Functional characterization of a recombinant sodium-dependent nucleoside transporter with selectivity for pyrimidine nucleosides (cNT1_{rat}) by transient expression in cultured mammalian cells

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We have demonstrated that monkey kidney (COS-1) cells have a single type of nucleoside transport process, which, because it was equilibrative, sodium-independent and could be inhibited by nitrobenzylthioinosine (NBMPR), was identified as the 'equilibrative sensitive' or 'es' transporter. Using NBMPR or dilazep to inhibit the endogenous nucleoside transport activity, we have transiently expressed a cDNA that encodes an inhibitor-insensitive, concentrative nucleoside transporter protein (cNT1_{rat}) of rat intestine in COS-1 cells. The production of recombinant cNT1_{rat} was examined by immunoblotting using an epitope-tagged construct and by analysis of inward fluxes of ³H-labelled nucleosides. Recombinant cNT1_{rat} was sodium-dependent and selective for pyrimidine nucleosides, with approximate *K_m* values

of 21 μ M, 12.5 μ M and 15 μ M for uridine, thymidine and adenosine, respectively. Although adenosine exhibited high affinity for the recombinant transporter, its *V_{max}* value was low. A variety of anti-viral and anti-cancer nucleoside drugs inhibited cNT1_{rat}-mediated uptake of uridine by transfected COS-1 cells although to different extents (Floxidine > Idoxuridine > Zidovudine > Zalcitabine > Cytarabine > Gemcitabine), suggesting that the concentrative pyrimidine-selective nucleoside transporters, of which cNT1_{rat} is a representative, may play a role in cellular uptake of these drugs. The cNT1_{rat}/COS-1 expression system is a useful tool for analysis of cNT1_{rat}-mediated transport processes.

INTRODUCTION

Many nucleosides and nucleoside analogues have important physiological activities and clinical applications. Adenosine is a local signalling molecule and is known to have a regulatory function in physiological and metabolic activities, including lipolysis, neurotransmitter release, platelet aggregation, coronary vasodilation, cardiac contractility and renal vasoconstriction [1]. Several important anti-cancer and anti-viral drugs are nucleoside analogues [2]. Because most nucleosides and nucleoside analogues are highly hydrophilic and do not readily cross the lipid bilayer of biological membranes in the absence of functional nucleoside transport processes, the presence of functional nucleoside transporter (NT) proteins in plasma membranes is an important prerequisite for physiological and pharmacological activities.

NT-mediated processes in mammalian cells can be divided into two classes, depending on whether they are equilibrative or concentrative. The equilibrative NTs are driven by the concentration gradient of nucleoside permeants, whereas the concentrative NTs are driven by transmembrane sodium gradients. Equilibrative NTs, which are widely distributed in mammalian cells and tissues [3–5], can be further classified into two subtypes: one subtype (*es*) is sensitive to inhibition by nanomolar concentrations of nitrobenzylthioinosine (NBMPR), whereas the other subtype (*ei*) is not sensitive to inhibition by NBMPR. The concentrative NTs comprise four major subtypes: N1/*cif* exhibits selectivity for purine nucleosides and uridine and has been found in many different cell types [6–10]; N2/*cit* is selective for pyrimidine nucleosides and adenosine and has been observed in

freshly isolated enterocytes and in brush-border vesicles from renal epithelial cells of bovine, rat and rabbit [11–14]; N3/*cib* has broad specificity for both purine and pyrimidine nucleosides and has been observed in cultured colorectal (Caco-2) cells [15] and differentiated human promyelocytic (HL-60) leukaemia cells [16,17]; N4/*cit* is pyrimidine-selective but also transports adenosine and guanosine and has been found only in brush-border vesicles from human kidney [18]. The four concentrative NTs are unaffected by high concentrations (> 10 μ M) of the classic inhibitors of equilibrative NT-mediated processes (NBMPR, dilazep, dipyridamole).

Many cell types simultaneously exhibit two or three NT-mediated processes and it is often difficult to study the characteristics of a particular process in the presence of others with overlapping permeant selectivities. Although a few cell types that naturally express a single transporter subtype have been used profitably for structure–activity studies, such a cell type has not been available for the concentrative pyrimidine-selective NTs. N2/*cit* and N4/*cit* activities have been studied only in freshly isolated mouse intestinal epithelial cells [11], brush-border membrane vesicles from kidney [19] and oocytes of *Xenopus laevis* microinjected with rabbit intestinal mRNA [20,21]. These preparations are time-consuming, and many of them contain more than one NT-mediated process. A system that exhibits a single transporter subtype would provide a more convenient and specific approach for analysis of functional characteristics.

A cDNA encoding a pyrimidine-selective NT protein has been cloned from a rat intestinal cDNA library by functional expression in *Xenopus* oocytes [22]. This protein, named concen-

Abbreviations used: araC, Cytarabine (1- β -D-arabinofuranosylcytosine); AZT, Zidovudine (3'-azido-3'-deoxythymidine); ddC, Zalcitabine (2',3'-dideoxycytidine); FUDR, Flouxidine (5-fluoro-2'-deoxyuridine); 3TC, 3'-thiacytidine; IUdR, Idoxuridine (5-iodo-2'-deoxyuridine); dFdC, Gemcitabine (2',2'-difluorodeoxycytidine); NT, nucleoside transporter; NBMPR, nitrobenzylthioinosine; RT, room temperature; DMEM, Dulbecco's modified Eagle's medium; X-Gal, 5-bromo-4-chloro-3-indolyl-D-galactopyranoside; SV40, simian virus 40.

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trative NT1 of rat (cNT1_{rat}), is predicted to have 648 amino acids with 14 transmembrane domains. When expressed in *Xenopus* oocytes, cNT1_{rat} exhibits high levels of nucleoside transport activity with N2/*cit*-like characteristics. Database searching revealed homology between cNT1_{rat} and a H⁺-dependent pyrimidine-selective NT of *Escherichia coli* (NUPC). Another sodium-dependent NT of rat liver with selectivity for purine nucleosides (SPNT) has just been reported [23]. These structurally related proteins appear to be members of a cation-dependent family of NTs [22].

Although the cDNAs encoding NT proteins have all been expressed in *Xenopus* oocytes [22,23], none has been successfully expressed in a mammalian expression system. The goal of this study was to transiently express cNT1_{rat} cDNA in a mammalian cell line at levels that allowed kinetic characterization of fluxes of [³H]nucleosides. A readily transfectable cell line (monkey kidney COS-1) was shown to exhibit a single inhibitor-sensitive (*es*) NT and was, therefore, used as the recipient of cNT1_{rat}, since the latter could be blocked completely with NBMPR. Kinetic studies were conducted with uridine, thymidine and adenosine, providing the first detailed analysis of N2/*cit*-mediated NT activity in intact mammalian cells. We also tested the inhibition ability of several clinically important anti-viral and anti-cancer nucleoside drugs, including Zidovudine (AZT), Zalcitabine (ddC), Flouxidine (FUDR), Idoxuridine (IUdR), Cytarabine (araC) and Gemcitabine (dFdC).

