Transport of A₁ Adenosine Receptor Agonist Tecadenoson by Human and Mouse Nucleoside Transporters: Evidence for Blood-Brain Barrier Transport by Murine Equilibrative Nucleoside Transporter 1 mENT1

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ABSTRACT

The high density of A₁ adenosine receptors in the brain results in significant potential for central nervous system (CNS)–related adverse effects with A₁ agonists. Tecadenoson is a selective A₁ adenosine receptor agonist with close similarity to adenosine. We studied the binding and transmembrane transport of tecadenoson by recombinant human equilibrative nucleoside transporters (hENTs) hENT1 and hENT2, and human concentative nucleoside transporters (hCNTs) hCNT1, hCNT2, and hCNT3 in vitro and by mouse mENT1 in vivo. Binding affinities of the five recombinant human nucleoside transporters for tecadenoson differed (hENT1 > hCNT1 > hCNT3 > hENT2 > hCNT2), and tecadenoson was transported largely by hENT1. Pretreatment of mice with a phosphorylated prodrug of nitrobenzylmercaptopurine riboside, an inhibitor of mENT1, significantly decreased brain exposure to tecadenoson compared with that of the untreated (control) group, suggesting involvement of mENT1 in transport of tecadenoson across the blood-brain barrier (BBB). In summary, ENT1 was shown to mediate the transport of tecadenoson in vitro with recombinant and native human protein and in vivo with mice. The micromolar apparent Kᵦ value of tecadenoson for transport by native hENT1 in cultured cells suggests that hENT1 will not be saturated at clinically relevant (i.e., nanomolar) concentrations of tecadenoson, and that hENT1-mediated passage across the BBB may contribute to the adverse CNS effects observed in clinical trials. In contrast, in cases in which a CNS effect is desired, the present results illustrate that synthetic A₁ agonists that are transported by hENT1 could be used to target CNS disorders because of enhanced delivery to the brain.

Introduction

Tecadenoson (N-[3-(R)-tetrahydrofuran]–6-aminopurine riboside) is a high-affinity and selective A₁ adenosine receptor agonist with potent antiarrhythmic effects in tachycardia involving the atrioventricular node such as paroxysmal supraventricular tachycardia (Lerman et al., 2001; Peterman and Sanoski, 2005). A₁ adenosine receptors are present on the surface of cells in organs throughout the body and their actions on these cells are diverse, including slowing of heart rate and atrioventricular nodal conduction, decreased cardiac arterial contractility, an antilipolytic effect in adipose tissue, and heart rate and atrioventricular nodal conduction, decreased cardiac

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ABBREVIATIONS: BBB, blood-brain barrier; CMM, complete minimal media; CNS, central nervous system; CNT1/2/3, concentrative nucleoside transporters 1, 2, and 3; ENT1/2/3/4, equilibrative nucleoside transporters 1, 2, 3 and 4; GLU, glucose; h, human; m, murine; NT, nucleoside transporter; NBMPR, nitrobenzylmercaptopurine riboside; NBMPR-P, nitrobenzylmercaptopurine riboside 5′-monophosphate; OD, optical density; RT, room temperature; tecadenoson, N-[3-(R)-tetrahydrofuran]–6-aminopurine riboside.
affinities for nucleosides than the CNTs (Young et al., 2008). ENT2 is insensitive to nanomolar concentrations of NBMPR, and transports both nucleosides and nucleobases (Yao et al., 2002). ENT3 is found in intracellular membranes (lysosomes) and exhibits broad permeant selectivity, whereas ENT4 is found in plasma membranes and transports monoamines and, to a lesser extent, adenosine in brain and heart (Zhang et al., 2005; Barnes et al., 2006). CNT1 transports pyrimidine nucleosides such as thymidine, cytidine, and uridine and, to a lesser extent, the purine nucleoside adenosine (Ritzel et al., 1997). CNT2 transports uridine and pyrimidine nucleosides such as adenosine, guanosine, and inosine (Ritzel et al., 1998). CNT3 transports both purine and pyrimidine nucleosides, including adenosine, guanosine, inosine, thymidine, cytidine, and uridine (Ritzel et al., 2001).

