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

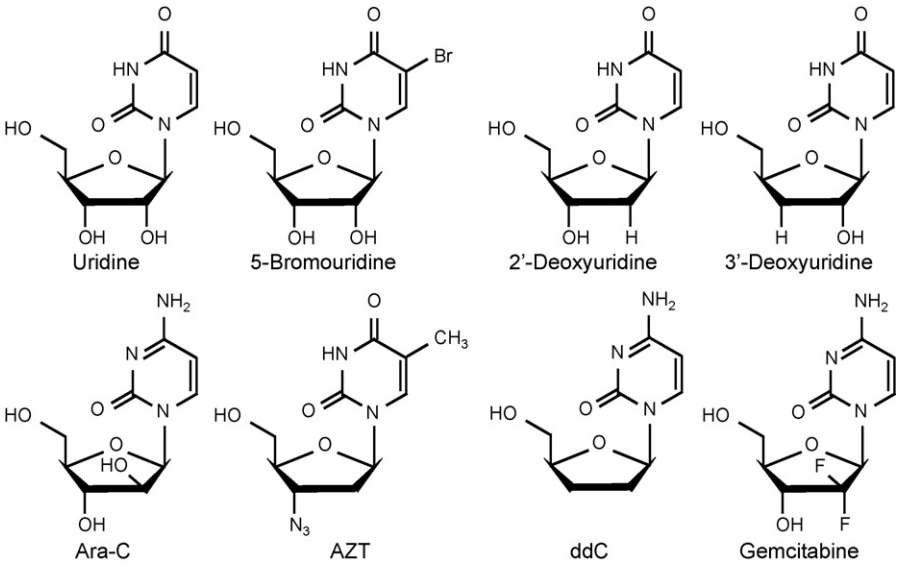

Fig. 1. Chemical structures of uridine analogues used.

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.

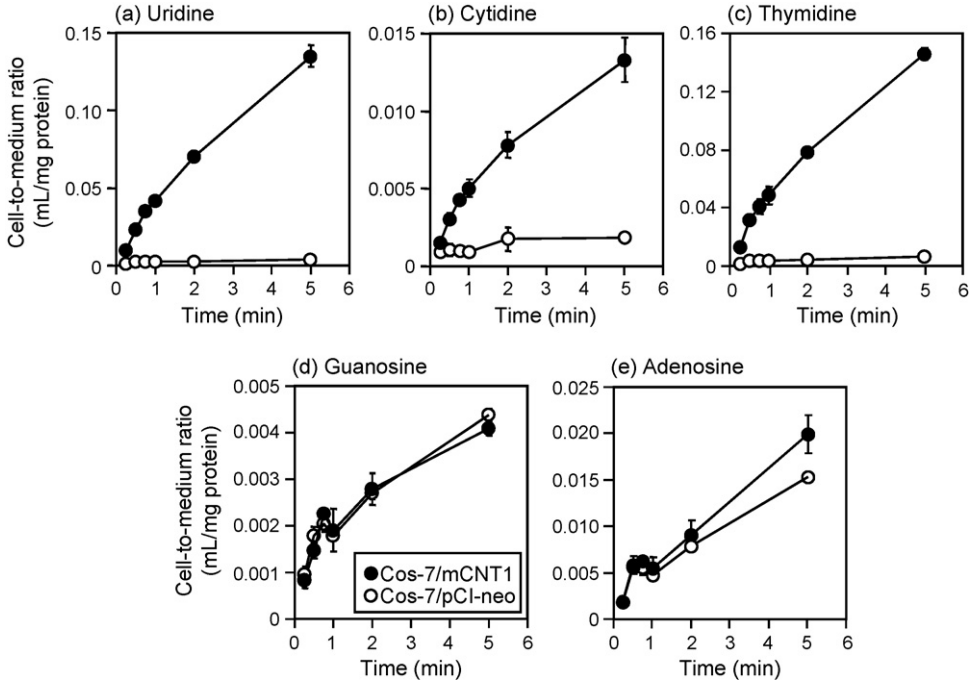

Fig. 2. Time courses of uptake of [^{3}H]nucleosides by Cos-7/mCNT1 and Cos-7/pCI-neo. After cells had been preincubated for 5 min in HEPES–HBSS containing 10 μM NBMPR, they were incubated with 33 nM [^{3}H]uridine, 50 nM [^{3}H]cytidine, 33 nM [^{3}H]thymidine, 66 nM [^{3}H]guanosine or 60 nM [^{3}H]adenosine in HEPES–HBSS for the indicated times at 37 °C. Each point represents the mean ± SD (n = 3).

