## Title

Functional characterization in yeast of genetic variants in the human equilibrative nucleoside, ENT1



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# Functional Characterization in Yeast of Genetic Variants in the Human Equilibrative Nucleoside Transporter, ENT1

by

Douglas Hiroshi Osato

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August 25, 2003

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The human equilibrative nucleoside transporter (ENT1, SLC29A1) facilitates the cellular uptake of naturally occurring nucleosides and synthetic nucleoside analogs. It was purified from erythrocytes in 1998, which led to the cloning of the cDNA from the placenta in 1997 [1,2]. Upon characterization in *Xenopus oocytes*, it showed *es*-type activity; that is it was broadly selective for purine and pyrimidine nucleosides, inhibitable by nitrobenzylmercaptopurine (NBMPR), and transport was cation-independent. The 456 amino acid protein is predicted to consist of 11 transmembrane domains, based on hydropathy analysis and native and engineered *N*-glycosylation sites. The secondary structure of ENT1 is also characterized as having a large extracellular loop between transmembrane domains 1 and 2, and a large intracellular loop between transmembrane domains 6 and 7. There is one site of glycosylation at N48 [2]. However, the glycosylation is not necessary for the function of the protein [3].

The structural domains containing the active site of the protein are not well understood. However, it is believed that transmembrane domains 1-6 are involved in the transport of nucleosides. Using chimeric constructs of human and rat ENT1, it was shown that transmembrane domains 3-6 were important for the inhibition of radio-labeled uridine uptake by dipyridamole and dilazep [4]. Met 33 in transmembrane domain 1 may also play a role in the inhibition of transport by dipyridamole and dilazep. When Met 33 is changed to the corresponding amino acid in the human equilibrative nucleoside transporter 2 (ENT2), Ile, the sensitivity of ENT1-M33I to dipyridamole and dilazep is reduced from wild type ENT1 towards that of wild type ENT2. Conversely, when ENT2-I33M is tested, the sensitivity of the transporter to inhibition by dipyridamole and dilazep

is increased from wild type ENT2 towards that of wild type ENT1 [5]. Also, mutations of Gly 179 in transmembrane domain 5 were able to disrupt the transport activity of the protein [6].

ENT1 is thought to be ubiquitously expressed, which has been supported by the presence of ENT1 mRNA in all tissues probed thus far. These tissues include the kidney, liver, intestines, spleen, bone marrow, adrenal gland, pancreas, brain, heart, thymus, placenta, and many others [7]. ENT1 has been localized primarily to the basolateral membrane of renal epithelial cells [8]. However, the sorting sequence for ENT1 is not known. It should be noted that mutations of G184 affected the targeting of ENT1 to the plasma membrane in *Saccharomyces cerevisiae* so that there was little to no detectable activity [6].

ENT1 has several physiological roles in the body. It plays a role in nucleotide biosynthesis by salvage pathways in cells, particularly those that lack *de novo* nucleotide biosynthetic pathways, such as enterocytes and erythrocytes. Inhibition studies indicate that ENT1, through its regulation of extracellular adenosine concentrations, is involved in adenosine-mediated processes, including coronary vasodilation, neurotransmitter release, renal vasoconstriction, platelet aggregation, and lipolysis [9]. Furthermore, ENT1 is a route of cellular uptake and efflux of many anti-cancer and anti-viral nucleoside analogs [10].

The kinetics of interaction of ENT1 with naturally occurring nucleosides and synthetic analogs have been examined. The $K_m$ values for the naturally occurring nucleosides fall in the range of 50 μM for adenosine to 680 μM for cytidine [11]. ENT1 is unable to transport nucleobases. Inhibitors of ENT1 include NBMPR, dilazep, and dipyridamole. At nanomolar concentrations of NBMPR and micromolar concentrations of dilazep and dipyridamole, these compounds are able to inhibit the transport of nucleosides by ENT1 [2]. ENT1 is known to transport the anti-cancer nucleoside analogs cytarabine, gemcitabine, cladribine, fludarabine, and fluorouridine [12,13]. Anti-viral agents transported by ENT1 include zalcitabine, didanosine, floxidine, and ribavirin [12-14].

Nucleoside analog drugs such as ribavirin and gemcitabine exhibit large inter-individual differences in their efficacy and side effects [15,16]. It is possible that these differences are due to variation in the levels of nucleoside analogs in their target tissues. Therefore, we hypothesized that genetic variation in ENT1 may contribute to inter-individual differences in tissue drug levels and response to nucleoside analogs.

As part of a study to identify variants in transporter genes that may govern differences in drug response, we identified variants of *ENT1* (localized to chromosome 6p21.1-21.2) in 247 ethnically diverse DNA samples obtained from the Coriell Institute of Medicine [17,18]. Fifteen variants of *ENT1* were identified, of which two were non-synonymous variants. The major goal of this study was to determine the haplotype structure of *ENT1* and the function of the two non-synonymous ENT1 variants.

Data on the variants of *ENT1* are available at the PharmGKB and UCSF Pharmacogenetics websites (http://www.pharmgkb.org and http://pharmacogenetics.ucsf.edu, respectively) and are summarized in Table 1. *ENT1* exhibited exceptionally low mutability even in non-coding regions relative to other transporters [18]. Coding region variants (four synonymous and two non-synonymous) were present in both loops and transmembrane domains (TMD) (Fig. 1a). The two non-synonymous variants, ENT1-I216T and ENT1-E391K, were found in 1.2% and 0.4% of the samples screened, respectively. ENT1-I216T was found in African Americans, Caucasian Americans, and Mexican Americans. ENT1-E391K was found only in African Americans.