Adverse effects related to the nervous system, including symptoms consistent with the known A1 adenosine receptor–mediated depressant effect on neurotransmission, have been reported in patients who received tecadenoson (Lerman et al., 2001). Since tecadenoson structurally resembles adenosine, a permeant for NTs, the objective of this work was to investigate the role of five of the seven human (h) NTs (hENT1/2, hCNT1/2/3) in tecadenoson transport, and to assess NT-mediated transport across the BBB in mice. The in vitro membrane transport properties of tecadenoson were studied with hNTs produced in recombinant form in the yeast Saccharomyces cerevisiae and in oocytes of Xenopus laevis and native hENT1 in cultured cells, whereas in vivo membrane transport properties were studied with murine (m) ENT1. In the in vivo studies, mice were treated with tecadenoson in the absence or presence of nitrobenzylmercaptopurine riboside 5′-monophosphate (NBMPR-P), a soluble prodrug of the potent inhibitor of mENT1, to study accumulation of tecadenoson in brain and plasma.

Materials and Methods

Chemicals. Tecadenoson and [3H]tecadenoson with a specific activity of 17 Ci/mmol were provided by CV Therapeutics (now Gilead Sciences Inc., Foster City, CA). [3H]Fludarabine (2-fluoroadenine arabinofuranoside; F-araA) (specific activity of 4.4 Ci/mmol) and [3H]Juridine (specific activity of 14.7 Ci/mmol) were from Moravek Biochemicals (Brea, CA). NBMPR-P synthesis was reported previously (Lynch et al., 1981). NBMPR, dilaizep, unlabeled nucleosides, and other chemicals were obtained from Sigma-Aldrich (Oakville, ON, Canada). Cell culture media and fetal bovine serum were from Gibco BRL (Burlington, ON, Canada). EcoLite was from ICN Pharmaceuticals (Montreal, QC, Canada).

Measurement of [3H]Juridine and [3H]Tecadenoson Uptake by Recombinant Human NTs Produced in S. cerevisiae. Construction of the yeast expression systems for hENT1/2 and hCNT1/2/3 was performed under conditions for which the individual hNTs have been shown to be present and functional in plasma membranes (Vickers et al., 1999, 2002; Zhang et al., 2003, 2005). Yeast strains were maintained in logarhythmic growth phase in complete minimal media (CMM) containing 0.67% yeast nitrogen base (Difco, Detroit MI), amino acids (as required to maintain auxotrophic selection), and 2% glucose (C/MM/GLU).

Transport of [3H]Juridine or [3H]Tecadenoson was assessed at room temperature (RT) with a high-throughput assay described previously (Zhang et al., 2003) that used 96-well plates and a semi-automated cell harvester (Micro96 Harvester; Skatron Instruments, Lier, Norway). Briefly, yeast were grown in CMM/GLU to an optical density (OD)_{500} of 0.8–1.2, washed three times with fresh CMM/GLU (pH 7.4), and re-suspended to an OD_{500} of 4 in CMM/GLU (pH 7.4). For uridine inhibition assays, 50–μl portions of CMM/ GLU (pH 7.4) with [3H]Juridine and a test compound (if present) at twice the desired concentration were preloaded into 96-well plates. For tecadenoson transportability assays, uptake of 1 μM [3H]tecadenoson was measured alone or with 10 mM nonradioactive uridine. The transport assays were initiated by adding an equal volume of yeast suspension at OD_{500} = 4 to each of the individual wells of the preloaded 96-well plates, which were placed on the semi-automated cell harvester. At graded time intervals, groups of transport reactions (usually 24) were terminated simultaneously by harvesting yeast on glass-fiber filters (Skatron Instruments) with continued washing with demineralized water to remove unincorporated permeant. The filter discs with yeast corresponding to a particular transport assay were placed into individual scintillation counting vials (1 disc/vial) to which 5 ml scintillation counting fluid (EcoLite; ICN Biomedicinal Inc., Aurora, OH) was added. Scintillation vials were allowed to sit at RT overnight with shaking before analysis.

