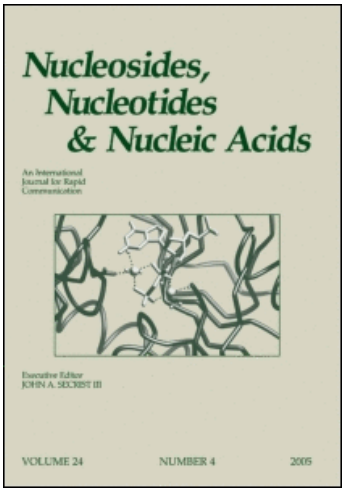

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Differential Transport of Cytosine-Containing Nucleosides by Recombinant Human Concentrative Nucleoside Transporter Protein hCNT1

Kathryn A. Graham[a]; Jackie Leithoff[a]; Imogen R. Coe[b]; Delores Mowles[a]; John R. Mackey[a]; James D. Young[c]; Carol E. Cass[a]
[a] Department of Oncology, University of Alberta Cross Cancer Institute, Edmonton, Alberta, Canada [b] Department of Biology, York University, Toronto, Ontario, Canada [c] Department of Physiology, University of Alberta, Edmonton, Canada

To cite this Article Graham, Kathryn A. , Leithoff, Jackie , Coe, Imogen R. , Mowles, Delores , Mackey, John R. , Young, James D. and Cass, Carol E.(2000) 'Differential Transport of Cytosine-Containing Nucleosides by Recombinant Human Concentrative Nucleoside Transporter Protein hCNT1', Nucleosides, Nucleotides and Nucleic Acids, 19: 1, 415 — 434
To link to this Article: DOI: 10.1080/15257770008033018
URL: http://dx.doi.org/10.1080/15257770008033018

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(1&2), 415–434 (2000)

DIFFERENTIAL TRANSPORT OF CYTOSINE-CONTAINING NUCLEOSIDES BY RECOMBINANT HUMAN CONCENTRATIVE NUCLEOSIDE TRANSPORTER PROTEIN hCNT1.

Kathryn A. Graham[1^], Jackie Leithoff[1^], Imogen R. Coe[2^], Delores Mowles[1], John R. Mackey[1], James D. Young[3], Carol E. Cass[1]*

[1]Department of Oncology, University of Alberta, Cross Cancer Institute, Edmonton, Alberta, Canada, T6G 1Z2.
[2]Department of Biology, York University, Toronto, Ontario, Canada M3J 1P3.
[3]Department of Physiology, University of Alberta, Edmonton, Canada, T6G 2H7.

Dedicated to the memory of Gertrude B. Elion

ABSTRACT: The transportability of cytosine-containing nucleosides by recombinant hCNT1 was investigated in transfected mammalian cells. Apparent K_m values for hCNT1-mediated transport of uridine, cytidine and deoxycytidine were, respectively, 59, 140 and 150 μM. Uridine transport was inhibited 89, 32 and 11%, respectively, by 500 μM gemcitabine, cytarabine and lamivudine, demonstrating that, unlike gemcitabine (a high-affinity hCNT1 permeant), cytarabine and lamivudine are poor hCNT1 permeants.

INTRODUCTION

Nucleoside transporter (NT) proteins[a], which mediate movement of nucleosides and their analogs into and out of cells, have been identified by molecular cloning and functional expression of their cDNAs[1,2]. The characteristics of the four different

^These authors contributed equally to this study.

*The author to whom correspondence should be addressed.

[a] The relationship between the nomenclature used for NT proteins and NT activities[3-7] is as follows: ENT1 (Equilibrative Nucleoside Transporter 1) mediates *es* (equilibrative, sensitive to nitrobenzylthioinosine) transport activity; ENT2 (Equilibrative Nucleoside Transporter 2) mediates *ei* (equilibrative, insensitive to nitrobenzylthioinosine) transport activity; CNT1 (Concentrative Nucleoside Transporter 1) mediates *cit* (concentrative, insensitive to nitrobenzylthioinosine, thymidine) transport activity; and CNT2 (Concentrative Nucleoside Transporter 2) mediates *cif* (concentrative, insensitive to nitrobenzylthioinosine, formycin B) transport activity.

mammalian nucleoside transporters so identified have revealed the existence of two previously unrecognized membrane protein families, the equilibrative nucleoside transporter (ENT) and the concentrative nucleoside transporter (CNT) proteins[3-7]. The equilibrative processes, *es* and *ei*, mediated by the ENT1 and ENT2 proteins, respectively, transport a broad spectrum of nucleosides bidirectionally and are energetically driven by the concentration gradient. The concentrative processes, *cit* and *cif*, mediated by the CNT1 and CNT2 proteins, respectively, are unidirectional sodium-dependent symporters that transport a restricted group of nucleosides into cells and are energetically driven by the inwardly directed sodium gradient[1,8]. The ENT-mediated processes are widely distributed among mammalian cell types whereas the CNT-mediated processes are found in specialized cell types, including intestinal and renal epithelia[1].

Concentrative nucleoside transport processes of mammalian cells typically coexist with equilibrative, and sometimes other concentrative, processes with overlapping permeant selectivities, making the identification and functional characterization of the proteins responsible for individual processes difficult. Recent advances in methods for expression of cDNAs encoding NT proteins in *Xenopus laevis* oocytes[3,4,6,7], transfected mammalian cells[5,9,10] and the yeast *Saccharomyces cerevisiae*[11] have enabled the identification and functional analysis of individual nucleoside transport processes in the absence of other confounding activities. The existence of the *cit*-like transporter in humans was established by the isolation of a cDNA encoding an NT protein with selectivity for pyrimidine nucleosides and adenosine when expressed in *Xenopus* oocytes[3,4,6,7]. The mammalian CNT proteins are predicted to have 648-658 amino acid residues and an uncertain number of transmembrane domains (8-14 transmembrane domains, with 13 favored)[6-8,12]. The human (h) and rat (r) proteins with *cit*-like activity (hCNT1, rCNT1) are 83% identical in their amino acid sequences[6-8,12].

When expressed in *Xenopus* oocytes, recombinant r and hCNT1, respectively, exhibit high-affinity sodium-dependent transport of uridine, thymidine and adenosine, although the low V_{max} values observed for adenosine, relative to those observed for uridine and thymidine, indicate that adenosine is a "low-activity" permeant[6,8,13]. The antiviral nucleosides, zidovudine and zalcitabine, are low-affinity permeants[6,14] whereas the anticancer nucleoside, gemcitabine, is a high-affinity permeant[15]. High-affinity

transport of gemcitabine by hCNT1 has also been shown in studies with transiently transfected cultured HeLa cells[16]. Studies with cultured cells transfected with rCNT1 demonstrated that the antiviral nucleoside, lamivudine, and the anticancer nucleoside, cytarabine, are poor inhibitors of rCNT1-mediated transport of uridine[9,17]. The inability of rCNT1 to transport cytarabine was confirmed by direct measurements of cytarabine uptake[17]. The transportability of lamivudine and cytarabine by hCNT1 has not been established.

