Mutation of Residue 33 of Human Equilibrative Nucleoside Transporters 1 and 2 Alters Sensitivity to Inhibition of Transport by Dilazep and Dipyridamole*

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Human equilibrative nucleoside transporters (hENT) 1 and 2 differ in that hENT1 is inhibited by nanomolar concentrations of dipyridamole and dilazep, whereas hENT2 is 2 and 3 orders of magnitude less sensitive, respectively. When a yeast expression plasmid containing the hENT1 cDNA was randomly mutated and screened by phenotypic complementation in Saccharomyces cerevisiae to identify mutants with reduced sensitivity to dilazep, clones with a point mutation that converted Met33 to Ile (hENT1-M33I) were obtained. Characterization of the mutant protein in S. cerevisiae and Xenopus laevis oocytes revealed that the mutant had less than one-tenth the sensitivity to dilazep and dipyridamole than wild type hENT1, with no change in nitrobenzylmercaptapurine ribonucleoside (NBMPR) sensitivity or apparent uridine affinity. To determine whether the reciprocal mutation in hENT2 (Ile33 to Met) also altered sensitivity to dilazep and dipyridamole, hENT2-I33M was created by site-directed mutagenesis. Although the resulting mutant (hENT2-I33M) displayed >10-fold higher dilazep and dipyridamole sensitivity and >8-fold higher uridine affinity compared with wild type hENT2, it retained insensitivity to NBMPR. These data established that mutation of residue 33 (Met versus Ile) of hENT1 and hENT2 altered the dilazep and dipyridamole sensitivities in both proteins, suggesting that a common region of inhibitor interaction has been identified.

Cellular uptake and release of nucleosides and nucleoside analog drugs is mediated by integral membrane nucleoside transporter proteins (1–4). These proteins are involved in salvage of extracellular nucleosides for nucleotide biosynthesis in mammalian cells, especially those that lack de novo synthesis pathways such as enterocytes and hemopoietic cells. They are critical for the cellular uptake of cytotoxic nucleoside analogs used in the treatment of human hematologic malignancies, solid tumors, and viral diseases (5, 6). Nucleoside transporters also affect the cell surface concentration of adenosine, which is a signaling molecule that binds to G protein-coupled cell surface adenosine receptors, affecting physiological processes such as coronary vasodilation, renal vasoconstriction, neuromodulation, platelet aggregation, and lipolysis (7, 8).

Mammalian nucleoside transporters are classified into two structurally and functionally distinct families: the concentrative nucleoside transporters (CNTs)1 and the equilibrative nucleoside transporters (ENTs). CNTs mediate Na+-dependent transport against the nucleoside concentration gradient and are found primarily in specialized cells such as intestinal and renal epithelia. Three CNT isoforms, a pyrimidine-nucleoside preferring (CNT1), a purine-nucleoside and uridine preferring (CNT2), and a broadly selective (C1T3) protein, have been identified by molecular cloning from mammalian tissues (9–14). Mammalian ENTs are responsible for facilitated diffusion of nucleosides across cell membranes and have a broad tissue distribution. Two ENT isoforms have been identified by molecular cloning and functional expression from mammalian tissues and mediate nucleoside transport processes that are functionally distinguished by their differential sensitivity to inhibition by NBMPR (1–4). NBMPR-sensitive nucleoside transport processes that bind NBMPR with high affinity, (Kd = 0.1–1 μM), have been assigned the functional designation es (equilibrative sensitive) and are mediated by ENT1 proteins. NBMPR-insensitive nucleoside transport processes are resistant to inhibition by micromolar concentrations of NBMPR, are functionally designated as ei (equilibrative insensitive), and are mediated by ENT2 proteins. ENTs are pharmacological targets for the coronary vasodilators dilazep, dipyridamole, and drafazine, which have been shown to inhibit transport and NBMPR binding (3, 15–17). Adenosine interacts with G protein-coupled cell surface receptors of endothelial and smooth muscle cells to induce vasodilation. Transporter-mediated adenosine uptake is the major means by which this interaction is terminated, a mechanism that is blocked by coronary vaso-

1 The abbreviations used are: CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; NBMPR, nitrobenzylmercaptapurine ribonucleoside (6-[(4-nitrobenzyl)thiol]-9-β-D-ribofuranosyl purine); TM, transmembrane; h, human; m, mouse; r, rat; CMM, complete minimal medium; GLU, glucose; MTX, methotrexate; PGK, phosphoglycerate kinase; SAA, sulfanilamide.

