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Interaction of fused-pyrimidine nucleoside analogs with human concentrative nucleoside transporters: High-affinity inhibitors of human concentrative nucleoside transporter 1

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ABSTRACT

Human concentrative nucleoside transporters (hCNTs) mediate electrogenic secondary active transport of physiological nucleosides and nucleoside drugs into cells. Six fused-pyrimidine ribonucleosides and one 2′-deoxynucleoside were assessed for their abilities to inhibit [^{3}H]uridine transport in the yeast *Saccharomyces cerevisiae* producing recombinant hCNT1, hCNT2 or hCNT3. Six of the analogs inhibited hCNT1 with K_i values < 1 μM whereas only two analogs inhibited hCNT3 with K_i values < 1 μM and none inhibited hCNT2. To assess if the inhibitory analogs were also permeants, currents evoked were measured in oocytes of *Xenopus laevis* producing recombinant hCNT1, hCNT2 or hCNT3. Significant inward currents, indicating permeant activity, were generated with (i) three of the analogs in hCNT1-producing oocytes, (ii) none of the analogs in hCNT2-producing oocytes and (iii) all of the analogs in hCNT3-producing oocytes. Four were not, or were only very weakly, transported by hCNT1. The thienopyrimidine 2′-deoxynucleoside (dMeThPmR, **3**) and ribonucleoside (MeThPmR, **4**) were the most active inhibitors of uridine transport in hCNT1-producing oocytes and were an order of magnitude more effective than adenosine, a known low-capacity transport inhibitor of hCNT1. Neither was toxic to cultured human leukemic CEM cells, and both protected CEM cell lines with hCNT1 but not with hENT1 against gemcitabine cytotoxicity. In summary, dMeThPmR (**3**) and MeThPmR (**4**) were potent inhibitors of hCNT1 with negligible transportability and little apparent cytotoxicity, suggesting that pending further evaluation for toxicity against normal cells, they may have utility in protecting normal hCNT1-producing tissues from toxicities resulting from anti-cancer nucleoside drugs that enter via hCNT1.

1. Introduction

Human nucleoside transporters (hNTs) are broadly distributed in cells and tissues and thus are involved in cellular uptake and cytotoxic activity of many anticancer nucleoside drugs. In humans, multiple hNTs play a role in salvage and disposal of nucleosides, in modulation of local concentrations of adenosine, a signaling nucleoside, and in disposition of anticancer and antiviral nucleoside drugs [1,2]. Equilibrative bi-directional transport of nucleosides across membranes is mediated by hENTs, and concentrative inwardly-directed sodium- and/or proton-coupled transport of nucleosides against their concentration gradients is mediated by hCNTs [1,3].

To date, four hENT (hENT1-4) and three hCNT (hCNT1-3) subtypes have been identified by molecular cloning and functional expression of their cDNAs [4–10]. All of the hCNTs transport uridine but exhibit different preferences for other permeants, with selectivities by hCNT1 for pyrimidine nucleosides, hCNT2 for purine nucleosides and hCNT3 for both purine and pyrimidine nucleosides [8–10]. The differences in permeant selectivities are also seen in the transportabilities of gemcitabine (2′-deoxy-2′,2′-difluorocytidine) and AZT (3′-azido-3′-deoxythymidine), both of which are permeants for hCNT1 and hCNT3 but not for hCNT2 [11,12]. hCNTs are distributed in specialized cells, such as intestinal and renal cells, and

Abbreviations: hENTs, human equilibrative nucleoside transporters; hCNTs, human concentrative nucleoside transporters; NBMPR, nitrobenzylmercaptopurine riboside (6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine); IC_{50}, the concentration of test compound that inhibited growth of treated cells by 50% relative to that of untreated cells; V_h, holding potential; AZT, 3′-azido-3′-deoxythymidine; CMM, complete minimal medium; GLU, glucose.

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also in some cancer cells [8,10,13]. These differences suggest unique roles for hCNTs.

New approaches for optimizing selective transport of nucleoside drugs into diseased tissues vs. normal tissues should include development of targeted inhibitors for the concentrative transporters since some clinically used anti-cancer nucleoside drugs cause toxicity to normal tissues, most commonly gastrointestinal, bone marrow, liver and kidney. A family of potent and highly specific equilibrative nucleoside transport inhibitors, including nitrobenzylmercaptopurine ribonucleoside (NBMPR), were identified many years ago [14,15] by their potent effects on the nucleoside transport process now known to be mediated by hENT1.

In recent studies [16,17], phloridzin analogs and some naturally occurring flavone compounds were identified as inhibitors of hCNT3-mediated transport of uridine in stable transfectants subjected to quantitative structure activity studies (QSAR). Although uridine inhibition was observed at μM concentrations, these compounds were not high-affinity inhibitors. Additionally, because these experiments assessed inhibition of uridine transport, it is not possible to distinguish between compounds that were inhibitors because they competed with uridine for transport into cells and those that were inhibitors because they bound to the transporter without being transported (i.e., high affinity, low capacity inhibitors).

In this study we assessed six fused-pyrimidine ribonucleosides and one 2′-deoxynucleoside for inhibition of CNT-mediated transport using three *in vitro* model systems (*Saccharomyces cerevisiae*, *Xenopus laevis* oocytes and cultured human leukemia cells). The analogs were first tested for their relative abilities to inhibit uridine transport in yeast producing recombinant hCNT1, hCNT2 or hCNT3 in concentration-effect experiments. The analogs were then tested for their permeant and inhibitor activities in oocytes of *X. laevis* producing recombinant hCNT1, hCNT2 or hCNT3 by measuring nucleoside-evoked membrane currents following the addition of fixed concentrations of each of the fused-pyrimidine analogs alone or in combination with known permeants (uridine, adenosine). Several analogs were identified as high affinity, low capacity inhibitors of hCNT1 and the most potent were tested in hCNT1-producing oocytes for inhibition of uridine-induced currents at varying concentrations. These analogs were also tested in both transport and cytotoxicity experiments with wild type CEM cells, which possess only hENT1 activity, and with stably transfected CEM cells, which possess only hCNT1 activity. Two novel high-affinity inhibitors of hCNT1 were identified, the thienopyrimidine 2′-deoxynucleoside (dMeThPmR, **3**) and its ribonucleoside analog (MeThPmR, **4**), and both exhibited little, if any, toxicity to the human leukemic cell lines used in this study.

2. Materials and methods

2.1. Materials

[5,6-^{3}H]Uridine (21.3 Ci/mmol) and [^{3}H]gemcitabine (11 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). Tissue culture (96- and 12-well) plates, RPMI 1640 cell culture media, and fetal bovine serum (FBS) were from Invitrogen (Burlington, Ontario). Ecolite was from ICN Pharmaceuticals (Montreal, Quebec). The Cell Titer 96 Aqueous One Solution Cell Proliferation Assay Kit was from Promega (Madison, WI). Yeast nitrogen base, amino acids and glucose were from Difco (Detroit, MI). Filter mats for yeast studies were from Molecular Devices (Sunnyvale, CA). Unless otherwise noted, chemicals were obtained from Sigma–Aldrich (Oakville, ON, Canada).

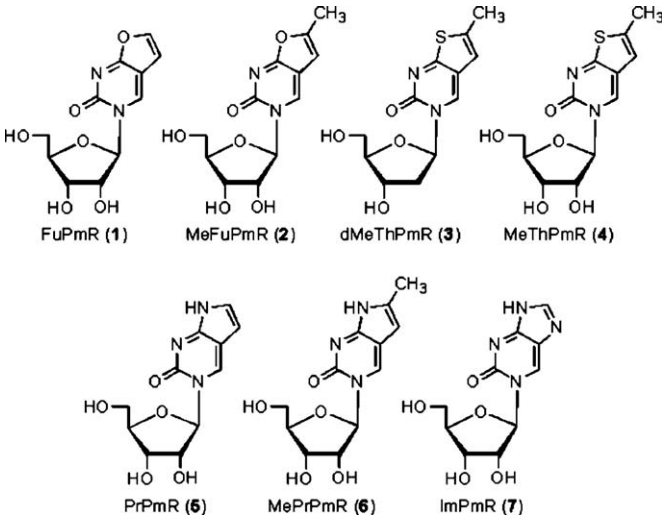

Fig. 1. Structures of the fused-pyrimidine nucleoside analogs.