The objective of the current study was to assess the transportability of cytosine-containing nucleosides by the human transporter, hCNT1, in a human genetic background, focusing on cytidine, deoxycytidine and three analogs (cytarabine, gemcitabine, lamivudine (Fig. 1) currently in clinical use in either antiviral or anticancer therapy. The capacity of hCNT1 for transport of cytosine-containing nucleosides has not been defined, with the exception of gemcitabine, the transport of which was recently investigated kinetically for recombinant human ENT and CNT proteins produced in *Xenopus* oocytes[15,16]. In this study transient transfection was used to introduce an expression plasmid containing hCNT1 into recipient cell lines that lacked endogenous CNT-mediated activity and thus could be rendered transport defective pharmacologically by treatment with an inhibitor of ENT-mediated processes. Expression of hCNT1 cDNA was demonstrated in transiently transfected cells by (i) immunological reactivity of epitope-tagged recombinant hCNT1 in cell lysates, and (ii) acquisition of sodium-dependent uridine transport activity. The nucleosides cytidine and deoxycytidine were assessed to determine their ability to inhibit hCNT1-mediated transport of [^{3}H]uridine and their transportability by directly measuring hCNT1-mediated fluxes of ^{3}H-labeled nucleoside. The introduction of hCNT1 into otherwise NT-defective human cells imparted the ability to mediate inwardly directed sodium-dependent (and thus concentrative) transport of extracellular cytidine and deoxycytidine by a saturable process that was subjected to kinetic analysis to determine apparent affinities of hCNT1 for both compounds. A comparison of the inhibitory capacities of the three cytosine-containing drugs indicated that cytarabine and lamivudine, unlike gemcitabine[15,16], have low affinities for hCNT1 and thus are not permeants of hCNT1. The present results, together with those reported elsewhere for gemcitabine[15,16], indicate that the presence of functional hCNT1 at the cell surface will enhance cellular uptake, and therefore potential pharmacological activity, of gemcitabine, but not that of either cytarabine or lamivudine.

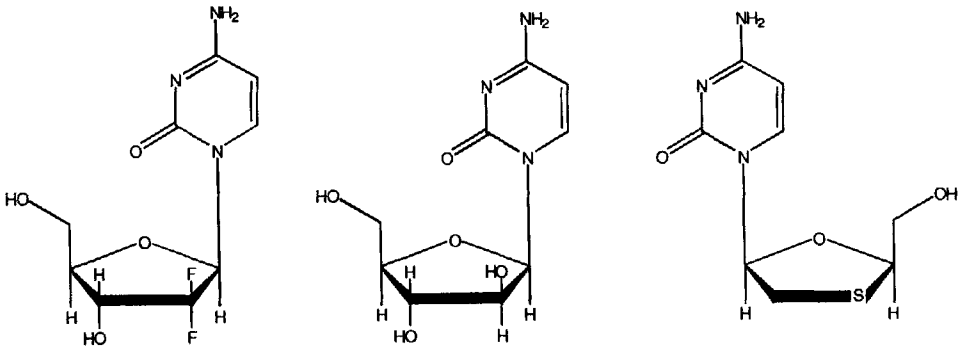

FIG. 1 Structures of the deoxycytidine analogs gemcitabine, cytarabine, and lamivudine.

MATERIALS AND METHODS

Cell Culture. The monkey kidney COS-1 and human cervical carcinoma HeLa cell lines were obtained from the American Type Culture Collection (Manassas, VA). The origin of the mouse L1210/B23.1 cell line is described elsewhere[18]. COS-1 and HeLa cells were propagated as adherent cultures in Dulbecco's modified Eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI) 1640 medium, respectively, supplemented with 10% calf serum. L1210 cells were propagated as suspension cultures in RPMI 1640 supplemented with 10% horse serum. Stock cultures were maintained without antibiotics at 37°C in 5% CO_2, subcultured every 3-4 days, and demonstrated to be free of mycoplasma. Cell numbers were determined using a Coulter Counter Model Z2 (Coulter Electronics Inc.; Luton, England). Cell culture reagents were purchased from Life Technologies (Gaithersburg, MD).

Plasmids. The hCNT1 cDNA was subcloned into the pcDNA3 mammalian expression vector. hCNT1 was amplified from its original cloning vector, pMHK2[6], by the polymerase chain reaction (PCR) with Taq Polymerase (Life Technologies), using the primers CNTa (5'-ATG CGG TAC CTG GAA GGT CTG GG) and CNTb (5'-GCA TTC TAG AAT CTA AGT CCT GTG GC), which contain *Kpn*I and *Xba*I restriction sites, respectively (underlined). The resulting PCR product was digested with *Kpn*I and *Xba*I (Life Technologies) and subcloned into pcDNA3 (Invitrogen Corp., Carlsbad, CA) to

produce pcDNA3-hCNT1. The nucleotide sequence of the entire insert was confirmed by standard cycle sequencing using an automated 310 Genetic Analyzer (PE Biosystems; Foster City, CA). An additional construct expressing the hemagglutinin (HA) tag[19,20] at the C-terminus of hCNT1 was prepared similarly by PCR amplification using primer CNTc (5'-CAG AGA TCT ATG GAG AAC GAC CCC TCG AGA CG) that encodes a *Bgl*II restriction site (underlined) at the 5' end of the gene and primer CNTd (5'-CCA TCC GTC GAC GCA TGC CTA AGC GTA GTC TGG GAC GTC GTA TGG GTA TTC CGC ATG AGC (ATG AGC TTC CTC ATG)$_3$ ATG CCC ATT CTG TGC ACA GAT CGT GTG GTT G) that encodes the 3' end of hCNT1, the HA immunological marker and the *Sal*I restriction site (underlined). The resulting PCR product was digested with *Bgl*II and *Sal*I and subcloned into compatible *Bam*HI and *Xho*I sites of pcDNA3, yielding the plasmid pcDNA3-hCNT1-HA.

Transient Transfection of COS-1 and HeLa Cells. The plasmid constructs were transiently transfected into COS-1 or HeLa cells as previously described[9]. Actively proliferating cultures (5 x 10^5 COS or 1.5 x 10^6 HeLa cells per 100-mm dish) were transfected with plasmid DNA (5 μg/dish) using DEAE-dextran (Pharmacia Biotech Inc., Quebec, Canada). After 24 h, the transfected cultures were trypsinized and pooled, and the resulting cell suspensions were replated into 60-mm dishes to ensure that each culture contained the same proportion of transfected cells. Uptake assays were routinely performed after a further 48 h of incubation with cultures that typically contained a minimum of 5 x 10^5 cells/dish. Such cultures were used for uptake and immunoblotting assays (described below).