The relative affinities of the hNTs for tecadenoson, adenosine, and uridine were assessed by measuring the concentration dependence of their inhibition of uptake of [3H]Juridine as described previously (Zhang et al., 2002, 2005, 2006a) as follows. Yeast producing recombinant hENT1, hENT2, hCNT1, hCNT2, or hCNT3 were incubated at RT with graded concentrations of test nucleoside in the presence of 1 μM [3H]Juridine for 15 minutes (hENT1/2, hCNT1/2) or 5 minutes (hCNT3). Each experiment was repeated at least three times. Nonspecifically associated radioactivity was determined in the presence of 10 mM nonradioactive uridine, and the resulting values were subtracted from total uptake values.

For inhibition assays, data were subjected to nonlinear regression analysis using GraphPad Prism software (version 4.0; GraphPad Software Inc., San Diego, CA) to obtain values for the concentrations of drug that inhibited transport by 50% relative to that of control (IC_{50} values) for uridine and tecadenoson. IC_{50} (inhibitory constant) values were determined using the Cheng-Prusoff equation (Cheng and Prusoff, 1973), which assumes competitive inhibition by the test compounds, in which IC_{50} = IC_{50} + (1/[L/K_i]), where L = [3H]Juridine concentration (always 1 μM). Because this method is independent of V_{max} values, it can be used to compare permeant affinities of recombinant NTs that are produced at different levels in the host organism. The K_{i} values for transport of uridine for each of the recombinant transporters produced in yeast were reported previously (Zhang et al., 2003, 2005, 2006b).

Transport of Tecadenoson by Recombinant hNTs Produced in Xenopus laevis Oocytes. Linearized plasmids with cDNAs encoding hCNT1, hCNT2, or hCNT3 or hENT1 in the Xenopus oocyte expression vector pGEMHE were transcribed with T7 polymerase in the presence of m7GppGpp cap using the mMESSAGE mMACHINE Ambion (Life Technologies), Carlsbad, CA) transcription system. Defolliculated stage VI Xenopus oocytes were micro-injected with 20 nl water or 20 nl water containing capped RNA transcript (20 ng) and incubated in modified Barth’s medium at 18°C for 4 days prior to the assay of transport activity (Yao et al., 2000).

For electrophysiological studies of recombinant hCNT1, hCNT2, or hCNT3, nucleoside-evoked membrane currents were measured at RT using a GeneClamp 500B oocyte clamp (Molecular Devices, Sunnyvale, CA) in the two-electrode, voltage-clamp mode (Smith et al., 2004, 2007). The GeneClamp 500B was interfaced to an IBM-compatible PC via a Digidata 1322A A/D converter and controlled by pCLAMP software (version 9.0; Molecular Devices). The microelectrodes were filled with 3 M KCl and had resistances that ranged from 0.5 to 2.5 MΩ. Oocytes were penetrated with the microelectrodes and their membrane potentials were monitored for periods of 10–15 minutes. Oocytes were discarded when membrane potentials were unstable, or more positive than ~30 mV. All steady-state current measurements were performed at a holding potential (V_{H}) of ~90 mV. Current responses were generated by perfusing individual hCNT1-, hCNT2-, or hCNT3-producing oocytes with uridine, adenosine, or tecadenoson at 100 or 500 mM in a sodium-containing transport medium containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl_2, 1 mM MgCl_2, and 10 mM HEPES, pH 7.5. The same experiment was performed in control water-injected oocytes. Current signals were filtered at 20 Hz (four-pole Bessel filter) and sampled at 20-millisecond intervals. Results are given as means ± S.E.M. for 4–6 individual oocytes. The experiments was performed twice on oocytes from different frogs, yielding closely similar results.

Kinetics of [3H]tecadenoson transport was characterized in hENT1-producing Xenopus oocytes as follows. Flux measurements in hENT1-producing and control water-injected oocytes were performed at RT as described previously (Yao et al., 2011) on groups of 12 oocytes in 200 μl of the same transport medium used for electrophysiological studies. At the end of 2-minute incubation periods, extracellular label was removed by six rapid washes in ice-cold transport medium, and individual oocytes were dissolved in 5% (w/v) sodium dodecyl sulfate for quantification of oocyte-associated radioactivity by
liquid scintillation counting. Results are given as means ± S.E.M. for 10–12 oocytes. Kinetic parameters (Km and Vmax) (± S.E.) for mediated transport corrected for basal uptake in control water-injected oocytes were determined using SigmaPlot software (Systat Software, San Jose, CA). Experiments were performed three times on oocytes from different frogs, yielding closely similar results.

