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Specificity of Concentrative Nucleoside Transporters

by

Karin Miller Gerstin

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Chemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO

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To my husband, Ed, my partner, soul mate and best friend,

for your unconditional love,

your unwavering belief in me and for never being wrong about me

To my son, Trevor,

for reminding me everyday what is truly important in life

To the memory of my grandmother, Helen,

who always believed that each of her grandkids would accomplish great things

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I thank my parents-in-law, Pat and Leo, for raising a wonderful son with whom I have the pleasure of sharing my life. I thank my family for their love, support, encouragement and prayers over the years. My sister, Cindy, who had the courage to go back to school and overcome many obstacles along the way to answer her calling, has been a role-model for me all of my life. My brother, Kevin, whose hard work and dedication to his family, is an inspiration to me. My parents, Jan and Dick, always believed I could achieve whatever I set my mind to and were supportive of me following my dream even when it took me away from home.

My son, Trevor, is a constant source of joy in my life; watching his growth and development has renewed in me a love of learning and discovery. He amazes and inspires me everyday. Finally, I never could have survived graduate school without the love, support, and constant encouragement of my husband, Ed; he is always there to pick me up when I fall down. Thank you for sharing my dreams.

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ABSTRACT

Specificity of Concentrative Nucleoside Transporters

Karin Miller Gerstin

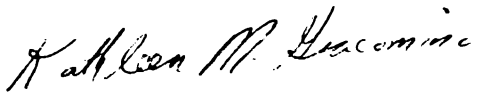
Nucleosides and many nucleoside analogs, clinically important in the treatment of viral infections and cancer, cross cellular membranes via nucleoside transporters. These transporters are therefore thought to play a role in the absorption, elimination and tissue-specific targeting of nucleosides and nucleoside analogs. The goals of this dissertation were to characterize the substrate selectivity of two nucleoside transporter isoforms and to elucidate the mechanisms of selectivity. Data from these studies contribute to the broader goal of understanding the *in vivo* role of these transporters.

In the first part of this dissertation, the cloning of the first nucleoside transporter from rabbit is described. Using a homology-cloning approach, a cDNA (rbSPNT1) highly similar to other cloned concentrative nucleoside transporters was isolated. Functional testing in *Xenopus laevis* oocytes suggested that the transporter was purine-selective.

In the next part of this dissertation, substrate selectivity profiles were determined for the rat pyrimidine-selective nucleoside transporter (CNT1) and the rat and human orthologs of the purine-selective nucleoside transporter (SPNT). Using two-electrode voltage clamped oocytes, expressing either CNT1 or SPNT, naturally-occurring nucleosides and synthetic nucleoside analogs were tested as potential substrates. Data obtained corroborated the established selectivity of rCNT1 for pyrimidine nucleosides and extended the profile to include uridine analogs with substitutions at the 3, 4 and 5 but not 6 positions of the base. In contrast, uridine analogs with substitutions in the 5 position

were poorly transported by rat and human SPNT. Many interspecies differences were observed between these two orthologs in a comparison of their specificities. For example, two clinically important nucleoside analogs, cladribine and vidarabine, were transported by the rat but not the human ortholog. In general, the rat ortholog translocated a wider array of compounds than the human ortholog.

In the final part of this dissertation, the molecular determinants of pyrimidine selectivity of rCNT1 were examined using site-directed mutagenesis of an amino acid residue previously determined to be important in pyrimidine selectivity. The data suggest that the ability of the side chain at this position to hydrogen bond was not sufficient to confer pyrimidine selectivity, but that the size of the side chain was important.



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CHAPTER 1

INTRODUCTION TO NUCLEOSIDE TRANSPORTERS

Transporters are membrane-bound proteins that interact with specific molecules and selectively move them across biological membranes. Plasma membrane transporters play many important physiological roles, particularly in processes of absorption and secretion. Transporters are responsible for the absorption in the intestine of nutrients such as nucleosides, amino acids, glucose, and vitamins (1). They participate in the maintenance of the homeostasis of these and other important molecules in the body and specific tissues through the active reabsorption of these molecules in such tissues as the kidney, intestine, and choroid plexus. Transporters present in the liver and kidney are important in the secretion of the metabolites of endogenous and exogenous compounds. Transporters of the intestine and brain (i.e., P-glycoprotein) have been shown to play a protective role through their secretion of xenobiotics and toxic compounds (2, 3). Transporters also play a role in the termination of nerve signals through the reuptake of neurotransmitters such as serotonin and glutamate from the synaptic cleft (4).

Transporters have also been shown to play a role in the disposition of many clinically relevant drugs. Transporters present in the small intestine have been shown to participate in the absorption of certain drug compounds (1). For example, the oligopeptide transporter PEPT-1, has been shown to transport the beta-lactam antibiotics and angiotensin converting enzyme inhibitors and is thought to play a role in the absorption of these orally administered drugs (5). PEPT-1 as well as other intestinal

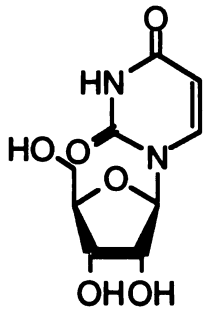
transporters are being targeted with pro-drugs of poorly absorbed oral drugs to improve their absorption (e.g. valacyclovir and valganciclovir) (6). Transporters present in the kidney such as the organic cation and organic anion transporters have been shown to actively secrete many drugs (e.g., methotrexate and penicillin) (7, 8). P-glycoprotein has been shown to limit the absorption of many drugs by actively effluxing them out of the intestinal epithelia back into the intestinal lumen (3, 9). It has also been shown to limit the diffusion of chemotherapeutic agents into cancer cells, thus conferring drug-resistance to these cells (2).

Some transporters are also the targets of several well-known drugs such as Prozac (fluoxetine). Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), targets the serotonin transporter and thereby inhibits the uptake of serotonin from the synapse, thus lengthening the action of serotonin (10).

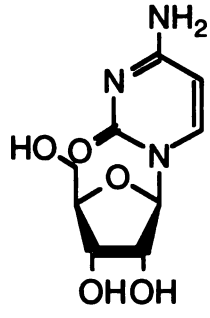
Nucleosides and many nucleoside analogs are hydrophilic compounds and therefore diffuse slowly across biological membranes. Thus, they require transporter proteins to cross membranes. Although most animal cells possess *de novo* synthesis pathways for nucleosides and nucleotides, cells of the intestinal mucosa, bone marrow, erythrocytes and leukocytes lack these pathways and are therefore dependent on nucleoside salvage. Nucleoside transporters are critical to the salvage of nucleosides through reabsorption of these molecules in these tissues (11).

Nucleosides consist of either purine (adenine, guanine, hypoxanthine) or pyrimidine (thymine, uracil, cytosine) bases plus a ribose (or 2'-deoxyribose) in the 9 or 1 position for purines and pyrimidines, respectively (Figure 1). These molecules, upon phosphorylation to triphosphorylated nucleotides, are the building blocks of DNA and

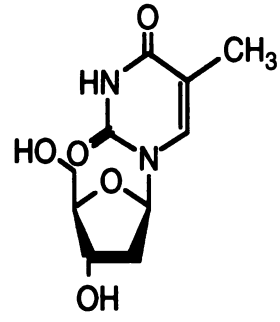
Pyrimidine Nucleosides



Uridine

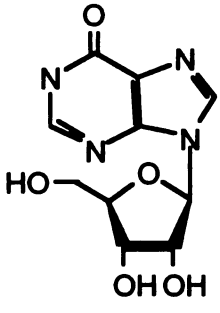


Cytidine

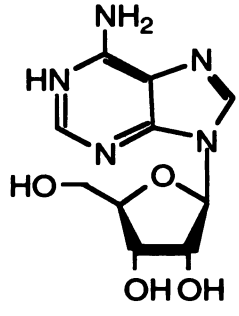


Thymidine

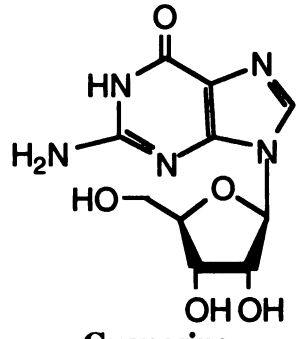
Purine Nucleosides



Inosine



Adenosine



Guanosine

Figure 1. Structures of naturally-occurring nucleosides.

RNA. In addition, ATP and GTP are important energy stores critical to driving chemical reactions. Adenosine, ADP, UDP and UTP interact with purine receptors and thereby mediate many diverse biological effects. Adenosine has been shown to cause vasodilation and hypotension while ATP can increase blood pressure in several animal models. ATP and adenosine are also important neurotransmitters and neuromodulators, respectively, in the peripheral and central nervous systems. UTP has been shown to participate in the contraction of vascular smooth muscle (12).

Nucleoside analogs are important in the treatment of viral infections and cancer. For example, ribose-modified 2',3'-dideoxyinosine (ddI, didanosine) and adenine arabinoside (ara-A, vidarabine) are important in the treatment of HIV and HSV, respectively, and base-modified 2-chloro-2'-deoxyadenosine (2CdA, cladribine) is used in the treatment of leukemia (13-16). One mechanism of action of nucleoside analogs is to interfere with DNA or RNA synthesis of viruses or quickly proliferating cells (e.g., cancer cells) by causing chain termination. Alternatively, they cause DNA strand breaks or perturb the intracellular pools of nucleotides (17).