Ten haplotypes of *ENT1* were identified (Fig. 1b). Haplotypes were named using two criteria: evolutionary relationship and frequency in the sample [17]. As shown in the cladogram in Fig. 1c, haplotype *1 accounted for 91.3% of the total sample. The next most frequent haplotype, *2, accounted for only 2% of the total sample. Only haplotypes *1, *3B, and *6 were present in three or more ethnicities. Haplotypes *2, *3A, *4, *5, *7, and *8 were found only in African Americans. All 60 Asian and 14 Pacific Islander chromosomes were of the *1 haplotype. *ENT1* thus has little genetic variation.

To assay the activity of the two non-synonymous variants of ENT1, ENT1-I216T and ENT1-E391K, we expressed the transporters in a yeast mutant strain in which the endogenous nucleoside transporter, *FUI1*, had been inactivated. As shown in Fig. 2a, the

reference and variant transporters all allowed uptake of the cytotoxic nucleoside analogs, tubercidin and 5-fluorouridine, thereby inhibiting yeast growth and demonstrating that the ENT1 variants and the reference ENT1 transported tubercidin and 5-fluorouridine. The similar sizes of the haloes indicate that the rate of transport of the reference transporter and the two variants were similar. NBMPR inhibited uptake of these compounds, indicating that the variant ENT1 transporters and the reference transporter are inhibited by NBMPR.

The activity of ENT1 and its variants was also assessed by measuring inhibition of growth of cells in culture. In cells expressing ENT1, 5-fluorouridine (100 μM) inhibited the growth of cells (assayed at 6 hours after addition of inhibitor to exponentially growing cells). In contrast, there was no growth inhibition in cells containing an empty vector (data not shown). These data demonstrate that growth inhibition by 5-fluorouridine is dependent on ENT1. Inhibition studies were carried out at various drug concentrations (Fig. 2b). The $IC_{50}$ values for 5-fluorouridine inhibition of growth of yeast expressing the reference ENT1, ENT1-I216T, and ENT1-E391K were $0.96 \pm 0.07$ μM, $1.12 \pm 0.14$ μM, and $0.97 \pm 0.09$ μM, respectively, and were not significantly different ($p>0.05$, unpaired Student's t-test, data shown is mean ± SD). Similarly, the $IC_{50}$ values for tubercidin inhibition of growth of yeast cells expressing the reference ENT1, ENT1-I216T, and ENT1-E391K were not different ($1.17 \pm 0.14$ μM, $1.07 \pm 0.09$ μM, and $0.90 \pm 0.08$ μM, respectively).

To directly measure uptake across the plasma membrane of yeast cells expressing ENT1 and its variants, we performed uptake studies using a filtration assay. The six naturally occurring nucleosides (adenosine, guanosine, thymidine, uridine, cytidine, and inosine) and three of the nucleoside analog drugs (ribavirin, gemcitabine, and cytarabine) all inhibited the uptake of $^3$H-inosine by the reference and variant transporters. The three classic high affinity inhibitors of ENT1 (NBMPR, dipryidamole, and dilazep) also inhibited uptake of $^3$H-inosine by all three transporters. In general, the variant transporters behaved similarly to reference ENT1 with respect to inhibition of uptake by nucleosides and nucleoside analogs (Table 2). $IC_{50}$ values were determined for uridine, inosine, ribavirin, cytarabine, and gemcitabine inhibiting the uptake of $^3$H-inosine by the reference transporter and by ENT1-I216T. The naturally occurring nucleosides, uridine and inosine, were more potent inhibitors of the reference ENT1 and ENT1-I216T in comparison to the synthetic nucleoside analogs. However, no significant differences were observed in the potency of any of the compounds in inhibiting the reference ENT1 and ENT1-I216T (Figure 3 and Table 3, $p>0.05$, unpaired Student's t-test).

In other studies, we observed that substitutions at evolutionarily conserved positions are more likely to affect function than those at evolutionarily unconserved positions [18,19]. Furthermore, we observed that substitutions that lead to radical chemical changes in amino acids (assessed by Grantham values [20], in which large values indicate radical changes and small values indicate moderate changes) are more likely to affect function than less radical substitutions. Our finding that the two non-synonymous variants did not affect function is consistent with these observations: E391K and I216T both affect

evolutionarily unconserved sites and have moderate Grantham values (56 and 89, respectively) [20].

In conclusion, no significant differences in the functional characteristics of the two non-synonymous variants of ENT1 in comparison to the reference ENT1 were observed. This observation may suggest that ENT1 is critical for human fitness and that selection acts to preserve its function. However, because ENT1 exhibits low intrinsic mutability, as shown by the low $\pi$ values in Table 1b [18], and high prevalence of a single haplotype in ethnically diverse populations (Fig. 1b and 1c), there is little variation in *ENT1* on which selection might act. It is therefore unclear whether ENT1 is critical for human fitness. A recent study demonstrates that mice lacking *ENT1* do not exhibit gross phenotypic abnormalities, indicating that ENT1 is not critical for mouse viability [Robert Messing, personal communication]. Irrespective of the reason why ENT1 has remarkably little variation, our study suggests that coding region non-synonymous variants of ENT1 do not contribute to variation in response to anti-viral and anti-cancer nucleoside analog drugs. Future studies are needed to determine whether variation in non-coding regions of ENT1, such as the promoter region, may contribute to variation to response to nucleosides and nucleoside analog drugs.