To monitor transfection efficiency, separate cultures were transfected by the same protocol with a β-galactosidase expression vector (pcDNA3-β-gal) and stained for β-galactosidase activity as described[9].

Uptake of ^{3}H-Labeled Nucleosides by Transfected Cultures. Measurements of uptake of [^{3}H]uridine, [^{3}H]cytidine and [^{3}H]deoxycytidine (Moravek Biochemicals; Brea, CA) by transfected adherent cultures of COS-1 or HeLa cells were performed as previously described[9]. Radiochemicals were 98-99% pure as assessed by high-performance liquid chromatography using water-methanol gradients on a C18 reverse phase column. Briefly, replicate cultures were exposed at room temperature to sodium-containing (20 mM Tris/HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 6H_20$, 2 mM $CaCl_2$, 5 mM

glucose, 130 mM NaCl, pH 7.4) or sodium-free (20 mM Tris/HCl, 3 mM K_2HPO_4, 1 mM $MgCl_2 \cdot 6H_20$, 2 mM $CaCl_2$, 5 mM glucose, 130 mM N-methyl-D-glucamine/HCl, pH 7.4) transport buffer containing ^{3}H-labeled nucleosides (10 μM) for precisely timed intervals. Assays were terminated by immersion of the culture dish in an excess volume of ice-cold transport buffer. Each culture dish was processed separately. Assays were routinely performed in the presence of 100 μM dilazep (a gift from F. Hoffmen La Roche and Co., Basel, Switzerland) to block equilibrative transport of the test nucleoside. To determine uptake at time zero, cells were incubated for 10 min at 4°C with sodium-containing or sodium-free transport buffer that contained 100 μM dilazep and then immediately thereafter for a period of $\leq$ 2 s with ice-cold sodium-containing or sodium-free transport buffer that contained 100 μM dilazep and the ^{3}H-labeled nucleoside.

The ability of various nucleosides to inhibit transport of ^{3}H-labeled nucleosides was determined by conducting uptake assays in the presence of unlabeled test nucleosides. Uridine, cytidine, 2'-deoxycytidine, guanosine, adenosine and cytarabine (1-β-D-arabinosylcytosine, araC) were purchased from Sigma Chemical Co. (St. Louis, MO), gemcitabine (2',2'-difluorodeoxycytidine, Gem) was a gift from Eli Lilly Inc. (Indianapolis, IN) and lamivudine (2'-dideoxy-3'-thiacytidine, 3TC) was a gift from D.L. Tyrrell (University of Alberta). Data were analyzed using Prism GraphPad Software (San Diego, CA). Transport rates were derived from initial rates of uptake by linear regression analysis of time courses of influx of ^{3}H-labeled-nucleoside. Kinetic constants (apparent K_m and V_{max} values) of ^{3}H-nucleoside transport by COS-1 and HeLa cells were determined by nonlinear regression analysis.

Detection of Recombinant hCNT1 by Immunoblotting. HeLa cells transfected with either pcDNA3-hCNT1-HA or pcDNA3-β-gal were harvested by resuspending the cell monolayers in reducing polyacrylamide gel electrophoresis loading buffer[21]. The samples were sheared with a 21-gauge needle, heated to 37°C for 30 min, and subjected to electrophoresis on 12.5% SDS polyacrylamide gels. Pre-stained low molecular weight protein markers (BioRad Laboratories, Missasauga, Ont) were used as standards. Proteins were electroblotted onto polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA), and analyzed using standard protocols[21]. The HA-tagged proteins were detected using rat anti–HA monoclonal antibodies (clone CF10 at a 1/5000 dilution, Boehringer Mannheim, Laval, Que) and horse radish peroxidase (HRP)-labeled

donkey anti-rat polyclonal antibodies (1/5000 dilution, Jackson Laboratories, West Grove, PA). The HRP label was visualized on X-ray film using ECL Chemiluminesence (Amersham Pharmacia Biotech, Baie d'Urfe, Que) according to the manufacturer's instructions.

Analysis of Antiproliferative Activity. The ability of cytarabine to inhibit the proliferation of L1210/B23.1 cells was assessed as follows. Cells from actively proliferating cultures were seeded into 12-well tissue culture plates (1×10^5 cells/well) in the absence or presence of graded concentrations of cytarabine. Cell numbers were determined by electronic particle counting at 24 and 48 h of incubation. The cytarabine concentrations that inhibited proliferation by 50% (IC_{50} values) were derived from linear regression analysis of growth rates plotted as a function of cytarabine concentrations.

RESULTS

The cDNA encoding hCNT1 was subcloned into a mammalian expression vector (pcDNA3) that was used previously for successful transient expression of rCNT1 in COS-1 cells[9]. The initial experiments were conducted in both the COS-1 (of monkey origin) and HeLa (of human origin) cell lines, and the subsequent analysis of transportability of cytosine-containing nucleosides by recombinant hCNT1 was conducted in the human cell line. Since COS-1 and HeLa cells exhibit equilibrative nucleoside transport activity[5,22], dilazep, which is a potent inhibitor of the equilibrative nucleoside transporters[23], was routinely used at high concentrations (100 μM) to pharmacologically block endogenous activity in recipient cells during functional studies of recombinant hCNT1. When COS-1 (Fig. 2A) or HeLa (Fig. 2B) cells were transfected with pcDNA3-hCNT1 and assayed for the uptake of 10 μM [^{3}H]uridine, cell-associated radioactivity accumulated rapidly relative to that observed in cultures transfected with the "empty" vector (controls). The latter cultures exhibited low levels of cell-associated radioactivity which, because they were similar to values observed in the presence of excess non-radioactive uridine (data not shown), were probably due to non-facilitated diffusion and/or nonspecific binding. The stimulation of uridine uptake in the pcDNA3-hCNT1-transfected cultures indicated that recombinant hCNT1 was functional. An increase in cell-associated radioactivity was not observed in the pcDNA3-hCNT1-transfected cultures that were incubated in medium that lacked sodium (data not shown),