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dilator binding to the human ENT isoforms hENT1 and hENT2.

hENT2 shares 50% amino acid identity with hENT1 and is 2 and 3 orders of magnitude less sensitive, respectively, to inhibition by dipyridamole and dilazep than hENT1, whereas both rat isoforms (rENT1 and rENT2) are completely insensitive to these inhibitors (18, 19). Human and rat ENT1 and ENT2 proteins share a common membrane architecture, recently confirmed by hydroxyproline analysis and glycosylation-scanning mutagenesis (20), with 11 transmembrane (TM) segments, a large glycosylated loop between TM segments 1 and 2, and a large intracellular loop between TM segments 6 and 7. In a previous study, chimeric recombinant proteins were created between hENT1 and rENT1 to identify the structural domains of hENT1 that are responsible for interaction with dilazep and dipyridamole (21). The inhibitor sensitivities of the chimeras suggested that TM segments 3–6 contain the major site(s) of interaction with secondary contributions from TM segments 1–2, providing the first insight into the regions of hENT1 that are important for interaction with dilazep and dipyridamole. The individual amino acid residues responsible for interaction with dilazep and dipyridamole have not yet been identified.

The goal of the current study was to identify amino acid residues involved in dilazep and dipyridamole interaction by using a phenotypic complementation assay to screen a library of randomly mutated yeast expression plasmids containing the hENT1 DNA (pYPHENT1) for functional thymidine transport-competent mutants with reduced sensitivity to dilazep. The complementation assay is based on the ability of recombinant hENT1 produced in Saccharomyces cerevisiae to import thymidine under conditions of dTMP starvation (23). In brief, yeast cells transformed with pYPHENT1 using a lambda acid sequence procedure (24) were plated directly onto CMM/GLU plates containing methotrexate (MTX) at 50 μg/ml and sulfanilamide (SA) at 6 mg/ml (CMM/GLU/MTX/SA). Colonies formed with an efficiency of ~10⁵ transformants/μg of DNA after incubation at 30 °C for 3.5 days in the presence of 10 μm thymidine, and complementation was prevented when 10 μm dilazep was also present. Hydroxymethylated-pYPHENT1 (20) was transformed into KTK cells, which were then plated onto CMM/GLU/MTX/SA with 10 μm thymidine and 10 μm dilazep and incubated at 30 °C for 3.5 days. Colonies with apparent resistance to dilazep inhibition of complementation were isolated, grown in 5 ml of liquid CMM/GLU for 2 days, and restreaked onto CMM/GLU/MTX/SA plates with 10 μm thymidine and 10 μm dilazep. The mutant hENT1 cDNAs were amplified from the yeast colonies by PCR, subcloned back into nonmutated pYPGE15, and sequenced.

