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Capillary bioreactors based on human purine nucleoside phosphorylase: A new approach for ligands identification and characterization

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ABSTRACT

The enzyme purine nucleoside phosphorylase (PNP) is a target for the discovery of new lead compounds employed on the treatment severe T-cell mediated disorders. Within this context, the development of new, direct, and reliable methods for ligands screening is an important task. This paper describes the preparation of fused silica capillaries human PNP (HsPNP) immobilized enzyme reactor (IMER). The activity of the obtained IMER is monitored on line in a multidimensional liquid chromatography system, by the quantification of the product formed throughout the enzymatic reaction. The K_M value for the immobilized enzyme was about twofold higher than that measured for the enzyme in solution (255 ± 29.2 μM and 133 ± 14.9 μM, respectively). A new fourth-generation immucillin derivative (DI4G; $IC_{50} = 40.6 \pm 0.36$ nM), previously identified and characterized in HsPNP free enzyme assays, was used to validate the IMER as a screening method for HsPNP ligands. The validated method was also used for mechanistic studies with this inhibitor. This new approach is a valuable tool to PNP ligand screening, since it directly measures the hypoxanthine released by inosine phosphorolysis, thus furnishing more reliable results than those one used in a coupled enzymatic spectrophotometric assay.

1. Introduction

Purine nucleoside phosphorylase (PNP) is a key enzyme of the purine salvage pathway and catalyzes the cleavage of (deoxy)ribonucleosides, in the presence of inorganic phosphate (P_i), to the corresponding purine bases and ribose(deoxyribose)-1-phosphate. The description of a unique and rare form of immune deficiency found in children lacking the PNP enzyme has demonstrated its importance for immune system integrity. PNP-deficiency results in gradual specific T-cell loss function after birth, suggesting that PNP activity may be required for normal human T-cell proliferation [1–3].

The biochemical link between PNP and T-cell deficiency relies on the failure to degrade deoxyguanosine (dGuo) and its conversion to deoxyguanosine triphosphate (dGTP). PNP catalyzes dGuo phosphorolysis to guanine and deoxyribose 1-phosphate. However, dGuo is also a substrate for deoxycytidine kinase (dCK), which catalyzes dGuo conversion to deoxyguanosine monophosphate (dGMP). Nevertheless, dGuo has higher affinity for PNP than for dCK. Therefore, dGuo mainly undergoes phosphorolysis by PNP catalysis. When the PNP enzyme is inhibited within cells, the dGuo concentration increases and it is transformed into dGMP by dCK action. The dGMP formed within these conditions is further converted to deoxyguanosine triphosphate (dGTP) by cellular kinases. Accumulated dGTP inhibits the activity of ribonucleotide reductase, thus preventing the conversion of ribonucleoside diphosphates to the corresponding deoxyribonucleoside diphosphates. Deoxyribonucleotides depletion ultimately results in the inhibition of DNA synthesis and cell replication, thereby leading to suppressed immature T-cell proliferation [4–6]. Such T-cells require DNA synthesis for cell division, and the excess of dGTP inhibits ribonucleotide-diphosphate reductase, causing imbalance in deoxynucleotide pools and T-cell death via a characteristic mechanism of apoptosis induction [5,7,8]. Consequently, PNP inhibitors represent a new class of selective immunosuppressive agents that might be useful in the treatment of a wide variety of T-cell-mediated disorders, such as psoriasis, rheumatoid arthritis, and T-cell proliferative disorders, such as organ transplant rejection and adult T-cell leukemia [5,9–11].

PNP inhibitors can also be used in order to avoid anticancer and antiviral drug cleavage. Since this enzyme is highly active in the human blood and tissues, some nucleoside analogues, employed

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as potential chemotherapeutic agents, may be degraded before significant doses reach the target cells [4,12,13].

