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

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Received July 31, 2003; accepted September 10, 2003 This article is available online at http://molpharm.aspetjournals.org

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 [3H]uridine by intact yeast. The K_m values of uridine analogs were obtained from inhibitor-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.

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 permenant 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., 1997, 1998, 2001).

Received July 31, 2003; accepted September 10, 2003

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; FdUrd, 5-fluoro-2'-deoxyuridine; dUrd, deoxyuridine; araU1, 1-(β-D-arabinofuranosyl)uracil; ddUrd, dideoxyuridine; OmeUrd, O-methyluridine; 5'ClUrd, 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.
Materials and Methods

Strains and Media. Fu1::TRP1 (MATa, gal, ura3–52, trpl, lys2, ade2, his2Δ2000, and Δ fu1::TRP1), which contains a disruption in the gene encoding the endogenous Urd permease (FuU1) (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 Fu1::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 GAG 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-hCNT-Myc (5’-CGA GGT ACC TCA CTG ACG ATC CTC TTC TGA GAT GAG TTT TTT TTC TTC TGC TCG TGC ACA GAT CTT GTG GAT G-3’), and 3’-Xhol-hCNT3 (5’-CGA GGT ACC TCA CTG ACG ATC CTC TTC TGA GAT GAG TTT TTT TTC TTC TGC TCG TGC ACA GAT CTT GTG GAT 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 pYPhC1NT1, pYPhC1NT-Myc, and pYPhC3NT3. 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 (ρ600) of 0.7 to 1.0, collected by centrifugation (1000g, 5 min, 4°C), washed three times with breaking buffer (10 mM Tris, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1% (w/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 (500g, 5 min, 4°C), and membrane fractions were obtained by centrifugation of lysates (100,000g, 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.
Urd Uptake in Yeast Producing Recombinant hCNTs. The uptake of [3H]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 A560 of 0.8 to 1.5, washed twice with fresh media, pH 7.4, and resuspended in CMM/GLU, pH 7.4, to an A560 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 [3H]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 hours, the plates were placed on the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) and every 24 hours, the plates were placed on the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) and every 24 hours, the plates were placed on the semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) for 24 hours, respectively, and [3H]Urd uptake was measured. All experiments were carried out in quadruplicate. The amount of [3H]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 IC50 (inhibitory concentration 50%) values for compounds that inhibited uptake of [3H]Urd by using concentration-effect curves with at least 11 points distributed over the relevant range of concentrations. Ks (inhibitory constant) values were determined from the Cheng and Prusoff equation (Cheng and Prusoff, 1973), in which Ks = IC50/(1 + ([1/Ki])).

Gibbs free energy (ΔG°) was calculated from ΔG° = -RTln(Ks), 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° as described elsewhere (de König and Jarvis, 2001).

### Table 1

<table>
<thead>
<tr>
<th>Urn Compounds</th>
<th>hCNT1</th>
<th>hCNT3</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ks (μM)</strong></td>
<td>ΔG°</td>
<td>Ks (μM)</td>
</tr>
<tr>
<td>Urd</td>
<td>3.1 ± 0.3</td>
<td>31.5</td>
</tr>
<tr>
<td>Base Modification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FUrU</td>
<td>0.9 ± 0.2</td>
<td>34.5</td>
</tr>
<tr>
<td>IUrU</td>
<td>0.9 ± 0.1</td>
<td>34.5</td>
</tr>
<tr>
<td>3MeUrU</td>
<td>73.1 ± 16.8</td>
<td>23.6</td>
</tr>
<tr>
<td>Sugar Modification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2′dUrU</td>
<td>7.0 ± 0.3</td>
<td>29.4</td>
</tr>
<tr>
<td>5′dUrU</td>
<td>48.6 ± 7.9</td>
<td>24.6</td>
</tr>
<tr>
<td>3′dUrU</td>
<td>420 ± 68</td>
<td>19.3</td>
</tr>
<tr>
<td>araU</td>
<td>&gt;1000</td>
<td>&gt;2000°</td>
</tr>
<tr>
<td>1′-(β-D-Arabinofuranosyl)uracil</td>
<td>&gt;1000</td>
<td>&gt;2000°</td>
</tr>
<tr>
<td>2′,5′-dUrd</td>
<td>94.2 ± 14.6</td>
<td>23.0</td>
</tr>
<tr>
<td>2′OMeU</td>
<td>113 ± 22</td>
<td>22.5</td>
</tr>
<tr>
<td>5′OMeU</td>
<td>210 ± 42</td>
<td>21.0</td>
</tr>
<tr>
<td>3′OMeU</td>
<td>&gt;1000°</td>
<td>&gt;1000°</td>
</tr>
<tr>
<td>5′ClUrd</td>
<td>8.5 ± 1.1</td>
<td>28.9</td>
</tr>
<tr>
<td>2′AzdUrd</td>
<td>11.5 ± 0.5</td>
<td>28.2</td>
</tr>
<tr>
<td>3′AzdUrd</td>
<td>&gt;1000°</td>
<td>&gt;1000°</td>
</tr>
<tr>
<td>5′AzdUrd</td>
<td>326 ± 31</td>
<td>19.9</td>
</tr>
</tbody>
</table>

| Base and Sugar Modifications |       |       |       |       |
| 5-Ethyl-2′-deoxyuridine | 17.2 ± 2.4 | 27.2 | 26.2 ± 1.2 | 26.2 |
| FuUrU         | 2.0 ± 0.1 | 32.5 | 2.3 ± 1.4 | 32.2 |
| RedUrU        | 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° |       |