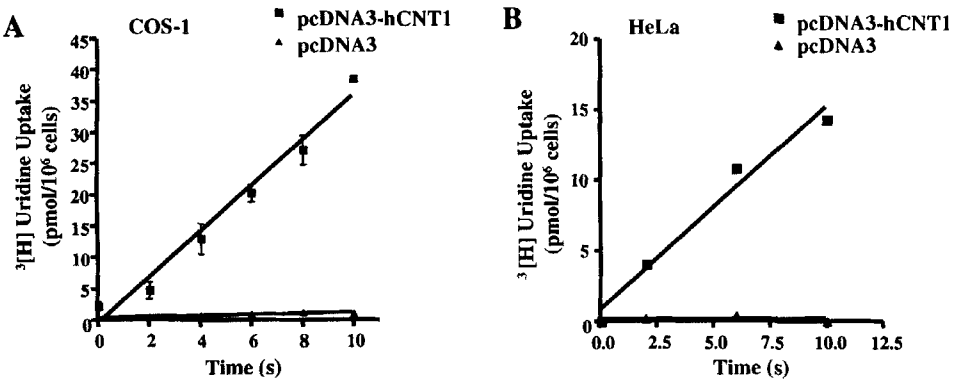

FIG. 2 Stimulation of initial rates of uptake of [^{3}H]uridine into adherent cultures of COS-1 **(A)** and HeLa **(B)** cells by transient transfection with pcDNA3-hCNT1. Uptake of 10 μM [^{3}H]uridine, in the presence of sodium, was measured in cultures transfected with either pcDNA3-hCNT1 (squares) or pcDNA3 (triangles), as described in Materials and Methods. Assays were performed in the presence of 100 μM dilazep to inhibit endogenous equilibrative (*es*, *ei*) transport activity. The data are plotted as linear regression lines with r^2 values of 0.98 (pcDNA3-hCNT1) and 0.93 (pcDNA3) in COS-1 cells and 0.97 (pcDNA3-hCNT1) and 0.93 (pcDNA3) in HeLa cells. Each value represents the mean (± S.D.) of three determinations; error bars are not shown where S.D. values were small.

confirming that the stimulation was sodium-dependent and therefore concentrative, as expected for an hCNT1-mediated transport process.

To confirm that the transiently transfected recipient cells produced recombinant hCNT1, a construct that encoded hCNT1 fused with the HA immunoepitope[19,20] was introduced into HeLa cells (Fig. 3). Lysates of cells transfected with pcDNA3-hCNT1-HA (encoding epitope-tagged recombinant hCNT1) or pcDNA3-β-gal (encoding non-immunoreactive bacterial protein) were subjected to polyacrylamide gel electrophoresis, and the proteins were then transferred to membranes and probed with anti-HA antibodies. A 72-kDa band was observed in the immunoblots of lysates from cells transfected with the hCNT1-HA-containing vector but not in those from cells transfected with the β-gal-containing vector. Thus, the hCNT1-HA-transfected cells produced an immunoreactive recombinant protein of the expected electrophoretic mobility.

In many cell types, nucleoside transport across the plasma membrane occurs more rapidly than subsequent metabolism and rapid-assay technologies are required to

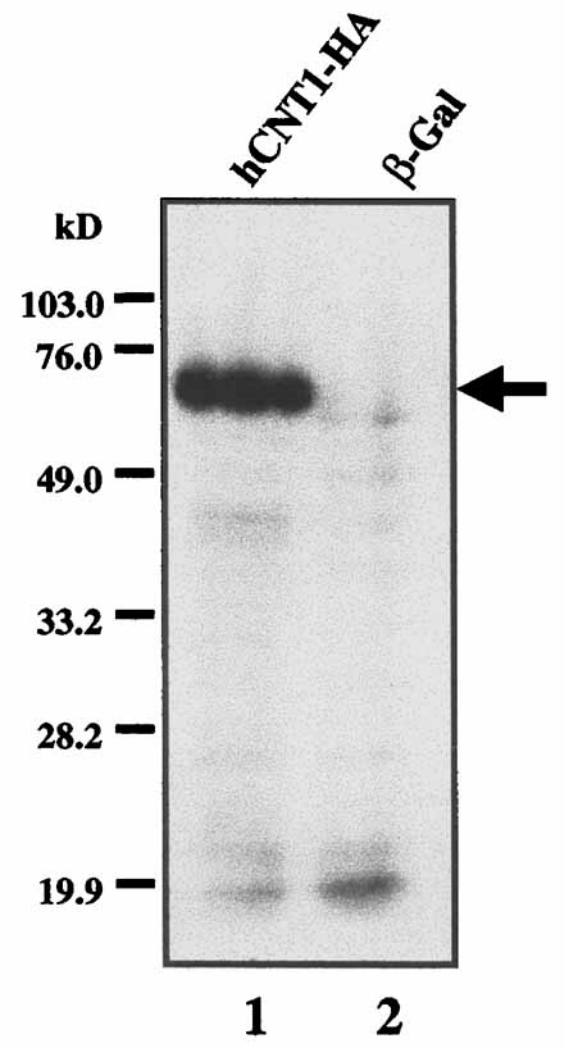

FIG. 3 Production of recombinant hCNT1 protein in transiently transfected HeLa cells. Whole cell lysates were prepared from cells transfected with either pcDNA3-hCNT1-HA (lane 1) or pcDNA3-β-gal (lane 2) and subjected to polyacrylamide gel electrophoresis and immunoblotting with an anti-HA antibody as described in Materials and Methods. The arrow indicates the location of hCNT1-HA protein.

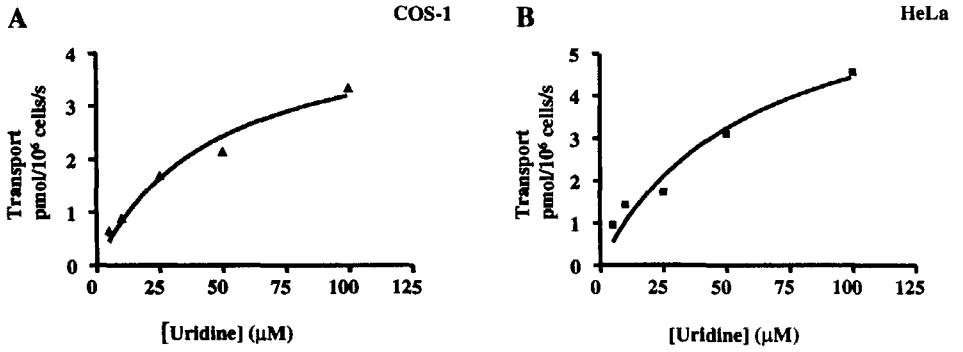

FIG. 4 Demonstration of saturable transport of uridine mediated by recombinant hCNT1 in transiently transfected cells. Cultures of COS-1 **(A)** and HeLa **(B)** cells that had been transiently transfected with pcDNA3-hCNT1 were exposed to graded concentrations of [^{3}H]uridine in the presence of 100 μM dilazep (to inhibit *es-* and *ei-*mediated transport activity) and time courses of accumulation of cell-associated radioactivity were measured as shown in Fig 2. Initial uptake rates were determined by linear regression analysis of time courses and are plotted as a function of the concentration of [^{3}H]uridine present in transport assays. Each value represents the mean of triplicate values (±S.D.); error bars are not shown where S.D. values were smaller than the symbols.

