added over 5 days (202 mg, 3.20 mmol after 24 h, 404 mg, 6.41 mmol after 96 h and 101 mg, 1.60 mmol after 120 h). The suspension was then filtered on celite and evaporated under reduced pressure to get the title compound as a very pale yellow powder (43 mg, 0.15 mmol, 96% yield). R_f: 0.10 (CH₂Cl₂-MeOH, 4:1). ¹H NMR (DMSO-*d*₆, 400 MHz): δ (ppm) 3.63 (br s, 2H, H^{5a}, H^{5b}), 3.94 (br q, *J* = 2.3 Hz, 1H, H⁴), 4.12 (dd, *J* = 5.4, 2.0 Hz, 1H, H³), 4.59 (t, *J* = 6.4 Hz, 1H, H²), 5.16 (br s, 1H, OH³), 5.30 (br s, 1H, OH²), 5.56 (br s, 1H, OH⁵), 5.85 (d, *J* = 7.3 Hz, 1H, H¹), 6.50 (br s, 2H, N⁸H₂), 7.79 (s, 1H, H²), 12.04 (br s, 1H, N¹H). ¹³C NMR (DMSO-*d*₆, 100 MHz): δ (ppm) 61.8 (C⁵), 71.0 (C²), 71.2 (C³), 86.2 (C⁴), 87.2 (C¹), 120.9 (C⁵), 143.0 (C²), 147.5 (C⁴), 151.1 (C⁸), 155.4 (C⁶). MS (ESI⁺): *m/z* 284.0 [M+H]⁺, 306.1 [M+Na]⁺, 567.0 [2M+H]⁺, 589.0 [2M+Na]⁺.

8-(*i*-Propylamino)inosine (5): To a solution of 2',3',5'-*O*-triacetyl-8-bromoinosine^[26a] (**13**, 189 mg, 0.40 mmol) in dry 1,4-dioxane (0.9 mL) was added a large excess of isopropylamine (3.22 mL, 37.4 mmol) under a N₂ atmosphere. The mixture was stirred at 80 °C and monitored by TLC (CH₂Cl₂-MeOH, 4:1) until complete consumption of the starting material. The solvent was removed under reduced pressure and the residue was purified by semipreparative HPLC to give the title compound as a white powder (32 mg, 25% yield). R_f: 0.32 (CH₂Cl₂-MeOH, 4:1). ¹H NMR (DMSO-*d*₆, 400 MHz): δ (ppm) 1.18 (d, *J* = 3.4 Hz, 3H, CH(CH₃)₂), 1.20 (d, *J* = 3.5 Hz, 3H, CH(CH₃)₂), 3.61-3.71 (m, 2H, H^{5a}, H^{5b}), 3.95 (br q, *J* = 2.1 Hz, 1H, H⁴), 3.97-4.07 (m, 1H, NHCH), 4.12 (dd, *J* = 5.5, 2.0 Hz, 1H, H³), 4.54 (dd, *J* = 7.1, 5.8 Hz, 1H, H²), 4.96-5.48 (m, 2H, OH², OH³), 5.63 (br s, 1H, OH⁵), 5.89 (d, *J* = 7.4 Hz, 1H, H¹), 6.59 (d, *J* = 7.6 Hz, 1H, N⁸H), 7.81 (s, 1H, H²), 12.01 (br s, 1H, N¹H). ¹³C NMR (DMSO-*d*₆, 100 MHz): δ (ppm) 22.8, 23.1 (CH(CH₃)₂), 44.4 (NHCH), 61.8 (C⁵), 70.9 (C²), 71.2 (C³), 86.0 (C⁴), 87.0 (C¹), 122.2 (C⁵), 142.5 (C²), 147.9 (C⁴), 150.5 (C⁸), 155.9 (C⁶). MS (ESI⁺): *m/z* 326.0 [M+H]⁺, 348.2 [M+Na]⁺, 673.1 [2M+Na]⁺.

