Residue 33 of Human Equilibrative Nucleoside Transporter 2 is a Functionally Important Component of Both the Dipyridamole and Nucleoside Binding Sites

Frank Visser, Jing Zhang, R. Taylor Raborn, Stephen A. Baldwin, James D. Young and Carol E. Cass

From the Department of Oncology, Cross Cancer Institute and the University of Alberta, Edmonton, Alberta T6G 1Z2, Canada
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To whom correspondence should be addressed: Dr. Carol E. Cass, Department of Oncology, Cross Cancer Institute, 11560 University Avenue T6G 1Z2, Edmonton, Alberta T6G 1Z2, Canada (Tel: 780-432-8320, Fax: 780-432-8425, Email: carol.cass@cancerboard.ab.ca)

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The abbreviations used are: (h)ENT, (human) equilibrative nucleoside transporter; NBMPR, nitrobenzylmercaptopurine ribonucleoside (6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosyl purine); pCMBS, p-chloromercuribenzy1 sulphonate; TM, transmembrane domain; CMM, complete minimal medium; EC50, concentration of reagent resulting in 50% effectiveness; IC50, concentration of inhibitor for 50% inhibition of total mediated transport.
ABSTRACT

Human equilibrative nucleoside transporters 1 and 2 (hENT1 and hENT2) differ functionally in that hENT2 generally displays lower affinity for its nucleoside permeants and is less sensitive to inhibition by the coronary vasodilators dilazep and dipyridamole. In previous work, we demonstrated that mutation of residues 33 (Met vs Ile) of hENT1 and hENT2 altered sensitivity to dilazep and dipyridamole and that the hENT2 mutant (I33M) displayed a $K_m$ value for uridine that was lower than that of hENT2 and similar to that of hENT1 (Visser et al. (2002) J Biol Chem 277: 395-401). In this study we report results of an in-depth investigation of the role of residue 33 in hENT2. We found that hENT2-I33M displayed decreased $K_m$ values for both pyrimidine and purine nucleosides and increased $V_{max}$ values for purine nucleosides. Cys or Ser at position 33 had similar effects on the kinetic parameters of hENT2 as Met, indicating that hydrophobic (Met, Cys) or hydrogen-bonding energy (Ser) contributed to permeant binding by these residues. hENT2-I33M and I33C displayed increased sensitivities to dipyridamole compared to wildtype hENT2, hENT2-I33A and I33S, suggesting interaction of the sulfur atom of Met and Cys with aromatic moieties on dipyridamole. hENT2-I33C was inhibited by the membrane-impermeant sulfhydryl reactive reagent p-chloromercuribenzenyl sulphonate (pCMBS) and uridine, adenosine and dipyridamole protected against inhibition. Our results indicated that residue 33 resides in an extracellular domain as predicted by the current hENT2 topology model and suggested that is a functionally important component of both the permeant and dipyridamole binding sites.
**INTRODUCTION**

Integral membrane proteins mediate the transport of hydrophilic nucleosides and anticancer or antiviral nucleoside analogs across biological membranes (Cass et al., 1999). Equilibrative nucleoside transporters (ENTs) in mammalian cells mediate facilitated diffusion of nucleosides down their concentration gradients. Four ENT family members have been identified by molecular cloning: hENT1, hENT2, hENT3 and hENT4. hENT1 and hENT2 display equilibrative sensitive (es) and equilibrative insensitive (ei) transport activities, respectively, based on their differential sensitivities to the inhibitor nitrobenzylmercaptopurine ribonucleoside (NBMPR) (Crawford et al., 1998; Griffiths et al., 1997a; Griffiths et al., 1997b). Neither hENT3 or hENT4 have been functionally characterized, but hENT3 is believed to be a transporter of intracellular membranes (Acimovic and Coe, 2002; Hyde, 2001). Although hENT1 and hENT2 mediate the transport of a broad variety of nucleoside permeants, kinetic analyses have revealed that hENT2 generally displays lower affinities (higher $K_m$ values) for its permeants and, unlike hENT1, can also transport nucleobases (Ward et al., 2000; Yao et al., 2002). The amino acid residues responsible for these functional differences have not been identified.

ENT proteins control extracellular concentrations of adenosine, a ligand for cell-surface adenosine receptors that facilitates a variety of physiological responses, such as coronary vasodilation, renal vasoconstriction, platelet aggregation, and neuromodulation (Van Belle, 1993). hENT1 and hENT2 are the pharmacological targets of the coronary vasodilators dilazep and dipyridamole and differ in their sensitivities to these inhibitors by two to three orders of magnitude with hENT1 being more sensitive (Visser et al., 2002).

Despite limited sequence identities, all members of the ENT family share a common putative topology model consisting of 11 transmembrane domains (TMs), a large extracellular
loop between TMs 1 and 2 and a large cytoplasmic loop between TMs 6 and 7 (Sundaram et al., 2001). The current level of knowledge of the structure and function of these transporter proteins is limited. A number of studies on chimeric constructs involving domain swaps between different members of the ENT family have implicated TMs 3 – 6 as a region involved in permeant and inhibitor binding (Sundaram et al., 1998; Sundaram, 2001; Yao et al., 2001; Yao et al., 2002). In addition, Cys 140 in TM 4 of rat ENT2 has been demonstrated by sulfhydryl modification experiments to form part of the permeant translocation pore, and the corresponding residue of hENT1, Gly 154, is critical for NBMPR sensitivity (SenGupta and Unadkat, 2004; Yao, 2001). Other mutagenesis studies have identified Gly 179 in TM 4 and Leu 92 in TM 2 of hENT1 as residues that, when mutated, impair inhibitor binding and transporter function (Endres et al., 2004; SenGupta et al., 2002). Single residues in TMs 5, 7 and 8 of the LdNT transporters, which are ENT family members from the parasitic protozoan *Leishmania donovani*, were demonstrated to play important roles in transporter function (Arastu-Kapur et al., 2003; Vasudevan et al., 2001). Furthermore, by use of the substituted cysteine accessibility method, TM 5 of LdNT1.1 was shown to line the permeant translocation pathway (Valdes et al., 2004).