2.5. Statistical analysis

The data were expressed as means ± SD. Cellular uptake was determined as the cell/medium ratio, which was obtained by dividing the uptake amount by the concentration of the substrate in the transport medium, unless otherwise stated. The Michaelis constant (K_m) and maximal transport rate (V_{max}) were calculated on the basis of the Eadie–Hofstee equation. Comparisons among groups were performed by means of analysis of variance (ANOVA, followed by Fisher's PLSD test), differences with a p-value of 0.05 or less being considered statistically significant.

3. Results

3.1. Uptake characteristics of mCNT1 for naturally occurring nucleosides

By Cos-7/mCNT1, [^{3}H]uridine, [^{3}H]cytidine and [^{3}H]thymidine, pyrimidine nucleosides, were apparently taken up into the cells, but their uptake by Cos-7/pCI-neo was negligible (Fig. 2). On the other hand, the uptake of [^{3}H]guanosine and [^{3}H]adenosine, purine nucleosides, time-dependently increased, but there was no difference in their uptake between the two cell types.

To obtain the kinetic profiles of mCNT1, we characterized the uptake of uridine, cytidine and thymidine as representative ligands by mock- and mCNT1-transfected cells. Under the conditions in the absence of extracellular Na^+, the uptake of [^{3}H]uridine, [^{3}H]cytidine and [^{3}H]thymidine by the transfectant cells decreased to the levels in the mock cells (Fig. 3). As depicted in Fig. 4, the uptake of these pyrimidine nucleosides by mCNT1 showed clear concentration dependency with saturation at high concentrations, and Eadie–Hofstee analysis gave a linear regression line, indicating the involvement of a single transport system, that is, mCNT1. The K_ms for uridine, cytidine and thymidine were all calculated to be approximately 10 μM (Table 1), this being almost comparable with the corresponding values for hCNT1 (33 ± 2, 23 ± 2 and 27 ± 2 μM, respectively) (Slugoski et al., 2007). Their V_{max}s were estimated to be 0.154, 0.0197 and 0.242 pmol/mg protein per 30 s, respectively, and the uptake clearances (V_{max}/K_ms) were 0.0145, 0.00170 and 0.0199 mL/mg protein per 30 s, respectively.

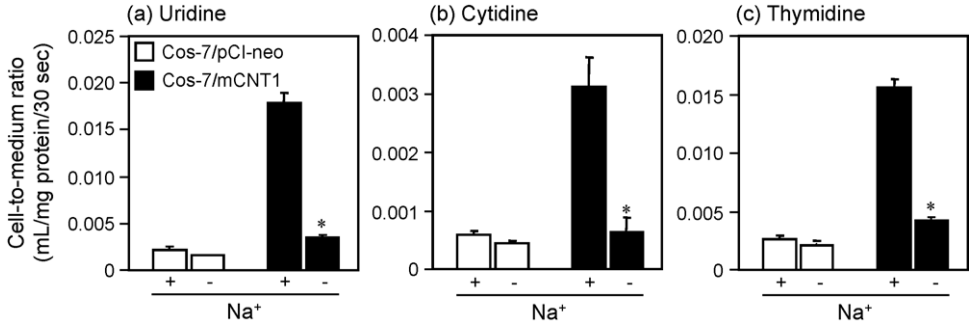

Fig. 3. Effect of extracellular Na^+ on uptake of [^{3}H]pyrimidine nucleosides by Cos-7/mCNT1 and Cos-7/pCI-neo. After cells had been preincubated in HEPES–HBSS or choline-replaced HEPES–HBSS containing 10 μM NBMPR for 5 min, they were incubated with 33 nM [^{3}H]uridine, 50 nM [^{3}H]cytidine or 33 nM [^{3}H]thymidine in an appropriate buffer for 30 s at 37 °C. Each point represents the mean ± SD (n = 3). *p < 0.01 (*vs.* each value of Na^+ (+)).

