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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].

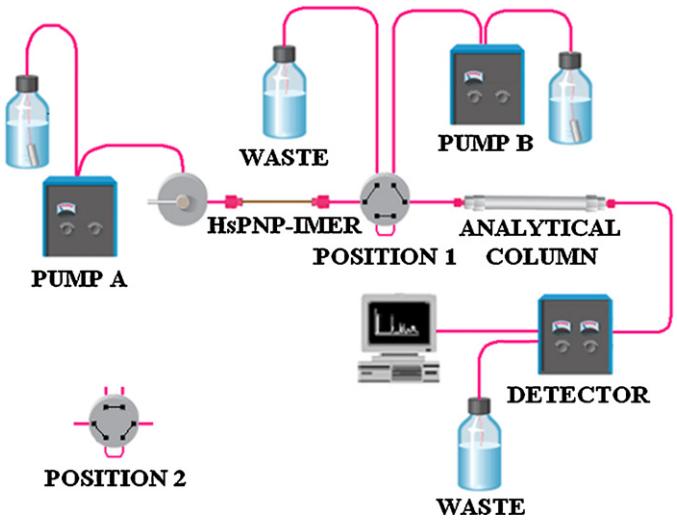

Fig. 1. Schematic diagram of the column switching system. Position 1: uncoupled columns. Position 2: coupled columns. Conditions as described in Table 1.

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