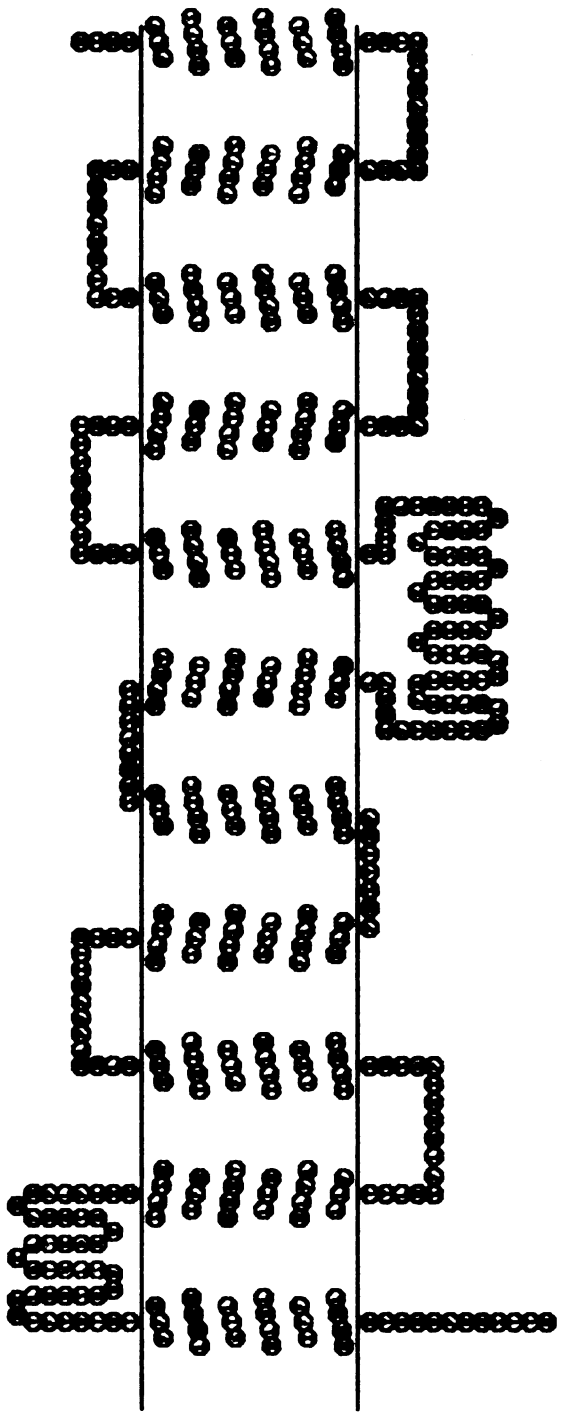
## Figure Legends

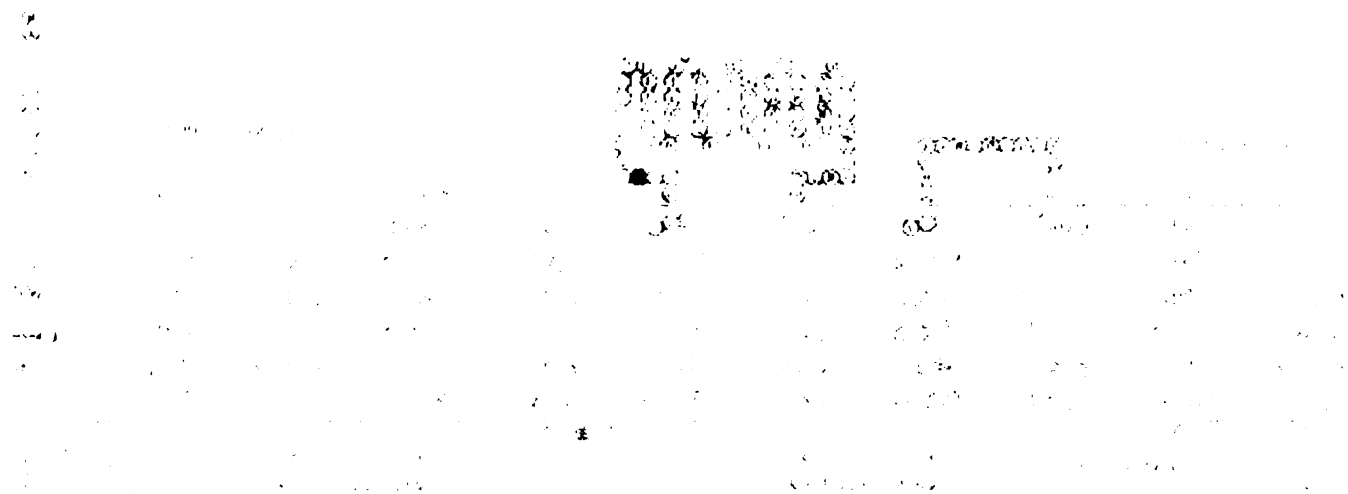
**Figure 1. Variants and haplotypes of hENT1.** (a) Secondary structure of ENT1 with coding region SNPs. The transmembrane topology schematic was rendered using TOPO (S.J. Johns [UCSF, San Francisco, CA, USA] and R.C. Speth [Washington State University, Pullman, WA, USA]) transmembrane protein display software, available at the UCSF Sequence Analysis Consulting Group website (http://www.sacs.ucsf.edu/TOPO/topo.html). Non-synonymous amino acid changes are shown in red; synonymous changes in green. (b) Sequences of the haplotypes at each of the 8 non-singleton variable sites. The first row denotes the sequence of the common allele at each site. ENT1-I216T occurs in haplotype **6* and ENT1-E391K occurs in haplotype **7*. Haplotypes were predicted using PHASE software version 1.0.1 as previously described [17,21]. (c) Cladogram showing the relationships between haplotypes. Haplotypes with red-filled circles contain a non-synonymous variant; those with blue-filled circles do not. A pink halo indicates that this haplotype is present in at least three ethnic groups. Lines between haplotypes indicate the type of change (black, non-coding; green, synonymous; red, non-synonymous). The relative areas of the circles are proportional to the relative frequencies of each haplotype.