In previous work, we found that mutation of Met 33 of hENT1 to Ile, the corresponding residue in hENT2, resulted in ~10-fold reduced affinities for dilazep and dipyridamole, whereas the reciprocal mutation of Ile 33 of hENT2 to Met resulted in ~10-fold increased sensitivities to these inhibitors (Visser et al., 2002). hENT1-M33I displayed similar kinetic parameters for uridine transport to those of wildtype hENT1, whereas hENT2-I33M displayed kinetic parameters that were more similar to those of hENT1 than hENT2. In this work, we utilized an improved method for the functional characterization of recombinant hENT1 and hENT2 in yeast (Vickers et al., 2004; Visser et al., 2002; Zhang et al., 2003) to determine the
kinetic properties of hENT1-M33I, hENT2-I33M and a series of hENT2 mutants at residue 33 for a variety of different nucleoside permeants. These experiments revealed that hENT2-I33M had higher transport activities than wildtype hENT2 for all the permeants tested whereas hENT1-M33I was functionally similar to wildtype hENT1. The residue 33 hENT2 mutants were also tested for their sensitivities to dipyridamole and the membrane impermeant sulfhydryl-reactive reagent p-chloromercuribenzyl sulphonate (pCMBS). The results of these studies indicated that residue 33 was accessible from the extracellular side of the membrane and suggested that it is a common functional determinant of the nucleoside and dipyridamole binding sites.
MATERIALS AND METHODS

Strains and media – KY114 (MATα, gal, ura3-52, trp1, lys2, ade2, hisd2000) was the parental yeast strain used to generate fui1::TRP1, which contains a disruption in the gene encoding the endogenous uridine permease (FUI1) (Vickers et al., 2000). Other strains were generated by transformation of the *Saccharomyces cerevisiae*/*Escherichia coli* shuttle vector pYPGE15 (Brunelli, 1993) using a standard lithium acetate method (Gietz, 1992). cDNA inserts were under the transcriptional control of the constitutive PGK promoter. Yeast strains were maintained in complete minimal medium (CMM) containing 0.67% yeast nitrogen base (Difco, Detroit, MI), amino acids (as required to maintain auxotrophic selection) and 2% glucose. Plasmids were propagated in the *E. coli* strain TOP10F’ (Invitrogen, Carlsbad, CA) and maintained in Luria broth with ampicillin (0.1 mg/ml).

Plasmid Construction and Site-directed Mutagenesis – The cDNAs encoding hENT1, hENT1-M33I, hENT2 and hENT2-I33M were subcloned into pYPGE15 to generate pYPhENT1, pYPhENT1-M33I, pYPhENT2, pYPhENT2-I33M as previously described (Visser et al., 2002). pYPhENT2-I33C, pYPhENT2-I33A and pYPhENT2-I33S were generated using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). All constructs were verified by DNA sequencing using an ABI PRISM 310 sequence detection system (Perkin Elmer Life Sciences, Wellesley, MA).

Nucleoside transport in *S. cerevisiae* – Yeast cells containing pYPhENT1, pYPhENT2 or plasmid with one of the constructs encoding the various mutant transporters were grown in CMM media to $A_{600} = 0.5-1.0$, washed twice in fresh medium and resuspended to $A_{600} = 4.0$. All transport assays were performed at room temperature and pH 7.4. All unlabeled nucleosides and nucleoside analogs, dilazep, dipyridamole and NBMPR were obtained from...
Sigma, St. Louis, MO. The radiolabeled compounds [5,6-\textsuperscript{3}H]-uridine, [5-\textsuperscript{3}H(N)]-cytidine, [methyl-\textsuperscript{3}H]-thymidine, [2,8-\textsuperscript{3}H]-adenosine, [2,8-\textsuperscript{3}H]-inosine, [8-\textsuperscript{3}H]-guanosine, [6-\textsuperscript{3}H]-5-fluorouridine, [5-\textsuperscript{3}H]-2’,2’-difluoro-2’-deoxycytidine (gemcitabine), [5-\textsuperscript{3}H]-cytosine-\beta-D-arabinofuranoside (cytarabine), [8-\textsuperscript{3}H]-2-chloro-2’-deoxyadenosine (cladribine) and [8-\textsuperscript{3}H]-2-fluoro-arabinofuranosyl adenine (fludarabine) were purchased from Moravek Biochemicals, Brea, CA. A final specific activity of 0.5 µCi/µl was used in all transport reactions. Fifty-µl portions of yeast culture were added to 50-µl portions of 2X concentrated [\textsuperscript{3}H]-nucleoside in 96-well microtiter plates. At a given time point, the yeast cells were collected on a filtermat using a Micro96 Cell Harvester (Skatron Instruments, Norway) and rapidly washed with deionized water. The individual filter circles corresponding to individual wells of microtiter plates were removed from the filtermats using forceps and transferred to vials for liquid scintillation counting. Trace uridine transport activity in fui1::TRP1 yeast due to the presence of the endogenous uracil/uridine permease, FUR4, was subtracted by determining background uptake in the presence of 10 mM thymidine, which does not interact with any endogenous transport systems.