Due to transporters playing a role in many aspects of drug disposition and drug response, it is important to understand the selectivity of these transporters with respect to endogenous compounds as well as clinically relevant drugs. The first step in this process is to clone the relevant transporters. The second step is to functionally characterize the cloned transporters in isolation. Chapter 2 of this dissertation reports the cloning and initial characterization of a nucleoside transporter from rabbit. Chapters 3 and 5 are focused on the detailed characterization of the substrate selectivity of nucleoside

transporters from rat and human. Chapter 4 explores the molecular determinants of substrate selectivity of nucleoside transporters from rat.

Mechanisms of Transport

There are several mechanisms by which transporters translocate substrates across a membrane: facilitated diffusion and primary and secondary active transport. Transporters using a facilitated diffusion mechanism simply use the electrochemical gradient of the substrate and move the substrate in accordance with that gradient. Some examples of this type of transporter are the organic cation transporters (OCT), organic anion transporters (OAT), equilibrative nucleoside transporters (ENT), and glucose transporters (GLUT). A second mechanism is primary active transport. These carriers are directly coupled to the expenditure of energy. Some examples of this type of transporter are P-glycoprotein (P-gp or MDR) and Na^+/K^+ATPase. Secondary active transport employs the energy stored in a concentration gradient maintained by a primary active transport process. In animal cells, the Na^+ gradient provides the driving force for many secondary active transporters; however, there are a number of transporters driven by the transport of H^+, Cl^-, K^+ and/or OH^-. For example, the Na^+-coupled nucleoside transporters use the inwardly-directed Na^+ gradient (maintained by Na^+/K^+ ATPase) to drive the transport of nucleosides. In bacteria and yeast that driving force is typically provided by a H^+ gradient. The analogous transporter in bacteria to the Na^+/nucleoside cotransporter mentioned above is coupled to H^+ transport (18). Symporters transport the driving ion (Na^+ or H^+) in the same direction as the substrate (e.g., nucleoside). Anti-porters transport the driving ion and substrate in opposite directions. Secondary active transport allows the

substrate to be transported in an uphill or concentrative manner, that is, against its concentration gradient (19).

Pharmacological Characterization of Nucleoside Transporters

In the 1970s and 1980s, the first nucleoside transport processes were characterized in various tissue preparations, cultured cells, and cell lines. There were found to be two basic mechanisms by which nucleosides were transported: facilitated diffusion (equilibrative transport) and secondary active transport (concentrative transport) (Table 1). Two distinct equilibrative transport processes, differing in their sensitivity to a highly specific inhibitor, nitrothiobenzylinosine (NBMPR), were subsequently characterized. The *es* transporter (equilibrative, sensitive) was inhibited by nanomolar concentrations of NBMPR. The *ei* (equilibrative, insensitive) was insensitive to micromolar concentrations of NBMPR. Both transporters exhibit a broad substrate selectivity, translocating both purine and pyrimidine nucleosides and many nucleoside analogs. These transporters have since been found in many cancer cell lines as well as normal tissues and are believed to be present (*es*, *ei* or both) in all mammalian cell types (20, 21).

Concentrative nucleoside transporters were characterized in whole tissue preparations of choroid plexus and vesicle preparations of kidney and intestine (22-28). They have a more limited tissue distribution than the equilibrative nucleoside transporters and are found primarily in specialized epithelia of the kidney, intestine, and choroid plexus but are also found in parts of the brain and liver and in the heart, lung, spleen and skeletal muscle (29). Since their initial characterization, six distinct concentrative

Table 1. Pharmacologically characterized nucleoside transport processes.

Transporter	Substrate Selectivity	Na+-Dependent	NBMPR Sensitive
Equilibrative			
es	Purine nucleosides & Pyrimidine nucleosides	No	Yes
ei	Purine nucleosides & Pyrimidine nucleosides	No	No
Concentrative			
N1	Purine nucleosides & Uridine	Yes	No
N2	Pyrimidine nucleosides & Adenosine	Yes	No
N3	Purine nucleosides & Pyrimidine nucleosides	Yes	No
N4	Pyrimidine nucleosides, Adenosine & Guanosine	Yes	No
N5	Adenosine & Adenosine analogs	Yes	Yes
N6	Guanosine	Yes	Yes

nucleoside transport processes have been observed in tissue preparations from several mammalian species and cell lines. These processes differ from each other with respect to their preferred substrates. The N1 process (or *cif* for concentrative, insensitive to inhibition to NBMPR, model substrate formycin B) is purine-selective, transporting purine nucleosides and uridine. The N2 process (or *cit* for concentrative, insensitive, thymidine) is considered pyrimidine-selective, transporting pyrimidine nucleosides and adenosine. N3 is broadly-selective (or *cib*, concentrative, insensitive, broadly-selective) and transports both purine and pyrimidine nucleosides (29). N4-N6 are less well characterized. N4 (*cit*) was found in vesicle preparations of human kidney and was found to be similar to N2 (also *cit*) but also allows the purine nucleoside, guanosine as a substrate (30, 31). N5 (*cs*) and N6 (*csg*) are, unlike N1-N4, sensitive to nanomolar concentrations of NBMPR. The substrate selectivities of N5 and N6 are not well defined but it appears that adenosine and some adenosine analogs are substrates of N5 and that guanosine is the preferred substrate of N6 (32, 33). To date, es, ei, N1, N2 and N3 have been successfully cloned.

Equilibrative and concentrative nucleoside transporters are co-expressed in several specialized epithelia (e.g. intestine and kidney) and are thought to work together in the transepithelial flux of nucleosides. Figure 2 shows a cartoon of the epithelial cells of the intestine and depicts a model of the transepithelial flux of nucleosides from the intestinal lumen to the blood (absorption). Vesicle preparations of brush border membrane (BBM) and basolateral membrane (BLM) from intestinal epithelia suggest that concentrative nucleoside transporters are present in the BBM and equilibrative transporters are present in the BLM (34). The localization of the concentrative NTs has

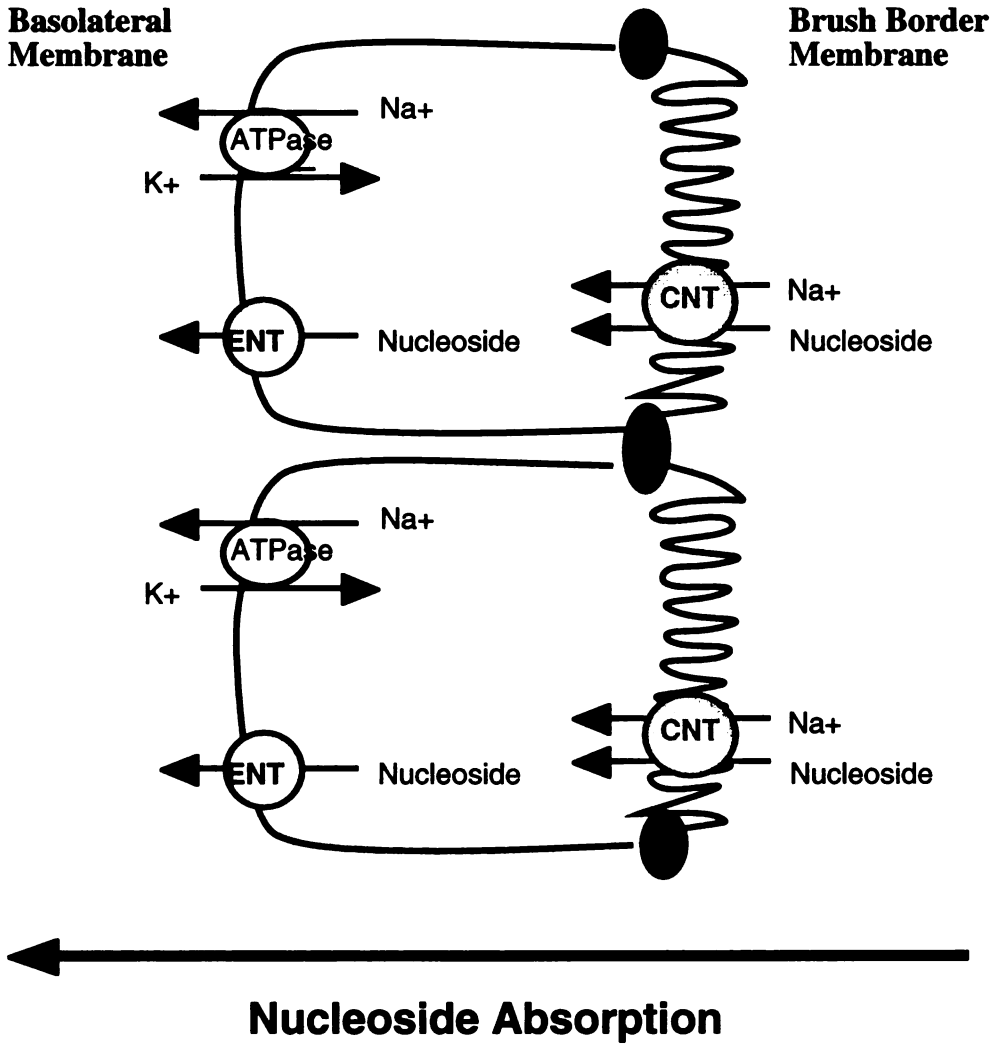


Figure 2. Model of the transepithelial flux of nucleosides. In this 2-step model, nucleosides are cotransported across the apical or brush border membrane with a sodium ion by concentrative nucleoside transporters (CNT). Nucleosides are then transported across the basolateral membrane, down their concentration gradient by equilibrative nucleoside transporters (ENT).

been confirmed recently with the use of GFP-tagged transporters transfected into polarized cells (35). Nucleosides in the intestinal lumen are transported across the BBM by concentrative NTs using the Na^+ gradient maintained by Na^+/K^+ATPase. Nucleosides are then transported, down their concentration gradient, across the BLM by the equilibrative NTs. In this way, the two types of NTs work together to achieve the vectorial transport (absorption, in this case) of nucleosides.