Uridine Transport in S. cerevisiae—The plasmids pYPHENT1, pYPHENT1-M33I, pYPHENT1-M33I, and pYPHENT2-M33I were transformed into full-length TRP1 yeast, a strain that lacks the endogenous uridine permissive FUU1 (25). The transport of [3H]uridine (Moravek Biochemicals, Brea, CA) by logarithmically proliferating yeast was measured as described previously using the “oil stop” method (30, 31) with the following modifications. Yeast were harvested into ice-cold stage VI oocytes washed once with fresh medium, and resuspended to an A₆₀₀ of 2.0 in fresh medium. All transport assays were performed at room temperature and pH 7.0. 1-ml portions of yeast culture were distributed into 15-ml plastic centrifuge tubes to which 5–10-μl portions of stock dilazep, dipyridamole, or NBBMP (Sigma) solution or solvent alone (H₂O, ethanol, or dimethyl sulfoxide) were added to achieve the desired final concentration. To allow for steady-state equilibration, the yeast were incubated in the presence of inhibitor for 30 min before addition of radiolabeled perment (32–35). Transport reactions were initiated by the rapid addition of a small volume of [3H]uridine to a final concentration of 2 μM. Transport reactions were terminated at graded time intervals by pipetting triplicate 200-μl portions of yeast suspension into 1.5-ml microcentrifuge tubes containing 200 μl of transport oil; the tubes were immediately centrifuged at 12,000 × g for 2 min. The supernatants were removed by aspiration, the resulting pellets were solubilized with 5% Triton X-100 for 24 h, and the radioactive content was determined by liquid scintillation counting.

Functional Expression of hENT1 and hENT1-M33I in X. laevis Oocytes—In vitro synthesized transcripts were prepared from pKS(+)–hENT1 and pKS(+)–hENT1-M33I (SP6 MEGAscript Kit, Ambion, Austin, TX) in linear and injectable form from X. laevis as described previously (14). Mock-injected oocytes were injected with water alone. Transport assays were performed as described previously (21, 28) on groups of 10 oocytes at 20 °C using [3H]uridine (Amersham Life BioSciences) (1 μCi/ml) in 200 μl of transport buffer containing 100 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, and 10 mM HEPES, pH 7.5. The initial rates of uridine uptake (10 μM) were determined using incubation periods of 5 min.
FIG. 1. Time courses of [3H]uridine uptake for recombinant hENT1 and hENT2 produced in S. cerevisiae. Yeast cells containing pYPhENT1 (circles), pYPGE15 (triangles), or pYPGE15 (squares) were incubated with 2 mM [3H]uridine for the indicated time periods. The inset shows the time courses for the first 10 s of [3H]uridine influx. Each point represents mean uridine uptake (± S.E., n = 3); S.E. values are not presented where the size of the point is larger than the S.E.

RESULTS

Uridine Transport by Recombinant hENT1 and hENT2 in Yeast—Time courses for influx of [3H]uridine were measured into fui1::TRP1, a uridine transport-defective strain of yeast (25), that contained pYPhENT1 or pYPGE15 to determine incubation times that provided significant signal-to-noise ratios while also maintaining the initial rates of uptake (Fig. 1). The time course for nonmediated uridine influx was obtained by assessing uridine uptake into pYPGE15-containing yeast and yielded a rate of 0.11 ± 0.01 pmol/mg protein/s. Time courses for uridine uptake into pYPhENT1 and pYPGE15-containing yeast for the first 10 s (Fig. 1, inset) gave rates of 1.03 ± 0.40 and 1.63 ± 0.45 pmol/mg protein/s, respectively. Uptake time courses over 40 min were linear for both pYPhENT1- and pYPGE15-containing yeast and yielded rates, respectively, of 0.93 ± 0.02 and 1.4 ± 0.02 pmol/mg protein/s. Uptake rates over the first 10 s were not different from the rates calculated from 40-min time courses, indicating that initial rates representing uridine transport were maintained over long periods of time. The extended linear time courses were likely due to efficient substrate “trapping” by conversion of uridine to UMP by uridine kinase, thereby minimizing backflow of [3H]uridine from the small intracellular compartment to the much larger extracellular volume. Uridine transport rates were determined for all subsequent experiments using incubation times of 10 or 20 min.

