Mutation of leucine-92 selectively reduces the apparent affinity of inosine, guanosine, NBMPR [*S*⁶-(4-nitrobenzyl)-mercaptopurine riboside] and dilazep for the human equilibrative nucleoside transporter, hENT1

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We developed a yeast-based assay for selection of hENT1 (human equilibrative nucleoside transporter 1) mutants that have altered affinity for hENT1 inhibitors and substrates. In this assay, expression of hENT1 in a yeast strain deficient in adenine biosynthesis (*ade2*) permits yeast growth on a plate lacking adenine but containing adenosine, a hENT1 substrate. This growth was prevented when inhibitors of hENT1 {e.g. NBMPR [*S*⁶-(4-nitrobenzyl)-mercaptopurine riboside], dilazep or dipyridamole} were included in the media. To identify hENT1 mutants resistant to inhibition by these compounds, hENT1 was randomly mutagenized and introduced into this strain. Mutation(s) that allowed growth of yeast cells in the presence of these inhibitors were then identified and characterized. Mutants harbouring amino acid changes at Leu⁹² exhibited resistance to NBMPR and dilazep but not dipyridamole. The IC₅₀ values of NBMPR and dilazep for [³H]adenosine transport by one of these mutants L92Q (Leu⁹² → Gln) were approx. 200- and 4-fold greater when compared with

the value for the wild-type hENT1, whereas that for dipyridamole remained unchanged. Additionally, when compared with the wild-type transporter, [³H]adenosine transport by L92Q transporter was significantly resistant to inhibition by inosine and guanosine but not by adenosine or pyrimidines. The *K_m* value for inosine transport was approx. 4-fold greater for the L92Q mutant (260 ± 16 μM) when compared with the wild-type transporter (65 ± 7.8 μM). We have identified for the first time an amino acid residue (Leu⁹²) of hENT1 that, when mutated, selectively alters the affinity of hENT1 to transport the nucleosides inosine and guanosine and its sensitivity to the inhibitors NBMPR and dilazep.

Key words: dilazep, hENT1 (human equilibrative nucleoside transporter 1), nitrobenzylthioinosine, purines, pyrimidines, resistance.

INTRODUCTION

Owing to the wide tissue distribution and broad nucleoside substrate selectivity, hENT1 (human equilibrative nucleoside transporter 1) [1] plays an important role in the pharmacological activity and disposition of nucleosides (e.g. adenosine) [2–6] and nucleoside drugs (e.g. ribavirin and gemcitabine) [7,8]. Additionally, hENT1 is important in the salvage pathway of nucleoside biosynthesis, and in modulating the levels of the physiological regulator adenosine. hENT1 is a member of the ENT (equilibrative nucleoside transporter) family, a family of transporters characterized by their sodium-independent facilitative transport of nucleosides and sensitivity to inhibition by NBMPR [*S*⁶-(4-nitrobenzyl)-mercaptopurine riboside], dilazep or dipyridamole. NBMPR is a potent inhibitor (IC₅₀ ~ 0.4–8 nM) of hENT1 (*es*, equilibrative-sensitive) [1, 9–11], whereas it inhibits hENT2 (*ei*, equilibrative-insensitive) with lesser potency (IC₅₀ ~ 2.8 μM) [11]. Similarly, hENT1 is more sensitive to inhibition by dilazep (IC₅₀ ~ 19–60 nM) and dipyridamole (IC₅₀ ~ 5–140 nM) when compared with hENT2 (IC₅₀ ~ 134 and 6–356 μM respectively) [10–13]. cDNAs, encoding putative nucleoside transporters hENT3 [2] and hENT4 [14], have been identified through sequence homology to expressed sequence tags of unknown function, but these putative transporters are yet to be functionally characterized.

The CNT (concentrative nucleoside transporter) family is characterized by sodium-dependent concentrative transport of nucleosides [15] and, except for a single putative transporter (*csg*), by their lack of sensitivity to inhibition by NBMPR [16]. cDNAs

encoding three members of the CNT family have been isolated [17–19]. Of these, two members, hCNT1 and hCNT2, have been extensively characterized after expression of recombinant proteins in various expression systems [20–24]. Interestingly, human and rat CNT1 and CNT2 appear to be expressed concurrently with the ENTs in epithelial cells of organs important in disposition of nucleoside drugs (e.g. the intestine, kidney and liver) to allow vectorial transport of nucleosides and nucleoside drugs in these tissues [24–26].