2.2. Fused-pyrimidine nucleosides

Methods for synthesis of the seven nucleoside analogs shown in Fig. 1 and their absorption and emission properties will be reported separately (manuscript under preparation). The known furopyrimidine nucleosides were prepared by the methods of Robins and Barr [18,19]. The names, abbreviations and arbitrarily assigned analog numbers are: 1-(β-D-ribofuranosyl)furo[2,3-*d*]pyrimidin-2(1*H*)-one (FuPmR, **1**); 5-methyl-1-(β-D-ribofuranosyl)furo[2,3-*d*]pyrimidin-2(1*H*)-one (MeFuPmR, **2**); 1-(2-deoxy-β-D-*erythro*-pentofuranosyl)-5-methylthieno[2,3-*d*]pyrimidin-2(1*H*)-one (dMeThPmR, **3**); 5-methyl-1-(β-D-ribofuranosyl)thieno[2,3-*d*]pyrimidin-2(1*H*)-one (MeThPmR, **4**); 1-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(1*H*, 4*H*)-one (PrPmR, **5**); 5-methyl-1-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(1*H*,4*H*)-one (MePrPmR, **6**); 1-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyrimidin-2(1*H*, 4*H*)-one (ImPmR, **7**).

2.3. Yeast strains and media

Yeast strains were maintained in complete minimal medium (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). Plasmids were propagated in *E. coli* strain TOP10F (Invitrogen, Carlsbad, CA) and maintained in Luria broth with 100 μg/mL ampicillin as described earlier [20,21].

2.4. Transport inhibition assays in yeast with recombinant transporters

Saccharomyces cerevisiae 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) [22] and therefore lacks endogenous uridine transport activity, was the parental yeast strain used to generate the hCNT expression system and were characterized in detail earlier [23,24].

Fui1::TRP1 yeast were separately transformed with plasmids (pYPhCNT1, pYPhCNT2, pYPhCNT3) with cDNAs encoding, respectively, hCNT1, hCNT2 or hCNT3 as described previously [20,21,23,24] and were used to examine the ability of fused-pyrimidine nucleosides to inhibit initial rates of uptake of 1 μM [^{3}H]uridine. Transport experiments were conducted in 96-well plates with a semiautomated cell harvester (Micro96 HARVESTER; Skatron Instruments, Lier, Norway) as described previously [21].

Yeast producing hCNT1, hCNT2 or hCNT3 were grown in CMM/GLU to an absorbance at 600 nm (A600) of 0.5–1.0, washed twice in fresh medium, and resuspended at an A600 of 4.0. Yeast producing hCNT1, hCNT2 or hCNT3 were incubated at room temperature for 15, 20 or 5 min respectively with graded concentrations of test compounds (0–100 μM) in the presence of 1 μM [^{3}H]uridine. Transport reactions were initiated by rapid mixing of 50-μL yeast suspensions with or without various concentrations of test compounds with 50 μL of two times concentrated [^{3}H]uridine in 96-well microtiter plates. Yeast cells were collected on filtermats using a Micro96 Cell Harvester and rapidly washed with deionized water. The individual filter circles corresponding to wells of the microtiter plates were removed from filtermats with forceps and transferred to vials for quantification of radioactivity by scintillation counting. Each experiment was repeated at least three times with six replicates per condition. Data were subjected to nonlinear regression analysis using Prism software (Version 4.0; GraphPad Software Inc., San Diego, CA) to obtain IC_{50} values that were used to calculate K_i values [25].

2.5. In vitro transcription and expression in oocytes of Xenopus laevis

cDNAs encoding hCNT1, hCNT2 or hCNT3 (GenBank™ accession numbers U62968, AF036109 and AF305210, respectively) in the enhanced *Xenopus* plasmid expression vector pGEM-HE with flanking 5′- and 3′-untranslated regions (UTRs) from the *Xenopus* β-globin gene were linearized with *Nhe1* (hCNT1/3) or *Sph1* (hCNT2) and transcribed with T7 polymerase using the mMESSAGE mMACHINE™ (Ambion, Austin, TX) transcription system as described previously [8]. The remaining template was removed by digestion with RNase-free DNase1. Stage V–VI oocytes were isolated by collagenase treatment (2 mg/mL for 2 h) of ovarian lobes from female frogs (Biological Sciences Vivarium, University of Alberta) that had been anaesthetized by immersion in 0.3% (w/v) tricaine methanesulphonate (pH 7.4). The remaining follicular layers were removed by phosphate treatment (100 mM K_2HPO_4) and manual defolliculation. Oocytes were injected with either 10 nL of water containing 10 ng of RNA transcripts encoding hCNT1, hCNT2 or hCNT3 or 10 nL of water alone. Prior to measurement of currents, the injected oocytes were incubated for 4–7 days at 18 °C in modified Barth's solution (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM Hepes, 2.5 mM sodium pyruvate, 0.1 mg/mL penicillin and 0.05 mg/mL gentamycin sulfate, pH 7.5), which was changed daily. Endogenous uridine-transport activity in water injected oocytes has previously been shown to be very low [9].

2.6. Electrophysiological studies

Electrophysiology experiments were performed in sodium-containing transport medium composed of 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes (pH 7.5). Nucleoside-evoked membrane currents were measured in hCNT-producing oocytes at room temperature (~20 °C) using a GeneClamp 500B oocyte clamp (MDS Analytical Technologies, Sunnyvale, CA) in the two-electrode, voltage-clamp mode. The GeneClamp 500B was interfaced to an IBM-compatible PC *via* a Digidata 1322A A/D converter and controlled by pCLAMP software (Version 9.0, MDS Analytical Technologies). The microelectrodes were filled with 3 M KCl and had resistances that ranged from 0.5 to 2.5 MΩ (megaohms). Oocytes were penetrated with the microelectrodes and their membrane potentials were monitored for periods of 10–15 min. Oocytes were discarded when membrane potentials were unstable, or more positive than −30 mV. All steady-state current measurements were performed at a holding potential (V_h) of −90 mV. Following stabilization of the baseline potential, uridine or nucleoside analog was added to the perfusion chamber in sodium-containing transport medium (pH 7.5). For representative current recordings, hCNT-producing oocytes were perfused with 5 mL of uridine- or nucleoside analog-containing solution, each at a concentration of 100 μM. In experiments investigating the mean current generated, or in competition studies, 500 μL of solution containing uridine or nucleoside analog was manually added to the perfusion chamber due to limitations in the quantity of nucleoside analog available. Current signals were filtered at 20 Hz (four-pole Bessel filter) and sampled at intervals of 20 ms. In experiments measuring mean uridine- or nucleoside analog-induced currents, the concentration of the permeant to be tested was 100 μM. In competition studies, hCNT1- or hCNT3-producing oocytes were tested for sufficient levels of expression with 100 μM uridine. Currents were then measured following the addition of (i) 25 μM uridine, adenosine or nucleoside analog, or (ii) 25 μM uridine + 25 μM adenosine or nucleoside analog in the same oocyte. In experiments measuring the inhibition of hCNT1-mediated uridine-induced currents (25 μM) in the presence of increasing concentrations of inhibitor, IC_{50} values were determined using Sigmaplot 10 software (Jandel Scientific Software, San Rafael, CA). Corresponding K_i values were calculated as described for yeast experiments. No currents were observed in control water-injected oocytes (data not shown), consistent with results described previously [26].

2.7. Cell culture

The human CCRF-CEM leukemia, hereafter termed CEM, cell line was obtained from William T. Beck (University of Illinois at Chicago, Chicago, IL). CEM/ARAC8C, a nucleoside transport-deficient derivative of CEM [27], referred to as CEM-ARAC, was a gift from Dr. B. Ullman (Oregon Health and Science University, Portland, OR). CEM-ARAC/hCNT1 was derived from CEM-ARAC by stable transfection with a pcDNA3 plasmid that contained the coding sequence for hCNT1 [28]. All cell lines were free of mycoplasma. CEM and CEM-ARAC/hCNT1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, ON) as suspension cultures. Cells were maintained in the absence of antibiotics, incubated at 37 °C in a humidified atmosphere (5% CO_2), and subcultured at 2–4 day intervals to maintain active proliferation. Cell numbers were determined with a Coulter Z2 electronic particle counter equipped with a size analyzer (Beckman Coulter Canada, Mississauga, ON).