accurately define time courses of uptake of isotopically labeled nucleosides that are required for computation of transport rates. In the experiments of Fig. 2 with 10 μM [^{3}H]uridine and in others with a broad range of uridine concentrations (data not shown), linear uptake time courses were obtained between 0 and 10 sec and it was thus feasible to measure initial rates of uptake (i.e., transport) of [^{3}H]uridine mediated by recombinant hCNT1 over a range of different extracellular permeant concentrations. The hCNT1-dependent uptake of [^{3}H]uridine observed in hCNT1-producing COS-1 and HeLa cells (Fig. 4) exhibited a hyperbolic dependency on extracellular permeant concentration, indicating saturability of the transport process at higher permeant concentrations. The Michaelis-Menten analysis yielded apparent K_m values of 45 and 59 μM and V_{max} values of 4.7 and 7.1 pmol/10^6 cells/s, respectively, for COS-1 and HeLa cells. The K_m values were in close agreement with the value (45 _M) reported for recombinant hCNT1 in *Xenopus* oocytes[6]. V_{max} values were dependent on expression levels and thus could not be compared between experimental systems.

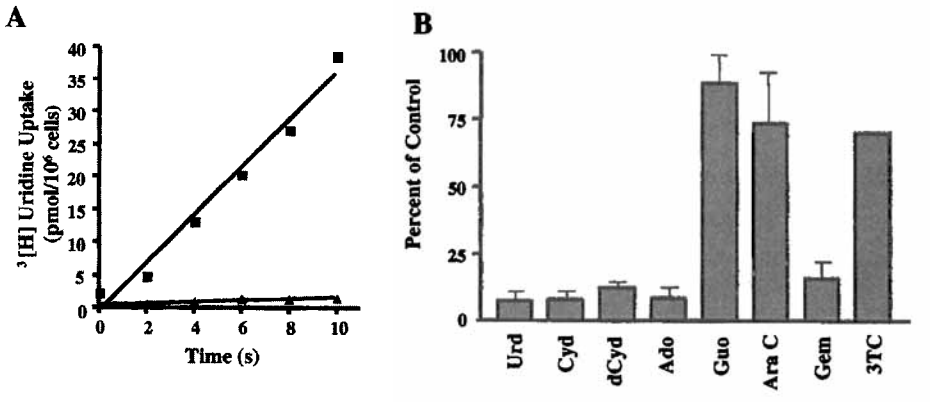

FIG. 5 Inhibition of hCNT1-mediated transport of [^{3}H]uridine into HeLa cells by non-radioactive uridine and other nucleosides. **(A)** Uptake of 10 μM [^{3}H]uridine by HeLa cells transiently transfected with pcDNA3-hCNT1 was determined as in Fig. 1B in the absence (squares) or presence (triangles) of 1 mM non-radioactive uridine (Urd). **(B)** The transport rates obtained from the time courses of Panel A and from similar time courses (not shown) in the absence (uninhibited control values) or presence of 1 mM cytidine (Cyd), deoxycytidine (dCyd), adenosine (Ado), guanosine (Guo), cytarabine (araC), gemcitabine (Gem) or lamivudine (3TC) were used to compute the experimental values expressed as "percent of control". Each value represents the mean (±S.D.) of three determinations; error bars are not shown where S.D. values were small.

Subsequent experiments were conducted in HeLa cells to define the nucleoside transport activity associated with recombinant hCNT1 when produced in a human genetic background. Uptake of 10 μM [^{3}H]uridine by transfected HeLa cells was inhibited almost completely by unlabeled 1 mM uridine (Fig. 5A); since the inhibitory concentration of uridine was about 20-fold higher than its K_m value, the residual uptake was likely due to a combination of hCNT1-mediated transport and non-facilitated diffusion. The ability of other nucleosides, also at concentrations of 1 mM, to inhibit [^{3}H]uridine uptake by hCNT1 was assessed by the same procedure (Fig. 5B). Adenosine, cytidine and deoxycytidine all inhibited hCNT1-mediated uptake of [^{3}H]uridine to the same extent as unlabeled uridine, whereas guanosine, which is known to be transported by hCNT2, but not by hCNT1[1,24-27], had no effect on hCNT1-mediated transport of uridine. The ability of 1 mM gemcitabine, cytarabine and lamivudine to inhibit [^{3}H]uridine uptake was also assessed. Gemcitabine inhibited [^{3}H]uridine uptake by

transiently transfected HeLa cells almost completely and to the same extent as was observed for 1 mM unlabeled uridine. In contrast, [^{3}H]uridine uptake was only partially inhibited by cytarabine and lamivudine, suggesting that these nucleosides could be poorly transported, if at all, by recombinant hCNT1.

The concentration-effect relationships for inhibition of hCNT1-mediated transport of [^{3}H]uridine by cytidine and deoxycytidine were investigated in the experiments of Fig. 6. The concentrations that reduced transport by 50% (IC_{50} values) were almost the same (cytidine 37 μM; deoxycytidine, 31 μM) and yielded apparent K_i values, respectively, of 41 and 34 μM when analyzed by the equation for competitive inhibition[28], using the K_m value for hCNT1-mediated uridine transport determined in HeLa cells (see Fig. 4). The transportability of cytidine and deoxycytidine was then determined directly by measurements of initial rates of uptake of ^{3}H-labeled permeants at different concentrations by transiently transfected cultures of HeLa cells. Michaelis-Menten analysis indicated transporter saturability for both cytidine and deoxycytidine; the representative experiment shown in Fig. 7 yielded, respectively, apparent K_m values of 140 μM and 150 μM and V_{max} values of 18 and 6 pmol/10^6 cells/s. In other experiments (not shown) conducted similarly, apparent K_m values that ranged from 50 to 250 _M were obtained for both compounds. The discrepancies between K_m and K_i values may have resulted from (i) variability in transfection efficiencies between experiments, (ii) the low affinities of hCNT1 for these compounds, which necessitated the use of high concentrations thereby increasing the likelihood of non-facilitated diffusion, and/or (iii) a small amount of transport mediated by another transport process.

