Transport of A1 adenosine receptor agonist tecadenoson by human and mouse nucleoside transporters: Evidence for blood-brain barrier transport by mENT1


Gilead Sciences, Inc., (E-IL) 333 Lakeside Dr, Foster City, CA 94070, USA.
Novartis Institute of BioMedical Research (KHL), 4560 Horton Street, Emeryville, CA 94608, USA.
Departments of Oncology (VLD, JZ, CEC), Pharmacology (WPG) and Physiology (SYMY, KMS, EK, JDY), University of Alberta, Edmonton, AB T6G 1Z2, Canada
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Corresponding author:
Carol E. Cass, PhD, FRSC, FCAHS
Professor Emeritus of Oncology, University of Alberta
Phone: 780-432-8524; fax: 780-432-8425
Edmonton, AB, Canada
carol.cass@ualberta.ca

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Abbreviations:
AUC, area under the concentration-time curve; BBB, blood-brain barrier; CL, apparent total body clearance of the drug from plasma; CNS, central nervous system; Cmax, maximum drug concentration; CNT1/2/3, concentrative nucleoside transporters 1, 2 and 3; ENT1/2/3/4, equilibrative nucleoside transporters 1, 2, 3 and 4; h, human; m, mouse; NTs, nucleoside transporters; IC50, concentration of drug that inhibits transport by 50%; NBMPR, nitrobenzylmercaptopurine riboside; RT, room temperature; Tmax, time to reach maximum concentration; t1/2, elimination half-life; Vss, apparent volume of distribution at steady state.
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 nucleoside transporters (hNTs) hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in vitro and by mouse mENT1 in vivo. Binding affinities of the five recombinant hNTs 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 (NBMPR), an inhibitor of mENT1, significantly decreased brain exposure to tecadenoson compared to 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 µM apparent Kₘ value of tecadenoson for transport by native hENT1 in cultured cells suggests that (i) hENT1 will not be saturated at clinically relevant (i.e., nM) concentrations of tecadenoson, and (ii) hENT1-mediated passage across the BBB may contribute to the adverse CNS effects observed in clinical trials. In contrast, in cases where 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)-tetrahydrofuranyl]-6-aminopurine riboside), is a high affinity and selective A₁ adenosine receptor agonist with potent antiarrhythmic effects in tachycardia involving the atrioventricular (AV) 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 AV nodal conduction, decreased cardiac arterial contractility, an anti-lipolytic effect in adipose tissue, and inhibition of neuronal activity (Dhalla et al., 2003). Since the density of A₁ adenosine receptors in the heart is less than in the central nervous system (CNS), there is a significant potential for CNS-related adverse effects with A₁ agonists that are able to cross the blood-brain barrier (BBB). Tecadenoson structurally resembles adenosine, an endogenous substrate for nucleoside transporters (NTs) found in all organs, including brain. Although a contribution of ENT1 has been shown in BBB transport of some synthetic A₁ adenosine receptor agonists in vitro in a coculture of bovine capillary endothelial cells and rat astrocytes, the results were not confirmed in an in situ rat brain perfusion model (Schaddelee et al., 2003; Schaddelee et al., 2005).

The transporters for physiologic nucleosides and nucleoside analogs include the equilibrative nucleoside transporters (ENTs: hENT1/2/3/4 in humans, mENT1/2/3/4 in mice) and the concentrative nucleoside transporters (CNTs: hCNT1/2/3 in humans, mCNT1/2/3 in mice). For recent reviews of nucleoside transport in humans and rodents, see (Damaraju et al., 2003; King et al., 2006; Zhang et al., 2007; Young et al., 2008; Parkinson et al., 2011).

The following summary applies to the transporters of humans and rodents. ENT1 is sensitive to nM concentrations of the inhibitor nitrobenzylmercaptopurine riboside (NBMPR), and transports both purine and pyrimidine nucleosides, but has lower affinities for nucleosides than the CNTs (Young et al., 2008). ENT2 is insensitive to nM 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 purine 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 Xenopus laevis oocytes 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 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/mm mol 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/mm mol) and [3H]uridine (specific activity of 14.7 Ci/mm mol) were from Moravek Biochemicals (Brea, CA). Nitrobenzylmercaptopurine riboside 5'-monophosphate (NBMPR-P) synthesis was reported previously (Lynch et al., 1981). NBMPR, dilazep, unlabeled nucleosides and other chemicals were obtained from Sigma Chemical Company (Mississauga, ON). Cell culture media and fetal bovine serum (FBS) were from Gibco BRL (Burlington, ON). Ecolite was from ICN Pharmaceuticals (Montreal, QC).


