International Journal of Pharmaceutics 388 (2010) 168–174

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Transport characteristics of mouse concentrative nucleoside transporter 1

Miho Niitani, Kentaro Nishida, Hiroto Okuda, Katsuhito Nagai, Sadaki Fujimoto, Kazuki Nagasawa*

Department of Environmental Biochemistry, Kyoto Pharmaceutical University, 5 Nakauchi-cho, Misasagi, Yamashina-ku, Kyoto 607-8414, Japan

ARTICLE INFO

Article history:
Received 17 October 2009
Received in revised form
18 December 2009
Accepted 24 December 2009
Available online 7 January 2010

Keywords:
Concentrative nucleoside transporter 1
Mouse
Substrate specificity
Nucleoside analogue

ABSTRACT

Concentrative nucleoside transporter 1 (CNT1, SLC28A1) is a key molecule for determining the pharmacokinetic/pharmacodynamic profile of a candidate compound derived from a pyrimidine nucleoside, but there is no available information on the differences in the functional profile of this ortholog between man and mouse. Here, using a clone of mouse CNT1 (mCNT1), we investigated its transport characteristics and substrate specificity for synthetic nucleoside analogues, and compared them with those of human CNT1 (hCNT1). In mCNT1-transfected Cos-7 cells, pyrimidine, but not purine, nucleosides showed sodium- and concentration-dependent uptake, and uridine uptake was competitively inhibited by uridine analogues, the rank order of the inhibitory effects being 5-bromouridine > 3′-deoxyuridine > 2′-deoxyuridine. *cis*- and *trans*-Inhibition studies involving synthetic nucleoside drugs revealed that gemcitabine and zidovudine greatly inhibited [^{3}H]uridine uptake mediated by mCNT1 in the both cases, while cytarabine and zalcitabine showed small *cis*-inhibitory effect, and no *trans*-inhibitory effect on the uptake. These results demonstrate that the transport characteristics of mCNT1 are almost the same as those of hCNT1, suggesting that mice may be a good animal model in evaluation of pyrimidine nucleoside analogues as to their applicability in human therapy.

1. Introduction

Nucleosides play a critical role in the maintenance of cellular physiology. They are hydrophilic compounds and thus are taken up into cells *via* specific transport systems, i.e., nucleoside transporters (NTs). Mammalian cells possess multiple NTs that are either equilibrative or concentrative, for which six isoforms have been molecularly identified (Baldwin et al., 2005; Cass et al., 1998; Ritzel et al., 2001). Concentrative NTs (CNTs) require an inwardly directed Na^+-gradient as a driving force and are classified into three isoforms based on their substrate specificity. CNT1 (SLC28A1) and CNT2 can transport both uridine and adenosine, but prefer pyrimidine and purine nucleosides, respectively, while CNT3 is broad selective (Cass et al., 1998; Ritzel et al., 2001).

In addition to the large specificity differences among paralogs of CNTs, there are striking specificity differences among species orthologs particularly with respect to synthetic nucleoside analogues (Gerstin et al., 2002; Kong et al., 2004). Understanding the complex relationship between transporter proteins and nucleoside analogue drugs could facilitate efforts at rational drug design, and preclinical experimentation with relevant animal models, including transgenic animals. Smith et al. (2004) revealed detail characteristics of human CNT1 (hCNT1) using its gene expression system, and pyrimidine nucleosides were good substrates for hCNT1. On the other hand, rat CNT1 (rCNT1) was demonstrated to transport adenosine, a purine nucleoside, in addition to pyrimidine ones (Yao et al., 1996a,b). Thus there is a critical species difference in substrate specificity between hCNT1 and rCNT1. Transport characteristics of mouse CNT1 (mCNT1) have been extensively investigated using intact cultured cells (Kato et al., 2005; Peng et al., 2005; Soler et al., 2001). Recently, mCNT1 has been molecularly identified (GenBank accession no. BC061230) (Strausberg et al., 2002), and the proteins are speculated to consist of 649 amino acids with 13 trans-membrane domains (TMDs) as the same as human CNT1 (hCNT1) (Gray et al., 2004), but there is no detailed information on the substrate specificity of mCNT1.

