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Residues Met89 and Ser160 in the Human Equilibrative Nucleoside Transporter 1 Affect Its Affinity for Adenosine, Guanosine, S^6-(4-Nitrobenzyl)-mercaptopurine Riboside, and Dipyridamole

Christopher J. Endres and Jashvant D. Unadkat

Department of Pharmaceutics, University of Washington, Seattle, Washington

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ABSTRACT

The human equilibrative nucleoside transporter 1 (hENT1) is an important modulator of the physiological action of adenosine. We identified amino acid residues involved in adenosine transport using a yeast-based assay to rapidly screen and identify randomly generated hENT1 mutants that exhibited decreased sensitivity to inhibition of adenosine transport by various hENT1 competitive inhibitors. We identified Met89 and Ser160 as important in the affinity of hENT1 for various substrates and inhibitors. Mutation to Met89Cys or Ser160Cys significantly ($p < 0.05$) increased the S^6-(4-nitrobenzyl)-mercaptopurine riboside (NBMPR) IC_{50} values by approximately 4- and 6-fold, respectively (42 ± 13 and 65 ± 1.6 nM) compared with the wild-type transporter (11 ± 0.7 nM). The double mutant Met89Cys/Ser160Cys synergistically increased the NBMPR IC_{50} value to approximately 19-fold of that of the wild-type transporter. In contrast, compared with wild-type hENT1, the sensitivity to dipyridamole inhibition was significantly ($p < 0.05$) increased by only the Ser160Cys (~2.6-fold) or the double mutant Met89Cys/Ser160Cys (~4.7-fold) but not by the Met89Cys mutant. Mutation to Met89Cys or Ser160Cys increased the K_m of adenosine (~8- and 3-fold) and the K_i of guanosine (~6- and 2-fold). The double mutant increased both the K_m value of adenosine and the K_i value of guanosine by ~8-fold and seemed to confer no additional reduction in adenosine or guanosine affinity than that by mutation of Met89 alone. Together, these data indicate that transmembrane domains (TMDs) 2 (Met89) and 4 (Ser160) of hENT1 interact and are important in conferring sensitivity to NBMPR. In contrast, Ser160 and Met89 of hENT1, respectively, play a dominant role in conferring sensitivity to dipyridamole and adenosine/guanosine affinity.

The endogenous nucleoside adenosine is an important regulator in autocrine and paracrine signaling through its interactions with adenosine receptors (A_1, A_{2A}, A_{2B}, and A_3) located in the plasma membrane (Mubagwa and Flameng, 2001). The extracellular adenosine concentration, and therefore its physiological and pharmacological activity, is modulated by nucleoside transporters that actively (concentrative nucleoside transporters) or facilitatively (equilibrative nucleoside transporters, ENTs) transport adenosine into the cell (Kong et al., 2004). For example, ENTs modulate extracellular adenosine concentration at the adenosine receptors in ethanol-induced ataxia (Choi et al., 2004), in adenosine-mediated cardioprotection in ischemia/reperfusion injury (Chaudary et al., 2004; Taniguchi et al., 2004), and in adenosine-mediated neuromodulation (Snell et al., 2004). ENTs also transport a number of antiviral and anticancer drugs such as ribavirin (Jarvis et al., 1998) and gemcitabine (Mackey et al., 1998). Therefore, a better understanding of the molecular mechanisms by which adenosine and other nucleosides (including nucleoside drugs) bind to and are translocated by nucleoside transporters may aid in the development of new drugs that either modulate adenosine availability to adenosine receptors or are improved antiviral or anticancer drugs.

hENT1, an equilibrative nucleoside transporter, is the most widely expressed member of the ENT family and is ubiquitously expressed (Pennycooke et al., 2001) including in the intestine (Chandrasena et al., 1997) and the kidney (Franco et al., 1990). hENT1 has broad nucleoside substrate selectively (Ward et al., 2000) and has an affinity for adenosine in the low micromolar range (Ward et al., 2000). hENT1

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ABBREVIATIONS: ENT, equilibrative nucleoside transporter; NBMPR, S^6-(4-nitrobenzyl)-mercaptopurine riboside; TMD, transmembrane domain.

is characterized by potent inhibition by NBMPR (Ward et al., 2000) and by inhibition by the non-nucleosides dipyridamole and dilazep (Visser et al., 2002).

A number of hENT1 amino acid residues have been identified which, when mutated, alter the affinity of hENT1 toward its substrates or inhibitors (Fig. 1 and Table 1). Using two different yeast expression assays, our laboratory has identified a number of these amino acid residues (SenGupta et al., 2002; Endres et al., 2004). The "adenosine rescue" assay is one of these and relies on the transport of extracellular adenosine by heterologously expressed hENT1 for yeast growth and survival. This assay can identify randomly generated hENT1 mutants that are capable of transporting adenosine despite the presence of the hENT1 inhibitors such as NBMPR, dipyridamole, or dilazep. In this article, we report the use of this adenosine rescue assay to identify hENT1 amino acid residues Met89 and Ser160 which, when either singly or simultaneously mutated, selectively reduce hENT1 affinity for adenosine or guanosine (but not other natural nucleosides) and hENT1 sensitivity to inhibition by NBMPR or dipyridamole but not dilazep. In addition, we have identified for the first time that these two residues seem to act synergistically in reducing the sensitivity of the transporter to NBMPR inhibition.