Cloned Equilibrative Nucleoside Transporters

The first equilibrative transporter to be cloned was the human *es* transporter (hENT1), cloned from placenta in 1997 (Table 2) (36). This cDNA codes for a protein of 456 amino acid residues and is predicted to contain 11 transmembrane domains with an intracellular N-terminus and an extracellular C-terminus. hENT1 was found to have no sequence similarity to any other transporter proteins including the concentrative nucleoside transporters described below. Consistent with the functionally characterized *es* transport process, the uptake of uridine by hENT1 was independent of the presence of Na^+ ions and was potently inhibited by NBMPR. Uridine transport was also inhibited by all of the naturally-occurring nucleosides, consistent with the inhibition profile of the *es* transporter . The *ei* transporter was subsequently identified through a database search using the hENT1 clone (37). HNP36, a delayed-early proliferative response gene product, was found to have 44% identity to the carboxy two thirds of hENT1. A full-length HNP36 was cloned from human placenta and termed hENT2. hENT2, when expressed in *Xenopus laevis* oocytes, was found to be a broadly-selective nucleoside transporter, insensitive to micromolar concentrations of NBMPR and not dependent on Na^+ ions. It

Table 2. Cloned nucleoside transporters.

Transporter	Subtype	Species	Amino Acids	Reference
Equilibrative				
hENT1	*es*	Human	456	34
rENT1	*es*	Rat	457	36
hENT2	*ei*	Human	456	35
rENT2	*ei*	Rat	456	36
Concentrative				
rCNT1	N2	Rat	648	37
hCNT1	N2	Human	650	40
pkCNT1	N2	Pig	647	44
rSPNT	N1	Rat	659	39
hSPNT1	N1	Human	658	41
mCNT2	N1	Mouse	659	42
rbSPNT1	N1	Rabbit	658	43
CeCNT3	Novel‡	C. elegans	575	45
hfCNT	N3*	Pacific hagfish	683	46
hCNT3	N3	Human	691	47
mCNT3	N3	Mouse	703	47

‡ Not-classified. N1-like but also accepts thymidine as a substrate

* No functional data reported.

was therefore determined to be an *ei* transporter. The rat orthologs to these two transporters were cloned from jejunum cDNA (38). rENT1 was cloned by PCR amplification using primers derived from the hENT1 sequence and a rat EST. The resulting rENT1 sequence was 78% identical to hENT1 and hydropathy analysis suggested the two orthologs possess similar secondary structures. rENT1 was sensitive to nanomolar concentrations of NBMPR, similar to hENT1, but was not inhibited by dilazep and dipyridamole (two potent inhibitors of hENT1). rENT2 (identified as the rat ortholog of HNP39) was also PCR amplified from rat jejunum and was 49% identical to rENT1. rENT2 was broadly-selective and not inhibited by micromolar concentrations of NBMPR.

Cloned Concentrative Nucleoside Transporters

During the 1990's, concentrative nucleoside transporters were cloned. To date, three isoforms of this transporter have been cloned from various species (Table 2). The first of these was cloned using an expression cloning method in *Xenopus laevis* oocytes. In this method, pools of cRNA transcribed from cDNA generated from a tissue expressing the transporter of interest are injected into oocytes. The oocytes are then assayed for nucleoside transport activity. Positive pools are then iteratively subdivided to isolate a single clone. The first two isoforms were isolated by this method. Subsequent species orthologs of these two isoforms were isolated by homology cloning methods in which the sequence information from the cloned cDNAs was used to generate primers to consensus regions. These primers were used to amplify an internal fragment of a related cDNA using polymerase chain reaction (PCR). The ends of the new clones were obtained

with Rapid Amplification of cDNA Ends (RACE). The third isoform was identified through database searches of the *C. elegans* and human genomic databases.

The first concentrative nucleoside transporter was cloned from a cDNA library from rat jejunum in 1994 (39). This transporter, termed rCNT1, was found to correspond to the pyrimidine-selective transport process N2, transporting the pyrimidine nucleosides and adenosine. Transcripts of rCNT1 were found in kidney and intestine and in subsequent studies, in many regions of the brain (40). The deduced primary sequence of this transporter contained 648 amino acids. Hydropathy analysis suggested 11-14 putative transmembrane domains. Subsequent studies have suggested that the C-terminus is glycosylated and therefore, extracellular giving rise to the predominant 13 transmembrane domain model of the secondary structure (unpublished data). rCNT1 has no similarity to other mammalian Na^+-coupled transporters such as the glucose transporter (SGLT) and was, thus, a new gene family. Only the NupC transporter, a proton-dependent nucleoside transporter from *E. coli* was found to have any similarity (27%) to rCNT1 and is thought to be another member of this family (39).

The second mammalian concentrative nucleoside transporter was cloned in 1995 from rat liver (41). This transporter, corresponding to the purine-selective transporter N1, was termed rSPNT (also known as rCNT2). This transporter was found to preferentially transport purine nucleosides and uridine. In rat, rSPNT was found to be expressed in more tissues than rCNT1 but interestingly, was not found in the kidney. By Northern blot analysis rSPNT transcripts were found in liver, jejunum, spleen and heart. The deduced primary sequence contains 659 amino acids and is 64% identical to rCNT1 with the

termini being the most divergent. This high degree of identity suggests that they are members of the same gene family (41).

In the last few years orthologs to these two transporters have been cloned from several species. Most importantly, the human orthologs were cloned in 1997. hCNT1, the human ortholog of rCNT1, was cloned from a human kidney cDNA library using hybridization screening and RT-PCR methods (42). The protein encoded by hCNT1 was 650 amino acid residues in length and 72% identical to rCNT1. hCNT1 was functional when expressed in oocytes and its inhibition profile was consistent with the pyrimidine-selective rCNT1. hSPNT1, the human ortholog of rat SPNT was also cloned from human kidney through a PCR-based homology cloning method (43). hSPNT1 encodes a protein of 658 amino acid residues and is 81% identical to rSPNT. Although hSPNT1, when expressed in oocytes, exhibited a substrate selectivity similar to that of rSPNT, hSPNT1 had a broader tissue distribution than its rat ortholog. In addition to the liver, intestine and heart, hSPNT1 transcripts were detected in skeletal muscle, pancreas and kidney. Subsequently, SPNT orthologs were cloned from mouse and rabbit and a CNT1 ortholog was cloned from pig (44-46).

Most recently, the broadly selective transporter (N3 transport process) was cloned from *C. elegans*, Pacific hagfish, human and mouse (47-49). The molecular identity of this transporter was pursued for many years. In 1994, a low activity nucleoside transporter (termed SNST1) was isolated from a rabbit kidney cDNA library through screening with a 1.6kb fragment of the rabbit SGLT1 cDNA (50). Although SNST1 was approximately 61% identical to SGLT1, when expressed in *X. laevis* oocytes it transported uridine at low levels but not glucose or any glucose analogs. Uridine uptake

was inhibited by uridine, cytidine, adenosine and guanosine, consistent with a broadly-selective transporter. For several years it was thought that perhaps this cDNA was the broadly-selective nucleoside transporter. However, there were some discrepancies between the N3 transport process characterized in rabbit choroid plexus and ileum and SNST1. For example, by Northern blot analysis, the SNST1 transcript was shown to be abundant in heart but not present in intestine or brain where one would expect to find N3 (22-24, 51). The function of SNST1 continues to be somewhat controversial but the consensus appears to be that it is a glucose transporter due to its high identity to the SGLTs. In later years a chimera of rCNT1 and rSPNT (and subsequently a single amino acid mutant of rCNT1) was constructed that had the selectivity of the broadly-selective N3-type transporter (52, 53). This suggested that an isoform closely related to rCNT1 or perhaps a polymorphism of rCNT1 could be responsible for the broadly-selective transporter. However, homology cloning methods attempted in our laboratory did not yield the cDNA for this transporter but did result in the cloning of the first concentrative nucleoside transporter from rabbit (rbSPNT1) (Chapter 2). The first broadly-selective transporter to be cloned and functionally characterized was from *C. elegans*, CeCNT3. (A potential broadly-selective transporter was cloned from Pacific hagfish a year earlier, however no functional characteristics were reported. (48)) Database searches recently identified the human and mouse broadly-selective transporter. The cDNA encoding the 691 amino acid residue human transporter (hCNT3) was then isolated from mammary gland by RT-PCR. The primary sequence of this clone was 57% identical to the hagfish ortholog and 48% and 47% identical to the other human isoforms, hCNT1 and hSPNT1, respectively. CNT3 appears to have a more widespread tissue distribution than either

CNT1 or SPNT. hCNT3 transcripts were detected at high levels in tissues such as pancreas, bone marrow, trachea and intestine and at lower levels in kidney, liver, lung, placenta and many other tissues. Phylogenic analysis of the CNT sequences suggest that CNT3 and CNT1/CNT2 (SPNT) are distinct subfamilies (49).