Instrumentation and chromatographic conditions

Semipreparative HPLC was performed using an AKTA Basic100 instrument (Amersham Pharmacia Biotech, Little Chalfont, UK)

equipped with a UV-Vis detector (Pharmacia, Uppsala, Sweden); chromatographic conditions were as follows: column, Jupiter[®] 10 μm Proteo 90A (250 x 10.0 mm, Phenomenex); flow rate, 5 mL/min; detector, λ = 260 and 250 nm; mobile phase MeOH-H₂O, isocratic elution at 3% MeOH for 2 min, then gradient elution from 3% to 100% MeOH in 30 min. ¹H and ¹³C NMR spectra were recorded at 400.13 and 100.61 MHz, respectively, on a Bruker AVANCE 400 spectrometer equipped with the XWIN-NMR software package (Bruker, Karlsruhe, Germany) at 300 K. ¹H and ¹³C chemical shifts (δ) are given in parts per million and were referenced to the solvent signals (δ_H=2.50 and δ_C=39.50 ppm for DMSO-*d*₆). ¹³C NMR signal multiplicities were based on APT (attached proton test) spectra. Electrospray ionization mass spectra (ESI-MS) were acquired on a ThermoFinnigan LCQ Advantage spectrometer (Hemel Hempstead, Hertfordshire, UK).

Analytical HPLC was performed on an Agilent HPLC series 1100 system (Santa Clara, CA, USA), equipped with mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment and diode array detector. For data acquisition and analysis, the HP ChemStation for LC software version Rev. A.06.03 [509] was used. A TSKgel Amide-80 (3 μm, 80 Å, 2 × 150 mm) column from Tosoh Bioscience (Montgomeryville, PA, USA) was employed. The mobile phase was composed of a mixture of ACN and 15 mM ammonium formate 92/8 (v/v), pH 7.0. Chromatographic conditions consisted of an isocratic elution for 17 min, a temperature of 25 °C, an injection volume of 10 μL and a constant flow rate of 300 μL/min. In all experiments, compounds were detected at 254 nm. The linearity of the method was assessed for Ino and Hpx in the concentration range 0.5-50 μM. Eight concentration levels were considered, and three independent determinations were performed for each point. A fixed amount (5 μM) of the internal standards dAdo and Acv was added to each sample. Calibration curves were obtained by the least squares method plotting Hpx/dAdo and Ino/Acv peak area ratios (y-axis) against Hpx and Ino concentrations (x-axis), respectively.

Sample preparation: For the calibration curve determination, stock solutions of 10 mM Ino (in water), Hpx, dAdo and Acv (in DMSO) were prepared. Working solutions (0.025 mM, 0.25 mM and 1 mM for Ino and Hpx, 0.25 mM for dAdo and Acv) were obtained by dilution of the stock solutions in water. The working solutions of compounds and internal standards were then combined and made up to a 500 μL final volume with ACN in order to reach the required concentrations.

For the enzymatic assay, 10 mM Ino (in water) and inhibitor (in DMSO) stock solutions were diluted to different concentrations with 10 mM ammonium phosphate, pH 7.5 to reach a volume of 480 μL. The addition of 20 μL of enzyme solutions, prepared by diluting 1:100 the HsPNP stock solution (see reagents and chemicals), allowed to obtain the final volume (500 μL) for the reaction.

Enzymatic activity assay

The procedure followed for the enzymatic assays was adapted to the available laboratory equipment starting from the one described by Cattaneo and colleagues.^[16] Briefly, microcentrifuge tubes containing 480 μL solutions of Ino and

phosphate buffer for the kinetic assay or Ino, phosphate buffer and inhibitor for the inhibition assay, were conditioned for 5 min at 37 °C under continuous stirring. After, 20 µL enzyme solution (see sample preparation) were added to each tube (0.08 µg *HsPNP*/microcentrifuge tube). The reaction was run for 5 min at 37 °C under continuous stirring and subsequently stopped by pipetting 25 µL of the reaction mixture in a new microcentrifuge tube containing 475 µL ACN and the internal standards (1.05 µM of dAdo and Acv). The obtained solutions were mixed by pipetting 150 µL up and down for three times and the inactivated enzyme was precipitated by centrifugation (5000 rpm for 10 min). Finally, the supernatant was analyzed by HILIC-UV.

