AUTOFLUORESCENT FUSED-PYRIMIDINE NUCLEOSIDES: SYNTHESIS AND EVALUATION AS PERMEANTS AND INHIBITORS OF HUMAN NUCLEOSIDE TRANSPORTERS

Ireneusz NOWAK[a1], Vijaya L. DAMARAJU[b1], Carol E. CASS[b2], James D. YOUNG[c] and Morris J. ROBINS[a2,*]

[a] *Department of Chemistry and Biochemistry, Brigham Young University, Provo 84602-5700, Utah, U.S.A.; e-mail: [1] i_nowak@hotmail.com, [2] morris_robins@byu.edu*

[b] *Department of Oncology, University of Alberta, Edmonton, Alberta, Canada T6G 1Z2; e-mail: [1] vijaya.damaraju@albertahealthservices.ca, [2] carol.cass@albertahealthservices.ca*

[c] *Department of Physiology, University of Alberta, Edmonton, Alberta, Canada T6G 2H7; e-mail: james.young@ualberta.ca*

Received March 9, 2011
Accepted August 23, 2011
Published online November 30, 2011

Dedicated to Professor Antonín Holý on the occasion of his 75th birthday in appreciation of a long and rewarding friendship and in recognition of his outstanding contributions to nucleic acid chemistry, biochemistry, and medicinal applications.

Nucleosides with an aromatic five-membered ring heterocycle (N, O, or S) fused at C4–C5 of pyrimidin-2-one were prepared by ring closures with 5-(alkyn-1-yl)pyrimidin-2-one intermediates, heterocyclic atom replacements, and ring closure with a 5-aminocytidine derivative. Ultraviolet absorption and emission properties of the autofluorescent products enabled studies on permeation and inhibition of the trans-cellular trafficking effected by human equilibrative nucleoside transporters (hENTs). Some of the autofluorescent nucleosides were shown to be potent and selective inhibitors of human concentrative nucleoside transporters (hCNTs) in a companion study reported elsewhere.

Keywords: Alkynylpyrimidine cyclizations; Autofluoresent nucleoside analogues; Nucleoside transport studies; Ring-fused pyrimidines; Sonogashira coupling.

Pyrimidine nucleoside analogues and derivatives have been applied extensively in biochemistry, pharmacology, and medicine. We reported Sonogashira coupling of terminal alkynes with 5-iodouracil compounds in 1981 and demonstrated that copper(I)-catalyzed cyclization of the 5-(alkyn-1-yl) products gave fluorescent furo[2,3-*d*]pyrimidin-2-one analogues[1]. Considerable interest has been focused on such nucleosides with furan, pyrrole, and thiophene rings fused at C4–C5 of the pyrimidine ring

doi:10.1135/cccc2011061

(Fig. 1). Bicyclic hybrids of this type have been found to target bacterial enzymes, and oligonucleotides containing pyrrolopyrimidine nucleobase analogues in a CpG motif were recognized by toll-like receptor 9 (TLR9) and stimulated the immune system[2]. The autofluorescence properties we noted[1] have been employed in probes for analysis of DNA and RNA hybridization[3] and for detection of single nucleotide polymorphisms[4]. Potent and selective antiviral activities were identified by extensive structure activity relationship studies with longer-chain bicyclic derivatives[5], and the selectivities and potencies were found to depend on the chain length and nature of alkyl/aryl substituents at C6 of the fused five-membered rings[5]. Because such appendages affect hydrophobic properties that can increase interactions with cellular membrane components and/or simple diffusion, we chose the parent bicyclic ring analogues (and those with a minimal 6-methyl substituent) for studies with human nucleoside transporters. We recently reported real-time analysis of nucleoside transport into living cells with confocal microscopy using furo[2,3-*d*]pyrimidin-2-one 2′-deoxy- and ribonucleoside fluorophores[6], and other applications of fluorescent probes for measurement of uptake of nucleoside drugs into cancer cells[7,8].

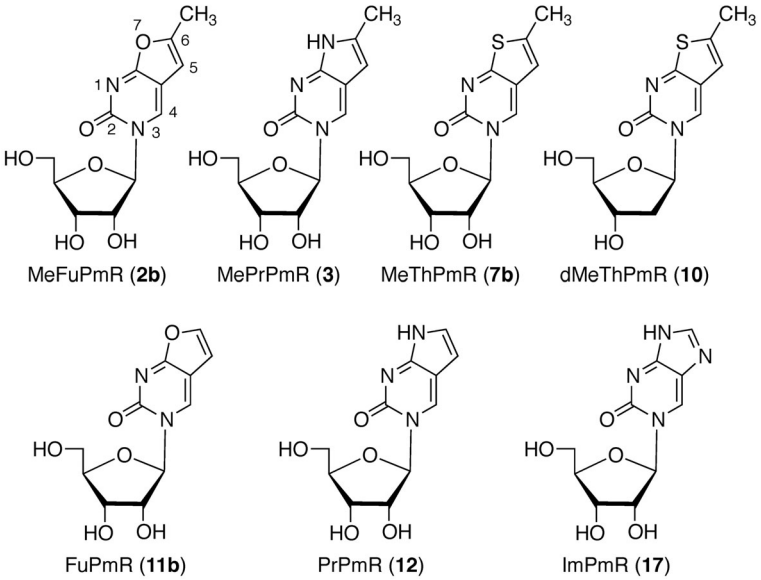


FIG. 1
Structures, abbreviations, ring numbering, and compound numbers of fused-pyrimidine nucleosides

The role of human equilibrative and concentrative nucleoside transporters (hENTs and hCNTs) as biomarkers for prediction of response to nucleoside drugs is gaining increased recognition. The presence of hENT1 in membranes has been shown to be of major importance for entry of nucleoside analogue drugs into cells and is a useful predictor of drug activities[9]. Studies on patients with pancreatic cancer have demonstrated positive correlations between clinical responses to gemcitabine (2′-deoxy-2′,2′-difluorocytidine) and abundances of hENT1 protein in the cancer tissues[10]. Immunohistochemistry (IHC) has been used to estimate hENT1-mediated transport of gemcitabine into cancer cells, and while that methodology is applicable to paraffin-embedded or frozen tissue samples, it is time consuming and less useful for routine monitoring of nucleoside transport capacity of freshly isolated cells. The abundance of hENT1 on cell membranes can be enumerated by equilibrium binding with tritium-labeled 6-*S*-(4-nitrobenzyl)thioinosine ([^{3}H]NBMPR) but procedures that involve measurement of radioactivity are less attractive. High-affinity autofluorescent analogues that identify cell-surface abundances of hENT1 enable rapid quantification with some clinical samples and provide initial predictions of potential treatment outcomes with nucleoside drugs[9c]. We recently described a new approach for real-time measurement of nucleoside trafficking[6] with a novel fluorescent cell-surface hENT1 probe[11]. Autofluorescent permeants and inhibitors of other nucleoside transporters will provide tools for new investigations in transporter research. We now report preparation of the autofluorescent analogues shown in Fig. 1 and further evaluation of their activities as permeants and/or inhibitors of human nucleoside transporters. Five were shown to be permeants for hENT1 in the present study. In a related study using a *Xenopus* oocyte expression system, all the compounds inhibited hCNT1-mediated uptake of uridine, two were shown to be potent inhibitors of hCNT1 with negligible permeant activity, and some were shown to be permeants for hCNT3 [12]. (Note that the bicyclic rings were numbered incorrectly in ref.[12], in which N3 was erroneously numbered N1 as in the pyrimidine nucleoside precursors.)