Random Mutagenesis and Screening—MTX and SAA prevent the conversion of dUMP to dTMP by yeast thymidylate synthase and thus cause depletion of intracellular dTMP pools and inhibition of growth (22). KTK yeast producing recombinant hENT1 and H. simplex thymidine kinase can salvage thymidine via transporter-mediated uptake when low concentrations (e.g. 10 μM) are present in the growth medium, thereby allowing yeast to circumvent MTX/SAAs-mediated growth arrest. Because thymidine salvage can be blocked by the inclusion of 10 μM dilazep in the complementation growth medium (23), this inhibition of thymidine rescue was used to screen a hENT1 random mutant library for functional proteins with reduced affinity for dilazep. pYPhENT1 was treated in vitro with the mutagen hydroxylamine, transformed into KTK yeast, and screened for dilazep resistance. Dilazep-resistant yeast colonies were isolated, and the hENT1 cDNA was amplified and subcloned into nonmutated pYPGE15. Twenty-one resistant mutant cDNA clones were sequenced and shown to be identical, with a point mutation in codon 33 that converted Met to Ile.

A Comparison of Sequences of Inhibitor-sensitive and -insensitive Mammalian ENTs—Recombinant human and mouse ENT1 proteins are highly sensitive to transport inhibition by dipiridamole, whereas recombinant human and mouse ENT2 proteins are much less sensitive (18, 36). For example, the reported IC50 values for mENT1 and mENT2 produced in X. laevis oocytes were 75 and 2204 nM, respectively, which corresponds to a 29.4-fold difference (36). A transport-deficient cultured cell line stably transfected with recombinant hENT1 or hENT2 exhibited a 70-fold difference between the two proteins in sensitivity to dipiridamole with IC50 values of 5 and 356 nM, respectively (19). The rat ENT isoforms (rENT1 and rENT2) are completely insensitive to dipiridamole and dilazep transport inhibition when produced in X. laevis oocytes (18).

Multiple sequence alignment of the predicted amino acid sequences for the human, mouse, and rat ENT1 and ENT2 proteins revealed that the identity of the amino acid at residue 33 was consistent with the dilazep and dipiridamole sensitivity of the recombinant transporters (Fig. 2). Residue 33 is a Met in human and mouse ENT1, the most inhibitor-sensitive transporters, whereas it is an Ile in rat ENT1 and ENT2 proteins, all of which exhibit transport activity that is insensitive to inhibition by dilazep and dipiridamole (18, 28, 36, 37). The predicted topology model for hENT1 suggests that position 33 is the last residue in the first TM segment and may therefore be solvent-accessible and/or in the plane of the extracellular bilayer/solvent interface (20, 28).

Effect of Met→Ile Interconversion at Residue 33 of hENT1 and hENT2 on Uridine Transport Inhibition by Dilazep, Dipyridamole, and NBMPR—Uridine transport was measured in fui1::TRP1 yeast containing pYPhENT1 or pYPENT1-M33I in the presence or absence of a single high concentration of dilazep, dipiridamole, or NBMPR (Fig. 3A). hENT1-mediated uridine transport was inhibited >80% by 0.1 μM dilazep and 0.3 μM dipiridamole, whereas hENT1-M33I was capable of transport at 60% of the maximal rate in the presence of both inhibitors. These results suggested that hENT1-M33I was substantially less sensitive to dilazep and dipiridamole than wild type hENT1. In contrast, uridine transport was completely inhibited by 0.1 μM NBMPR in yeast with either recombinant protein, suggesting that residue 33 was not involved in the binding of NBMPR. Although hENT2 can be inhibited by high concentrations of dilazep and dipiridamole, it is 2 and 3 orders of magnitude less sensitive, respectively, to these compounds than hENT1 (19). To investigate the role of residue 33 in inhibitor sensitivity of hENT2, Ile33 was converted to Met using site-directed mutagenesis, and the effects of dilazep, dipiridamole and NBMPR on uridine transport were determined in fui1::TRP1 yeast containing either pYPHENT2 or pYPENT2-I33M (Fig. 3B). Dilazep (10 μM) and dipiridamole (1 μM) had no effect on hENT2...
mediated uridine transport, whereas both strongly inhibited hENT2-I33M-mediated transport. In contrast, uridine transport in yeast with either mutant or wild type hENT2 remained insensitive to NBMPR, a result that was consistent with the lack of an effect of the opposite conversion on NBMPR sensitivity of hENT1. These data, together with the data from Fig. 3A, indicated that residue 33 plays a key role in dilazep and dipyridamole inhibition of transport of both hENT1 and hENT2 and is not involved in NBMPR inhibition of transport.