EXPERIMENTAL

Materials

Dulbecco's modified Eagle's medium (DMEM) and calf serum were from Gibco/BRL; Nu-serum culture I supplement was from Collaborative Biomedical; restriction enzymes and DNA modification enzymes were from Gibco/BRL or New England Biolabs; DMSO, NBMPR, dipyrindamole, AZT, ddC, IUdR, FUDR and araC were from Sigma; DEAE-dextran and deoxy-ribonucleoside triphosphates (dNTPs) were from Pharmacia; 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-Gal) was from Boehringer Mannheim Biochemical; D-[5,6-³H]uridine, D-[methyl-³H]thymidine, D-[2,8-³H]adenosine, Bes and EcoLite scintillant were from ICN Biomedicals Inc; pCMVGal was from Clontech; pcDNAI/Amp vector was from Invitrogen Corp; dFdC was a gift from Eli Lilly Inc.; 3'-thiacytidine (3TC) was a gift from Dr. D. L. Tyrrell (University of Alberta); dilazep was a gift from F. Hoffman La Roche & Co. (Basel, Switzerland); anti-*c-myc* monoclonal antibody was from Berkeley Antibody Company; goat anti-(mouse IgG) horseradish peroxidase conjugate was from Bio-Rad; and other materials were obtained from standard commercial sources. Radiolabelled nucleosides were purified with a Varian 5000 HPLC instrument using water-methanol gradients on a C18 reverse-phase column.

Generation of expression construct

For expression in mammalian cells, the full coding sequence of cNT1_{rat} was removed from its original cloning vector pGEM3Z by *Afl*III/*Xba*I digestion. Subsequently, the 5'-overhang was blunt-ended with the Klenow fragment and ligated into pCDNAI/Amp at the *Eco*RV site. The 3'-end of cNT1 was ligated into the vector at the *Xba*I site. The structure of pCDNAI/Amp-cNT1 was confirmed by restriction mapping.

Construction of *c-myc*-tagged cNT1_{rat}

The addition of the *c-myc* tag to the C-terminus of cNT1_{rat} was

accomplished by PCR modification of the cNT1_{rat} cDNA. The 5'-primer was 5'-GTA ATA CGA CTC ACT ATA GGG C. The 3'-primer was GCT CTA GAG CTA CAA GTC TTC TTC AGA-AATAAGCTTTTGTTT TGT GCA GAC TGT GTG GTT GTA-3 and included sequences corresponding to cNT1 (bold letters) and the *c-myc* tag (underlined), as well as a stop codon and *Xba*I restriction site. The PCR product was (i) digested with *Eco*RI and *Xba*I, (ii) subcloned into pBluescript KS, and (iii) its nucleotide sequence at the 3' end (about 300 bp, including the *c-myc* tag) was confirmed by DNA sequencing. The unconfirmed 5'-portion of the PCR product (about 1700 bp) was removed by digesting with *Eco*RV and *Bam*HI. The original cNT1 in pGEM3Z was removed by digestion with *Afl*III (5'-end) and *Bam*HI (3'-end). The 5'-end was then blunt-ended, and the resulting fragment was used to replace the corresponding 5'-portion of the PCR product. The *c-myc*-tagged cNT1_{rat} cDNA was subcloned into pCDNAI/Amp at *Xho*I and *Xba*I to produce pCDNAI/Amp-cNT1_{rat}-*myc*. Sequence analysis established that pCDNAI/Amp-cNT1_{rat}-*myc* lacked the first 78 nucleotides of the predicted open reading frame of cNT1_{rat} cDNA and thus was expected to initiate translation at a secondary start site (the ATG located at residues 93–95 of cNT1_{rat} cDNA).

Protein immunoblotting

Cells were washed once with PBS and harvested in 1 ml of ice-cold preparation buffer (0.25 M sucrose, 1 mM PMSF and 10 mM Hepes) by gentle scraping. The cell suspension was disrupted on ice with a Teflon homogenizer (35 strokes) and centrifuged (900 *g*, 10 min, 4 °C). The resulting supernatant was further centrifuged (30000 *g*, 15 min, 4 °C), and the pellet, which consisted of cell membranes, was resuspended in ice-cold preparation buffer and then stored at –70 °C.

Membrane proteins (25 µg) were solubilized in an equal volume of sample buffer [20 % (v/v) glycerol, 4 % (w/v) SDS, 20 mM Tris/HCl, 0.01 % (w/v) Bromophenol Blue, 2 % mercapto-ethanol] and were separated by SDS/PAGE (12 % gel) and transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore) [24]. Membrane blots were immunostained with anti-*c-myc* monoclonal antibody [1:2000 (v/v) dilution] and goat anti-(mouse IgG) horseradish peroxidase conjugate [1:3000 (v/v) dilution]. Immunoreactive proteins were detected with the enhanced chemiluminescence (ECL) kit (Amersham Life Sciences), according to the manufacturer's instructions, and using Kodak XAR-5 film. The molecular mass standards were: phosphorylase *b*, 106 kDa; BSA, 80 kDa; ovalbumin, 49.5 kDa; carbonic anhydrase, 32.5 kDa; soybean trypsin inhibitor, 27.5 kDa; lysozyme, 18.5 kDa.

Determination of protein concentrations

Protein quantification was performed by the bicinchoninic acid (BCA) assay [25]. The protein concentration of the unknown sample was determined from a standard curve constructed with BSA.

Cell cultures

Monkey kidney COS-1 cells from the American Type Culture Collection were grown as adherent cultures in DMEM containing 5 % (v/v) calf serum and 5 % (v/v) NuSerum. Stock cultures were maintained in the latter medium without antibiotics at 37 °C in 95 % air/5 % CO₂ with 95 % humidity. Cell concentrations were determined using a Coulter Counter Model ZF electronic particle counter (Counter Electronics Inc.). Cultures were removed from dishes with trypsin-EDTA (0.05 % trypsin,

0.02% EDTA in 0.15 M NaCl) and subcultured to 0.2×10^5 cells/T-25 flask every 3–4 days.

Transient transfection of COS-1 cells

pCDNAI/Amp-cNT1 DNA was transiently transfected in COS-1 cells using the procedure described by Ausubel et al. [24]. Actively proliferating cells were plated and grown to an estimated 70% confluence in 100-mm-diam. dishes. The cultures were washed once with sterile PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , pH 7.4), and 5 ml of fresh prewarmed DMEM medium that contained 10% (v/v) NuSerum and 100 μM chloroquine was added to each dish. The DNA (5 μg) was diluted to 0.1 ml in PBS and mixed with 0.1 ml of DEAE-dextran (10 mg/ml) and a 0.2-ml portion of the DNA/dextran mixture was then added dropwise to each dish. The cultures were incubated at 37 °C for 4 h, after which 2 ml of PBS containing 10% (v/v) DMSO was added for 2 min at room temperature (RT). The cultures were washed three times with PBS and, after addition of growth medium [DMEM containing 5% (v/v) calf serum and 5% (v/v) NuSerum], allowed to recover for 24 h at 37 °C in 95% humidified air/5% CO_2 . The cultures were then trypsinized, pooled and centrifuged (100 g, 5 min, RT), and the resulting cell pellet was suspended in growth medium, plated into 60-mm-diam. dishes at 5×10^5 cells/dish and incubated at 37 °C in 95% humidified air/5% CO_2 . Transport studies utilizing transfected cells were conducted 72 h after transfection.

