Several mammalian nucleoside transporters have been identified at the molecular level. Human and rat equilibrative nucleoside transporter 2 (hENT2 and rENT2, respectively) was previously reported to have the dual ability of transporting both nucleosides and nucleobases. In the present study, we characterized the transport of a variety of nucleosides and nucleobases via recombinant mouse ENT2 (mENT2). Cloned mENT2 mediated the uptake of nucleosides and purine nucleobases, but not pyrimidine nucleobases. The mENT2-mediated uptake of adenosine was significantly inhibited by nucleosides and nucleobases, irrespective of purine and pyrimidine. The $K_m$ values for the uptake of nucleosides and purine nucleobases mediated by mENT2 varied between 1.24 and 16.3 μM, and the transport clearances of adenosine and hypoxanthine via the transporter were greater than those of other substrates. Therefore, we concluded that mENT2 is nucleoside and purine nucleobase transporter, and pyrimidine nucleobases are blockers for the transporter, differing from hENT2 and rENT2 that were reported to also transport pyrimidine nucleobases.

Key words nucleoside; nucleobase; nucleoside transporter; ENT2; chemotherapy

Materials and Methods

Several mammalian nucleoside transporters are important precursors for nucleotide synthesis. Individual nucleosides and nucleobases also serve a variety of specialized functions. Various structural analogues of nucleoside and nucleobase are cytotoxic and have found expanding therapeutic use as antineoplastic agents. Nucleosides and nucleobases are hydrophilic and require specific transporters for permeation through the cell membrane. Several mammalian nucleoside transporters, which are expressed in the plasma membrane of cells, have been cloned. The detailed transport characteristics of human and rat equilibrative nucleoside transporter 2 (hENT2 and rENT2, respectively) have been examined, and they transported a broad range of natural nucleosides and their analogues in Na$^+$-independent and nitrobenzylmercapturine riboside (NBMPR)-resistant fashions. Furthermore, hENT2 and rENT2 were previously reported to transport not only nucleosides but also purine and pyrimidine nucleobases. However, the transport of nucleosides and nucleobases via mouse ENT2 (mENT2) was not characterized in detail, except for the sensitivity of natural nucleosides and cardioprotective agents to uridine uptake mediated by the transporter. The transport mechanisms of various nucleosides and nucleobases in mouse primary-cultured cells have been investigated. Furthermore, tumor cells-bearing mouse has been used for the developments of improved chemotherapeutic strategies. Therefore, the functional characterization of mENT2 using a variety of nucleosides and nucleobases is physiologically and pharmacologically important. Recently, we clarified that the uracil uptake by mENT2-overexpressing Cos-7 cells (Cos-7/ENT2) for 2 min was similar to that by mock cells (Cos-7/pCI-neo), although uracil was reported to be a substrate for hENT2 and rENT2. These findings hypothesized that there are species differences in transport characteristics of ENT2.

In this study, therefore, we evaluated the transport of nucleosides and nucleobases mediated by recombinant mENT2.

Materials and Methods

Chemicals NBMPR was purchased from Sigma Chemical Co. (MO, U.S.A.), and all nucleosides and nucleobases were from Wako Pure Chemical Ind. (Osaka, Japan). Radioactive nucleosides and nucleobases were obtained from American Radiolabeled Chemicals Inc. (MO, U.S.A.), except for cytosine (MORAVEK Biochem. INC., CA, U.S.A.). All other reagents were of commercial or analytical grade requiring no further purification.

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 (MP Biologicals, LLC., CA, U.S.A.) at 37°C under a humidified atmosphere of 5% CO$_2$ in air.

Generation of mENT2-Transfectants As described previously, a cDNA of mENT2 subcloned into pCI-neo expression vector (Promega Co., WI, U.S.A.) was introduced into Cos-7 cells using LipofectAMINE2000 (Invitrogen, CA, U.S.A.). The peptide encoded by cloned mENT2 was identical to that previously reported.

Uptake Assay The uptake assaying was performed by the modified method of Nagasawa et al. The uptake reaction was initiated by adding 3H-labeled nucleoside or nucleobase (1 μCi/ml), or 14C-labeled hypoxanthine (0.1 μCi/ml) to the cells. In the case of nucleoside uptake, the cells were treated with 100 nM NBMPR to completely block the uptake mediated by ENT1 in Cos-7 cells. After an appropriate time interval, the reaction was terminated by the addition of ice-cold phosphate-based saline containing an excess concentration of each unlabeled substrate. The intracellular concentrations of nucleosides and nucleobases were determined with a liquid scintillation counter. Protein concentrations were measured by the method of Bradford with bovine serum albumin (Sigma Chemical Co.) as the standard.