Kinetic studies: The kinetic properties of a great number of enzymes can be described by the Michaelis-Menten equation:

$$v = \frac{V_{\max} [S]}{K_M + [S]}$$

where v is the reaction rate, $V_{\max}$ is the maximum reaction rate, $[S]$ represents the concentration of the enzymatic substrate and K_M or Michaelis-Menten constant is the substrate concentration that allows to reach half of the $V_{\max}$. Another important kinetic parameter to assess enzyme activity is the turnover number K_{cat} , which expresses the number of substrate molecules converted into product

Ino kinetic constants were defined by performing the enzymatic assay in presence of increasing Ino concentrations (seven concentrations in the range 0.01-1 mM, mean of three replicates) and a fixed amount of P_i (10 mM ammonium phosphate, pH 7.5). To calculate reaction rates of *HsPNP*, µmol of produced Hpx were derived taking into account the Hpx/dAdo calibration line and were divided by the reaction time (5 min). The Michaelis-Menten trend was obtained by plotting the rate of the enzymatic reaction, expressed as µmol of produced Hpx/min, against the substrate (Ino) concentration expressed as mM. The kinetic parameters $V_{\max}$ (maximal reaction rate, expressed as µmol/min), K_M (Michaelis-Menten constant, expressed as mM) and K_{cat} (turnover number, expressed as 1/sec) were calculated by Prism® software (GraphPad Software, San Diego, CA, USA) using a non-linear regression curve fitting. To define $V_{\max}$ and K_M , the Michaelis-Menten model $Y = V_{\max} \cdot X / (K_M + X)$ was chosen. The enzyme kinetics equation $Y = E_t \cdot K_{\text{cat}} \cdot X / (K_M + X)$ was used to determine K_{cat} . The term E_t represents the concentration of enzyme catalytic sites and it was calculated considering that *HsPNP* (MW = 96354 Da) is homotrimeric.

Inhibition studies: The enzymatic assay was applied to the screening of ten nucleoside analogues (acyclovir (1), 8-methylaminoinosine (2), 8-*N,N*-dimethylaminoinosine (3), 8-aminoinosine (4), 8-*i*-propylaminoinosine (5), 8-bromoinosine (6), 6-*i*-propylaminopurine-9-ribose (7), 6-*i*-propoxypurine-9-ribose (8), 6-dimethylaminopurine-9-ribose (9) and 6-chloropurine-9-ribose (10)) as potential PNP inhibitors. The assay was carried out using a high inhibitor concentration (0.5 mM) and fixed concentrations of Ino (close to the K_M level: 0.1 mM - *HsPNP*) and P_i (10 mM ammonium phosphate, pH 7.5). *HsPNP* residual activity (%) was calculated comparing the produced Hpx in presence of each inhibitor to the reaction carried out in the same conditions, but in absence of inhibitor (100% residual activity). Each resulting percentage is the mean

of three replicates. Inhibition constants (K_i) were assessed in the same conditions of the screening assay, except for the amount of inhibitors. Five concentrations of each inhibitor (0.01-1 mM, three replicates) were incubated with *HsPNP* and reactions without inhibitor were carried out in parallel. Residual activity (%) of *HsPNP* was calculated considering the Hpx/dAdo calibration line, the dilution factor and the assay volume (500 µL) and plotted against the inhibitor concentration (µM) in a logarithmic scale. K_i and IC_{50} (inhibitor concentration that gives 50% of the maximal response) parameters were calculated by Prism® software (GraphPad Software, San Diego, CA, USA) according to the One site - Fit K_i (1) and One site - Fit $LogIC_{50}$ (2) equations, respectively:

$$(1) \text{ } LogIC_{50} = \text{Log}(10^{LogK_i \cdot (1 + [Ino] / InoK_M)})$$

$$(2) \text{ } Y = (\text{Top} - \text{Bottom}) / (1 + 10^{(X - LogIC_{50})}) + \text{Bottom}$$

where K_i and IC_{50} are expressed in M, $[Ino]$ is the constant concentration of substrate (M), $InoK_M$ is the Michaelis-Menten constant of the substrate (M), top and bottom represent the plateaus reached on the y-axis (%).

Computational chemistry

Molecular modeling and graphics manipulations were performed using Maestro 11.0 (Schrödinger, LLC, New York, NY, 2017) and UCSF-Chimera 1.8.1 (<http://www.cgl.ucsf.edu/chimera>) software packages running on a E4 Computer Engineering E1080 workstation provided with an Intel Xeon processor. GOLD Suite 5.5 docking package (CCDC Software Limited: Cambridge, UK)^[28] was used for all docking calculations. All quantum chemical calculations were performed with the Gamess-US program.^[29] Figures were generated using Pymol 2.0 (Schrödinger, LLC, New York, NY, 2017).