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; Vickers et al., 2002; Zhang et al., 2003; Zhang et al., 2005). Yeast strains were maintained in logarithmic 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 (CMM/GLU).

Transport of [3H]uridine 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) 600 of 0.8-1.2, washed three times with fresh CMM/GLU (pH 7.4), and re-suspended to an OD 600 of 4 in CMM/GLU (pH 7.4). For uridine inhibition assays, fifty-µl portions of CMM/GLU (pH 7.4)
with $[^3H]$uridine 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 non-radioactive uridine in. The transport assays were initiated by adding an equal volume of yeast suspension at $OD_{600} = 4$ 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 (one disc/vial) to which five ml of scintillation counting fluid (EcoLite, ICN Biomedical 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 $[^3H]$uridine as described previously (Zhang et al., 2003; Zhang et al., 2005; Zhang et al., 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 $[^3H]$uridine for 15 min (hENT1/2, hCNT1/2) or 5 min (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. $K_i$ (inhibitory constant) values were determined
using the Cheng-Prusoff equation (Cheng and Prusoff, 1973), which assumes competitive inhibition by the test compounds, in which $K_i = IC_{50}/[1 + (L/K_m)]$ where L = [3H]uridine 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 $K_m$ values for transport of uridine for each of the recombinant transporters produced in yeast were reported previously (Zhang et al., 2003; Zhang et al., 2005; Zhang et al., 2006b).

**Transport of tecadenoson by recombinant human nucleoside transporters produced in *Xenopus laevis* oocytes.**

Linearized plasmids with cDNAs encoding either hCNT1, 2 or 3 or hENT1 in the *Xenopus* oocyte expression vector pGEMHE were transcribed with T7 polymerase in the presence of m7GpppG cap using the mMESSAGE mMACHINE™ (Ambion) transcription system. Defolliculated stage VI *Xenopus* oocytes were microinjected with 20 nl of water or 20 nl of water containing capped RNA transcript (20 ng) and incubated in modified Barth’s medium at 18°C for four days prior to the assay of transport activity (Yao et al., 2000).

For electrophysiological studies of recombinant hCNT1, 2 or 3, nucleoside-evoked membrane currents were measured at RT using a GeneClamp 500B oocyte clamp (Molecular Devices, Sunnyvale, CA, USA) in the two-electrode, voltage-clamp mode (Smith et al., 2004; Smith et al., 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 - 2.5 MΩ (megaohms). Oocytes were penetrated with the microelectrodes and their membrane potentials were monitored for periods of 10 - 15 min. 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, 2 or 3-producing oocytes with uridine, adenosine or tecadenoson at 100 and 500 mM in a sodium-containing transport medium containing 100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 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 intervals of 20 ms. Results are given as means ± S.E.M. for four to six individual oocytes. The experiment 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 two-min incubation periods, extracellular label was removed by six rapid washes in ice-cold transport medium, and individual oocytes were dissolved in 5% (w/v) SDS 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 (Jandel Scientific Software, USA). 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 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) as suspension cultures. Cells were maintained in the absence of antibiotics, incubated at 37°C in a humidified atmosphere (5% CO₂), and subcultured at two to four-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 one min and one hr. 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. Radioactivity in solubilized extracts was measured by liquid scintillation counting. Uptake values were expressed as pmol/10⁶ cells and graphs were generated using the software GraphPad Prism version 4.0 (GraphPad Software Inc., San Diego, CA). 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 s 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 a single intravenous (IV) bolus dose of 0.1 mg/kg tecadenoson in saline. Animals were pre-treated with saline (Group 1) or 25 mg/kg NBMPR-P in saline (Group 2) via intraperitoneal (IP) injection 20 min prior to the treatment with tecadenoson. Two other groups (3, 4) of mice received a single IV bolus dose of 60 mg/kg fludarabine in water. Group 3 was pre-treated with saline and Group 4 received 25 mg/kg NBMPR-P in saline 20 min prior to treatment with fludarabine. Animals were sacrificed at various time points over six-h (tecadenoson) and eight-h (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 coupled to tandem mass spectrometry (LC/MSMS). For LC-MSMS analysis, whole brain samples were homogenized in a three-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 ten-fold in methanol:water (1:1). The samples were then analyzed using an API5000 LC-MSMS (AB Sciex Instruments, Foster City, CA). A LC Column (Luna 3µ C18(2); 20x4.0mm), 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 Figure 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 Figure 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 > four-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 (two-min) and long (60-min) uptake intervals in the
presence and absence of 10 µM uridine (Figure 3, Panels 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 \[^{3}H\]tecadenoson by recombinant hENT2-producing yeast showed no uptake over the first two min and moderate uptake over 60 min (Figure 3, Panels C and D), thus showing that hENT2 transports tecadenoson albeit poorly.