Because their potential in clinical applications, many base, nucleoside, and nucleotide analogues have been synthesized and tested as PNP inhibitors. A variety of methods has been applied to evaluate the activity of different compounds against the PNP enzyme, including capillary electrophoresis with UV detection [14], liquid chromatography (LC) with and without radiolabeled substrates [15], and spectrophotometric coupled assays [13]. The most widely employed assay was developed by Kalckar [16], and consists of a coupled assay in which the hypoxanthine (Hypo) released by inosine (Ino) phosphorolysis is oxidized by xanthine oxidase (XOx) to generate uric acid, which is spectrophotometrically monitored at 293 nm. However, in a screening process, it is important to evaluate the selectivity of the active compounds towards XOx [13].

Within this context, the development of a direct enzymatic assay for PNP ligands screening is an important task. It can lead to reduced costs, as only one enzyme is employed. In addition, a selective response regarding ligand potency can be obtained in a single assay, demanding low sample consumption. Furthermore, it is important to keep in mind that, in coupled assays, any interference in the activity of the second enzyme, by inhibition or unspecific inactivation, can generate false-positive results. All these drawbacks can be avoided by the use of a direct method. Attained to this purpose, here we describe a direct, specific, and effective method that employs immobilized PNP enzyme for the direct on-line quantification of Hypo formed by Ino phosphorolysis. This PNP direct assay also allows the use of the same amount of enzyme for several analysis, which increases method reproducibility and minimizes costs [17].

A number of works have been reported on the application of immobilized PNP from microbial origin, as biosensors [18–21] and in biocatalysis [22]. Structural studies on PNP from pig brain have also been reported [23]. Thereby, to the best of our knowledge, this is the first report describing the immobilization of human PNP (HsPNP) for screening purposes and characterization of a new ligand.

Different procedures may be employed to covalently bind an enzyme to a variety of diverse matrices. The selected procedure and matrix should maintain enzyme activity with increased stability [17,24]. When the produced immobilized enzyme reactor (IMER) is employed as a LC column, rapid evaluation of thermodynamic and kinetic constants can be achieved, as well as screening and determination of mechanisms of action of new inhibitors [17,24].

Our group has previously described the covalent immobilization of human and *Trypanosoma cruzi* glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzymes [25–27] onto fused silica capillaries. This support offers some benefits, such as a large surface area to volume ratio within the capillary, low back pressure, and high enzymatic reaction rate. The obtained IMERs demonstrated optimum activity, catalytic efficiency, and stability. Moreover, it prevented interactions with ligands in identification screenings. Based on these results, silica capillaries of HsPNP IMER was selected as a screening inhibitor device.

This work also reports the synthesis of a fourth-generation immucillin derivative (DI4G) and its characterization as a potent synthetic inhibitor of HsPNP. It was firstly evaluated in a free enzyme assay in order to be used to validate the IMER as a screening method for HsPNP ligands.

2. Materials and methods

2.1. Reagents and chemicals

All chemicals were analytical or reagent grade and were used without further purification. Ino and Hypo were purchased from Sigma (St. Louis, USA). The DI4G was synthesized based on a previously described procedure [28], and solubilized in dimethyl sulfoxide (DMSO). HsPNP expression and purification were conducted as reported elsewhere [29].

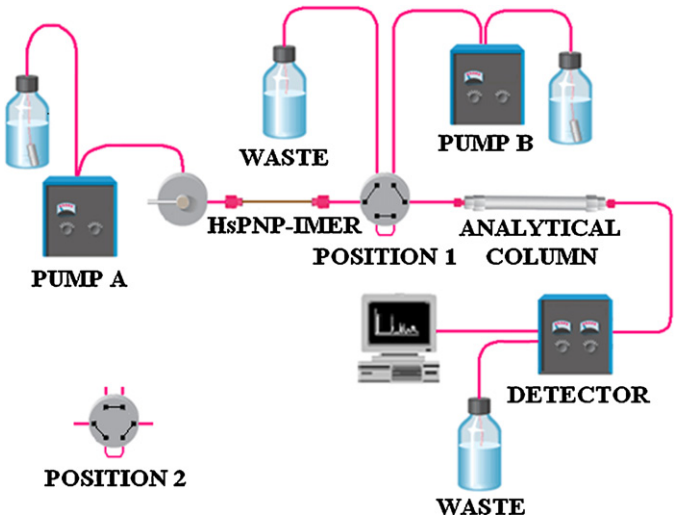

Fig. 1. Schematic diagram of the column switching system. Position 1: uncoupled columns. Position 2: coupled columns. Conditions as described in Table 1.