The efficiency of transfection was estimated using a β -galactosidase staining procedure [26]. pCMVGal DNA was transiently transfected in COS-1 cells using the same procedure as described above for cultures transfected with pCDNAI/Amp-cNT1. pCMVGal-transfected COS-1 cells were incubated with fixative solution (0.5% glutaraldehyde in PBS) for 15 min at RT and then washed three times with PBS. The cells were then incubated overnight in a staining mixture (1 mg/ml X-Gal, 20 mM potassium ferricyanide, 20 mM potassium ferrocyanide and 2 mM MgCl_2 in PBS). Cells expressing β -galactosidase stained an Aqua-Blue colour and the transfection efficiency was estimated by counting stained and unstained cells in several microscopic fields. The transfection efficiency varied between experiments (20–30%).

Uptake assays

Nucleoside uptake assays (two or three cultures/condition) were conducted at RT in sodium-containing transport buffer (20 mM Tris/HCl, 3 mM K_2HPO_4 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mM CaCl_2 , 5 mM glucose and 130 mM NaCl, pH 7.4, 300 ± 15 mOsm) or sodium-free transport buffer (20 mM Tris/HCl, 3 mM K_2HPO_4 , 1 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 2 mM CaCl_2 , 5 mM glucose and 130 mM NMDG/HCl, pH 7.4, 300 ± 15 mOsm). Cells were washed once with transport buffer and were (i) processed immediately or (ii), in some experiments, incubated with NBMPR or dilazep at RT for 30 min before the uptake assay. Uptake intervals were started by adding 1.5 ml of transport buffer containing ^3H -labelled permeant and inhibitors. Uptake intervals were ended by rapidly aspirating the permeant solution and immersing the culture dishes in 1.5 litres of ice-cold transport buffer. The dishes were drained, and the cells were solubilized in 1 ml of 5% (v/v) Triton X-100 and combined with 5.0 ml of EcoLite scintillant for radioactivity measurements.

Uptake at time zero was determined by incubating cells for 10 min at 4 °C with transport buffer that contained NBMPR or dilazep and, immediately thereafter, for ≤ 3 s with ice-cold transport buffer that contained ^3H -labelled permeant and either

NBMPR or dilazep. For inhibition experiments, cells were exposed to non-radioactive nucleosides at the same time as ^3H -labelled permeant. Initial rates of uptake were derived by analysis of time courses of uptake of radioactive nucleosides by linear regression analysis. Kinetic parameters were estimated from Woolf, Eadie–Hofstee and Lineweaver–Burk plots (all as cited in [27]) and the resulting K_m and V_{max} values were averaged.

NBMPR binding assay

NBMPR binding by COS-1 cells was determined under equilibrium conditions as described in [28]. Specific NBMPR binding was defined as the difference between cell-associated radioactivity in the presence and the absence of 10 μM non-radioactive NBMPR. NBMPR binding parameters were determined from Scatchard plots of associated/free versus bound NBMPR [29].

RESULTS

Characterization of NT activity in COS-1 cells

COS-1 cells are widely used as recipients in transient transfection assays because the presence of the simian virus 40 (SV40) large

Figure 1 Initial rates of uptake of [^3H]uridine by COS-1 cells

Assays were conducted in Na^+ -containing buffer as described in the Experimental section with cultures that contained 2×10^6 cells/dish (A) or 1.6×10^6 cells/dish (B). Each value represents the mean $\pm$ S.D. of three determinations, and error bars are not shown where S.D. values were small. (A) Uptake of 1 μM [^3H]uridine was measured at 1-min intervals in the absence (open symbols) or presence (solid symbols) of 1 mM non-radioactive uridine. The transport rates were 0.240 and 0.002 pmol/10⁶ cells per s, respectively. (B) Uptake of 10 μM [^3H]uridine was measured at 2-s intervals in the absence (open symbols) or presence (solid symbols) of 10 nM NBMPR. The transport rates were 2.3 and 0.1 pmol/10⁶ cells per s, respectively.

Figure 2 Effects of NBMPR, dilazep and dipyrindamole on uridine transport by COS-1 cells

Initial rates of uptake of $100 \mu\text{M}$ [^3H]uridine by COS-1 cells were determined as in Figure 1 in cultures (about 2×10^6 cells/dish) that were assayed in the absence or presence of graded concentrations of either NBMPR (■), dilazep (▲) or dipyrindamole (●). The initial rates determined in the absence of NT inhibitors served as control values (100%, $9.7 \text{ pmol}/10^6$ cells per s) and those determined in the presence of various concentrations of NT inhibitors were calculated as percentages of control values. The concentrations that inhibited transport rates by 50% (IC_{50}) for NBMPR, dilazep and dipyrindamole were 1, 4 and 28 nM, respectively.

T antigen allows production of large quantities of plasmids that have the SV40 origin of replication [24]. Before attempting to express cNT1_{rat} cDNA in COS-1 cells by transient transfection, we first examined the transport of physiological nucleosides in COS-1 cells. Three nucleosides (uridine, thymidine and adenosine) were used as ^3H -labelled permeants and, because similar results were obtained with each, only the data obtained with [^3H]uridine are presented in detail.

To determine if permeation of nucleosides by COS-1 cells was mediated, the time course of uptake of $1 \mu\text{M}$ [^3H]uridine was measured in the presence and absence of an excess of non-radioactive uridine (Figure 1A). Initial rates of uptake of tracer uridine were significantly reduced in the presence of a high concentration of non-radioactive uridine, indicating that uptake was transporter-mediated. The transport process was sodium-independent since similar results were obtained when the experiment of Figure 1(A) was repeated in sodium-free buffer (results not shown). Uptake of $1 \mu\text{M}$ [^3H]thymidine and [^3H]adenosine was also shown to be mediated and sodium-independent (results not shown).

NBMPR has proven useful in distinguishing between functionally distinct NT subtypes [1]. At nanomolar concentrations, NBMPR-sensitive (*es*) NTs are inhibited whereas NBMPR-insensitive (*ei*) NTs are not [1]. We first examined the effects of a low concentration (10 nM) of NBMPR on the initial rates of uptake of $10 \mu\text{M}$ ^3H -labelled nucleoside by COS-1 cells. The data for uridine are shown in Figure 1(B), where it is evident that influx was almost completely blocked by NBMPR. Uptake of $10 \mu\text{M}$ [^3H]thymidine and [^3H]adenosine were also blocked by NBMPR (results not shown). The results of Figure 1 also demonstrate the linearity of time courses for uptake of [^3H]uridine measured at 1-min (Figure 1A) and 2-s (Figure 1B) intervals.