**Figure 2. Cytotoxic assays of ENT1, ENT1-I216T, and ENT1-E391K.** (a) Halo assays of the reference and variant transporters. Yeast strains were grown in SD-His medium (lacking histidine to select for maintenance of the plasmid.) to $OD_{600} = 1$. Approximately $10^6$ cells were spread on an SD-His plate (lacking histidine to select for maintenance of the plasmid.). Disks were spotted with (clockwise from upper left): tubercidin (2.5 mM), 5-fluorouridine (5 mM), 5-fluorouridine and NBMPR (5 mM and 100 μM, respectively), and water. Plates were incubated overnight at 30°C. (b) Growth inhibition curve for yeast expressing the ENT1 reference transporter. Yeast in liquid culture ($OD_{600} = 0.13$) containing 5-fluorouridine or tubercidin was incubated at 30°C for 6 hours and measured for cell density at 600 nm. Data are representative of experiments carried out 3 times. Each value represents mean ± SD. The growth inhibition curves for yeast expressing ENT1-I216T and ENT1-E391K are similar to data shown for ENT1 reference. The yeast strain, IH4644 (an *fui1* derivative of W303), was used for studying ENT1 and its variants. Complete deletion of the coding region of the uridine transporter gene, *FUI1*, in W303 was carried out using a PCR method as described [22]. Variants were constructed by site-directed mutagenesis using a PCR-based method [17]. Each variant was subcloned into the yeast expression vector pSH92 (a kind gift from Shai Shaham, University of California, San Francisco), which contains a constitutively active *TDH* promoter and a *HIS*3 gene marker. The resulting plasmids were introduced into IH4644 by a standard lithium acetate method with selection for $His^+$ [23].

**Figure 3. Kinetics of inhibition of $^{3}$H-inosine uptake by ribavirin, cytarabine, and gemcitabine in yeast expressing the ENT1 reference transporter.** Uptake of inosine (1 μM unlabelled inosine, 0.05 μM $^{3}$H-inosine) in the presence of increasing concentrations of ribavirin, cytarabine, or gemcitabine by reference ENT1 (●) or ENT1-I216T (■) was measured after incubation for 20 min. Data are representative of experiments carried out 7 times. Fifty μL of yeast cell suspension ($OD_{600}$ = 11) was added to 50 μL of buffer containing $^{3}$H-inosine and various concentrations of the inhibitors. After 20 min of incubation, samples were filtered through a Millipore nitrocellulose membrane and washed three times with 5 mL of ice-cold water. The filters were air dried, and the radioactivity was measured by liquid scintillation counting.

1 (a)