Statistical Analysis The data were expressed as means±S.E. or S.E.M. The Michaelis constant ($K_m$) and
maximal transport rate \( (V_{\text{max}}) \) were calculated on the basis of the Eadie–Hofstee equation. Comparison among groups was performed by means of analysis of variance (ANOVA, followed by Fischer's PLSD), differences with a \( p \) value of 0.05 or less being considered statistically significant.

RESULTS AND DISCUSSION

Initially, the uptake of nucleosides and nucleobases by Cos-7/ENT2 and Cos-7/pCI-neo was measured. Transfection of mENT2 cDNA into Cos-7 cells resulted in an increase in the uptake of nucleosides and purine nucleobases, but not pyrimidine nucleobases, in a time-dependent manner (Fig. 1). More prolonged incubation (>30 min) of pyrimidine nucleobases did not result in their uptake via mENT2 (data not shown). Therefore, mENT2 was nucleoside and purine nucleobase transporter.

Next, the ability of nucleosides and nucleobases to inhibit mENT2-mediated uptake of adenosine was examined. The adenosine uptake via mENT2 was significantly inhibited not only by an excess concentration of nucleosides and purine nucleobases but also by the concentration of pyrimidine nucleobases (Table 1). Thus, pyrimidine nucleobases were blockers, but not substrates, for mENT2. On the other hand, the substrates for mENT2, except for adenosine, were poor inhibitors for adenosine uptake mediated by the transporter. Furthermore, the potency of effect of nucleosides and purine nucleobases on adenosine uptake via mENT2 differed according to the inhibitors, suggesting that nucleosides and purine nucleobases may interact with a site that is distinct form, but likely overlap, in the transporter.

![Fig. 1. Time Courses of Uptake of Nucleosides and Nucleobases by Cos-7/ENT2 and Cos-7/pCI-neo](image)

Cos-7/ENT2 and Cos-7/pCI-neo were incubated with 1 \( \mu \)M adenosine in HEPES-HBSS (pH 7.4) containing the indicated nucleoside (1 \( \mu \)M) or nucleobase (1 \( \mu \)M) for 2 min at 37 °C. The net uptake via mENT2 was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/ENT2. Each point represents the mean±S.E. (n=3).

![Fig. 2. Concentration Dependence of Uptake of Nucleosides and Purine Nucleobases Mediated by mENT2](image)

Cos-7/ENT2 and Cos-7/pCI-neo were incubated with the indicated concentrations of nucleosides or purine nucleobases in HEPES-HBSS (pH=7.4) for the indicated times at 37 °C. The net uptake via mENT2 was calculated by subtracting the uptake by Cos-7/pCI-neo from that by Cos-7/ENT2. Each point represents the mean±S.E.M. (n=3).

Table 1. Effects of Nucleosides and Nucleobases on Adenosine Uptake Mediated by mENT2

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>Uptake rate (% of control)</th>
<th>Nucleobase</th>
<th>Uptake rate (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>100±3.63</td>
<td>Control</td>
<td>100±4.46</td>
</tr>
<tr>
<td>Adenosine</td>
<td>0.925±0.0296***</td>
<td>Adenine</td>
<td>78.1±2.47**</td>
</tr>
<tr>
<td>Inosine</td>
<td>57.9±0.386***</td>
<td>Hypoxanthine</td>
<td>70.5±1.83***</td>
</tr>
<tr>
<td>Cytidine</td>
<td>80.1±3.26*</td>
<td>Cytosine</td>
<td>28.9±0.725***</td>
</tr>
<tr>
<td>Thymidine</td>
<td>64.1±1.14***</td>
<td>Thymine</td>
<td>43.6±0.324***</td>
</tr>
<tr>
<td>Uridine</td>
<td>71.1±2.477**</td>
<td>Uracil</td>
<td>62.6±1.135***</td>
</tr>
</tbody>
</table>

