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Uridine Binding Motifs of Human Concentrative Nucleoside Transporters 1 and 3 Produced in *Saccharomyces cerevisiae*

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ABSTRACT

An extensive series of structural analogs of uridine that differed in substituents in the sugar and/or base moieties were subjected to inhibitor-sensitivity assays in a yeast expression system to define uridine structural determinants for inhibitors of human concentrative nucleoside transporters 1 and 3 (hCNT1 and hCNT3). The production of recombinant hCNT1 and hCNT3 in a nucleoside-transporter deficient strain of yeast was confirmed by immunoblotting, and uridine transport parameters (K_m, V_{max}) were determined by defining the concentration dependence of initial rates of uptake of [^{3}H]uridine by intact yeast. The K_i values of uridine analogs were obtained from inhibitory-effect curves and converted to binding energies. hCNT1 and hCNT3 recognized uridine through distinguishable binding motifs. hCNT1 was sensitive to modifications at C(3), less sensitive at C(5′) or N(3), and much less sensitive at C(2′). hCNT3 was sensitive to modifications at C(3′), but much less sensitive at N(3), C(5′) or C(2′). The changes of binding energy between transporter proteins and different uridine analogs suggested that hCNT1 formed hydrogen bonds (H-bonds) with C(3′)-OH, C(5′)-OH, or N(3)-H of uridine, but not with C(2′)-OH, whereas hCNT3 formed H-bonds to C(3′)-OH, but not to N(3)-H, C(5′)-OH, and C(2′)-OH. Both transporters barely tolerated modifications at C(3′) or inversion of configurations at C(2′) or C(3′). The binding profiles identified in this study can be used to predict the potential transportability of nucleoside analogs, including anticancer or antiviral nucleoside drugs, by hCNT1 and hCNT3.

Nucleoside transporters (NTs) are required for most natural and synthetic nucleosides to cross cell membranes (Cass et al., 1999). NT-mediated permeation is a determinant of cellular uptake of physiological nucleosides and antineoplastic and antiviral nucleoside drugs (Baldwin et al., 1999). NTs also affect extracellular concentrations of adenosine, which acts as a signaling molecule to affect many physiological processes, including neurotransmission, vasodilation, platelet aggregation, and lipolysis (Latini and Pedata, 2001; Burnstock, 2002). Mammalian NTs are classified into two structurally unrelated protein families, the concentrative and equilibrative nucleoside transporters (CNTs and ENTs) (Baldwin et al., 1999; Cass et al., 1999), which exhibit different mechanisms of transport. In mammals, the ENTs transport a broad range of both purine and pyrimidine nucleosides and have a ubiquitous tissue and cell distribution, whereas the CNTs seem to exhibit more limited permeant selectivities and tissue distributions.

Three human CNTs (hCNTs) with different permeant selectivities have been identified (Ritzel et al., 1997, 1998, 2001). hCNT1 and hCNT2 prefer pyrimidine nucleosides and purine nucleosides, respectively, although hCNT1 transports adenosine and hCNT2 transports uridine (Urd). hCNT3 transports a broad range of pyrimidine and purine nucleosides, including anticancer nucleoside drugs (Ritzel et al.,

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ABBREVIATIONS: NT, nucleoside transporter; CNT, concentrative nucleoside transporter; ENT, equilibrative nucleoside transporter; hCNT human concentrative nucleoside transporter; Urd, uridine; CMM, complete minimal media; PCR, polymerase chain reaction; TTBS, 0.2% Tween 20, Tris-buffered saline; GLU, glucose; AZT, 3′-azido-3′-deoxythymidine; EtOAc, ethyl acetate; DMSO, dimethyl sulfoxide; FUrd, 5-fluorouridine; IUrd, 5-iodouridine; 3MeUrd, 3-methyluridine; dUrd, deoxyuridine; araU, 1-(β-D-Arabinofuranosyl)uracil; ddUrd, dideoxyuridine; OmeUrd, *O*-methyluridine; 5′CldUrd, 5′-chloro-5′-deoxyuridine; 2′AzdUrd, 2′-azido-2′-deoxyuridine; 3′AzdUrd, 3′-azido-3′deoxyuridine; 5′AzdUrd, 5′-azido-5′-deoxyuridine; iPUrd, 2′,3′-*O*-isopropylideneuridine; FdUrd, 5-fluoro-2′-deoxyuridine; BrdUrd, 5-bromo-2′-deoxyuridine.

2001). hCNT1 and hCNT2, which contain 650 and 659 amino acid residues, respectively, share 72% amino acid identity and are predicted to have 13 putative transmembrane segments with an exofacial glycosylated tail at the carboxyl terminus (Hamilton et al., 2001). hCNT3 is 48 and 47% identical to hCNT1 and hCNT2, respectively, and its predicted topology is similar to that predicted for other hCNT family members. The hCNTs are mainly found in specialized cells, such as intestinal and renal epithelia (Ritzel et al., 1998, 2001; Ngo et al., 2001); they have also been observed in leukemic cells and a few cancer cell lines (Mackey et al., 1998). Differences in tissue distribution of hCNTs suggest unique physiological roles for the hCNTs and may reflect different nucleoside-transport capacities of various tissues. Normal and tumor tissues exhibited different expression of hCNT transcripts (Pennycooke et al., 2001). hCNT1 mRNA distributions in some cancer cell lines were found to correspond to drug cytotoxicity patterns (Lu et al., 2002), suggesting that nucleoside chemotherapy could be optimized based on differences among individuals in the abundance of hCNTs.

The differences in permeant selectivities among hCNT1, hCNT2, and hCNT3 are also reflected in their different abilities to transport anticancer and antiviral nucleoside drugs. For example, gemcitabine, which is used in therapy of metastatic solid tumors, is transported by recombinant hCNT1 and hCNT3 in oocytes (Mackey et al., 1999; Ritzel et al., 2001), but not by recombinant hCNT2 (Mackey et al., 1999). Studies in oocytes have shown that AZT (3′-azido-3′-deoxythymidine), an anti-HIV drug, is a low-affinity permeant of recombinant hCNT1 (Huang et al., 1994) and a moderate permeant of recombinant hCNT3 (Ritzel et al., 2001), but is not a permeant of recombinant hCNT2 (Ritzel et al., 1998). These differences in transportability of different nucleoside drugs by hCNT1, hCNT2, and hCNT3 imply that different hCNTs have unique binding site(s) for nucleoside drugs. We hypothesize that each hCNT will exhibit a unique binding profile for nucleoside analogs because of differences in their permeant binding and translocation sites.

Urd is a permeant of all human NTs identified so far. Urd analogs such as 5-fluoro-5′-deoxyuridine (a cytotoxic prodrug metabolite of capecitabine) are commonly used in the treatment of advanced human cancers, especially colorectal and breast cancers. Despite the common application of Urd analogs in anticancer therapy, the interactions of these drugs with hCNTs and the mechanisms of selectivity at the transporter level remain unclear. To investigate the Urd recognition motifs of individual hCNTs in a NT-free background, hCNTs were expressed in a yeast strain that lacks NT activity and the capacity of the transporter proteins to mediate uptake of radiolabeled Urd was determined. We developed a high-throughput inhibitor sensitivity assay with the hCNT yeast expression system that was used to quantify the inhibitory effects of a series of Urd analogs with various sugar and base modifications. The resulting K_i values were used to calculate the binding energies for the interactions of various inhibitors for hCNTs, thereby identifying structural determinants of Urd for interactions with the nucleoside binding sites of hCNT1 and hCNT3.