**Kinetic Properties of Uridine Transport for hENT1, hENT1-M33I, hENT2, and hENT2-I33M** —The effect of mutating residue 33 (Met versus Ile) of hENT1 and hENT2 on the kinetics of uridine transport was assessed by determining the concentration dependence of initial rates of uridine uptake (Table I). hENT1 and hENT1-M33I showed similar kinetic parameters for uridine transport with \( K_m \) values of 110 ± 12 and 110 ± 28 \( \mu \text{M} \), respectively, and \( V_{\text{max}} \) values of 5893 ± 1399 and 5215 ± 562 \( \text{pmol/mg protein/min} \), respectively, suggesting that uridine interaction with hENT1 was unaffected by the mutation. In contrast, \( K_m \) values were 729 ± 53 and 87.2 ± 13.8 \( \mu \text{M} \), respectively, for hENT2 and hENT2-I33M, indicating an 8.4-fold increase in the apparent affinity for uridine. \( V_{\text{max}} \) values of 8370 ± 1091 and 6555 ± 1616 \( \text{pmol/mg protein/min} \) were obtained, respectively, for wild type and mutant hENT2. The \( V_{\text{max}} \) values for the mutant and wild type hENT1 and hENT2 proteins were not significantly different (\( p > 0.05 \)) based on an unpaired two-tailed \( t \) test, suggesting that expression of the recombinant proteins in yeast was not affected by mutation of residue 33. The \( V_{\text{max}}/K_m \) ratios for mutant and wild type hENT1 were similar (47 and 53 \( \text{pmol/mg protein/min/} \mu \text{M} \), respectively), whereas the ratios for mutant hENT2 were much larger than those for wild type hENT2 (75 and 12 \( \text{pmol/mg protein/min/} \mu \text{M} \), respectively).

**Concentration-Effect Relationships for Dilazep, Dipyridamole, and NBMPR** —The relative changes in inhibitor sensitivities of mutant and wild type hENT1 and hENT2 were determined by assessing the concentration dependence of uridine transport inhibition for the recombinant proteins produced in full-TPI yeast. The yeast were incubated with graded concentrations of inhibitors and then assayed for \( [\text{H}] \) uridine transport (Fig. 4). The Hill coefficients determined from these relationships were not significantly different from unity based on a \( t \) test against the theoretical value of 1.00 resulting in \( p > 0.05 \), which was consistent with (i) the presence of a single class of binding sites and (ii) the findings of previous studies (21, 23).

The IC\(_{50}\) values obtained from the data of Fig. 4 and the kinetic constants of Table I were used to compute apparent \( K_i \) values, assuming that dilazep, dipyridamole, and NBMPR inhibit uridine transport in a reversible and strictly competitive manner at the concentration equal to the IC\(_{50}\) value (Table II) (17, 33, 35–40). The transport of uridine by wild type hENT1 was potently inhibited by dilazep (\( K_i, 18.7 \pm 2.0 \text{ mM} \)), whereas hENT1-M33I-mediated transport was an order of magnitude less sensitive to dilazep inhibition (\( K_i, 195 \pm 51 \text{ mM} \)). In contrast, hENT2-I33M was 46-fold more sensitive to dilazep inhibition than wild type hENT2 with \( K_i \) values of 2.91 ± 0.79 and 134 ± 40 \( \mu \text{M} \), respectively. Thus, the mutations at residue 33 decreased the differences in dilazep sensitivity between hENT1 and hENT2. The mutant proteins displayed a 15-fold difference (hENT1-M33I > hENT2-I33M), whereas the wild type proteins displayed a 7000-fold difference (hENT1 > hENT2) in sensitivity to inhibition by dilazep.