For determination of nucleoside concentration-effect relationships, unlabeled nucleosides and [\textsuperscript{3}H]-adenosine were added simultaneously to yeast suspensions. For dipyridamole concentration-effect relationships, the yeast suspensions were first incubated for 15-30 min with dipyridamole to allow for equilibration of the inhibitor with its binding sites before the addition of radiolabeled permeant as previously described (Visser et al., 2002).

\textit{pCMBS experiments} - Yeast containing pYPhENT1, pYPhENT2 or one of the various mutant plasmids were grown in CMM media to A\textsubscript{600} of 0.5-1.0, washed twice in ice-cold fresh medium (pH 7.4) and resuspended to an A\textsubscript{600} of 2.0. All reactions were performed on ice.
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(Yao, 2001). The yeast cells were distributed to microcentrifuge tubes to which pCMBS (Toronto Research Chemicals, Toronto, ON) was added alone or together with uridine, adenosine, dilazep, dipyridamole or NBMPR. Following a 30-min incubation period, the cells were centrifuged and washed 3X with ice-cold medium to remove unreacted pCMBS, nucleosides and inhibitors. The cells were resuspended to an A$_{600}$ of 4.0 and distributed to 96-well microtiter plates for nucleoside transport assays.
RESULTS

Initial rates of nucleoside transport by recombinant hENT1 and hENT2 produced in yeast – Fui1::TRP1 yeast cells containing either pYPhENT1, pYPhENT2 or pYPGE15 (vector without insert) were incubated in the presence of 1 or 1000 µM [³H]-labeled uridine, cytidine, thymidine, adenosine, inosine or guanosine for various intervals within 0 to 30 s and 0 to 60 min (data not shown). For yeast with recombinant hENT1, the rates of uptake of 1 or 1000 µM uridine, cytidine, adenosine, inosine and guanosine from 0 to 30 s were linear and not significantly different from the rates observed from 0 to 10 min, and for uridine and adenosine from 0 to 60 min. For yeast with hENT2, rates of uptake of 1 or 1000 µM uridine, adenosine and inosine were linear from 0 to 30 s and not significantly different from the rates observed at time points up to 10 min. Uptake of [³H]-cytidine and [³H]-guanosine by yeast producing hENT2 was significant but with poor signal-to-noise ratios, and subsequent kinetic experiments did not yield reproducible data. Uptake of [³H]-thymidine by yeast producing either hENT1 or hENT2 was very poor even though thymidine is a known permeant of both transporters (Griffiths et al., 1997a; Griffiths et al., 1997b) and unlabeled thymidine was a potent inhibitor of nucleoside transport in yeast with either transporter (data not shown). The low uptake of thymidine was likely because fui1::TRP1 yeast do not possess thymidine kinase and thus cannot metabolically “trap” thymidine once inside the cell. However, metabolism did not appear to be rate-limiting for uptake of the other nucleoside permeants since functional differences were observed between hENT1, hENT2 and mutants thereof, indicating that the transported permeants were rapidly trapped and the intracellular concentrations of free nucleosides were, therefore, negligible. For yeast containing pYPGE15, the rates of uptake for all [³H]-labeled nucleosides were low and similar to those of yeast producing recombinant hENT1 or hENT2 in the presence of 10 mM...
unlabeled thymidine or uridine. For all subsequent experiments initial rates of nucleoside transport were estimated from values of total uptake at 10 min minus values observed in the presence of 10 mM unlabeled thymidine.

**Nucleoside transport by hENT1, hENT1-M33I, hENT2 and hENT2-I33M** – Yeast cells containing either pYPPhENT1, pYPPhENT1-M33I, pYPPhENT2, pYPPhENT2-I33M or pYPGE15 were incubated in the presence of 10 µM [³H]-labeled uridine, cytidine, adenosine, inosine or guanosine for 10 min (Fig. 1A). hENT1 and hENT1-M33I displayed similar rates of uptake for all of the nucleosides whereas hENT2-I33M displayed rates of uptake that were higher than those of wildtype hENT2 and similar to those of hENT1. This experiment demonstrated total uptake observed at a 10-min timepoint and did not distinguish between mediated and non-mediated uptake.

Uptake of the [³H]-labeled nucleoside analog drugs (5-fluorouridine, gemcitabine, cytarabine, cladribine and fludarabine) into yeast cells containing pYPPhENT1, pYPPhENT2, pYPPhENT2-I33M or pYPGE15 was also determined (Fig. 1B). Consistent with what was observed for the naturally occurring nucleosides, hENT2-I33M displayed rates of uptake that were higher than those of hENT2 and similar to those of hENT1 with the exception of the cytidine analogs gemcitabine and cytarabine, of which hENT2-I33M-mediated uptake was higher then either wildtype protein.

**Inhibition of adenosine transport mediated by recombinant hENT1 and hENT2 by physiological permeants** – Concentration-effect relationships for inhibition of 1 µM [³H]-adenosine transport by recombinant hENT1 and hENT2 in yeast by graded concentrations of either uridine, cytidine, thymidine, adenosine, inosine or guanosine were determined. The resulting IC₅₀ values were used to calculate $K_i$ values using the equation of Cheng and Prusoff:
\[ K_i = \frac{IC_{50}}{1 + [S]/K_m} \] where \([S]\) is the permeant concentration (Cheng and Prusoff, 1973). The results are given in Tables I (hENT1 series) and II (hENT2 series). The \(K_i\) values obtained for inhibition of adenosine transport were similar to the observed \(K_m\) values for transport of the same permeant, indicating that a common transporter (hENT1 or hENT2) was responsible for uptake of the permeants tested. Furthermore, the \(K_i\) values served as surrogate measures of the affinities of the transporter for its permeants, which enabled assessment of hENT2 interactions with cytidine and guanosine (Table II).