Materials and Methods

Strains and Media. Fui1::TRP1 (MATα, gal, ura3–52, trp1, lys2, ade2, hisd2000, and Δ fui1::TRP1), which contains a disruption in the gene encoding the endogenous Urd permease (FUI1) (Vickers et al., 2000), was the parental yeast strain used to generate the hCNT expression system (Vickers et al., 2002; Visser et al., 2002). Other strains were generated by transformation of the yeast-*Escherichia coli* shuttle vector pYPGE15 (Brunelli and Pall, 1993) into Fui1::TRP1 by using a standard lithium acetate method (Ito et al., 1983). Yeast strains were maintained in complete minimal media (CMM) containing 0.67% yeast nitrogen base (Difco, Detroit MI), amino acids (as required to maintain auxotrophic selection), and 2% glucose (CMM/GLU). Agar plates contained CMM with various supplements and 2% agar (Difco, Detroit, MI). Plasmids were propagated in *E. coli* strain TOP10F′ (Invitrogen, Carlsbad, CA) and maintained in Luria broth with 100 μg/ml ampicillin.

Plasmid Construction. For *Saccharomyces cerevisiae* expression, the hCNT1 and hCNT3 open reading frames were amplified from vectors (pCDNA3-hCNT1 and pCDNA3-hCNT3) by PCR methodology with the following primers (restriction sites underlined, c-Myc tag sequence in italic): 5′-XbaI-hCNT1 (5′-CTG TCT AGA ATG GAGA ACG ACC CCT CGA GAC G-3′), 3′-KpnI-hCNT1 (5′-CGA GGT ACC TCA CTG TGC ACA GAT CGT GTG GTT G-3′), 3′-KpnI-hCNT1-Myc (5′-CGA GGT ACC TCA *CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC* CTG TGC ACA GAT CGT GTG GTT G-3′), 5′-BglII-hCNT3 (5′-CTG AGA TCT ATG GAG CTG AGG AGT ACA GCA G-3′), and 3′-XhoI-hCNT3 (5′-CGA CTC GAG TCA AAA TGT ATT AGA GAT CCC ATT G-3′). The amplified open reading frames were inserted into the yeast expression vector pYPGE15, which is a high copy-number episomal vector that expresses the inserted DNA under the transcriptional control of a constitutive promoter (phosphoglycerate kinase promoter) to generate pYPhCNT1, pYPhCNT1-Myc, and pYPhCNT3. The PCR reactions were performed using *Pwo* polymerase (Roche Diagnostics, Laval, PQ, Canada) and the resulting PCR products were verified by DNA sequencing by using an ABI PRISM 310 sequence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA).

Preparation of Yeast Membranes and Immunostaining. Yeast membranes were prepared by a method described previously (Vickers et al., 2000). Briefly, yeast were grown to an optical density at 600 nm (A_{600}) of 0.7 to 1.0, collected by centrifugation (1000*g*, 5 min, 4°C), washed three times with breaking buffer [10 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% (v/v) 2-mercaptoethanol, pH 7.4] that contained additional protease inhibitors (complete protease inhibitors; Roche Diagnostics), and lysed by vortexing in the presence of glass beads (425–600 μm, Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) for 15 min at 4°C. Unbroken cells and glass beads were removed from lysates by centrifugation (500*g*, 5 min, 4°C), and membrane fractions were obtained by centrifugation of lysates (100,000*g*, 60 min, 4°C). The resulting membrane pellets were resuspended in breaking buffer that contained protease inhibitors. The samples were either used immediately or frozen (−80°C) in breaking buffer.

Yeast membranes were subjected to SDS-polyacrylamide gel electrophoresis (Vickers et al., 1999), after which proteins were transferred to polyvinylidene fluoride membranes (Immobilon-P; Millipore, Bedford, MA). The transfer membranes were incubated overnight at 4°C first in TTBS (0.2% Tween 20, Tris-buffered saline) containing 5% (w/v) skim milk powder and then in TTBS with the primary antibodies and 5% (w/v) skim milk powder. The membranes were then washed three times with TTBS, incubated with TTBS-containing species-specific horseradish-peroxidase secondary antibodies (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) and 5% (w/v) skim milk powder, washed with TTBS, and visualized with enhanced chemiluminescence (Amersham Biosciences Inc., Piscataway, NJ) and autoradiography. The primary antibodies used were monoclonal antibodies against the c-myc epitope tag

(9E10; Babco, Richmond, CA.) and against hCNT3. The latter were raised against an immunogenic epitope (the amino acid sequence REHTNTKQDEEQVTVEQDSPRNREH) that corresponded to residues 45 to 69 of hCNT3, a region predicted to be located in a large intracellular loop close to the amino terminus.

Urd Uptake in Yeast Producing Recombinant hCNTs. The uptake of [^{3}H]nucleosides (Moravek Biochemicals, Brea, CA) into logarithmically proliferating yeast was measured using a modified transport assay (Vickers et al., 2002). Yeast were grown in CMM/GLU to an A_{600} of 0.8 to 1.5, washed twice with fresh media, pH 7.4, and resuspended in CMM/GLU, pH 7.4, to an A_{600} of 4.0. Transport reactions were initiated by rapid mixing of 100 μl of yeast suspension with 100 μl of CMM/GLU, pH 7.4, containing [^{3}H]Urd (final concentration, 1 μM) preloaded in a 96-well cell culture plate. The 96-well plates were placed on the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) and every 24 transport reactions were terminated simultaneously at graded time intervals by harvesting yeast on glass-fiber filters (Skatron Instruments) with continued washing with demineralized water. The filters were air-dried for about 5 min, and the portions of the filter that corresponded to individual assays were excised and placed in scintillation vials. The amounts of radioactivity associated with the filters were determined by liquid scintillation counting.

The inhibitory capacities of Urd and Urd analogs against the recombinant hCNTs were assessed by measuring their relative abilities to inhibit the uptake of [^{3}H]Urd in the "inhibitor-sensitivity" assay as follows. Yeast cells were incubated with graded concentrations of a particular test compound and 1 μM [^{3}H]Urd in CMM/GLU, pH 7.4, for 30 and 10 min for recombinant hCNT1 and hCNT3, respectively, and [^{3}H]Urd uptake was measured. All experiments were carried out in quadruplicate. The amount of [^{3}H]Urd associated with yeast in the presence of 10 mM Urd was also determined to quantify nonspecifically associated radioactivity, which was subtracted from total radioactivity for each transport assay. Data were fitted to theoretical inhibition curves by nonlinear regression with the use of the GraphPad Prism, version 3.0, software to obtain IC_{50} (inhibitory concentration 50%) values for compounds that inhibited uptake of [^{3}H]Urd by using concentration-effect curves with at least 11 points distributed over the relevant range of concentrations. K_i (inhibitory constant) values were determined from the Cheng and Prusoff equation (Cheng and Prusoff, 1973), in which $K_i = IC_{50}/[1 + (L/K_m)]$ and L = [^{3}H]Urd concentration, which was always 1 μM. Gibbs free energy (ΔG^0) was calculated from $\Delta G^0 = -RT\ln(K_i)$, in which R is the gas constant and T is the absolute temperature. The thermodynamic stability of transporter-inhibitor complexes was quantitatively estimated from ΔG^0 as described elsewhere (de Koning and Jarvis, 2001).

