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Short communication

Development and characterization of immobilized human organic anion transporter-based liquid chromatographic stationary phase: hOAT1 and hOAT2

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Abstract

This paper reports the development of liquid chromatographic columns containing immobilized organic anion transporters (hOAT1 and hOAT2). Cellular membrane fragments from MDCK cells expressing hOAT1 and S2 cells expressing hOAT2 were immobilized on the surface of the immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The resulting stationary phases were characterized by frontal affinity chromatography, using the marker ligand [^{3}H]-adefovir for the hOAT1 and [^{14}C]-*p*-aminohippurate for the hOAT2 in the presence of multiple displacers. The determined binding affinities (K_d) for eight OAT1 ligands and eight OAT2 ligands were correlated with literature values and a statistically significant correlation was obtained for both the hOAT1 and hOAT2 columns: $r^2 = 0.688$ ($p < 0.05$) and $r^2 = 0.9967$ ($p < 0.0001$), respectively. The results indicate that the OAT1 and OAT2 have been successfully immobilized with retention of their binding activity. The use of these columns to identify ligands to the respective transporters will be presented.
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Keywords: hOAT1; hOAT2; Drug transporters; Affinity chromatography

1. Introduction

Transport proteins are found in the liver, kidney and intestine and play an essential role in the absorption and excretion of endogenous and exogenous compounds. These proteins have become a target in drug development and drug discovery [1]. The solute carrier (SLC) family contains 298 members in humans of which the majority are highly specific [2]. However, some of the families are polyspecific, including SLC 21 and SLC 22.

The SLC 22 superfamily has 12 members in humans, these include organic cation transporters, carnitine transporters and several organic anion transporters (OATs). Several families of multispecific organic anion transporters have recently been identified by molecular cloning, including hOAT1 [3], hOAT2 [4], hOAT3 [5] and hOAT4 [6]. Among the OAT families, hOAT1 (*SLC22A6*), hOAT3 (*SLC22A8*) and hOAT4 (*SLC22A11*) are predominantly expressed in the human kidney, whereas hOAT2 (*SLC22A7)* is highly expressed in the human liver and weakly in the human kidney [7,8].

OATs play an important role in the distribution and excretion of endogenous (cyclic nucleotides and dicarboxylates) and exogenous (mycotoxins, sulfate, glucoronide and glycine conjugates) drugs, as they are typically found at boundary epithelia [1,9,10]. They are predominantly anion exchangers and in the kidney, for example, are functionally coupled to sodium driven mono- and di-carboxylate transporters [1]. It has been reported that in some cases interactions with the OATs may result in pharmacokinetic drug–drug interactions or nephrotoxicity [11,12].

While OATs are multispecific, the subtypes appear to have different selectivities. The hOAT1 contributes to uptake of a

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range of small organic anions across the basolateral membrane of the renal proximal tubule and drives their urinary elimination. Several recent studies indicated that the OAT1 is involved in the tubular secretion of many important therapeutics such as β-lactam antibiotics, nonsteroidal anti-inflammatory drugs and antiviral nucleotide analogs [12–14].

Identified hOAT2 substrates include methotrexate, prostaglandin E2, cAMP, azidodeoxythymidine (AZT) and tetracycline [15–17]. However, there is still limited data concerning the substrate selectivity and transport mechanism of hOAT2.

The measurement of the binding affinities of OAT substrates or inhibitors is a key step in the study of transport mechanism and in the identification of drug–transporter interactions. Currently, OAT affinities are measured by cellular uptake studies, which are used to determine IC_{50} and K_is values [11]. Although this method provides reliable results, it is time consuming.

This laboratory previously developed an alternative method for the study of binding interactions between compounds and receptors or drug transporters [18,19]. This approach is based upon liquid chromatography utilizing stationary phases containing immobilized membranes from cells expressing the target protein. For example, binding to the human organic cation transporter (hOCT1) has been studied using a column created using membranes from a cell line expressing hOCT1. The membranes were immobilized on an immobilized artificial membrane (IAM) liquid chromatographic stationary phase. The OCT1-IAM stationary phases were used in frontal affinity chromatography studies to determine the binding affinities (K_d values) of OCT substrates and inhibitors and were able to identify competitive and enantioselective interactions between ligands and the OCT1 transporter.