2.8. Nucleoside uptake assays in cultured cells

Nucleoside uptake assays to measure initial rates of transport were conducted at room temperature for 60 s in transport medium (20 mM Tris–HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2$ ($6H_2O$), 2 mM $CaCl_2$, 5 mM glucose and 130 mM NaCl, pH 7.4; 300 ± 15 mOsM) in CEM cells or CEM-ARAC/hCNT1, as described for suspension cultures [29] using [^{3}H]uridine or [^{3}H]gemcitabine. Cells were lysed with 5% Triton X-100 and mixed with Ecolite scintillation fluid to measure cell-associated radioactivity (Beckman LS 6500 scintillation counter; Beckman Coulter Canada). All experiments were repeated three times with 3–4 replicates/condition.

2.9. Chemosensitivity testing with cultured cells

Cytotoxicity assays were conducted using the CellTiter 96 proliferation assay (Promega, Madison, WI) as described previously [30]. This assay is based on the reduction of a tetrazolium compound to a soluble formazan derivative by the dehydrogenase enzymes of metabolically active cells. The absorbance (490 nm) is directly proportional to the number of living cells in culture. CEM

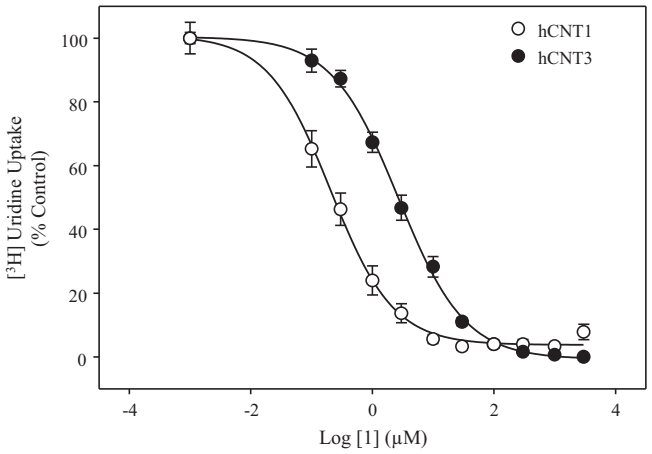

Fig. 2. Inhibition of uridine uptake in yeast producing recombinant hCNT1 and hCNT3 by fused-pyrimidine nucleoside analog FuPmR (**1**). Uptake of 1 μM [^{3}H]uridine into yeast producing hCNT1 (○) and hCNT3 (●) was measured at ambient temperature in the presence or absence of graded concentrations of **1**, as described in Section 2. Values, expressed as % control (untreated cells) shown in the graph are the means (±S.E.M) of three separate experiments, each conducted with six replicates and where error bars are not shown, they were smaller than the symbols.

or CEM-ARAC/hCNT1cells were seeded in 96-well tissue culture plates (10^4 cells/well, six replicates/condition), incubated for 2 h with or without 1 μM NBMPR, dMeThPmR (**3**) or MeThPmR (**4**), and then exposed to graded concentrations (0–100 μM) of gemcitabine in the presence or absence of 1 μM NBMPR, **3** or **4** for 72 h after which they were treated with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent for assessment of cytotoxicity. Each experiment was repeated at least three times with six replicates per condition. Chemosensitivity was expressed as the effective gemcitabine concentration at which cell proliferation was inhibited by 50% (EC_{50} values) and was determined from concentration-effect relationships using GraphPad Prism.

3. Results

3.1. Inhibition by fused-pyrimidine nucleosides of uridine transport by recombinant hCNTs produced in yeast

The inhibitory activity of the fused-pyrimidine nucleoside analogs on transporter-mediated uptake of [^{3}H]uridine was examined using hCNT-producing yeast as described in Section 2. The analogs were screened for concentration-dependent inhibition of [^{3}H]uridine transport in experiments using hCNT-producing yeast to determine the IC_{50} (inhibitor concentration that produced 50% inhibition of uridine transport) values for each of the three recombinant transporters. Representative concentration-effect curves for inhibition of hCNT1 and hCNT3-mediated uridine transport by FuPmR (**1**) are shown in Fig. 2 where it is evident that **1** produced dose-dependent inhibition of [^{3}H]uridine transport at μM concentrations. The IC_{50} values obtained from similar experiments with yeast producing hCNT1, hCNT2 or hCNT3 with all seven analogs are summarized in Table 1. Apparent K_i values, which provide a measure of relative affinities of the various transporters for the analogs, were calculated from the observed IC_{50} values and previously reported kinetic constants for uridine [3] and are presented in Table 1. Six of the seven analogs were potent inhibitors of hCNT1 with K_i values less than 1 μM whereas only three had K_i values less than 1 μM for hCNT3 and none were good inhibitors of hCNT2.

3.2. Transport of nucleoside analogs in Xenopus oocytes

The transportabilities of the fused-pyrimidine nucleoside analogs were examined in oocytes producing recombinant hCNT1, hCNT2 or hCNT3 using the two-microelectrode voltage clamp. Representative recordings comparing currents evoked by the addition of uridine, MeFuPmR (**2**) or dMeThPmR (**3**), each at a concentration of 100 μM in sodium-containing (100 mM NaCl pH 7.5) transport medium, are shown in Fig. 3. Currents are shown for hCNT1- (Fig. 3A) and hCNT3- (Fig. 3B) producing oocytes. As currents generated in oocytes producing hCNT2 were not observed or negligible, traces are not shown. No currents were observed in control water-injected oocytes (data not shown). Fig. 4A depicts mean currents observed following the addition of uridine or nucleoside analog for ≥3 oocytes in sodium-containing transport medium (100 mM NaCl pH 7.5). Inward currents were generated by the addition of uridine (100 μM) to hCNT1-, hCNT2- and hCNT3-producing oocytes, consistent with the known permeant specificities of these transporters [8–10]. Inward currents were also generated by the application of 100 μM FuPmR (**1**), MeFuPmR (**2**) or PrPmR (**5**) in oocytes producing hCNT1, whereas dMeThPmR (**3**), MeThPmR (**4**), MePrPmR (**6**) and ImPmR (**7**) had little effect (i.e., no or very small inward currents). Currents were not observed or negligible in oocytes producing hCNT2 following addition of any of the analogs, whereas currents were generated by all of the analogs in oocytes producing hCNT3, with the largest seen with **1** and the smallest with **6**. For all of the analogs tested, no currents were evident in control water-injected oocytes (data not shown).

3.3. Inhibition studies in Xenopus oocytes

Because **3**, **4**, **6**, and **7** had not generated significant currents in hCNT1-producing oocytes and thus were not significantly transported by hCNT1, they were further tested for their abilities to inhibit hCNT1-mediated uptake of uridine (Fig. 4B). Currents

Table 1
Effects of fused-pyrimidine nucleoside analogs on the uptake of uridine in *S. cerevisiae* producing recombinant hCNT1, hCNT2 or hCNT3.

Compound	hCNT1	hCNT2	hCNT3
	IC_{50} ± SE μM (K_i)		
FuPmR (**1**)	0.12 ± 0.01 (0.11 ± 0.01)	393 ± 42 (380 ± 41)	5.3 ± 1.2 (3.3 ± 0.8)
MeFuPmR (**2**)	0.37 ± 0.08 (0.33 ± 0.07)	1419 ± 246 (1372 ± 238)	25.0 ± 1.2 (15.7 ± 0.8)
dMeThPmR (**3**)	0.33 ± 0.08 (0.30 ± 0.07)	133 ± 11 (129 ± 11)	52.0 ± 7.6 (32.7 ± 4.8)
MeThPmR (**4**)	0.18 ± 0.03 (0.16 ± 0.02)	360 ± 24 (348 ± 23)	7.5 ± 0.06 (4.7 ± 0.4)
PrPmR (**5**)	0.39 ± 0.03 (0.35 ± 0.03)	239 ± 29 (231 ± 28)	1.2 ± 0.6 (0.8 ± 0.4)
MePrPmR (**6**)	0.26 ± 0.05 (0.23 ± 0.05)	175 ± 43 (169 ± 42)	0.7 ± 0.2 (0.4 ± 0.1)
ImPmR (**7**)	0.19 ± 0.07 (0.17 ± 0.06)	270 ± 22 (261 ± 21)	1.3 ± 0.1 (0.8 ± 0.1)

Presented are IC_{50} values (±S.E.), which represent the inhibitor concentrations at which initial rates of uptake (*i.e.*, transport) of 1 μM [^{3}H]uridine were inhibited by 50% as determined by computer-generated concentration-effect curves (see Fig. 2). The corresponding K_i values (±S.E.M.) are given in parentheses, where $K_i = IC_{50}/(1 + ([S]/K_m)$ [25]. In these calculations, $[S] = 1$ μM and K_m (uridine/hCNT1) = 9.2 μM; K_m (uridine/hCNT2) = 29.1 μM and K_m (uridine/hCNT3) = 1.7 μM [3].