Buffer component and all the chemical materials used during the immobilization procedure were of analytical grade and were supplied by Sigma, Merck (Darmstadt, Germany), Synth (São Paulo, Brazil), or Acros (Geel, Belgium). Water purified in a Milli-Q system (Millipore, São Paulo, Brazil) was employed in all experiments. The silica-fused capillary (0.375 mm × 100 μm I.D.) used for enzyme immobilization and IMERs preparation was acquired from Polymicro Technologies (Phoenix, USA). Before being used on LC analysis, the buffer solutions were filtered on cellulose nitrate membranes (0.45 μm) provided by Phenomenex. Stock solutions of the evaluated inhibitors were prepared in water/methanol (1 mM) or DMSO (10 mM) and diluted in water to give a concentration lying in the 0.01–100 μM range.

2.2. Apparatus

The solution assays were carried out in an UV-2550 UV–vis spectrophotometer (Shimadzu, Kyoto, Japan).

Enzyme immobilization was accomplished using a syringe-pump 341B (Sage Instruments, Boston, USA).

The chromatographic experiments were performed using a Shimadzu LC system (Shimadzu, Kyoto, Japan), consisting of two LC 10 AD VP pumps, one of which had a FCV-10AL valve for solvent selection, an UV–vis detector (SPD-M10AV VP), and an autosampler equipment with a 500 μL loop (SIL 10 AD VP). The column containing the immobilized HsPNP enzyme (HsPNP-IMER) was coupled on line to an octyl silica column (Luna Phenomenex®, 10 nm, 10 μm, 10 cm × 0.46 mm I.D.). A three-way switching sample-valve Valco Nitronic 7000 EA (Supelco, St. Louis, USA) was employed to connect the two columns as depicted in Fig. 1. Data acquisition was accomplished on a Shimadzu SCL 10 AVP system interfaced with a computer equipped with a LC Solutions (v. 2.1) software (Shimadzu, Kyoto, Japan).

2.3. Enzyme immobilization

Enzyme immobilization adopted procedure was the same previously described for GAPDH enzyme [26]. Briefly, by means of a syringe pump working at a 130 μL min^{-1} flow rate, the fused silica

Table 1
The LC conditions used in the separation between Hypo and Ino.

Pump (eluent)	Time (min)	Event	Valve position
1 (A)	0.0–1.2	Analytes elution	1
2 (B)	0.0–1.2	Conditioning of the analytical column	1
1 (A)	1.2–8.5	Transference of the analytes from the IMER to the analytical column	2
1 (A)	8.5–20	HsPNP-IMER conditioning	1
2 (B)	8.5–20	Separation of Hypo and Ino by the analytical column	1

Pump 1: flow rate 0.05 mL min^{-1}, eluent A Tris–HCl 100 mM pH 7.0; pump 2: flow rate 0.5 mL min^{-1}, eluent B: 1% (v/v) solution of triethylamine, pH 6.0 (acidified with acetic acid): MeOH (95:5, v/v)]. Detection at 260 nm.

capillary tube (30 cm × 100 μm I.D.) was cleaned by washing with 2.0 mL 2.0 M HCl solution, followed by 1.0 mL distilled water. After rinsing, the capillary was dried in an oven at 95 °C for at least 1 h. Then, 1.0 mL 3-aminopropyltriethoxysilane (10%, v/v) solution in water was passed through the capillary, which was subsequently placed in an oven at 95 °C for 30 min. The capillary was stored overnight at room temperature.