RESULTS AND DISCUSSION

Chemistry

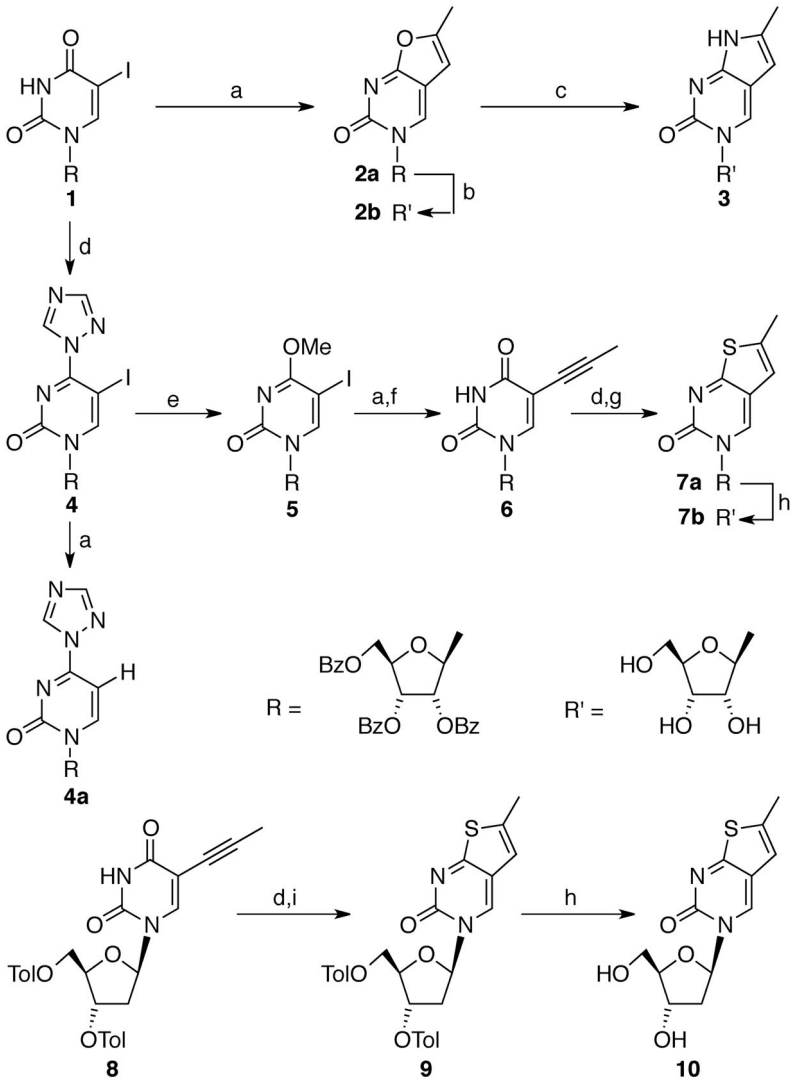
Heating a mixture of 2′,3′,5′-tri-*O*-benzoyl-5-iodouridine[13] (**1**; Scheme 1), propyne, triethylamine, tetrakis(triphenylphosphine)palladium, and copper(I) iodide in dichloromethane (50 °C, sealed flask) for 3.5 h resulted

in copious precipitation of furopyrimidine derivative **2a** (the usual[1] 5-(propyn-1-yl) Sonogashira coupling product was not observed). The highly fluorescent **2a** also was produced by similar treatment of **1** for 75 min, and no intermediate was detected by TLC. Apparently, cuprous-ion catalyzed cyclization of the 5-alkynyl intermediate occurred rapidly under these reaction conditions. We previously observed[14] that the rates of such cyclizations were much slower in DMF/Et_3N mixtures. The limited solubility of **2a** in CH_2Cl_2/Et_3N might also shift the equilibrium from a cuprous-alkyne complex toward **2a**.

The fused furan ring in **2a** is sensitive to opening with bases. Deprotective cleavage of *O*-acetyl groups had been effected by brief exposure to cold methanolic ammonia[6,15], but removal of the more stable *O*-benzoyl groups from **2a** was incomplete under conditions that resulted in attack on the ring system. As expected[16], heating **2a** in NH_3/MeOH produced 6-methyl-3-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(3*H*,7*H*)-one (**3**). Our weakly basic procedure with lithium trifluoroethoxide in trifluoroethanol[17] was the only one of several attempted deprotection methods that gave 6-methyl-3-(β-D-ribofuranosyl)furo[2,3-*d*]pyrimidin-2(3*H*)-one (**2b**) without significant byproducts.

The thiophene analogue **7b** was then targeted. Subjection of **1** to the Divakar–Reese procedure[18] gave the 4-(triazol-1-yl) intermediate **4**. However, attempted Sonogashira coupling of **4** and propyne resulted in reductive deiodination with nearly quantitative conversion to 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)pyrimidin-2(1*H*)-one (**4a**). The π-deficient triazole ring was displaced from **4** using methanol superheated at 110 °C, which gave **5** with an electron-donating 4-methoxyl group. Intermediate **5** was a good substrate for Sonogashira coupling with propyne, and the product was subjected to acidic hydrolysis to give the 5-(propyn-1-yl)uracil derivative **6**. Conversion of **6** into its 4-triazolyl derivative followed by treatment with thioacetic acid[19] gave the benzoyl-protected thiophene compound **7a**, which was much more stable under basic conditions than its furan analogue **2a**. The benzoyl esters were cleaved with dilute methanolic potassium hydroxide to give 6-methyl-3-(β-D-ribofuranosyl)thieno[2,3-*d*]pyrimidin-2(3*H*)-one (**7b**). A similar sequence was used for preparation of the 2′-deoxy analogue **10**. The propynyl derivative **8** [20] underwent smooth conversion into its 5-(propyn-1-yl)-4-(1,2,4-triazol-1-yl) intermediate, which was treated directly with potassium thioacetate in DMF. Nucleophilic displacement of the triazolyl group by sulfur, loss of the acetyl group, and ring closure occurred *in situ*. Direct treatment of the resulting thiophene derivative **9** with methanolic potassium hydroxide

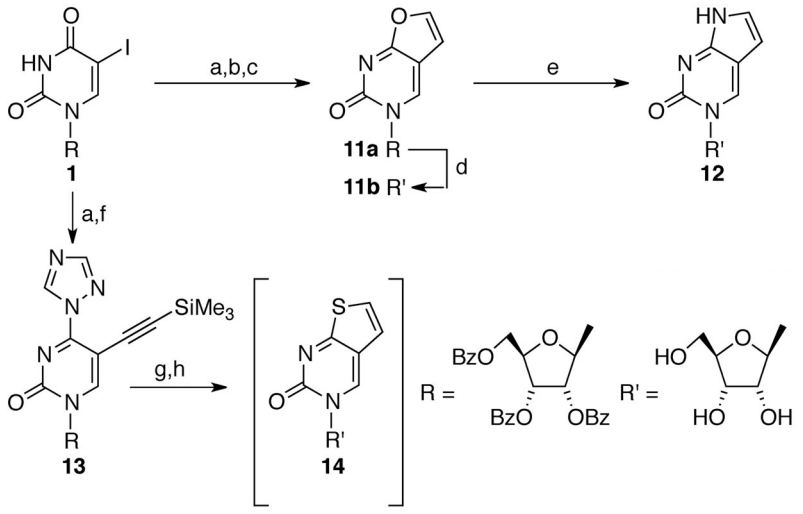
cleaved the 4-methylbenzoyl esters to give 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)-6-methylthieno[2,3-*d*]pyrimidin-2(3*H*)-one (**10**).



SCHEME 1

Reagents and conditions: (a) $(Ph_3P)_4Pd/CuI/Et_3N/propyne/CH_2Cl_2/50$ °C; (b) $CF_3CH_2OLi/TFE/80$ °C; (c) $NH_3/MeOH/80$ °C; (d) $POCl_3/1,2,4$-triazole/Et_3N/CH_3CN; (e) MeOH/110 °C; (f) TFA/H_2O; (g) $AcSH/CH_3CN$; (h) (i) KOH/MeOH, (ii) AcOH; (i) AcSK/DMF

Analogues without a 6-methyl substituent were significantly less stable. Sonogashira coupling of trimethylsilylacetylene and **1** followed by fluoride-promoted removal of the TMS group and copper(I) iodide catalyzed cyclization produced the furopyrimidine derivative **11a** (Scheme 2). Mild solvolysis of **11a** with trifluoroethanol[17] gave 3-(β-D-ribofuranosyl)furo-[2,3-*d*]pyrimidin-2(3*H*)-one (**11b**), and methanolic ammonia at 80 °C converted **11a** into 3-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(3*H*,7*H*)-one (**12**). The unsubstituted thieno analogue **14** was not isolated in pure form. The Sonogashira coupling intermediate from **1** was converted into the 4-(1,2,4-triazol-1-yl)-5-TMSethynyl derivative **13** and subjected to tandem *in situ* substitution of triazole with thioacetate, fluoride-promoted desilylation, and cyclization. That sequence was the most successful of several attempts, but gave a mixture containing two major products (TLC). Partial separation followed by mild solvolysis gave **14** as the presumed major (unstable) product with ^{1}H NMR signals similar to those of the other bicyclic analogues. The geminal H5′/H5″ signals in spectra of **2b**, **3**, **7b**, **11b**, **12**, and **14** are separated by Δδ 0.16–0.19 ppm, whereas those signals in spectra of their 5-(alkyn-1-yl)uracil nucleoside precursors have narrow separations.