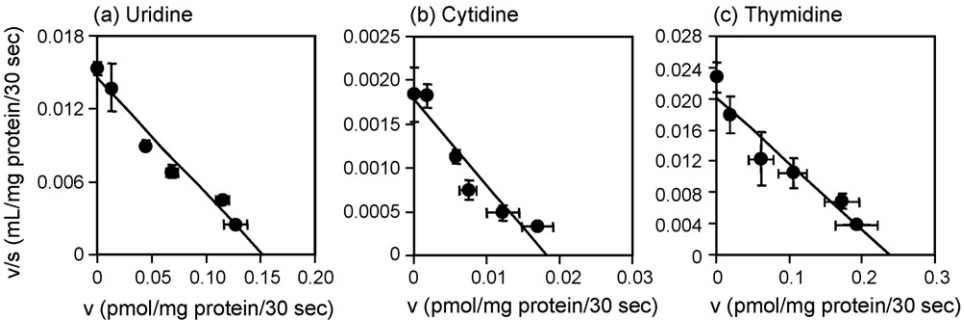

Fig. 4. Concentration dependency of uptake of [^{3}H]pyrimidine nucleosides mediated by mCNT1. After cells had been preincubated in HEPES–HBSS containing 10 μM NBMPR for 5 min, they were incubated with 0.033, 1, 5, 10, 25 or 50 μM of a [^{3}H]pyrimidine nucleoside in HEPES–HBSS for 30 s at 37 °C. The mCNT1-mediated uptake of nucleosides was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/mCNT1. Each point represents the mean ± SD (n = 3).

Table 1
Apparent kinetic constants for uptake of [^{3}H]pyrimidine nucleosides mediated by mCNT1.

Substrate	K_m (μM)	V_{max} (pmol/mg protein/30 s)	V_{max}/K_m (mL/mg protein/30 s)
Uridine	10.7 ± 1.6	0.154 ± 0.013	0.0145 ± 0.0012
Cytidine	11.5 ± 1.1	0.0197 ± 0.0035	0.00170 ± 0.00014
Thymidine	12.2 ± 0.8	0.242 ± 0.029	0.0199 ± 0.0030

Kinetic constants were calculated with the Eadie–Hofstee equation using the data shown in Fig. 4. Each value represents the mean ± SD (n = 3).

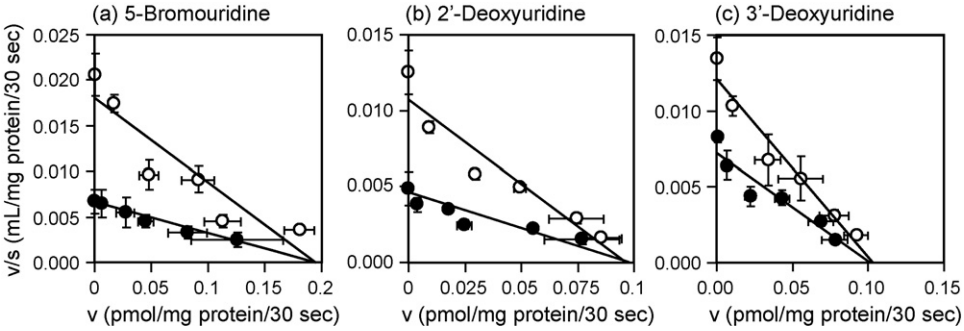

Fig. 5. *cis*-Inhibitory effects of uridine analogues on [^{3}H]uridine uptake mediated by mCNT1. After cells had been preincubated in HEPES–HBSS containing 10 μM NBMPR for 5 min, they were incubated with 0.033, 1, 5, 10, 25 or 50 μM of [^{3}H]uridine in HEPES–HBSS in the presence (closed symbols) or absence (opened symbols) of 15 μM 5-bromouridine, 50 μM 2′-deoxyuridine or 100 μM 3′-deoxyuridine for 30 s at 37 °C. The mCNT1-mediated uptake of nucleosides was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/mCNT1. Each point represents the mean ± SD (n = 3).

3.2. Substrate specificity of mCNT1 for nucleoside analogues

Using 5-bromouridine, 2′-deoxyuridine and 3′-deoxyuridine (Fig. 1), the effect of uridine analogues on uridine uptake by mCNT1 was examined. These three analogues dose-dependently decreased the [^{3}H]uridine uptake and in their Eadie–Hofstee plots, the intersection of the two linear regression lines was on the X-axis in each case (Fig. 5). This together with the finding that the K_m, but not the V_{max}, values of uridine uptake significantly increased with the addition of the analogues (Table 2) demonstrated that 5-bromouridine, 2′-deoxyuridine and 3′-deoxyuridine competitively inhibited the uridine uptake by mCNT1, the inhibition constants (K_i) being calculated to be 9.40 ± 1.72, 23.3 ± 5.59 and 161.7 ± 29.8 μM, respectively.