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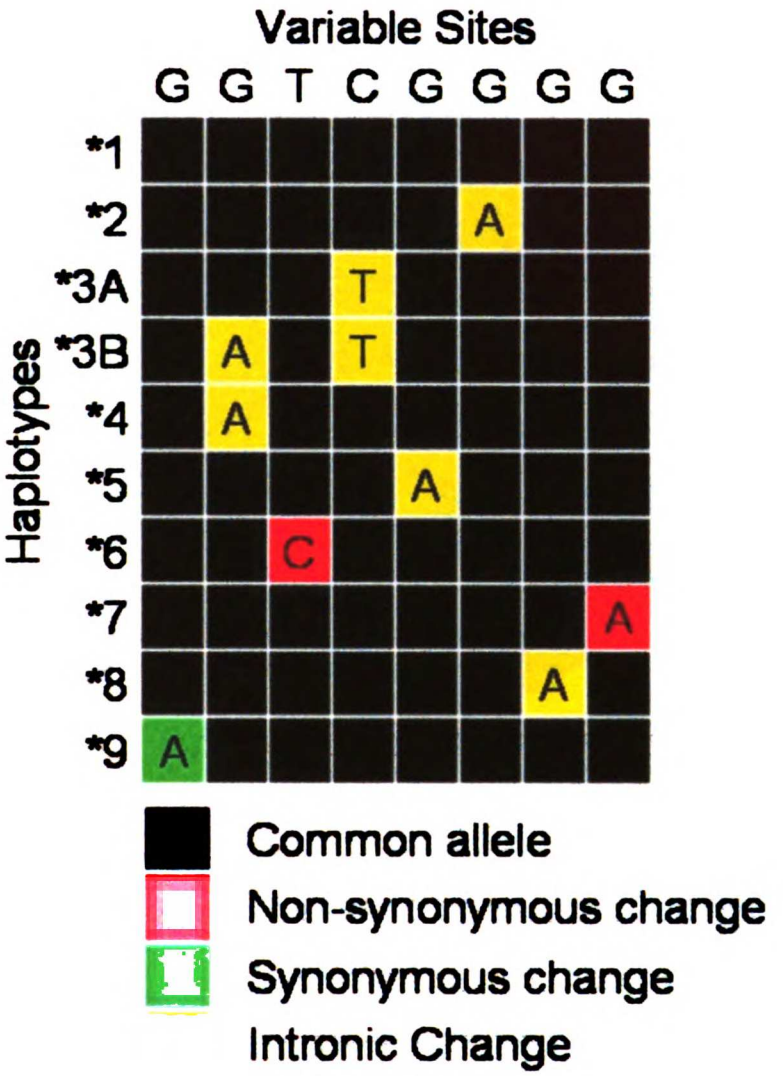
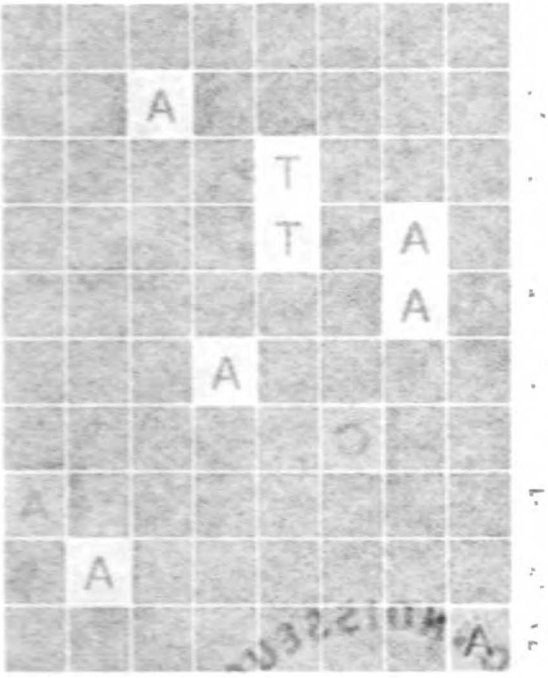
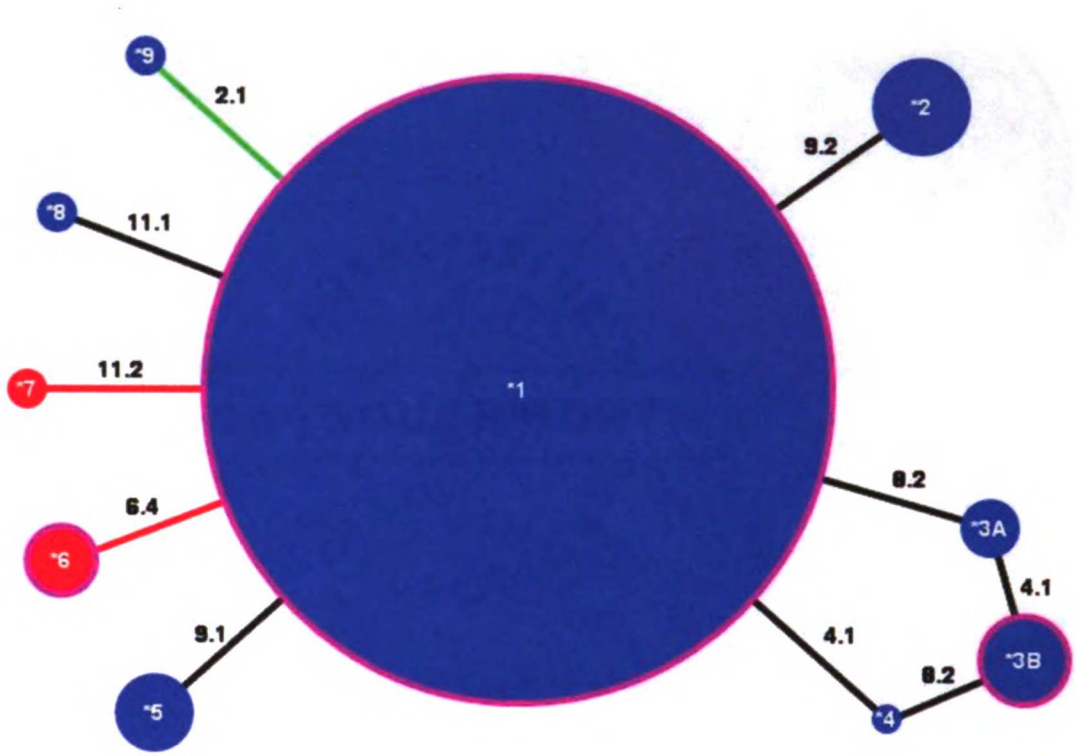

Variable Sites
G G T C G G G G
Haplotypes
*1
*2
*3A
*3B
*4
*5
*6
*7
*8
*9
A
T
T
A
A
C
A
A
A
Common allele
Non-synonymous change
Synonymous change
Intronic Change