In the current study, this experimental approach has been extended to the development of hOAT1-IAM and hOAT2-IAM stationary phases. The columns were prepared using MDCK (canine kidney) cells stably transfected with hOAT1 [20] and S2 (mouse kidney) cells stably transfected with hOAT2 [21]. Columns were prepared from both stationary phases and tested to determine the binding activity and specificity of the immobilized membranes containing hOAT1 and hOAT2. The columns were characterized using frontal displacement chromatography with [^{3}H]-adefovir for hOAT1 [12,11] and [^{14}C]-PAH for hOAT2 [4] as the marker ligand and known hOAT1 and hOAT2 ligands as the displacers. The results demonstrate that the hOAT1 and hOAT2 membranes were successfully immobilized on the IAM stationary phase and they retained the ability to specifically bind known ligands from which K_d values could be determined.

2. Experimental

2.1. Materials

Glutarate, *p*-aminohippurate (PAH), probenecid, indomethacin, 3′-azido-3′-deoxythymidine (AZT), ketoprofen, methotrexate (MTX), diclofenac, mefenamic acid, cholate, leupeptin, phenyl methyl sulfonyl fluoride (PMSF), EDTA, Trizma, glycerol, pepstatin, sodium chloride, $MgSO_4$, $CaCl_2$, and 6-carboxyfluorescein were purchased from Sigma (St. Louis, MO, USA). Ammonium acetate and 0.1 M ammonium hydroxide solution were purchased from Fisher Scientific (Pittsburgh, PA, USA). 9-(2-Phosphonylmethoxyethyl) adenine ([2,8-^{3}H] adefovir; 10 Ci/mmol) and adefovir were purchased from Moravek Biochemicals (Brea, CA, USA). [^{14}C]-PAH was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). Minimum essential medium with Earle's salts and L-glutamine (MEM), fetal bovine serum (FBS) and geneticin were purchased from Gibco (Carlsbad, CA, USA). RITC 80-7 medium was kindly provided by Dr. Naohiko Anzai (Kyorin University, Tokyo, Japan). Immobilized artificial membrane stationary phase (IAM-PC, 12 μm particle size, 300 Å pore size) was purchased from Regis Technologies Inc. (Morton Grove, IL, USA). HR 5/2 glass columns were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.2. Methods

2.2.1. Cell lines

The hOAT1 membranes were obtained from a previously described hOAT1-MDCK cell line were maintained as previously described [20]. In brief, cells were maintained in EMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum, 1 mM Na-pyruvate and 200 μg/ml geneticin (G418) in a humidified incubator at 37 °C with 95% O_2 and 5% CO_2. Cells were split 1:10 every 7 days and were subcultured into new flask.

The hOAT2 membranes were obtained from S2 cells stably expressing hOAT2 and established as described previously [22]. In brief, cells were grown in RITC 80-7 medium containing 5% fetal bovine serum, 10 μg/ml transferrin, 0.08 U/ml insulin, 10 ng/ml recombinant epidermal growth factor and 400 μg/ml geneticin in a humidified incubator at 33 °C and 5% CO_2 atmosphere. The cells were subcultured in a medium containing 0.05% trypsin–EDTA solution.

2.2.2. Preparation of the hOAT1-IAM and hOAT2-IAM columns

The hOAT1-MDCK cells and hOAT2-S2 cells (100×10^6 cells) were placed in 10 ml of homogenization buffer (Tris–HCl [50 mM, pH 7.4] containing 50 mM NaCl, 8 μM leupeptin, 10 μM PMSF and 8 μM pepstatin). The suspension was homogenized for 3×10 s at the setting of 12.5 on a Model PT-2100 homogenizer (Kinematica AG, Luzern, Switzerland). The homogenate was centrifuged at 27,000 rpm for 35 min at 4 °C and the resulting pellet containing the cellular membranes was collected and resuspended in 10 ml of solubilization buffer (Tris–HCl [50 mM, pH 7.4], containing 2% cholate, 50 mM NaCl, 10 μM PMSF, 8 μM pepstatin A, 10 μM leupeptin, 10 μM aprotinin and 10% glycerol). The resulting mixture was rotated at 150 rpm using an orbit shaker (Lab-line Model 3520, Melrose Park, IL, USA) for 18 h at 4 °C and then it was centrifuged at 20,000 × rpm for 22 min. Subsequently, 160 mg of the IAM stationary phase was suspended in the supernatant containing hOAT1-cholate solution, and the resulting mixture was rotated at room temperature for 3 h at 150 rpm using an orbit shaker