Fig. 6 shows their *cis*-inhibitory effects of synthetic pyrimidine analogues, Ara-C, AZT, ddC and gemcitabine, on uridine uptake by mCNT1. All of the drugs dose-dependently decreased the [^{3}H]uridine uptake. The K_m value of uridine uptake significantly

Table 2
Apparent kinetic constants for uptake of [^{3}H]uridine mediated by mCNT1 in the presence or absence of a uridine analogue.

Analogue	K_m (μM)	V_{max} (pmol/mg protein/30 s)	V_{max}/K_m (mL/mg protein/30 s)
5-Bromouridine			
–	10.8 ± 1.2	0.192 ± 0.022	0.0178 ± 0.0020
+	29.5 ± 5.0*	0.193 ± 0.061	0.00643 ± 0.00012*
2′-Deoxyuridine			
–	8.86 ± 0.65	0.0945 ± 0.0087	0.0107 ± 0.0051
+	29.7 ± 5.1*	0.110 ± 0.003	0.00373 ± 0.00072*
3′-Deoxyuridine			
–	8.91 ± 0.37	0.106 ± 0.011	0.0119 ± 0.0017
+	14.6 ± 1.8*	0.103 ± 0.001	0.00713 ± 0.00083*

Kinetic constants were calculated with the Eadie–Hofstee equation using the data shown in Fig. 5. Each value represents the mean ± SD (n = 3).
* $p < 0.05$ (*vs.* each value of uridine analogue (–)).

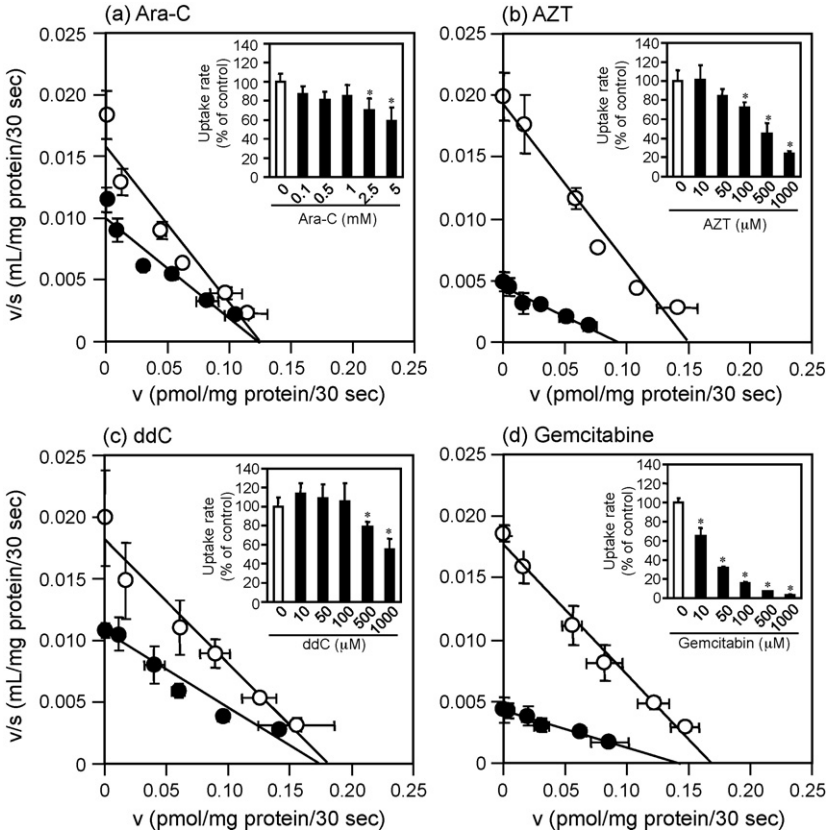

Fig. 6. *cis*-Inhibitory effects of synthetic nucleoside drugs on [^{3}H]uridine uptake mediated by mCNT1. After cells had been preincubated in HEPES–HBSS containing 10 μM NBMPR for 5 min, they were incubated with 0.033, 1, 5, 10, 25 or 50 μM of [^{3}H]uridine in HEPES–HBSS in the presence (closed symbols) or absence (opened symbols) of 5 mM Ara-C, 1 mM AZT, 1 mM ddC or 100 μM gemcitabine (Eadie–Hofstee plots) the indicated concentrations of a drug (insets) for 30 s at 37 °C. The mCNT1-mediated uptake of nucleosides was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/mCNT1. Each point represents the mean ± SD (n = 3).

increased in the presence of Ara-C, AZT, ddC and gemcitabine, while only AZT decreased the V_{max} value of uridine uptake (Table 3), implying that Ara-C, ddC and gemcitabine were competitive inhibitors of mCNT1-mediated uridine uptake, and AZT was a competitive and non-competitive inhibitor. Their inhibitory effects increased in the order of gemcitabine > AZT > ddC > Ara-C, this order corresponding with their K_is, 39.2 ± 14.0, 612 ± 225, 2308 ± 1172 and 9650 ± 3659 μM, respectively.