A glutaraldehyde solution 1% (v/v) in 50 mM phosphate buffer, pH 7.0 (2.0 mL) was passed through the aminopropylsilica (APS) capillary with the aid of a syringe pump operating at a 130 μL min^{-1} flow rate. Then, the capillary tubing was rinsed with the same phosphate buffer (0.5 mL at 130 μL min^{-1}) and then, HsPNP enzyme (0.2 mL at 1.6 mg mL^{-1}) in the storage solution (85% ammonium sulfate solution) was passed twice through the capillary. The HsPNP-IMERs were kept at 4 °C with the two ends of the capillary tubing immersed in 50 mM phosphate buffer, pH 7.0, whenever not in use.

2.4. Chromatographic conditions

The analytical columns were packed by the ascending slurry method, using methanol for slurry preparation (50 mL) and for packing. Packing was carried out at a pressure of 7500 p.s.i. as described elsewhere [30,31]. Afterwards, the columns were conditioned with methanol at a 1.0 mL min^{-1} flow rate for 12 h. The highest selectivity between Ino and Hypo was obtained using the octyl column and a 1% (v/v) solution of triethylamine, pH 6.0 (acidified with acetic acid)/MeOH (95:5, v/v) as mobile phase, at a 0.5 mL min^{-1} flow rate.

The flow rate used for the HsPNP-IMER was 0.05 mL min^{-1}. This flow rate was selected on the basis of efficient catalytic activity of others IMERS prepared by our group [25–27]. The buffer used as mobile phase in the HsPNP-IMER was 100 mM Tris–HCl, pH 7.0. The time-width for transferring the enzyme reaction products from the HsPNP-IMER to the analytical columns was evaluated by injecting duplicate aliquots of a 20 μL solution containing Ino (500 μM) and Hypo (500 μM).

Hypo and Ino were chromatographically separated using a multidimensional chromatography system [32], in which the HsPNP-IMER was placed, at room temperature, at the first dimension and coupled to the analytical octyl silica column (Luna Phenomenex®, 100 Å, 10 μm, 10 cm × 0.46 mm I.D.) by a three-way switching sample-valve (Fig. 1). The chromatographic conditions are listed in Table 1.

2.5. Method validation

The Hypo calibration curve was obtained using appropriate standard Hypo solutions. Sample solutions were prepared in triplicates at the following concentrations: 2.5, 5.0, 10, 20, 40, 80, 100, 200, and 400 μM. To prepare these solutions, 50 μL aliquots of the appropriate standard Hypo solution were added to 50 μL of 100 mM Tris–HCl, pH 7.0. The solutions were vortex mixed for 10 s for homogenization and 90 μL aliquots were transferred to an autoinjector vial. 20 μL samples were injected into the LC system having an empty fused silica capillary at the first dimension. Hypo calibration curves were constructed by plotting the peak area against the injected Hypo concentration.

The intra- and inter-day precision and accuracy of the method were evaluated by analyzing quality control samples at three different concentrations, namely 3.0, 160, and 300 μM. To this end, five samples of each concentration were prepared and analyzed on three non-consecutive days. The acceptance criteria for the limit of quantification were that the precision of three samples should be under 20% variability, while the limit of detection was calculated taking a signal-to-noise ratio of 3. The selectivity of the method was assessed by blank (Tris–HCl buffer) injection [33].

2.6. Kinetic studies

2.6.1. Free enzyme solution assays

All enzyme activity assays in solution were carried out under initial rate conditions at 25 °C in 50 mM Tris–HCl, pH 7.6 (500 μL total reaction volumes), and each individual datum is the average of duplicate or triplicate measurements.

Apparent steady-state kinetic parameters determination for Ino phosphorolysis to Hypo (ε = 1000 M cm^{-1} at 280 nm), in the presence of P_i and HsPNP, was spectrophotometrically monitored by a time-dependent decrease in absorbance [34–37]. The experiment was conducted by varying Ino concentration (10–800 μM) at fixed saturating P_i concentration (20 mM).