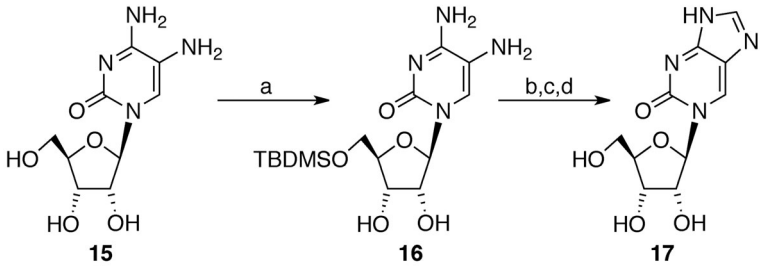


SCHEME 2

Reagents and conditions: (a) $(Ph_3P)_4Pd/CuI/Et_3N$/TMS-acetylene/CH_2Cl_2/50 °C; (b) NH_4F/THF/MeOH/H_2O/50 °C; (c) CuI/Et_3N/DMF/80 °C; (d) CF_3CH_2OLi/TFE/90 °C; (e) NH_3/MeOH/80 °C; (f) $POCl_3$/1,2,4-triazole/Et_3N/CH_3CN; (g) $AcSK/NH_4F/H_2O$/pyridine; (h) Dowex 1 X2 ($^-OCH_2CF_3$)/TFE/90 °C

Vicinal proton signals for the –CH=CH–X system in spectra of [**14**] (X = S), $^3J_{HH}$ = 5.9 Hz (0.26 ppm signal separation), **12** (X = N), $^3J_{HH}$ = 3.9 Hz (0.75 ppm signal separation), and **11b** (X = O), $^3J_{HH}$ = 2.9 Hz (0.95 ppm signal separation), show effects of the relative S, N, and O electronegativities on values of the coupling constants (decreasing) and signal separations (increasing).

The imidazo-pyrimidine analogue **17** [21] was prepared by a slight modification of a published procedure (Scheme 3). Heating 5-aminocytidine[22] (**15**) in diethoxymethyl acetate was reported[21a] to give **17**. However, the solubility of **15** is very limited in diethoxymethyl acetate, and mixtures were obtained. Protection of **15** with *tert*-butyldimethylsilyl chloride in pyridine gave the TBDMS ether **16**. Heating 16 in diethoxymethyl acetate at 145 °C and successive trifluoroacetic acid-catalyzed desilylation, neutralization, and chromatography on silica gel and then on Dowex 1 X2 (OH^-) resin gave 3-(β-D-ribofuranosyl)imidazo[4,5-*d*]pyrimidin-2(3*H*,5/7*H*)-one (**17**).



SCHEME 3
Reagents and conditions: (a) TBDMSCl/pyridine; (b) $(EtO)_2CHOAc$/145 °C; (c) TFA/H_2O/0 °C; (d) NH_3/MeOH

Nucleoside Transport

Concentration-dependent inhibition of transporter-mediated uptake of [^{3}H]uridine by the compounds in Fig. 1 was examined using recombinant hENTs produced in yeast as described in Experimental. Values for 50% inhibitory concentrations (IC_{50}) with the seven analogues for hENT1 and hENT2 are listed in Table I along with previously reported[12] IC_{50} values for hCNT1, hCNT2, and hCNT3 (which are included for completeness). All of the compounds were potent inhibitors of hCNT1 with IC_{50} values less than 1 μM, whereas only one had an IC_{50} value less than 1 μM for hCNT3 and none were effective inhibitors of hCNT2 or of the equilibrative transporters hENT1 and hENT2 [12].

Live-cell imaging of uptake of the analogues into BeWo and CEM cells was evaluated using confocal microscopy at an excitation wavelength of 351 nm. The extracellular medium emitted green fluorescence instantly upon addition of an analogue to the well, and the fluorescence intensity of the medium changed but slightly during the observation time. Figure 2a illustrates that cells had increased fluorescence intensities after exposure to the analogues. Significant intracellular fluorescence enhancement was observed within 1 min, and the fluorescence intensities continued to increase over a period of 10 min (data not shown). Data presented are fluorescence intensities after subtracting values in the presence of NBMPR for each of the analogues. Longer time-courses exhibited two-phase accumulation of intracellular fluorescence with rapid initial rates of uptake during the first phase (data not shown). The almost complete inhibition of uptake of the analogues by NBMPR (10 μM) indicated that transport was mediated primarily by hENT1. Similar results with CEM/hENT1 cells were observed with compounds **10** (Fig. 2b) and **7b** (Fig. 2c).

Because **7b** and **10** were shown[12] to be inhibitors of hCNT1 with negligible transport activity, we analyzed uptake of **7b** (50 μM) using confocal microscopy with a panel of CEM cell lines (which exhibited some autofluorescence). Uptake was measured with CEM/hENT1 cells (in the absence or presence of NBMPR), with nucleoside-transport deficient CEM/ARAC cells, and with CEM/hCNT1 cells (Fig. 3a–3d). CEM/hENT1 cells exhibited uptake of **7b** (Fig. 3a) as shown by the enhanced green fluorescence inside the cells, and NBMPR blocked that uptake (Fig. 3b). CEM/ARAC cells (Fig. 3c) and CEM/hCNT1 cells (Fig. 3d) showed no uptake, which demonstrated that hENT1 was essential for the transport of **7b** and that **7b** did not enter cells via hCNT1.

In summary, our current and recently reported[12] studies with yeast producing recombinant hNTs revealed that all seven compounds in Fig. 1 were high-affinity inhibitors of hCNT1, moderate-affinity inhibitors of hCNT3, and low-affinity inhibitors of hENT1, hENT2, and hCNT2. Laser scanning confocal microscopy allowed direct visualization of autofluorescence of the analogues in living BeWo and CEM/hENT1 cells (including blockage of uptake by the potent hENT1 inhibitor NBMPR). The results indicated that transport of **7b** was mediated predominantly by hENT1 (Fig. 3a and 3b).

Live-cell imaging was undertaken with a panel of CEM cell lines that are deficient in nucleoside transporter activity or that have either hENT1 or hCNT1 as the sole activity. The absence of uptake of the autofluorescent nucleoside analogues in transport-deficient cells compared with their transportability in hENT1-containing cells suggests that they could be used for

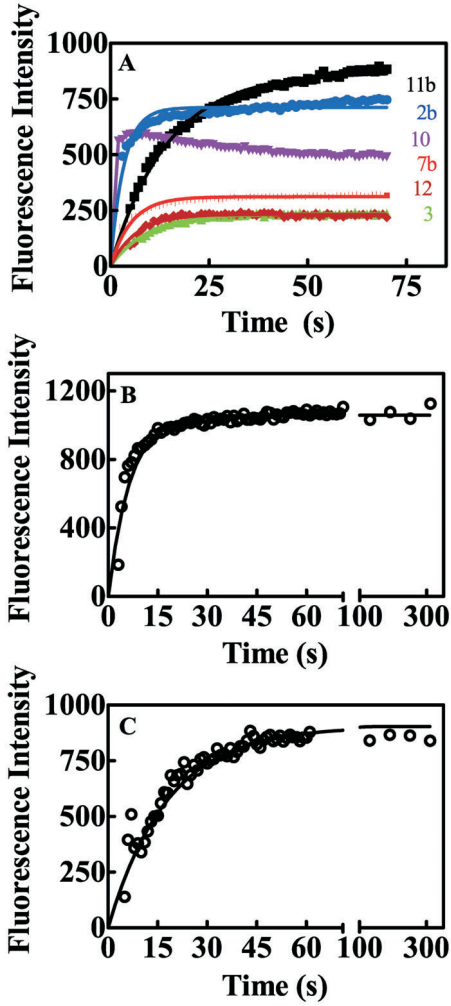


FIG. 2

Time course of uptake of autofluorescent analogues by cultured cells: Compounds **11b**, **2b**, **10**, **7b**, **12**, and **3** were added individually to BeWo cells and representative time courses of uptake of the autofluorescent analogues are shown (A). The whole-cell fluorescence intensities of the analogues and the extracellular fluorescence intensities at different time points were measured with six to eight cells and one extracellular region (similar in size to a cell). The extracellular fluorescence values were subtracted, and the resulting mean values ± SE after correcting for values in the presence of NBMPR were plotted against time (compound numbers are shown beside the curves). Analogous experiments were performed with **10** (B) and **7b** (C) in CEM/hENT1 cells. Time-dependent increases in fluorescence were observed with both compounds