Urd Analogs. Uridine, 5-fluorouridine, 5-iodouridine, 3-methyluridine, 2′-deoxyuridine, 5-ethyl-2′-deoxyuridine, 5-fluoro-2′-deoxyuridine, 5-bromo-2′-deoxyuridine, and thymidine were obtained from Sigma-Aldrich; AZT was obtained from Aldrich Chemical Co. (Milwaukee, WI); 3′-*O*-methyluridine, 2′,3′-*O*-isopropylideneuridine, 2′,3′-dideoxyuridine and 2′,5′-dideoxyuridine were obtained from R. I. Chemical, Inc. (Orange, CA). 1-(β-D-Arabinofuranosyl)uracil (Codington et al., 1960; Hampton and Nichol, 1966), 2′-azido-2′-deoxyuridine (Verheyden et al., 1971), 2′-*O*-methyluridine (Robins et al., 1974), 1-(β-D-xylofuranosyl)uracil (Yung and Fox, 1961), 3′-deoxyuridine (Rhie and Pfleiderer, 1994), 3′-azido-3′-deoxyuridine (Matray and Gryaznov, 1999), 5′-deoxyuridine (Wang et al., 1977), 5′-chloro-5′-deoxyuridine (Robins et al., 1991), 5′-azido-5′-deoxyuridine (Horwitz et al., 1962), and 5′-*O*-methyluridine (Hovinen, 1997) were prepared as described in the references noted. 3′,5′-Dideoxyuri-

TABLE 1

K_i and Gibbs free energy values for inhibition of hCNT1- and hCNT3-mediated Urd uptake in *S. cerevisiae* by Urd analogs

The transport of 1 μM [^{3}H]Urd into yeast (fui1::TRP) expressing either pYPhCNT1 or pYPhCNT3 was measured over 30 or 10 min, respectively, in the presence of graded concentrations of Urd or Urd analogs. Average IC_{50} values (mean ± S.E., n = 3–4) were determined using GraphPad Prism, version 3.0, software and were converted to K_i values by the Cheng and Prusoff equation (Cheng and Prusoff, 1973) using K_m values (mean ± S.E., n = 3) of 9.2 ± 3.8 and 8.7 ± 1.1 μM for recombinant hCNT1 and hCNT3, respectively. ΔG^0 was calculated from $\Delta G^0 = -RT\ln(K_i)$.

Urd Compounds	hCNT1		hCNT3	
	K_i μM	ΔG^0 kJ/mol	K_i μM	ΔG^0 kJ/mol
Urd	3.1 ± 0.3	31.5	6.7 ± 1.0	29.5
Base Modification				
FUrd	0.9 ± 0.2	34.5	1.2 ± 0.2	33.8
IUrd	0.9 ± 0.1	34.5	3.4 ± 0.2	31.2
3MeUrd	73.1 ± 16.8	23.6	22.0 ± 0.2	26.6
Sugar Modification				
2′dUrd	7.0 ± 0.3	29.4	5.0 ± 1.3	30.3
5′dUrd	48.6 ± 7.9	24.6	15.2 ± 2.1	27.5
3′dUrd	420 ± 68	19.3	258 ± 41	20.5
araU	>1000[a]		>2000[a]	
1-(β-D-Xylofuranosyl)uracil	>1000[a]		>2000[a]	
3′,5′ddUrd	>2000[b]		>2000[a]	
2′,3′ddUrd	>2000[a]		>2000[b]	
2′,5′ddUrd	94.2 ± 14.6	23.0	36.2 ± 3.2	25.4
2′OMeUrd	113 ± 22	22.5	143 ± 8.6	22
5′OMeUrd	210 ± 42	21.0	135 ± 11	22.1
3′OMeUrd	>1000[a]		>1000[b]	
5′CldUrd	8.5 ± 1.1	28.9	5.7 ± 0.6	29.9
2′AzdUrd	11.5 ± 0.5	28.2	33.3 ± 2.0	25.6
3′AzdUrd	>1000[b]		>1000[b]	
5′AzdUrd	326 ± 31	19.9	82.2 ± 4.8	23.3
iPUrd	>2000[a]		>2000[b]	
Base and Sugar Modifications				
5-Ethyl-2′-deoxyuridine	17.2 ± 2.4	27.2	26.2 ± 1.2	26.2
FdUrd	2.0 ± 0.1	32.5	2.3 ± 1.4	32.2
BrdUrd	0.8 ± 0.3	34.8	3.9 ± 0.9	30.9
5-Fluoro-5′-deoxyuridine	27.6 ± 4.9	26.0	22.0 ± 2.9	26.6
Thymidine	2.6 ± 0.1	31.9	26.5 ± 0.8	26.1
AZT	293 ± 44	20.2	>2000[a]	

[a] Inhibition of less than 50% was observed.
[b] No obvious inhibition was observed.

dine was prepared from 3′-deoxyuridine by the following procedure: to a solution of 2′-*O*-(*tert*-butyldimethylsilyl)-3′-deoxyuridine (Bender et al., 2000) (0.60 g, 1.75 mmol) in *N*,*N*-dimethylformamide (8 ml), was added a solution of methyltriphenylphosphonium iodide (1.6 g, 3.5 mmol) in *N*,*N*-dimethylformamide (4 ml). The mixture was stirred at room temperature for 25 min and then partitioned between H_2O (50 ml) and CH_2Cl_2 (50 ml). The aqueous phase was extracted with CH_2Cl_2 (2 × 30 ml), and the combined organic phase was concentrated. The residue was purified by silica gel column chromatography [ethyl acetate (EtOAc)/hexanes, 1:3 → 1:1] to give 2′-*O*-(*tert*-butyldimethylsilyl)-5′-iodo-3′,5′-dideoxyuridine (700 mg, 88%). This material (700 mg, 1.55 mmol), Bu_3SnH (1.25 ml, 1.36 g, 4.65 mmol) and α,α′-azobisisobutyronitrile (50 mg) were dissolved in deoxygenated toluene (15 ml), and the solution was heated at 100°C for 1 h. Volatiles were evaporated in vacuo, and the residue was purified by chromatography (silica gel, EtOAc/hexanes, 1:3 → 1:1) to give a colorless oil (~430 mg, 85%). This material was dissolved in acetonitrile (10 ml), and 48% aqueous hydrofluoric acid (0.5 ml) was added with stirring. After 1.5 h, the mixture was added to a column of silica gel. Elution with EtOAc gave 3′,5′-dideoxyuridine (260 mg, 70%) as a colorless syrup: ^{1}H NMR (CD_3OD, 500 MHz) δ 1.42 (d, J = 6.3 Hz, 3H), 1.67 to 1.74 (ddd, J = 5.4, 10.7, 13.7 Hz, 1H), 1.99 to 2.04 (ddd, J = 1.5, 4.8, 13.2 Hz, 1H), 4.32 to 4.35 (m, 1H), 4.46 to 4.53 (m, 1H), 5.69 (d, J = 1.0 Hz, 1H), 5.70 (d, J = 7.8 Hz, 1H), 7.60 (d, J = 7.8 Hz, 1H); ^{13}C NMR (CD_3OD, 125 MHz) δ 20.4, 77.8, 78.5, 95.0, 102.4, 142.0, 152.3, 166.5; MS (EI) m/z 212 (M^+, 10), 194, 141, 113, 101, 72, 57 (100%); HRMS m/z 212.0800 (M^+ [$C_9H_{12}N_2O_4$] = 212.0797).