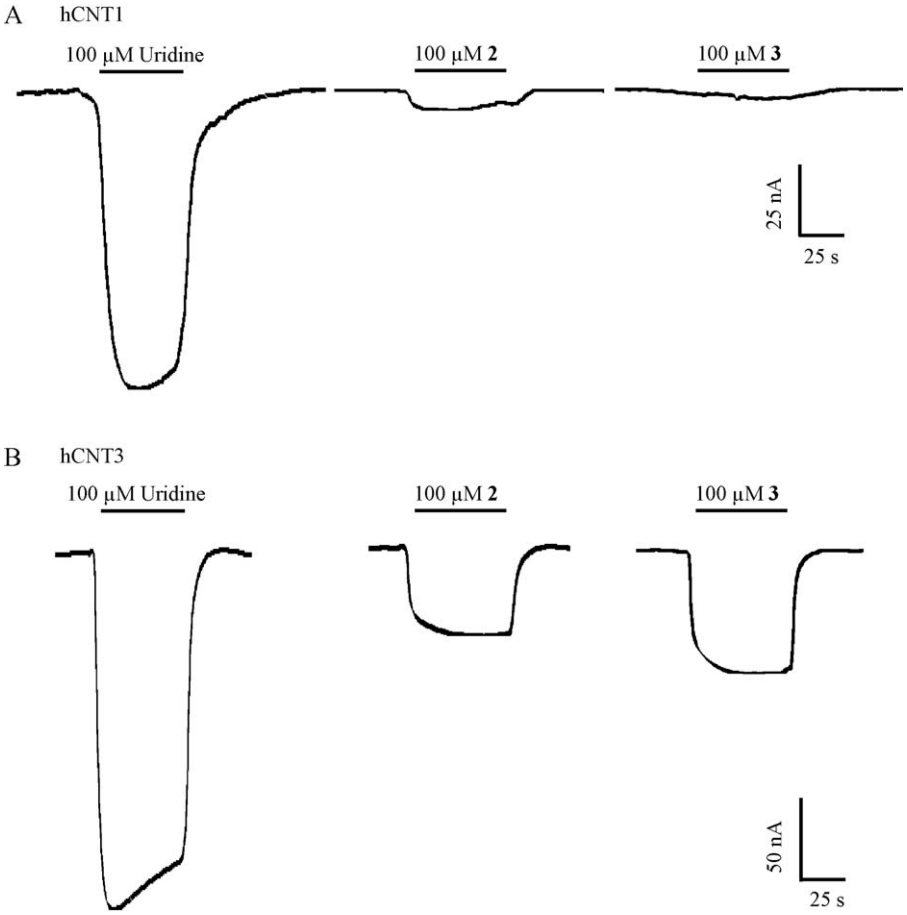

Fig. 3. Steady-state currents of hCNT1 and hCNT3. Representative uridine or nucleoside analog current traces are shown for single hCNT1- (Panel A) or hCNT3- (Panel B) producing oocytes clamped at −90 mV in sodium-containing transport medium (pH 7.5). The *bar* denotes perfusion of hCNT1- or hCNT3-producing oocytes with uridine, **3** or **4**, each at a concentration of 100 μM. No currents were observed in control water-injected oocytes (data not shown).

generated by the addition of 25 μM uridine in oocytes producing hCNT1 were significantly higher than those produced in the same oocyte by simultaneous addition (each at a concentration of 25 μM) of (i) uridine and adenosine (a known inhibitor of hCNT1), or (ii) uridine and **3**, **4**, **6** or **7**. Nucleoside analogs **3** and **4** inhibited hCNT1-mediated uridine currents to a greater extent than **6** and **7**.

3.4. Concentration-effect relationships for inhibition of hCNT1-mediated uridine influx by the thienopyrimidine analogs 3 and 4 in Xenopus oocytes

The concentration dependences of inhibition of hCNT1-mediated uridine-induced currents by **3** and **4** in *Xenopus* oocytes was determined in the experiments of Fig. 5. Adenosine, which is a known inhibitor of hCNT1 [31], was included for comparison. Increasing concentrations of (i) adenosine (0–50 μM) or (ii) **3** or **4** (0–25 μM) were simultaneously added with 25 μM uridine to hCNT1-producing oocytes and the resulting currents were measured. Adenosine inhibited uridine transport by hCNT1 with an IC_{50} value of 11.1 ± 2.3 μM (Fig. 5A), consistent with previously published results [31]. dMeThPmR (**3**) and MeThPmR (**4**) blocked uridine transport by hCNT1 with approximately 10-fold greater potencies (Fig. 5B and C). IC_{50} values of 1.7 ± 0.5 μM (for **3**) and 1.2 ± 0.3 μM (for **4**) were obtained for inhibition of hCNT1-induced uridine currents. Corresponding K_i values calculated using the previously reported K_m value of 32 μM for uridine-induced currents in hCNT1-producing oocytes [26] were 6.24 ± 1.29 μM (for adenosine), 0.93 ± 0.28 μM (for **3**) and 0.69 ± 0.15 μM (for **4**).

3.5. Effects of 3 and 4 on uridine transport in cultured CEM (hENT1) and CEM-ARAC/hCNT1 cells

dMeThPmR (**3**) and MeThPmR (**4**), which were identified as inhibitors of hCNT1 in the yeast radiotracer and *Xenopus* oocyte electrophysiology experiments, were tested in the CEM and CEM/hCNT1 cell lines, each of which possesses a single nucleoside transporter subtype, to evaluate their effects on hCNT1-mediated transport in human cells. Fig. 6A shows the effects of 5 μM uridine, **3** and **4** on uridine transport in the two cell lines. Both **3** and **4** inhibited uridine transport in CEM-ARAC/hCNT1 cells but not in CEM cells, which exhibit only hENT1-mediated transport of nucleosides, confirming that the two analogs inhibited hCNT1-mediated transport of uridine and demonstrating that they were without effect on hENT1. Concentration-effect studies in CEM-ARAC/hCNT1 cells yielded IC_{50} values of 99 ± 8 nM (for **3**) and 89 ± 4 nM (for **4**) (Fig. 6B), indicating high-affinity inhibition of hCNT1-mediated uridine transport by both thienopyrimidine compounds.

Gemcitabine, a nucleoside analog used clinically in therapy of solid tumors, is a permeant of hENT1, hENT2, hCNT1 and hCNT3 [12] and we assessed if the two analogs (**3** and **4**) inhibited hENT1 and hCNT1-mediated transport of [^{3}H]gemcitabine. Accumulation during 1-h exposures to 1 μM [^{3}H]gemcitabine was examined in CEM and CEM-ARAC/hCNT1 cells in the absence or presence of 1 μM NBMPR (high-affinity inhibitor of hENT1), **3** or **4** (Fig. 6C). Both **3** and **4** inhibited gemcitabine transport and accumulation in CEM-ARAC/hCNT1 cells, but not in CEM cells, whereas NBMPR inhibited transport in CEM cells and not in CEM-ARAC/hCNT1 cells.