hENT1 is a multipass membrane protein and it is believed to contain 11 transmembrane domains (TMDs) [1]. Its putative secondary-structure topology is characterized by an intracellular N-terminus [27], and two large loops between TMDs 1 and 2, and TMDs 6 and 7. The loop between TMDs 1 and 2 has been shown to be glycosylated and extracellular [27]. Owing to the intrinsic difficulty in obtaining crystallographic data from multipass integral membrane proteins, numerous strategies have been employed to correlate the primary and putative secondary structural components of the ENTs to their function. The two most successful strategies have employed either random mutagenesis or chimaeric protein constructs. Studies involving chimaeric rat ENT1/ENT2 transporters expressed in *Xenopus laevis* oocytes have implicated TMDs 3–6 of rat ENT1 in NBMPR binding [28], and TMDs 5–6 of rat ENT2 in nucleobase translocation [29]. Previous studies from our laboratory have shown that Gly¹⁷⁹ in TMD 5 and Gly¹⁵⁴ in TMD 4 are critical for nucleoside transport function, and mutations at these positions reduce the ability of NBMPR to inhibit hENT1 [9,30]. However, alternative approaches have

Abbreviations used: CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; hENT, human ENT; NBMPR, *S*⁶-(4-nitrobenzyl)-mercaptopurine riboside; TMD, transmembrane domain.

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revealed that other amino acids outside domains 3–6 are important in the binding of hENT1 inhibitors. For example, Visser et al. have identified two amino acids, Met³³ [10] and Asn³³⁸ [31], which, when mutated, result in decreased inhibitor affinity. Mutation of Met³³ in TMD 1 decreases dipyridamole and dilazep inhibition of hENT1, whereas mutation of Asn³³⁸ in TMD 8 results in reduced inhibition of hENT1 by draflazine, solufazine, dipyridamole and dilazep.

None of the above studies have identified amino acid residues of hENT1 which, when mutated, selectively alter the affinity of hENT1 for the natural nucleosides inosine or guanosine. In the present study, we report the discovery of such an amino acid residue, Leu⁹². Using random PCR mutagenesis and functional complementation in yeast followed by targeted site-directed mutagenesis, we have determined that mutation of Leu⁹² alters the binding and/or translocation of the purine nucleosides inosine and guanosine, but not adenosine or pyrimidines. In addition, mutation of this amino acid residue selectively affects the sensitivity of the transporter to the inhibitors NBMPR and dilazep but not to dipyridamole.

EXPERIMENTAL

Generation of a random mutant library

hENT1 from the plasmid pYES [9] was amplified using the decreased fidelity PCR method of Cadwell and Joyce [32]. Putative hENT1 TMDs 1–6, 7–11 and 2–11 were targeted for random mutation by performing mutagenic PCR with the following primers respectively: 5'-GCCCTTGACCATGACAACAGT-CACCAGCTCAGGACAGATAC-3' (sense) and 5'-GCTTGA-GCTGCTGGTAGTAGCGGTAGAATTCCAGGCGGGGAG-3' (antisense) (TMDs 1–6); 5'-AAGCTTGAAGGACCCGGGGAG-CAGGAGACCAAGTTGGACCTCATT-3' (sense) and 5'-TAG-GGATAGGCTTACCTTCGAATGGGTGACCTCGAAGCTCG-CCCT-3' (antisense) (TMDs 7–11); 5'-CACCTTGCCTGAGC-GCAAC-3' (sense) and 5'-GTTAGAGCGGATGTGGG-3' (antisense) (TMDs 2–11). The mutagenic PCR reactions were performed at 94 °C for 5 min; 5 cycles of 94 °C for 30 s, 52.5 °C for 30 s, and 72 °C for 4 min, followed by 72 °C for 7 min. These products were then further amplified by 30 cycles of non-mutagenic PCR conditions using the same primers, durations and temperatures. Plasmid pYES was cut with *Bsu36I*–*EcoRI*, *SmaI*–*BstEII* or *BamHI*–*XbaI* and transformed [33] along with the randomly mutated PCR fragments into yeast strain W303-1A (MATa *ade2-1*, *can1-100*, *cyh2*, *his3-11,15*, *leu1-c*, *leu2-3,112*, *trp1-1*, *ura3-1*). The randomly mutated fragments were incorporated into the cut plasmid by *in vivo* yeast homologous recombination [9]. Yeast cells containing successfully recombined plasmids were selected on SD-Ura [2 % (w/v) dextrose, 1 % (w/v) yeast nitrogen base, 1 % (w/v) amino acid mix–uracil] plates by the presence of the URA3-selectable marker on the pYES plasmid.