TABLE I
Effects of autofluorescent nucleoside analogues on the uptake of uridine into *S. cerevisiae* producing recombinant hENT1, hENT2, hCNT1, hCNT2, or hCNT3

Compound	hENT1[a]	hENT2[a]	hCNT1[b]	hCNT2[b]	hCNT3[b]
	IC_{50} ± SE, μM				
FuPmR (**11b**)	161 ± 42	1757 ± 280	0.12 ± 0.01	393 ± 42	5.3 ± 1.2
MeFuPmR (**2b**)	424 ± 31	714 ± 18	0.37 ± 0.08	1419 ± 246	25.0 ± 1.2
dMeThPmR (**10**)	33 ± 2	1036 ± 53	0.33 ± 0.08	133 ± 11	52.0 ± 7.6
MeThPmR (**7b**)	79 ± 12	526 ± 25	0.18 ± 0.03	360 ± 24	7.5 ± 0.1
PrPmR (**12**)	101 ± 14	>3000	0.39 ± 0.03	239 ± 29	1.2 ± 0.6
MePrPmR (**3**)	313 ± 24	1235 ± 42	0.26 ± 0.05	175 ± 43	0.7 ± 0.2
ImPmR (**17**)	1450 ± 270	201 ± 21	0.19 ± 0.07	270 ± 22	1.3 ± 0.1

[a] IC_{50} values (± SE) for inhibition of initial rates of uptake (i.e., transport) of [^{3}H]uridine (1 μM) were determined by computer-generated concentration-effect curves. Each experiment was conducted with six replicates. [b] We recently reported these data[12] for hCNT1, hCNT2, and hCNT3 (obtained by methods similar to those presently described for the hENTs).

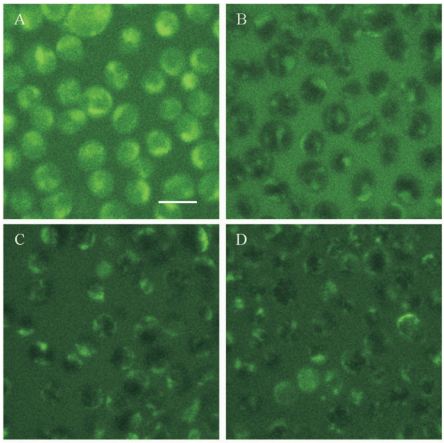


FIG. 3
Live-cell imaging to measure uptake of **7b** by cultured cells with different transport capabilities: Uptake of compound **7b** into CEM/hENT1 cells occurred in the absence of NBMPR (a). Uptake of **7b** into CEM/hENT1 cells was blocked in the presence of NBMPR (10 μM) (b). Uptake of **7b** into CEM/ARAC (c) and CEM/hCNT1 (d) cells was not observed. Images were taken 300 s after the addition of **7b** (500 μM) to cells as described in Experimental

rapid assessment of hENT1 abundance in cell samples. The lack of uptake in hCNT1-viable cells showed that **7b** was not transported by hCNT1.

Our results clearly demonstrate that selected fused-pyrimidine nucleosides have a number of potential applications. The autofluorescence of such analogues allows measurement of uptake in live cells as shown earlier[6], and presently with six of the seven analogues. Compounds **7b** and **10** also are inhibitors of hCNT1 as well as permeants of hCNT3 [12]. Therefore, they have potential for analysis of nucleoside uptake by hCNT3.

EXPERIMENTAL

Chemistry

All chemicals and solvents were of reagent grade and were used as received from suppliers unless specified. Solutions in $CDCl_3$ were used for ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra unless noted. Chemical shifts are given in ppm (δ-scale), coupling constants (*J*) in Hz. ^{13}C NMR peaks with the same chemical shift for more than one carbon are specified, and a shift range (ovlp) is given for overlapping signals from multiple carbon atoms. Solutions in methanol were used for ultraviolet (UV) absorption and emission spectra. Purities (>95%) of final products (except **14**) conformed to our reported criteria[23] for evaluation of impurities in nucleoside samples.

3-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-6-methylfuro[2,3-*d*]pyrimidin-2(3*H*)-one (**2a**)

A 30-ml pressure flask equipped with a Teflon valve was charged with 2′,3′,5′-tri-*O*-benzoyl-5-iodouridine[13] (**1**; 2.80 g, 4.11 mmol) and CH_2Cl_2 (10 ml) and Et_3N (5 ml) were added. N_2 was bubbled through the solution for 15 min, the solution was cooled to –20 °C, and propyne gas (~2 l balloon) was bubbled through. $(Ph_3P)_4Pd$ (160 mg, 0.14 mmol) and CuI (60 mg, 0.32 mmol) were added and the mixture was sealed and stirred at 50 °C for 1.5 h during which time copious precipitation of crystalline material occurred. The mixture was filtered and the filter cake was washed with CH_2Cl_2 to dissolve the precipitated product. Volatiles were evaporated from the filtrate and the residue was chromatographed (CH_2Cl_2). Crystallization of the purified residue (CH_2Cl_2/MeOH) gave **2a** (2.17 g, 89%). ^1H NMR: 2.31 (s, 3 H), 4.69 (dd, *J* = 2.9, 12.2, 1 H), 4.81–4.84 (m, 1 H), 4.95 (dd, *J* = 2.4, 12.2, 1 H), 5.69 (s, 1 H), 5.83 (t, *J* = 4.9, 1 H), 5.92 (t, *J* = 5.9, 1 H), 6.61 (d, *J* = 4.4, 1 H), 7.35–7.66 (m, 9 H), 7.92–8.13 (m, 6 H), 8.06 (s, 1 H). ^{13}C NMR: 14.1, 63.3, 70.5, 75.1, 80.5, 90.0, 99.3, 108.8, 128.4–129.9 (ovlp), 133.6, 133.65, 133.69, 133.8, 154.5, 156.6, 165.1, 165.3, 166.0, 172.3. FAB-MS *m/z*: 617 ([M + Na^+] 10%), 445 (100%). HRMS ($C_{33}H_{26}N_2NaO_9$): calculated 617.1531, found 617.1527.

6-Methyl-3-(β-D-ribofuranosyl)furo[2,3-*d*]pyrimidin-2(3*H*)-one (**2b**)

A 250-ml pressure flask equipped with a Teflon valve was charged with **2a** (4.0 g, 6.73 mmol) and a solution of $LiOCH_2CF_3/CF_3CH_2OH$ prepared by addition of BuLi (1.6 M, 8.0 ml, 12.8 mmol) to TFE (40 ml). The solution was stirred at 80 °C for 19 h, neutralized with AcOH, and concentrated. Chromatography (EtOAc → EtOAc/MeOH, 3:1) gave purified

2b (1.82 g, 96%). A sample for analysis was obtained by PTLC [EtOAc/MeOH (4:1) followed by CH_2Cl_2/MeOH (3:1)]. ^{1}H NMR (CD_3OD): 2.37 (q, *J* = 1.5, 3 H), 3.84, 4.01 (2 × dd, *J* = 2.4, 12.2, 2 × 1 H), 4.11–4.20 (m, 3 H), 5.96 (d, *J* = 1.5, 1 H), 6.36 (d, *J* = 1.5, 1 H), 8.77 (s, 1 H). ^{13}C NMR (CD_3OD): 13.9, 61.1, 69.7, 77.0, 85.9, 94.1, 101.3, 109.9, 138.5, 157.2, 157.7, 173.3. FAB-MS *m/z*: 283 ([M + H$^+$] 100%). HRMS ($C_{12}H_{15}N_2O_6$): calculated 283.0925, found 283.0924.