**Kinetic parameters of hENT1 and hENT1-M33I** – The concentration dependence of \([^3H]-labeled uridine, cytidine, adenosine, inosine and guanosine transport was determined for yeast cells containing either pYPvENT1 or pYPvENT1-M33I (Table I). Both wildtype and mutant hENT1 conformed to simple Michaelis-Menten kinetics for all nucleosides tested. Recombinant hENT1-M33I displayed apparent \(K_m\) values that were similar to those of hENT1 whereas the mutant consistently displayed lower \(V_{max}\) values than hENT1, which likely reflected a lower abundance of the mutant protein in the plasma membrane. The \(V_{max}:K_m\) ratios, which reflect transporter efficiencies for the various nucleoside permeants, for hENT1-M33I were similar to those of hENT1. These results suggested that there were no apparent functional differences between mutant and wildtype hENT1.

**Kinetic parameters of hENT2 and various residue 33 mutants** – The concentration dependence of \([^3H]-labeled uridine, cytidine, adenosine, inosine and guanosine transport was determined for yeast cells containing pYPvENT2 or pYPvENT2-I33M (Table II). As was observed for hENT1, both wildtype hENT2 and hENT2-I33M conformed to simple Michaelis-Menten kinetics for all the nucleoside permeants tested. Recombinant hENT2-I33M displayed \(K_m\) values for the pyrimidine nucleosides uridine and cytidine that were similar to those of
hENT1 and ~25 % of those of wildtype hENT2. Although the $K_m$ values of hENT2-I33M for adenosine, inosine and guanosine were lower than those of wildtype hENT2, they were higher than those of hENT1. The $V_{\text{max}}$ values of hENT2-I33M for the purine nucleosides, particularly adenosine, were significantly higher than those of either hENT1 or hENT2. The $V_{\text{max}}:K_m$ ratios of hENT2-I33M for all the nucleoside permeants tested were higher than those of hENT2 and similar to those of hENT1, providing an explanation for the differences in nucleoside uptake results observed in Fig. 1A.

To test the effects of substituting different amino acid side chains at residue 33 of hENT2, additional mutations were generated (hENT2-I33A, I33C and I33S) and the kinetic parameters of uridine and adenosine transport were determined (Table II). Representative rate vs concentration plots and Eadie Hofstee plots for adenosine transport by hENT2 and all the residue 33 mutants are presented in Fig. 2. hENT2-I33A displayed similar $K_m$ and $V_{\text{max}}$ values to those of hENT2, whereas the values for hENT2-I33C and I33S were similar to those of hENT2-I33M in that they displayed $K_m$ values for uridine that were 20 to 30 % of wildtype and $V_{\text{max}}$ values for adenosine that were 2 to 3 - fold higher. hENT2-I33M and hENT2-I33C displayed modestly lower $K_m$ values for adenosine compared to hENT2 whereas hENT2-I33S only displayed an increase in its $V_{\text{max}}$ value. These results suggested that Met, Cys and Ser side chains at residue 33 of hENT2 were all capable of promoting similar interactions with uridine and adenosine.

Concentration-effect relationships for dipyridamole inhibition of hENT2 and various residue 33 mutants – hENT2-I33M was previously shown to be more sensitive to dipyridamole than wildtype hENT2 (Visser et al., 2002). Yeast cells producing either hENT2 or one of the residue 33 mutants were incubated in the presence of 1 $\mu$M [3H]-uridine in the absence (control) or presence of graded concentrations of dipyridamole (Fig. 3). The resulting IC$_{50}$ values were

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used to calculate $K_i$ values using the equation of Cheng and Prusoff as was done in Tables I and II (Table III). hENT2 and hENT2-I33A displayed similar $K_i$ values whereas hENT2-I33M and I33C were 14 and 18-fold more sensitive, respectively, and hENT2-I33S was only 1/5 as sensitive to dipyridamole inhibition as wildtype. The $K_i$ values were used to calculate the Gibbs free energy for dipyridamole binding by hENT2 and the residue 33 mutants using the equation: 

$$\Delta G^o = -RT\ln(K_i)$$  

where $R$ is the gas constant and $T$ is the temperature (Table III). In hENT2-I33M and I33C, the Met and Cys side chains each contributed an additional 7 kJ/mol to dipyridamole binding.

Sulfhydryl modification of hENT2 and various residue 33 mutants – Yeast cells producing hENT2, hENT2-I33M, hENT2-I33C, hENT2-I33A or hENT2-I33S were incubated with graded concentrations of p-chloromercuriphenyl sulphonate (pCMBS), a membrane-impermeant sulfhydryl-reactive reagent, followed by measurement of 1 µM $[^3H]$-uridine uptake (Fig. 4). hENT2, hENT2-I33A, hENT2-I33S and hENT2-I33M-mediated uridine uptake was insensitive to concentrations of pCMBS up to 3 mM, whereas hENT2-I33C-mediated uridine uptake was inhibited in a dose-dependent manner to a maximum inhibition of 64% and an average IC$_{50}$ value of 8.8 ± 1.0 µM (n=3). The average Hill slope of hENT2-I33C inhibition by pCMBS was 1.08 ± 0.14, suggesting that modification of a single Cys residue was responsible for the observed effect. The observation that hENT2-I33A and I33S were insensitive to pCMBS suggested that the substitution of an amino acid residue with a small side chain did not induce a conformational change leading to the exposure of an endogenous pCMBS-reactive Cys residue. Furthermore, the observation that wildtype hENT2 was insensitive to inhibition by pCMBS was consistent with previously published work (Yao, 2001). The current putative topology model of hENT2 places residue 33 as the last position on the extracellular end of TM 1 (Griffiths et al.,
1997b; Sundaram et al., 2001; Yao, 2001) and the observation that residue 33 was accessible to pCMBS supported this model.