Materials and Methods

Screening of Random Mutants by Phenotypic Complementation. We screened our library of randomly generated hENT1 expressed in yeast strain W303-1A (MATa *ade2-1*, *can1-100*, *cyh2*, *his3-11,15*, *leu1-c*, *leu2-3,112*, *trp1-1*, and *ura3-1)* for random mutants resistant to inhibition by NBMPR, dipyridamole, and dilazep in an adenosine rescue plate assay described previously (Endres et al., 2004). In brief, the yeast cells transformed with random mutants were replica-plated onto GR-Ura-Ade [2% galactose, 1% raffinose, 1% yeast nitrogen base (Difco, Detroit, MI), and 1% amino acid mix-uracil-adenine) plates containing 0 or 150 μM adenosine and the presence or absence of 2 μM NBMPR, 50 μM dipyridamole, or 10 μM dilazep (Sigma-Aldrich, St. Louis, MO). These plates were incubated at 30°C for 4 days and scored for growth. The yeast-expressing random mutants that showed resistance to inhibition of adenosine complementation were identified, and the plasmid's contribution to the inhibitor-resistant phenotype was confirmed by rescreening. Candidate plasmids that successfully reproduced the plate assay phenotype were sequenced using the BigDye Terminator reaction kit (Applied Biosystems, Foster City, CA) and analyzed by the University of Washington DNA Sequencing and Gene Analysis Center (Seattle, WA).

Generation and Expression of hENT1 Point Mutants. hENT1 point mutations were kinetically characterized in the plasmid pCEs and expressed in the yeast strain YPL1 (MATa *fui1*Δ::*HIS3, ura3-52*, *lys2-801*, and *HIS3*Δ*)* (SenGupta et al., 2002). Plasmid pCEs was created from the plasmid pABC3 containing the yeast PDR5 constitutive promoter (Nakamura et al., 2001). In brief, the 2 μM yeast origin of replication was amplified from the plasmid pYES (SenGupta et al., 2002) with overhanging EcoRI and XhoI restriction sites. This fragment was then ligated into plasmid pABC3 and cut with EcoRI and XhoI to create plasmid pCE1. The cDNA encoding hENT1 was amplified from pYES with HindIII and NotI overhanging restriction sites and ligated into pCE1 cut with HindIII and NotI. The single Met89Cys/Leu/Gln/Thr/Val and Ser160Asn/Cys and Met89Leu/Ser160Asn (Leu/Asn), Met89Gln/Ser160Asn (Gln/Asn), Met89Thr/Ser160Asn (Thr/Asn), Met89Val/Ser160Asn (Val/Asn), and Met89Cys/Ser160Cys (Cys/Cys) double point mutants were created in the plasmid pCES by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers used to introduce point mutations at Met89 were 5′-ATGACCCTAGCCXXX-CTGCCCCTGCTGTTA-3′ (sense) and 5′-TAACAGGGGCAGXXXG-GCACATAGGGTCAT-3′ (antisense), where the underlined sense nucleotides were TGT, TTG, CAA, ACT, and GTT for mutation to cysteine, leucine, glutamine, threonine, and valine, respectively. The primers used to introduce point mutations at Ser160 were 5′-GCCATCCTG-CAGGGCXXXCTGTTTGGTCTGGCT-3′ (sense) and 5′-AGCCAGAC-CAAACAGXXXGCCCTGCAGGATGGC-3′ (antisense), where the underlined sense nucleotides were ATT and TGT for mutation to asparagine and cysteine, respectively. The primers used to create Thr387Ile were 5′-CCCCGCCGCTACATTGTGGTCTTCGAGCAC-3′ (sense) and 5′-GTGCTCGAAGACCACAATCAGGTAGCGGCGGGG-3′ (antisense). All plasmids were sequenced for fidelity and transformed into yeast strain YPL1.

^{3}H-Nucleoside Transport Experiments. Yeast strain YPL1 harboring plasmids containing wild-type and mutant hENT1 were grown overnight in 5 ml of SD-Ura liquid media by shaking at 30°C. These mid-late log growth-phase yeast cells were then pelleted, and the supernatant was then decanted and the pellet resuspended in an equivalent volume of transport buffer (10 mM HEPES, pH 7.4, 100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$). Then, 200-μl aliquots of this cell suspension were pelleted and the supernatant discarded; 200 μl of transport buffer containing radiolabeled substrate (2 μCi [^{3}H]adenosine or [^{3}H]guanosine; 0.25 and 1.6 μM final concentration, respectively) (Moravek Biochemicals, Brea, CA) was added to each cell pellet to start the uptake experiment. After the predetermined uptake time (20 and 10 min for [^{3}H]adenosine and [^{3}H]guanosine, respectively), three 50-μl aliquots of cells were rapidly filtered, and each filter was rapidly washed with

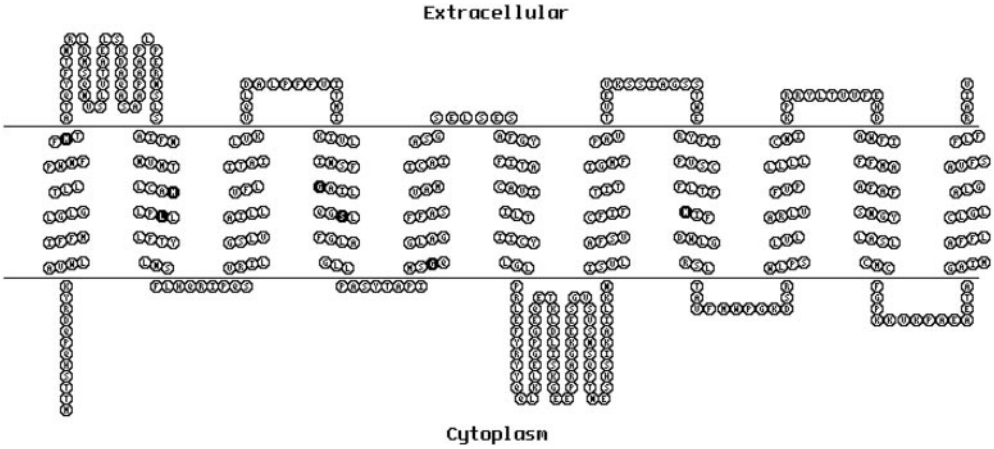

Fig. 1. Putative secondary structure of hENT1. The putative secondary structure of hENT1 is shown and was generated using TOPO2 (Sequencing Analysis Consulting Service, University of California, San Francisco, San Francisco, CA). The N terminus of hENT1 is intracellular, and amino acid residue numbering increases from left to right. Residues implicated in substrate or inhibitor binding are shown as darkened circles. Met89 and Ser160 are in TMDs 2 and 4, respectively, in which previous amino acid residues implicated in substrate and inhibitor binding have been identified.