6-Methyl-3-(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(3*H*,7*H*)-one (**3**)

A solution of **2b** (300 mg, 1.06 mmol) in NH_3/MeOH (~14%, 15 ml) was heated in a sealed flask at 80 °C for 24 h. Volatiles were evaporated and the residue was chromatographed (EtOAc/MeOH, 3:1) to give **3** (280 mg, 94%). A sample for analysis was obtained by PTLC [EtOAc/MeOH (3:1) followed by CH_2Cl_2/MeOH (3:1)]. ^{1}H NMR (CD_3OD): 2.29 (s, 3 H), 3.83, 4.00 (2 × dd, *J* = 2.4, 12.2, 2 × 1 H), 4.10–4.20 (m, 3 H), 5.98 (d, *J* = 0.9, 1 H), 6.02 (d, *J* = 2.4, 1 H), 8.77 (s, 1 H). ^{13}C NMR (CD_3OD): 13.7, 61.5, 70.0, 77.1, 85.9, 93.9, 99.0, 122.3, 136.4, 140.6, 157.3, 160.3. FAB-MS *m/z*: 282 ([M + H$^+$] 50%), 301 (100%). HRMS ($C_{12}H_{16}N_3O_5$): calculated 282.1085, found 282.1091.

1-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-5-iodo-4-methoxypyrimidin-2(1*H*)-one (**5**)

Et_3N (2.0 ml, 1.45 g, 14.4 mmol) was added dropwise to a stirred, cooled (~0 °C) mixture of 1,2,4-triazole (1.01 g, 14.7 mmol), $POCl_3$ (0.31 ml, 510 mg, 3.32 mmol), and MeCN (9 ml). A solution of **1** (1.00 g, 1.47 mmol) in CH_2Cl_2 (10 ml) was added and stirring was continued at ambient temperature for 30 min. Volatiles were evaporated and the residue was chromatographed (EtOAc → EtOAc/MeOH, 10:1) to give 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-iodo-4-(1,2,4-triazol-1-yl)pyrimidin-2(1*H*)-one (**4**; 820 mg, 76%). Sonogashira treatment of **4** with propyne resulted in deiodination to give 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-4-(1,2,4-triazol-1-yl)pyrimidin-2(1*H*)-one (**4a**; 89%). ^{1}H NMR: 4.73–4.92 (m, 3 H), 5.84 (t, *J* = 5.1, 1 H), 5.92 (t, *J* = 5.4, 1 H), 6.50 (d, *J* = 4.9, 1 H), 6.97 (d, *J* = 7.3, 1 H), 7.37–7.69 (m, 9 H), 7.98 (d, *J* = 7.3, 2 H), 8.10, 8.19 (2 × d, *J* = 7.3, 2 × 1 H), 8.12, 9.25 (2 × s, 2 × 1 H). ^{13}C NMR: 63.4, 70.8, 74.7, 80.8, 90.2, 95.2, 128.2–129.7 (ovlp), 131.75, 131.81, 131.9, 133.5–133.6 (ovlp), 143.2, 146.5, 153.86, 153.95, 159.4, 165.0, 165.1, 165.8.

A solution of **4** (1.6 g, 2.18 mmol) in MeOH (15 ml) was heated in a pressure flask at 110 °C for 4 h. Volatiles were evaporated and the residue was chromatographed (EtOAc/hexanes, 1:2 → 1:1) to give 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-iodo-4-methoxypyrimidin-2(1*H*)-one (**5**; 1.23 g, 81%). ^{1}H NMR: 4.00 (s, 3 H), 4.71–4.88 (m, 3 H), 5.72 (t, *J* = 5.9, 1 H), 5.91 (dd, *J* = 3.9, 5.9, 1 H), 6.52 (t, *J* = 3.9, 1 H), 7.35–7.63 (m, 9 H), 7.96–7.98 (m, 4 H), 7.99 (s, 1 H), 4.12–8.14 (m, 2 H). ^{13}C NMR: 56.0, 57.6, 63.8, 71.3, 74.7, 81.0, 88.4, 128.3–129.9 (ovlp), 133.5–133.6 (ovlp), 147.5, 154.4, 165.2, 165.3, 166.0, 168.5. FAB-MS *m/z*: 719 ([M + Na$^+$] 20%), 445 (100%). HRMS ($C_{31}H_{24}IN_2NaO_9$): calculated 719.0497, found 719.0501.

2′,3′,5′-Tri-*O*-benzoyl-5-(propyn-1-yl)uridine (**6**)

Sonogashira treatment of **5** (500 mg, 0.72 mmol) with propyne as described for **1** → **2a**, evaporation of volatiles, and chromatography of the residue (EtOAc/hexanes, 1:2 → 1:1) gave 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-4-methoxy-5-(propyn-1-yl)pyrimidin-2(1*H*)-one (400 mg, 92%). This material was treated directly with TFA/H_2O (2:1, 3 ml), the mixture was stirred at ambient temperature for 4 h, and volatiles were evaporated. The residue was

chromatographed (EtOAc/hexanes, 1:2 → 1:1) to give **6** (350 mg, 90%). ^{1}H NMR: 1.85 (s, 3 H), 4.70–4.81 (m, 3 H), 5.75 (t, *J* = 6.1, 1 H), 5.88 (dd, *J* = 3.9, 5.9, 1 H), 6.37 (d, *J* = 5.9, 1 H), 7.35–7.64 (m, 9 H), 7.65 (s, 1 H), 7.92–8.14 (m, 6 H), 8.26 (br s, 1 H). ^{13}C NMR: 4.4, 63.9, 69.6, 71.2, 73.8, 80.6, 87.6, 91.1, 102.0, 128.1–129.8 (ovlp), 133.3, 133.6, 133.7, 141.0, 149.2, 161.6, 165.1, 165.2, 166.0. FAB-MS *m/z*: 595 ([M + H$^+$] 10%), 445 (100%). HRMS ($C_{33}H_{27}N_2O_9$): calculated 595.1711, found 595.1710.

3-(2,3,5-Tri-*O*-benzoyl-β-D-ribofuranosyl)-6-methylthieno[2,3-*d*]pyrimidin-2(3*H*)-one (**7a**)

Et_3N (3.0 ml, 2.18 g, 21.6 mmol) was added dropwise to a stirred, cooled (~0 °C) mixture of 1,2,4-triazole (1.62 g, 23.48 mmol), $POCl_3$ (0.50 ml, 820 mg, 5.36 mmol) and MeCN (14 ml). A solution of **6** (1.40 g, 2.35 mmol) in MeCN (14 ml) was added to this mixture, and stirring was continued at ambient temperature for 2 h. Volatiles were evaporated and the residue was chromatographed (EtOAc/hexanes, 1:1 → EtOAc) to give 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-(propyn-1-yl)-4-(1,2,4-triazol-1-yl)pyrimidin-2(3*H*)-one (1.48 g, 97%). A solution of this material (1.4 g, 2.16 mmol) and thioacetic acid (1.0 ml, 1.07 g, 14.0 mmol) was stirred at ambient temperature for 3 h. Volatiles were evaporated and the residue was chromatographed (CH_2Cl_2 → EtOAc) to give **7a** (1.1 g, 83%). ^{1}H NMR: 2.35 (s, 3 H), 4.68 (dd, *J* = 3.4, 12.7, 1 H), 4.83–4.86 (m, 1 H), 5.00 (dd, *J* = 2.9, 12.7, 1 H), 5.86 (dd, *J* = 4.4, 5.4, 1 H), 5.91 (d, *J* = 1.5, 1 H), 5.93 (t, *J* = 5.9, 1 H), 6.60 (d, *J* = 4.4, 1 H), 7.35–7.66 (m, 9 H), 7.91–8.14 (m, 6 H), 8.19 (s, 1 H). FAB-MS *m/z*: 633 ([M + Na$^+$] 10%), 445 (100%). HRMS ($C_{33}H_{26}N_2NaO_8S$): calculated 633.1302, found 633.1291.