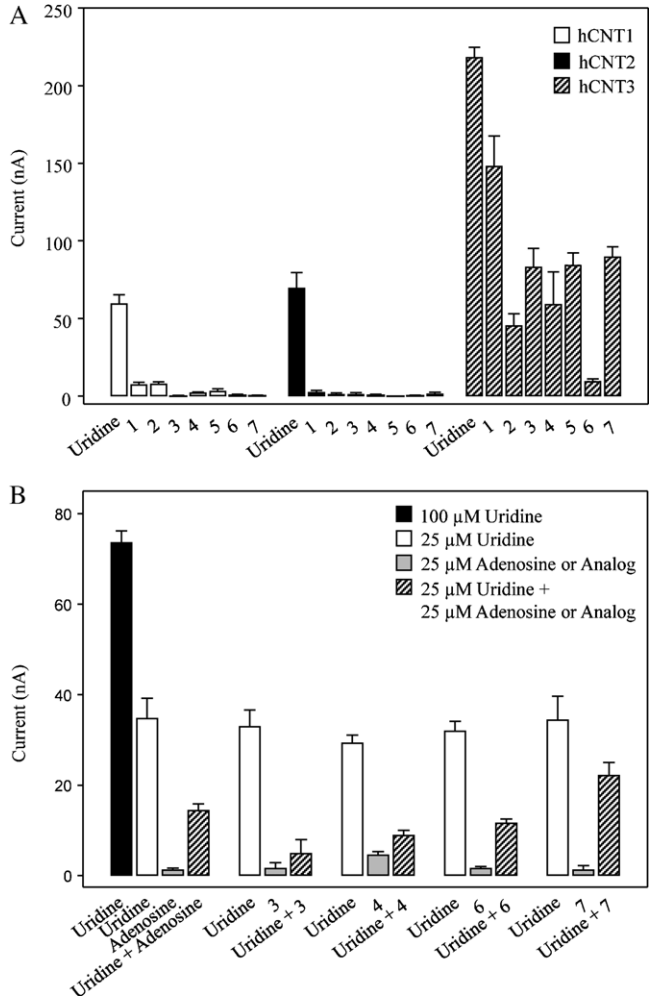

Fig. 4. Transport of fused-pyrimidine nucleoside analogs by recombinant hCNT1, hCNT2 and hCNT3 in *Xenopus* oocytes. Panel A shows currents generated by perfusing hCNT1- (*open bars*), hCNT2- (*black bars*), or hCNT3- (*hatched bars*) producing oocytes with 100 μM uridine or nucleoside analogs in sodium-containing medium (100 mM NaCl pH 7.5; V_h = −90 mV). Values are means ± S.E.M. for three different oocytes. The same experiments were also performed in control water-injected oocytes (data not shown); no currents were observed. In panel B, oocytes were tested for functional expression of hCNT1 (controls) by the addition of 100 μM uridine in Na^+-containing transport medium (pH 7.5; V_h = −90 mV). The current generated following the addition of 25 μM uridine was compared to the current generated when the same oocyte was exposed to 25 μM adenosine, **3**, **4**, **6** or **7** in the presence of 25 μM uridine. For comparison, the currents generated in the presence of 25 μM adenosine, **3**, **4**, **6** or **7** alone are also shown. Values are means ± S.E.M. for three different oocytes.

These results demonstrated that **3** and **4** were potent inhibitors of hCNT1-mediated, but not of hENT1-mediated, uptake of gemcitabine.

Compounds **3** and **4**, which were shown to be the most potent inhibitors, were tested further to assess the nature of the inhibition of uridine transport in CEM-ARAC/hCNT1 cells. In the experiments of Fig. 7, the effects of fixed concentrations of compounds **3** or **4** on uridine transport rates were assessed. Both compounds appeared to be competitive inhibitors of uridine transport yielding K_i values of 3.8 ± 0.6 and 2.7 ± 0.4 μM, respectively, for **3** and **4**.

3.6. Cytotoxicities of 3 and 4 in CEM and CEM-ARAC/hCNT1 cells

There was no evidence of toxicity at any of the concentrations tested up to100 μM when CEM and CEM-ARAC/hCNT1 cells were exposed to **3** or **4** for 72 h (data not shown). This result suggested that it might be possible to use **3** and **4** to manipulate hCNT1-mediated transport of nucleoside analogs similar to the manipulation of hENT1-mediated transport by NBMPR [32]. In the experiments of Table 2, CEM and CEM-ARAC/hCNT1 cells were

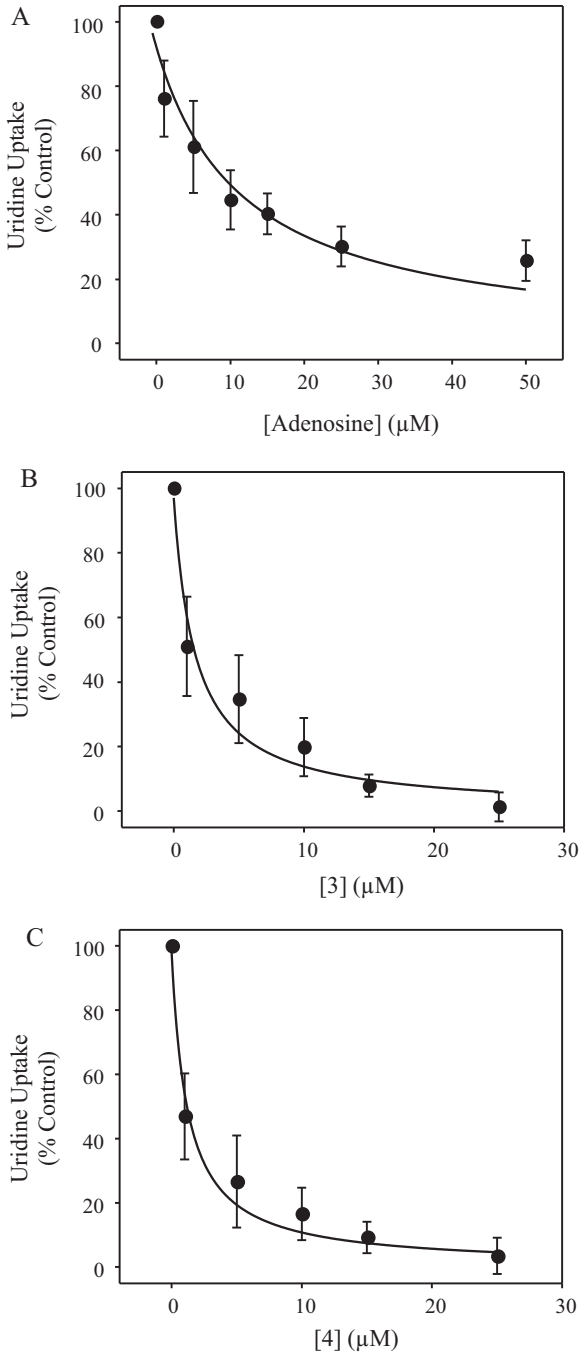

Fig. 5. Inhibition of hCNT1-mediated uridine influx in *Xenopus* oocytes. Uridine-induced currents (25 μM; 100 mM NaCl pH 7.5) were measured in hCNT1-producing oocytes (Panel A) in the presence of increasing concentrations of adenosine (0–50 μM) at a membrane potential of −90 mV. Values were obtained from individual oocytes (n = 8) normalized to the currents generated in the presence of 25 μM uridine alone (control). The average uptake of adenosine (in the absence of uridine) at each tested concentration was normalized to the current generated in the presence of 25 μM uridine alone. From the averaged inhibition of uridine-induced currents, the average uptake of adenosine (in the absence of uridine) at each tested concentration was subtracted to give the mean inhibition-uptake as a percent of uridine control and the calculated error (S.E.M.). In panels B and C, uridine-induced currents (25 μM; 100 mM NaCl pH 7.5) were measured in hCNT1-producing oocytes (n = 6) in the absence or presence of increasing concentrations (0–25 μM) of **3** (Panel B) or **4** (Panel C) at a membrane potential of −90 mV. Values were calculated as in panel A.