Transport of \[^{3}H\]tecadenoson by recombinant hENT1 was also demonstrated in Xenopus oocytes. Figure 4 shows a representative experiment comparing the concentration dependence (0 - 1 mM) of \[^{3}H\]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 min\(^{-1}\)). In contrast, uptake by hENT1-producing oocytes was rapid and saturable, the mediated component of transport (defined as the difference in uptake between hENT1-producing and control water-injected oocytes) giving calculated apparent K\(_{m}\) and V\(_{max}\) values (± S.E.) of 196 ± 9 µM and 46.7 ± 0.8 pmol/oocyte.2 min\(^{-1}\), respectively.

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

Since \[^{3}H\]tecadenoson transport by recombinant hNTs in yeast and Xenopus oocytes was shown to be mediated largely by hENT1, uptake of \[^{3}H\]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 (one min) and accumulation (one h) of 10 µM \[^3\text{H}\]tecadenoson and \[^3\text{H}\]fludarabine were similar during short and prolonged exposures (Figure 6, Panel A). The concentration dependence of initial rates of uptake of \[^3\text{H}\]tecadenoson exhibited saturation as tecadenoson concentrations were increased from 0 to 300 µM (Figure 6, Panel B). Apparent kinetic parameters obtained from Michaelis-Menten analysis after correction of uptake values for non-saturable uptake in the presence of excess unlabelled uridine (i.e., diffusion) yielded \(K_m\) and \(V_{\text{max}}\) values (+ S.E.) of 24 ± 6 µM and 3.4 ± 1 pmol/10^6 cells/s respectively. The lower apparent \(K_m\) value in CEM cells (24 µM) versus that in \textit{Xenopus} oocytes (196 µM) is in accord with differences noted for other permeants (Young et al., 2008; Parkinson et al., 2011).

In the \textit{in vivo} experiments, plasma and brain concentrations of tecadenoson (0.1 mg/kg IV) and fludarabine (60 mg/kg IV) from saline and NBMPR-P-treated mice were measured over six and eight-hr time courses. In these experiments, fludarabine was used as a positive control for NBMPR-dependent uptake into cells \textit{in vivo} (Adjei et al., 1992). Brain and plasma concentration-time profiles of tecadenoson and fludarabine are presented in Figure 7 (Panel A: top, brain tecadenoson; bottom, plasma tecadenoson. Panel B: top, brain fludarabine; bottom, plasma fludarabine) and the calculated pharmacokinetic parameters are shown in Tables 2 and 3. Prior treatment of mice with NBMPR-P reduced maximum drug concentration (\(C_{\text{max}}\)) 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 h (AUC\(_{0-4h}\)) was reduced from 22 ± 2 to 7 ± 1 ng.h/g. Similar observations were made in mice treated with fludarabine—i.e., in the group pre-treated with NBMPR-P, \(C_{\text{max}}\) of fludarabine was 801 ± 378 ng/g compared to 6050 ± 378 ng/g in the group pre-treated with saline and the AUC\(_{0-4h}\) of fludarabine was 2490 ± 418 ng.h/g in the NBMPR-P pre-treated group compared to 11300 ± 1550 ng.h/g in the saline pre-
treated group. These data suggest involvement of mENT1 in transport of tecadenoson across the BBB.
Discussion

A₁ adenosine receptors are present at high density in the CNS, and there is a significant potential for CNS-related adverse effects with A₁ 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 gender 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 A₁ adenosine receptors (Parkinson et al., 2011). The relatively hydrophilic nature of tecadenoson due to its ribose moiety and evidence of P-glycoprotein mediated efflux in multidrug resistance protein-1 (MDR1)-transfected cell lines (unpublished results) predicts poor brain penetration for tecadenoson. However, at higher doses (15 and 30 μg/kg), CNS-related side effects were observed in a clinical trial, suggesting that tecadenoson crosses the BBB and activates central A₁ adenosine receptors (Lerman et al., 2001).