6-Methyl-3-(β-D-ribofuranosyl)thieno[2,3-*d*]pyrimidin-2(3*H*)-one (**7b**)

A suspension of **7a** (600 mg, 0.98 mmol) in a solution of KOH (250 mg, 4.46 mmol) in MeOH (50 ml) was stirred at 60 °C for 1 h. The clear solution was then neutralized (AcOH), volatiles were evaporated, and the residue was chromatographed (EtOAc → EtOAc/MeOH, 3:1) to give **7b** (200 mg, 68%) that was recrystallized (MeOH) to give purified **7b**. ^{1}H NMR (DMSO-d_6): 2.41 (d, *J* = 1.0, 3 H), 3.65, 3.84 (2 × ddd, *J* = 2.0, 4.9, 12.2, 2 × 1 H), 3.94–4.02 (m, 3 H), 5.03 (d, *J* = 5.9, 1 H), 5.31 (t, *J* = 5.1, 1 H), 5.64 (d, *J* = 4.9, 1 H), 5.80 (d, *J* = 2.0, 1 H), 6.74 (q, *J* = 1.5, 1 H), 8.98 (s, 1 H). ^{13}C NMR (DMSO-d_6): 16.1, 59.3, 67.8, 74.7, 84.1, 91.6, 117.0, 118.4, 136.4, 138.4, 151.9, 178.7. FAB-MS *m/z*: 299 ([M + H$^+$] 30%), 141 (100%). HRMS ($C_{12}H_{15}N_2O_5S$): calculated 299.0696, found 299.0709.

3-(2-Deoxy-β-D-*erythro*-pentofuranosyl)-6-methylthieno[2,3-*d*]pyrimidin-2(3*H*)-one (**10**)

The procedure described above with **6** was applied to 1-[2-deoxy-3,5-di-*O*-(4-methylbenzoyl)-β-D-*erythro*-pentofuranosyl]-5-(propyn-1-yl)uracil[20] (**8**; 500 mg, 1.0 mmol) to give 1-[2-deoxy-3,5-di-*O*-(4-methylbenzoyl)-β-D-*erythro*-pentofuranosyl]-5-(propyn-1-yl)-4-(1,2,4-triazol-1-yl)pyrimidin-2(1*H*)-one, which was stirred with potassium thioacetate (180 mg, 1.58 mmol) in DMF (15 ml) at ambient temperature for 1 h. Volatiles were evaporated and the residue was chromatographed (CH_2Cl_2) to give 3-[2-deoxy-di-*O*-(4-methylbenzoyl)-β-D-*erythro*-pentofuranosyl]-6-methylthieno[2,3-*d*]pyrimidin-2(3*H*)-one (**9**; 470 mg, 91% for two steps), a suspension of which in a solution of KOH (130 mg, 2.32 mmol) in MeOH (15 ml) was stirred for 2 h. The solution was neutralized (AcOH), volatiles were evaporated, and the residue was chromatographed (EtOAc → EtOAc/MeOH, 3:1) to give **10** (210 mg, 82%). ^{1}H NMR (DMSO-d_6): 2.07 (td, *J* = 5.9, 13.2, 1 H), 2.41 (d, *J* = 1.0, 3 H), 2.42 (ddd, *J* =

4.4, 6.3, 13.2, 1 H), 3.59–3.71 (m, 2 H), 3.92–3.94 (m, 1 H), 4.20–4.24 (m, 1 H), 5.14 (t, *J* = 5.1, 1 H), 5.30 (d, *J* = 3.9, 1 H), 6.11 (t, *J* = 5.9, 1 H), 6.78 (q, *J* = 1.5, 1 H), 8.83 (s, 1 H). ^{13}C NMR (DMSO-d_6): 16.1, 41.2, 60.6, 69.5, 87.6, 88.3, 117.1, 118.4, 136.1, 138.0, 151.7, 178.5. FAB-MS *m/z*: 305 ([M + Na^+] 30%), 587 ([2 M + Na^+] 100%). HRMS ($C_{12}H_{14}N_2NaO_4S$): calculated 305.0567, found 305.0570. UV (absorption) max 274 (ε 5200), 358 (ε 2800), 367 nm (ε 2700); min 303 nm (ε 200). UV/VIS (emission) max 390, 400, 461, 496 nm.

3-(β-D-Ribofuranosyl)furo[2,3-*d*]pyrimidin-2(3*H*)-one (**11b**)

$(Ph_3P)_4Pd$ (120 mg, 0.10 mmol) and CuI (45 mg, 0.24 mmol) were added to a solution of **1** (2.1 g, 3.1 mmol) and TMS–acetylene (0.6 ml) in Et_3N (3.0 ml) and CH_2Cl_2 (10 ml), and the mixture was stirred at 50 °C for 2 h, during which time copious quantities of crystalline material precipitated. The mixture was filtered and the filter cake was washed with CH_2Cl_2 to dissolve the precipitated product. Volatiles were evaporated from the filtrate and the residue was chromatographed (CH_2Cl_2) to give 2′,3′,5′-tri-*O*-benzoyl-5-(trimethylsilylethynyl)-uridine (2.0 g, 99%). A solution of that material (5.2 g, 8.0 mmol) and NH_4F (300 mg, 8.1 mmol) in THF (100 ml), MeOH (820 ml), and H_2O (10 ml) was stirred at 50 °C for 30 min. Volatiles were evaporated and the residue was chromatographed (CH_2Cl_2/hexanes, 1:2 → CH_2Cl_2). Crystallization of the purified residue (EtOAc/hexanes) gave 2′,3′,5′-tri-*O*-benzoyl-5-ethynyluridine (1.59 g, 34%), which was stirred with CuI (520 mg, 2.74 mmol) in Et_3N (7 ml) and DMF (16 ml) at 80 °C for 12 h. Volatiles were evaporated, CH_2Cl_2 was added, and the mixture was filtered using a short column of silica. Volatiles were evaporated, EtOAc was added, and the solution was filtered through silica. Evaporation of volatiles gave clean (TLC) **11a** [17] (1.0 g, 63%).

A 250-ml pressure flask equipped with a Teflon valve was charged with **11a** (1.0 g, 1.72 mmol) and a $LiOCH_2CF_3$/CF_3CH_2OH solution prepared by addition of BuLi (1.6 M, 2.0 ml, 3.2 mmol) to TFE (10 ml). The solution was stirred at 90 °C overnight, neutralized (AcOH), concentrated, and chromatographed (EtOAc → EtOAc/MeOH, 4:1) to give **11b** (340 mg, 74%). Successive PTLC with EtOAc/MeOH (4:1) and CH_2Cl_2/MeOH (3:1) gave TLC-homogeneous **11b**. ^{1}H NMR (DMSO-d_6): 3.66, 3.82 (2 × d, *J* = 12.4, 2 × 1 H), 3.94–4.02 (m, 3 H), 5.00–5.80 (br s, 3 H), 5.85 (d, *J* = 2.0, 1 H), 6.81 (d, *J* = 2.9, 1 H), 7.76 (d, *J* = 2.9, 1 H), 9.01 (s, 1 H). ^{13}C NMR (DMSO-d_6): 69.0, 77.4, 84.6, 93.8, 101.3, 114.4, 114.9, 149.1, 154.6, 163.7, 181.0. EI-MS *m/z*: 268 ([M^+] 5%), 136 (100%). HRMS ($C_{11}H_{12}N_2O_6$): calculated 268.0695, found 268.0701. UV (absorption) max 328 nm (ε 2200); min 267 nm (ε 400). UV/VIS (emission) max 400 nm.

3-(β-D-Ribofuranosyl)pyrrolo[2,3-*d*]pyrimidin-2(3*H*,7*H*)-one (**12**)

A solution of **11a** (200 mg, 0.35 mmol) in NH_3/MeOH (~14%, 10 ml) was heated in a sealed pressure flask at 80 °C for 3 h. Volatiles were evaporated and the residue was chromatographed (EtOAc/MeOH, 3:1) to give **12** (64 mg, 69%). PTLC (EtOAc/MeOH, 3:1) provided a sample of **12** for analysis. ^{1}H NMR (CD_3OD): 3.85, 4.02 (2 × dd, *J* = 2.4, 12.7, 2 × 1 H), 4.12–4.21 (m, 3 H), 6.02 (d, *J* = 2.0, 1 H), 6.35 (d, *J* = 3.9, 1 H), 7.10 (d, *J* = 3.9, 1 H), 9.04 (s, 1 H). ^{13}C NMR (CD_3OD): 61.4, 69.9, 77.2, 85.9, 94.1, 102.5, 111.1, 128.8, 139.1, 157.2, 159.5. EI-MS *m/z*: 267 ([M^+] 5%), 135 (100%). HRMS ($C_{11}H_{13}N_3O_5$): calculated 267.0855, found 267.0847. UV (absorption) max 273 (ε 3600), 340 nm (ε 2800); min 248 (ε 2000), 292 nm (ε 800). UV/VIS (emission) max 424 nm.