Screening of random mutants by phenotypic complementation

The yeast cells transformed with random mutants were replica plated on to GR-Ura-Ade [2 % (w/v) galactose, 1 % (w/v) raffinose, 1 % (w/v) yeast nitrogen base (Difco, Detroit, MI, U.S.A.), 1 % (w/v) amino acid mix–uracil–adenine] plates containing 150 µM adenosine, and the presence or absence of the hENT1 inhibitors 2 µM NBMPR, 50 µM dipyridamole or 10 µM dilazep (Sigma, St. Louis, MO, U.S.A.). 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 picked from the corresponding inhibitor

plates, and spotted on to SD-Ura plates. These candidates were then re-screened as above. Mutant hENT1 plasmids were recovered from yeast colonies that showed reproducible inhibitor-resistant phenotypes [34], amplified in *Escherichia coli* strain XL1-Blue, and then re-transformed into yeast strain W303-1A. These were re-screened as above to confirm the plasmid's contribution to the inhibitor-resistant phenotype.

Sequencing of candidate plasmids

Candidate plasmids that successfully reproduced the plate assay phenotype were sequenced using the BigDye Terminator reaction kit (Applied Biosystems, Foster City, CA, U.S.A.), and analysed by the University of Washington Biochemistry Sequencing Facility.

Expression of mutant hENT1 for transport experiments

Plasmids containing mutant hENT1 were transformed into yeast strain YPL-1 (MATa *fui1Δ::HIS3*, *ura3-52*, *lys2-801*, *HIS3Δ*) [9] for subsequent transport experiments. Presence of a functional nucleoside transporter was confirmed by the lack of growth of the candidate clones on a GR-Ura plate in the presence of 175 µM tubercidin (Sigma).

Generation of hENT1 point mutants

The single-point mutant L92Q was created by generating two overlapping double-stranded DNA fragments [9] by *Pfu* enzyme-mediated polymerase-chain reaction using pYES as the template and the following primers: 5'-GAACTGAGCAA-GGACGCCCA-3' (sense), 5'-GTAGGTGAATAACAGTTGGG-GCAGCATGGCACA-3' (antisense); and 5'-TGTGCCATGC-TGCCCCAACTGTTATTCACCTAC-3' (sense), 5'-GTTAGAG-CGGATGTGGG-3' (antisense). These overlapping PCR fragments containing the coding sequence of hENT1 and flanking sequences homologous to the cloning sites in pYES were transformed along with pYES cut with *PvuII* and *XbaI* into yeast strain YPL1 [9]. Plasmids that successfully recombined were selected for on SD-Ura plates. The presence of hENT1 containing the point mutation was confirmed by PCR and sequencing.

[³H]Nucleoside transport experiments

Yeast strain YPL1 harbouring plasmids containing wild-type and mutant hENT1 were grown overnight in SD-Ura liquid media by shaking at 30 °C. The cells were then pelleted and washed three times with deionized water. The cells were resuspended to an A₆₀₀ 0.5 with GR-Ura, and induced for 15 h by shaking at 30 °C. The induced yeast cells were pelleted, the supernatant decanted and resuspended in an equivalent volume of transport buffer (10 mM Hepes, pH 7.4/ 100 mM choline chloride/2 mM KCl/1 mM CaCl₂/1 mM MgCl₂). These cell suspensions (196 µl) were added to 2 µl of DMSO containing either hENT1 inhibitors or DMSO alone; 2 µl of substrates were added (1 µM final concentration; Moravsek, Brea, CA, U.S.A.) as described for each specific experiment. After the allotted uptake time, three 50 µl aliquots of cells were rapidly filtered on to 0.45 µm HA-type membrane filters (Millipore, Billerica, MA, U.S.A.), followed by three quick 3 ml washes each with transport buffer. Cells deposited on filter membranes were then solubilized with 5 % (v/v) Triton X-100 and the radioactivity on each filter determined by scintillation counting (Packard Tricarb 1600 Scintillation Counter). The uptake of radiolabelled substrate due to diffusion was determined by performing control uptake reactions in the

presence of 2 mM adenosine. Uptake data were corrected for cell concentration, which was determined by measuring A_{600} of the cellular suspensions in transport buffer.

[³H]Adenosine transport inhibition experiments

Inhibition of [³H]adenosine transport by NBMPR, dipyridamole, and dilazep was studied by preincubation with the inhibitor for 20 min, and the activity of [³H]adenosine transport was determined for 20 min in the presence of the inhibitor. Natural nucleoside inhibition experiments were performed as above with no preincubation. IC₅₀ values of the inhibitors were determined as above using a range of concentrations of the inhibitor (NBMPR, 0.01–5000 nM; dipyridamole, 0.02–10 000 nM; and dilazep, 0.01–1000 nM).