2.6.2. HsPNP-IMER kinetic assays

The HsPNP-IMER activity was directly evaluated by quantification of Hypo produced using the calibration curve. 20 μL solutions containing Ino at concentrations ranging from 1 to 2800 μM were injected, in duplicate. Two different phosphate concentrations (5 mM or 400 μM) were used to assess the effect of P_i concentration on enzyme activity.

2.7. HsPNP-IMER stability and repeatability studies

The HsPNP-IMER stability was determined by quantifying the produced Hypo via daily injections of 20 μL of a solution containing Ino 382.5 μM and 5 mM phosphate buffer, pH 7.0, which was later accomplished on a weekly basis. The analyses were carried out in triplicate. The same conditions were used to evaluate the repeatability of the enzyme immobilization procedure. In this way, the activity of six freshly prepared IMERs was assessed by quantifying Hypo in duplicate analyses.

2.8. Inhibitory potency (IC_{50}) determination

2.8.1. Inhibition assays in solution

The DI4G concentration that reduces HsPNP enzyme activity by half (IC_{50}) was determined by measuring initial rates at Ino and P_i concentrations close to their K_M values [34–36] in either

the absence or presence of DI4G (10–50 nM). The inhibition pattern was determined by measuring initial velocity rates at varying Ino concentrations, a fixed non-saturating P_i concentration, and fixed-varying inhibitor levels. The concentrations employed in the inhibition studies were as follow: varying Ino (20–1100 μM) at fixed P_i concentration (260 μM) and fixed-varying DI4G concentration (0–50 nM), and each datum is the average of duplicate. It should be pointed out that DI4G was solubilized in DMSO for all the experiments, and the effect of the latter on HsPNP enzyme activity was always taken into account.

2.8.2. HsPNP-IMER inhibition assays

The DI4G compound, firstly identified as a potent HsPNP inhibitor by the solution assays, was employed to validate the use of the HsPNP-IMER in screening and characterization of unknown ligands. The DI4G 10 mM stock solution was prepared in DMSO. The assay solutions were obtained by addition of 50 μL of a solution containing the substrates (10 mM phosphate buffer, pH 7.0 and 765 μM Ino) and different volumes of the DI4G solutions (0.05, 10, and 500 μM) in H_2O/DMSO (9:1, v/v). The final volume was completed to 100 μL with H_2O/DMSO (9:1, v/v) solution, maintaining DMSO concentration fixed in all samples. 20 μL aliquots of these samples were injected in duplicate into the HsPNP-IMER, placed at the multidimensional chromatographic system. The percentage inhibition for this compound was calculated by comparing the obtained Hypo concentration to those obtained in the absence of the inhibitor. Thus, the following expression was employed: $\%I = 100 - (C_i/C_0 \times 100)$, where C_i is the Hypo concentration obtained in the presence of the compounds, and C_0 is the Hypo concentration calculated in the absence of the evaluated compounds. An inhibition curve was constructed for this compound by plotting the percentage inhibition versus the inhibitor concentration utilized in the assay. The linear regression parameters were calculated, and the IC_{50} was extrapolated. The type of inhibition and K_i value for DI4G were determined under the same experimental conditions for three different inhibitor concentrations (30, 120, and 180 nM) with four different Ino concentrations (80, 200, 400, and 1600 μM). The phosphate concentration selected was 5 mM.

2.9. Data analysis

The kinetic parameter values and their respective standard errors were calculated by fitting the data to the appropriate equations, using the Sigma-Plot nonlinear regression function (SPSS Inc.). Hyperbolic saturation curves [38] were fitted to Eq. (1) at varying concentrations of a given substrate and a single fixed-saturating concentration of the other:

$$v = \frac{VA}{K_a + A} \quad (1)$$

In Eq. (1), v is the measured reaction velocity, V is the maximal velocity, A is the substrate concentration (either Ino or P_i), and K_a is the substrate's Michaelis constant.