3 ml of transport buffer. Cells deposited on filter membranes were then solubilized with 5% Triton X-100, and the radioactivity on each filter was determined by scintillation counting (Tricarb 1600 Scintillation Counter; PerkinElmer Life and Analytical Sciences, Boston, MA). Uptake data were normalized to yeast cell concentration, which was determined by measuring the optical density at 600 nm of the cellular suspensions in transport buffer. The uptake of radiolabeled substrate caused by diffusion and nonspecific binding was subtracted from the net uptake values by performing control uptake reactions in the presence of 10 μM NBMPR.

Percentage inhibition experiments were performed by the inclusion of various concentrations of inhibitors or competing substrates in the transport solutions. IC_{50} experiments were conducted by including increasing concentrations of inhibitors NBMPR (0–10 μM), dipyridamole (0–5 μM), dilazep (0–1 μM), or guanosine (0–3 mM) in the transport solutions.

Kinetic parameters were calculated as described previously by tracer displacement (Malo and Berteloot, 1991; Chenu and Berteloot, 1993; Endres et al., 2004). In brief, increasing concentrations of unlabeled adenosine (0–2 mM) were included in the transport buffer, and the kinetics of substrate transport were calculated from the inhibition (tracer displacement) profile as described below.

Data Analysis. The Student's t test was used to compare transport values of wild-type and mutant transporters. IC_{50} values were estimated by fitting the following modified Hill equation to background-subtracted transport velocity data using nonlinear regression (WinNonLin):

$$E = E_{max}\left(1 - \frac{c^{\gamma}}{c^{\gamma} + IC_{50}{}^{\gamma}}\right) \quad (1)$$

where E is the observed transport velocity, c is the inhibitor concentration, E_{max} is the maximal inhibition of transport by the inhibitor, IC_{50} is the inhibitor concentration at which 50% inhibition of transport is observed, and γ is the Hill coefficient.

The t test using pooled variances was used to test the null hypothesis that the sum of the mean IC_{50} values of the single mutations and the mean IC_{50} value of the double mutant were equivalent (additive effect).

Kinetic parameters (V_{max} and K_m) were estimated by fitting

$$v^* = \frac{V_{max}T}{K_m + S_{cold} + T} + K_dT \quad (2)$$

to the tracer displacement data using nonlinear regression (WinNonLin), where v^* is the velocity of transport of the labeled nucleoside, S_{cold} is the concentration of the unlabeled nucleoside, T is the concentration of the labeled nucleoside, and V_{max}, K_m, and K_d (diffusion constant) are the transport parameters.

The guanosine K_i value was estimated using the method of Cheng and Prusoff (1973) assuming competitive inhibition of adenosine by guanosine:

$$K_i = \frac{IC_{50}}{1 + \frac{S}{K_m}} \quad (3)$$

where S is the substrate concentration. The guanosine IC_{50} value and adenosine K_m value for the various transporters were experimentally determined as described above. Because $[S] \ll K_m$ in our experimental protocol, eq. 3 reduces to $K_i \approx IC_{50}$.

Results

Screening of Random Mutant Libraries and Identification of Met89 and Ser160. Using our adenosine rescue assay, we screened our library of randomly generated hENT1 mutants for mutations that rendered hENT1 resistant to inhibition by NBMPR, dipyridamole, or dilazep. This screening assay used plates containing 0 or 150 μM adenosine and 150 μM adenosine plus 2 μM NBMPR, 50 μM dipyridamole, or 10 μM dilazep. NBMPR, dipyridamole, and dilazep are well-known competitive inhibitors of hENT1, and we have shown previously that these concentrations prevent adenosine rescue in yeast expressing wild-type hENT1 yet allow for the detection of random mutants expressing inhibitor-resistant hENT1. As expected, in the absence of 150 μM adenosine, neither the wild-type transporter nor the random mutant candidate NDDC8 grew, whereas in the presence of 150 μM adenosine, both transported sufficient adenosine to overcome the *ade2* phenotype and grew (Fig. 2). In addition, 2 μM NBMPR, 50 μM dipyridamole, and 10 μM dilazep prevented adenosine rescue in yeast expressing wild-type hENT1, whereas yeast candidate NDDC8 grew strongly in the presence of 2 μM NBMPR or 50 μM dipyridamole and weakly in the presence of 10 μM dilazep (Fig. 2). This suggested that an amino acid change (or changes) in this random mutant contributed to a reduced affinity for these inhibitors.

Sequencing of the random mutant candidate NDDC8 identified three amino acid changes: Met89Thr, Ser160Asn, and Thr387Ile. We individually created these amino acid changes and expressed these hENT1 mutants in yeast strain YPL1 for kinetic characterization. [^{3}H]Adenosine transport was linear through 30 min for yeast expressing wild-type and all mutant transporters (data not shown), and all subsequent [^{3}H]adenosine transport experiments were carried out for 20 min. The ability of 10 nM NBMPR, 70 nM dipyridamole, or 400 nM dilazep to inhibit [^{3}H]adenosine transport in yeast expressing wild-type or Met89Thr, Ser160Asn, or Thr387Ile mutant hENT1 was then examined. Yeast expressing only

TABLE 1

Summary of amino acid residues in hENT1 important for sensitivity to inhibitors or affinity for various substrates

The fold decrease in sensitivity to inhibition (K_i or IC_{50}) or affinity (K_m) relative to the wild-type transporter is shown.