Quantum chemical calculations: The initial tautomeric structure of **4** was built with the Avogadro program^[30] and the geometry optimized by using B3LYP method with 6-311G** basis set. The level of theory was then increased using the second-order Møller-Plesset perturbation theory (MP2)^[19] with the triple- ζ quality Gaussian-type basis sets augmented with polarization functions (def2-TZVP).^[31] This kind of calculations allowed us to obtain more reliable electrons wave functions and stationary nuclei coordinates. To mimic aqueous solutions, the IEF-PCM model^[32] was applied. The IEF-PCM model defines the cavity within the solvent as the union of a series of interlocking atom spheres and is considered more effective when a higher accuracy in the results is required for the solvent-solute interactions.^[33] To ensure that the optimized geometries represented the local minima and that there were only positive eigenvalues, vibrational frequencies and approximate description of each normal modes were also carried out using MP2/6-311G** in both gas and liquid phase. The vibrational calculations were carried out within the harmonic approximation. BPRs of the tautomer in vacuum and water at room temperature ($T = 298.15$ K) were calculated using the Boltzmann distribution formula:

$$n_i = \frac{e^{\frac{-(G_i - G_0)}{RT}}}{\sum_n e^{\frac{-(G_n - G_0)}{RT}}} \times 100$$

where n_i is the population (in %) of the i th tautomer, G_i the Gibbs energy of the i th tautomer and G_0 the Gibbs energy of the most stable tautomer.

Protein and ligand preparation: The crystal structure of HsPNP in complex with the transition-state analogue I-DADMe-ImmH (PDB codes 3BGS),^[18] recovered from Brookhaven Protein Database,^[34] was employed for the automated docking experiment. The protein was processed through the Protein Preparation Wizard in Maestro. Apart from two structural water molecules and a phosphate anion that are critical in creating catalytic activity, all crystallographic water molecules and other chemical components were deleted. The right bond orders as well as charges and atom types were assigned, and the hydrogen atoms were added. Arginine and lysine side chains were considered as cationic at the guanidine and ammonium groups, and the aspartic and glutamic residues were considered as anionic at the carboxylate groups. H257 in HsPNP was modeled in the neutral form with the proton at NE leaving ND1 to interact with 5'-OH of the ribose ring.^[35] Moreover, an exhaustive sampling of the orientations of groups, whose H-bonding network needs to be optimized, was performed. Finally, the protein structure was refined with a restrained minimization with the OPLS2005 force field^[36] by imposing a 0.3 Å rmsd limit as the constraint.

The core structure of compound **4** was built using the Molecular Builder module in Maestro. Atom N7 of the ligand was protonated because transition state analysis^[9a] has established its protonation to N7H before reaching the transition state. The ligand was then preprocessed with LigPrep 3.3 (Schrödinger, LLC, New York, NY, 2017) and optimized by MacroModel 10.7 (Schrödinger, LLC, New York, NY, 2017), using the MMFFs force field with the steepest descent (1000 steps) followed by truncated Newton conjugate gradient (500 steps) methods. Partial atomic charges were computed using the OPLS-AA force field.

Docking simulations: Docking of **4** to HsPNP was performed with the GOLD software,^[28] which uses a genetic algorithm for determining the docking modes of ligands and proteins. The coordinates of the cocrystallized transition-state analogue I-DADMe-ImmH were chosen as active-site origin. The active-site radius was set equal to 6 Å. Two explicit water molecules were allowed to toggle on or off during the individual docking runs (i.e., these waters were not automatically present in the binding site, but were included if their presence strengthened the interaction of the ligand with the receptor, as determined by the scoring function).^[37] Each GA run used the default parameters of 100 000 genetic operations on an initial population of 100 members divided into five subpopulations, with weights for crossover, mutation, and migration being set to 95, 95, and 10, respectively. GOLD allows a user-definable number of GA runs per ligand, each of which starts from a different orientation. For these experiments, the number of GA runs was set to 100 without the option of early termination, and scoring of the docked poses was performed with the original ChemPLP scoring function rescoring with ChemScore.^[38] The final receptor–ligand complex for each ligand was visually inspected and chosen interactively by selecting the highest scoring pose that was consistent with experimentally-derived information about the binding mode of the ligand.