**Permeant and inhibitor protection of hENT2-I33C from pCMBS modification** –

Yeast cells producing hENT2-I33C were incubated in the presence or absence of 0.1 mM pCMBS either alone or together with (i) 1 mM adenosine or uridine, (ii) 10 µM dilazep or dipyridamole or (iv) 1 µM NBMPR and then assayed for [3H]-uridine uptake (Fig. 5A). The presence of either adenosine, uridine or dipyridamole protected hENT2-I33C from pCMBS inhibition whereas dilazep and NBMPR did not protect against the pCMBS-dependent inhibition. Yeast cells producing hENT2-I33C that had been incubated with 0.1 mM pCMBS and subsequently incubated with 1 mM dithiothreitol exhibited full recovery of uridine uptake activity, demonstrating involvement of a sulfhydryl group in the pCMBS-dependent inhibition.

To determine the extent to which either adenosine, uridine, dipyridamole or dilazep protected yeast cells producing hENT2-I33C from pCMBS inhibition, graded concentrations of compound were tested in the experiments of Fig. 5B. hENT2-I33C was protected from pCMBS inhibition in a dose-dependent manner by uridine (EC50, 320 ± 50 µM, n=3), adenosine (EC50, 67.1 ± 8.9 µM, n=3) and dipyridamole (EC50, ≥ 10 µM, n=3), whereas dilazep had no protective effects at concentrations up to 1 mM. The highest dipyridamole concentration used for protection from pCMBS modification was 10 µM because this was the solubility limit on ice, the temperature at which the reactions were performed. The data suggested that binding of adenosine, uridine or dipyridamole prevented pCMBS from inhibiting hENT2-I33C and implied that residue 33 was involved in the binding of these compounds or at the least forms part of the immediate binding environment of these compounds.
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DISCUSSION

In this study, we have demonstrated that fui1::TRP1 yeast cells display little or no endogenous transport activity for uridine, cytidine, thymidine, adenosine, inosine and guanosine and are therefore a powerful heterologous expression system for the comprehensive functional analysis of recombinant hENT1 and hENT2. The observed affinity parameters, $K_i$ or $K_m$, for uridine were similar to those reported in other studies for recombinant hENT1 and hENT2 produced in yeast (Endres et al., 2004; Osato et al., 2003; SenGupta and Unadkat, 2004; Vickers et al., 2004). However, these parameters differed from those obtained in other recombinant expression systems such as *Xenopus laevis* oocytes and transfected mammalian cells (Griffiths et al., 1997a; Griffiths et al., 1997b; Ward et al., 2000; Yao et al., 1997). These discrepancies are likely due to differences in post-translational processing of the transporter protein and in the lipid composition of plasma membranes. Nonetheless, the relative affinities of hENT1 and hENT2 for their permeants were consistent with those reported in transfected cells with the exception of inosine and thymidine for hENT2 (Ward et al., 2000). Recombinant hENT1 and hENT2 were previously reported to have similar apparent affinities for uridine (Griffiths et al., 1997a; Griffiths et al., 1997b; Ward et al., 2000) although earlier studies of endogenous $es$ and $ei$ transport systems in cultured cells and rat erythrocytes had demonstrated lower affinities of the $ei$ transporter (i.e., ENT2) for uridine (Boleti et al., 1997; Jarvis and Young, 1986).

hENT2-I33M, I33C and I33S all displayed increased affinities for uridine (Table II), suggesting that residue 33 is an important functional determinant for the binding of uridine and other pyrimidine nucleosides. Furthermore, the observation that hENT2-I33M displayed increased apparent $V_{\text{max}}$ values for all purine nucleoside permeants and that hENT2-I33M, I33C and I33S all displayed notably increased apparent $V_{\text{max}}$ values for adenosine also suggested that
residue 33 was also an important functional determinant for the purine nucleoside transport activity of hENT2. That increased $V_{\text{max}}$ values were observed with adenosine and not with uridine suggested that the observed effects were not due to an increase in the plasma membrane abundance of the protein but rather to an increase in catalytic activity brought about by increases in the rate of conformational turnover and/or increases in protein flexibility. However, hENT2-I33S did not display a reduced $K_m$ value for adenosine (Table II), as was observed for hENT2-I33M and I33C, suggesting that residue 33 contributed to permeant recognition and transport. The Met and Cys side chains contain highly polarizable sulphur atoms (Gellman, 1991), that likely interacted with hydrophobic moieties on the base portions of uridine and adenosine whereas the Ser side chain evidently contributed hydrogen-bond interactions to uridine binding. Furthermore, Met was favored over Ile in this regard, probably because of its relatively high degree of conformational flexibility. The apparent ability of this residue to interact with different parts of the permeant molecule likely stems from the conformational flexibility in TM 1 brought about by the highly conserved glycine residues G22 and G24 (Hyde, 2001).