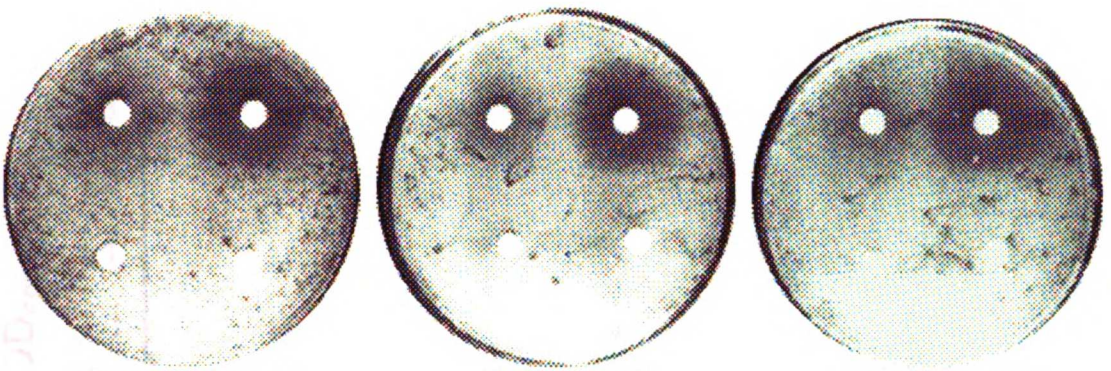

A
T
T
A
A
A
A
A

1 (c)



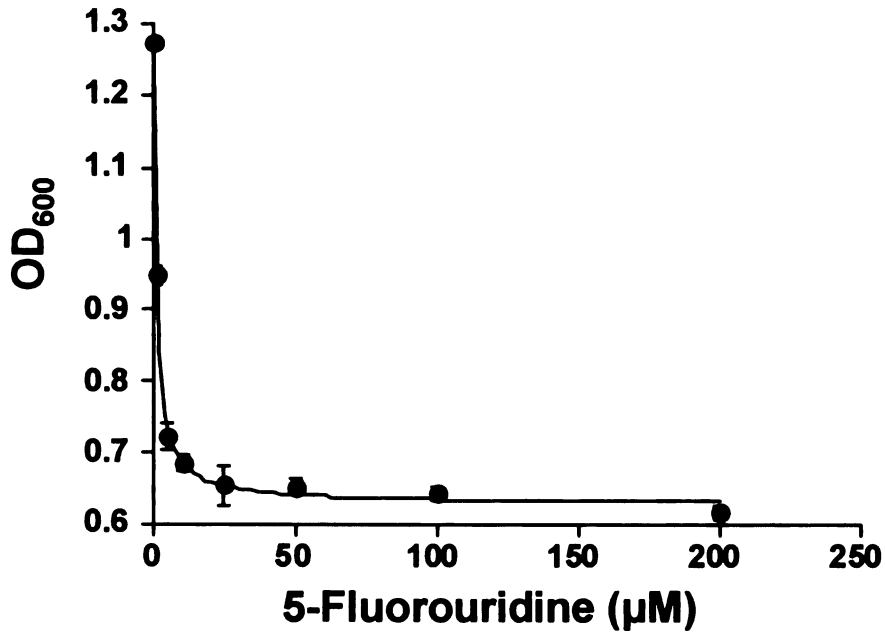
SOUTHWORTH
COLLECTION

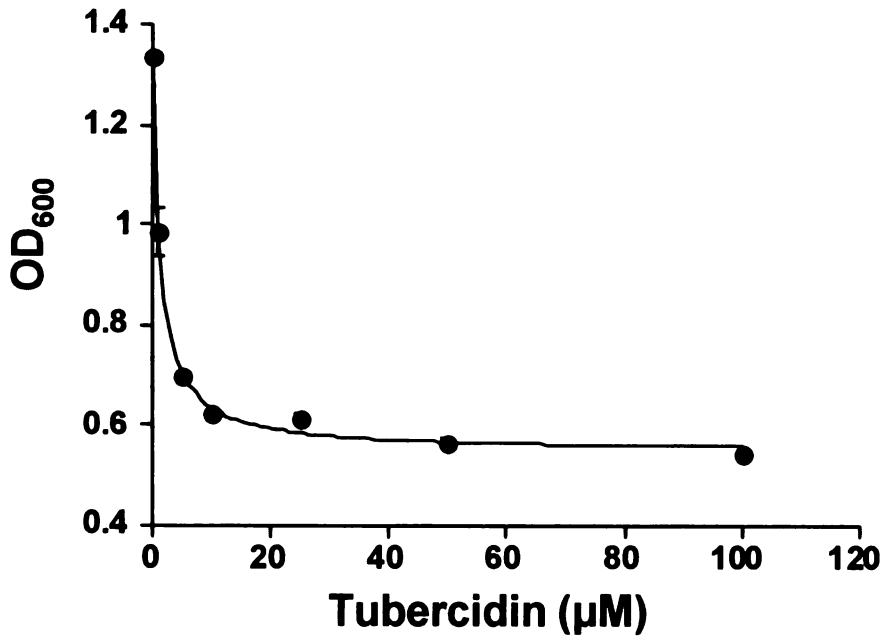
2 (a)



ENT1 Reference ENT1-I216T ENT1-E391K

2 (b)


1.3
1.2
1.1
1
0.9
0.8
0.7
0.6
OD600
0
50
100
150
200
250
5-Fluorouridine (μM)


1.4
1.2
1
0.8
0.6
0.4
OD600
0
20
40
60
80
100
120
Tubercidin (μM)