Amino Acid Residue in hENT1	Inhibitors				Substrates				Reference
	NBMPR	Dipyridamole	Dilazep	Soluflazine	Adenosine	Inosine	Guanosine	Cytidine	
Met33	0.62	11	10	4.5	N.D.	N.D.	N.D.	N.D.	Visser et al., 2002a
Met89Cys	3.7	N.S.D.	N.S.D.	N.D.	7.9	N.D.	5.7	N.D.	Tables 2–4
Leu92Gln	222	N.S.D.	3.8	N.D.	N.S.D.	4	N.D.	N.D.	Endres et al., 2004
Gly154Ser	2500	3	7	N.D.	3	N.D.	N.D.	3	SenGupta and Unadkat, 2004
Ser160Cys	5.8	2.6	N.S.D.	N.D.	2.7	N.D.	1.8	N.D.	Tables 2–4
Gly179	6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	SenGupta et al., 2002
Asn338	2.4	10	3.4	14	N.D.	N.D.	N.D.	N.D.	Visser et al., 2002b
Met33/Asn338	1.4	13	67	7.2	N.D.	N.D.	N.D.	N.D.	Visser et al., 2002b
Met89/Ser160	19	4.7	N.S.D.	N.D.	7.8	N.D.	7.7	N.D.	Tables 2–4

N.S.D., not significantly different; N.D., not determined.

Met89Thr or Ser160Asn transporters were significantly resistant to inhibition by NBMPR or dipyridamole compared with the wild-type transporter (Fig. 3). In addition, all three mutants were significantly resistant to inhibition by dilazep, although the degree of difference in the Thr387Ile mutant was much smaller than that in the Met89Thr and Ser160Asn mutant. This suggested that mutation of both Met89Thr and Ser160Asn, but not Thr387Ile, contributed to the resistant phenotype in the plate assay toward NBMPR and dipyridamole (Fig. 4).

Characterization of the Inhibition of Yeast Expressing Met89 or Ser160 Mutations by hENT1 Inhibitors. In addition to Met89Thr and Ser160Asn, we created and characterized conserved (Met89Leu and Met89Val) and nonconserved (Met89Gln) mutations in hENT1 at this position. We also characterized the mutations Met89Cys and Ser160Cys to investigate the accessibility of these amino acid residues to sulfhydryl reactive reagents. Because the resistance to inhibition of [^{3}H]adenosine transport by the Met89Thr and Ser160Asn in the presence of 10 nM NBMPR seemed to be the greatest, we then investigated the inhibitory capacities (IC_{50} values) of NBMPR toward wild-type and mutant hENT1-mediated [^{3}H]adenosine transport. The NBMPR IC_{50} values of the Met89Cys, threonine, and valine mutants and Ser160Asn and cysteine single mutants were 1.6- to 7.5-fold greater and significantly different from that of the wild-type protein, whereas the IC_{50} values of Met89Leu and glutamine showed that these mutants were approximately 2- to 3-fold more sensitive to NBMPR inhibition (Table 2).

We then created and characterized the effect of simultaneous mutation at Met89 and Ser160 on NBMPR. We were surprised to find that the simultaneous mutation of Met89 and Ser160 to cysteine/cysteine resulted in a 19-fold reduction in NBMPR sensitivity compared with the wild-type transporter (Table 2). We investigated whether the 19-fold increase in NBMPR IC_{50} value (210 ± 77.1 μM) in the double mutant could be explained by an additive effect of the two single amino acid changes (3.7- and 5.8-fold increase to 41.6 ± 13.1 and 65.0 ± 1.55 μM, respectively). The reduction of affinity (19-fold increase in IC_{50} value) of NBMPR for hENT1 in the double mutant was approximately 2-fold greater and significantly different from that of the additive effect (9.5-fold increase in additive IC_{50}) of the individual single mutants ($p < 0.05$, Student's t test, using pooled variances). This indicates that the increase in NBMPR IC_{50} value in the double mutant is significantly greater than that expected by the additive effect of the individual mutations and suggests a synergistic interaction between these amino acid residues when mutated to cysteine. When the Met89 mutations leucine, glutamine, threonine, and valine were simultaneously mutated with Ser160Asn, the NBMPR IC_{50} value was increased approximately 1.2- to 7.7-fold.

We further characterized the IC_{50} values of dipyridamole and dilazep in the Met89Cys, Ser160Cys, and Met89Cys/

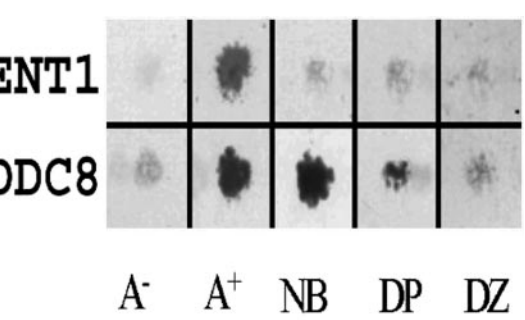

Fig. 2. Phenotypic complementation plate assay. Plasmids were expressed in yeast strain W303–1A, spotted on a master plate (SD-Ura), and replica-plated in media in the absence (A−) or presence (A+) of 150 μM adenosine or 150 μM adenosine and the following hENT1 inhibitors: 2 μM NBMPR (NB), 50 μM dipyridamole (DP), or 10 μM dilazep (DZ). Shown are the growths after 4 days of incubation at 30°C of wild-type hENT1 and the random mutant candidate NDDC8 containing the amino acid changes Met89Thr, Ser160Asn, and Thr387Ile. Unlike wild-type hENT1, this random mutant candidate shows growth in the presence of NBMPR or dipyridamole but not in the presence of dilazep.