Transport of [3H]Tecadenoson and [3H]Fludarabine by Native hENT1 in CEM Cells. The human CCRF-CEM acute lymphoblastic leukemia, hereafter termed CEM, cell line was obtained from William T. Beck (University of Illinois at Chicago, Chicago, IL). CEM cells were grown in Roswell Park Memorial Institute 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON, Canada) as suspension cultures. Cells were maintained in the absence of antibiotics, incubated at 37°C in a humidified atmosphere (5% CO2), and subcultured at 2- to 4-day intervals to maintain active proliferation.

Short time transport and long time accumulation of 10 µM each of [3H]tecadenoson and [3H]fludarabine were measured at RT in CEM cells in transport buffer (pH 7.4) containing 20 mM Tris, 3 mM K2HPO4, 1 mM MgCl2, 1.4 mM CaCl2, and 5 mM glucose with 144 mM NaCl for 1 minute and 1 hour. At the end of uptake intervals, permeant-containing solutions were removed by aspiration; cells were quickly rinsed twice with transport buffer and solubilized with 5% TritonX-100 (Fisher Scientific, Ottawa, ON, Canada). Radioactivity in solubilized extracts was measured by liquid scintillation counting. Uptake values were expressed as pmol/106 cells and graphs were generated using GraphPad Prism software (version 4.0; GraphPad Software Inc.). Each experiment was conducted two or more times with triplicate measurements.

For kinetic studies, CEM cells were incubated with increasing concentrations of [3H]tecadenoson (0–300 µM) for 5 seconds at RT and spun quickly and washed. Intracellular radioactivity was measured as described above and analyzed using GraphPad Prism software.

Animal Studies. Two groups of male CD1 mice received single i.v. bolus doses of 0.1 mg/kg tecadenoson in saline. Animals were pretreated with saline (group 1) or 25 mg/kg NBMPR-P in saline (group 2) via i.p. injection 20 minutes prior to treatment with tecadenoson. Two other groups (groups 3 and 4) of mice received single i.v. bolus doses of 60 mg/kg fludarabine in water. Group 3 was pretreated with saline and group 4 received 25 mg/kg NBMPR-P in saline 20 minutes prior to treatment with fludarabine. Animals were sacrificed at various time points over 6-hour (tecadenoson) and 8-hour (fludarabine) time courses, and blood and brains were harvested. Plasma and brain concentrations of tecadenoson and fludarabine, as well as plasma concentrations of NBMPR, were measured by liquid chromatography-tandem mass spectrometry. For liquid chromatography-tandem mass spectrometry analysis, whole brain samples were homogenized in a 3-fold dilution in 3% (w/v) sodium fluoride solution with 1% (v/v) HCl. The homogenates were processed by protein precipitation, centrifuged, and the supernatants diluted 10-fold in methanol/water (1:1). The samples were then analyzed using an

**Fig. 1.** Structures of adenosine, fludarabine, and tecadenoson.

**Fig. 2.** Transport inhibition assays in yeast producing human recombinant hENT1. Relative abilities of uridine (□), adenosine (△), or tecadenoson (○) to inhibit the uptake of 1 µM [3H]uridine were assessed as described in the Materials and Methods. Concentration dependencies of uridine, adenosine, and tecadenoson inhibition of hENT1-mediated [3H]uridine uptake in yeast producing hENT1 are shown. The values presented (mean ± S.E.M.) are derived from four replicates for each concentration; error bars are not shown where values were smaller than the size of data points. Each data point represents the mean ± S.E. from three or more experiments.
API5000 liquid chromatographer coupled to a tandem mass spectrometer (AB Sciex Instruments, Foster City, CA). A LC column (Luna 3 μm C18(2); 20 × 4.0 mm), injection volume 10 μl, and mobile phase of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid (90:10) were used. Plasma sample processing was similar to that of brain homogenates. Pharmacokinetic parameters for tecadenoson and fludarabine in plasma and brain were calculated using WinNolin 5.01 software (Pharsight, Mountain View, CA).