To further characterize the NBMPR sensitivity of NT-mediated processes of COS-1 cells, we examined transport of $100 \mu\text{M}$ ^3H -labelled permeant either in the absence of NT inhibitors or in the presence of graded concentrations of NBMPR, dilazep or dipyrindamole. The concentration-effect relationships are shown in Figure 2. The concentrations at which 50% inhibitions were

Figure 3 Scatchard analysis of NBMPR binding by COS-1 cells

NBMPR binding by COS-1 cells was determined under equilibrium conditions. Replicate cultures (three per condition, each with 0.5×10^6 cells) were preincubated in Na^+ -containing buffer in the presence or absence of $10 \mu\text{M}$ non-radioactive NBMPR at RT for 15 min, after which a portion of binding medium (Na^+ -containing buffer with one of several graded concentrations of [^3H]NBMPR) was then added to each culture. Cultures were incubated at RT for 30 min, when the binding assays were ended by aspiration of the binding medium, and the cells were air dried and prepared for determination of cell-associated radioactivity by liquid scintillation counting as described in the Experimental section. Free NBMPR concentrations were determined from the radioactive content of the binding medium at the end of the assay. Specific NBMPR binding was defined as the difference between cell-associated (bound) radioactivity in the presence and the absence of $10 \mu\text{M}$ non-radioactive NBMPR. Each value represents the mean $\pm$ S.D. of three determinations, and error bars are not shown where S.D. values were small.

achieved (IC_{50}) for NBMPR, dilazep and dipyrindamole were 1.0, 4 and 28 nM, respectively, and the curves were monophasic, suggesting that COS-1 cells possess a single type of NT-mediated process. In similar experiments with thymidine, IC_{50} values of 0.7, 6 and 27 nM were found, respectively, for inhibition of transport by NBMPR, dilazep and dipyrindamole (results not shown).

Because NBMPR inhibits *es* NTs by binding with high affinity to transport-inhibitory sites believed to be associated with the transporter protein [28], we also examined high-affinity binding of [^3H]NBMPR to COS-1 cells (Figure 3). The Scatchard plot was linear, suggesting that COS-1 cells had only one population of NBMPR-binding sites, with K_d and B_{max} values, respectively, of 0.8 nM and $1.2 \text{ pmol}/10^6$ cells. The correspondence of the K_d and IC_{50} values was consistent with the transport inhibition resulting from interaction of NBMPR with the high-affinity binding sites known to be associated with *es* NTs in the plasma membrane. The number of NBMPR-binding sites in COS-1 cells was 7×10^5 sites per cell.

The foregoing results demonstrated that COS-1 cells had a single NT-mediated process, which was equilibrative, NBMPR-sensitive and sodium-independent. Detailed kinetic studies, which were conducted with only one ^3H -labelled permeant (uridine), yielded K_m and V_{max} , respectively, of $184 \mu\text{M}$ and $44 \text{ pmol}/10^6$ cells per s. Thus, because *es* NTs could be completely blocked with either NBMPR or dilazep, COS-1 cells were used as recipients for expression of cDNAs encoding NT proteins such as cNT1_{rat} , which lack sensitivity to NBMPR or dilazep (S. Y. M. Yao, C. E. Cass and J. D. Young, unpublished work).

Transient expression of cNT1_{rat} in COS-1 cells

COS-1 cells were transfected by a modification of the DEAE-dextran method and, 24 h after transfection, the cells were trypsinized, pooled and replated to eliminate any differences in

Figure 4 Uptake of uridine by COS-1 cells transiently transfected with pCDNAI/Amp-cNT1_{rat}

Actively proliferating cells were transfected with pCDNAI/Amp-cNT1_{rat} or pCDNAI/Amp by the DEAE-dextran method and uptake of 10 μ M [³H]uridine was determined 72 h after transfection as described in the Experimental section. All of the transfected cultures were subjected to the same transfection conditions and assay procedures. Transfected cells were incubated with 1 μ M NBMPR at RT for 30 min before uptake measurements and uptake assays were conducted in the presence of 1 μ M NBMPR. Each value represents the mean $\pm$ S.D. of three determinations (1.1×10^6 cells/dish), and error bars are not shown where S.D. values were small. (A) Uptake of [³H]uridine in Na⁺-containing buffer by pCDNAI/Amp-cNT1_{rat}-transfected cells was determined in the absence (■) or presence (●) of 1 mM uridine; uptake by cells transfected with pCDNAI/Amp (▲) served as a control. (B) Uptake of 10 μ M [³H]uridine by cells transfected with pCDNAI/Amp-cNT1_{rat} (closed symbols) or pCDNAI/Amp (open symbols) was determined in Na⁺-containing buffer (triangles) or in Na⁺-free buffer (circles) in the presence of 1 μ M NBMPR.

transfection efficiencies among the individual cultures. Since the peaks of plasmid replication and protein production in transfected COS-1 cells occur 48–72 h and 72–96 h post-transfection, respectively [24], cells were used for uptake assays 72 h after transfection. In the early uptake assays with transfected cells (e.g. Figure 4), the experiments were conducted with cultures that had been pre-incubated with 1 μ M NBMPR at RT for 30 min to inhibit endogenous *es*-mediated NT activity. In subsequent experiments, dilazep, which is more soluble than NBMPR, was used as the inhibitor.

Representative time courses of uptake of 10 μ M [³H]uridine by COS-1 cells transfected with pCDNAI/Amp-cNT1_{rat} or, as a control, the pCDNAI/Amp vector are shown in Figure 4. Uridine uptake over a 30-min interval was substantially greater in cells that had been transfected with pCDNAI/Amp-cNT1_{rat} than that in cells transfected with the vector alone, indicating production

Figure 5 Analysis of recombinant cNT1_{rat}-myc protein by protein immunoblotting

Detergent-solubilized membrane fractions were prepared from COS-1 cells that had been transfected 72 h earlier with either pCDNAI/Amp-cNT1_{rat}-myc (lane 1) or pCDNAI/Amp (lane 2); the proteins contained therein were resolved (25 μ g/lane) on an SDS/12%-PAGE gel and transferred to a polyvinylidene difluoride membrane for immunostaining with anti-c-myc antibodies as described in the Experimental section. The signal that appears between lanes 1 and 2 is an artifact; the faint bands seen in corresponding positions in lanes 1 and 2 are due to non-specific binding of antibody. The positions of molecular-mass markers (in kDa) are indicated on the left.

of functional, recombinant cNT1_{rat}. The uptake mediated by the recombinant transporter was clearly mediated, since uptake was greatly reduced by the addition of 1 mM non-radioactive uridine to the solution that contained 10 μ M [³H]uridine (Figure 4A). In addition, uptake was Na⁺-dependent, since uridine uptake by transfected cells incubated in Na⁺-free buffer was greatly reduced relative to that by cells in Na⁺-containing buffer (Figure 4B). However, in the absence of a sodium gradient, a small amount of uridine uptake was seen in cells transfected with pCDNAI/Amp-cNT1_{rat} relative to that seen in cells transfected with vector alone, suggesting the presence of uncoupled uridine transport activity. The uptake observed in the experiments of Figure 4 was in the presence of excess (1 μ M) NBMPR, confirming that the recombinant transporter belongs to the NBMPR-insensitive group of NTs.