*** \( p < 0.001 \), ** \( p < 0.01 \), * \( p < 0.05 \), significantly different from each control.
fore, mENT2 might be important physiologically in transferring adenosine and hypoxanthine across the plasma membrane in skeletal muscle and heart during strenuous exercise and recovery process, and in neurons to maintain their electrical and synaptic activities. The \( K_m \) values for the uptake of nucleosides and pyrimidine nucleobases via ENT2 showed by us were less than those by other groups.\(^{10—12,14} \) Although we can not clearly give the reason for the difference in this kinetic parameter, it might be due to the different expression systems (Cos-7 cells, Xenopus oocytes or NT-deficient PK15 cells) rather than species difference. Namely, we previously reported that ENT2 predominantly contributed to transport of adenosine (\( K_m = 4.4 \mu M \)) and uridine (\( K_m = 6.4 \mu M \)) as a high-affinity transport system in primary-cultured rat cortical neurons.\(^{21} \) Therefore, it is difficult to explain the high-affinity uptake via mENT2 observed in the present study by species difference. On the other hand, ENTs were recently reported to be metabolism-driven transporters,\(^{15} \) and the salvage pathways for nucleotide synthesis are not necessary for NT-deficient cells (Xenopus oocytes and NT-deficient PK15 cells), suggesting that the sufficient activity of intracellular metabolism in transfectants may lead to the high-affinity transport mediated by ENT2.

There were distinctive differences in nucleobase transport among mouse, human and rat, namely hENT2 and rENT2, but not mENT2, transport pyrimidine nucleobases.\(^{13} \) Furthermore, the order of affinity of ENT2 for nucleosides, especially guanosine and cytidine, differed between mouse and human.\(^{15} \) Therefore, the attention might be necessary in the case of evaluating the transport mechanisms of pyrimidine nucleobases and these nucleosides and improving the chemotherapeutic strategies with the antimitabolites of their analogues using mouse primary-cultured cells and tumor cells-bearing mouse. Furthermore, we also compared the peptide encoded by ENT2 among species, namely same amino acid residues in human and rat ENT2, but not mouse ENT2, were explored. As a result, some of five amino acid residues in hENT2, that is, Ile129, Ser325, Gln326, Leu360 and Arg383, might be involved in the uptake of pyrimidine nucleobases and their analogue drugs, such as 5-fluorouracil, mediated by the transporter.

In summary, we demonstrated that mENT2 transports nucleosides and pyrimidine nucleobases, especially adenosine and hypoxanthine, and pyrimidine nucleobases are blockers for the transporter, which differed from the transport characteristics of hENT2 and rENT2.

**Acknowledgements** This work was supported by the “Academic Frontier” Project organized by Kyoto Pharmaceutical University.

### REFERENCES


### Table 2. Kinetic Constants for Uptake of Nucleosides and Purine Nucleobases Mediated by mENT2

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>( K_m ) (( \mu M ))</th>
<th>( V_{max} ) (pmol/mg protein/min)</th>
<th>( V_{max}/K_m ) (( \mu M )/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adenosine</td>
<td>1.24±0.153</td>
<td>108±12.2</td>
<td>88.2±7.84</td>
</tr>
<tr>
<td>Guanosine</td>
<td>7.10±0.873</td>
<td>364±33.0</td>
<td>51.6±1.88</td>
</tr>
<tr>
<td>Inosine</td>
<td>5.81±1.56</td>
<td>200±9.35</td>
<td>39.6±10.1</td>
</tr>
<tr>
<td>Cytidine</td>
<td>7.84±0.730</td>
<td>53.2±4.01</td>
<td>6.81±0.170</td>
</tr>
<tr>
<td>Uridine</td>
<td>6.67±0.607</td>
<td>73.2±6.44</td>
<td>11.0±0.584</td>
</tr>
<tr>
<td>Adenine</td>
<td>16.3±4.29</td>
<td>725±89.9</td>
<td>47.8±6.39</td>
</tr>
<tr>
<td>Guanine</td>
<td>12.5±1.70</td>
<td>363±46.0</td>
<td>29.1±0.260</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td>2.8±0.602</td>
<td>256±55.1</td>
<td>91.4±19.7</td>
</tr>
</tbody>
</table>

Kinetic constants were calculated by means of the Eadie–Hofstee equation using the data in Fig. 2. Each value represents the mean±S.E.M. (n=3).