Determination of the kinetics of [³H]nucleoside transport

Transport experiments were performed as above with the following modifications. After induction, cells were aliquoted and pelleted. The experiment was initiated by adding to the cell pellet 200 µl of transport buffer containing 0.18 µM [³H]inosine or 0.50 µM [³H]adenosine and different concentrations of unlabelled substrate (0–13 mM inosine or 0–2 mM adenosine).

Intracellular metabolism of adenosine during transport experiments

To investigate the degree of intracellular metabolism of adenosine during transport experiments, [³H]adenosine transport was performed in the presence of DMSO or 200 µM guanosine. The filtered cells were then suspended in methanol, spiked with unlabelled 100 µM adenosine and lysed by mechanical agitation with 0.45–0.55 mm glass beads. The lysate was filtered through 0.22 µm Millex-HV filters (Millipore), and analysed by HPLC [Alltech EconoSil C18 5 µm column, 50% (v/v) methanol mobile phase, 1.0 ml/min flow rate, UV detection at 254 nm]. The HPLC eluent was collected every 30 s for 10 min, and the radioactive content of each eluent fraction was counted. The percentage of non-metabolized adenosine metabolism in the cells was determined by the ratio of radioactivity that co-eluted with the unlabelled adenosine to the total radioactivity eluted.

Data analysis

IC₅₀ values were estimated by non-linear regression analysis of the concentration and inhibition data using

$$E = E_{\max} - (E_{\max} - E_0) \left(\frac{c}{c + \text{IC}_{50}} \right) \quad (1)$$

where E is the observed transport, $E_{\max}$ the maximal inhibition of transport by the inhibitor, E_0 the residual uptake observed at the maximum concentration of the inhibitor and c the inhibitor concentration.

The $V_{\max}$ and K_m values of [³H]nucleoside transport were estimated by non-linear regression analysis of the labelled substrate displacement curves as described previously [35,36]:

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

where v^* is the velocity of transport of the labelled nucleoside, T the concentration of the labelled nucleoside, K_d the diffusion constant and S_{cold} the concentration of the unlabelled nucleoside.

Figure 1 Phenotypic complementation plate assay

Plasmids were expressed in yeast strain W303-1A, spotted on a master plate (SD-Ura), and replica plated on to GR-Ura-Ade (A⁻), GR-Ura-Ade + 150 µM adenosine (A⁺), GR-Ura-Ade + 150 µM adenosine + 2 µM NBMPR (NB), GR-Ura-Ade + 150 µM adenosine + 50 µM dipyridamole (DP), or GR-Ura-Ade + 150 µM adenosine + 10 µM Dilazep (DZ). Growths after 4 days of incubation are shown at 30 °C of wild-type hENT1 (ENT), wild-type hCNT1 (CNT), and the random mutant candidate containing the single amino acid change L92P. Unlike wild-type hENT1, L92P shows growth in the presence of NBMPR and DZ. Results for L92P were typical for all four random mutant candidates showing NBMPR and dilazep-resistant phenotype.

Parameter estimates were performed using the Gauss–Newton iterative method and y^{-2} weighting using WinNonlin™ (Pharsight Corporation, Mountain View, CA, U.S.A.). The mean and standard deviation of the parameter estimates from at least three separate experiments were calculated.

Student's t test was used to test the null hypothesis that the transport of nucleosides by the wild-type and mutant transporter was equivalent.

RESULTS

Phenotypic complementation screening of random mutants

Yeast strain W303-1A carries a mutation in the ADE2 gene, and will not survive when grown in the absence of adenine. However, these cells will survive when grown in the presence of adenosine if adenosine is transported into the cell by a functional nucleoside transporter such as hENT1 (adenosine rescue) [37]. To identify randomly generated hENT1 mutants resistant to inhibition by NBMPR, dipyridamole or dilazep, we screened yeast strain W303-1A expressing these mutants in the adenosine rescue plate assay in the presence of inhibitors. hCNT1 was included as a negative control in this assay, as this transporter has negligible activity when expressed in yeast [20]. Consistent with this, yeast expressing hCNT1 did not survive when grown in the absence of adenine and presence of 150 µM adenosine, whereas the wild-type and mutant hENT1 transporters exhibited strong growth (Figure 1). Inhibition of growth of wild-type hENT1-expressing yeast cells in the presence of adenosine and 2 µM NBMPR, 50 µM dipyridamole or 10 µM dilazep was observed. Four candidate random mutant colonies were identified, and all of them showed strong resistance to inhibition by NBMPR and weaker, but detectable, resistance to inhibition by dilazep. These mutants transported adenosine despite the presence of inhibitors that would otherwise inhibit the transport of the wild-type, and therefore grew in the presence of NBMPR and dilazep. To confirm this resistance to inhibition, the plasmids were rescued and re-transfected into W303-1A yeast. Sequencing revealed that one candidate had a single amino acid change L92P (Leu⁹² → Pro), two had L92P and additional amino acid changes, and one had

Table 1 IC₅₀ values of NBMPR, dipyridamole and dilazep for [³H]adenosine transport by the wild-type and L92Q hENT1 transporter

Values represent the means $\pm$ S.D. for three independent experiments of inhibition of 1 μ M [³H]adenosine transport.