Protein immunoblotting analysis of recombinant c-myc-tagged cNT1 proteins expressed in COS cells

To analyse production of the cNT1_{rat} protein in transfected COS-1 cells, an epitope tag based on human *c-myc* (10 amino acid residues, EQKLISEEDL) was fused to the C-terminus of the cNT1_{rat} transporter since antibodies against native cNT1_{rat} were not available. To test if the presence of the *c-myc* tag altered the function (or processing) of the recombinant transporter, COS-1 cells transfected with either pCDNAI/Amp-cNT1_{rat}-myc, pCDNAI/Amp-cNT1_{rat} or pCDNAI/Amp were assayed for uptake of 10 μ M [³H]uridine 72 h after transfection. Uptake of uridine during the 10-min interval was 329 ± 27.4 , 425 ± 20.2 and 16 ± 0.3 pmol/10⁶ cells, respectively, for cells transfected with vectors that contained either the cNT1_{rat}-myc insert, the cNT1_{rat} insert, or no insert. Thus, since the presence of the epitope tag reduced uridine uptake by only 25%, a substantial quantity of the recombinant cNT1_{rat}-myc that had been produced was correctly processed. The epitope-tagged recombinant protein was identified in immunoblots prepared from detergent-solubilized membranes from the variously transfected cells (Figure 5).

Table 1 Effects of high concentrations of physiological nucleosides on uridine uptake mediated by recombinant cNT1_{rat}

Uptake of 10 μ M [3 H]uridine by pCDNAI/Amp-cNT1_{rat}-transfected cells during a 5-min incubation was determined in Na⁺-containing buffer either without (control) or with 1 mM non-radioactive uridine, thymidine, adenosine, cytidine, deoxycytidine or guanosine. Each value represents the mean $\pm$ S.D. of three determinations in cultures that contained 1.2×10^6 (Experiment 1) or 0.5×10^6 (Experiment 2) cells/dish.

	Uridine uptake (pmol/10 ⁶ cells)	Percentage of control (%)
Experiment 1		
Control	126.0 $\pm$ 5.6	100.0 $\pm$ 4.5
+ Uridine	6.3 $\pm$ 0.5	4.7 $\pm$ 0.4
+ Thymidine	9.3 $\pm$ 0.6	7.3 $\pm$ 0.5
+ Adenosine	12.9 $\pm$ 0.7	10.2 $\pm$ 0.2
+ Guanosine	81.5 $\pm$ 17.7	64.7 $\pm$ 14.0
Experiment 2		
Control	81.4 $\pm$ 12.1	100.0 $\pm$ 14.7
+ Cytidine	12.6 $\pm$ 0.8	15.5 $\pm$ 1.1
+ Deoxycytidine	16.6 $\pm$ 0.8	20.3 $\pm$ 1.0

Figure 6 Concentration–effect relationships for inhibition by cytidine and deoxycytidine of recombinant cNT1_{rat}-mediated uridine transport

Cells were transfected with pCDNAI/Amp-cNT1_{rat} and processed as described for Figure 4, except that dilazep (10 μ M) was substituted for NBMPR. Uptake of 10 μ M [3 H]uridine during a 5-min incubation was determined in Na⁺-containing buffer either without additives (control, 134 pmol/10⁶ cells, 100%) or with various concentrations of cytidine (■) or deoxycytidine (▲). Uptake values are presented as a percentage of control values. Each value represents the mean $\pm$ S.D. of three determinations (0.75×10^6 cells/dish), and error bars are not shown where S.D. values were small.

Immunostaining with anti-c-myc monoclonal antibodies revealed (i) a single band with an apparent molecular mass of 45 kDa in the fraction isolated from cells transfected with pCDNAI/Amp-cNT1_{rat}-myc, and (ii) nothing in the fraction from cells transfected with pCDNAI/Amp, confirming production of the recombinant myc-tagged cNT1_{rat} protein in cells transfected with pCDNAI/Amp-cNT1_{rat}-myc.

Inhibition studies with physiological nucleosides

The experiments of Table 1 examined the ability of various physiological nucleosides to inhibit uridine uptake mediated by recombinant cNT1_{rat}. Non-radioactive nucleosides were added at concentrations of 1 mM to transport assay mixtures to test if they were able to inhibit cNT1_{rat}-mediated uptake of 10 μ M

Figure 7 Uptake of [3 H]adenosine, [3 H]guanosine and [3 H]uridine mediated by recombinant cNT1_{rat} in transiently transfected COS-1 cells

Cells were transfected with pCDNAI/Amp-cNT1_{rat} (closed symbols) or pCDNAI/Amp (open symbols) and processed in uptake experiments as described for Figure 4, except that dilazep (10 μ M) was substituted for NBMPR. All measurements were conducted in Na⁺-containing buffer. Each value represents the mean $\pm$ S.D. of three determinations (1.07×10^6 cells/dish, **A**; 1.05×10^6 cells/dish, **B**), and error bars are not shown where S.D. values were small. (**A**) 10 μ M [3 H]uridine (squares); 10 μ M [3 H]adenosine (triangles). (**B**) 10 μ M (squares), 100 μ M (triangles) and 1000 μ M (circles) [3 H]guanosine.

[3 H]uridine during a 5-min incubation. All of these studies were conducted in the presence of 10 μ M dilazep to block endogenous NT activity in COS-1 cells. cNT1_{rat}-mediated uptake of [3 H]-uridine was reduced by the presence of non-radioactive uridine to 5% of control values. Reductions to 7–20% of control values were seen with thymidine, adenosine, cytidine and deoxycytidine, whereas guanosine reduced uptake to 65% of the control value. The inhibitory effects of cytidine and deoxycytidine were further investigated in the experiments of Figure 6, which examined the concentration-dependence of inhibition of uridine uptake. Although both were relatively potent inhibitors, with 50% reductions in uridine uptake seen at concentrations of about 50 μ M, the maximal reductions achieved at high concentrations were less than those observed with high concentrations of uridine itself.