3

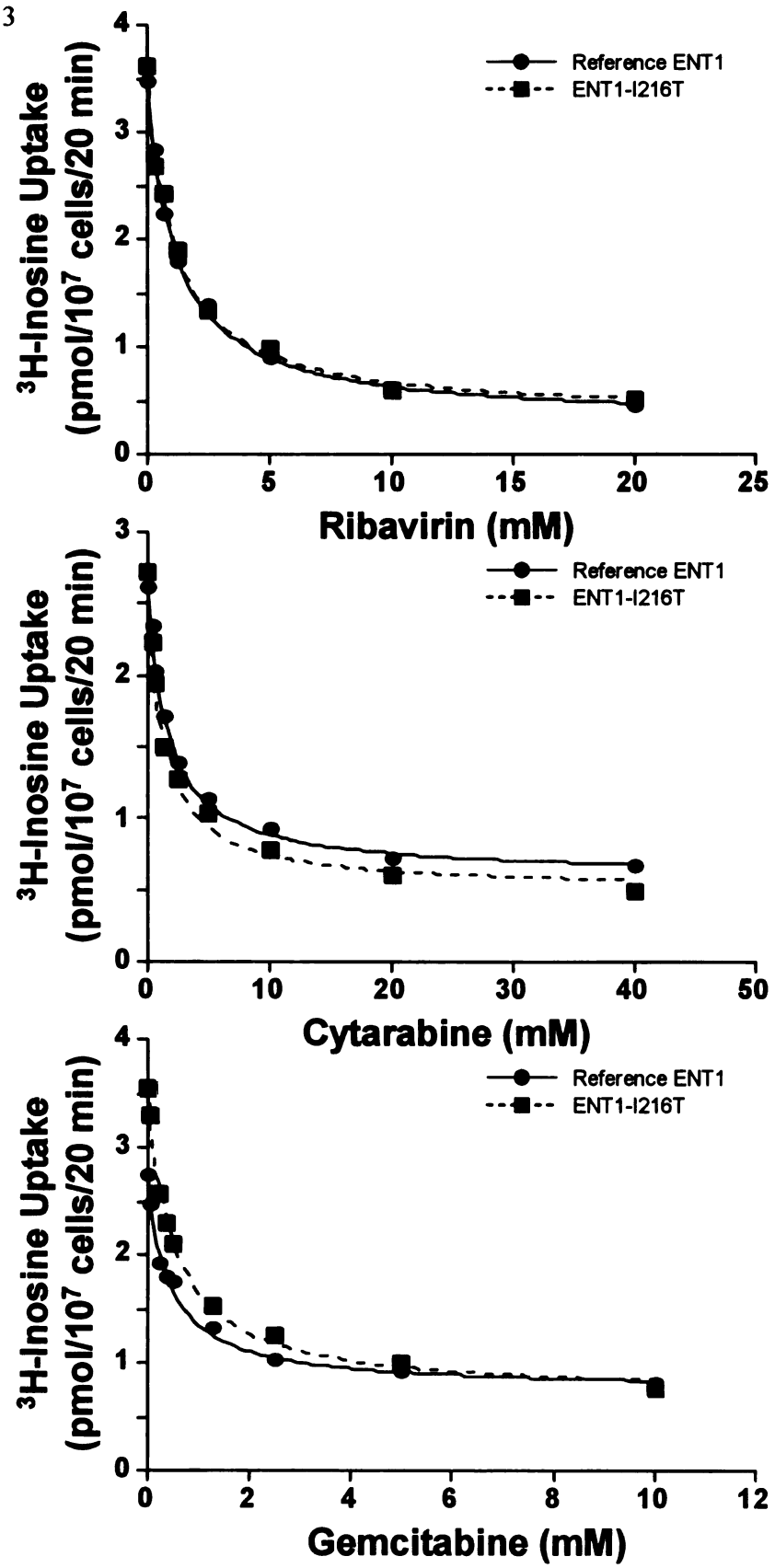

Reference ENT1
ENT1-I216T
$^3$H-Inosine Uptake (pmol/10$^7$ cells/20 min)
Ribavirin (mM)
Cytarabine (mM)
Gemcitabine (mM)

Table 1[a]