The observation that hENT1 and hENT1-M33I did not display functional differences suggested that residue 33 did not contribute to permeant interactions in hENT1 (Table I). This was likely due to minor structural differences between hENT1 and hENT2. In particular, the large extracellular loop that extends from TM 1 is considerably more hydrophobic in hENT1 than hENT2 and may affect the conformation and solvent accessibility of residue 33. Nonetheless, we have observed that mutations of W29 have specific effects on the permeant selectivity of hENT1 (unpublished observations). Because TM 1 is likely alpha-helical, as would be predicted from the crystal structures of other major facilitator superfamily proteins such as lac permease (Abramson et al., 2003), W29 is predicted to be in close proximity to
residue 33, suggesting that this region of hENT1 is involved in permeant recognition and transport.

The substitution of Cys or Met at residue 33 increased sensitivity to dipyridamole by 18 and 14-fold, respectively, whereas the substitution of Ile (wildtype), Ala or Ser were less favourable for dipyridamole inhibition (Fig. 3, Table III). That hENT2-I33S exhibited increased transport activity but decreased dipyridamole sensitivity was consistent with the conclusion that this residue could not engage in hydrogen bond interactions with the more hydrophobic dipyridamole molecule. Met or Cys at residue 33 likely participated in interactions with the pi electron cloud of the aromatic moieties on the dipyridamole structure, which was supported by the fact that the strength of the interaction (7 kJ/mol) is consistent with the strength of similar interactions observed in alpha-helices (3 to 8 kcal/mol) and is based on the high degree of polarizability of the sulphur atom (Pal and Chakrabarti, 2001; Viguera and Serrano, 1995).

hENT2-I33C was the only hENT2 mutant that displayed a dose-dependent sensitivity to the membrane-impermeant sulfhydryl reactive reagent pCMBS. These results independently confirmed the location of this residue on the putative topology model of hENT2, which places it as the last residue on the extracellular end of TM 1 (Sundaram et al., 2001). hENT2-I33C was protected from pCMBS modification by uridine, adenosine and dipyridamole. These results suggested that the binding of permeants or dipyridamole prevented pCMBS from reacting either by directly blocking access to residue 33 or by altering accessibility to residue 33 by a long-range conformational effect on the tertiary structure of hENT2. Given the functional significance of residue 33, we postulate a direct interaction between residue 33 and either nucleoside permeants or dipyridamole. Earlier studies had provided contradictory evidence for both competitive and allosteric binding of dipyridamole to hENT1 (Griffith and Jarvis, 1996).
However, the current results, which suggested that dipyridamole and permeants bind to the outward-facing conformation of mammalian ENTs, support the conclusion that dipyridamole and nucleoside permeant bind to the same or an overlapping site (Jarvis, 1986; Jarvis et al., 1983; Paterson et al., 1980). That Met was favored at residue 33 for permeant and inhibitor binding is consistent with the ability of ENTs to bind a large variety of chemically unrelated permeants and inhibitors. Met residues of the signal-recognition particle 45 (SRP45) and calmodulin have been implicated as critical for recognition of a wide variety of unrelated protein targets (Gellman, 1991).

Although our previous study (Visser et al., 2002) had suggested an important role for residue 33 in dilazep binding, dilazep was unable to protect against pCMBS inhibition of hENT2-I33C even though sensitivity to dilazep inhibition was retained. Dilazep and dipyridamole are believed to bind to the same or overlapping sites of hENT1 (Koren et al., 1983; Sundaram et al., 1998), which was supported by our previous study in which residue 33 mutations similarly affected the potency of these two inhibitors (Visser et al., 2002). The results of the current study suggest that dilazep may bind adjacent to residue 33 in a manner that does not occlude this residue.

In conclusion, our in-depth study of the impact of mutations of residue 33 in hENT2 in the yeast expression system has yielded novel information about the role of this residue in permeant and inhibitor interactions with hENT2 and hENT1. We confirmed our earlier conclusion (Visser et al., 2002) that residue 33 is a determinant of the overall functional differences between hENT1 and hENT2. We demonstrated that residue 33 is extracellular, thereby validating the predicted topology model for hENT2. Our results established residue 33 as a common exofacial determinant of the binding sites for nucleosides and inhibitors, providing
molecular evidence that dipyridamole competes with nucleosides for binding to hENT2. Although these conclusions are consistent with the data presented in this study, a crystal structure of the transporter would be necessary to further address the role of residue 33 in permeant and inhibitor interactions with hENT2.
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Yao SY, Ng AM, Muzyka WR, Griffiths M, Cass CE, Baldwin SA and Young JD (1997) Molecular cloning and functional characterization of nitrobenzylthioinosine (NBMPR)-
sensitive (es) and NBMPR-insensitive (ei) equilibrative nucleoside transporter proteins (rENT1 and rENT2) from rat tissues. *J Biol Chem* **272**:28423-30.


FOOTNOTES:

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Reprint requests:
Dr. Carol E. Cass
Department of Oncology
Cross Cancer Institute
11560 University Avenue T6G 1Z2
Edmonton, Alberta T6G 1Z2
Canada

LEGENDS FOR FIGURES

Fig. 1. Nucleoside and nucleoside analog uptake rates by hENT1, hENT1-M33I, hENT2 and hENT2-I33M. Yeast cells containing pYPENT1, pYPENT1-M33I, pYPENT2, pYPENT2-I33M or pYPGE15 (no insert) were incubated for 10 min with the following [3H]-labeled nucleosides or nucleoside analogs: (A) uridine, cytidine, adenosine, inosine, guanosine, and (B) 5-fluorouridine, gemcitabine, cytarabine, cladribine or fludarabine at a concentration of 10 µM. The representative uptake values are presented as the means ± standard error of triplicate determinations. Three separate experiments gave similar results. For each single experiment, all five yeast strains were assayed simultaneously for all the permeants indicated so that direct uptake rate comparisons could be made.