Data obtained in the inhibition studies were properly fitted to Eq. (2), which describes a competitive inhibition, where I is the inhibitor concentration and K_{is} is the slope inhibition constant.

$$v = \frac{VA}{K_a(1 + I/K_{is}) + A} \quad (2)$$

3. Results and discussion

3.1. Multidimensional on-line HsPNP-IMER chromatographic system

In 1947, Kalckar [16] published the most cited and used method for PNP activity assay [13]. This coupled assay uses XOx to produce uric acid from Hypo released by Ino phosphorolysis. The employment of a coupled assay has been justified on the proximity basis of the absorbance maximum of Ino and Hypo (at 249 and 252 nm, respectively). Uric acid absorbs at 293 nm and thus, can be spectrophotometrically monitored. The main drawback of this assay is that one needs to evaluate selectivity between PNP and XOx on searching for potential ligands/inhibitors of PNP [13]. A number of different analytical procedures have been described for measuring PNP activity [13,29]. However, a direct method for measuring quantitatively the production of Hypo is still necessary. In this work, a multidimensional LC direct assay for assessing PNP activity was developed using immobilized HsPNP enzyme as a target for drug development in high-throughput drug screening.

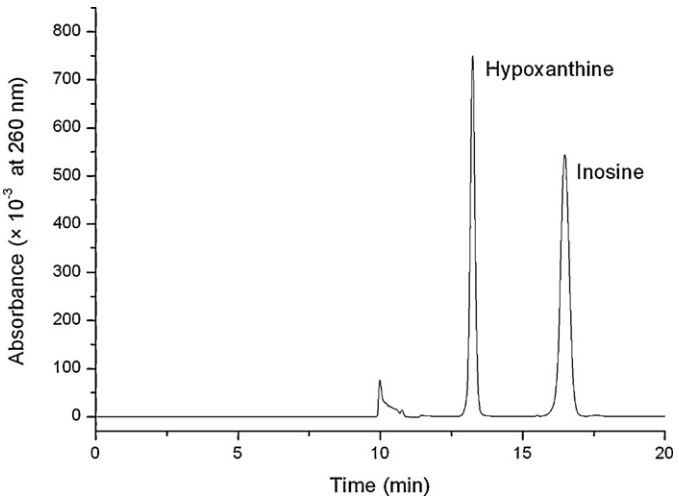

Fig. 2. Hypo and Ino chromatographic separation. Conditions as depicted in Table 1.

Due to the lack of selectivity by the IMER, a multidimensional chromatography system was assembled by coupling an octyl silica column to the HsPNP-IMER [32]. This was necessary in order to obtain the chromatographic separation of Ino and Hypo, the optimized chromatographic conditions are summarized in Table 1. The column switching system employed in this study is illustrated in Fig. 1.

A representative chromatogram of the separation of Hypo and Ino is shown in Fig. 2.

In the optimized conditions, the retention factor (k) for Hypo and Ino were, respectively, 0.15 and 0.43, with a separation factor (α) of 2.86 and resolution (R_s) of 2.67. A linear relationship was established for the injected concentration versus the peak area ($y = 1.18 \times 10^3 + 7.25 \times 10^3 x$, $r = 0.999$) using Hypo solutions with concentrations ranging from 2.5 to 400 μM.

Inter- and intra-day variability were determined by relative standard deviation (RSD) as ranging from 0.28 to 3.21%, with accuracy values between 86.4 and 103%. The limit of quantification was of 2.5 μM (RSD = 2.43% with 86.4% accuracy), while the limit of detection was of 0.5 μM.

3.2. Kinetic studies with the free and immobilized enzymes

The Ino kinetic curves were obtained by varying Ino concentration while keeping the phosphate concentration constant. For the tested concentration range, the curves were best fitted to a Michaelis–Menten hyperbolic function (data not shown).