Substrate Selectivity and Interspecies Differences

The availability of homologous transporter clones from several species has allowed researchers to begin to study the interaction of various nucleosides and nucleoside analogs with these transporters. Detailed characterization of substrate selectivity profiles and kinetic parameters of nucleoside analogs will enable a better understanding of the *in vivo* distribution in preclinical testing in animal models of new drugs in this class. Many studies reported in the literature addressing the substrate selectivity of these cloned nucleoside transporters use competitive inhibition methodologies. Frequently, researchers infer that the ability of a compound to interfere with the uptake of a known substrate suggests that the inhibitor is also a substrate. However, direct methods are required to determine if an inhibitor is also translocated. Uptake of a radiolabeled compound or an electrophysiological method in which a current induced by the transport of a substrate by an electrogenic transport process (e.g. a Na^+-coupled transport process) is measured in a heterologous expression system, are examples of direct methods. To date, few studies are reported in the literature that use a direct method, such as the uptake of a radiolabeled nucleoside analogs in cells or oocytes expressing one of the cloned nucleoside transporters. In HeLa cells expressing either hSPNT1 or rSPNT, 2',3'-dideoxyinosine (ddI) and 2-chloro-2'-deoxyadenosine (2CdA)

were shown to inhibit uptake of inosine by both transporters (IC_{50} of 19 μM and 46 μM for ddI and 371 μM and 14 μM for 2CdA for hSPNT1 and rSPNT, respectively). Although ddI interacts with both human and rat SPNT, studies with radiolabeled ddI suggest that it is not significantly translocated by either transporter. 2CdA appears to be a substrate of rSPNT but not hSPNT1 (54, 55). These limited studies suggest that there may be significant interspecies differences in the interactions of important nucleoside analogs with purine-selective nucleoside transporters. These differences suggest that the rat may not be very useful in predicting the distribution of certain nucleoside analogs in humans. It is therefore important to do comprehensive studies of the substrate selectivity of these cloned transporters. Detailed study of the spectrum of selectivity of these transporters using radiolabeled uptake assay is cost-prohibitive and limited by the availability of labeled compounds. Electrophysiological methods such as the two-electrode voltage clamp enable the study of a large number of unlabeled compounds in a short period of time and are therefore more amenable to comprehensive analysis. The two electrode voltage clamp of oocytes expressing rCNT1, rSPNT and hSPNT1 is used to study the substrate selectivity and kinetics of these transporters (Chapters 3 and 5).

Molecular Determinants of Substrate Selectivity

The availability of the cDNAs coding for these transporters has allowed researchers to begin to study the structure activity relationships of these proteins. In the first of these studies, the substrate discrimination region was identified through the construction of a series of chimeras of the rCNT1 and rSPNT. When transmembrane domains (TM) 7 and 8 (of the 13 transmembrane domain model or TM 8 and 9 of the

earlier 14 TM model) of rCNT1 were replaced with the corresponding TM of rSPNT, the phenotype switched from pyrimidine-selective to purine-selective (52). This suggested that TMs 7 and 8 formed part of the substrate discrimination region. The replacement of only TM 7 of rCNT1 with that of rSPNT resulted in a broadly-selective transporter (52, 53). An alignment of TM 7 from rSPNT and rCNT1 revealed only 5 differences in amino acid sequence. Single replacement of four of the five amino acid residues of rCNT1 with the corresponding residue from rSPNT resulted in a transporter that retained the rCNT1 phenotype, that is, it was pyrimidine-selective. However, when Ser-318 was replaced with glycine, the selectivity of rCNT1 was broadened to include purine nucleosides as substrates (56). These data were confirmed and extended subsequently in similar studies with the human clones (48). The reason for this switch in selectivity was not determined but several hypotheses were proposed. The first was that the decrease in size of the side chain at position 318 due to the S318G substitution increased the size of the permeation pathway allowing the larger purine nucleosides access to the binding site. The second was that a hydrogen bond formed between the hydroxyl group of the serine residue and possibly another amino acid within the protein prevented the purine nucleosides from accessing the binding site. These hypotheses are tested in Chapter 4.

Summary of Experimental Chapters

Chapter 2 – Cloning of a purine-selective nucleoside transporter, rbSPNT1, from rabbit.

Substantial species differences in the transport kinetics of nucleosides and therapeutic nucleoside analogs have been observed in various experimental systems. The goal of the studies in Chapter 2 was to clone the nucleoside transporters present in rabbit,

an important preclinical animal model, and to functionally characterize the clone(s). A PCR-based homology cloning approach in conjunction with RACE was used to isolate a full-length cDNA. Characterization of this transporter was accomplished through heterologous expression in *Xenopus laevis* oocytes. A full-length cDNA encoding a purine-selective, Na^+-dependent nucleoside transporter, rbSPNT1, was isolated from rabbit small intestine. The encoded protein is 658 amino acid residues in length. Hydropathy analysis suggests that rbSPNT1 has 11 to 14 transmembrane domains. In *Xenopus laevis* oocytes expressing rbSPNT1, the uptake of uridine and inosine was enhanced significantly; uridine transport was inhibited by purine, but not pyrimidine nucleosides. mRNA transcripts for rbSPNT1 were detected primarily in intestine, lung and kidney and at lower levels in liver, brain and heart. A full-length functional nucleoside transporter was cloned. Sequence analysis and functional characterization suggest that rbSPNT1 is the rabbit homolog of the purine-selective nucleoside transporter, N1. The cloned rbSPNT1 can be used to understand the molecular mechanisms responsible for the observed species differences in the transport of nucleosides and therapeutic nucleoside analogs.

Chapter 3- Electrophysiological analysis of the substrate selectivity of a sodium-coupled nucleoside transporter (rCNT1) expressed in Xenopus laevis *oocytes.*

Nucleoside transporters that mediate cellular uptake of therapeutic nucleoside analogs are major determinants of the pharmacokinetic properties of these compounds. Understanding the substrate selectivity of these transporters is critical in the development of therapeutic nucleoside analogs with optimal pharmacokinetic properties, including

high oral bioavailability and tissue-specific distribution. In general, substrate selectivity of nucleoside transporters has been evaluated indirectly by inhibition studies. The purpose of this study was to directly measure the transport of nucleoside analogs by the sodium-coupled pyrimidine-selective transporter rCNT1 using electrophysiology methods. We used a two-electrode voltage clamp assay to investigate the substrate selectivity of rCNT1; nineteen structurally diverse nucleosides and nucleoside analogs were studied. Uridine-induced currents in voltage-clamped oocytes expressing rCNT1 were sodium-, voltage-, and concentration-dependent ($K_{0.5}$ = 21 μM), and were blocked by adenosine. Uridine-induced currents increased ~ 5-fold upon hyperpolarization of membrane potential from –10 to –150 mV. Uridine, thymidine, and cytidine induced currents in rCNT1-expressing oocytes, whereas guanosine, inosine, and adenosine did not. Uridine, deoxyuridine, and cytidine analogs with modifications at the 3, 4, or 5 position were found to be substrates of rCNT1, whereas uridine and cytidine analogs modified at the 6 position were not. In addition, it was found that the 5'-hydroxyl group of the sugar is not required for transport by rCNT1. These results enhance our understanding of the structural basis for substrate selectivity of nucleoside transporters and should prove useful in the development of therapeutic nucleoside analogs.

Chapter 4 – Molecular determinants of the pyrimidine-selectivity of a nucleoside transporter (rCNT1).

The recent cloning of purine-selective and pyrimidine-selective Na^+-dependent nucleoside transporters has enabled the study of specific amino acid residues involved in substrate recognition of these proteins. Site-directed mutagenesis studies revealed that a

single substitution, serine to glycine at position 318, was sufficient to broaden the substrate-selectivity of the rat pyrimidine-selective transporter (rCNT1) to accept purine nucleosides as substrates. The goal of the present study was to determine the molecular mechanisms responsible for the broadened selectivity of rCNT1 by investigating whether the size of the side chain or its ability to form a hydrogen bond was responsible for the selectivity change. In order to determine the mechanism of the selectivity change incurred by the serine to glycine substitution, we constructed a series of mutants in which the residue at position 318 was changed to residues of increasing size or to residues that can form hydrogen bonds. Our data suggest that the size of the side chain at position 318 is important for conferring pyrimidine-selectivity, that is, the smaller glycine side chain allowed both pyrimidine and purine nucleosides as substrates whereas the larger side chains of alanine, threonine and serine (in wildtype rCNT1) allowed only pyrimidine nucleosides and adenosine. The hydroxyl group of the serine residue may be important for efficient adenosine transport by rCNT1.