Stock solutions of test compounds were either prepared in water or dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and the final concentration of DMSO in transport reactions was 0.1% if DMSO was used as a solvent.

Results

Detection of Recombinant hCNT1-myc and hCNT3 in Yeast Membranes. The production of recombinant hCNT1-myc and hCNT3 in *S. cerevisiae* was verified by immunoblotting by using either anti-myc or anti-hCNT3 antibodies (Fig. 1). A 75- and an 80-kDa immunoreactive species were detected in membranes of pYPhCNT1-myc-containing (Fig. 1A) and pYPhCNT3-containing (Fig. 1B) yeast, respectively, that were not present in membranes of pYPGE15-containing yeast. The electrophoretic mobilities of the detected proteins were consistent with the predicted molecular masses of hCNT1-myc and hCNT3. The apparent abundance of hCNT3 was probably much greater than that of hCNT1-myc given that the amounts of membrane loaded for electrophoresis for detection of hCNT1-myc and hCNT3 were 20 and 5 μg, respectively, and the exposure times for autoradiography for hCNT1-myc and hCNT3 were 10 min and 2 s, respectively.

Urd Transport by Recombinant hCNT1 and hCNT3 in Yeast. The time course for Urd uptake into fui1::TRP1 that contained pYPGE15 was reported previously to yield a rate of 0.11 ± 0.01 pmol/mg protein/s (Visser et al., 2002). To determine the initial rates of Urd uptake into fui1::TRP1 yeast that contained either pYPhCNT1 or pYPhCNT3, time courses for influx of [^{3}H]Urd were measured in the experiments of Fig. 2. The Urd uptake time course for pYPhCNT1-containing yeast was linear for up to 60 min with a mean rate (±S.E.) of 10.43 ± 0.22 pmol/mg protein/min. The uptake time course for pYPhCNT3-containing yeast was linear for about 12 min with a mean rate (±S.E.) of 282.6 ± 14.27 pmol/mg protein/min. The rate calculated from the time course for Urd uptake into pYPhCNT3-containing yeast over the first 60 s (Fig. 2, inset) was the same as that calculated from the 12-min time course, indicating that initial rates of Urd transport were maintained over long periods. The extended linear time courses were probably caused by efficient intracellular metabolism of Urd by conversion of Urd to UMP by Urd kinase, thereby maintaining the concentration gradient of Urd between the extracellular medium and the intracellular compartment. Urd uptake into pYPhCNT1- and pYPhCNT3-containing yeast was greatly reduced by addition of 10 mM cold Urd to assay mixtures, with mean rates (±S.E.) of 0.46 ± 0.1 and 0.58 ± 0.18 pmol/mg protein/min, respectively, indicating that most of the observed uptake was mediated by functional transporters.

The C-terminal tag of c-myc on hCNT1 had no obvious impact on the function of hCNT1 protein because time courses of Urd uptake mediated by the fui1::TRP1 strain transformed with pYPhCNT1-myc gave a mean rate (±S.E.) of 9.25 ± 0.36 pmol/mg protein/min, which was similar to the rate obtained from pYPhCNT1-containing yeast in the experiments of Fig. 2. Initial rates of ^{3}H-labeled cytidine and thymidine into pYPhCNT1-containing yeast were also measured, giving mean rates (±S.E.) of 6.21 ± 0.41 pmol/mg protein/min and 2.42 ± 0.36 pmol/mg protein/min, respectively. In contrast, rates of uptake of ^{3}H-labeled guanosine and adenosine were low, indicating that these nucleosides were not good permeants for recombinant hCNT1. Recombinant hCNT3 exhibited broad transportabilities for naturally occurring nucleosides, with mean uptake rates (±S.E.) of 312 ± 2, 283 ± 4, and 336 ± 4 pmol/mg protein/min for ^{3}H-labeled cytidine, guanosine, and adenosine, respectively. These results demonstrated that the recombinant hCNT1 and hCNT3 produced in yeast showed characteristics similar

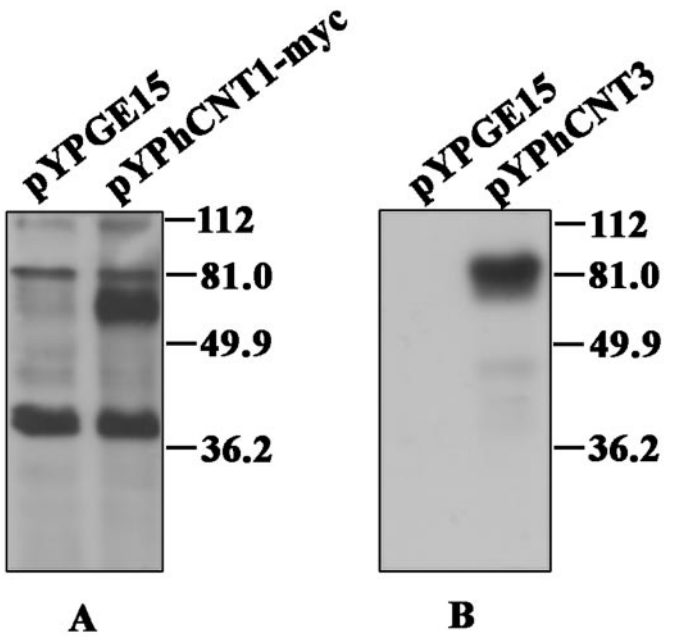

Fig. 1. Immunoblotting to detect recombinant hCNT1-myc and hCNT3 in yeast. Yeast cells (*fui1*::TRP1) were transformed with either pYPGE15, pYhCNT1-myc, or pYhCNT3 to form new yeast strains named *fui1*::TRP1 + pYPGE15, *fui1*::TRP1 + pYhCNT1-myc, and *fui1*::TRP1 + pYhCNT3. Yeast membranes (20 μg in A, 5 μg in B) were subjected to SDS-polyacrylamide gel electrophoresis, after which proteins were transferred to polyvinylidene fluoride membranes that were subjected to immunoblotting with either 9E10 anti-myc monoclonal antibodies (A) or anti-hCNT3 monoclonal antibodies (B). The positions of the molecular mass markers are indicated in kilodaltons at right.

to those reported previously (Ritzel et al., 1997, 2001). Urd transport rates were determined for all subsequent experiments by using incubation times of 30 and 10 min for recombinant hCNT1 and hCNT3, respectively, thereby providing large signal-to-noise ratios and initial rates of uptake.