Since tecadenoson, an A₁ adenosine receptor agonist, resembles adenosine, a permeant for NTs, the objective of this work was to investigate the role of individual nucleoside transporters in tecadenoson transport, and to assess NT-mediated transport across the BBB. We studied transmembrane transport of tecadenoson by recombinant human nucleoside transporters hENT1, hENT2, hCNT1, hCNT2, and hCNT3 in vitro and by native mouse mENT1 in vivo. Although all five recombinant hNT proteins bound tecadenoson with relatively high to moderate affinities, transport of tecadenoson was observed primarily with hENT1, to a smaller extent by hENT2 and not at all with the three hCNTs. Thus, introduction of a modification at the 6 position of the adenine moiety did not seriously limit interaction of tecadenoson with hENT1.

Our results showed that tecadenoson was a high-affinity inhibitor of hENT1, but a low-to-moderate affinity inhibitor of the other four hNTs. The presence of the C(3')-OH position of the sugar moiety in tecadenoson may partially explain why the modified
adenosine analog retained the ability to inhibit the five hNT subtypes, although the modification at the 6 position of the adenine moiety had a major effect on transportability by hENT2 and 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; Ritzel et al., 1998; Ritzel et al., 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 non-transported inhibitor of hCNT2 and hCNT3. Although hCNT3 has shown high tolerance to modifications in the uracil moiety of uridine (Zhang et al., 2003; Zhang et al., 2005), it exhibited a pronounced reduction in its interaction with tecadenoson, which has modification in the adenine moiety.

Kinetic experiments in CEM cells were carried out to measure the apparent affinity of native hENT1 for tecadenoson to determine if hENT1 could also play a role in uptake of tecadenoson at concentrations that are pharmacologically relevant in the \textit{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, the 5'-monophosphoester of NBMPR, has been employed in mice as a readily soluble form of NBMPR; NBMPR-P \textit{per se} does not inhibit nucleoside transport, but
NBMPR released by dephosphorylation *in vivo* is responsible for inhibition of nucleoside uptake by ENTs- ENT1 at nanomolar and ENT2 at 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 one hr. 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-gp may have contributed to the small, NBMPR-insensitive portion of influx and elimination of 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 hr 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 mENT1 *in vivo*.
vivo. The micromolar $K_m$ value exhibited by native hENT1 for tecadenoson suggests that the transporter would be active at clinically relevant concentrations of tecadenoson (nM 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 where a CNS effect is desired, our results illustrate how synthetic A$_1$ agonists targeting CNS disorders would exploit ENT1 to enhance delivery to the brain.
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Author contributions:

Participated in research design: Cass, Damaraju, Gati, Karpinski, Lepist, Leung, Young, and Zhang.

Conducted experiments: Smith, Yao, and Zhang.

Performed data analysis: Damaraju, Lepist, Smith, Yao, and Zhang.

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

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Figure legends:

Figure 1: Structures of adenosine, fludarabine and tecadenoson

Figure 2: Transport inhibition assays in yeast producing human recombinant hENT1
Relative abilities of uridine (□), adenosine (Δ) or tecadenoson (○) to inhibit the uptake of 1 µM [3H]uridine were assessed as described in Materials and Methods. Shown are concentration dependencies of uridine, adenosine, and tecadenoson inhibition of hENT1-mediated [3H]uridine uptake in yeast producing hENT1, and 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.

Figure 3: Studies of tecadenoson uptake by recombinant hENT1 and hENT2 in yeast.
The uptake of 1 µM [3H]tecadenoson by yeast that were transformed with pYPhENT1 or pYPhENT2 was measured alone (open symbols) or with excess 10 mM non-radioactive uridine (closed symbols) for 60 min. Panels A and C show time courses for the first two min and Panels B and D for 60 min of [3H]tecadenoson uptake by yeast producing recombinant hENT1 or hENT2, respectively.

Figure 4: Kinetics of tecadenoson transport by recombinant hENT1 produced in Xenopus oocytes.
Measured as initial rates of transport (2-min fluxes), values for the concentration dependence of uptake of [3H]tecadenoson in Xenopus oocytes producing hENT1 (solid circles) and in control water-injected oocytes (open circles) are means (± S.E.M.) of 10-12 individual oocytes. Calculated kinetic parameters (Km and Vmax) for the mediated component of transport, defined as the difference in uptake by hENT1-producing and control water-injected oocytes, are given in the text.
Figure 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.

Figure 6: Transport of tecadenoson and fludarabine by native hENT1 in CEM cells.

Panel A: Short (1 min) and long (1 h) 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. Panel B:

Concentration dependence of transport of [³H]tecadenoson measured as 5-sec fluxes in CEM cells. The flux values shown are means ± S.E.M. of three independent experiments. Each experiment was performed in triplicate.