### Results

Structures of adenosine, fludarabine, and tecadenoson are shown in Fig. 1. The relative binding affinities of hNTs for tecadenoson, and for comparison uridine and adenosine, were determined using an inhibitor sensitivity assay in which graded concentrations of test compound were assessed for their ability to inhibit uptake of 1 μM [3H]uridine mediated by recombinant hNTs produced in yeast. Representative concentration-effect relationships in yeast producing hENT1 are shown in Fig. 2. Mean IC₅₀ values (± S.E.) and corresponding mean Kᵢ values (± S.E.) calculated from such data for uridine, adenosine, and tecadenoson inhibition of hENT1-, hENT2-, hCNT1-, hCNT2-, and hCNT3-mediated uridine transport are presented in Table 1.

With an inverse relationship between Kᵢ values and apparent affinities, recombinant hENT1 produced in yeast displayed >4-fold higher apparent affinity for tecadenoson (Kᵢ = 11 μM) than for uridine (Kᵢ = 50.8 μM) and comparable affinity for adenosine (Kᵢ = 17 μM). hENT2 showed moderate affinity for tecadenoson (Kᵢ = 189 μM), somewhat higher than that for uridine (Kᵢ = 242 μM) and somewhat lower than that for adenosine (Kᵢ = 106 μM). Adenosine is a weak (low Vₘₐₓ) permeant for hCNT1 (Smith et al., 2004) but, as illustrated in Table 1, was bound by hCNT1 with high affinity (Kᵢ = 2 μM). hCNT1 bound tecadenoson with a somewhat lower affinity (Kᵢ = 37 μM) than that for uridine (Kᵢ = 5 μM). The affinity of hCNT2 for tecadenoson was lower (Kᵢ = 231 μM) than that for either adenosine (Kᵢ = 5 μM) or uridine (Kᵢ = 31 μM). hCNT3 had approximately 40-fold greater affinity for adenosine and uridine (Kᵢ = 3 and 2 μM, respectively) than that for tecadenoson (Kᵢ = 126 μM). The rank order of relative affinities of the recombinant hNTs for tecadenoson was hENT1 > hCNT1 > hCNT3 > hENT2 > hCNT2.

Direct uptake experiments in hENT1-producing yeast showed mediated transport of 1 μM [3H]tecadenoson over both short (2-minute) and long (60-minute) uptake intervals in the presence and absence of 10 μM uridine (Fig. 3, A and B). The almost complete inhibition of uptake of tecadenoson by the presence of uridine indicated that most, if not all, of the observed uptake was mediated by hENT1. Transport of [3H]tecadenoson by recombinant hENT2-producing yeast showed no uptake over the first 2 minutes and

### Table 1

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<th>Adenosine</th>
<th>Tecadenoson</th>
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<td>Kᵢ μM</td>
<td>IC₅₀ μM</td>
<td>Kᵢ μM</td>
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<td>52 ± 5</td>
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<td>242 ± 22</td>
<td>106 ± 5</td>
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<td>31 ± 4</td>
<td>5.5 ± 1.6</td>
</tr>
<tr>
<td>hCNT3</td>
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<td>3.4 ± 0.6</td>
<td>3.3 ± 0.4</td>
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### Notes

- **CNT**: concentrative nucleoside transporter; **ENT**: equilibrative nucleoside transporter; **h**: human.
- **Materials and Methods**: Data are presented as means ± S.E.M. (n ≥ 3).
injected) oocytes are presented as mean currents (± S.E.) for 4–6 different oocytes. Moderate uptake over 60 minutes (Fig. 3, C and D), thus showing that hENT2 transports tecadenoson albeit poorly.