Fig. 2. Concentration-dependence of adenosine transport by hENT2 and various residue 33 mutants. (A) Yeast cells containing pYPENT2 (squares), pYPENT2-I33M (circles), pYPENT2-I33C (right-side up triangles), pYPENT2-I33A (upside down triangles) or pYPENT2-I33S (diamonds) were incubated for 10 min with increasing concentrations of [3H]-adenosine. The transport rates presented were derived from the difference between the uptake observed in the absence and presence of 10 mM unlabeled thymidine at each uridine concentration. K_m and V_max values were obtained by nonlinear regression analysis using GraphPad Prism version 4.0 software, and the average values from 3 - 5 separate experiments are presented in Table II. (B) Eadie-Hofstee plot of the data presented in panel A. Each point is presented as the mean ± standard error (n=4-9), and where the size of the point is larger than the standard error, it is not shown.
Fig. 3. Concentration dependence of dipyridamole inhibition of hENT2, hENT2-I33M, hENT2-I33C, hENT2-I33A and hENT2-I33S. Yeast cells containing pYPheNT2 (squares), pYPheNT2-I33M (circles), pYPheNT2-I33C (right-side up triangles), pYPheNT2-I33A (upside down triangles) or pYPheNT2-I33S (diamonds) were incubated with 1 µM [3H]-uridine for 10 min in the absence (control) or presence of graded concentrations of dipyridamole. IC50 values were determined by nonlinear regression analysis using GraphPad Prism version 4.0 software, and the average values from 3 separate experiments are presented in Table III. Each point is presented as the mean ± standard error (n=4), and where the size of the point is larger than the standard error, it is not shown.

Fig. 4. Concentration-dependence of pCMBS inhibition of hENT2 and various residue 33 mutants. Yeast cells containing pYPheNT2 (squares), pYPheNT2-I33M (circles), pYPheNT2-I33C (right-side up triangles), pYPheNT2-I33A (upside down triangles) or pYPheNT2-I33S (diamonds) were incubated in the absence or presence of graded concentrations of pCMBS followed by incubation with 1 µM [3H]-uridine for 10 min. Each point represents the mean ± standard error (n=4), and where the size of the point is larger than the standard error, it is not shown. IC50 values were determined by nonlinear regression using GraphPad Prism version 4.0 software, and average values are presented in the text. Three separate experiments gave similar results.

Fig. 5. Protection of hENT2-I33C from pCMBS modification by various permeants and inhibitors. (A) Yeast cells producing hENT2-I33C were incubated in the absence (control) or presence of 0.1 mM pCMBS with or without 1 mM uridine (Urd), 1 mM adenosine (Ado), 10
µM dilazep (Dil), 10 µM dipyridamole (Dip) or 1 µM NBMPR. Some of the yeast cells incubated with 0.1 mM pCMBS alone were subsequently incubated with 1 mM dithiothreitol. The yeast cells were then incubated for 10 min with 1 µM [³H]-uridine in the absence of the test protecting agents. Uridine uptake is presented as the mean ± standard error, n=4 and was analyzed using GraphPad Prism version 4.0 software. Three separate experiments gave similar results. (B) Yeast cells producing hENT2-I33C were incubated in the absence or presence of 0.1 mM pCMBS with or without graded concentrations of uridine (circles), adenosine (squares), dilazep (diamonds) or dipyridamole (triangles) followed by incubation for 10 min with 1 µM [³H]-uridine in the absence of the test protecting agents. Uridine transport rates in the absence of 0.1 mM pCMBS were set as 100 % of control whereas rates in the presence of 0.1 mM pCMBS were set as 0 % of control. EC₅₀ values were determined by nonlinear regression using GraphPad Prism version 4.0 software, and average values are presented in the text. Each point represents the mean ± standard error (n=4), and where the size of the point is larger than the standard error, it is not shown. Three separate experiments gave similar results.
Table I

Kinetic properties of hENT1 and hENT1-M33I

The $K_m$, $V_{max}$ and $K_i$ values shown are the means ± S.E. of 3-5 separate experiments. Representative curves for uridine are presented in Fig. 2A.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Permeant</th>
<th>Apparent $K_m$</th>
<th>Apparent $V_{max}$</th>
<th>$V_{max}$/Km</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu$M</td>
<td>pmol/mg/min</td>
<td>pmol/mg/min/\mu M</td>
<td>$\mu$M</td>
</tr>
<tr>
<td>hENT1</td>
<td>uridine</td>
<td>44.1 ± 2.6</td>
<td>1060 ± 20</td>
<td>24.0</td>
<td>51.9 ± 2.8</td>
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<tr>
<td></td>
<td>cytidine</td>
<td>234 ± 47</td>
<td>1280 ± 70</td>
<td>5.4</td>
<td>346 ± 49</td>
</tr>
<tr>
<td></td>
<td>thymidine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>81.6 ± 3.1</td>
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<tr>
<td></td>
<td>adenosine</td>
<td>17.8 ± 0.8</td>
<td>1330 ± 20</td>
<td>74.7</td>
<td>10.3 ± 0.4</td>
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<tr>
<td></td>
<td>inosine</td>
<td>28.5 ± 2.6</td>
<td>1300 ± 30</td>
<td>45.6</td>
<td>34.6 ± 1.9</td>
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<tr>
<td></td>
<td>guanosine</td>
<td>47.5 ± 4.8</td>
<td>1080 ± 30</td>
<td>22.7</td>
<td>48.6 ± 3.6</td>
</tr>
<tr>
<td>hENT1-M33I</td>
<td>uridine</td>
<td>30.0 ± 1.4</td>
<td>707 ± 10</td>
<td>23.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>cytidine</td>
<td>150 ± 38</td>
<td>814 ± 68</td>
<td>5.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>12.2 ± 0.5</td>
<td>1010 ± 10</td>
<td>82.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inosine</td>
<td>24.0 ± 4.0</td>
<td>804 ± 36</td>
<td>33.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>guanosine</td>
<td>49.8 ± 4.7</td>
<td>784 ± 36</td>
<td>15.7</td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined
Table II