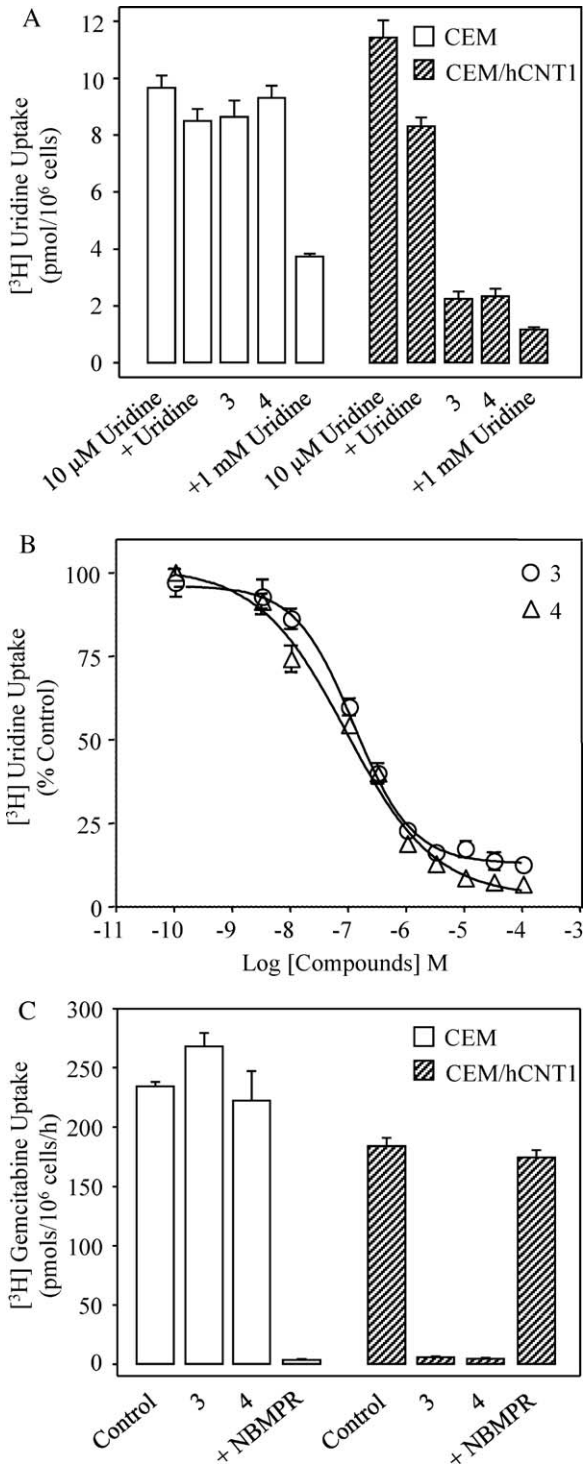

Fig. 6. Inhibition of uridine transport and gemcitabine uptake by **3** and **4** in CEM and CEM-ARAC/hCNT1. Panel A shows uptake of 10 μM [^{3}H]uridine into CEM (*open bars*) and CEM-ARAC/hCNT1 (*hatched bars*) cells at room temperature (60 s fluxes) in the absence or presence of 5 μM uridine, **3** or **4** or 1 mM uridine. In panel B, inhibition of uptake of 1 μM [^{3}H]uridine into CEM-ARAC/hCNT1 cells (60 s fluxes) by graded concentrations of **3** (○) or **4** (Δ) was assessed as described in Section 2. Panel C shows inhibition of 1 μM [^{3}H]gemcitabine accumulation (1-h incubations) in CEM (*open bars*) and CEM-ARAC/hCNT1 cells (*hatched bars*) in the absence (control) or presence of 1 μM NBMPR, **3** or **4**. For Panels 1–3, each bar or data point represents the means (±S.E.M) of three separate experiments with three replicates per experiment and error bars are not shown where values are smaller than the symbols.

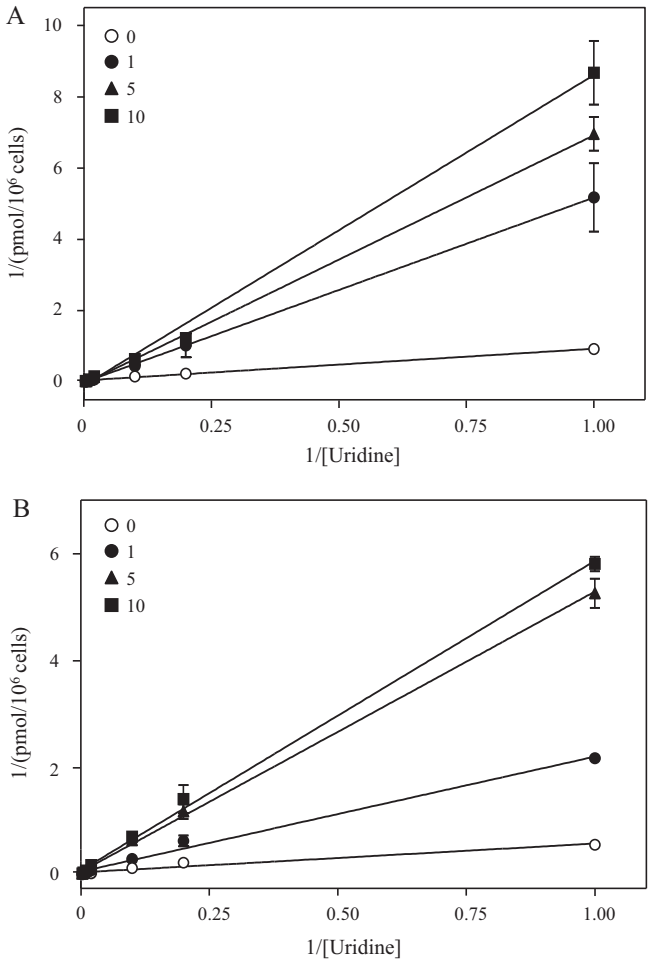

Fig. 7. Competitive inhibition of [^{3}H]uridine transport in CEM-ARAC/hCNT1 cells. Panels A and B show the effects of 0 (○), 1 (●), 5 (▲) or 10 (■) μM concentrations of **3** or **4** on rates of uptake of 1 μM [^{3}H]uridine by CEM-ARAC/hCNT1 cells. Data presented (means ± S.E.M) is from one representative experiment conducted twice with three replicates and error bars are not shown where values are smaller than the symbols.

pretreated with 1 μM NBMPR, **3** or **4** for 2 h followed by exposure to graded concentrations of gemcitabine for 72 h in the absence or presence of 1 μM NBMPR, **3** or **4**. CEM cells, which possess only hENT1 activity, were protected from gemcitabine toxicity by treatment with 1 μM NBMPR, as evidenced by an increase in the IC_{50} value. NBMPR, a high affinity inhibitor of hENT1, blocks influx of gemcitabine into cells (Table 2). Treatment with either **3** or **4** showed similar increases in IC_{50} values, indicating protection against gemcitabine toxicity in CEM-ARAC/hCNT1 cells but not in CEM cells (IC_{50} values were similar in the absence and presence of **3** or **4**), consistent with the observation that these compounds inhibited the entry and accumulation of gemcitabine into CEM/hCNT1 cells. It is important to note that short term (1 h) inhibition of accumulation of gemcitabine is not similar to long-term cytotoxicity studies since gemcitabine can diffuse into cells at higher concentrations.

4. Discussion

As understanding of the CNTs increases, their importance in absorption and disposition of nucleoside drugs is becoming more evident. Mammalian CNTs are found in highly differentiated tissues such as liver and the epithelial lining of the intestine and

Table 2
Gemcitabine cytotoxicity to CEM and CEM-ARAC/hCNT1 in absence or presence of NBMPR, **3** or **4**.

Compound	CEM	CNT1
	IC50 (nM) ± S.E.	
Gemcitabine	3 ± 0.6	27 ± 3
Gemcitabine + 1 μM NBMPR	337 ± 40	29 ± 5
Gemcitabine + 1 μM 3	2.9 ± 0.5	128 ± 16
Gemcitabine + 1 μM 4	1.8 ± 0.3	243 ± 70

Actively proliferating CEM and CEM-ARAC/hCNT1 cells in 96-well plates were exposed to 1 μM NBMPR, **3** or **4** for 2 h and then treated with graded concentrations of gemcitabine (in the presence or absence of 1 μM NBMPR, **3** or **4**) for 72 h as described in Section 2. IC_{50} values were obtained from the MTS-cytotoxicity relationships and are means (±S.E.M) of three experiments.

kidney [33], although they appear to be present at low levels in other tissues. The high abundance of CNTs in liver and renal tissues may contribute to the renal and hepatic toxicities observed when patients are treated with nucleoside analog drugs.

Clinically, the abundance of hCNTs in some cancers may have adverse effects on cancer chemotherapy. For example, in a recent study of breast cancer patients, the presence of cytoplasmic (with some nuclear) hCNT1 staining was suggested to have a negative prognostic value for disease-free survival (DFS) and risk of relapse [34]. In a study involving chronic lymphocytic leukemia patients, a significant indicator of clinical fludarabine resistance was high expression of hCNT3 mRNA [35]. Recently a role for hCNT3 in prediction of gemcitabine sensitivity was shown in patients with pancreatic cancer [36] in a study that concluded that high hENT1 and high hCNT3 abundance was associated with better response and overall survival than either hENT1 or hCNT3 abundance alone.