Kinetic Properties of Recombinant hCNT1 and hCNT3. The experiments of Fig. 3 showed that recombinant hCNT3 had similar apparent affinity but higher capacity for Urd (K_m = 8.7 ± 1.1 μM, V_{max} = 1,400 ± 286 pmol/mg protein/min; mean ± S.E., n = 3) than recombinant hCNT1 (K_m = 9.2 ± 3.8 μM, V_{max} = 86.9 ± 12.9 pmol/mg protein/min, mean ± S.E., n = 3). The higher capacity of hCNT3 could be explained by its probable higher abundance in yeast membranes, which was detected in the immunoblotting experiment of Fig. 1. The K_m values of recombinant hCNT1 and hCNT3 in yeast were slightly lower than those obtained with other expression systems, such as *Xenopus laevis* oocytes and cultured mammalian cells (Ritzel et al., 1997; Loewen et al., 1999; Graham et al., 2000; Ritzel et al., 2001), which might be caused by different recombinant expression systems.

Interaction of Urd Analogs with Recombinant hCNT1 and hCNT3. To obtain an understanding of the structural regions of Urd that interact with the transporters, Urd analogs with modifications of the base and/or sugar moieties were tested for their ability to inhibit uptake of 1 μM [^{3}H]Urd mediated by recombinant hCNT1 or hCNT3. The structures of the analogs that were studied are shown in Fig. 4 and representative concentration-effect curves of some of the analogs for inhibition of hCNT1-mediated Urd transport are shown in Fig. 5. In all cases, the Hill coefficients were close to −1, indicating a single class of inhibitor binding sites, and apparent K_i values were therefore determined from the IC_{50} values. The mean K_i values (±S.E.) and the corresponding Gibbs free energy values are listed in Table 1.

hCNT1. The C(5) of Urd did not form part of a stringent binding motif because FUrd, IUrd, and BrdUrd were potent inhibitors with somewhat higher affinities than Urd itself for hCNT1 (t test, $P < 0.05$). Thymidine, which is 5-methyl-2′-dUrd, was a high-affinity inhibitor of Urd uptake with a K_i value (2.6 ± 0.1 μM) close to that of Urd (3.1 ± 0.3 μM). 5-Ethyl-2′-deoxyuridine exhibited a ΔG^0 value of 27.2 kJ/mol, compared with that of Urd (31.5 kJ/mol), suggesting that the ethyl group, with a larger volume than a fluoro or a

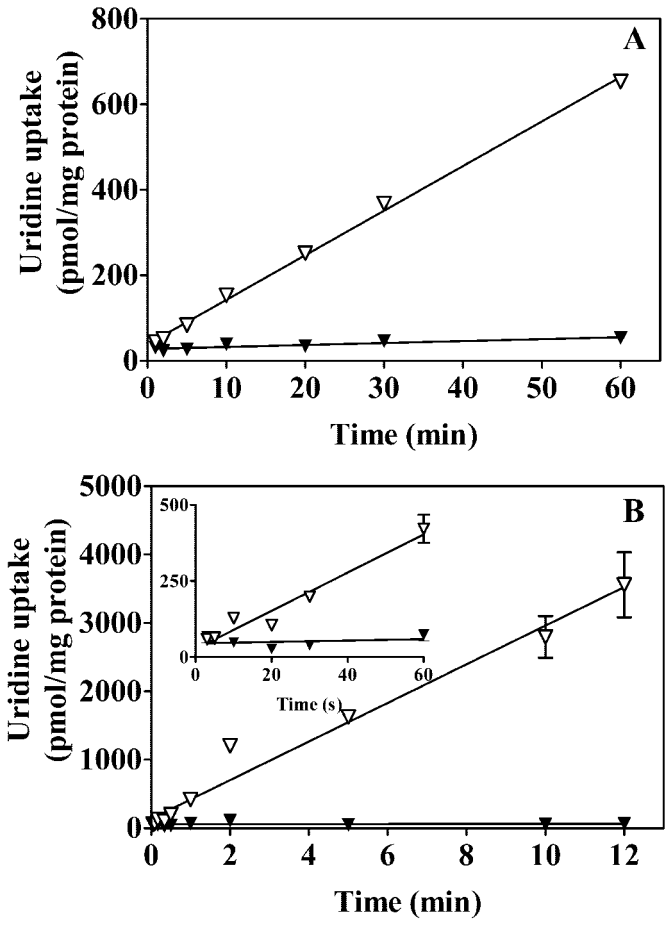

Fig. 2. Time courses of [^{3}H]Urd uptake by recombinant hCNT1 (A) and hCNT3 (B) produced in yeast. The uptake of 1 μM [^{3}H]Urd by yeast that were transformed with either pYhCNT1 or pYhCNT3 was measured in CMM/GLU, pH 7.0, in the presence of 100 mM NaCl, alone (open symbols) or with 10 mM nonradioactive Urd (closed symbols). Each point is the mean ± S.E. of triplicate determinations. S.E. values are not shown where they were smaller than the data points. Each graph represents one of three identical experiments that gave qualitatively similar results.