For the free enzyme (in solution), the apparent steady-state kinetic parameters for Ino were $k_{cat} = 43.2 \pm 1.65\ s^{-1}$ and $K_M = 133 \pm 14.9$ μM, and the catalytic efficiency was $k_{cat}/K_M = 3.2 \pm 0.4 \times 10^5\ M\ s^{-1}$. To pave the way for the HsPNP-IMER assay, the kinetic studies with the free enzyme were monitored

by the change in the UV spectrum at 280 nm [34–36]. Since the k_{cat} value found by this approach is similar to the one reported for human erythrocyte PNP [39] using the traditional Ino phosphorolysis XOx coupled assay, we chose to use the first approach, as it seems more appropriate here.

The dependence of phosphorolysis [13], as well as of ligand affinity [40] by PNPs on P_i has been previously exploited, and supported the evaluation of P_i concentration effect on HsPNP-IMER activity. The kinetic studies for the Ino substrate were carried out at two different phosphate concentrations: 5 mM and 400 μM. The obtained K_M values were 255 ± 29.2 μM and 610 ± 123 μM, respectively. Since the results evidenced that the catalytic efficiency of the HsPNP-IMER is higher at high phosphate concentration, 5 mM phosphate was used in all subsequent experiments. The comparison between the K_M values obtained for free and immobilized enzymes revealed that the K_M for the HsPNP-IMER is about 1.9 larger than the one obtained from the free enzyme assay. However, it must be noticed that in the IMER phosphorolysis occurs on flow, and that the contact time between the enzyme and the substrate is shorter. In addition, the immobilization procedure might affect the quaternary structure, flexibility of the dynamic domains, and accessibility to the substrate binding sites [27,41]. These results, however, represent a crucial step on the development of a model system for rapid evaluation of thermodynamic and kinetic constants.

3.3. HsPNP-IMER stability and repeatability assays

In order to determine the HsPNP-IMER stability over time, Hypo production was monitored as described in materials and methods. The immobilized enzyme retained its activity for nearly two months, making its use feasible for a number of different assays.

The repeatability of the immobilization procedure used was assessed by measuring, in duplicate, the initial activity of six different freshly prepared IMERs. The relative standard deviation for the initial activity of the six IMERs (average of the produced Hypo 65.9 ± 10.2 μM and RSD = 15.4%, n = 6) demonstrates the robustness of the immobilization method.

3.4. Inhibition studies

The determination of transition-state structures for PNP-catalyzed phosphorolysis has contributed to the synthesis of second-generation immucillin derivatives, with dissociation constants in the pico-molar range [42]. Subsequently, a series of acyclic immucillin analogues were synthesized, which displayed a dissociation constant similar to those of first-generation immucillins [28]. The latter derivatives had a simple synthetic pathway and were named third-generation PNP inhibitors [43]. More recently, a fourth generation transition-state analogues have been produced [3].

Inhibition studies carried out in solution with a fourth-generation immucillin derivative DI4G (Fig. 3) allowed the identification of a new and potent HsPNP ligand/inhibitor.

Data from DI4G inhibition studies conducted in solution were fitted to Lineweaver–Burk double reciprocal plots (Fig. 4A), and the results demonstrated that DI4G is a competitive inhibitor of HsPNP with respect to Ino substrate, yielding a K_i value of 11.8 ± 1.47 nM. An IC_{50} value of 40.6 ± 0.36 nM was determined using substrate concentrations near to the K_M values for HsPNP.

Thus, DI4G was used to validate the HsPNP-IMER as a screening method to HsPNP ligands. The IC_{50} obtained with the IMER was 119 ± 11.5 nM, as expected, an IC_{50} of higher value than the one of the free enzyme assay. Even though, it demonstrates the use of the HsPNP-IMER for ligand recognition assay, as well as the dose–response inhibition pattern. In agreement with the free enzyme assay, results from the Lineweaver–Burk plots for the

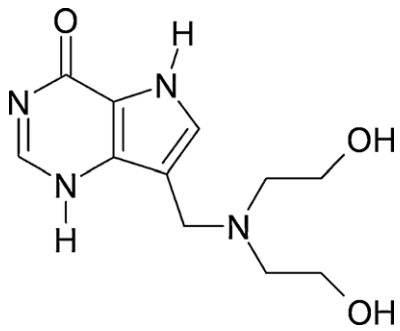

Fig. 3. Structure of the synthesized fourth-generation immucillin derivative (DI4G).