The lack of effectiveness of cytarabine and lamivudine, relative to that of gemcitabine, to inhibit hCNT1-mediated transport of uridine (see Fig. 5B) was further examined in experiments in which the concentration of [^{3}H]uridine was less than its K_m value and the concentrations of test inhibitor were in considerable excess (Table 1). Gemcitabine, which is a high-affinity permeant of recombinant hCNT1[15,16], was present in assay mixtures at 10- and 50-fold higher concentrations than [^{3}H]uridine whereas cytarabine and lamivudine were present at 50- and 500-fold higher concentrations. In the presence of 0.1 mM test compound, almost complete inhibition of hCNT1-mediated transport of [^{3}H]uridine was observed for gemcitabine, whereas partial inhibitions were observed for the other two cytosine-containing compounds (cytarabine > lamivudine) at

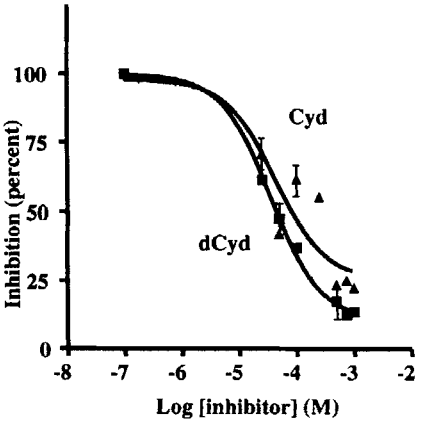

FIG. 6 Inhibition of hCNT1-mediated transport of [^{3}H]uridine into HeLa cells by cytidine and deoxycytidine. Time courses of uptake 10 μM [^{3}H]uridine by pcDNA3-hCNT1 transfected HeLa cells were determined in the absence or presence of graded concentrations of cytidine or deoxycytidine and the initial rates obtained therefrom were used to compute "percent of control" values as in Fig 5. Each value represents the mean (± S.D.) of three determinations; error bars are not shown where S.D. values were small.

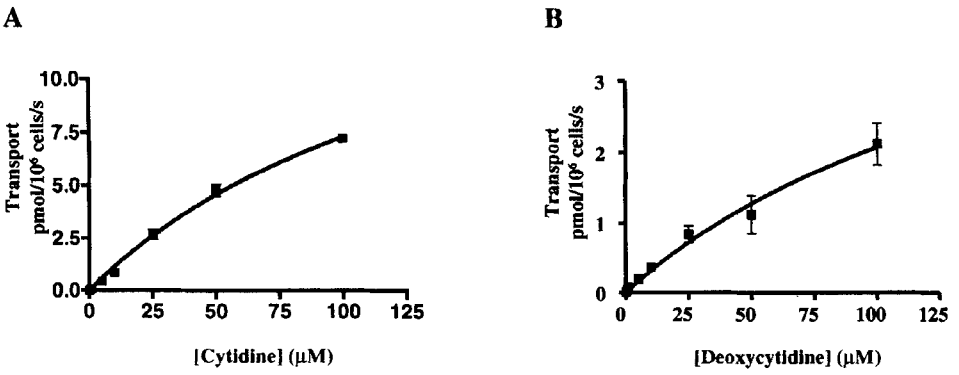

FIG. 7 Demonstration of saturable transport of cytidine and deoxycytidine mediated by recombinant hCNT1 in transiently transfected HeLa cells. Cultures of HeLa cells that had been transiently transfected with pcDNA3-hCNT1 were exposed to graded concentrations of [^{3}H]cytidine **(A)** and [^{3}H]deoxycytidine **(B)** in the presence of 100 μM dilazep (to inhibit *es*- and *ei*-mediated transport activity) and time courses of accumulation of cell-associated radioactivity were measured as shown in Fig 2. Initial uptake rates were determined in triplicate by linear regression analysis of time courses and are plotted as a function of permeant concentrations. Each point represents the mean (± S.D.) rate of uptake derived from linear analysis of experiments performed in triplicate; error bars are not shown where S.D. values were small.

TABLE 1. A comparison of the abilities of cytarabine, gemcitabine and lamivudine to inhibit uridine transport by recombinant hCNT1. Time courses of uptake of 10 μM [^{3}H]uridine by pcDNA3-hCNT1transfected HeLa cells were determined in the absence or presence of the indicated concentrations of cytidine (Cyd), cytarabine (araC), gemcitabine (Gem) or lamivudine (3TC) and means of triplicate values (± S.D.) for the initial rates obtained therefrom were used to compute "percent of control" values as in Fig 5.

Drug	Concentration (mM)	Uridine Transport Rate	
		Absolute rate (pmole/10^6 cells/s)	Relative rate (Percent of Control)
Cyd	0	2.9±.045	100
	0.1	0.77±0.05	27
	0.5	0.12±0.04	3
Ara C	0	2.6 ± 0.04	100
	0.5	1.6 ± 0.12	62
	0	4.6 ± 1.04	100
	5.0	1.5 ± 0.17	33
3TC	0	6.4 ± 0.07	100
	0.5	5.2 ± 0.31	81
	5.0	5.7 ± 0.7	89
Gem	0	2.8 ± 0.18	100
	0.1	0.4 ± 0.02	14
	0.5	0.3 ± 0.07	11

concentrations as high as 5 mM. For comparison, the ability of cytidine to inhibit uridine transport was also examined. When tested at 0.1 mM, cytidine was a less effective inhibitor than gemcitabine, a result that was consistent with the observed differences in K_m values of cytidine and gemcitabine. When tested at 0.5 mM, both compounds reduced uridine transport to low levels (3-11%); the low rates of uptake observed in these experiments were attributed to a combination of residual hCNT1-mediated activity, non-facilitated diffusion and/or non-specific binding. The marked differences in hCNT1-mediated transportability suggested that cells with different levels of hCNT1 activity would be differentially sensitive to the pharmacological activities of cytarabine, gemcitabine and lamivudine.

TABLE 2. A comparison of the anti-proliferative effects of cytarabine and gemcitabine against L1210 leukemia cell lines with different nucleoside transport activities. IC_{50} values for gemcitabine (Gem) against the three cell lines are from Mackey *et al.*[16] and those for cytarabine (AraC) against L1210/DU5 and L1210/DNC3 are from Crawford *et al.*[17] and against L1210/B23.1 were determined as described in Materials and Methods. The cytotoxicity ratio is defined as the relative decrease of the IC_{50} value for the transport-competent cell lines (B23.1, DU5) compared with that for the transport-deficient cell line (DNC3) and was calculated by dividing the IC_{50} for DNC3 by the IC_{50} for either B23.1 or DU5.