For both hENT1 and hENT2, the relative differences between the mutant and wild type proteins in sensitivity to dipyridamole were similar to those observed for dilazep (Table II). \( K_i \) values of 47.9 ± 8.9 and 528 ± 165 \( \text{nm} \) were obtained for dipyridamole inhibition of transport for wild type and mutant hENT1, respectively, translating into an 11-fold decrease in sensitivity. The dipyridamole sensitivities of hENT2 (\( K_i, 6230 \pm 900 \text{ nm} \)) and hENT2-I33M (\( K_i, 461 \pm 74 \text{ nm} \)) differed by 13.5-fold. Wild type hENT2 was 128-fold less sensitive to dipyridamole than hENT1, which is consistent with the results of previous studies (19), whereas the mutant proteins displayed approximately equal sensitivities to dipyridamole.

The results of Fig. 3 suggested that mutant and wild type hENT1 were highly sensitive to NBMPR because complete inhibition of transport was observed for both at 0.1 \( \mu \text{M} \) NBMPR. In the experiments of Table II, \( K_i \) values of 5.83 ±
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1.08 and 3.34 ± 0.97 nM were obtained for hENT1 and hENT1-M33I, respectively, demonstrating that both were potently inhibited by NBMPR, with no statistically significant difference in $K_i$ values. The NBMPR sensitivities of hENT2 and hENT2-I33M were not determined because the experiments of Fig. 3B had established that neither protein was inhibited by NBMPR.

In a previous study (21), recombinant chimeric proteins were constructed by domain substitutions between hENT1, which is sensitive to inhibition by dilazep and dipyridamole, and its rat isoform, rENT1, which is insensitive to both compounds and functionally characterized in X. laevis oocytes. The results suggested that TM segments 1–6 of hENT1 are required for interaction with dilazep and dipyridamole, with TM segments 3–6 being the major site of interaction and TM segments 1–2 making a secondary contribution. Because residue 33 is predicted to be the last residue in TM segment 1, recombinant hENT1-M33I was produced in X. laevis oocytes (Fig. 5) to assess the functional characteristics of the mutated protein in the same recombiant expression system as the chimera study. When oocytes producing mutant and wild type hENT1 were assayed for uridine uptake in the presence of graded concentrations of dipyridamole, IC$_{50}$ values were 3640 ± 1410 and 300 ± 79 nM, respectively, corresponding to a 12.1-fold lower sensitivity for the mutant protein. This relative decrease in sensitivity was similar to that observed when the recombinant proteins were produced in yeast.

DISCUSSION

The results of molecular cloning and functional expression studies on recombinant ENTs are consistent with the findings of studies on es- and ei-type transport processes in cultured cell lines and erythrocytes. The human and mouse es-type transporters, which correspond to the hENT1 and mENT1 proteins, are highly sensitive to dilazep and dipyridamole (3, 16, 41, 42). In contrast, rat es and human, mouse, and rat ei transporters are relatively insensitive to transport inhibition by dilazep and dipyridamole, and these observed effects have been correlated with the transport-inhibition phenotypes of recombinant rENT1, hENT2, mENT2, and rENT2 (3, 41, 42). The current study provides evidence that mutation of residue 33 of the hENT1 and hENT2 proteins affects interaction with dilazep and dipyridamole significantly. The identity of this residue (Met versus Ile) corresponds with the relative dilazep and dipyridamole sensitivities of the known mammalian ENTs, being a Met in human and mouse ENT1 and an Ile in rat ENT1 and human, mouse, and rat ENT2 proteins (Fig. 2) (18, 19, 21, 28, 36, 37).