When Cos-7/mCNT1 cells were pre-loaded with AZT or gemcitabine, but not Ara-C or ddC, the [^{3}H]uridine uptake decreased in a dose-dependent manner, indicating AZT and gemcitabine had a *trans*-inhibitory effect on uridine uptake *via* mCNT1 (Fig. 7).

4. Discussion

When mCNT1 was expressed by Cos-7 cells, it could transport pyrimidine nucleosides Na^+-dependently (Figs. 2–4) with high affinity, the K_m values being approximately 10 μM for each of uridine, cytidine and thymidine, while adenosine, a purine nucleoside, was not a substrate of mCNT1. These findings indicate that the obtained mCNT1 is a mouse CNT1 that well-matches previously characterized hCNT1 (Slugoski et al., 2007). This is supported by the evidence that the sequences of TMDs 7–9, which are critical for the substrate specificity of CNT1 (Loewen et al., 1999), are identical between mCNT1 and hCNT1 (data not shown). On the other hand, it

Table 3
Apparent kinetic constants for uptake of [^{3}H]uridine mediated by mCNT1 in the presence or absence of a synthetic nucleoside drug.

Drug	K_m (μM)	V_{max} (pmol/mg protein/30 s)	V_{max}/K_m (mL/mg protein/30 s)
Ara-C			
–	8.19 ± 2.0	0.127 ± 0.019	0.0158 ± 0.0017
+	12.7 ± 1.5*	0.125 ± 0.007	0.100 ± 0.008*
AZT			
–	7.86 ± 0.22	0.151 ± 0.008	0.0192 ± 0.0016
+	22.0 ± 5.2*	0.0962 ± 0.0050*	0.00450 ± 0.00085*
ddC			
–	11.2 ± 2.8	0.191 ± 0.025	0.0176 ± 0.0035
+	16.5 ± 1.5*	0.172 ± 0.003	0.0105 ± 0.0012*
Gemcitabine			
–	9.74 ± 1.26	0.170 ± 0.019	0.0175 ± 0.0013
+	36.3 ± 8.5*	0.150 ± 0.016	0.00423 ± 0.00078*

Kinetic constants were calculated with the Eadie–Hofstee equation using the data shown in Fig. 6. Each value represents the mean ± SD (n = 3).
* $p < 0.05$ (*vs.* each value of drug (–)).