Transport of [3H]tecadenoson by recombinant hENT1 was also demonstrated in Xenopus oocytes. Figure 4 shows a representative experiment comparing the concentration dependence (0–1 mM) of [3H]tecadenoson uptake in hENT1-producing oocytes and control water-injected oocytes. Uptake in water-injected oocytes was slow and exhibited linear concentration dependence consistent with simple diffusion of tecadenoson across the membrane lipid bilayer (5.0 ± 0.9 pmol/oocyte-2 minutes⁻¹). In contrast, uptake by hENT1-producing oocytes was rapid and saturable, with the mediated component of transport (defined as the difference in uptake between hENT1-producing and control water-injected oocytes) giving calculated apparent Kₘ and Vₘₐₓ values (± S.E.) of 196 ± 9 μM and 46.7 ± 0.8 pmol/oocyte-2 minutes⁻¹, respectively.

Inward Na⁺ currents measured in Xenopus oocytes using the two-electrode, voltage-clamp (Smith et al., 2004, 2007) assessed the relative abilities of hCNT1/2/3 to transport tecadenoson (Fig. 5). Figure 5 shows mean (± S.E.M.) nucleoside-evoked membrane currents produced by perfusion with 100 or 500 μM uridine, adenosine, or tecadenoson in hCNT1-, hCNT2-, or hCNT3-containing oocytes compared with those in control water-injected cells.

Since [3H]tecadenoson transport by recombinant hNTs in yeast and Xenopus oocytes was shown to be mediated largely by hENT1, uptake of [3H]tecadenoson by native hENT1 was compared with that of an adenosine analog used in cancer therapy using a cultured human cell line, CEM, which possesses hENT1 as its only NT activity. Transport (1 minute) and accumulation (1 hour) of 10 μM [3H]tecadenoson and [3H]fludarabine were similar during short and prolonged exposures (Fig. 6A). The concentration dependence of initial rates of uptake of [3H]tecadenoson exhibited saturation as tecadenoson concentrations were increased from 0 to 300 μM (Fig. 6B). Apparent kinetic parameters obtained from Michaelis-Menten analysis after correction of uptake values for nonsaturable uptake in the presence of excess unlabeled uridine (i.e., diffusion) yielded Kₘ and Vₘₐₓ values (± S.E.) of 24 ± 6 μM and 3.4 ± 1 pmol/10⁶ cells per second, respectively. The lower apparent Kₘ value in CEM cells (24 μM) versus that in Xenopus oocytes (196 μM) is in accord with differences noted for other permeants (Young et al., 2008; Parkinson et al., 2011).

In the in vivo experiments, plasma and brain concentrations of tecadenoson (0.1 mg/kg i.v.) and fludarabine (60 mg/kg i.v.) from saline and NBMPR-P-treated mice were measured over 6 and 8-hour time courses. In these experiments, fludarabine was used as a positive control for NBMPR-dependent uptake into cells in vivo (Adjei et al., 1992). Brain and plasma concentration-time profiles of tecadenoson and fludarabine are presented in Fig. 7 and the calculated pharmacokinetic parameters are shown in Tables 2 and 3. Prior treatment of mice with NBMPR-P reduced maximum drug concentration (Cₘₐₓ) of tecadenoson in brain from 23 ± 3 ng/g to 4 ± 1 ng/g, and the area under the concentration-time curve during 0 to 4 hours was reduced from 22 ± 2 to 7 ± 1 ng·h/g. Similar observations were made in mice treated with fludarabine—that is, in the group pretreated with NBMPR-P, Cₘₐₓ of fludarabine was 801 ± 378 ng/g compared with 6050 ± 378 ng/g in the group pretreated with saline and the area under the concentration-time curve during 0 to 4 hours of fludarabine was 2490 ± 418 ng·h/g in the NBMPR-P pretreated group compared with 11300 ± 1550 ng·h/g in the saline pretreated group. These data suggest involvement of mENT1 in transport of tecadenoson across the BBB.