In this study, we elucidated transport characteristics for naturally occurring nucleosides and synthetic nucleoside analogues (Fig. 1) in mCNT1-transfected Cos-7 cells, and compared them with those of hCNT1.

2. Materials and methods

2.1. Chemicals

NBMPR (nitrobenzylmercaptopurine riboside) and uridine were purchased from Sigma Chemical Co. (MO, USA), and adenosine, cytarabine (Ara-C), cytidine, 2′-deoxyuridine, guanosine, inosine, thymidine, zalcitabine (ddC), and zidovudine (AZT) were from Wako Pure Chemical Ind. (Osaka, Japan). Gemcitabine was a gift

* Corresponding author. Tel.: +81 75 595 4648; fax: +81 75 595 4756.
E-mail address: nagasawa@mb.kyoto-phu.ac.jp (K. Nagasawa).

0378-5173/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.12.057

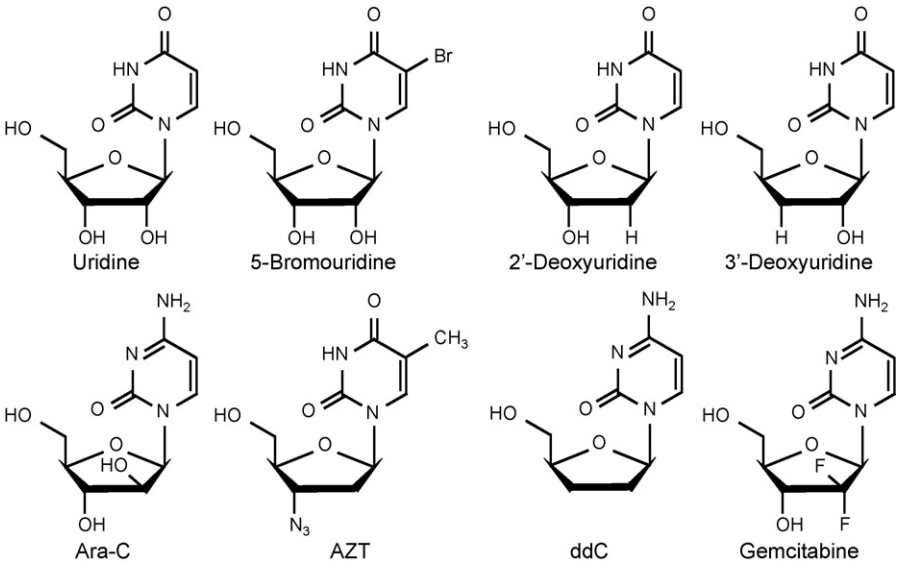

Fig. 1. Chemical structures of uridine analogues used.

from Eli Lilly Inc. (Scarborough, Ontario, Canada). 3′-Deoxyuridine and 5-bromouridine were obtained from Toronto Research Chemicals, Inc. (North York, Ontario, Canada) and Tokyo Chemical Ind. (Tokyo, Japan), respectively.

[^{3}H]Adenosine ([2,8-^{3}H]adenosine, 30 Ci/mmol), [^{3}H]cytidine ([5,6-^{3}H]cytidine, 20 Ci/mmol), [^{3}H]guanosine ([8-^{3}H]guanosine, 15 Ci/mmol), [^{3}H]inosine ([8-^{3}H]inosine, 20 Ci/mmol), [^{3}H]thymidine ([6-^{3}H]thymidine, 24 Ci/mmol), and [^{3}H]uridine ([5,6-^{3}H]uridine, 40 Ci/mmol) were purchased from American Radiolabeled Chemicals Inc. (MO, USA).

2.2. Cell culture

Cos-7 cells were maintained in Dulbecco's modified Eagle's MEM (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum at 37 °C under a humidified atmosphere of 5% CO_2 in air. The density and viability (>90%) were determined by means of the trypan blue exclusion test.