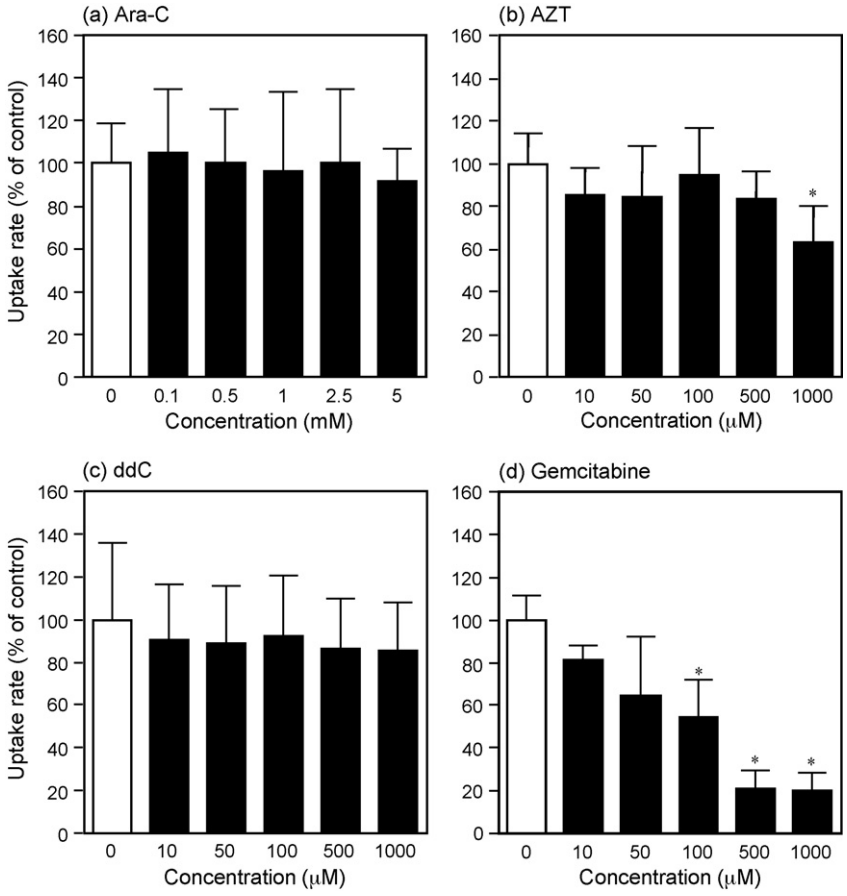

Fig. 7. *trans*-Inhibitory effects of synthetic nucleoside drugs on [^{3}H]uridine uptake mediated by mCNT1. Cells were loaded with the indicated concentrations of an analogue in HEPES–HBSS containing 10 μM NBMPR for 15 min, and then washed once with warmed HEPES–HBSS. Thereafter, they were incubated with 33 nM [^{3}H]uridine in HEPES–HBSS for 30 s at 37 °C. The mCNT1-mediated uptake of nucleosides was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/mCNT1. Each column represents the mean ± SD (n = 3).

appears that rCNT1 also has the same sequences of TMDs 7–9 with hCNT1 (Loewen et al., 1999). Thus, these results suggest that in addition to TMDs 7–9, other sequences of CNT1 may be important to determine whether adenosine is a substrate of CNT1.

To reveal the substrate specificity of mCNT1, we examined the *cis*-inhibitory effects of synthetic uridine analogues on mCNT1-mediated uridine uptake. 5-Bromouridine, 2′-deoxyuridine and 3′-deoxyuridine competitively inhibited the uridine uptake, the K_i values being 9.4, 23 and 162 μM, respectively. The K_i values of 5-bromouridine and 2′-deoxyuridine were the same as the K_m value of uridine uptake, while that of 3′-deoxyuridine was greater than the K_m value, this being the same tendency as in the case of hCNT1 (Smith et al., 2004; Zhang et al., 2005, 2003). Thus, it is suggested that the C(3′)-OH residue may play a very important role in substrate recognition by mCNT1, but that the C(5)-H and C(2′)-OH residues might not, as the case of hCNT1, as already demonstrated (Chang et al., 2004; Patil et al., 2000; Zhang et al., 2005, 2003). To obtain the direct evidence for this, transport characteristics of these analogues by mCNT1 should be assessed. On the other hand, the transport ability of mCNT1 as to pyrimidine nucleosides, as indicated by the V_{max} values, was in the order of thymidine > uridine > cytidine, and this was not the same order as in the case of hCNT1, uridine > cytidine > thymidine (Slugoski et al., 2007), suggesting different transport abilities of the species.

Using synthetic nucleoside analogues, Ara-C, AZT, ddC and gemcitabine, known as substrates for hCNT1 (Kong et al., 2004; Smith et al., 2004; Zhang et al., 2005, 2003), the substrate specificity of mCNT1 was further characterized. As depicted in Fig. 6, Ara-C, ddC and gemcitabine competitively inhibited mCNT1-mediated uridine uptake, but AZT showed mixed-type, competitive and non-competitive, inhibition of the uptake. Comparing their K_i values, the apparent affinity order for mCNT1 is gemcitabine > AZT > ddC > Ara-C, and this is supported by the previous findings that gemcitabine, AZT and ddC, and Ara-C is a high-, moderate- and low-affinity substrate of hCNT1, respectively (Graham et al., 2000; Smith et al., 2004). Furthermore, we think that these differences in the inhibition efficacy against the uridine uptake mediated by mCNT1 among the analogues might be explained by those in their *trans*-inhibitory effects, pre-loading of AZT and gemcitabine, but not Ara-C or ddC, decreasing the uridine uptake (Fig. 7). These results suggested that these synthetic nucleoside analogues were, at least in part, substrates for mCNT1 as the same as hCNT1 (Kong et al., 2004). As shown in Fig. 1, AZT and ddC are 3′-deoxy nucleoside derivatives, while gemcitabine has two fluorides and Ara-C has a stereoisomeric OH residue at the C(2′) position. Thus, it is speculated that differing from the case of uridine analogue, the C(3′)-OH did not contribute to substrate recognition by CNT1, while the residue and its stereoisomerism at the C(2′)-position might be important for determination of the affinity of cytidine and thymidine analogues for CNT1, and further detail investigations are needed to confirm this.