Transporter	NBMPR (nM)	Dilazep (nM)	Dipyridamole (nM)
hENT1	0.54 $\pm$ 0.03	0.74 $\pm$ 0.08	7.3 $\pm$ 2.0
L92Q	120 $\pm$ 69	2.8 $\pm$ 0.23	11 $\pm$ 3.8
<i>P</i> value	0.037	< 0.001	0.226

L92Q and additional amino acid changes. The presence of a common amino acid, Leu⁹², in all four mutants was a strong indication that mutations of this amino acid were responsible for the phenotypic effect, and the presence of a single amino acid change in one of the candidates confirmed this observation. Leu⁹² is conserved amongst ENT1 and ENT2 in human, mouse and rat and is located in putative TMD 2 in a region highly conserved amongst ENT1–ENT3.

Inhibition of [³H]adenosine transport by nucleoside transporter inhibitors

To characterize in detail the effect of Leu⁹² mutation on hENT1 function, we created the single amino acid mutants L92P and L92Q by point mutagenesis. The inhibition of [³H]adenosine transport by the wild-type, L92P and L92Q transporters was measured in the presence and absence of NBMPR, dilazep or dipyridamole. Transport values were obtained after correcting for uptake due to diffusion, and expressed as a percentage of transport in the absence of the inhibitor. Transport of [³H]adenosine was linear for 60 min (results not shown) and all subsequent adenosine transport experiments were performed for 20 min. Both mutants showed similar degrees of resistance to inhibition by NBMPR. Inhibition of [³H]adenosine transport by the wild-type transporter in the presence of 10 nM NBMPR was 82%, whereas that for the two mutants varied from 0 to 20% ($n = 3$). The two mutants showed a similar degree of inhibition by 1 nM dilazep (29–32%, $n = 3$), which was significantly lower than that of the wild-type transporter (68%, $n = 3$). The two mutants showed no significant difference from wild-type transporter with respect to inhibition by 10 nM dipyridamole. Since both L92Q and L92P showed virtually identical degrees of resistance to inhibition by NBMPR and dilazep, kinetics of transport were further characterized with the L92Q mutant. Determination of the IC₅₀ values of NBMPR and dilazep for [³H]adenosine transport showed that L92Q was 228- and 4-fold less sensitive to inhibition by NBMPR and dilazep respectively compared with the wild-type transporter (Table 1). As expected, the IC₅₀ value of dipyridamole for L92Q was unchanged when compared with the wild-type transporter.

Inhibition of [³H]adenosine transport by natural nucleosides

To determine if L92Q also affected the ability of hENT1 to transport nucleosides, the sensitivity of wild-type and L92Q transporter to inhibition by six naturally occurring nucleosides was first measured. The ability of 200 μ M or 2 mM inosine or guanosine to inhibit [³H]adenosine transport by the L92Q mutant was reduced approx. 2-fold relative to the wild-type transporter (Table 2). The wild-type transporter was 55 and 54% inhibited by 200 μ M inosine and guanosine respectively, whereas the L92Q mutant was 22 and 19% inhibited ($n = 6$). The other four natural nucleosides, cytidine, thymidine, adenosine and uridine, showed no significant

Table 2 Inhibition of [³H]adenosine transport by natural nucleosides

[³H]Adenosine (1 μ M) transport by the wild-type hENT1, L92Q or L92P mutants was measured in the absence and presence of natural nucleosides adenosine (A), inosine (I), guanosine (G), uridine (U), cytidine (C) and thymidine (T), and expressed as a percentage (means $\pm$ S.E.M.; $n = 3$ –6) of the control [absence of inhibitor; wild-type: 0.71–28 amol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹, L92Q: 65–78 amol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹, L92P: 60–160 amol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹] transport after correcting for diffusion. * $P < 0.05$ (two-sided), significant differences from the wild-type transporter. N.D., not determined.