In Vitro Effect of 8-aminoinosine (**4**) on CCRF-CEM cells

Cells and reagents: CCRF-CEM cell line (ATCC® CCL-119™), a T-lymphoblast from human acute lymphoblastic leukemia, was purchased from the Rio de Janeiro Cell Bank, Brazil. Roswell Park Memorial Institute Medium (RPMI-1640), Hank's Balanced Salt Solution, foetal bovine serum (FBS), antibiotic solution (10,000U/mL penicillin, 10mg/mL streptomycin), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2'-deoxyguanosine monohydrate (dGuo) were all supplied by Sigma–Aldrich (São Paulo, Brazil). Dimethyl sulfoxide (DMSO) and other reagents were from analytical grade.

Cell culture conditions: Culture medium RPMI-1640 was supplemented with 4.5 mg/ml glucose, 0.1 mg/ml penicillin, 0.14 mg/ml streptomycin and 10% inactivated FBS. Cultured cells were maintained at 37°C in an atmosphere containing 95% air and 5% CO₂. Every 2 to 3 days cultures were centrifuged (1500 rpm / 10 minutes) and subsequently resuspended in fresh medium at 3 x 10⁵ to 1 x 10⁶ viable cells/mL.

Measurement of cell proliferation by MTT assay:

Metabolically active cells were assessed using the MTT reduction colorimetric assay, as previously reported by Mosmann.^[39] Cells ($n = 4$) were seeded in 96-well plates (Corning) at density of 1 x 10⁶ cells/mL, distributed in a total volume of 100 µL/well. CCRF-CEM cells were placed in contact with dGuo (0 or 10 µM) and 8-aminoinosine (**4**) (0 to 250 µM) for 96 hours. Stock solutions of dGuo and 8-aminoinosine (**4**) were prepared in DMSO and diluted in culture medium at 1% to obtain desired concentrations. Cell culture medium (DMSO 1% - v/v) containing or not 10 µM dGuo was used as 100% of cell viability control. Microplates were centrifuged at 1500 rpm for 10 minutes. Samples were aspirated, and cells were treated with 100 µL/well of MTT solution (0.5 mg/mL in HBSS). Plates were incubated with MTT reagent at 37 °C and 5% CO₂. After 3 hours of incubation, microplates were centrifuged again and MTT solution was aspirated. Cells were lysed by DMSO (100 µL/well) and cell viability spectrophotometrically measured ($\lambda = 570$ nm), after vigorously plate mixing for 60 seconds, using a microplate reader (TP-Reader™, Thermoplate, Brazil). The cytotoxicity was expressed as the percentage of surviving cells in relation to respective control groups.^[40]

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[1] M. Friedkin, H. M. Kalckar, *J. Biol. Chem.* **1950**, *184*, 437–448.

[2] M. L. Markert, *Immunodef. Rev.* **1991**, *3*, 45–81.