Chapter 5 - Species differences in the substrate selectivity of the human and rat orthologs of the concentrative nucleoside transporter, SPNT.

Nucleoside analogs are currently in clinical use for the treatment of viral infections and cancer. In order to understand the role that nucleoside transporters play in the *in vivo* distribution of nucleoside analogs, the scope of the substrate specificity must be determined. In the present work we have addressed the substrate specificity of the human and rat orthologs of the purine-selective, Na^+-dependent nucleoside transporter, SPNT, using the two-electrode voltage clamp technique with *Xenopus laevis* oocytes

expressing these transporters. We examined transport of naturally occurring nucleosides and nucleobases, base-modified nucleoside analogs and ribose-modified nucleoside analogs. Currents were induced in oocytes expressing rSPNT and hSPNT1 by the purine nucleosides and uridine. The rank order of magnitude of nucleoside-induced currents was guanosine > uridine > adenosine > inosine and guanosine > uridine > inosine > adenosine for rSPNT- and hSPNT1-expressing oocytes, respectively. Uridine analogs with modifications to the 5-position of the base induced little or no current suggesting that these compounds are only poorly transported by the SPNT orthologs. Cladribine (2CdA) induced currents in rSPNT-expressing oocytes ($K_{0.5}$ = 57 ± 12 μM) but not in hSPNT1-expressing oocytes. Of the ribose-modified nucleoside analogs tested, adenine arabinoside and 2',3'-dideoxyadenosine induced currents in rSPNT-expressing oocytes (approximately 70% and 30% of that induced by guanosine, respectively) but not in hSPNT1-expressing oocytes. Overall, these data suggest that there are pronounced differences in the substrate specificities of these two transporters with the rat ortholog accepting a wider array of substrates. These findings suggest that the rat may be an inappropriate animal model for use in the evaluation of new nucleoside analogs in pre-clinical drug development.

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CHAPTER 2

CLONING OF A PURINE-SELECTIVE NUCLEOSIDE TRANSPORTER, rbSPNT1, FROM RABBIT[1]

Introduction

Membrane transporters in the epithelial lining of the intestinal lumen play an important role in the salvage of endogenous nucleosides and may be critical in the absorption of therapeutically important nucleoside analog drugs. Concentrative, or Na^+-dependent, nucleoside transporters have been characterized in intestinal epithelia from several species (1-3). Recently, purine- and pyrimidine-selective Na^+-dependent nucleoside transporters (termed SPNT (cNT2) and cNT1, respectively) were cloned from rat, human and pig (4-8).

Functional studies indicate that there are significant interspecies differences in the kinetic parameters of various nucleosides in interacting with cloned transporters from rat and human (4, 7, 9, 10). In contrast, studies in isolated rabbit tissues suggest that concentrative nucleoside transporters from rabbit may be more similar kinetically to human transporters (3, 11). To study the molecular mechanisms responsible for interspecies differences in nucleoside transport, it is necessary to clone the relevant transporters and examine their kinetic characteristics in heterologous expression systems. Using homology cloning methods, we isolated a purine-selective nucleoside transporter,

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termed rbSPNT1, from rabbit intestine. This study reports its cloning and initial functional characterization.

Methods

Cloning. A full-length cDNA was obtained from rabbit small intestine using a PCR-based homology cloning method. Total RNA was isolated from various tissues of New Zealand white rabbits using the TRIzol reagent (Gibco BRL). First-strand cDNA synthesis was performed using the SUPERSCRIPT Preamplification System for First Strand cDNA Synthesis (Gibco BRL) with total RNA from rabbit ileum as template. Primers were designed to the consensus regions (amino acid residues 272-282 and amino acid residues 534-544 of the rSPNT protein sequence). These primers were used in PCR amplification with ileum cDNA as the template under the following conditions: 94°C for 1 minute; 50°C for 2 minutes; 72°C for 2.5 minutes for 30 cycles. A single fragment of approximately 0.8 kb was obtained. Gene-specific primers were designed to regions of this fragment for 5' and 3' RACE (Rapid Amplification of cDNA Ends). First-strand cDNA synthesis from ileum total RNA was performed using the 3'RACE System (Gibco BRL) per the manufacturer's protocol. The resulting cDNA was amplified with the first gene-specific primer (5'-TAAAACTGCCTCGTGGGAAGGAGAG-3') and the Abridged Universal Amplification Primer (AUAP) under the following conditions: 94°C for 1 minute; 50°C for 2 minutes; 72°C for 1.5 minutes for 30 cycles. A nested PCR was carried out using the product from the first amplification (diluted 1:40 with water) as the template with the AUAP primer and the second gene-specific primer (5'-GCTATAGCTCTTGTTGCGAATGTCG-3') under the following conditions: 94°C for 1

minute; 55°C for 2 minutes; 72°C for 3 minutes for 35 cycles. The resulting 1.1 kb product was subcloned and sequenced. The sequence of this fragment contained a poly-adenylation signal (AATAAA), a poly-A tail, and a region which overlapped with the initial 0.8 kb fragment, suggesting that the entire 3' end of the same clone had been isolated. The 5'end of this cDNA was then obtained using the 5'RACE System (Gibco BRL). First-strand cDNA synthesis was carried out using total RNA from rabbit jejunum and ileum with the spnt1 primer (5'-CCCTATGGAAGTAAGATTGGCAAATCCACAGAG-3'). PCR was performed using a gene-specific anti-sense primer (5'-GATGAGCAGAGGTGCCTCTGTCATA-3') and the Anchor Primer under the following conditions: 94°C for 1 minute; 50°C for 2 minutes; 72°C for 2 minutes for 30 cycles. A nested PCR was performed using a second gene-specific primer (5'-ACGCATAGTGACTTGCAAAAACCAGG-3') and the AUAP primer under the same conditions. Two bands of approximately 1.0 and 1.1 kb resulted from the nested PCR derived from the jejunum cDNA. Both fragments were subcloned and sequenced; the longer of the two appeared to be a splice variant and will be investigated further in the future. A start codon and Kozak consensus sequence were found in the longest open reading frame. Alignment with SPNT and hSPNT1 suggested that the entire 5' end of the coding region had been obtained. Primers were then designed to amplify the full-length cDNA. A sense primer (5'-TCCTTCCCTGAGGAGCCATTAGGAATC-3') was designed to bases 1-27 of the 5'RACE product. An anti-sense primer (5'-CTAAATGCTCACAGCAATTCCTACCC-3') was designed to the 3'RACE product within the untranslated region, upstream of the poly-adenylation signal. PCR was carried out using these primers and jejunum cDNA

under the following conditions: 94°C for 1 minute; 55°C for 2 minutes; 72°C for 3 minutes for 35 cycles. The resulting 2.2 kb cDNA was subcloned into the pGEM-T vector (Promega) and sequenced.

Tissue Distribution. The tissue distribution of rbSPNT1 mRNA was determined by RT-PCR. Gene-specific primers were designed to amplify a 276 bp fragment of rbSPNT1. Total RNA or mRNA was isolated from various tissues (including kidney, intestine, liver, heart, brain, spleen and lung), and first-strand cDNA was synthesized using the SUPERSCRIPT Preamplification System for First-Strand cDNA Synthesis. cDNA was synthesized using approximately 0.5 μg mRNA from each tissue with the exception of spleen and liver for which total RNA was used (approximately 1 μg and 2.2 μg, respectively). PCR was then performed using the gene-specific primers and the resulting products were separated on a 1% agarose gel.

Radiotracer Transport Assays. Functional studies were carried out using the *Xenopus laevis* oocyte expression system. Defolliculated oocytes were injected with approximately 50 ng of cRNA that had been transcribed from the cDNA *in vitro* as previously described (12). Uptake assays were performed with radio-labeled and unlabeled nucleosides 2 days post cRNA injection (13).

Results and Discussion

The cDNA sequence and deduced amino acid sequence are shown in Figure 1A. The complete 2302 bp cDNA contains an ORF of 1977 bp that encodes a 658 amino acid protein. Hydropathy analysis of the deduced protein sequence revealed 11-14 potential transmembrane domains (Figure 1B), suggesting a topology similar to other members of this family of transporters (14). Further sequence analysis using the Genetics Computer

Group (GCG) software (Wisconsin Package, versions 9 and 10) identified four potential N-linked glycosylation sites on asparagine residues 538, 605, 624, and 653. Six potential PKC phosphorylation sites were identified on serine residues 5, 198, and 522 and on threonine residues 36, 295, and 604.

Alignment of the protein sequence with those of previously cloned concentrative nucleoside transporters using the Bestfit program of the GCG software showed that rbSPNT1 is 84% identical to hSPNT1 and 82% identical to rSPNT. It is less identical to the cNT1 (N2) clones (65% identical to hCNT1a and 65% identical to rCNT1), suggesting that rbSPNT1 is the rabbit homolog of human and rat SPNT. Furthermore, an alignment of amino acids 296 to 358 from this protein sequence with the corresponding region of the other cloned transporters shows that the residues determined to be critical for substrate discrimination (13, 15) are identical to those residues conserved across all SPNT sequences (Figure 2). This observation further supports the identification of rbSPNT1 as the rabbit N1 homolog.