L1210 Leukemia Cell Line	NT Activity	AraC		Gem	
		IC_{50} (μM)	Cytotoxicity Ratio	IC_{50} (μM)	Cytotoxicity Ratio
DNC3	null	6.12	1	13	1
B23.1	*es*	0.019	322	0.1	130
DU5	*cit* (rCNT1)	0.80	8	0.01	1300

Table 2 compares cytotoxicities of cytarabine and gemcitabine in a panel of murine cell lines that lacked the capacity for nucleoside transport altogether or possessed either a single broadly selective transport activity (murine *es*) or a single pyrimidine-nucleoside concentrative transport activity (rCNT1). The antiviral drug lamivudine was not included since it has low toxicities against uninfected cells[29]. The nucleoside-transport deficient cells (L1210/DNC3) survive prolonged exposures to cytarabine[17] or gemcitabine[16] at concentrations substantially higher than those that were effective against the cells used in this study, which exhibited only *es* activity (L1210/B23.1). The latter results confirmed the previous conclusions[30] that *es*-type processes accept both drugs as permeants and their cellular uptake requires the presence of functional transporters at the cell surface. In contrast, cells that exhibit only CNT1 activity (L1210/DU5) are relatively resistant to cytarabine[17] and highly sensitive to gemcitabine[16], as would be predicted from the observed differences in CNT1-dependent transportability of these drugs.

DISCUSSION

The human concentrative transporter, hCNT1, is selective for pyrimidine nucleosides and adenosine as demonstrated in the *Xenopus*-oocyte expression system[6].

When expressed in *Xenopus* oocytes, recombinant hCNT1 exhibited K_m values of 45 μM for uridine, 7 μM for thymidine and 15 μM for adenosine and was inhibited by 1 mM cytidine and deoxyadenosine but not by guanosine or inosine. The low V_{max} values observed for adenosine suggest that it may function *in vivo* as an inhibitor rather than a permeant. Deoxyadenosine was also shown to be a potent inhibitor (K_i, 46 μM) of hCNT1-mediated uridine transport but was not itself actually transported. Zidovudine and zalcitabine at 1 mM inhibited uridine transport and zidovudine was a low-affinity permeant (K_m > 0.5 mM); zalcitabine transport was not examined[6,13]. In a separate study[15], gemcitabine was shown to be a high-affinity permeant (K_m, 24 μM) of recombinant hCNT1 in the *Xenopus*-oocyte expression system. High-affinity hCNT1-mediated transport of gemcitabine (K_m, 18 μM) has also been shown in transiently transfected HeLa cells[16].

The demonstration that gemcitabine is a high-activity permeant for recombinant hCNT1[15,16] suggested that other cytosine-containing nucleosides would also be permeants. The present study, therefore, assessed the transportability of cytidine, deoxycytidine, cytarabine, lamivudine and, for comparison, gemcitabine, using the approach of transient expression of hCNT1 cDNA in cultured human cells. There have been no previous reports of direct measurement of hCNT1-mediated uptake of cytidine or deoxycytidine in human cells (or preparations therefrom), although there are several in which cytidine inhibited sodium-dependent transport of either [^{3}H]uridine or [^{3}H]thymidine by an apparent *cit*-type transport process[31,32].

Functional expression of hCNT1 cDNA was verified by demonstration of sodium-dependent uridine transport in COS-1 and HeLa cells transiently transfected by procedures used previously in cultured COS-1 cells for expression of rCNT1 cDNA[9]. The K_m values for recombinant hCNT1-mediated uridine transport in COS-1 and HeLa cells were, respectively, 45 and 59 μM, and were essentially the same (45 μM) as was obtained previously[6] in the *Xenopus*-oocyte expression system. The V_{max} values obtained in these studies are not comparable since transport rates are dependent on the amount of recombinant protein produced and correctly processed to the plasma membrane and expression levels differ substantially between different systems and often between experiments conducted with the same system. The presence of the hCNT1 protein in

HeLa cell membranes was demonstrated by immunoblotting after mock transfection or transfection with a cDNA construct that encoded an epitope-tagged version of hCNT1. A 72-kDa band was observed in the immunoblots from cells transfected with the hCNT1-HA-containing vector but not in those from cells transfected with the control vector, indicating that the hCNT1-HA-transfected cells produced an immunoreactive recombinant protein of the expected electrophoretic mobility.

When cytidine and deoxycytidine were assessed for their ability to inhibit hCNT1-mediated transport of ^{3}H-uridine, apparent K_i values of 41 and 34 μM, respectively, were obtained. While these results indicated that cytidine and deoxycytidine have the capacity to bind to recombinant hCNT1 with similar affinities, their activity as permeants could only be established by direct measurement of hCNT1-mediated fluxes. Transient transfection of HeLa cells with hCNT1 cDNA resulted in an increased capacity for saturable, sodium-dependent transport of cytidine and deoxycytidine with apparent K_m values, respectively, of 140 _M and 150 _M. These results indicated that hCNT1 accepts cytidine and deoxycytidine equally well as permeants. In the studies that compared the abilities of gemcitabine, cytarabine and lamivudine to inhibit hCNT1-mediated transport of uridine, only gemcitabine, which has previously been shown to be a high-affinity permeant (K_m values, 24 and 18 μM) of recombinant hCNT1[15,16], inhibited transport completely. The marginal inhibitions observed with cytarabine and lamivudine indicated that hCNT1 binds these compounds with low affinity and therefore plays little role, if any, in their uptake into human cells. The order of preference (gemcitabine > cytidine = deoxycytidine >> cytarabine) for interaction of the compounds with hCNT1 indicates that the 2'-position of the sugar is a key determinant of permeant binding to hCNT1.

Mediated transport of gemcitabine and cytarabine into cells is required for cytotoxicity as shown by comparing their cytotoxicities against cells that are nucleoside-transport defective with those against cells with drug-transport capacity (see Table 2). Human and murine cell lines that have been made nucleoside-transport defective either genetically or pharmacologically exhibit high-level resistance to both gemcitabine and cytarabine (for review, see Mackey *et al.*[30]), an indication that their cellular uptake requires the presence of functional transporters in the plasma membrane. Cells with either *es*-type (ENT1) or *cit*-type (rCNT1) transporters are highly sensitive to

gemcitabine cytotoxicity[16] whereas cells with *es*-type (ENT1) transporters are highly sensitive to cytarabine cytotoxicity[30] (see also Table 2) and cells with *cit*-type (rCNT1) transporters are relatively resistant to cytarabine cytotoxicity[17]. Direct flux analysis has established that cytarabine is not a permeant of rCNT1. The present study demonstrated that hCNT1 has relatively low affinity for cytarabine, indicating that the presence of functional hCNT1 in human cells, like that previously shown for rCNT1 in murine cells[17], will have little, if any, impact on cytarabine cytotoxicity. Although lamivudine was not included in the cytotoxicity studies because of its low toxicity against uninfected cells[29], its poor inhibitory activity against hCNT1-mediated uridine uptake indicated that hCNT1 is also not an important determinant of lamivudine efficacy.