![Fig. 5. Electrophysiological transport studies in Xenopus oocytes. Sodium currents induced by uridine, adenosine, and tecadenoson at 100 and 500 μM in Xenopus oocytes producing recombinant hCNT1, hCNT2, or hCNT3 and in control (water-injected) oocytes are presented as mean currents (± S.E.) for 4–6 different oocytes.](image)

![Fig. 6. Transport of tecadenoson and fludarabine by native hENT1 in CEM cells. (A) Short (1 minute) and long (1 hour) time courses of uptake of 10 μM [3H]tecadenoson (open bars) and [3H]fludarabine (dashed bars) in CEM cells are presented as means ± S.E.M. of three independent experiments each conducted with three replicates. (B) Concentration dependence of transport of [3H]tecadenoson measured as 5-second fluxes in CEM cells. The flux values shown are means ± S.E.M. of three independent experiments. Each experiment was performed in triplicate.](image)

![Fig. 7. Effects of treatment of mice with prodrug of ENT1 inhibitor on brain and plasma levels of tecadenoson and fludarabine. Mean (± S.D., n = 4) brain (A) and plasma (C) concentrations of tecadenoson after 0.1 mg/kg i.v. dose in mice with (□ and dashed lines) and without NBMPR-P (●). Mean (± S.D., n = 4) brain (B) and plasma (D) concentrations of fludarabine after 60 mg/kg i.v. dose in mice with (□ and dashed lines) and without NBMPR-P (●).](image)
A$_1$ adenosine receptors are present at high density in the CNS, and there is a significant potential for CNS-related adverse effects with A$_1$ agonists that are able to cross the BBB. NTs are located throughout the CNS, although there seem to be differences in distribution that may be species and even sex specific (Lu et al., 2004). In the human brain, hENT1 is present in the BBB, and appears to be distributed regionally in a pattern that correlates with the existence of A$_1$ adenosine receptors (Parkinson et al., 2011). The relatively hydrophilic nature of tecadenoson due to its ribose moiety and evidence of P-glycoprotein tors (Parkinson et al., 2011). The relatively hydrophilic nature of tecadenoson may partially explain why the modified adenosine analog hNTs. The presence of the C(3’)-OH position of the sugar moiety in tecadenoson with hENT1. A modification at the 6 position of the adenine moiety did not seriously limit interaction of tecadenoson with the three hCNTs. Thus, introduction of a modification at the 6 position of the adenine moiety had a major effect on transportability of hCNT1/2/3.

In electrophysiology experiments with oocytes, relative to uridine, and similar to previous findings with each of the three hCNTs (Ritzel et al., 1997, 1998, 2001; Smith et al., 2004), adenosine generated large inward currents with hCNT3, intermediate currents with hCNT2, and small but still significant currents with hCNT1. In contrast, tecadenoson was without effect, even at the highest concentration of 500 µM. Inability to generate inward currents, and the corresponding absence of uridine-, adenosine-, and tecadenoson-evoked membrane currents in control water-injected oocytes, established that, unlike hENT1, the A1 adenosine receptor agonist was not transported by hCNT1/2/3. Together with the inhibition data presented in Table 1, these results indicate that tecadenoson was a high-affinity non-transported inhibitor of hCNT1, and a low-affinity nontransported inhibitor of hCNT2 and hCNT3. Although hCNT3 has shown high tolerance to modifications in the uracil moiety of uridine (Zhang et al., 2003, 2005), it exhibited a pronounced reduction in its interaction with tecadenoson, which is modified in the adenine moiety.

Kinetic experiments in CEM cells were carried out to measure the apparent affinity of native hENT1 for tecadenoson to determine whether hENT1 could also play a role in uptake of tecadenoson at concentrations that are pharmacologically relevant in the in vivo setting. The $K_m$ values for hENT1-mediated transport of tecadenoson in CEM cells were 24 ± 6, well above the clinically relevant concentrations (i.e., nM range) of tecadenoson (Lerman et al., 2001).

NBMPR-P is a readily soluble prodrug form of NBMPR that does not itself inhibit nucleoside transport, whereas NBMPR released by dephosphorylation of NBMPR-P in vivo is responsible for inhibition of nucleoside uptake by ENT1 and ENT2 at nanomolar and micromolar concentrations, respectively (Ogbunude et al., 1984; Wiebe et al., 1990; Rahn et al., 1997; Wright et al., 2000). In the in vivo experiment, brain uptake of both fludarabine and tecadenoson was reduced by NBMPR. The highest levels of tecadenoson in brain were seen at the earliest time points, and the time to reach maximum concentration ($T_{\text{max, brain}}$) for fludarabine was observed around 1 hour. The shift in the peak concentration of fludarabine in brain is likely to reflect its phosphorylation and subsequent trapping within cells that continues beyond the first time point. The absence of this feature in the data for tecadenoson suggests that tecadenoson was not phosphorylated inside cells, although definitive data are not available. Pretreatment of mice with NBMPR-P significantly decreased brain uptake of tecadenoson, suggesting involvement of mENT1 in transport of tecadenoson across the BBB. In addition, components of passive diffusion and transport by P-glycoprotein may have contributed to the small, NBMPR-insensitive portion of influx and elimination of tecadenoson in brain.