3-(β-D-Ribofuranosyl)thieno[2,3-*d*]pyrimidin-2(3*H*)-one (**14**)

Coupling of **1** and TMS–acetylene (described in the first step of **1** to **11a**) was followed by conversion of 2′,3′,5′-tri-*O*-benzoyl-5-(trimethylsilylethynyl)uridine (3.0 g, 4.6 mmol) into 1-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-5-(trimethylsilylethynyl)-4-(1,2,4-triazol-1-yl)pyrimidin-2(3*H*)-one (**13**) (as described for the first step in the conversion of **6** to **7a**). The crude material was chromatographed (EtOAc/hexanes, 1:2 → EtOAc) and recrystallized from EtOAc/hexanes to give **13** (3.98 g, 92%). A solution of **13** (900 mg, 1.28 mmol), NH_4F (300 mg, 8.11 mmol), and KSAc (600 mg, 5.26 mmol) in pyridine (14 ml) and H_2O (1.5 ml) was stirred at ambient temperature for 40 min. Volatiles were evaporated and the residue was chromatographed (EtOAc/hexanes, 1:1 → 3:1) to give a mixture (3:2, 465 mg) of the presumed fused-thieno intermediate and an unidentified product. Solvolysis of the benzoyl esters with Dowex 1 X2 (OH^-) in trifluoroethanol at 80 °C for 2 h and PTLC (EtOAc → EtOAc/MeOH, 4:1) gave **14** as an unstable material. ^{1}H NMR (DMSO-d_6): 3.66, 3.84 (2 × d, J = 12, 2 × 1 H), 3.92–4.04 (m, 3 H), 5.20–5.60 (br s, 3 H), 5.81 (d, J = 2.0, 1 H), 7.06 (d, J = 5.9, 1 H), 7.32 (d, J = 5.9, 1 H), 9.19 (s, 1 H).

3-(β-D-Ribofuranosyl)imidazo[4,5-*d*]pyrimidin-2(3*H*,5/7*H*)-one
[1-(β-D-Ribofuranosyl)purin-2(1*H*,7/9*H*)-one] (**17**)

A solution of 5-aminocytidine[22] **15** (200 mg, 0.78 mmol) and TBDMSCl (140 mg, 0.93 mmol) in dried pyridine (2 ml) was stirred at ambient temperature overnight. Volatiles were evaporated and the residue was chromatographed (EtOAc → EtOAc/MeOH, 3:1) to give 5′-*O*-TBDMS-5-aminocytidine **16** (260 mg, 90%). A solution of **16** (200 mg, 0.70 mmol) in $(EtO)_2CHOAc$ (5 ml) was heated at 145 °C for 1.5 h and volatiles were evaporated. The residue was chromatographed (EtOAc → EtOAc/MeOH, 10:1) and the resulting blue fluorescent residue (100 mg) was stirred at 0 °C for 1 h with TFA/H_2O (9:1, 1 ml). Volatiles were evaporated, the residue was dissolved in NH_3/MeOH (~14%, 10 ml), and the solution was stirred at ambient temperature for 1 h. Volatiles were evaporated and the residue was chromatographed (MeOH → H_2O). The UV-active fractions were pooled, concentrated, and applied to a column of Dowex 1 X2 (OH^-) resin in H_2O. Elution (H_2O → MeOH → MeOH/AcOH, 3:1) and evaporation of volatiles from the UV-active fractions gave **17** (46 mg, 32%). ^{1}H NMR (DMSO-d_6): 3.66, 3.83 (2 × d, J = 11.7, 2 × 1 H), 3.94–4.06 (m, 3 H), 5.01, 5.38, 5.55 (3 × br s, 3 × 1 H), 5.87, 8.19, 9.12 (3 × s, 3 × 1 H), 12.40 (br s, 1 H). ^{13}C NMR (DMSO-d_6): 59.3, 67.9, 75.0, 83.8, 91.4, 123.0, 135.9, 146.8, 154.2, 160.3. FAB-MS *m/z*: 291 ([M + Na^+] 20%), 131 (100%). HRMS ($C_{10}H_{12}N_4NaO_5$): calculated 291.0700, found 291.0701. UV (absorption) max 322 nm (ε 4100); min 270 nm (ε 2100). UV/VIS (emission) max 390 nm.

Nucleoside Transport

Materials: [5,6-^{3}H]Uridine (21.3 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Tissue culture (96) plates, Roswell Park Memorial Institute (RPMI) 1640 cell culture media, and fetal bovine serum (FBS) were from Invitrogen (Burlington, Ontario). Ecolite was from ICN Pharmaceuticals (Montreal, Quebec). 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, NBMPR, (*p*-nitrobenzylmercaptopurine riboside) [6-*S*-(4-nitrobenzyl)-6-thioinosine] and other chemicals were from Sigma–Aldrich (Oakville, Ontario).

The hENT1-containing human CCRF-CEM leukemia cell line (referred to as CEM/hENT1) was obtained from W. T. Beck (University of Illinois at Chicago). CEM/ARAC-8C, a nucleoside transport-deficient derivative of CCRF-CEM [24] (referred to as CEM/ARAC) was a gift from Dr. B. Ullman (Oregon Health and Science University, Portland). The origin and characterization of the hCNT1-containing cell line (referred to as CEM/hCNT1), which was produced by stable transfection of CEM/ARAC with a vector containing a cDNA encoding hCNT1, are described elsewhere[25]. BeWo human choriocarcinoma cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown in RPMI 1640 medium supplemented with 10% FBS as suspension (CEM) or adherent (BeWo) cultures. Cells were determined to be free of mycoplasma, maintained in the absence of antibiotics, incubated at 37 °C in a humidified atmosphere (5% CO_2), and subcultured at two- to four-day intervals to maintain active proliferation.

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[26].

Methods: Measurement of [^{3}H]uridine uptake mediated by recombinant hENTs produced in *Saccharomyces cerevisiae*: Construction of the yeast expression systems for hENT1 and hENT2 has been described earlier[27a]. Yeast producing individual recombinant hENTs were maintained in logarithmic growth phase CMM/GLU.

Transport experiments were conducted with a high-throughput assay described previously[26a] with 96-well plates and a semi-automated cell harvester (Micro96 Harvester; Skatron Instruments, Lier, Norway). Relative affinities of the transporters for the auto-fluorescent nucleoside analogues were assessed by measuring the concentration dependence of their inhibition of uptake of [^{3}H]uridine as follows. Yeast producing recombinant hENT1 or hENT2 were incubated with graded concentrations of the analogues in the presence of [^{3}H]uridine (1 μM) for 10 min. Each experiment was repeated at least three times. Nonspecifically associated radioactivity was determined in the presence of non-radioactive uridine (10 mM), and the resulting values were subtracted from the total uptake values. Data were subjected to nonlinear regression analysis using GraphPad Prism Software version 3.0 (GraphPad Software Inc., San Diego, CA) to obtain IC_{50} values (inhibitor concentrations that produced 50% inhibition of uridine transport).

Live-cell imaging of the uptake of autofluorescent nucleoside analogues into BeWo and CEM cells with confocal microscopy: Time-lapse microscopy was performed with a Zeiss LSM510 Confocal Laser Scanning microscope (Carl Zeiss, Jena) mounted on an Axiovert 100M inverted microscope as described[6]. Coverslips with adhered BeWo cells (attached during growth) or CEM cells (attached with poly-L-lysine and air dried) were washed three times with phosphate-buffered saline (PBS, pH 7.4) and glued onto the edge of an open hole located in the middle of a 2-μm thick metal slide to form a well. PBS alone or PBS containing NBMPR (10 μM) was added to the well, which was maintained at ambient temperature for 5 min before the confocal measurements. The solution in the well was gently removed by suction, and time-lapse image capturing was begun a few seconds before adding PBS (200 μl) containing the individual analogue into the well. The live-cell imaging was continued for 10 min. Fluorescence intensities were measured by placing a circle around the stored digital image of a targeted cell or intracellular part. Integrated intensities were exported to a linked Microsoft Excel worksheet. Background fluorescence (defined as the fluorescence intensity of

a comparable measurement circle in a nearby extracellular region) and fluorescence in the presence of NBMPR were determined and subtracted from each cellular measurement to calculate specific uptake values.