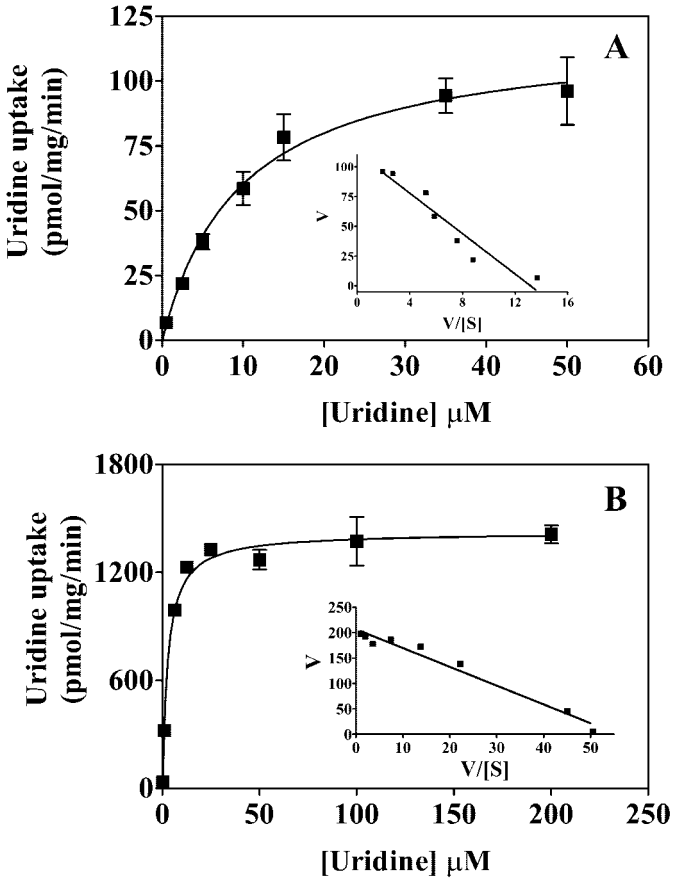

Fig. 3. Kinetic properties of recombinant hCNT1 (A) and hCNT3 (B) produced in yeast. Initial rates of Urd uptake in yeast transformed with pYhCNT1 or pYhCNT3 were measured in transport medium containing 100 mM NaCl. The mediated component of Urd transport (uptake of [^{3}H]Urd at a particular Urd concentration minus uptake at that concentration in the presence of 10 mM nonradioactive permeants) was plotted as a function of concentration and subsequently converted to V versus V/S plots (insets) to determine the kinetic properties of the transporters (PRISM; GraphPad Software Inc., San Diego, CA). Each value is the mean ± S.E. of six to nine determinations, and S.E. values are not shown where they were smaller than the data points. Each kinetic curve represents one of three identical experiments that gave qualitatively similar results.

bromo group, may have sterically reduced the ability of the analog to efficiently contact the transporter protein. In contrast, the 3-position of the base moiety [N(3)-H], which is a potential hydrogen bond donor, contributed a recognition determinant for binding to hCNT1. The low affinity of 3MeUrd, with a 24-fold increase in K_i value compared with that of Urd, demonstrated the importance of the 3-position for binding to the transporter. The difference of 8 kJ/mol binding energy of 3MeUrd might be caused by the loss of a weak hydrogen bond.

hCNT1 displayed relatively high affinities for 2′dUrd, 2′AzdUrd, FdUrd, and BrdUrd (Fig. 4), suggesting that the 2′-hydroxyl group was not an important determinant for interaction of Urd with hCNT1. However, araU (Fig. 4), an epimer of Urd with the 2′-hydroxyl group above the plane of the sugar ring, exhibited a pronounced reduction in its interaction with the transporter ($K_i > 1$ mM). The inverted orientation of the hydroxyl group evidently produced an analog that could no longer interact with the transporter protein. The 36-fold difference in potency of 2′OMeUrd to inhibit Urd uptake might result from the bulkier size of the C(2′)-O-CH_3 group [δ (ΔG^0) = 9 kJ/mol, relative to ΔG^0 of Urd].

There was an apparent interaction between hCNT1 and the 5′-hydroxyl group because its removal (5′dUrd) produced a difference of 6.9 kJ/mol in ΔG^0 with a 16-fold increase in K_i value, suggesting that hydrogen bonding could be important. The further change of 4.7 kJ/mol in ΔG^0 value upon substitution of an azido group for a hydrogen atom at the C(5′) of 5′dUrd [δ (ΔG^0) = 11.5 kJ/mol, relative to ΔG^0 of Urd] was also consistent with the loss of hydrogen bonding between Urd and hCNT1. Because 2′dUrd was a high-affinity inhibitor of hCNT1, the difference of 8.5 kJ/mol in ΔG^0 for 2′,5′ddUrd relative to Urd was most probably caused by the removal of the 5′-hydroxyl group.

Although hCNT1 exhibited lower apparent affinity for 5′OMeUrd (K_i = 210 ± 42 μM) with a difference in ΔG^0 of 10.5 kJ/mol relative to Urd, the substitution of a chloro group for the 5′-hydroxyl group restored high affinity binding to hCNT1 (5′CldUrd, K_i = 8.5 ± 1.1 μM). The slightly higher apparent affinity observed with 5-fluoro-5′-deoxyuridine than with 5′dUrd was evidently caused by gained energy by addition of the fluoro group at the 5-position of the base.

Although removal of the 3′-hydroxyl group shifted the concentration-effect curve far to the right (Fig. 5), 3′dUrd inhibited Urd transport mediated by hCNT1 at high concentrations, with a K_i value of 420 ± 68 μM. The difference in

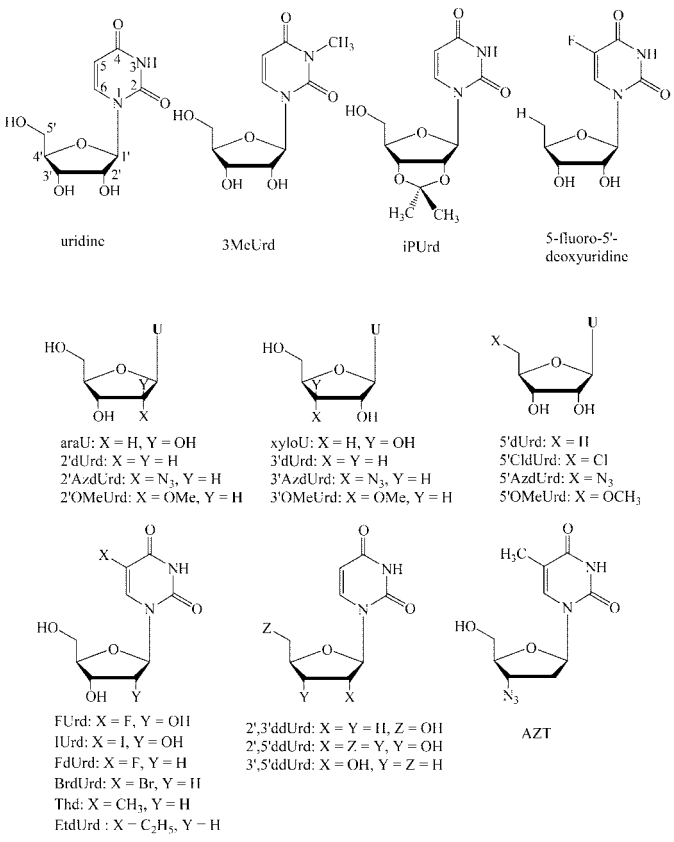

Fig. 4. Structures of Urd and some Urd analogs. Numbering for Urd is indicated. XyloU, 1-(β-D-xylofuranosyl)uracil; Thd, thymidine; EtdUrd, 5-ethyl-2′-deoxyuridine.