- [3] J. G. Cory, Chiba P. in *Inhibitors of ribonucleoside diphosphate reductase activity* (Eds.: J. G. Cory, A. H. Cory), Pergamon Press, Oxford, **1989**, pp. 245-264.
- [4] S. Eriksson, L. Thelander, M. Akerman, *Biochemistry* **1979**, *18*, 2948-2952.
- [5] a) J. A. Montgomery, *Med. Res. Rev.* **1993**, *13*, 209-228; b) J. C. Sircar, R. B. Gilbertsen, *Drugs Future* **1988**, *13*, 653-668; c) G. A. Omura, *Curr. Pharm. Des.* **2000**, *6*, 943-959.
- [6] a) G. B. Evans, R. H. Furneaux, A. Lewandowicz, V. L. Schramm, P. C. Tyler, *J. Med. Chem.* **2003**, *46*, 3412-3423; b) V. L. Schramm, *Biochim. Biophys. Acta Mol. Basis Dis.* **2002**, *1587*, 107-117.
- [7] L. L. Bennett, P. W. Allan, P. E. Noker, L. M. Rose, S. Niwas, J. A. Montgomery, M. D. Erion, *J. Pharmacol. Exp. Ther.* **1993**, *266*, 707-714.
- [8] S. E. Ealick, Y. S. Babu, C. E. Bugg, M. D. Erion, W. C. Guida, J. A. Montgomery, J. A. d. Secrist, *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 11540-11544.
- [9] a) A. Lewandowicz, V. L. Schramm, *Biochemistry* **2004**, *43*, 1458-1468; b) E. A. Taylor, K. Clinch, P. M. Kelly, L. Li, G. B. Evans, P. C. Tyler, V. L. Schramm, *J. Am. Chem. Soc.* **2007**, *129*, 6984-6985; c) J. M. Mason, A. S. Murkin, L. Li, V. L. Schramm, G. J. Gainsford, B. W. Skelton, *J. Med. Chem.* **2008**, *51*, 5880-5884; d) A. S. Murkin, K. Clinch, J. M. Mason, P. C. Tyler, V. L. Schramm, *Bioorg. Med. Chem. Lett.* **2008**, *18*, 5900-5903.
- [10] a) P. C. Kline, V. L. Schramm, *Biochemistry* **1993**, *32*, 13212-13219; b) R. W. Miles, P. C. Tyler, R. H. Furneaux, C. K. Bagdassarian, V. L. Schramm, *Biochemistry* **1998**, *37*, 8615-8621.
- [11] A. Lewandowicz, E. A. T. Ringia, L.-M. Ting, K. Kim, P. C. Tyler, G. B. Evans, O. V. Zubkova, S. Mee, G. F. Painter, D. H. Lenz, *J. Biol. Chem.* **2005**, *280*, 30320-30328.
- [12] a) A. Al-Kali, V. Gandhi, M. Ayoubi, M. Keating, F. Ravandi, *Future Oncol.* **2010**, *6*, 1211-1217; b) S. Mohammad, S. L. Giattino, R. T. Keenan, *Curr. Treat. Options Rheumatol.* **2015**, *1*, 143-155; c) Q.-H. Wen, L.-H. Wang, X.-A. Zeng, D.-B. Niu, M.-S. Wang, *Int. J. Biol. Macromol.* **2018**, *118*, 588-598.
- [13] R. A. Copeland in *Evaluation of Enzyme Inhibitors in Drug Discovery* (Ed: R. A. Copeland), John Wiley & Sons Inc., Hoboken, **2013**, pp. 88-96.
- [14] A. Bzowska, E. Kulikowska, D. Shugar, *Pharmacol. Ther.* **2000**, *88*, 349-425.
- [15] M. C. de Moraes, R. G. Ducati, A. J. Donato, L. A. Basso, D. S. Santos, C. L. Cardoso, Q. B. Cass, *J. Chromatogr. A* **2012**, *1232*, 110-115.
- [16] G. Cattaneo, D. Ubiali, E. Calleri, M. Rabuffetti, G. C. Hofner, K. T. Wanner, M. C. De Moraes, L. K. B. Martinelli, D. S. Santos, G. Speranza, G. Massolini, *Anal. Chim. Acta* **2016**, *943*, 89-97.
- [17] J. D. Stoeckler, C. Cambor, V. Kuhns, S.-H. Chu, R. E. Jr. Parks, *Biochem. Pharmacol.* **1982**, *31*, 163-171.
- [18] A. Rinaldo-Matthis, A. S. Murkin, U. A. Ramagopal, K. Clinch, S. P. Mee, G. B. Evans, P. C. Tyler, R. H. Furneaux, S. C. Almo, V. L. Schramm, *J. Am. Chem. Soc.* **2008**, *130*, 842-844.
- [19] C. Möller, M. S. Plesset, *Phys. Rev.* **1934**, *46*, 618-622.
- [20] M. Alvarez-Ros, M. A. Palafox, *J. Mol. Struct.* **2013**, *1047*, 358-371.
- [21] a) A. Psoda, D. Shugar, *Biochim. Biophys. Acta* **1971**, *247*, 507-513; b) G. C. Medeiros, G. J. Thomas, Jr., *Biochim. Biophys. Acta* **1971**, *238*, 1-4.