Oocytes injected with rbSPNT1 cRNA exhibited an enhanced Na^+-dependent uptake of [^{3}H]-uridine and [^{3}H]-inosine over uninjected oocytes (controls) (Figure 3A). [^{3}H]-Uridine (11 μM) uptake in the presence of Na^+ in cRNA-injected oocytes was 0.35 ± 0.042 pmol/oocyte/30 minutes, whereas the uptake of [^{3}H]-uridine was 0.08 ± 0.015 pmol/oocyte/30 minutes in uninjected oocytes, representing an enhancement in uptake of approximately 4-fold. [^{3}H]-Inosine (11 μM) uptake in the presence of Na^+ was enhanced approximately 16-fold in cRNA-injected oocytes compared to uninjected oocytes (1.32 ± 0.456 pmol/oocyte/30 minutes versus 0.08 ± 0.006 pmol/oocyte/30 minutes). [^{3}H]-Uridine (11 μM) uptake in the presence of Na^+ was measured in the presence of each

A.

```
1     TTCTTCCCTGAGGAGCCATTAGGAATCCCACAAGCTGGGTTCAGAAGAGAGGATGGAGAAAGCCAGTGGAAGAAAGTCTCTTGCTCTGTCCACAGCGGAG
1                                                   M  E  K  A  S  G  R  K  S  L  A  L  S  T  A  E
                                                                    ‡
101   AACGGCATAGAGAACGCAGGGCTGGAGCTCACGGAAGAAGGGATAAATTCTGAGCAAACCAGGAGGATGGAAGTGCAAGGACACAGTCTCAGCGATGATG
17    N  G  I  E  N  A  G  L  E  L  T  E  E  G  I  N  S  E  Q  T  R  R  M  E  V  Q  G  H  S  L  S  D  D  V
                                                                    ‡
201   TGAGGCCTGCCACTCACCAAAGGAGTTATCTACAGCCTCTCACCAAGGCAAGAACTTTCTGCCAAAGACATGCAAGCTTGTTCAAGAAGATCCTGTTGGG
51      R  P  A  T  H  Q  R  S  Y  L  Q  P  L  T  K  A  R  T  F  C  Q  R  H  A  S  L  F  K  K  I  L  L  G

301   CCTGTTGTGTCTGGCGTATGCTGCCTATTTCTTGGCAGCTTGCATCTTGGATTTCCAGAGGGCACTGGCCTTATTTGTCATCACCTGTTTGGTGATCTTG
84     L  L  C  L  A  Y  A  A  Y  F  L  A  A  C  I  L  D  F  Q  R  A  L  A  L  F  V  I  T  C  L  V  I  L

401   GTCCTGCTTCTCCACTTTCTGAAAAAGTTCTTGGGTAAAAAATTAACAAGATGCCTGAAGCCCTTTAAAAATTCCCAGCTGAGACTTTGGATAAAACGGG
117   V  L  L  L  H  F  L  K  K  F  L  G  K  K  L  T  R  C  L  K  P  F  K  N  S  Q  L  R  L  W  I  K  R  V

501   TGTTCGCAGGAGTCTCCTTGGTTGGCCTTATCCTGTGGCTGGCACTAGACACAGCTCAAAGGCCAGAGCAGCTGATCTCCTTTGCAGGAATCTGCATGTT
151     F  A  G  V  S  L  V  G  L  I  L  W  L  A  L  D  T  A  Q  R  P  E  Q  L  I  S  F  A  G  I  C  M  F

601   CGTCCTCATCCTCTTTGCGTGCTCCAAACACCACAGTGCAGTGTCCTGGAGGACAGTGTTTTGGGGCCTGGGTCTTCAGTTTGTCTTTGGATTATTGGTC
184     V  L  I  L  F  A  C  S  K  H  H  S  A  V  S  W  R  T  V  F  W  G  L  G  L  Q  F  V  F  G  L  L  V
                                                    ‡
701   ATCAGAACGGATCCTGGATTTATTGCATTTCAGTGGCTGGGAGATCAGGTCCAGATTTTCCTGGCCTACACCGTCGCTGGCTCCAGTTTTGTCCTTGGGG
217   I  R  T  D  P  G  F  I  A  F  Q  W  L  G  D  Q  V  Q  I  F  L  A  Y  T  V  A  G  S  S  F  V  L  G  D

801   ATACCCTGGTCAATGATGTCTTTGCTTTTCAGTCGTTACCAATCATCATTTTCTTTGGATGCGTGATGTCCATTCTCTACTACCTGGGCCTTGTGCAGTG
251     T  L  V  N  D  V  F  A  F  Q  S  L  P  I  I  I  F  F  G  C  V  M  S  I  L  Y  Y  L  G  L  V  Q  W

901   GGTTGTTCAGAAGATCGCCTGGTTTTTGCAAGTCACTATGCGTACCACTGCCACAGAGACCCTAGCTGTAGCAGGAAACATCTTTGTGGGTATGACAGAG
284   V  V  Q  K  I  A  W  F  L  Q  V  T  M  R  T  T  A  T  E  T  L  A  V  A  G  N  I  F  V  G  M  T  E
                                       ‡
1001  GCACCTCTGCTCATCCGCCCCTACCTTGCAGACTTGACCCTCTCTGAAATCCATGCAGTGATGACTTCAGGGTTTGCCACCATTTCAGGCACTGTGTTGG
317   A  P  L  L  I  R  P  Y  L  A  D  L  T  L  S  E  I  H  A  V  M  T  S  G  F  A  T  I  S  G  T  V  L  G

1101  GAGCCTTCATATCCTTTGGGATTGATGCATCATCCTTGATTTCTGCTTCTGTGATGGGTGCCCCTTGTGCACTTGCCTTGTCAAAGCTGGTATATCCAGA
351     A  F  I  S  F  G  I  D  A  S  S  L  I  S  A  S  V  M  G  A  P  C  A  L  A  L  S  K  L  V  Y  P  E

1201  AGAGGAGGAGTCCAAGTTCAAGAGTAAAGAGGGGGTAAAACTGCCTCGTGGGAAGGAGAGCAATGTCCTAGAAGCTGCCAGCAATGGAGCGACAGATGCT
384   E  E  E  S  K  F  K  S  K  E  G  V  K  L  P  R  G  K  E  S  N  V  L  E  A  A  S  N  G  A  T  D  A

1301  ATAGCTCTTGTTGCGAATGTCGCAGCCAACCTGGTTGCCTTTCTGGCCGTGTTGGCCTTCATCAATGCTGCCCTTTCCTGGTTGGGGGAACTGGTGGACA
417   I  A  L  V  A  N  V  A  A  N  L  V  A  F  L  A  V  L  A  F  I  N  A  A  L  S  W  L  G  E  L  V  D  I

1401  TACAGGGGCTCACTTTCCAGGTCATCTGCTCCTATATCCTAAGGCCCATGGTTTACATGATGGGTGTAGAGTGGACAGACTGTCCAATGGTGGCTGAGAT
451     Q  G  L  T  F  Q  V  I  C  S  Y  I  L  R  P  M  V  Y  M  M  G  V  E  W  T  D  C  P  M  V  A  E  M

1501  GGTGGGAATCAAGTTCTTCACAAATGAGTTCGTGGCCTATCAGCAACTGTCTCAATACAAGAAAAAACGTCTCTCTGGAATGGAAGAATGGATTGATGGC
484   V  G  I  K  F  F  T  N  E  F  V  A  Y  Q  Q  L  S  Q  Y  K  K  K  R  L  S  G  M  E  E  W  I  D  G

1601  CAGAAACAGTGGATTTCTGTGAGAGCTGAAGTCATTACAACATTTTCACTCTGTGGATTTGCCAATCTTAGTTCCATAGGAATAACACTGGGAGGCTTGA
517 Q  K  Q  W  I  S  V  R  A  E  V  I  T  T  F  S  L  C  G  F  A  N  L  S  S  I  G  I  T  L  G  G  L  T

1701  CTTCAATGGTGCCCCCACCGAAAAAGTGATCTGTCCAAAGTTGTGATCCGGGCCCTCTTCACAGGGTCCTGTGTATCCTTTATCAGTGCCTGTGTGGCAGG
551     S  M  V  P  H  R  K  S  D  L  S  K  V  V  I  R  A  L  F  T  G  S  C  V  S  F  I  S  A  C  V  A  G

1801  AATCCTCTATGTTCCCCGGGGAGCTGAAACTGACTGTGTCTCCTTCCTAAGCACAAGTTTCACCAACAGAAGCTATGAGACCTATGTGTGCTGCAGGGAG
584   I  L  Y  V  P  R  G  A  E  T  D  C  V  S  F  L  S  T  S  F  T  N  R  S  Y  E  T  Y  V  C  C  R  E
                                                                    Δ
1901  CTCTTTCAGAATACTTATCTGAATGGCACCAATCCACCTTCTTTTTCTGGTGCCTGGGAAGACAAGGCATTCAGTGCCATGGCCCTTGCTAACTGCTGTG
617   L  F  Q  N  T  Y  L  N  G  T  N  P  P  S  F  S  G  A  W  E  D  K  A  F  S  A  M  A  L  A  N  C  C  G
                                 Δ
2001  GATTCTACAACAATACTGTCTGTGCCTAAGGATGGTTGGTCCATTTCTACCACAGTTTTAATCTTAACAGCTTTTTGGTGCAAAGGTGTTTGTTTACTCA
651     F  Y  N  N  T  V  C  A  *
          Δ
2101  GGATTCCAACCCTTTAACCAACTCTCCAAGATCCTTAACAGTAAATGTAAAAGATTCATTTGGTTCAGTGCATTCCACTAATGAAAATTAGCATTCATCA
2201  TTCCACTATTTCACTGAACCATAAAGGTACTCATGGGTAGGAATTGCTGTGAGCATTTAGTTGAAAAAATAAATTTTATAATATCAAAAAAAAAAAAAAA
2301  AA
```

B.