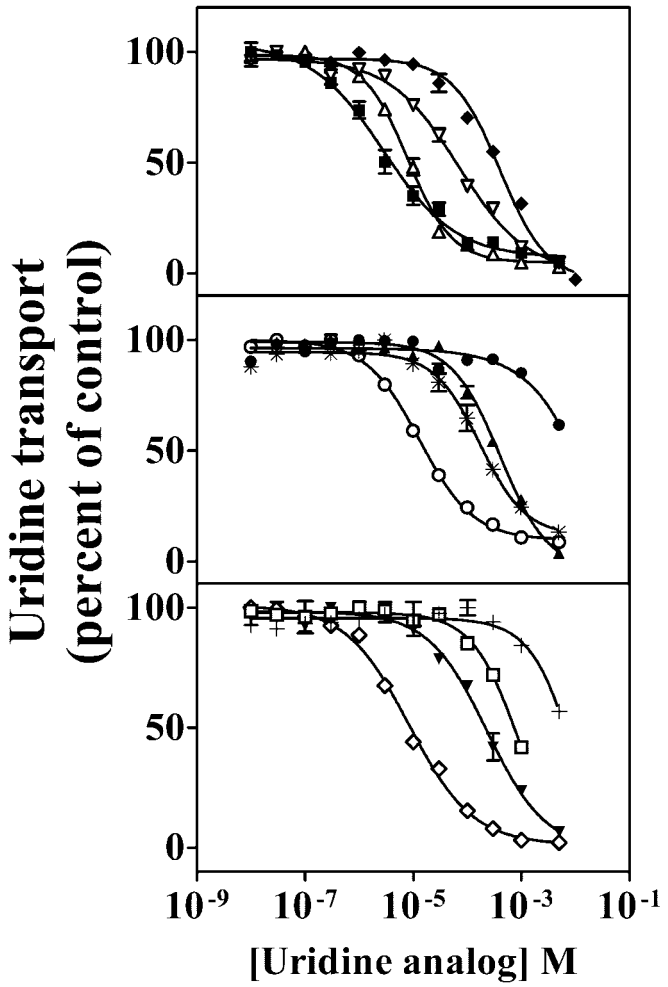

Fig. 5. Inhibition of recombinant hCNT1-mediated Urd uptake by some Urd analogs. The uptake of 1 μM [^{3}H]Urd into fui1::TRP yeast expressing pYPhCNT1 was measured over 30 min in the presence of graded concentrations of test compounds. The test compounds were Urd (■), 2′dUrd (▽), 5′dUrd (△), 3′dUrd (♦), 5′OMedUrd (*), 3′OMedUrd (●), 2′AzdUrd (○), 5′AzdUrd (▲), 5′CldUrd (◇), araU (□), iPUrd (+), and AZT (▼). Uptake values in the presence of Urd compounds are given as the percentage of uptake values in their absence. Each data point represents the means ± S.E. of quadruplicate determinations; error bars are not shown where they are smaller than the symbol. Three or four independent experiments gave similar results and results from representative experiments are shown.

binding energy for this ligand was 12.2 kJ/mol relative to that of Urd, suggesting loss of hydrogen bonding. Removal of the hydroxyl groups from both C(3′) and C(5′), which could result in additional loss of hydrogen bonding, seriously limited interaction of this ligand with the transporter, as indicated by the extremely low affinity ($K_i > 2$ mM) of hCNT1 for 3′,5′ddUrd. Although 2′dUrd was a high-affinity inhibitor of hCNT1-mediated Urd transport, additional removal of the 3′-hydroxyl group (2′,3′ddUrd) abolished its inhibitory effect. The possible involvement of the 3′-hydroxyl group in H-bonding was also apparent from the effects of substitution of an azido group or *O*-methyl group at C(3′) because neither 3′AzdUrd nor 3′OMeUrd inhibited hCNT1-mediated Urd transport. Although hCNT1 strongly bound Urd with the 3′-hydroxyl group below the sugar ring plane, 1-(β-D-xylofuranosyl)uracil (Fig. 4), with the 3′-hydroxyl group oriented upwards, had little effect on hCNT1-mediated Urd transport ($K_i > 1$ mM). Similarly, iPUrd (Fig. 4) failed to inhibit Urd transport, presumably because the 3′ position was no longer available and the bulky isopropylidene group was a steric barrier for interaction with hCNT1.

Recombinant hCNT1 has previously been shown to accept AZT (Fig. 4) as a permeant (apparent $K_m = 0.49$ mM) (Huang et al., 1994). It seemed that any modifications at C(3′) abolished the inhibitory effect of the resulting Urd analogs to interact with hCNT1, with the notable exception of AZT. Albeit a poor inhibitor ($K_i = 293 \pm 44$ μM), AZT inhibited hCNT1-mediated Urd transport in yeast with a greater potency than that of 3′dUrd.

hCNT3. As observed with hCNT1, C(5) of the base moiety was not involved in binding to hCNT3 because introduction of either a fluoro or an iodo group at this position resulted in significantly higher affinities (*t* test, $P < 0.05$, relative to the K_i value for Urd). The differences in binding energies for FUrd and IUrd [$\delta(\Delta G^0) = 4.3$ and 1.7 kJ/mol, respectively, relative to that of Urd] may reflect interactions between the halogen and adjacent amino acid residues of the transporter protein. Methylation of N(3) resulted in a 3-fold decrease in affinity, relative to that of Urd. The observed $\delta(\Delta G^0)$ of less than 3 kJ/mol between 3MeUrd and Urd was probably caused by steric effects.

hCNT3 tolerated modifications at C(2′) well. The K_i value for 2′dUrd was similar to that for Urd. Further evidence for noninvolvement of the 2′-hydroxyl group was that FdUrd and BrdUrd exhibited slightly lower K_i values than that of Urd. As observed with hCNT1, the inverted orientation of the 2′-hydroxyl group abolished binding of hCNT3 to araU ($K_i >$ 2 mM) and the substitution of the 2′-hydroxyl group by an azido or *O*-methyl group caused small changes in binding energy that might have been caused by steric effects.

In contrast to hCNT1, hCNT3 did not seem to have strong interaction with the 5′-hydroxyl group. The changes in ΔG^0 of 2 and 4.1 kJ/mol with 5′dUrd and 2′,5′ddUrd, respectively, relative to that of Urd, did not match the energy contained in a hydrogen bond. As was observed with hCNT1, hCNT3 displayed higher apparent affinity for 5′CldUrd ($K_i = 5.7 \pm 0.6$ μM) than for 5′-dUrd ($K_i = 15.2 \pm 2.1$ μM). hCNT3 exhibited high tolerance for this modification at the 5′ position with a chloride, but lower tolerance for modification with other substituents. Replacement of the 5′-hydroxyl group with an azido group or *O*-methyl group at C(5′) yielded analogs with further reductions in binding energies [$\delta(\Delta G^0) = 4$ to 6 kJ, relative to 5′dUrd].

hCNT3 had a low apparent binding affinity for 3′dUrd ($K_i = 258 \pm 41$ μM) relative to that of Urd with a difference of 9 kJ/mol in binding energy, indicating the possible loss of hydrogen bonding. Of the three hydroxyl groups in the sugar moiety, the 3′-hydroxyl group below the plane of the sugar ring seemed to be most important for hydrogen bonding with hCNT3. Retention of this feature was a structural requirement for high-affinity interaction with hCNT3. Any further modifications at C(3′) abolished the capacity of the Urd analogs to bind to hCNT3 in the present assay because modified nucleosides, including 2′,3′ddUrd, 3′,5′ddUrd, AZT, 3′OMeUrd, iPUrd, and 3′AzdUrd did not significantly inhibit Urd transport by recombinant hCNT3.