## Table 2

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<td>7 ± 1</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>0.88 ± 0.25</td>
<td>6050 ± 378</td>
<td>11,300 ± 1550</td>
</tr>
<tr>
<td>Fludarabine + NBMPR-P</td>
<td>1.63 ± 1.6</td>
<td>801 ± 83</td>
<td>2490 ± 418</td>
</tr>
</tbody>
</table>

AUC$_{0-4\text{h}}$, area under the concentration-time curve during 0 to 4 hours; NBMPR-P, nitrobenzylmercaptopurine riboside 5’-monophosphate; $T_{\text{max}}$, time to reach maximum concentration.

## Table 3

<table>
<thead>
<tr>
<th>Compound</th>
<th>$V_m$</th>
<th>$\text{CL}$</th>
<th>$t_{1/2}$</th>
<th>AUC$_{0-\infty}$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>l/hg</td>
<td>l/h per kg</td>
<td>h</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Tecadenoson</td>
<td>2.8 ± 0.6</td>
<td>6.2 ± 0.9</td>
<td>0.7 ± 0.1</td>
<td>16.4 ± 2.3</td>
</tr>
<tr>
<td>Tecadenoson + NBMPR-P</td>
<td>2.2 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>50.3 ± 5.5</td>
</tr>
<tr>
<td>Fludarabine</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.5</td>
<td>39,600 ± 4560</td>
</tr>
<tr>
<td>Fludarabine + NBMPR-P</td>
<td>2.6 ± 0.3</td>
<td>0.9 ± 0.1</td>
<td>2.2 ± 0.6</td>
<td>65,700 ± 10,300</td>
</tr>
</tbody>
</table>

AUC$_{0-\infty}$, area under the concentration-time curve extrapolated to infinity; CL, apparent total body clearance of the drug from plasma; NBMPR-P, nitrobenzylmercaptopurine riboside 5’-monophosphate; $t_{1/2}$, elimination half-life; $V_m$, apparent volume of distribution at steady state.

Role of ENT1 in Tecadenoson Transport
tecadenoson from brain (Fig. 7A). Plasma profiles for tecadenoson for the saline- and NBMPR-P-treated groups were also different from those of fludarabine. The first hour of the plasma elimination curves for fludarabine might be explained as NBMPR-independent clearance from plasma (in the absence of contributions due to efflux from cellular compartments, where accumulation continues). Subsequently, fludarabine elimination data may be largely attributable to NBMPR-dependent release from cells, rather than to elimination from plasma. The apparent immediate NBMPR-dependence of tecadenoson elimination from plasma might, again, support the suggestion that tecadenoson did not accumulate in cells through phosphorylative trapping, but was eliminated almost from the outset through NBMPR-dependent loss from cells into the plasma compartment.

In summary, our results showed that transport of tecadenoson was mediated by recombinant and native hENT1 and recombinant hENT2 in vitro and by native hENT1 in vivo. The micromolar IC50 value exhibited by native hENT1 for tecadenoson suggests that the transporter would be active at clinically relevant concentrations of tecadenoson (nanomolar levels), and thus would contribute to adverse CNS effects such as those previously observed in the clinical trial reported by Lerman et al. (2001). In contrast, in cases in which a CNS effect is desired, our results illustrate how synthetic A1 agonists targeting CNS disorders would exploit ENT1 to enhance delivery to the brain.

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Performed data analysis: Lepist, Damaraaju, Zhang, Yao, Smith.

Wrote or contributed to writing of the manuscript: Lepist, Damaraaju, Gati, Yao, Young, Cass.

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