- [22] A. R. Munns, P. Tollin, *Acta Crystallogr. B* **1970**, *26*, 1101-1113.
- [23] G. A. Kicska, L. Long, H. Hörig, C. Fairchild, P. C. Tyler, R. H. Furneaux, V. L. Schramm, H. L. Kaufman, *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 4593-4598.
- [24] M. Huang, Y. Wang, J. Gu, J. Yang, K. Noel, B. S. Mitchell, V. L. Schramm, L. M. Graves, *Leuk. Res.* **2008**, *32*, 1268-1278.
- [25] J. Elguero, C. Marzin, A. R. Katritzky, P. Linda, *The Tautomerism of Heterocycles* (Ed.: J. Elguero), Academic Press, New York, **1976**, pp. 87-102.
- [26] a) D. Ubiali, C. D. Serra, I. Serra, C. F. Morelli, M. Terreni, A. M. Albertini, P. Manitto, G. Speranza, *Adv. Synth. Catal.* **2012**, *354*, 96-104; b) D. Ubiali, C. F. Morelli, M. Rabuffetti, G. Cattaneo, I. Serra, T. Bavaro, A. M. Albertini, G. Speranza, *Curr. Org. Chem.* **2015**, *19*, 2220-2225.
- [27] T. Sasaki, K. Minamoto, Y. Fujiki, N. Shiomi, Y. Uda, *Nucleic Acids Symp. Ser.* **1984**, *15*, 53-56.
- [28] G. Jones, P. Willett, R. C. Glen, A. R. Leach, R. Taylor, *J. Mol. Biol.* **1997**, *267*, 727-748.
- [29] M. W. Schmidt, K. K. Baldrige, J. A. Boatz, S. T. Elbert, M. S. Gordon, J. H. Jensen, S. Koseki, N. Matsunaga, K. A. Nguyen, S. Su, *J. Comput. Chem.* **1993**, *14*, 1347-1363.
- [30] M. D. Hanwell, D. E. Curtis, D. C. Lonie, T. Vandermeersch, E. Zurek, G. R. Hutchison, *J. Cheminformatics* **2012**, *4*, 17.
- [31] F. Weigend, R. Ahlrichs, *Phys. Chem. Chem. Phys.* **2005**, *7*, 3297-3305.
- [32] E. Cancès, B. Mennucci, J. Tomasi, *J. Chem. Phys.* **1997**, *107*, 3032-3041.
- [33] W. Sang-Aroon, V. Ruangpornvisuti, *Int. J. Quantum Chem.* **2008**, *108*, 1181-1188.
- [34] H. M. Berman, T. Battistuz, T. N. Bhat, W. F. Bluhm, P. E. Bourne, K. Burkhardt, Z. Feng, G. L. Gilliland, L. Iype, S. Jain, P. Fagan, J. Marvin, D. Padilla, V. Ravichandran, B. Schneider, N. Thanki, H. Weissig, J. D. Westbrook, C. Zardecki, *Acta Crystallogr. D Biol. Crystallogr.* **2002**, *58*, 899-907.
- [35] J. S. Hirschi, K. Arora, C. L. Brooks, 3rd, V. L. Schramm, *J. Phys. Chem. B* **2010**, *114*, 16263-16272.
- [36] Z. Song, F. A. Kovacs, J. Wang, J. K. Denny, S. C. Shekar, J. R. Quine, T. A. Cross, *Biophys. J.* **2000**, *79*, 767-775.
- [37] M. L. Verdonk, G. Chessari, J. C. Cole, M. J. Hartshorn, C. W. Murray, J. W. M. Nissink, R. D. Taylor, R. Taylor, *J. Med. Chem.* **2005**, *48*, 6504-6515.
- [38] M. L. Verdonk, J. C. Cole, M. J. Hartshorn, C. W. Murray, R. D. Taylor, *Proteins* **2003**, *52*, 609-623.
- [39] T. Mosmann, *J. Immunol. Methods* **1983**, *65*, 55-63.
- [40] W. A. da Silva, L. C. R. P. da Silva, V. R. Campos, M. C. B. V. de Souza, V. F. Ferreira, A. C. P. B. dos Santos, P. C. Sathler, G. S. de Almeida, F. R. F. Dias, L. M. Cabral, *Future Med. Chem.* **2018**, *10*, 527-540.

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In this study, we developed a simple, economic, direct and non-radioactive enzymatic assay coupled to hydrophilic interaction liquid chromatography and UV detection (HILIC-UV) for evaluating and screening qualitatively and quantitatively human purine nucleoside phosphorylase (*Hs*PNP) inhibitors. One leading compound, the 8-amininosine (4), was identified and validated through experimental and computational protocols. Due to the homologous feature of different PNPs, we suppose that our assay should be readily used for PNPs from other sources, accelerating the study and drug discovery of molecules with a selective PNP inhibitory activity.