Kinetic properties of hENT2, hENT2-I33M, hENT2-I33A, hENT2-I33C and hENT2-I33S

The $K_m$, $V_{max}$ and $K_i$ values shown are the means ± S.E. of 3-5 separate experiments. Representative curves for uridine and adenosine are presented in Fig. 2B and 3, respectively.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Permeant</th>
<th>Apparent $K_m$</th>
<th>Apparent $V_{max}$</th>
<th>$V_{max}$/$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu M$</td>
<td>pmol/mg/min</td>
<td>pmol/mg/min/$\mu M$</td>
<td>$\mu M$</td>
</tr>
<tr>
<td>hENT2</td>
<td>uridine</td>
<td>195 ± 14</td>
<td>1940 ± 60</td>
<td>9.9</td>
<td>216 ± 17</td>
</tr>
<tr>
<td></td>
<td>cytidine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1380 ± 170</td>
</tr>
<tr>
<td></td>
<td>thymidine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>129 ± 9</td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>106 ± 6</td>
<td>3420 ± 60</td>
<td>32.2</td>
<td>93.7 ± 8.1</td>
</tr>
<tr>
<td></td>
<td>inosine</td>
<td>180 ± 37</td>
<td>2020 ± 150</td>
<td>11.2</td>
<td>192 ± 29</td>
</tr>
<tr>
<td></td>
<td>guanosine</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>394 ± 70</td>
</tr>
<tr>
<td>hENT2-I33M</td>
<td>uridine</td>
<td>49.0 ± 2.3</td>
<td>1110 ± 20</td>
<td>22.6</td>
<td>4.3</td>
</tr>
<tr>
<td></td>
<td>cytidine</td>
<td>393 ± 77</td>
<td>1700 ± 70</td>
<td>4.3</td>
<td>231</td>
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<tr>
<td></td>
<td>adenosine</td>
<td>52.0 ± 2.0</td>
<td>10000 ± 110</td>
<td>35.8</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>inosine</td>
<td>95.6 ± 6.5</td>
<td>3420 ± 70</td>
<td>35.8</td>
<td>28.3</td>
</tr>
<tr>
<td></td>
<td>guanosine</td>
<td>81.2 ± 15.6</td>
<td>2300 ± 110</td>
<td>35.8</td>
<td>28.3</td>
</tr>
<tr>
<td>hENT2-I33A</td>
<td>uridine</td>
<td>213 ± 28</td>
<td>1410 ± 70</td>
<td>6.6</td>
<td>19.3</td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>104 ± 11</td>
<td>2010 ± 50</td>
<td>19.3</td>
<td>19.3</td>
</tr>
<tr>
<td>hENT2-I33C</td>
<td>uridine</td>
<td>39.1 ± 5.0</td>
<td>1640 ± 40</td>
<td>41.9</td>
<td>41.9</td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>43.6 ± 2.2</td>
<td>6830 ± 80</td>
<td>157</td>
<td>157</td>
</tr>
<tr>
<td>hENT2-I33S</td>
<td>uridine</td>
<td>67.1 ± 5.7</td>
<td>2080 ± 40</td>
<td>31.0</td>
<td>31.0</td>
</tr>
<tr>
<td></td>
<td>adenosine</td>
<td>98.5 ± 4.4</td>
<td>10200 ± 130</td>
<td>104</td>
<td>104</td>
</tr>
</tbody>
</table>

ND = not determined
Table III

*Inhibition of hENT2, hENT2-I33M and hENT2-I33C-mediated [*H]-uridine transport by dipyridamole*

\(K_i\) values are the mean ± S.E. calculated using the equation of Cheng and Prusoff (Cheng and Prusoff, 1973) with the IC\(_{50}\) values obtained by nonlinear regression analysis of the curves presented in Fig. 5 using GraphPad Prism version 4.0 software.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(K_i)</th>
<th>Ratio(^\alpha)</th>
<th>(\Delta G^\circ) µM</th>
<th>(\Delta G^\circ) kJ/mol</th>
</tr>
</thead>
<tbody>
<tr>
<td>hENT2</td>
<td>3.77 ± 0.25</td>
<td>1.00</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>hENT2-I33M</td>
<td>0.263 ± 0.011</td>
<td>14.3</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>hENT2-I33A</td>
<td>4.95 ± 0.55</td>
<td>0.76</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>hENT2-I33C</td>
<td>0.206 ± 0.018</td>
<td>18.3</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>hENT2-I33S</td>
<td>18.6 ± 4.7</td>
<td>0.20</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

\(^\alpha\)Ratio = \(K_i\) hENT2/\(K_i\) mutant
FIG. 1

Panel A: Uptake (pmol/mg/min) for uridine, cytidine, adenosine, inosine, and guanosine. Differentations are indicated by different bar colors: hENT1, hENT1-M33I, hENT2, hENT2-I33M, and no insert.

Panel B: Uptake (pmol/mg/min) for 5-fluorouridine, gemcitabine, cytarabine, cladribine, and fludarabine. Differentations are indicated by different bar colors: hENT1, hENT2, hENT2-I33M, and no insert.