HsPNP-IMER data (Fig. 4B) indicated that HsPNP inhibition by DI4G is competitive with respect to Ino, giving a K_i = 64.3 ± 0.26 nM. The same considerations discussed at topic 3.2, regarding the differences between the K_M for the free and immobilized enzyme, should be brought back to mind while confronting the differences in the K_i values by the two models.

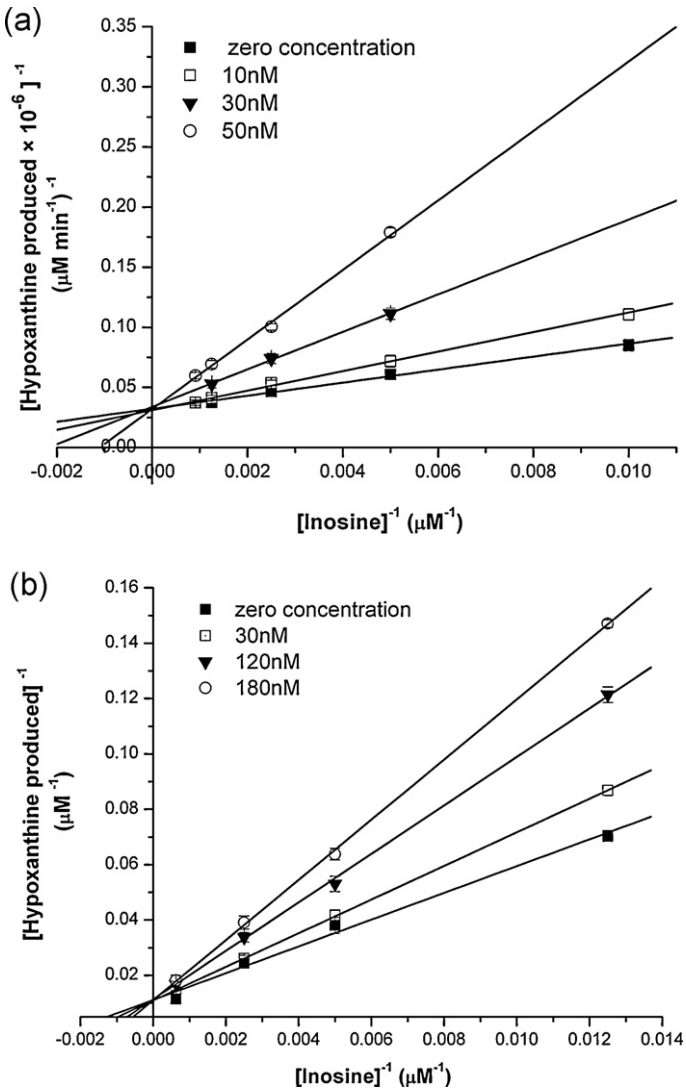

Fig. 4. Double-reciprocal plots of competitive inhibition of human purine nucleoside phosphorylase by DI4G with respect to hypoxanthine formation. (A) Inhibition pattern for the free enzyme assay (n = 2). (B) Inhibition pattern for the HsPNP-IMER assay, which are in agreement with those achieved with the free enzyme assay (n = 2). The concentrations of the inhibitor DI4G are indicated in the figures.

4. Conclusions

The direct quantification of Hypo produced by the HsPNP-IMER in a multidimensional chromatography system provides an easy and reliable tool for the identification and characterization of new ligands. In addition, this method allows the use of the same enzyme in numerous assays, besides requiring only a tiny amount of the evaluated compound. The short analysis time and the specificity and robustness of the developed IMER assay, associated with the use of an autosampler, enable screening of approximately one hundred compounds per day, by means of an automated system. Therefore, the method here reported can be described as a valuable asset in the search of new ligands from synthetic and natural products, it also represents a valuable tool for drug discovery research.

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