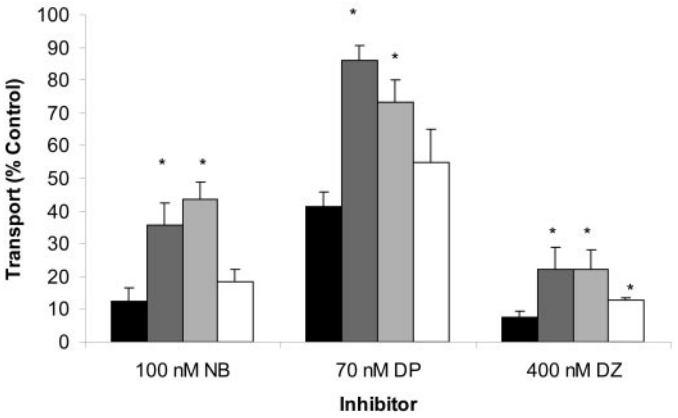

Fig. 3. Identification of amino acid changes responsible for NBMPR and dipyridamole inhibitor-resistant phenotype. The ability of 100 nM NBMPR (NB), 70 nM dipyridamole (DP), or 400 nM dilazep (DZ) to inhibit 0.25 μM [^{3}H]adenosine transport by YPL1 yeast expressing wild-type (■) or Met89Thr (▨), Ser160Asn (▤), or Thr387Ile (□) mutant hENT1 was determined. The values represent transport expressed as a percentage of control (transport in the absence of competing natural nucleoside, average values: 2.2, 0.87, 0.74, and 1.7 fmol/10^6 cells/min for wild type, Met89Thr, Ser160Asn, and Thr387Ile, respectively). Significant differences ($p < 0.05$) from the wild-type transporter are indicated by an asterisk. These data suggest that Met89 and Ser160 but not Thr387 substantially contribute to the NBMPR- and dipyridamole-resistant phenotype of NDDC8 on the plate assay.

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          80        90       100       150       160       170
           |         |         |         |         |         |
hENT1   FNNVMTLCAMLPLLLFTYLNSFLH...INSFGAILQGSLFGLAGLLPA
rENT1   FNNVMTLCAMLPLLIFTCLNSFLH...INSFGAILQASLFGLAGVLPA
mENT1   FNNVMTLCAMLPLLVFTCLNSFLH...INSFGAILQASLFGLAGVLPA
hENT2   FNNWVTLLSQLPLLLFTLLNSFLY...INSFSAVLQGSLFGQLGTMPS
rENT2   FNNWVTLLSQLPLLLFTLLNSFLY...INSFCAVLQGSLFGQLGTMPS
mENT2   FNNWVTLLSQLPLLLFTLLNSFLY...INSFCAVLQGSLFGQLGTMPS
rbENT2  FNNWVTLLSQLPLLLFTLLNSFLY...INSFCAVLQGSLFGQLGTMPS
hENT3   FNSYLAVASTVPSMLCLVANFLLV...LSGASTVFSSSIYGMTGSFPM
mENT3   FNSYLAVASTVPSLLFLVANFLLV...ISSSSTIFNSSVYGLTGSFPM
hENT4   IVFDMSLTYILVALAAVLLNNVLV...VAFGCTVQQSSFYGYTGMLPR
            TM2                          TM4
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Fig. 4. Multiple sequence alignment of human, mouse, rat, and rabbit ENT1, ENT2, ENT3, and ENT4. Sequences were aligned using ClustalW multiple-sequence alignment and the GONNET similarity matrix. The sequences were obtained from GenBank and had the following accession numbers: hENT1 (AF079117), rENT1 (NM_031684), mENT1 (AF218255), hENT2 (AF034102), rENT2 (NM_031738), mENT2 (AF183397), rbENT2 (AF323951), hENT3 (AF326987), mENT3 (AF326986), and hENT4 (NM_153247). Amino acid numbering is relative to hENT1. Met89 and Ser160 in hENT1 are shown in boldface type along with aligned homologous amino acids. Amino acid residue 89 is conserved as methionine in ENT1 and glutamine in ENT2 across the species shown, whereas Ser160 is highly conserved among all ENTs and species shown. Putative hENT1 TMDs 2 and 4 are underlined and were determined by the consensus predictions of five algorithms (TMPRED, TopPred2, TMHMM, HMMTop, and SOSUI).

Ser160Cys double mutants (Table 3). The IC_{50} values of dipyridamole toward [^{3}H]adenosine transport by the Ser160Cys and Met89Cys/Ser160Cys mutants were 2.6- and 4.7-fold greater and significantly different from that of the wild-type transporter. The IC_{50} value of dipyridamole toward [^{3}H]adenosine transport by the Met89Cys mutant was 2.0-fold that of the wild-type transporter, but this difference was not statistically significant. The IC_{50} values of dilazep toward [^{3}H]adenosine transport by all three mutants were 1.4- to 3.9-fold that of the wild-type transporter, but these differences were not statistically significant.

Characterization of the Inhibition and Kinetics of Natural Nucleoside Transport by Met89 and Ser160 Point Mutations. We then characterized the effect of the single and double point mutations at Met89 and Ser160 on the kinetics of various natural nucleosides. We first investigated the ability of the purines adenosine, guanosine, and inosine and pyrimidines uridine, cytidine, and thymidine to inhibit [^{3}H]adenosine transport by yeast expressing the wild-type Met89Cys and Ser160Cys single-mutant and Met89Cys/Ser160Cys double-mutant transporters. The ability of either low or high concentrations of adenosine (40 and 200 μM) and guanosine (200 μM and 2 mM) to inhibit [^{3}H]adenosine transport was significantly reduced in the yeast expressing the Met89Cys single and Met89Cys/Ser160Cys double mutants compared with the wild-type transporter (Fig. 5). In addition, yeasts expressing the double mutant were also marginally but significantly resistant to inhibition by low (200 μM) concentrations of uridine and both low and high (600 μM and 2 mM) concentrations of thymidine. At the high concentrations, the magnitude of this resistance to inhibition was not large; therefore, we chose to further characterize the kinetics of transport of the purines adenosine and guanosine.