The inhibition of uridine transport by adenosine, a purine nucleoside, has been taken as evidence that adenosine is a permeant of the pyrimidine-selective transporters (N2/cit, N4/cit). To demonstrate the transportability of adenosine by cNT1_{rat}, a direct comparison was made of uptake of [3 H]uridine and [3 H]adenosine in the same preparation of transfected cells (Figure 7A). The initial rate of uptake of 10 μ M uridine was 3.26 pmol/s per 10⁶ cells, while that of 10 μ M adenosine was

Table 2 Kinetic parameters for transport of uridine, thymidine and adenosine mediated by recombinant cNT1_{rat} in transfected COS-1 cells

Initial rates of uptake by COS-1 cells transfected with pCDNAI/Amp-cNT1 were determined for each of a series of graded concentrations of [³H]uridine (1–50 μ M), [³H]thymidine (1–20 μ M) and [³H]adenosine (1–30 μ M). Each set of kinetic parameters was derived from a separate experiment. The K_m and V_{max} values are the means ($\pm$ S.D.) of values determined from Woolf, Eadie–Hofstee and Lineweaver–Burk plots.

	K_m (μ M)	V_{max} (pmol/s per 10^6 cells)	V_{max}/K_m
Uridine	18.9 ± 1.8	12.3 ± 0.6	0.65
	23.0 ± 1.0	10.2 ± 5.0	0.43
Thymidine	13.9 ± 0.6	2.7 ± 0.12	0.20
	8.6 ± 2.3	2.9 ± 0.45	0.35
Adenosine	18.7 ± 2.9	0.2 ± 0.04	0.01
	11.3 ± 0.1	0.1 ± 0.01	0.01

Table 3 Effects of high concentrations of nucleoside drugs on uridine uptake mediated by recombinant cNT1_{rat}

Uptake of 10 μ M [³H]uridine by pCDNAI/Amp-cNT1_{rat}-transfected cells during a 5-min incubation was determined in Na⁺-containing buffer either without (control) or with 5 mM non-radioactive AZT, ddC, 3TC, FUdR, IUdR, araC and dFdC. Each value represents the mean $\pm$ S.D. of three determinations in cultures that contained 1.2×10^6 (Experiment 1) or 0.5×10^6 (Experiment 2) or 0.6×10^6 (Experiment 3) cells/dish.

	Uridine uptake	
	(pmol/ 10^6 cells)	(% of control)
Experiment 1		
Control	126.0 ± 5.6	100.0 ± 4.5
+ AZT	52.8 ± 3.1	42.9 ± 2.5
+ ddC	61.7 ± 1.3	48.9 ± 1.1
+ 3TC	106.5 ± 7.8	84.5 ± 6.2
Experiment 2		
Control	81.4 ± 12.1	100.0 ± 14.7
+ AraC	41.4 ± 5.0	51.0 ± 6.1
+ FUdR	5.6 ± 0.1	6.9 ± 0.1
+ IUdR	8.9 ± 0.8	11.0 ± 1.2
Experiment 3		
Control	120.0 ± 7.2	100.0 ± 6.0
+ dFdC	70.9 ± 2.9	59.0 ± 2.4

only 0.24 pmol/s per 10^6 cells. The 13-fold difference in transport rates indicated that adenosine, although a 'high-affinity' inhibitor, was a relatively poor permeant of recombinant cNT1_{rat}. The partial inhibition of uptake of [³H]uridine by guanosine seen in Table 1 suggested that guanosine was a poor permeant and/or a weak inhibitor of recombinant cNT1_{rat}. The inability of guanosine to serve as a substrate of cNT1_{rat} was confirmed by measuring uptake of various concentrations of [³H]guanosine by cNT1-transfected COS-1 cells (Figure 7B).

Kinetic studies

The kinetics of uptake of uridine, thymidine and adenosine were determined in cells transfected with the cNT1_{rat}-containing vector (Table 2). The transport assays, which were conducted in the presence of 10 μ M dilazep to block endogenous NTs, yielded linear 12-s time courses from which initial rates were extracted at all of the permeant concentrations tested (results not shown). Since preliminary studies had shown that transport velocities of uridine were reduced by more than 90 % in the presence of 1 mM non-radioactive uridine, the small contribution of diffusion to

Figure 8 Concentration–effect relationships for inhibition by IUdR and FUdR of recombinant cNT1_{rat}-mediated uridine uptake

Cells were transfected with pCDNAI/Amp-cNT1_{rat} and processed as described for Figure 4, except that dilazep (10 μ M) was substituted for NBMPR. Uptake of 10 μ M [³H]uridine during 5-min incubations was determined in Na⁺-containing buffer either without (control, 80 pmol/ 10^6 cells, 100%) or with various concentrations of IUdR ($\blacktriangle$) or FUdR ($\blacksquare$). Uptake values are presented as a percentage of control values. Each value represents the mean $\pm$ S.D. of three determinations (0.8×10^6 cells/dish), and error bars are not shown where S.D. values were small.

uptake rates was ignored. Recombinant cNT1_{rat} exhibited similar K_m values for uridine, thymidine and adenosine, indicating that each was a high-affinity permeant. However, V_{max} values for the three permeants differed greatly (uridine > thymidine > adenosine), with V_{max}/K_m ratios of 0.65, 0.20 and 0.009, respectively.

Inhibition studies with nucleoside drugs

Although a large number of nucleoside drugs have been shown to be accepted as permeants of *es* NTs, only a few have been tested with the pyrimidine-selective concentrative (N2/*cit*, N4/*cit*) transporters [1]. The availability of the COS-1/cNT1_{rat} expression system provided an opportunity to examine the transportability of nucleoside drugs by the N2/*cit* process, and several pyrimidine nucleoside drugs were tested for their ability to inhibit uridine uptake mediated by recombinant cNT1_{rat} (Table 3). Uptake of 10 μ M [³H]uridine was inhibited by the addition of 5 mM non-radioactive AZT, ddC, IUdR, FUdR, dFdC and araC, indicating that each of these drugs was either a substrate or an inhibitor of recombinant cNT1_{rat}. The extent of inhibition by 5 mM AZT, ddC and araC was about 50 %, whereas that by 5 mM IUdR and FUdR was 89 % and 93 %, respectively. Inhibition was not seen with 5 mM 3TC. The effects of IUdR and FUdR were further examined in the experiments of Figure 8, where uptake of 10 μ M [³H]uridine was measured in the presence of various concentrations of non-radioactive IUdR and FUdR. Uptake was reduced to 50 % by 40–60 μ M of FUdR or IUdR, indicating that both interacted with recombinant cNT1_{rat} with relatively high affinity. However, at the highest concentrations tested (500 μ M), uridine transport was reduced to 20–25 % of the control values, suggesting that a portion of transport mediated by the recombinant transporter was resistant to inhibition by either FUdR or IUdR.