Inhibitors of nucleoside transporters have potential therapeutic benefit in ischemic heart disease, hyperuricaemia, stroke, hypertension, gout and in modification of antimetabolite chemotherapy [37,38]. While many high-affinity inhibitors of hENTs have been identified and characterized to date (for reviews see [1,3]), specific high-affinity inhibitors for hCNTs are not available. Recently, adenosine derivatives modified at the 5′ position, ribofuranoside compounds having benzimidazole moieties, and purine nucleoside derivatives modified at the 8 position were shown to be inhibitors of hCNT2 [38–40]. Phloretin, the aglycone of phloridzin, was shown to be an inhibitor of hCNT3 [16], and phloridzin is a partial, low-affinity inhibitor of hCNT1 [30]. A more recent study using benzopyranone derivatives and related compounds with hCNTs identified a hCNT3-specific flavone inhibitor with an IC_{50} value of 0.6 μM that was 40-fold more potent than phloridzin [17]. The most effective hCNT1 inhibitor identified was a coumarin derivative with an IC_{50} value of 4 μM, and the most effective hCNT2 inhibitor was a flavone derivative with an IC_{50} value of 2 μM [41]. The conclusions in the above studies were drawn from uridine transport inhibition experiments only. Therefore, the possibility that the compounds were transported into cells and were inhibitory by competing with uridine (i.e., competitive permeants) rather than by binding to the transporter without being transported inside (i.e., high affinity, low capacity inhibitors) was not excluded.

In the present study, we assessed seven fused-pyrimidine nucleoside analogs as potential inhibitors of hCNTs. All seven of the analogs inhibited transporter-mediated uptake of [^{3}H]uridine by recombinant hCNTs produced in yeast, indicating molecular interactions of the compounds with each of the transporters. Electrophysiology experiments in oocytes were used to directly examine the ability of hCNT1, hCNT2 and hCNT3 to transport the fused-pyrimidine nucleosides. Of those that inhibited transport by the three hCNTs, only four were identified as high affinity, low capacity inhibitors with no or negligible transportabilities, and their effects were limited to hCNT1.

In hCNT1-producing oocytes, measurable inward currents were generated with **1**, **2** and **5**, whereas **3**, **4**, **6** and **7** did not generate significant currents. The latter four analogs were further tested for their abilities to act as high affinity, low capacity inhibitors of hCNT1, and all four reduced the magnitude of uridine-induced currents when added simultaneously with uridine. Because the two thienopyrimidine nucleosides (compounds **3** and **4**) inhibited uptake of uridine to the greatest extent, the concentration dependence of their inhibition of uridine uptake was determined and compared to that of adenosine, a weakly transported inhibitor of hCNT1 [26]. For example, in radioisotope studies, adenosine is transported by hCNT1 with a similar apparent K_m to that of uridine, but with a much lower V_{max} due to slow conversion of the hCNT1-adenosine complex from outward-facing to inward-facing conformations [26,31]. Competition experiments with hCNT1 have demonstrated that the current produced by a saturating concentration of uridine is substantially higher than that produced in the same oocyte by simultaneous perfusion of both uridine and adenosine [26]. Thus, adenosine functions as a low-capacity permeant, allowing it to sometimes act as a hCNT1 inhibitor. Both **3** and **4**, which exhibited K_i values in yeast and *Xenopus* oocyte heterologous expression systems of <1 μM (one-tenth of the values measured for adenosine), and even stronger apparent affinities in human cells, are the most potent hCNT1 inhibitors yet discovered. At a high concentration (100 μM), **3** and **4** generated negligible currents in hCNT1-producing oocytes relative to those generated by uridine (≤3 vs. 60 nA, respectively), thus effectively functioning as high affinity, low capacity inhibitors hCNT1 inhibitors. However, synthesis of radiolabelled derivatives for use in radiotracer flux experiments will be required to determine the extent to which they exhibit residual transportability.

In oocytes producing hCNT2, significant currents were not observed following exposure to any of the fused-pyrimidine nucleoside analogs tested, consistent with the purine and uridine specificities of hCNT2 [10]. Of the compounds tested, pyrrolopyrimidine nucleosides **5** and **6** and imidazopyrimidine nucleoside **7** most closely resembled conventional purine nucleosides, but with the pyrrolo- and imidazo-moieties displaced to C(3) and C(4) of the pyrimidine ring instead of the normal imidazole positioning at C(2) and C(3). In contrast, hCNT3 transported all of these analogs consistent with its broad nucleoside specificity [8]. All compounds inhibited hCNT1-mediated uridine transport in yeast, with K_i values ranging from 0.12 to 0.39 μM.

The two compounds (**3** and **4**) identified as high affinity, low capacity inhibitors of hCNT1 were tested in two human leukemia cell lines, CEM (possesses hENT1 activity) and CEM-ARAC/hCNT1 (possesses hCNT1 activity) to further evaluate their effects in both transport and cytotoxicity experiments. Both analogs inhibited uridine transport and gemcitabine accumulation in CEM-ARAC/hCNT1 cells but not in CEM cells. They also protected CEM-ARAC/hCNT1, but not CEM cells, against gemcitabine toxicity consistent with the observation that **3** and **4** are high affinity, low capacity inhibitors of hCNT1. The most pharmacologically relevant of the three systems (human cells) gave the lowest IC_{50} and K_i values for inhibition of hCNT1-mediated uridine transport (<100 nM).

In summary we have identified two novel high-affinity inhibitors of hCNT1 with no observed toxicities to cultured human leukemic cells. Further studies are needed to determine toxicities against other cell types, including normal cells. However, since the two compounds were highly selective for hCNT1, they may have potential as protective agent(s) towards normal tissues expressing hCNT1 in chemotherapy with nucleoside drugs that cause toxicities to tissues with hCNT1 (such as bone marrow, kidney and gastrointestinal tissues). These analogs may also have utility in

mechanistic studies of hCNT1 function. An added bonus is that such fused-pyrimidine nucleosides are autofluorescent [42]. The finding that most of the analogs investigated in the present study were efficiently transported by hCNT3 suggests that they may enable real-time measurements of hCNT3 function in living cells by confocal microscopy.