In this study, we characterized the transport characteristics and substrate specificity of mCNT1. The profiles on substrate specificity for naturally occurring and synthetic nucleoside analogues are almost the same as those of hCNT1, suggesting that mice may be a better animal model than rats in pharmacokinetic/pharmacodynamic profiling of pyrimidine nucleoside analogues as to their applicability in human therapy.

Acknowledgements

A part of this study was financially supported by Grant-in-Aid for Scientific Research (C) (21590107) and the Frontier Project from the Ministry of Education, Science and Culture of Japan.

References

Baldwin, S.A., Yao, S.Y., Hyde, R.J., Ng, A.M., Foppolo, S., Barnes, K., Ritzel, M.W., Cass, C.E., Young, J.D., 2005. Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. J. Biol. Chem. 280, 15880–15887.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Cass, C.E., Young, J.D., Baldwin, S.A., 1998. Recent advances in the molecular biology of nucleoside transporters of mammalian cells. Biochem. Cell Biol. 76, 761–770.

Chang, C., Swaan, P.W., Ngo, L.Y., Lum, P.Y., Patil, S.D., Unadkat, J.D., 2004. Molecular requirements of the human nucleoside transporters hCNT1, hCNT2, and hENT1. Mol. Pharmacol. 65, 558–570.

Gerstin, K.M., Dresser, M.J., Giacomini, K.M., 2002. Specificity of human and rat orthologs of the concentrative nucleoside transporter, SPNT. Am. J. Physiol. Renal Physiol. 283, F344–F349.

Graham, K.A., Leithoff, J., Coe, I.R., Mowles, D., Mackey, J.R., Young, J.D., Cass, C.E., 2000. Differential transport of cytosine-containing nucleosides by recombinant human concentrative nucleoside transporter protein hCNT1. Nucleosides Nucleotides Nucleic Acids 19, 415–434.

Gray, J.H., Owen, R.P., Giacomini, K.M., 2004. The concentrative nucleoside transporter family, SLC28. Pflugers Arch. 447, 728–734.

Kato, R., Maeda, T., Akaike, T., Tamai, I., 2005. Nucleoside transport at the blood–testis barrier studied with primary-cultured sertoli cells. J. Pharmacol. Exp. Ther. 312, 601–608.

Kong, W., Engel, K., Wang, J., 2004. Mammalian nucleoside transporters. Curr. Drug Metab. 5, 63–84.

Loewen, S.K., Ng, A.M., Yao, S.Y., Cass, C.E., Baldwin, S.A., Young, J.D., 1999. Identification of amino acid residues responsible for the pyrimidine and purine nucleoside specificities of human concentrative Na^+ nucleoside cotransporters hCNT1 and hCNT2. J. Biol. Chem. 274, 24475–24484.

Nagai, K., Nagasawa, K., Fujimoto, S., 2005. Transport mechanisms for adenosine and uridine in primary-cultured rat cortical neurons and astrocytes. Biochem. Biophys. Res. Commun. 334, 1343–1350.

Nagasawa, K., Nagai, K., Ishimoto, A., Fujimoto, S., 2003. Transport mechanism for lovastatin acid in bovine kidney NBL-1 cells: kinetic evidences imply involvement of monocarboxylate transporter 4. Int. J. Pharm. 262, 63–73.

Patil, S.D., Ngo, L.Y., Unadkat, J.D., 2000. Structure-inhibitory profiles of nucleosides for the human intestinal N1 and N2 Na^+-nucleoside transporters. Cancer Chemother. Pharmacol. 46, 394–402.

Peng, L., Huang, R., Yu, A.C., Fung, K.Y., Rathbone, M.P., Hertz, L., 2005. Nucleoside transporter expression and function in cultured mouse astrocytes. Glia 52, 25–35.

Ritzel, M.W., Ng, A.M., Yao, S.Y., Graham, K., Loewen, S.K., Smith, K.M., Ritzel, R.G., Mowles, D.A., Carpenter, P., Chen, X.Z., Karpinski, E., Hyde, R.J., Baldwin, S.A., Cass, C.E., Young, J.D., 2001. Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J. Biol. Chem. 276, 2914–2927.