DISCUSSION

Kinetic studies of the various Na⁺-dependent NTs have been hindered by the absence of convenient experimental systems that exhibit a single transporter subtype. cNT1_{rat} is a concentrative

Na⁺-nucleoside co-transporter of rat intestine that was identified by the isolation and functional expression of a novel cDNA in oocytes of *Xenopus laevis* [22]. The objective of the current work was to functionally express cNT1_{rat} cDNA in a transfectable mammalian cell line under conditions that allowed kinetic analysis of nucleoside fluxes induced by the recombinant transporter in the absence of other endogenous NT activities. Of the small number of genetically NT-defective cell types available [1,30], none is readily transfectable. Thus, we searched for a cell type that could be (i) transfected with high levels of expression, and (ii) made pharmacologically NT-defective by treatment with inhibitors of equilibrative NT activity. We found that nucleoside transport in cultured monkey kidney COS-1 cells, which can be transfected with high efficiency ($\geq 20\%$) and usually express foreign cDNAs at high levels [24], was mediated in large part, if not completely, by an equilibrative NBMPR-sensitive (*es*) transporter with characteristics similar to those reported for many other cell types [1].

The substantial differences in uptake observed between COS-1 cells transfected with the cNT1_{rat}-containing vector and cells transfected with vector alone indicated high levels of functional recombinant cNT1_{rat}. The induced transport was Na⁺-dependent, although inward fluxes in Na⁺-free buffer were consistently higher for cells transfected with the cNT1_{rat} vector than for cells transfected with vector alone (e.g. see Figure 4). The residual Na⁺-independent transport was not eliminated by treatment of cells with high concentrations of dilazep, a compound that inhibits both *es* and *ei* NTs [1], suggesting that it had not resulted from an increase in endogenous *ei* activity as part of a general cellular response to the transfection procedure. Thus, the residual Na⁺-independent NT activity may have been due to the uncoupled transport, or 'slippage', of permeant (uridine) in the absence of a gradient of the co-transported cation (Na⁺), as has been reported for other co-transporters [31].

The presence of recombinant transporter protein in COS-1 cell membranes was directly demonstrated by protein immunoblotting of preparations from cells transfected with a cDNA construct that encoded cNT1_{rat} with a c-myc epitope tag at the C-terminus. The epitope-tagged recombinant protein was functional, indicating apparently normal synthesis and processing of some (possibly all) of the newly synthesized protein. However, the apparent molecular mass (45 kDa) of recombinant cNT1_{rat}-myc was lower than that (71 kDa) calculated from the amino acid sequence of cNT1_{rat}. Further studies are required to determine the basis of this discrepancy in the predicted and apparent molecular mass of recombinant cNT1_{rat}-myc.

Recombinant cNT1_{rat} has been functionally produced in oocytes of *Xenopus laevis* and shown to (i) transport uridine and thymidine, and (ii) be inhibited by a variety of other pyrimidine nucleosides as well as adenosine, but not by guanosine or inosine [22]. Results of the transport studies conducted with recombinant cNT1_{rat} in COS-1 cells were consistent with the conclusion [22] from the *Xenopus* studies that recombinant cNT1_{rat} exhibits N2/*cit* permeant selectivity since inward fluxes of [³H]uridine were inhibited by pyrimidine nucleosides (uridine, thymidine, cytidine, deoxycytidine) and by adenosine but not by guanosine. Of interest was the observation that the maximal inhibitions achieved with uridine and thymidine were greater than those achieved with the other inhibitory nucleosides (compare results of Figures 4 and 6), raising the possibility that the recombinant transporter may exist in multiple forms that differ in their ability to recognize nucleoside permeants. cNT1_{rat} has four consensus sequences for protein kinase C phosphorylation, as well as multiple N-linked and O-linked glycosylation sites [22]. Studies to determine if recombinant cNT1_{rat} is subject to post-trans-

lational modifications that lead to functional differences are currently underway.

Uridine fluxes were much greater than adenosine fluxes when uptake was measured under identical conditions in the same preparation of transfectants, raising the possibility that adenosine, which was a 'high-affinity' inhibitor of uridine transport, was not itself a good permeant. This conclusion was confirmed by results of the kinetic studies, which yielded similar K_m values for uridine and adenosine but markedly different V_{max} values (uridine > adenosine). The V_{max} value for cNT1_{rat}-mediated transport of thymidine was intermediate between that of uridine and adenosine, even though all three permeants had similar K_m values. Permeants with similar K_m values but different V_{max} values have been observed for other transport systems [32–34].

Kinetic parameters for cNT1_{rat}-mediated transport of uridine and adenosine have recently also been determined in *Xenopus* oocytes (S. Y. M. Yao, A. M. L. Ng, M. W. L. Ritzel, C. E. Cass and J. D. Young, unpublished work). While the absolute V_{max} values can not be directly compared because of differences in cell size and amounts of recombinant protein, the K_m values, which represent a measure of transporter affinity, are directly comparable. The K_m values obtained in the two expression systems differed 2-fold for uridine and were essentially the same for adenosine, suggesting that recombinant cNT1_{rat} functioned similarly in mammalian and amphibian cells.

The transportability of adenosine by the N1/*cif* and N3/*cib* NT processes of murine leukaemia L1210 cells [35], rat renal brush-border membrane vesicles [12], rat hepatocytes [36] and rabbit intestinal brush-border membrane vesicles [11] has been established by direct measurements of [³H]adenosine fluxes. By contrast, the conclusion that adenosine is a N2/*cit* permeant was based on results from inhibition assays [11,12,19], and not from direct measurements of [³H]adenosine uptake by N2/*cit* NT processes. The ability to block uptake does not necessarily establish that a substance is a substrate, as is evidently the case for adenosine, which exhibited inhibitory activity similar to that of uridine and thymidine in the experiments of Table 1 yet was transported with a relatively low V_{max} value. A similar situation was reported [11] for tubercidin, which inhibited Na⁺-dependent thymidine transport in mouse intestinal cells, but was not itself transported.

Although inhibition studies have the limitations illustrated by the contradictory results obtained with adenosine (this study) and tubercidin [11], they can be efficiently used to predict (i) the permeant selectivity of a particular transporter, and (ii) transportability of non-radioactive test compounds. We examined the ability of a group of clinically important nucleoside analogues to inhibit cNT1_{rat}-mediated uridine uptake by COS-1 cells. The most potent inhibitors (Floxadine, Idoxuridine) were closely related 2'-deoxyribose analogues of thymidine with substituents at the 5-position of the pyrimidine ring, whereas the least potent inhibitors (Gemcitabine, Cytarabine, Zalcitabine, Zidovudine, 3'-thiacytidine) had variously modified sugar moieties, suggesting low tolerance of the transporter for modification of the sugar. The concentration-effect studies that were conducted with FUDR and IUdR (see Figure 8) revealed that the maximal inhibitions achieved with these analogues were 75–80%, again raising the possibility of multiple forms of recombinant cNT1_{rat}. cNT1_{rat} is found in intestine and kidney [22] and it is likely that cNT1_{rat} and other members of the cNT family are involved in the gastrointestinal absorption of pyrimidine nucleoside drugs.