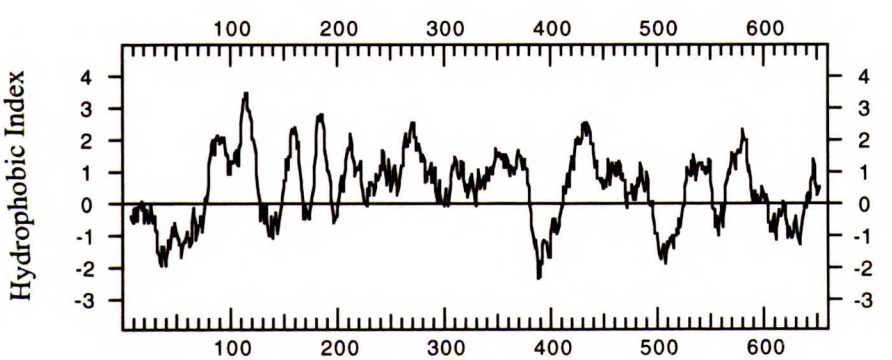


Figure 1. (A) Nucleotide and deduced amino acid sequence of rbSPNT1. The complete cDNA (2302 bp) containing a 1977 bp ORF is predicted to encode a protein of 658 amino acids. Thirteen putative transmembrane domains are underlined. Intracellular putative PKC phosphorylation sites (‡) and extracellular putative N-linked glycosylation sties (Δ) are indicated. (B) Hydropathy analysis of rbSPNT1. The Kyte-Doolittle method was used with a window of 13 residues.

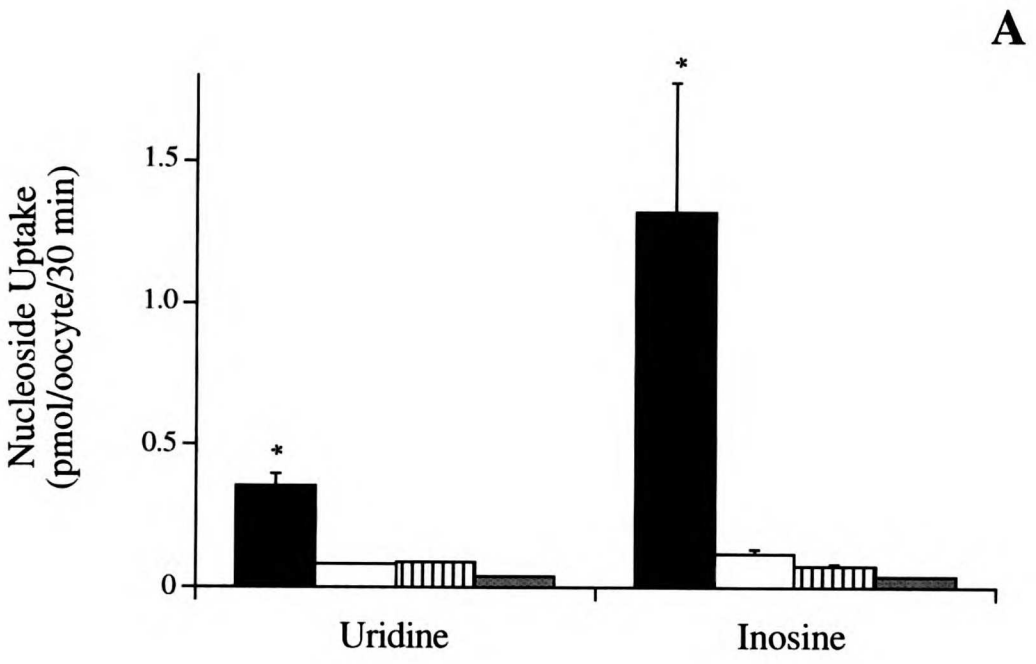
Purine-selective Transporters

```
 aa #    296                                                                358

rbSPNT1  MRTTATE TLAVAGNIFV GMTEAPLLIR PYLADLTLSE IHAVMTSGFA TIAGTVLGAF ISFGID
 hSPNT1  MGTTATE TLAVAGNIFV GMTEAPLLIR PYLGDMTLSE IHAVMTGGFA TISGTVLGAF IAFGVD
   SPNT  MGTTAAE TLAVAGNIFV GMTEAPLLIR PYLADMTLSE IHAVMTGGFA TIAGTVLGAF ISFGID
  mCNT2  MGTTAAE TLAVAGNIFV GMTEAPLLIR PYLADMTISE IHTVMTGGFA TIAGTVLGAF ISFGID
```

Pyrimidine-selective Transporters

```
 aa #    305                                                                367

  hCNT1  MGTTATE TLSVAGNIFV SQTEAPLLIR PYLADMTLSE VHVVMTGGYA TIAGSLLGAY ISFGID
   cNT1  MGTSATE TLSVAGNIFV SQTEAPLLIR PYLADMTLSE VHVVMTGGYA TIAGSLLGAY ISFGID
 pkCNT1  MGTTATE TLSVAGNIFV SQTEAPLLIR PYLADMTLSE IHVVMTGGYA TIAGSLLGAY ISFGID
```

Figure 2. Multiple sequence alignment of putative TMD 8 and 9 region. Amino acids of the purine-selective transporters, corresponding to residues 296 - 358 of rbSPNT1, were aligned with the corresponding amino acids of the pyrimidine selective transporters (amino acids 305 to 367 of hCNT1). Conserved differences in amino acid residues between purine- and pyrimidine-selective transporters shown in bold.


A
Nucleoside Uptake
(pmol/oocyte/30 min)
1.5
1.0
0.5
0
*
*
Uridine
Inosine

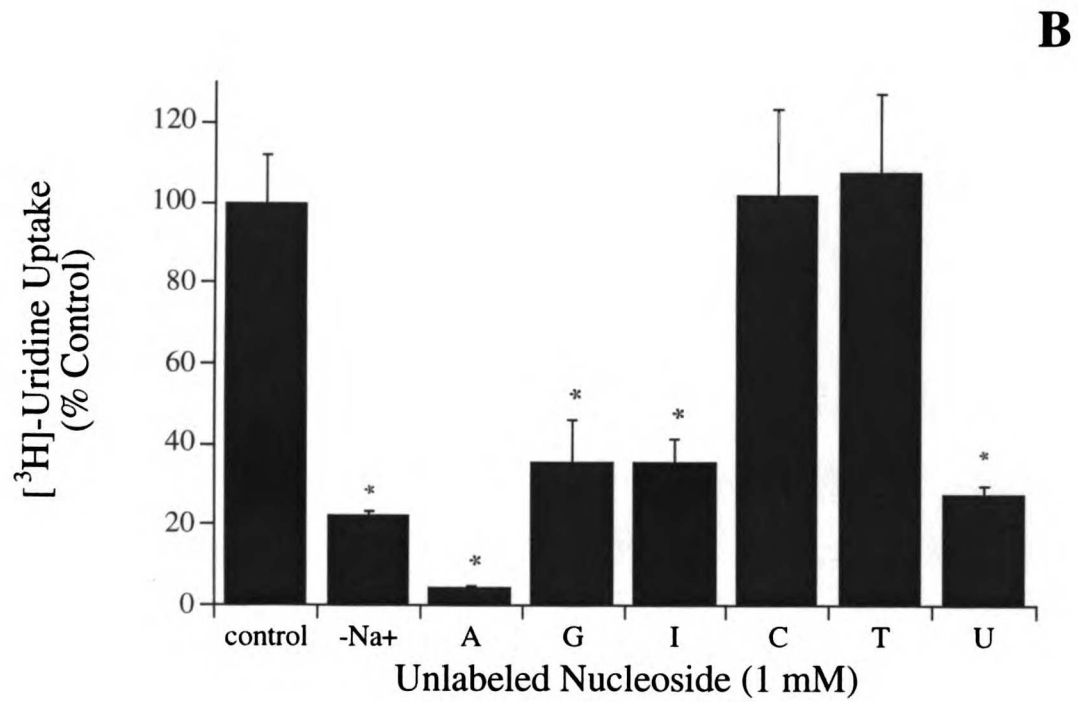

B
[3H]-Uridine Uptake
(% Control)
120
100
80
60
40
20
0
*
*
*
*
*
control
-Na+
A
G
I
C
T
U
Unlabeled Nucleoside (1 mM)