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References

[1] King AE, Ackley MA, Cass CE, Young JD, Baldwin SA. Nucleoside transporters: from scavengers to novel therapeutic targets. Trends Pharmacol Sci 2006; 27:416–25.
[2] Molina-Arcas M, Trigueros-Motos L, Casado FJ, Pastor-Anglada M. Physiological and pharmacological roles of nucleoside transporter proteins. Nucleos Nucleot Nucleic Acids 2008;27:769–78.
[3] Zhang J, Visser F, King KM, Baldwin SA, Young JD, Cass CE. The role of nucleoside transporters in cancer chemotherapy with nucleoside drugs. Cancer Metastasis Rev 2007;26:85–110.
[4] Baldwin SA, Beal PR, Yao SY, King AE, Cass CE, Young JD. The equilibrative nucleoside transporter family, SLC29. Pflugers Arch 2004;447:735–43.
[5] Baldwin SA, Yao SY, Hyde RJ, Ng AM, Foppolo S, Barnes K, et al. Functional characterization of novel human and mouse equilibrative nucleoside transporters (hENT3 and mENT3) located in intracellular membranes. J Biol Chem 2005;280:15880–7.
[6] Griffiths M, Beaumont N, Yao SY, Sundaram M, Boumah CE, Davies A, et al. Cloning of a human nucleoside transporter implicated in the cellular uptake of adenosine and chemotherapeutic drugs. Nat Med 1997;3:89–93.
[7] Griffiths M, Yao SY, Abidi F, Phillips SE, Cass CE, Young JD, et al. Molecular cloning and characterization of a nitrobenzylthioinosine-insensitive (ei) equilibrative nucleoside transporter from human placenta. Biochem J 1997;328(Pt 3):739–43.
[8] Ritzel MW, Ng AM, Yao SY, Graham K, Loewen SK, Smith KM, et al. Molecular identification and characterization of novel human and mouse concentrative Na^+-nucleoside cotransporter proteins (hCNT3 and mCNT3) broadly selective for purine and pyrimidine nucleosides (system cib). J Biol Chem 2001;276: 2914–27.
[9] Ritzel MW, Yao SY, Huang MY, Elliott JF, Cass CE, Young JD. Molecular cloning and functional expression of cDNAs encoding a human Na^+-nucleoside cotransporter (hCNT1). Am J Physiol 1997;272:C707–14.
[10] Ritzel MW, Yao SY, Ng AM, Mackey JR, Cass CE, Young JD. Molecular cloning, functional expression and chromosomal localization of a cDNA encoding a human Na^+/nucleoside cotransporter (hCNT2) selective for purine nucleosides and uridine. Mol Membr Biol 1998;15:203–11.
[11] Huang QQ, Yao SY, Ritzel MW, Paterson AR, Cass CE, Young JD. Cloning and functional expression of a complementary DNA encoding a mammalian nucleoside transport protein. J Biol Chem 1994;269:17757–60.
[12] Mackey JR, Yao SY, Smith KM, Karpinski E, Baldwin SA, Cass CE, et al. Gemcitabine transport in xenopus oocytes expressing recombinant plasma membrane mammalian nucleoside transporters. J Natl Cancer Inst 1999;91:1876–81.
[13] Ngo LY, Patil SD, Unadkat JD. Ontogenic and longitudinal activity of Na(+)-nucleoside transporters in the human intestine. Am J Physiol Gastrointest Liver Physiol 2001;280:G475–81.
[14] Cass CE, Gaudette LA, Paterson AR. Mediated transport of nucleosides in human erythrocytes. Specific binding of the inhibitor nitrobenzylthioinosine to nucleoside transport sites in the erythrocyte membrane. Biochim Biophys Acta 1974;345:1–10.
[15] Pickard MA, Brown RR, Paul B, Paterson AR. Binding of the nucleoside transport inhibitor 4-nitrobenzylthioinosine to erythrocyte membranes. Can J Biochem 1973;51:666–72.
[16] Gupte A, Buolamwini JK. Synthesis and biological evaluation of phloridzin analogs as human concentrative nucleoside transporter 3 (hCNT3) inhibitors. Bioorg Med Chem Lett 2009;19:917–21.
[17] Wang C, Pimple S, Buolamwini JK. Interaction of benzopyranone derivatives and related compounds with human concentrative nucleoside transporters 1, 2 and 3 heterologously expressed in porcine PK15 nucleoside transporter deficient cells. Structure-activity relationships and determinants of transporter affinity and selectivity. Biochem Pharmacol 2010;79:307–20.
[18] Robins MJ, Barr PJ. Efficient conversion of 5-iodo to 6-alkynyl and derived 5-substituted uracil bases and nucleosides. J Org Chem 1983;48:1854–62.
[19] Robins MJ, Barr PJ, Giziewicz J. Nucleic acid related compounds. Smooth and high-yield iodination and chlorination at C-5 of uracil bases and *p*-toluyl-protected nucleosides. Can J Chem 1982;60:554–7.
[20] Zhang J, Smith KM, Tackaberry T, Visser F, Robins MJ, Nielsen LP, et al. Uridine binding and transportability determinants of human concentrative nucleoside transporters. Mol Pharmacol 2005;68:830–9.
[21] Zhang J, Visser F, Vickers MF, Lang T, Robins MJ, Nielsen LP, et al. Uridine binding motifs of human concentrative nucleoside transporters 1 and 3 produced in *Saccharomyces cerevisiae*. Mol Pharmacol 2003;64:1512–20.
[22] Vickers MF, Yao SY, Baldwin SA, Young JD, Cass CE. Nucleoside transporter proteins of *Saccharomyces cerevisiae*. Demonstration of a transporter (FUI1) with high uridine selectivity in plasma membranes and a transporter (FUN26) with broad nucleoside selectivity in intracellular membranes. J Biol Chem 2000;275:25931–8.
[23] Vickers MF, Kumar R, Visser F, Zhang J, Charania J, Raborn RT, et al. Comparison of the interaction of uridine, cytidine, and other pyrimidine nucleoside analogues with recombinant human equilibrative nucleoside transporter 2 (hENT2) produced in *Saccharomyces cerevisiae*. Biochem Cell Biol 2002; 80:639–44.
[24] Visser F, Vickers MF, Ng AM, Baldwin SA, Young JD, Cass CE. Mutation of residue 33 of human equilibrative nucleoside transporters 1 and 2 alters sensitivity to inhibition of transport by dilazep and dipyridamole. J Biol Chem 2002;277:395–401.
[25] Cheng Y, Prusoff WH. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 1973;22:3099–108.
[26] Smith KM, Ng AM, Yao SY, Labedz KA, Knaus EE, Wiebe LI, et al. Electrophysiological characterization of a recombinant human Na^+-coupled nucleoside transporter (hCNT1) produced in *Xenopus* oocytes. J Physiol 2004;558: 807–23.
[27] Ullman B, Coons T, Rockwell S, McCartan K. Genetic analysis of 2′,3′-dideoxycytidine incorporation into cultured human T lymphoblasts. J Biol Chem 1988;263:12391–6.
[28] Lang TT, Young JD, Cass CE. Interactions of nucleoside analogs, caffeine, and nicotine with human concentrative nucleoside transporters 1 and 2 stably produced in a transport-defective human cell line. Mol Pharmacol 2004; 65:925–33.
[29] Boleti H, Coe IR, Baldwin SA, Young JD, Cass CE. Molecular identification of the equilibrative NBMPR-sensitive (es) nucleoside transporter and demonstration of an equilibrative NBMPR-insensitive (ei) transport activity in human erythroleukemia (K562) cells. Neuropharmacology 1997;36:1167–79.
[30] Damaraju VL, Visser F, Zhang J, Mowles D, Ng AM, Young JD, et al. Role of human nucleoside transporters in the cellular uptake of two inhibitors of IMP dehydrogenase, tiazofurin and benzamide riboside. Mol Pharmacol 2005; 67:273–9.
[31] Yao SY, Ng AM, Ritzel MW, Gati WP, Cass CE, Young JD. Transport of adenosine by recombinant purine- and pyrimidine-selective sodium/nucleoside cotransporters from rat jejunum expressed in *Xenopus laevis* oocytes. Mol Pharmacol 1996;50:1529–35.
[32] Wright AM, Gati WP, Paterson AR. Enhancement of retention and cytotoxicity of 2-chlorodeoxyadenosine in cultured human leukemic lymphoblasts by nitrobenzylthioinosine, an inhibitor of equilibrative nucleoside transport. Leukemia 2000;14:52–60.
[33] Damaraju VL, Damaraju S, Young JD, Baldwin SA, Mackey J, Sawyer MB, et al. Nucleoside anticancer drugs: the role of nucleoside transporters in resistance to cancer chemotherapy. Oncogene 2003;22:7524–36.
[34] Gloeckner-Hofmann K, Guillen-Gomez E, Schmidtgen C, Porstmann R, Ziegler R, Stoss O, et al. Expression of the high-affinity fluoropyrimidine-preferring nucleoside transporter hCNT1 correlates with decreased disease-free survival in breast cancer. Oncology 2006;70:238–44.
[35] Mackey JR, Galmarini CM, Graham KA, Joy AA, Delmer A, Dabbagh L, et al. Quantitative analysis of nucleoside transporter and metabolism gene expression in chronic lymphocytic leukemia (CLL): identification of fludarabine-sensitive and -insensitive populations. Blood 2005;105:767–74.
[36] Marechal R, Mackey JR, Lai R, Demetter P, Peeters M, Polus M, et al. Human equilibrative nucleoside transporter 1 and human concentrative nucleoside transporter 3 predict survival after adjuvant gemcitabine therapy in resected pancreatic adenocarcinoma. Clin Cancer Res 2009;15:2913–9.
[37] Buolamwini JK. Nucleoside transporter inhibitors: structure-activity relationships and potential therapeutic applications. Curr Med Chem 1997;4:35–66.
[38] Kikuchi N, Nonaka Y, Tatani K, Hiratochi M, Kuramochi Y, Isaji M, et al. Preparation of ribofuranoside compounds having benzimidazole moiety as CNT2 inhibitors. PCT Int Appl 2005;141.
[39] Nonaka Y, Tatani K, Hiratochi M, Kuramochi Y, Isaji M. Preparation of 5′-modified nucleoside derivatives as CNT-2-inhibitors. Patent number WO 2004101593; 2004, USA.
[40] Tatani KNY, Kikuchi N. Preparation of purine nucleoside derivatives modified in 8-position as inhibitors of concentrative nucleoside transporter 2. Patent number WO2006030803; 2006.
[41] Wang C, Pimple S, Buolamwini JK. Interaction of benzopyranone derivatives and related compounds with human concentrative nucleoside transporters 1, 2 and 3 heterologously expressed in porcine PK15 nucleoside transporter deficient cells. Structure-activity relationships and determinants of transporter affinity and selectivity. Biochem Pharmacol 2010; 79(3):307–20.
[42] Zhang J, Sun X, Smith KM, Visser F, Carpenter P, Barron G, et al. Studies of nucleoside transporters using novel autofluorescent nucleoside probes. Biochemistry 2006;45:1087–98.