We thank Brigham Young University and unrestricted gift funds from pharmaceutical companies for financial support of the chemistry; the nucleoside transport work was funded by grants from the Canadian Cancer Society Research Institute and the Canadian Institute of Health Research. We thank the Cell Imaging Facility at the Cross Cancer Institute for help with the confocal microscopy studies, and Gerry Barron, Delores Mowles, Tracey Tackaberry, and Marnie Wilson for expert technical assistance.

REFERENCES

1. a) Robins M. J., Barr P. J.: *Tetrahedron Lett.* **1981**, *22*, 421; b) Robins M. J., Barr P. J.: *J. Org. Chem.* **1983**, *48*, 1854.
2. Yu D., Putta M. R., Bhagat L., Li Y., Zhu F., Wang D., Tang J. X., Kandimalla E. R., Agrawal S.: *J. Med. Chem.* **2007**, *50*, 6411.
3. a) Liu C., Martin C. T.: *J. Mol. Biol.* **2001**, *308*, 465; b) Liu C., Martin C. T.: *J. Biol. Chem.* **2002**, *277*, 2725.
4. a) Srivatsan S. G., Weizman H., Tor Y.: *Org. Biomol. Chem.* **2008**, *6*, 1334; b) Sinkeldam R. W., Greco N. J., Tor Y.: *Chem. Rev.* **2010**, *110*, 2579.
5. a) McGuigan C., Brancale A., Barucki H., Srinivasan S., Jones G., Pathirana R., Blewett S., Alvarez R., Yarnold C. J., Carangio A., Velazquez S., Andrei G., Snoeck R., De Clercq E., Balzarini J.: *Drugs Future* **2000**, *25*, 1151; b) De Clercq E.: *Med. Res. Rev.* **2003**, *23*, 253; c) De Clercq E.: *Annu. Rev. Pharmacol. Toxicol.* **2011**, *51*, 1.
6. Zhang J., Sun X., Smith K. M., Visser F., Carpenter P., Barron G., Peng Y., Robins M. J., Baldwin S. A., Young J. D., Cass C. E.: *Biochemistry* **2006**, *45*, 1087.
7. Cai J., Damaraju V. L., Groulx N., Mowles D., Peng Y., Robins M. J., Cass C. E., Gros P.: *Cancer Res.* **2008**, *68*, 2349.
8. Paproski R. J., Ng A. M. L., Yao S. Y. M., Graham K., Peng Y., Robins M. J., Young J. D., Cass C. E.: *Mol. Pharmacol.* **2008**, *74*, 1372.
9. a) Wiley J. S., Jones S. P., Sawyer W. H., Paterson A. R.: *J. Clin. Invest.* **1982**, *69*, 479; b) Cass C. E., King K. M., Montano J. T., Janowska-Wieczorek A.: *Cancer Res.* **1992**, *52*, 5879; c) Gati W. P., Paterson A. R., Belch A. R., Chlumecky V., Larratt L. M., Mant M. J., Turner A. R.: *Leuk. Lymphoma* **1998**, *32*, 45; d) Mackey J. R., Baldwin S. A., Young J. D., Cass C. E.: *Drug Resist. Updat.* **1998**, *1*, 310; e) Gati W. P., Larratt L. M., Turner A. R.: *Proc. West. Pharmacol. Soc.* **2001**, *44*, 93.
10. a) Spratlin J., Sangha R., Glubrecht D., Dabbagh L., Young J. D., Dumontet C., Cass C., Lai R., Mackey J. R.: *Clin. Cancer Res.* **2004**, *10*, 6956; b) Farrell J. J., Elsaleh H., Garcia M., Lai R., Ammar A., Regine W. F., Abrams R., Benson A. B., Macdonald J., Cass C. E., Dicker A. P., Mackey J. R.: *Gastroenterology* **2009**, *136*, 187; c) Marechal R., Mackey J. R., Lai R., Demetter P., Peeters M., Polus M., Cass C. E., Young J., Salmon I., Deviere J., Van Laethem J. L.: *Clin. Cancer Res.* **2009**, *15*, 2913.
11. Robins M. J., Peng Y., Damaraju V. L., Mowles D., Barron G., Tackaberry T., Young J. D., Cass C. E.: *J. Med. Chem.* **2010**, *53*, 6040.

12. Damaraju V. L., Smith K. M., Mowles D., Nowak I., Karpinski E., Young J. D., Robins M. J., Cass C. E.: *Biochem. Pharmacol.* **2011**, *81*, 82.
13. Asakura J., Robins M. J.: *Tetrahedron Lett.* **1988**, *29*, 2855.
14. Robins M. J., Vinayak R. S., Wood S. G.: *Tetrahedron Lett.* **1990**, *31*, 3731.
15. Robins M. J., Miranda K., Rajwanshi V. K., Peterson M. A., Andrei G., Snoeck R., De Clercq E., Balzarini J.: *J. Med. Chem.* **2006**, *49*, 391.
16. a) Woo J., Meyer R. B., Jr., Gamper H. B.: *Nucleic Acids Res.* **1996**, *24*, 2470; b) Berry D. A., Jung K.-Y., Wise D. S., Sercel A. D., Pearson W. H., Mackie H., Randolph J. B., Somers R. L.: *Tetrahedron Lett.* **2004**, *45*, 2457.
17. Nowak I., Jones C. T., Robins M. J.: *J. Org. Chem.* **2006**, *71*, 3077.
18. Divakar K. J., Reese C. B.: *J. Chem. Soc., Perkin Trans. 1* **1982**, 1171.
19. Brancale A., McGuigan C., Algain B., Savy P., Benhida R., Fourrey J.-L., Andrei G., Snoeck R., De Clercq E., Balzarini J.: *Biorg. Med. Chem. Lett.* **2001**, *11*, 2507.
20. De Clercq E., Descamps J., Balzarini J., Giziewicz J., Barr P. J., Robins M. J.: *J. Med. Chem.* **1983**, *26*, 661.
21. a) Fox J. J., Van Praag D.: *J. Org. Chem.* **1961**, *26*, 526; b) Holý A.: *Collect. Czech. Chem. Commun.* **1979**, *44*, 2846.
22. Fukuhara T. K., Visser D. W.: *J. Am. Chem. Soc.* **1955**, 77, 2393.
23. Robins M. J., Sarker S., Wnuk S. F.: *Nucleosides Nucleotides* **1998**, *17*, 785.
24. Ullman B., Coons T., Rockwell S., McCartan K.: *J. Biol. Chem.* **1988**, *263*, 12391.
25. Lang T. T., Young J. D., Cass C. E.: *Mol. Pharmacol.* **2004**, *65*, 925.
26. a) Zhang J., Visser F., Vickers M. F., Lang T., Robins M. J., Nielsen L. P. C., Nowak I., Baldwin S. A., Young J. D., Cass C. E.: *Mol. Pharmacol.* **2003**, *64*, 1512; b) Zhang J., Smith K. M., Tackaberry T., Visser F., Robins M. J., Nielsen L. P. C., Nowak I., Karpinski E., Baldwin S. A., Young J. D., Cass C. E.: *Mol. Pharmacol.* **2005**, *68*, 830.
27. a) Vickers M. F., Kumar R., Visser F., Zhang J., Charania J., Raborn R. T., Baldwin S. A., Young J. D., Cass C. E.: *Biochem. Cell Biol.* **2002**, *80*, 639; b) Vickers M. F., Zhang J., Visser F., Tackaberry T., Robins M. J., Nielsen L. P. C., Nowak I., Baldwin S. A., Young J. D., Cass C. E.: *Nucleosides Nucleotides Nucleic Acids* **2004**, *23*, 361.