* Inhibition of less than 50% was observed.
* No obvious inhibition was observed.
Uridine Recognition Motifs of hCNT1 and hCNT3

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 3H-labeled cytidine and thymidine into pYPfCNT1-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 3H-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 3H-labeled cytidine, guanosine, and adenosine, respectively. These results demonstrated that the recombinant hCNT1 and hCNT3 produced in yeast showed characteristics similar 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 pYPfCNT1- and pYPfCNT3-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 fu1::TRP1 strain transformed with pYPfCNT1-myc gave a mean rate (±S.E.) of 9.25 ± 0.36 pmol/mg protein/min, which was similar to the rate obtained from pYPfCNT1-containing yeast in the experiments of Fig. 2. Initial rates of 3H-labeled cytidine and thymidine into pYPfCNT1-containing yeast were also measured, giving mean rates (±S.E.) of 0.26 ± 0.12 pmol/mg protein/min and 0.38 ± 0.14 pmol/mg protein/min, respectively. In contrast, rates of uptake of 3H-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 3H-labeled cytidine, guanosine, and adenosine, respectively. These results demonstrated that the recombinant hCNT1 and hCNT3 produced in yeast showed characteristics similar.

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 pYPfCNT1-myc-containing (Fig. 1A) and pYPfCNT3-containing (Fig. 1B) yeast, respectively, that were not present in membranes of pYPfGE15-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 fu1::TRP1 that contained pYPfGE15 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 fu1::TRP1 yeast that contained either pYPfCNT1 or pYPfCNT3, time courses for influx of [3H]Urd were measured in the experiments of Fig. 2. The Urd uptake time course for pYPfCNT1-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 pYPfCNT3-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 pYPfCNT3-containing yeast over the first 60 s (Fig. 2, inset) was the same as that calculated...
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 ($K_m = 8.7 \pm 1.1 \mu M$, $V_{max} = 1,400 \pm 286 \text{ pmol/mg protein/min}$; mean $\pm$ S.E., $n = 3$) than recombinant hCNT1 ($K_m = 9.2 \pm 3.8 \mu M$, $V_{max} = 86.9 \pm 12.9 \text{ pmol/mg protein/min}$, mean $\pm$ 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 $\mu M$ [3H]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 ($\pm$ 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 $\pm$ 0.1 $\mu M$) close to that of Urd (3.1 $\pm$ 0.3 $\mu M$). 5-Ethyl-2'-deoxyuridine exhibited a $G_0$ 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...
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 [$\delta (\Delta G^\circ) = 9$ kJ/mol, relative to $\Delta G^\circ$ 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 $\Delta G^\circ$ 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 $\Delta G^\circ$ value upon substitution of an azido group for a hydrogen atom at the C(5') of 5'dUrd [$\delta (\Delta G^\circ) = 11.5$ kJ/mol, relative to $\Delta G^\circ$ 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 $\Delta G^\circ$ 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 ($K_i = 210 \pm 42 \mu$M) with a difference in $\Delta G^\circ$ 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, $K_i = 8.5 \pm 1.1$ $\mu$M). 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 $\mu$M. The difference in

![Fig. 5. Inhibition of recombinant hCNT1-mediated Urd uptake by some Urd analogs. The uptake of 1 $\mu$M [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'OmeUrd ( ), 3'OmeUrd ( ), 2'AzdUrd ( ), 5'AzdUrd ( ), 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.](image-url)
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 hydrogen bonding was also apparent from the effects of substitution of an azido group or O-methyl group at C(3') because neither 3'AzUrd 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-xylouranosyl)uracil (Fig. 4), with the 3'-hydroxyl group oriented in the opposite direction, exhibited slightly lower affinity than that of Urd. As observed with hCNT1, the inverted orientation of the 3'-hydroxyl group was that FdUrd (0.6) 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 nucleosides, including 2',3'ddUrd, 3',5'ddUrd, AZT, 3'OMeUrd, iPdUrd, and 3'AzUrd 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'-hydroxyl 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 Kᵢ 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 (Ser319, Gln320, Ser353, and Leu354) 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 radio-labeled 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.

Acknowledgments

We thank Dr. S. M. Gryaznov for a gift of the protected 3-azido-3-deoxyribofuranose and Pat Carpenter for excellent technical assistance.

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