Figure 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 and plasma concentrations of tecadenoson after 0.1 mg/kg IV dose in mice with (□ and dashed lines) and without NBMPR-P (●) (Panel A); and mean (± S.D, n=4) brain and plasma concentrations of fludarabine after 60 mg/kg IV dose in mice with (□ and dashed lines) and without NBMPR-P (●) (Panel B).
Table 1. Inhibition of hENT1, hENT2, hCNT1, hCNT2 and hCNT3-mediated [3H]uridine transport by uridine, adenosine and tecadenoson. \( IC_{50} \) values for inhibition of [3H]uridine transport by recombinant hNTs in yeast were derived from computer-generated concentration-effect relationships and represent mean inhibitor concentrations at which initial rates of uptake of 1 µM [3H]uridine were inhibited by 50% (see Figure 2), and \( K_i \) (inhibitory constant) values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973) as described in Materials and Methods. Data are presented as means ± S.E.M, \( n \geq 3 \).

<table>
<thead>
<tr>
<th></th>
<th>Uridine</th>
<th>Adenosine</th>
<th>Tecadenoson</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( IC_{50} ) (µM)</td>
<td>( K_i ) (µM)</td>
<td>( IC_{50} ) (µM)</td>
</tr>
<tr>
<td>hENT1</td>
<td>( 52 \pm 5 ) (n = 4)</td>
<td>( 51 \pm 5 )</td>
<td>( 17 \pm 1 ) (n = 3)</td>
</tr>
<tr>
<td>hENT2</td>
<td>( 234 \pm 22 ) (n = 6)</td>
<td>( 242 \pm 22 )</td>
<td>( 106 \pm 5 ) (n = 3)</td>
</tr>
<tr>
<td>hCNT1</td>
<td>( 5.8 \pm 0.7 ) (n = 4)</td>
<td>( 5.2 \pm 0.6 )</td>
<td>( 2 \pm 0.4 ) (n = 3)</td>
</tr>
<tr>
<td>hCNT2</td>
<td>( 32 \pm 4 ) (n = 5)</td>
<td>( 31 \pm 4 )</td>
<td>( 5.5 \pm 1.6 ) (n = 3)</td>
</tr>
<tr>
<td>hCNT3</td>
<td>( 5.4 \pm 0.9 ) (n = 4)</td>
<td>( 3.4 \pm 0.6 )</td>
<td>( 3.3 \pm 0.4 ) (n = 3)</td>
</tr>
</tbody>
</table>
Table 2. Brain exposure of tecadenoson after 0.1 mg/kg IV bolus dose and fludarabine after 60 mg/kg IV bolus dose in mice with and without pretreatment with NBMPR-P. Data are presented as means ± S.D., n=4.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>$T_{\text{max}}$ (h)</th>
<th>$C_{\text{max}}$ (ng/g)</th>
<th>$\text{AUC}_{0-4\text{h}}$ (ng.h/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tecadenoson</td>
<td>0.11 ± 0.15</td>
<td>23 ± 3</td>
<td>22 ± 2</td>
</tr>
<tr>
<td>Tecadenoson + NBMPR-P</td>
<td>0.19 ± 0.17</td>
<td>4 ± 1</td>
<td>7 ± 1</td>
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<tr>
<td>Fludarabine</td>
<td>0.88 ± 0.25</td>
<td>6050 ± 378</td>
<td>11300 ± 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>
Table 3. Pharmacokinetic parameters of tecadenoson in plasma after 0.1 mg/kg IV bolus dose and fludarabine after 60 mg/kg IV bolus dose with and without pretreatment with NBMPR-P. Data are presented as means ± S.D., n=4.

<table>
<thead>
<tr>
<th>Compound</th>
<th>$V_{ss}$ (L/kg)</th>
<th>CL (L/h/kg)</th>
<th>$t_{1/2}$ (h)</th>
<th>AUC$_{0-\infty}$ (ng.h/ml)</th>
</tr>
</thead>
<tbody>
<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>
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<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>50.5 ± 5.5</td>
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<tr>
<td>Fludarabine</td>
<td>1.6 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.7 ± 0.5</td>
<td>39600 ± 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>65700 ± 10300</td>
</tr>
</tbody>
</table>
Figure 2

[^3]H Uridine uptake (% Control)

log[Compounds] mM
Figure 3

A

[BH]Tecadenoson uptake (pmol/mg protein)

Time (min)

B

[BH]Tecadenoson uptake (pmol/mg protein)

Time (min)

C

[BH]Tecadenoson uptake (pmol/mg protein)

Time (min)

D

[BH]Tecadenoson uptake (pmol/mg protein)

Time (min)