Discussion

Recombinant hCNTs have been functionally characterized in a number of model expression systems, including cultured cells (Mackey et al., 1998; Graham et al., 2000; Lai et al., 2002) and *X. laevis* oocytes (Ritzel et al., 1997, 2001; Mackey et al., 1999). *S. cerevisiae* has been used previously to characterize human ENT1 and ENT2 (Vickers et al., 1999, 2001, 2002; Visser et al., 2002). In the present study, hCNT1 and hCNT3 were successfully produced in yeast for the first time. The fui1::TRP strain, which lacks the endogenous Urd transporter (Fui1) (Vickers et al., 2000), enabled measurement of nucleoside uptake by recombinant hCNT1 or hCNT3 in the absence of endogenous transport activity. The ability of recombinant hCNT1 and hCNT3 to transport different naturally occurring nucleosides was tested and found to match their reported selectivities. Kinetic studies of Urd uptake mediated by pYPhCNT1- or pYPhCNT3-containing yeast demonstrated that both recombinant transporters had high affinity for Urd. These results indicated that the production of recombinant hCNT1 and hCNT3 in yeast provided a good model system for structure-function studies.

The structural regions of the Urd molecule involved in binding to hCNT1 and hCNT3 were probed by analysis of inhibition profiles and binding energies as described elsewhere (Wallace et al., 2002). Although hCNT1 and hCNT3 exhibited overall similarities, key differences in their ligand recognition profiles indicated differences in the permeant binding sites on the two concentrative transporters. hCNT1 and hCNT3 recognized Urd through distinguishable binding motifs. Because none of the hCNTs transport uracil or other nucleobases (Ritzel et al., 1997, 1998, 2001), indicating that the ribose moiety is essential for Urd binding, this study focused primarily on structural determinants in the sugar moiety for binding.

The regions of Urd most involved in interaction with hCNT1 were identified as C(3′)-OH, C(5′)-OH, and N(3)-H. The differences of 12.2, 10.5, and 7.9 kJ/mol in Gibbs free energy, respectively, when the 3′-hydroxyl, 5′-hydroxyl, and N(3)-H were modified, suggested that these groups are involved in hydrogen bonding with hCNT1. The total binding energy in the Urd-hCNT1 complex was calculated to be 31.5 kJ/mol, suggesting that the remaining structural features were less critical for hCNT1 binding of Urd. Neither C(5) in the base moiety nor C(2′) in the sugar moiety seemed to be involved in direct binding of Urd to hCNT1 because modifi-

cations at these positions did not cause substantial losses in binding energy.

The most critical functional group of Urd for binding to hCNT3 was the 3′-hydroxyl, which might participate in hydrogen bonding, whereas the 5′-hydroxyl and 2′-hydroxyl groups and the N(3)-H of the base moiety, which were not required for binding, were evidently not involved in hydrogen bonding to hCNT3. Thus, most of the binding energy must come from interactions between the base ring and hCNT3. It is possible that the carbonyl groups at C(2) and/or C(4) form hydrogen bonds, and the base ring might participate in hydrophobic interactions with amino acid residues in hCNT3. Urd analogs with modifications at C(2) and/or C(4) should be evaluated.

Almost any changes at the 3′ position, including removal of the hydroxyl group or inversion of its configuration, modification, or substitution, dramatically altered the interaction of the nucleosides with hCNT proteins. In contrast, substitution of a variety of groups for the 2′- or 5′-hydroxyl group allows binding of Urd analogs. The importance of the 3′-hydroxyl group of nucleosides for interaction with hCNTs, as well as hENTs, which are structurally unrelated proteins, is well established (Patil et al., 2000; Vickers et al., 2002). We thus hypothesize that the binding sites in the hCNTs recognize the 3′-hydroxyl group first and, through this binding, other parts of the nucleoside subsequently bind to the transporter proteins. The apparent stronger ability of hCNT1 than that of hCNT3 to bind AZT, a thymidine analog with a modification at C(3′), further indicates the differences in the binding sites of hCNT1 and hCNT3. Compared with Urd, the sugar moiety of AZT lacks both 2′- and 3′-hydroxyl groups and the hydroxyl group in the 3′ position is substituted with an azido group; these modifications reduce the capacity of AZT to interact with hCNT1. Because hCNT1 was able to interact with thymidine with high affinity and would not be predicted to bind 3′-azido-2′,3′-dideoxyuridine, the 5-methyl group of the base moiety might contribute to interaction of AZT with hCNT1. How hCNT1 interacts with the base moiety of thymidine needs further investigation.

Urd analogs with modifications at C(2′) displayed similar inhibitory profiles for recombinant hCNT1 and hCNT3. Both transporters tolerated very well the removal of the 2′-hydroxyl group but less well the substitution of the hydroxyl group with an azido group and even less well the addition of an *O*-methyl group. Neither of the transporters tolerated inversion of configuration of the 2′-hydroxy group. Although binding of the transporter proteins at C(2′) was not indicated, considerable cooperativity existed between C(2′) and nearby Urd recognition motifs. Changes at these positions could possibly weaken the permeant-transporter interaction by steric interference and physical separation. A bulkier substituent at C(2′) such as an azido or *O*-methyl group could make the 3′-hydroxyl function less accessible to residues at the binding sites of the transporters. C(5′) of the sugar moiety might have similar influences on interactions of hCNT1 with Urd analogs. Another similarity between hCNT1 and hCNT3 was the tolerance for modifications at C(5) of Urd with halogens. Substitution of a halogen at C(5) reduced K_i values, giving high-affinity analogs that were bound by both transporters.

Transmembrane domains 7 to 9 are thought to form the substrate translocation pore for CNT proteins and four critical residues (Ser^{319}, Gln^{320}, Ser^{353}, and Leu^{354}) in this region of hCNT1 determine permeant selectivities (Loewen et al., 1999). It is likely that certain amino acid residues within this region of hCNT1, which could serve as hydrogen-bond donors or acceptors within the postulated translocation pore, form part of the permeant binding sites and directly interact with functional group(s) of Urd. Currently, site-directed mutagenesis approaches are being applied to identify the amino acid residues that comprise the Urd binding sites of hCNT1 and hCNT3 proteins. Further understanding of Urd-hCNT interactions will depend on structural analysis of the purified hCNT proteins. With purified transporter proteins, the binding constants for interactions of permeants and/or inhibitors with the transporters might be predicted by computer simulation of their three-dimensional structures.

In summary, the hCNT yeast expression system, which can be used to characterize the binding profiles of nucleoside transporters, will enable rapid screening of interactions of newly developed nucleoside-derived drugs with hCNT proteins. The present work established profiles for the interaction of Urd analogs with the hCNT1 and hCNT3 proteins. The differences in binding motifs for hCNT1 and hCNT3 reflect differences in nucleoside-binding domains of the two transporters. Because a high-affinity ligand may inhibit nucleoside transport without being transported, further studies, such as direct assay of time courses of uptake of radiolabeled ligand, are required to determine whether the uridine compounds identified in this study are also permeants. Additional nucleosides with modifications in the base moiety should be assessed to generate more complete nucleoside-binding profiles to guide the rational design and use of nucleoside drugs in the treatment of human diseases. The interactions of nucleoside analogs or drugs with hCNT1 and hCNT3 might be predicted from the permeant-recognition models developed in the present study.

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