Inhibitor	Concentration (μ M)	Adenosine transport (% of control)		
		Wild-type	L92Q	L92P
A	40	50.0 $\pm$ 2.2	46.5 $\pm$ 3.2	N.D.
I	200	45.0 $\pm$ 11.4	78.4 $\pm$ 3.7*	64.7 $\pm$ 2.1*
	2000	7.8 $\pm$ 4.5	29.3 $\pm$ 5.7*	17.0 $\pm$ 2.0*
G	200	45.8 $\pm$ 13.2	80.8 $\pm$ 11.6*	80.2 $\pm$ 3.5*
	2000	17.7 $\pm$ 7.4	42.3 $\pm$ 9.5*	44.6 $\pm$ 10.9*
U	170	63.9 $\pm$ 7.3	76.3 $\pm$ 8.6	N.D.
	2000	19.5 $\pm$ 2.0	25.8 $\pm$ 4.0	N.D.
C	600	85.5 $\pm$ 13.5	72.7 $\pm$ 5.0	N.D.
	2000	48.5 $\pm$ 2.5	52.3 $\pm$ 6.8	N.D.
T	500	57.4 $\pm$ 6.9	45.7 $\pm$ 4.5	N.D.
	2000	22.0 $\pm$ 3.0	18.7 $\pm$ 3.3	N.D.

differences in inhibition between the wild-type and the mutant transporters.

To determine if this difference in sensitivity to purines extended to the L92P mutant, the inhibitory effect of either inosine or guanosine on [³H]adenosine transport by this mutant was also determined (Table 2). Consistent with the observations of the L92Q mutant, the ability of 200 μ M or 2 mM inosine and guanosine to inhibit [³H]adenosine transport by the L92P mutant was also reduced approx. 2-fold relative to the wild-type transporter.

To rule out the possibility that the observed resistant phenotype of the mutants was due to competition of [³H]adenosine metabolism by the unlabelled substrate, the intracellular metabolism of [³H]adenosine by L92Q-expressing cells was investigated after 20 min of [³H]adenosine transport in the absence and presence of 200 μ M guanosine. There was no significant difference in the non-metabolized proportion of [³H]adenosine in the presence (63.7 $\pm$ 3.3%) or absence (69.5 $\pm$ 4.5%) of 200 μ M guanosine ($P > 0.1$, two-sided, $n = 3$).

[³H]Nucleoside transport kinetics

To understand further the mechanism(s) by which the transport of purines was affected by mutations at Leu⁹², the kinetics of [³H]adenosine and [³H]inosine transport by both the wild-type and L92Q transporters were investigated. Inosine was chosen as a representative purine nucleoside over guanosine because of its greater aqueous solubility, thus allowing the use of a wider concentration range in determining the kinetic parameters $V_{\max}$ and K_m . Consistent with the reduced sensitivity to inhibition by inosine, the K_m value of the L92Q mutant for inosine was increased approx. 4-fold when compared with the wild-type transporter (260 and 65 μ M respectively, Table 3, $n = 3$). Furthermore, the $V_{\max}$ value of inosine transport was significantly greater for the L92Q (approx. 6.5-fold) when compared with the wild-type transporter [32 and 4.9 fmol $\cdot$ (10⁶ cells)⁻¹ $\cdot$ min⁻¹ respectively]. In contrast, there was no significant difference between the L92Q and wild-type transporter in their apparent affinity to transport adenosine ($K_m = 54$ and 44 μ M respectively, Table 3, $n = 5$). Consistent with this observation, the maximal

Table 3 Kinetic parameters for the transport of inosine and adenosine by the wild-type and L92Q transporters

The kinetic parameters, K_m (μM) and V_{max} [$\text{fmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$], of inosine transport into yeast cells expressing the wild-type hENT1 or the L92Q mutant were measured by incubating the cells for 10 min with [³H]inosine (0.18 μM) and different concentrations of unlabelled inosine (0–13 mM) or 20 min with [³H]adenosine (0.50 μM) and different concentrations of unlabelled adenosine (0–2 mM). The values represent means $\pm$ S.D. for 3–5 independent experiments.

	Inosine ($n = 3$)			Adenosine ($n = 5$)		
	K_m	V_{max}	$V_{\text{max}}/K_m \times 10^3$	K_m	V_{max}	$V_{\text{max}}/K_m \times 10^3$
hENT1	65 ± 7.8	4.9 ± 2.0	74 ± 29	44 ± 7.5	6.3 ± 3.1	150 ± 81.1
L92Q	260 ± 16	32 ± 4.1	120 ± 24	54 ± 15	44 ± 28	840 ± 570
<i>P</i>	< 0.001	< 0.001	0.083	0.224	0.016	0.028

capacity (V_{max}) of the L92Q mutant to transport adenosine was also significantly greater than that of the wild-type transporter [44 and $6.3 \text{ fmol} \cdot (10^6 \text{ cells})^{-1} \cdot \text{min}^{-1}$].