Because the inhibition by adenosine was most affected by Met89 and Ser160 mutations, we characterized in detail the kinetics of adenosine transport by the wild-type and these mutant transporters. Yeast expressing the Met89Cys and Met89Cys/Ser160Cys mutants had an approximate 8-fold and statistically significant increase in adenosine K_m value, whereas the Ser160Cys single mutant had an approximate 3-fold increase in adenosine K_m value (Table 4). The V_{max} value of [^{3}H]adenosine transport was 1.8- to 3.4-fold greater in the single and double mutants, but this difference was statistically significant for only the Met89Cys mutant.

We attempted to characterize the kinetics of [^{3}H]guanosine transport by the wild-type and mutant transporters. [^{3}H]-Guanosine transport rates by the wild-type transporter was linear through 10 min, and after 10 min of transport it was approximately 5-fold greater than that caused by diffusion (0.42 and 0.1 fmol/10^6 cells/min in the absence or presence of 10 μM NBMPR). [^{3}H]Guanosine transport rates by the Met89Cys and Ser160Cys single mutants were substantially lower than that of the wild-type transporter and undetectable for the Met89Cys/Ser160Cys double mutant (data not shown). The low [^{3}H]guanosine transport activity by the single mutants would make estimation of the K_m and V_{max} of guanosine transport difficult. As an alternative, we determined the IC_{50} value of guanosine toward [^{3}H]adenosine transport by these transporters. This would allow us to estimate the change in guanosine K_i value rather than K_m value as a result of single or double mutations. The guanosine K_i values for the Met89Cys, Ser160Cys, and Met89Cys/Ser160Cys mutants were approximately 5.7-, 1.8-, and 7.7-fold greater and significantly different from that of the wild-type transporter (178 ± 80 μM) (Table 4).

TABLE 2

Inhibitory capacity of NBMPR for wild-type or single- or double-mutant hENT1

The IC_{50} values (mean ± S.D.; n = 3) of NBMPR were estimated by nonlinear regression analysis of the inhibition by NBMPR of [^{3}H]adenosine transport by wild-type (WT), single, or double (e.g., Leu/Asp) mutant hENT1-expressing yeast.

	IC_{50}	Fold Change from WT
	nM	
WT	11.1 ± 0.65	1
Met89Cys	41.6 ± 13.1*	3.7
Met89Leu	3.24 ± 1.11*	0.3
Met89Gln	5.27 ± 0.58*	0.5
Met89Thr	83.4 ± 12.6*	7.5
Met89Val	17.7 ± 2.61*	1.6
Ser160Asn	53.5 ± 18.4*	4.8
Ser160Cys	65.0 ± 1.55*	5.8
Leu/Asn	13.8 ± 3.69	1.2
Gln/Asn	49.1 ± 12.7*	4.4
Thr/Asn	85.9 ± 20.7*	7.7
Val/Asn	83.9 ± 26.7*	7.5
Cys/Cys	210 ± 77.1*	19

*, significantly different from wild-type transporter ($p < 0.05$).

TABLE 3

Inhibitory capacity of dipyridamole and dilazep toward [^{3}H]adenosine transport by hENT1 wild-type, single, or double mutants

The IC_{50} values (mean ± S.D.; n = 3) of dipyridamole and dilazep were estimated by nonlinear regression analysis of the inhibition of [^{3}H]adenosine transport by hENT1 wild-type (WT), single, or Met89Cys/Ser160Cys (cysteine/cysteine) double mutant.

	Dipyridamole		Dilazep	
	IC_{50}	Fold Change from WT	IC_{50}	Fold Change from WT
	nM		*nM*	
WT	168 ± 63.1	1	39.8 ± 20.3	1
Met89Cys	334 ± 83.6	2.0	56.3 ± 13.1	1.4
Ser160Cys	436 ± 147*	2.6	155 ± 92.4	3.9
Cys/Cys	794 ± 113*	4.7	155 ± 71.5	3.9

*, significantly different from wild-type transporter ($p < 0.05$).

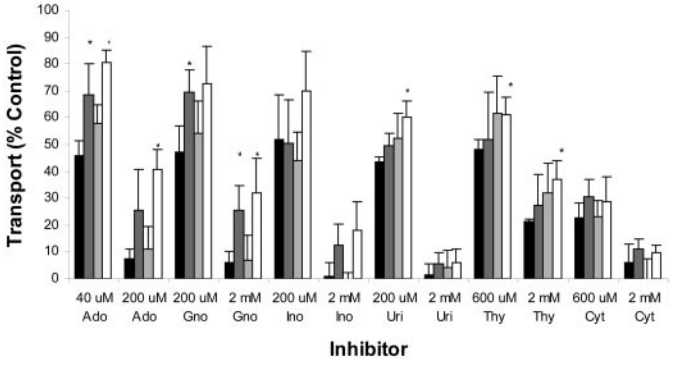

Fig. 5. Inhibition of wild-type and mutant hENT1 by natural nucleosides. The inhibition by various natural nucleosides of 0.25 μM [^{3}H]adenosine transport by yeast expressing wild-type (■) and Met89Cys (▨), Ser160Cys (▤), or Met89/Ser160Cys (□) mutant hENT1 was determined. Values represent transport of [^{3}H]adenosine expressed as a percentage (mean ± S.E., n = 3) of control (transport in the absence of competing natural nucleoside, average values: 2.7, 1.2, 1.5, and 0.59 fmol/10^6 cells/min for wild-type, Met89Cys, Ser160Cys, and Met89Cys/Ser160Cys, respectively). Significant differences ($p < 0.05$) from the wild-type transporter are indicated by an asterisk. The ability of both low (40 and 200 μM) and high (200 μM and 2 mM) concentrations of adenosine and guanosine, respectively, to inhibit [^{3}H]adenosine transport was significantly reduced in both the Met89Cys single- and Met89Cys/Ser160Cys double-mutant transporters.