ACKNOWLEDGMENTS: Supported by the Alberta Cancer Board and by Canadian Cancer Society and Terry Fox Foundation grants from the National Cancer Institute of Canada. IRC was a Postdoctoral Fellow of the Alberta Heritage Foundation for Medical Research and CEC was a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada during the course of this work. JDY is a Heritage Medical Scientist of the Alberta Heritage Foundation for Medical Research.

REFERENCES

(1) Cass, C. E. In *Drug Transport in Antimicrobial and Anticancer Chemotherapy*; Georgopapadakou, N. H., Ed.; Marcel Deker: New York, NY, **1995**, p 403-451.

(2) Cass, C. E.; Young, J. D.; Baldwin, S. A. *Biochem. Cell Biol.* **1998**, ***76,*** 761-770.

(3) Griffiths, M.; Beaumont, N.; Yao, S. Y.; Sundaram, M.; Boumah, C. E.; Davies, A.; Kwong, F. Y.; Coe, I.; Cass, C. E.; Young, J. D.; Baldwin, S. A. *Nature Medicine* **1997**, ***3,*** 89-93.

(4) Griffiths, M.; Yao, S. Y.; Abidi, F.; Phillips, S. E.; Cass, C. E.; Young, J. D.; Baldwin, S. A. *Biochem. J.* **1997**, ***328,*** 739-43.

(5) Crawford, C. R.; Patel, D. H.; Naeve, C.; Belt, J. A. *J. Biol. Chem.* **1998**, ***273,*** 5288-93.

(6) Ritzel, M. W. L.; Yao, S. Y. M.; Huang, M. Y.; Elliott, J. F.; Cass, C. E.; Young, J. D. *Am. J. Physiol.* **1997**, ***272,*** C707-C714.

(7) Ritzel, M. W.; Yao, S. Y.; Ng, A. M.; Mackey, J. R.; Cass, C. E.; Young, J. D. *Mol. Membr. Biol.* **1998**, ***15,*** 203-11.

(8) Baldwin, S. A.; Mackey, J. R.; Cass, C. E.; Young, J. D. *Mol. Med. Today* **1999**, ***5,*** 216-224.

(9) Fang, X.; Parkinson, F. E.; Mowles, D. A.; Young, J. D.; Cass, C. E. *Biochem. J.* **1996**, ***317,*** 457-65.

(10) Lang, T.; Young, J. D.; Caee, C. E. *FASEB J.* **1999**, ***13,*** A1391:349.

(11) Vickers, M. F.; Mani, R. S.; Sundaram, M.; Hogue, D. L.; Young, J. D.; Baldwin, S. A.; Cass, C. E. *Biochem. J.* **1999**, ***339,*** 21-32.

(12) Huang, Q. Q.; Yao, S. Y.; Ritzel, M. W.; Paterson, A. R.; Cass, C. E.; Young, J. D. *J. Biol. Chem.* **1994**, ***269,*** 17757-60.

(13) Yao, S. Y.; Cass, C. E.; Young, J. D. *Mol. Pharmacol.* **1996**, ***50,*** 388-93.

(14) Yao, S. Y.; Ng, A. M.; Ritzel, M. W.; Gati, W. P.; Cass, C. E.; Young, J. D. *Mol. Pharmacol.* **1996**, ***50,*** 1529-35.

(15) Mackey, J. M.; Yao, S. Y. M.; Smith, K. M.; Karpinski, E.; Baldwin, S. A.; Cass, C. E.; Young, J. D. *J. Natl. Cancer Inst.* **1999**, ***in press***.

(16) Mackey, J. R.; Mani, R. S.; Selner, M.; Mowles, D.; Young, J. D.; Belt, J. A.; Crawford, C. R.; Cass, C. E. *Cancer Res.* **1998**, ***58,*** 4349-57.

(17) Crawford, C. R.; Cass, C. E.; Young, J. D.; Belt, J. A. *Biochem. Cell Biol.* **1998**, ***76,*** 843-851.

(18) Vijayalakshmi, D.; Dagnino, L.; Belt, J. A.; Gati, W. P.; Cass, C. E.; Paterson, A. R. *J. Biol. Chem.* **1992**, ***267,*** 16951-6.

(19) Niman, H. L.; Houghten, R. A.; Walker, L. E.; Reisfeld, R. A.; Wilson, I. A.; Hogle, J. M.; Lerner, R. A. *Proc. Natl. Acad. Sci. USA* **1983**, ***80,*** 4949-4953.

(20) Wilson, I. A.; Niman, H. L.; Houghten, R. A.; Cherenson, A. R.; Connolly, M. L.; Lerner, R. A. *Cell* **1984**, ***37,*** 767-778.

(21) *Current Protocols in Molecular Biology*; Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K., Eds.; John Wiley & Sons, Inc.: New York, N.Y., **1997**.

(22) Cass, C. E.; Belt, J. A.; Paterson, A. R. *Prog. Clin. Biol. Res.* **1987**, ***230,*** 13-40.

(23) Paterson, A. R.; Harley, E. R.; Cass, C. E. *Biochem. J.* **1984**, ***224,*** 1001-8.

(24) Crawford, C. R.; Ng, C. Y.; Belt, J. A. *J. Biol. Chem.* **1990**, ***265,*** 13730-4.

(25) Dagnino, L.; Bennett, L. L., Jr.; Paterson, A. R. *J. Biol. Chem.* **1991**, ***266,*** 6312-7.

(26) Vijayalakshmi, D.; Belt, J. A. *J. Biol. Chem.* **1988**, ***263,*** 19419-23.

(27) Williams, T. C.; Jarvis, S. M. *Biochem. J.* **1991**, ***274,*** 27-33.

(28) Cheng, Y.-C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, ***22,*** 3099 - 3108.

(29) Rahn, J. J.; Kieller, D. M.; Lorne, D.; Tyrrell, J.; Gati, W. P. *Antimicrob. Agents Chemother.* **1997**, ***41 (5),*** 918-923.

(30) Mackey, J. R.; Baldwin, S. A.; Young, J. D.; Cass, C. E. *Drug Res. Updates* **1998**, ***1,*** 310-324.

(31) Gutierrez, M. M.; Brett, C. M.; Ott, R. J.; Hui, A. C.; Giacomini, K. M. *Biochim. Biophys. Acta.* **1992**, ***1105,*** 1-9.

(32) Patil, S. D.; Unadkat, J. D. *Am. J. Physiol. Gastro. Liver Physiol.* **1997**, ***35 (6),*** G1314-G1320.