DISCUSSION

Owing to its ubiquitous expression and broad substrate selectivity, hENT1 is important in the disposition, toxicity and pharmacological activity of various nucleoside drugs [7,8]. To design nucleoside drugs that have improved pharmacokinetic and pharmacodynamic properties, development of a tertiary structural model of hENT1 is necessary. With the known difficulty in obtaining such information for membrane transporters, a combination of structural modelling validated by detailed mutagenesis and functional studies of amino acids involved in substrate and inhibitor binding is necessary. We present results generated through a high-throughput low-cost yeast expression system to identify and characterize efficiently the functional ramifications of mutations in hENT1.

We first experimentally determined the concentrations of inhibitors used to screen the mutants by growing the wild-type transporter in the presence of adenosine and increasing concentrations of inhibitors. The highest concentration of inhibitor that permitted yeast growth (rescue) was defined as the threshold concentration. To identify mutant transporters resistant to inhibition, the random mutants and control transporters were then screened at concentrations of inhibitor approx. 2–4-fold greater than the threshold concentrations. Consistent with previous yeast-based rescue assays [10,38], the inhibitor concentrations that prevented the rescue of the wild-type transporter were much greater than the IC_{50} or K_i values of these inhibitors. This difference is most probably due to the differences in the end-point measured (IC_{50} value of substrate transport versus survival of ade2 phenotype yeast), experimental conditions (suspension versus plate assay) and the presence of 150 μM adenosine competing with inhibitor binding.

Using this high-throughput adenosine rescue plate assay, we identified four hENT1 mutants that were resistant to inhibition by NBMPR or dilazep but not to dipyrindamole. The possibility that the resistance is due to the lack of dependence on adenosine for growth was ruled out by growing yeast cells in the absence of adenosine (negative control). Functionality of the transporter was also confirmed by growing the yeast cells in the presence of adenosine and absence of inhibitor (positive control). Furthermore, the system was validated by growth of yeast cells harbouring wild-type hENT1 (pYES) and absence of growth of yeast cells expressing hCNT1 (due to its low activity and sodium dependence) in the presence of adenosine and absence of sodium.

Figure 2 Structures of hENT1 substrates and inhibitors

Structures of hENT1 substrates adenosine, guanosine and inosine and inhibitors NBMPR, dipyrindamole and dilazep are shown.

Our adenosine rescue plate assay appears to be quite sensitive in detecting small changes in inhibitor affinity. Mutation at Leu⁹² reduces the apparent affinity of dilazep approx. 4-fold, and this change could be detected as a weak growth on the plate assay. This sensitivity of the plate assay is consistent with the observations of other studies. For example, in a 5-fluorouridine cytotoxicity plate assay, to identify hENT1 random mutants resistant to inhibition by NBMPR [9], only a 6-fold decrease in NBMPR affinity of hENT1 mutant G179A resulted in resistance to NBMPR inhibition of 5-fluorouridine toxicity. Additionally, a 10-fold decrease in dipyrindamole and dilazep affinity in hENT1, mutated at Met³³, resulted in resistance to inhibition of growth by 10 μM dilazep in a yeast thymidine salvage plate assay [10]. These results suggest that plate assays, with end points of survival or death of yeast cells expressing mutant hENT1, are highly sensitive to detecting small changes in inhibitor affinity.

The resistance to inhibition by NBMPR and dilazep in the adenosine rescue plate assay was further confirmed quantitatively by both single-concentration inhibition studies and more detailed IC_{50} characterizations of adenosine transport. Our observation that these mutants were resistant to NBMPR or dilazep, but not dipyrindamole, suggests that these inhibitors have distinct as well as overlapping binding sites on hENT1. This observation is not surprising given the degree of structural differences in these inhibitors. NBMPR is an analogue of the nucleoside inosine, whereas both dipyrindamole and dilazep are structurally unrelated to nucleosides (Figure 2).

Since NBMPR is a nucleoside analogue, we hypothesized that the apparent affinity of the L92Q transporter for other nucleosides would also be changed. Indeed it was, but this change was selective for the purines, inosine and guanosine, and not for adenosine or the pyrimidines (Table 2). To discount the possibility that this

reduced inhibition by the purines could be a result of competitive inhibition of intracellular metabolism of adenosine in yeast, we determined the intracellular metabolism of transported adenosine, in the presence or absence of guanosine, in cells expressing the L92Q transporter. As the metabolism of adenosine in these cells was not significantly different in the presence or absence of guanosine, we concluded that the reduced inhibition of adenosine transport in the presence of the competing nucleosides (inosine or guanosine) was solely due to decreased hENT1 transport.