Slugoski, M.D., Loewen, S.K., Ng, A.M., Smith, K.M., Yao, S.Y., Karpinski, E., Cass, C.E., Baldwin, S.A., Young, J.D., 2007. Specific mutations in transmembrane helix 8 of human concentrative Na^+/nucleoside cotransporter hCNT1 affect permeant selectivity and cation coupling. Biochemistry 46, 1684–1693.

Smith, K.M., Ng, A.M., Yao, S.Y., Labedz, K.A., Knaus, E.E., Wiebe, L.I., Cass, C.E., Baldwin, S.A., Chen, X.Z., Karpinski, E., Young, J.D., 2004. Electrophysiological characterization of a recombinant human Na^+-coupled nucleoside transporter (hCNT1) produced in *Xenopus* oocytes. J. Physiol. 558, 807–823.

Soler, C., Valdes, R., Garcia-Manteiga, J., Xaus, J., Comalada, M., Casado, F.J., Modolell, M., Nicholson, B., MacLeod, C., Felipe, A., Celada, A., Pastor-Anglada, M., 2001. Lipopolysaccharide-induced apoptosis of macrophages determines the up-regulation of concentrative nucleoside transporters Cnt1 and Cnt2 through tumor necrosis factor-alpha-dependent and -independent mechanisms. J. Biol. Chem. 276, 30043–30049.

Strausberg, R.L., Feingold, E.A., Grouse, L.H., Derge, J.G., Klausner, R.D., Collins, F.S., Wagner, L., Shenmen, C.M., Schuler, G.D., Altschul, S.F., Zeeberg, B., Buetow, K.H., Schaefer, C.F., Bhat, N.K., Hopkins, R.F., Jordan, H., Moore, T., Max, S.I., Wang, J., Hsieh, F., Diatchenko, L., Marusina, K., Farmer, A.A., Rubin, G.M., Hong, L., Stapleton, M., Soares, M.B., Bonaldo, M.F., Casavant, T.L., Scheetz, T.E., Brownstein, M.J., Usdin, T.B., Toshiyuki, S., Carninci, P., Prange, C., Raha, S.S., Loquellano, N.A., Peters, G.J., Abramson, R.D., Mullahy, S.J., Bosak, S.A., McEwan, P.J., McKernan, K.J., Malek, J.A., Gunaratne, P.H., Richards, S., Worley, K.C., Hale, S., Garcia, A.M., Gay, L.J., Hulyk, S.W., Villalon, D.K., Muzny, D.M., Sodergren, E.J., Lu, X., Gibbs, R.A., Fahey, J., Helton, E., Ketteman, M., Madan, A., Rodrigues, S., Sanchez, A., Whiting, M., Madan, A., Young, A.C., Shevchenko, Y., Bouffard, G.G., Blakesley, R.W., Touchman, J.W., Green, E.D., Dickson, M.C., Rodriguez, A.C., Grimwood, J., Schmutz, J., Myers, R.M., Butterfield, Y.S., Krzywinski, M.I., Skalska, U., Smailus, D.E., Schnerch, A., Schein, J.E., Jones, S.J., Marra, M.A., 2002. Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc. Natl. Acad. Sci. U.S.A. 99, 16899–16903.

Yao, S.Y., Cass, C.E., Young, J.D., 1996a. Transport of the antiviral nucleoside analogs 3′-azido-3′-deoxythymidine and 2′,3′-dideoxycytidine by a recombinant nucleoside transporter (rCNT) expressed in *Xenopus laevis* oocytes. Mol. Pharmacol. 50, 388–393.

Yao, S.Y., Ng, A.M., Ritzel, M.W., Gati, W.P., Cass, C.E., Young, J.D., 1996b. Transport of adenosine by recombinant purine- and pyrimidine-selective sodium/nucleoside cotransporters from rat jejunum expressed in *Xenopus laevis* oocytes. Mol. Pharmacol. 50, 1529–1535.

Zhang, J., Smith, K.M., Tackaberry, T., Visser, F., Robins, M.J., Nielsen, L.P., Nowak, I., Karpinski, E., Baldwin, S.A., Young, J.D., Cass, C.E., 2005. Uridine binding and transportability determinants of human concentrative nucleoside transporters. Mol. Pharmacol. 68, 830–839.

Zhang, J., Visser, F., Vickers, M.F., Lang, T., Robins, M.J., Nielsen, L.P., Nowak, I., Baldwin, S.A., Young, J.D., Cass, C.E., 2003. Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in *Saccharomyces cerevisiae*. Mol. Pharmacol. 64, 1512–1520.