2.3. Cloning of mCNT1 cDNA and generation of the transfectant

The intestines were dissected out from ddY male mice aged 5 weeks (Japan SLC Inc., Shizuoka, Japan). Total RNA was extracted with Sepasol RNA-I super (Nacalai Tesque, Kyoto, Japan), and then purified with a GenElute™ Mammalian Total RNA kit (Sigma Chemical Co.) according to the manufacturer's instruction manual. Each total RNA was reverse transcribed into cDNA by means of Oligo-T priming and Moloney murine leukemia virus reverse transcriptase. A mCNT1 clone was obtained by the amplification of cDNA derived from mouse intestines, using the polymerase chain reaction (PCR) approach with exTaq (Takara, Shiga, Japan). A 5′-primer, 5′-GCTGAAGAGCCAAGCACATG-3′, and a 3′ primer, 5′-TGGATGAGCCCTGAGGCTAGG-3′, derived from the reported sequence of CNT1 in mouse muscle (GenBank accession no. BC061230) were used. The PCR product was subcloned into the pGEM-T vector (Promega Co., WI, USA) and sequenced. The cDNA sequence of mCNT1 obtained here was identical to previously reported one (GenBank accession no. BC061230). Based on these results, the mCNT1 cDNA obtained from mouse intestines was inserted into the pCI-neo expression vector (Promega Co.) using EcoRI and SalI (Toyobo, Osaka, Japan). For transfection, the mCNT1 cDNA inserted into the pCI-neo expression vector was purified with a Wizard® plus SV Miniprep DNA purification system (Promega Co.). An appropriate quantity of the required plasmid (26.32 ng of DNA/cm^2) was diluted with 100 μL of OPTI-MEM reduced serum medium containing 2.37 nmol/cm^2 of COATSOME EL-01-D (TFL-3; NOF Co., Tokyo, Japan), followed by incubation at room temperature for 30 min. DNA–liposome complexes were added to Cos-7 cells cultured up to 80% confluence, and after 48 h culture, the cells (Cos-7/mCNT1) were used for the uptake assay. Cos-7 cells transfected with the pCI-neo expression vector were used as mock cells (Cos-7/pCI-neo).

2.4. Uptake assay

The uptake experiments were performed by the method reported previously, using HBSS (136.9 mM NaCl, 5.4 mM KCl, 1.3 mM $CaCl_2$, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 4.2 mM $NaHCO_3$, 0.06 mM phenol red, 5.56 mM D-glucose and 25 mM HEPES, pH 7.2) as the transport buffer, and in the case of Na^+-free conditions, choline-replaced HBSS (choline buffer), in which NaCl, Na_2HPO_4 and $NaHCO_3$ were replaced by choline chloride, K_2HPO_4 and $KHCO_3$, respectively, was used (Nagai et al., 2005; Nagasawa et al., 2003). After the cells had been preincubated for 5 min in an appropriate buffer, that is, in HBSS or choline-replaced HBSS in the case of the presence or absence of extracellular Na^+, respectively, containing 10 μM NBMPR to completely block the NBMPR-sensitive uptake by them, the reaction was initiated by adding the indicated concentrations of ^{3}H-labeled nucleoside (1 μCi/mL) with or without an analogue compound as an inhibitor at the designated concentrations to the preincubated cells. After appropriate time intervals, the reaction was terminated by adding an excess volume of ice-cold choline buffer containing 1 mM of each unlabeled substrate. After the cells had been extensively washed three times with an excess volume of ice-cold phosphate-buffered saline to remove the extracellular substrates, they were scrapped from the wells and lysed in water. Thereafter, the uptake by both types of cells was determined with a liquid scintillation counter, following the method of Nagasawa et al. (2003). Protein concentrations were measured by the method of Bradford (1976) with bovine serum albumin (Sigma Chemicals Co.) as the standard.