In addition, the adenosine K_m and guanosine K_i values of the Met89Cys single mutant were not significantly different from those of the Met89Cys/Ser160Cys double mutant ($p > 0.05$).

Discussion

Using the adenosine rescue assay, we identified one random mutant hENT1 candidate (NDDC8) containing nonsynonymous amino acid changes at Met89Thr, Ser160Asn, and Thr387Ile. When we created these mutations individually, we found that the mutation at Met89 or Ser160 contributed to the resistance of hENT1 to inhibition by NBMPR, dipyridamole, or dilazep, whereas mutation at Thr387 did not contribute to resistance to NBMPR or dipyridamole but did contribute marginally to resistance to dilazep (Fig. 3).

Methionine is conserved at amino acid residue 89 in all mammalian ENT1s, whereas it is glutamine and threonine in the mammalian ENT2s and ENT3s. In contrast, Ser160 is conserved among all mammalian ENTs. To investigate the mechanisms by which these amino acids apparently reduced inhibitor affinity, we created various amino acid changes at these positions. At Met89, we created the amino acid residues present in hENT2 and hENT3 (glutamine and threonine, respectively). We also created substitutions of Met89 to valine (sterically conserved, but slightly more nonpolar than threonine), leucine (conserved), and cysteine. At Ser160, we created a sterically conserved, yet slightly more nonpolar, change from serine to cysteine. Then, we characterized in detail the inhibition by NBMPR of [^{3}H]adenosine transport in yeast expressing these various mutations at Met89 and Ser160. We focused on NBMPR, because only NBMPR is a nucleoside analog, whereas dipyridamole and dilazep are structurally unrelated to nucleosides. Substitution of Met89 to leucine or glutamine increased the sensitivity of these transporters to NBMPR, whereas substitution to cysteine or threonine decreased the sensitivity to NBMPR, and substitution to valine had very little effect on this sensitivity. Because theronine and valine are sterically very similar in size and differ in only a hydroxyl group, this suggests that the reduction of NBMPR affinity in the Met89Cys mutant is caused not by a steric effect of this mutation in the transporter but by that of the threonine hydroxyl group. This is similar to our observation with Ser160. The sterically conserved substitution of serine to cysteine also decreased the sensitivity to inhibition by NBMPR, suggesting that this is not a result of a steric impact on the protein structure.

We created the cysteine substitutions with the intent to investigate the accessibility of these amino acid residues to various sulfhydryl reagents. Unfortunately, various undetermined endogenous cysteines were reactive to these reagents, and we were unable to detect any differential reactivity between the wild-type transporter and the mutant transporters containing the cysteine substitutions. Despite this, we were surprised to find that the NBMPR IC_{50} value of yeast expressing the simultaneous mutation Met89Cys/Ser160Cys was approximately 2-fold greater and significantly different from what we would have expected from the additive effect of the individual mutations. We also characterized the effect of simultaneous mutation of Ser160Asn with the other Met89 substitutions. The Met89Leu mutation (which alone increased NBMPR affinity ~3-fold) seems to "counteract" the reduced NBMPR affinity that Ser160Asn contributes to, whereas the Met89Gln mutation does not. The Ser160Asn mutation seems to be dominant over the Met89Val mutation (which alone did not substantially contribute to a reduction in NBMPR affinity), whereas the reduction in NBMPR affinity in the Met89Thr/Ser160Asn mutant is essentially no different for either of the Met89Thr or Ser160Asn single substitutions.

We also characterized the effect of the single and simultaneous cysteine substitutions on dipyridamole and dilazep sensitivity. Consistent with what we observed in the plate assay and single concentration inhibition experiments with Met89Thr and Ser160Asn, the IC_{50} values of dipyridamole toward the Ser160Cys and the cysteine/cysteine mutants were increased, whereas none of the IC_{50} values for dilazep were significantly different from that of the wild-type transporter. Unlike NBMPR, Met89Cys mutation did not significantly decrease the ability of dipyridamole to inhibit hENT1, whereas the mutation Ser160Cys did. These data suggest that Ser160 is important for the interaction of dipyridamole with hENT1, whereas both Met89 and Ser160 are important for the interaction of NBMPR with hENT1.

Upon characterizing the ability of various natural nucleosides to inhibit [^{3}H]adenosine transport, we found that the ability of both low and high concentrations of adenosine and guanosine to inhibit [^{3}H]adenosine transport by hENT1 was reduced. Characterization of the transport kinetics of these two substrates for the mutant transporters revealed that substitution of Met89 to cysteine was sufficient to reduce the affinity (K_m) of adenosine approximately 8-fold but increased the V_{max} by approximately 3-fold. In contrast, Ser160Cys mutations had a significant but smaller (~3-fold) effect on the K_m of adenosine transport. The double mutant Met89Cys/Ser160Cys, however, demonstrated a K_m value for adenosine

TABLE 4

Kinetic parameters of [^{3}H]adenosine transport and guanosine inhibition of [^{3}H]adenosine transport by wild-type and mutant hENT1

The kinetic parameters (K_m and V_{max}) and guanosine inhibition (K_i) of [^{3}H]adenosine transport by wild-type (WT), single, and Met89Cys/Ser160Cys (cysteine/cysteine) double mutant hENT1-expressing yeast were determined by conducting [^{3}H]adenosine transport in the presence of various concentrations of unlabeled adenosine (0–2 mM) or guanosine (0–3 mM). All values represent the mean ± S.D. ($n = 3$) of the nonlinear regression parameter estimates.