(a): Summary of SNPs of the human equilibrative nucleoside transporter 1, *ENT1*

| Exon | SNP No. | cDNA Position[b] | NT Change | AA Position | AA Change | No. of Chromosomes | Total[d] (n=494)[e] | AA (n=200) | CA (n=200) | AS (n=60) | ME (n=20) | PA (n=14) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | None | | | | | | | | | |
| 2 | 2.1 | 84 | G to A | 28 | Syn | 2 | 0.004 | 0.005 | 0 | 0 | 0.05 | 0 |
| 3 | | | None | | | | | | | | | |
| 4 | 4.1 | (+19) | G to A | Intron 4 | | 10 | 0.02 | 0.04 | 0.005 | 0 | 0.05 | 0 |
| 5 | | | None | | | | | | | | | |
| 6 | 6.1 | (-17) | C to G | Intron 5 | | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |
| | 6.2 | (-9) | C to T | Intron 5 | | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |
| | 6.3 | 597 | G to A | 199 | Syn | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |
| | **6.4[c]** | **647** | **T to C** | **216** | **Ile to Thr** | **6** | **0.012** | **0.005** | **0.021** | **0** | **0.056** | **0** |
| | 6.5 | 687 | G to A | 229 | Syn | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |
| 7 | | | None | | | | | | | | | |
| 8 | 8.1 | (-5) | C to T | Intron 7 | | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |
| | 8.2 | (+4) | C to T | Intron 8 | | 13 | 0.026 | 0.055 | 0.005 | 0 | 0.05 | 0 |
| 9 | 9.1 | (+68) | G to A | Intron 9 | | 7 | 0.014 | 0.035 | 0 | 0 | 0 | 0 |
| | 9.2 | (+103) | G to A | Intron 9 | | 10 | 0.02 | 0.051 | 0 | 0 | 0 | 0 |
| 10 | 10.1 | 999 | T to C | 333 | Syn | 1 | 0.002 | 0 | 0.005 | 0 | 0 | 0 |
| 11 | 11.1 | (-33) | G to A | Intron 10 | | 2 | 0.004 | 0.01 | 0 | 0 | 0 | 0 |
| | **11.2** | **1171** | **G to A** | **391** | **Glu to Lys** | **2** | **0.004** | **0.01** | **0** | **0** | **0** | **0** |
| 12 | 12.1 | (+46) | C to A | Non-coding | | 1 | 0.002 | 0.005 | 0 | 0 | 0 | 0 |

(b): Estimates of the neutral parameter ($\theta$), nucleotide ($\pi$), and Tajima's $D$[f] for the human equilibrative nucleoside transporter, *ENT1*

| SNP type | Base pairs screened | n[e] | No. variable sites | $\theta$ (x $10^4$) | $\pi$ (x $10^4$) | *D* |
|---|---|---|---|---|---|---|
| All | 2684 | 494 | 15 | 8.24 ± 2.64 | 0.87 ± 1.12 | -2.03 |
| Non-coding | 1313 | 494 | 9 | 10.11 ± 3.88 | 1.39 ± 1.99 | -1.43 |
| Coding | 1371 | 494 | 6 | 6.46 ± 2.90 | 0.38 ± 0.97 | -1.20 |
| Synonymous | 334 | 494 | 4 | 17.67 ± 9.44 | 0.60 ± 2.47 | -0.93 |
| Non-synonymous | 1037 | 494 | 2 | 2.85 ± 2.08 | 0.30 ± 1.00 | -0.53 |

[a]All data in Tables 1a and 1b are from [18].

[b]cDNA numbers are relative to the ATG start site and based on the cDNA sequence from GenBank accession number U81375.1.

[c]Non-synonymous changes are shown in **bold**.

[d]Columns refer to the frequencies of each variable site in each ethnic group. Some samples contained amplicons that could not be sequenced. Therefore, allele frequencies are based on total samples analyzed for each variant. Total, entire sample; AA, African American; CA, Caucasian; AS, Asian; ME, Mexican; PA, Pacific Islander.

[e]*n* is the number of chromosomes in each ethnic group.

[f]$\theta$, $\pi$, and Tajima's *D* were calculated as described by Tajima [24].

Table 2: Inhibition of Inosine Uptake in Yeast Expressing ENT1, ENT1I216T, and ENT1-E391K[a]

| Inhibitor[b] | ENT1 Reference[c] | ENT1-I216T[c] | ENT1-E391K[c] |
|---|---|---|---|
| No Inhibitor | 100% | 100% | 100% |
| Adenosine | <15%[d] | <15% | <15% |
| Guanosine | <15% | <15% | <15% |
| Thymidine | <15% | <15% | <15% |
| Uridine | <15% | <15% | <15% |
| Cytidine | 25 ± 3% | 20 ± 3% | 28 ± 4% |
| Inosine | <15% | <15% | <15% |
| Ribavirin | 16 ± 2% | <15% | 18 ± 1% |
| Gemcitabine | 16 ± 1% | <15% | 22 ± 4% |
| Ara-C[e] | 15 ± 4% | 16 ± 3% | 32 ± 7% |
| Zalcitabine | 61 ± 4% | 56 ± 4% | 68 ± 2% |
| Uracil | 84 ± 5% | 100 ± 2% | 95 ± 9% |
| Dipyridamole | <15% | <15% | 19 ± 4% |
| NBMPR[f] | <15% | <15% | <15% |
| Dilazep | <15% | <15% | <15% |

[a]Data are listed as mean ± SE.

[b]Concentrations of inhibitors are 2 mM, with the exception of dipyridamole (5μM), NBMPR (500 nM), and dilazep (5 μM).

[c]Values represent percent of control, where control is the uptake of inosine in yeast expressing ENT1 and its variants in the absence of inhibitor.

[d]Less than 15% of control was considered the lower limit of detection.

[e] 9-β-D-arabinofuranosyl-cytosine

[f] 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine

Table 3: Inhibition of $^{3}$H-Inosine Uptake in Yeast Expressing Reference ENT1 and ENT1-I216T[a]

| | $IC_{50}$ (μM) | |
|---|---|---|
| Inhibitor | ENT1 | ENT1-I216T |
| Uridine | 43.0 ± 28.3[b] | 64.3 ± 17.5 |
| Inosine | 60.5 ± 14.7 | 73.3 ± 17.5 |
| Ribavirin | 1150 ± 170 | 1490 ± 410 |
| Cytarabine | 1430 ± 260 | 1340 ± 360 |
| Gemcitabine | 475 ± 138 | 520 ± 136 |

[a] Methods as per figure 3.

[b] Data are listed as mean ± SD.

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