Detailed kinetic analysis of the transport of [^3H]inosine by the L92Q mutant confirmed that the apparent affinity of the transporter for inosine was reduced approx. 4-fold when compared with the wild-type transporter. The V_{max} values of both inosine and adenosine transport were approx. 6–7-fold greater in the L92Q mutant. This suggests that mutation at this amino acid residue results in either increased expression or increased stability of hENT1 in yeast. However, basic principles of Michaelis–Menten kinetics indicate that the affinity of a transporter is independent of the magnitude of its expression. Based on these principles, and that the observed selective decrease in affinity for both substrates and inhibitors is independent of the total amount of protein present, this difference in V_{max} values has no effect on the conclusions drawn in the present study. Also, principles of Michaelis–Menten kinetics indicate that for competitive and non-competitive inhibition, the magnitude of inhibition of a transporter is independent of its magnitude of expression. As the single-concentration inhibition profile of guanosine was virtually identical with that of inosine, we conclude that the apparent affinity of guanosine is most probably affected to a similar degree. To our knowledge, this is the first time that a mutation in hENT1 has been shown to affect the affinity for inosine and guanosine but not for pyrimidines.

Mutating Leu⁹² appears to alter a significant determinant of hENT1 binding and/or translocation for NBMPR, dilazep, inosine and guanosine, which is not critical for the binding and/or translocation of dipyridamole, adenosine or pyrimidines. To explain this observation, we examined the difference in structure between the purines, adenosine, inosine and guanosine (Figure 2). Adenosine differs from guanosine and inosine at the C-6 and N-1 positions of the purine. It is possible that either the carbonyl group in inosine and guanosine, but not the amino group in adenosine, or the proton at the N-1 position of these purines interacts either directly or indirectly with Leu⁹². Consistent with this, the inhibitory capacity of NBMPR was also affected when Leu⁹² is mutated. NBMPR is an inosine analogue with a nitrobenzylthiol substitution at the C-6 position of the purine, a position where adenosine differs from inosine and guanosine. NBMPR is also deprotonated at the N-1 position of the purine, similar to adenosine. Therefore, we speculate that the nitrobenzylthiol substitution at the C-6 position of the purine in NBMPR interacts directly or indirectly with Leu⁹², resulting in a change in the ability of this compound to inhibit the transporter when this amino acid is mutated. Our observation that substitution of the hydrophobic amino acid, Leu⁹², with glutamine (polar) or proline (non-polar) results in similar phenotypes suggests a steric or indirect interaction between Leu⁹² and nucleoside substrates and inhibitors of hENT1. Alternatively stated, mutation of Leu⁹² may result in a structural change in hENT1 affecting neighbouring or other residues directly involved in substrate and inhibitor interaction. As Leu⁹² is adjacent to Pro⁹¹, this position of the α -helix may be sterically stressed, and sensitive to neighbouring mutation. However, the selective effect of the Leu⁹² mutation on the affinity of inosine and guanosine, but not adenosine, suggests that mutation of Leu⁹² does not produce a gross structural change in this transporter.

To date, very few results are available on the structure–function relationship of hENT1. Studies with rat ENT1 and ENT2 chimaeric proteins have identified TMDs 3–6 and 5–6 as components of inhibitor and nucleobase interactions respectively. Using a combination of random mutagenesis and phenotypic complementation screening, three amino acid residues have been identified in hENT1 that interact with the hENT1 inhibitors. Surprisingly, two of these residues, Met³³ and Asn³³⁸, are outside TMDs 3–6. Mutation of Met³³ in hENT1 to isoleucine increases the apparent K_i value of dilazep and dipyridamole approx. 10-fold without affecting the K_i value of NBMPR [10]. This residue appears to be located near the extracellular portion of TMD 1. Mutation of Asn³³⁸ in hENT1 to serine results in resistance to inhibition by the vasoactive drugs draflazine, solflazine, dilazep and dipyridamole, as well as NBMPR, and appears to be located in TMD 8 [31]. Mutation to alanine of the highly conserved residue, Gly¹⁷⁹, in TMD 5 increases the IC_{50} value of NBMPR for uridine transport approx. 6-fold [9]. None of the above studies has identified amino acid residues of hENT1, which when mutated, selectively alter the affinity of hENT1 for the natural nucleosides inosine or guanosine. In the present study, we have identified for the first time an amino acid residue, Leu⁹², located in TMD 2, which when mutated to glutamine selectively affects the apparent affinity of hENT1 for only the natural nucleosides inosine and guanosine. Moreover, this change also results in a selective decrease in the apparent affinity of the nucleoside transporter for the inhibitors NBMPR and dilazep, but not dipyridamole. Similar studies of other functional mutations in hENT1, together with structural studies (e.g. development of a helix packing model), should provide a starting point to begin building a putative three-dimensional model of the hENT1 substrate-binding site.

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