	Adenosine			Guanosine	
	K_m	Fold Change from WT	V_{max}	K_i	Fold Change from WT
	μM		*fmol/10^6 cells/min*	*μM*	
WT	20.6 ± 4.4	1	214 ± 85.8	178 ± 80	1
Met89Cys	162 ± 60.9*	7.9	726 ± 110*	1020 ± 110*	5.7
Ser160Cys	56.0 ± 19.3*	2.7	394 ± 80.6	313 ± 16.7*	1.8
Cys/Cys	160 ± 53.6*	7.8	397 ± 168	1370 ± 314*	7.7

*, significantly different from wild-type transporter ($p < 0.05$).

similar to that of Met89Cys mutant, indicating that this is the dominant mutation responsible for the change in affinity for adenosine. The transport of [^{3}H]guanosine by the single- and double-mutant transporter was not high enough for determination of its transport kinetics. Instead, we estimated the inhibitory capacity (K_i) of guanosine toward adenosine transport as a measure of its affinity for the transporter. As was the case for adenosine, substitution of Met89 to cysteine was the dominant mutation and reduced the "affinity" of guanosine approximately 6- to 8-fold.

The synergistic effect of simultaneous mutation of Met89 and Ser160 to cysteine suggests that these substituted residues interact (either directly or indirectly) to reduce NBMPR affinity. It has been suggested that in this situation, direct interaction between the inhibitor functional groups that interact with these amino acid residues can be identified by structure-activity studies of various inhibitor analogs (Kristensen et al., 2004). Our data indicate that the affinity of both adenosine and guanosine is not synergistically decreased in the double mutant and that Met89 is the dominant contributor to the reduction in affinity for these substrates. Because the affinity of inosine and the pyrimidines was not affected, this suggests that Met89 interacts with the purine ring of adenosine, guanosine, and NBMPR. In addition, this suggests that mutation of Ser160 alters the interaction of the nitrobenzylthiol group of NBMPR with the transporter, because mutation of Ser160 did not have any effect on any natural nucleosides affinity. To determine the specific functional groups of NBMPR that interact with Ser160, structure-activity studies with various NBMPR analogs must be conducted. These analogs have been described previously (Deghati et al., 2003), although none was available to us. It is possible that we did not observe the synergistic effect with Met89/Ser160Asn double mutations because the Ser160Asn substitution is sterically unconserved and may therefore introduce gross structural changes in the protein structure. On the other hand, substitution of both Met89 and Ser160 to cysteine introduces only subtle changes, which are unlikely to grossly alter the transporter structure.

Our data suggest that Met89 and Ser160 interact either directly or indirectly to alter NBMPR affinity. Met89 is in putative TMD 2, whereas Ser160 is in putative TMD 4. Previous studies have implicated other amino acid residues within these TMDs which when mutated have an effect on NBMPR and substrate affinity (Fig. 1 and Table 1). Our laboratory has shown that mutation of Leu92 (in TMD 2) increases the IC_{50} value of NBMPR and dilazep approximately 220- and 4-fold, respectively (Endres et al., 2004). In addition, this mutation increases the K_m value (reduced the affinity) of inosine but not adenosine approximately 4-fold compared with the wild-type transporter. Because helical-wheel analysis predicts that Leu92 and Met89 are on the same face of TMD 2, this is consistent with a model whereby Met89 directly interacts with the purine ring of adenosine, guanosine, and NBMPR and Leu92 directly interacts with the purine ring of inosine and NBMPR. Our group also characterized the effect of mutation of Gly154 (in TMD 4) to serine (the homologous amino acid residue in hENT2) and found that this increases the IC_{50} value for NBMPR, dipyridamole, and dilazep approximately 2500-, 3-, and 7-fold, respectively, and reduces the affinity of adenosine and cytidine approximately 3-fold (SenGupta and Unadkat, 2004). Although helical-wheel prediction expects Ser160 and Gly154 to be on nearly on opposite faces of the α helix, TMD 4 is clearly important in NBMPR binding. This work demonstrates for the first time the effect of simultaneous mutation of amino acid residues in these TMDs. It is possible that these TMDs are in proximity in the packing of the α-helices that form the tertiary structure of hENT1, and our observation of the synergistic effect of simultaneous mutation of Met89 and Ser160 on NBMPR affinity is a result of this. Indeed, while this work was under review, Arastu-Kapur et al. proposed a putative helical packing model of the ENT family in which TMDs 2 and 4 are adjacent and part of the putative aqueous pore (Arastu-Kapur et al., 2004).

In conclusion, we have shown for the first time that Met89 (TMD 2) and Ser160 (TMD 4) are synergistically important in the inhibition of hENT1 by NBMPR, suggesting that these TMDs interact. In contrast, Ser160 seems to play a dominant role in the ability of dipyridamole to inhibit hENT1, whereas Met89 plays a dominant role in the affinity of hENT1 for adenosine and guanosine. Identification of additional such critical amino acid residues will be necessary to validate a proposed model of the putative binding site(s) of hENT1 inhibitors and substrates. Building such a model is critical for developing drugs that target this transporter, an important physiological and pharmacological modulator of the effects of adenosine.

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Address correspondence to: Dr. Jashvant Unadkat, Department of Pharmaceutics, Box 357610, University of Washington, Seattle, WA 98195. E-mail: jash@u.washington.edu