In conclusion, we have produced functional, recombinant cNT1_{rat} in COS-1 cells. To the best of our knowledge, this is the first example of expression of a cDNA encoding an NT protein in mammalian cells at levels sufficient to allow detailed kinetic

analyses of tracer fluxes. The kinetic characteristics and permeant selectivities of recombinant cNT1_{rat} were similar in COS-1 cells and *Xenopus* oocytes ([22]; S. Y. M. Yao, A. M. L. Ng, M. W. L. Ritzel, C. E. Cass and J. D. Young, unpublished work). The permeant profiles obtained with both expression systems indicated that recombinant cNT1_{rat} is selective for pyrimidine nucleosides, transports adenosine poorly and does not transport guanosine. It is likely that native cNT1_{rat} functions similarly in intestinal and renal epithelia. The successful production of cNT1_{rat} in transiently transfected COS-1 cell has provided a model for further characterization of the cNT1_{rat} protein, and indicates that it is feasible to develop cNT1_{rat} stable transfectants in mammalian cells.

This work was jointly supported by Canadian Cancer Society operating grants from the National Cancer Institute of Canada (NCIC) to C. E. C. and J. D. Y. F. E. P. was a recipient of a Medical Research Council of Canada/CIBA Geigy fellowship. C. E. C. is a Terry Fox Cancer Research Scientist of the NCIC and J. D. Y. is a Heritage Medical Scientist of the AHFMR.

REFERENCES

- Cass, C. E. (1994) in *Drug Transport in Antimicrobial and Anticancer Chemotherapy* (Georgopapadakou, N. H., ed.), pp. 403–452, Marcel Dekker Inc., New York
- Cass, C. E., Gaudette, L. A. and Paterson, A. R. P. (1974) *Biochim. Biophys. Acta* **345**, 1–10
- Paterson, A. R. P., Clanachan, A. S., Craik, J. D., Gati, W. P., Jakobs, E. S., Wiley, J. S. and Cass, C. E. (1991) in *Role of Adenosine and Adenine Nucleotides in Biological Systems* (Imai, S. and Nakazawa, M., eds.), pp. 133–148, Elsevier Science Publishers BV, Amsterdam
- Kwong, F. Y. P., Fincham, H. E., Davies, A., Beaumont, N., Henderson, P. J. F., Young, J. D. and Baldwin, S. A. (1992) *J. Biol. Chem.* **267**, 21954–21960
- Kwong, F. Y. P., Wu, J. S. R., Shi, M. M., Fincham, H. E., Davies, A., Henderson, P. J. F., Baldwin, S. A. and Young, J. D. (1993) *J. Biol. Chem.* **268**, 22127–22134
- Hogue, D. L., Hodgson, K. C. and Cass, C. E. (1990) *Biochem. Cell Biol.* **68**, 199–209
- Huang, Q. Q., Harvey, C. M., Paterson, A. R. P., Cass, C. E. and Young, J. D. (1993) *J. Biol. Chem.* **268**, 20613–20619
- Dagnino, L. and Paterson, A. R. P. (1990) *Cancer Res.* **50**, 6549–6553
- Che, M., Nishida, T., Gatmaitan, Z. and Arias, I. M. (1992) *J. Biol. Chem.* **267**, 9684–9688
- Dagnino, L., Bennett, L. L. and Paterson, A. R. P. (1991) *J. Biol. Chem.* **266**, 6312–6317
- Vijayalakshmi, D. and Belt, J. A. (1988) *J. Biol. Chem.* **263**, 19419–19423
- Williams, T. C. and Jarvis, S. M. (1991) *Biochem. J.* **274**, 27–33
- Lee, C. W., Cheeseman, C. I. and Jarvis, S. M. (1990) *Am. J. Physiol.* **258**, F1203–F1210
- Williams, T. C., Doherty, A. J., Griffith, D. A. and Jarvis, S. M. (1989) *Biochem. J.* **264**, 223–231
- Belt, J. A., Marina, N. M., Phelps, D. A. and Crawford, C. R. (1993) *Adv. Enzyme Regul.* **33**, 235–252
- Belt, J. A., Harper, E. and Byl, J. (1992) *Proc. Am. Assoc. Cancer Res.* **33**, 20
- Lee, C. W., Sokoloski, J. A., Sartorelli, A. C. and Handschumacher, R. E. (1991) *Biochem. J.* **274**, 85–90
- Gutierrez, M. M. and Giacomini, K. M. (1993) *Biochim. Biophys. Acta* **1149**, 202–208
- Gutierrez, M. M., Brett, C. M., Ott, R. J., Hui, A. C. and Giacomini, K. M. (1992) *Biochim. Biophys. Acta* **1105**, 1–9
- Jarvis, S. M. and Griffith, D. A. (1991) *Biochem. J.* **278**, 605–607
- Terasaki, T., Kadowaki, A., Higashida, S., Nakayama, K., Tamai, I. and Tsuji, A. (1993) *Biol. Pharm. Bull.* **16**, 493–496
- Huang, Q. Q., Yao, S. Y. M., Ritzel, M. W. L., Paterson, A. R. P., Cass, C. E. and Young, J. D. (1994) *J. Biol. Chem.* **269**, 17757–17760
- Che, M., Ortiz, D. F. and Arias, I. M. (1995) *J. Biol. Chem.* **270**, 13595–13599
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (1994) *Current Protocols in Molecular Biology*, John Wiley and Sons, New York
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J. and Klenk, K. C. (1985) *Anal. Biochem.* **150**, 76–85
- Lin, W. and Culp, L. A. (1991) *BioTechniques* **11**, 344–351
- Matthews, J. D. (1993) *Fundamentals of Receptor, Enzyme, and Transport Kinetics*, pp. 134–135, CRC Press, Boca Raton, FL
- Boumah, C. E., Hogue, D. L. and Cass, C. E. (1992) *Biochem. J.* **288**, 987–996
- Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51**, 660–672
- Ullman, B. (1989) in *Resistance to Antineoplastic Drugs* (Kessel, D., ed.), pp. 293–315, CRC Press, Boca Raton, FL
- Stein, W. D. (1986) in *Transport and Diffusion across Cell Membranes*, pp. 397–400, Academic Press, London
- Tate, S. S., Yan, N. and Udenfriend, S. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 1–5
- Fincham, D. A., Mason, D. K., Paterson, J. Y. and Young, J. D. (1987) *J. Physiol. (London)* **389**, 385–409
- Fincham, D. A., Mason, D. K. and Young, J. D. (1988) *Biochim. Biophys. Acta* **937**, 184–194
- Dagnino, L., Bennett, L. L. and Paterson, A. R. P. (1991) *J. Biol. Chem.* **266**, 6308–6311
- Wu, X., Gutierrez, M. M. and Giacomini, K. M. (1994) *Biochim. Biophys. Acta* **1191**, 190–196