Mutation of Met$^{33}$ to Ile in hENT1 decreased the sensitivity of uridine transport to inhibition by dilazep and dipyridamole (as seen by the >10-fold increase in $K_i$ values) but did not alter the affinity for uridine (similar $K_m$ values) or the sensitivity to inhibition of uridine transport by NBMPR (similar $K_i$ values). In contrast, the sensitivity of hENT2 to dilazep and dipyridamole was increased >10-fold when Ile$^{33}$ was converted to Met, the affinity for uridine was increased 8.4-fold, and NBMPR sensitivity was not affected. These results, which implicated residue 33 in uridine interaction with hENT2 but not hENT1, suggested a difference in the permeant binding pockets of the two proteins. hENT1 and hENT2 are known to have different permeant binding properties because hENT2 is capable of transporting nucleobases and antiviral deoxyribonucleoside analogs, whereas hENT1 is not (45, 44).

The apparent $K_m$ value for uridine transport obtained for recombinant hENT1 in yeast (Table I) was $110 ± 12$ μM, whereas values of 200–260 μM have been obtained for recombinant hENT1 in other expression systems (cultured cells, X. laevis oocytes) and for the native protein in human erythrocytes (19, 28, 45). The basis for this discrepancy is uncertain but may have been due to the human protein being inserted into the yeast plasma membrane environment and/or an altered state of glycosylation, resulting in subtle changes in the conformation of the uridine-binding pocket.

Previous work in which chimeric recombinant proteins were created by substituting domains between inhibitor-sensitive hENT1 and inhibitor-insensitive rENT1 suggested that the region including residues 100–231 (which includes TM seg-
TABLE II

| Inhibitor | Inhibition of hENT1/hENT1-M33I (K_i) | Inhibition of hENT2/hENT2-I33M (K_i) |
|-----------|-------------------------------------|--------------------------------------|
|           | hENT1 | hENT1-M33I | Ratio^a | p value | hENT2 | hENT2-I33M | Ratio^b | p value |
| Dilazep   | 18.7 ± 2.0 | 195 ± 51 | 10.4 | 0.0260 | 134,000 ± 40,000 | 2910 ± 790 | 46.0 | 0.0306 |
| Dipyridamole | 47.9 ± 8.9 | 528 ± 165 | 11.0 | 0.0439 | 6230 ± 900 | 461 ± 74 | 13.5 | 0.0031 |
| NBMPR     | 5.83 ± 1.08 | 3.34 ± 0.97 | 0.57 | 0.1614 | ND | ND | ND | ND |

^a Ratio = K_i(hENT1-M33I)/K_i(hENT1) calculated from the average K_i values shown.

^b Ratio = K_i(hENT2-I33M)/K_i(hENT2) calculated from the average K_i values shown.

^c ND, not determined.

The current study established that residue 33 of hENT1 and hENT2 is important for dilazep and dipyridamole interaction. It is not clear whether residue 33 of hENT1 and hENT2 is directly involved in permeant or inhibitor binding or whether the effects observed when it was mutated were due to changes in the tertiary structure of these proteins. The alternatives are difficult to resolve in the absence of detailed structural data. Future studies include using different random mutagenesis and screening approaches to identify other residues that may be important for interaction with nucleoside transport inhibitors.

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The results of equilibrium binding studies in cells with the ex transport process, for which ENT1 proteins are believed to be responsible, have led to the conclusion that dilazep and dipyridamole are competitive inhibitors for a single or overlapping exofacial NBMPR and permeant binding site (17, 34, 39, 40, 47). However, results from other studies have suggested that dilazep and dipyridamole display characteristics of allostERIC ligands when present at high concentrations (33, 35, 39, 48). A unifying model that has been suggested for permeant and inhibitor binding to hENT1 describes two binding sites in which permeants, NBMPR, and other inhibitors such as dilazep and dipyridamole compete for a single high affinity site, which is subject to allosteric modulation by a distinct broad specificity low affinity site that binds nucleosides, nucleobases, and inhibitors when present at very high concentrations (3). The contribution of the potential allosteric binding site of hENT1 was likely to be negligible in the experiments of the current study because the Hill coefficients indicated the presence of a single class of binding sites. These results suggested that mutation of residue 33 affected dilazep and dipyridamole binding to the competitive binding site.

Future studies include using different random mutagenesis and screening approaches to identify other residues that may be important for interaction with nucleoside transport inhibitors.
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