Figure 3. (A) Nucleoside uptake in *Xenopus laevis* oocytes. Uptake of [^{3}H]-uridine (11 uM) or [^{3}H]-inosine (11 uM) in rbSPNT1 cRNA injected oocytes in the presence (solid bars) and absence (open bars) of Na^+ and uninjected control oocytes in the presence (striped bars) and absence (stippled bars) of Na^+. Asterisks indicate significant difference from control (uninjected oocytes in the presence of Na^+) ($P < 0.05$). (B) Inhibition of uridine uptake by endogenous nucleosides in *Xenopus laevis* oocytes. Uptake of [^{3}H]-uridine (11 uM) in rbSPNT1 cRNA injected oocytes in the presence of Na^+ (except where noted) and 1 mM unlabeled nucleosides abbreviated as follows: A, adenosine; G, guanosine; I, inosine; C, cytidine; T, thymidine; U, uridine. Asterisks indicate significant difference from control ($P < 0.01$).

of the endogenous nucleosides at a concentration of 1 mM. Uridine uptake was significantly inhibited ($P < 0.01$) by adenosine, guanosine, inosine and uridine but not thymidine or cytidine (Figure 3B). This inhibition profile is consistent with an N1 or purine-selective transporter. Interestingly, cytidine and thymidine do not seem to inhibit uptake of nucleosides by rbSPNT1 (no inhibition of uptake at concentrations of 1 mM) whereas they weakly inhibit uptake in the rat and human homologs (4, 7). This may suggest that rbSPNT1 is more purine-selective (i.e. less sensitive to even weak inhibition by pyrimidine nucleosides) than the other species homologs. Although detailed kinetic studies have not been performed, initial concentration-dependence studies of adenosine and inosine in inhibiting ^{3}H-uridine uptake suggest IC_{50} values in the low micromolar range (data not shown).

Bands corresponding to an amplified 276 bp fragment of rbSPNT1 RNA were observed in all tissues tested except spleen (Figure 4). The rbSPNT1 transcript appears to be most abundant in jejunum, ileum, total small intestine and lung. Moderate amounts of rbSPNT1 transcript were found in kidney medulla and kidney cortex and lesser amounts in heart, brain, and liver. rbSPNT1 appears to have a broader tissue distribution than its rat homolog; the rbSPNT1 transcript is present in kidney, lung and brain whereas the rSPNT transcript was not detected in these tissues by Northern analysis. However, the rbSPNT1 transcript seems to be present in lower abundance in liver and not at all in spleen. This is in contrast to the rSPNT transcript which is present in both of these tissues by Northern analysis and is abundant in liver (16). The distribution of the rbSPNT1 transcript appears to be more similar to that of the hSPNT1 transcript with the notable exception of the presence of the rbSPNT1 transcript in rabbit lung in abundance whereas

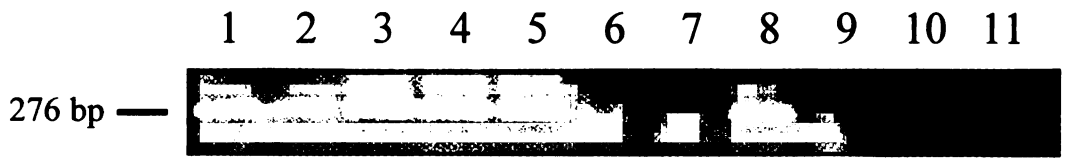


Figure 4. Tissue distribution of rbSPNT1 mRNA transcript by RT-PCR. Total RNA isolated from rabbit tissues was used as a template to synthesize cDNA. Gene-specific primers were used to amplify a 276 bp fragment. The resulting products were analyzed on a 1% agarose gel as follows: kidney medulla (lane 1), kidney cortex (lane 2), intestine (lane 3), jejunum (lane 4), ileum (lane 5), heart (lane 6), brain (lane 7), lung (lane 8), liver (lane 9), spleen (lane 10), water control (lane 11).

the hSPNT1 transcript was not detected in human lung by Northern analysis (7). Bands of approximately 0.7 and 0.8 kb were seen in many of the samples. Control samples in which no reverse transcriptase was added exhibited the 0.7 and 0.8 kb bands but not the 276 bp band, suggesting that these higher molecular weight bands were due to genomic contamination. In addition, primers to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were used to amplify a fragment of G3PDH of approximately 1.0 kb to verify the quality and quantity of cDNA for each tissue. Bands of approximately 1.0 kb were observed in PCR products from all tissues (data not shown).

Conclusions

A Na^+-dependent nucleoside transporter from rabbit small intestine, rbSPNT1, was cloned. RT-PCR analysis suggests that the rbSPNT1 transcript is expressed in numerous tissues. rbSPNT1 transported uridine and inosine; [^{3}H]-uridine transport was inhibited by purine but not pyrimidine nucleosides. This inhibition profile is consistent with a purine-selective transport process. Further characterization of this and other rabbit nucleoside transporters in comparison to rat and human cloned transporters will provide a better understanding of the interspecies differences in the intestinal uptake of nucleosides and nucleoside analogs that may be drugs or drug candidates. Such an understanding is important in pre-clinical drug evaluation in animal models.

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CHAPTER 3

ELECTROPHYSIOLOGICAL ANALYSIS OF THE SUBSTRATE SELECTIVITY OF A SODIUM-COUPLED NUCLEOSIDE TRANSPORTER (rCNT1) EXPRESSED IN *XENOPUS LAEVIS* OOCTYES[1]

Introduction

Nucleoside transporters mediate the transport of nucleosides in many cell types and organs. Studies using both cell culture models and tissue and plasma membrane preparations have identified two classes of nucleoside transporters in mammalian cells: equilibrative and concentrative (1). Equilibrative nucleoside transporters transport nucleosides down their concentration gradient, whereas concentrative nucleoside transporters couple uphill substrate transport to downhill sodium transport. The concentrative transporters are further categorized based on their interactions with endogenous purine and pyrimidine nucleosides; they are purine-, pyrimidine-, or broadly-selective. During the past decade, a number of nucleoside transporter genes have been isolated from mammalian, bacterial, and parasitic species (2, 3). In addition to their established role in the cellular uptake of endogenous nucleosides, these transporters play a role in the transport of nucleoside analogs as well (4). Numerous nucleoside analogs have been found to be clinically effective in the treatment of cancer and viral infections. For example, ribavirin and zidovudine (AZT) exhibit potent antiviral activity (5, 6). Likewise, the purine nucleoside 2-chlorodeoxyadenosine (2-CdA) has proven useful for

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treatment of indolent lymphoproliferative disorders, especially hairy-cell leukemia (7, 8). Many of the nucleoside analogs currently used clinically can cause toxic responses in some patients; responses that are thought to arise from their lack of selectivity for infected or cancerous cells. Furthermore, many nucleoside analogs are poorly available after oral administration. Therefore, the design and synthesis of new nucleoside analogs remains a very active area of research, with the goal of discovering more selective drugs with optimal oral bioavailability.

Because many nucleoside analogs are hydrophilic and diffuse very slowly across cellular membranes, nucleoside transporters play a primary role in their cellular uptake and release. Understanding the substrate selectivity of these transporters is critical for the development of new therapeutic nucleoside analogs with optimal pharmacokinetic properties, including high oral bioavailability and selective distribution. Indeed, there are a growing number of examples of synthetic analogs that are targeted to plasma membrane transporters to increase their bioavailability and to enhance their cell-specific delivery (9, 10). The assays currently used to investigate the substrate selectivity of nucleoside transporters either require radiolabeled compounds or rely on inhibition studies, which are indirect, because an inhibitor may not actually be transported.

In this study, we investigated the substrate selectivity of the concentrative, nucleoside transporter, rCNT1. rCNT1 was cloned from a rat jejunal cDNA library and was found to mediate the sodium-dependent uptake of uridine and thymidine in *Xenopus laevis* oocytes injected with rCNT1 cRNA (11). rCNT1 is expressed in the brain, intestine, liver, and kidney and may therefore play an important role in the distribution of therapeutic nucleoside analogs in these tissues (11-13). Understanding the structural

features of nucleoside analogs required for transport by rCNT1 is important in the rational design of therapeutic agents with optimal pharmacokinetic properties. In this study, we investigated the interaction of nineteen structurally diverse nucleosides and nucleoside analogs (Figure 1) with rCNT1. In particular, we used electrophysiology methods (14, 15) to directly determine the ability of rCNT1 to translocate these compounds. Structural features of nucleoside analogs important in substrate recognition and translocation by rCNT1 are discussed. This report demonstrates the electrogenic nature of nucleoside transport via rCNT1 and describes key positions on the nucleosides that affect the substrate selectivity of the transporter.

Materials and Methods

***In vitro* transcription and oocyte preparation.** rCNT1 cDNA was amplified by RT-PCR from rat intestine using gene-specific primers designed from the published sequence (11). The cDNA was then subcloned into the pOX vector (16). Capped cRNA was transcribed *in vitro* with T3 polymerase (mCAP RNA Capping Kit; Stratagene) from linearized rCNT1-pOX plasmids. Oocytes were harvested from oocyte positive *Xenopus laevis* (Nasco, Fort Atkinson, WI) and were dissected and treated with collagenase D (Boehringer-Mannheim Biochemicals, Indianapolis, IN) in a calcium-free ORII solution (2mg/ml), as previously described (17). Healthy stage V and VI oocytes were injected with 50 nl of capped rCNT1 cRNA (1 μg/μl). Oocytes were maintained at 18°C in modified Barth's medium.

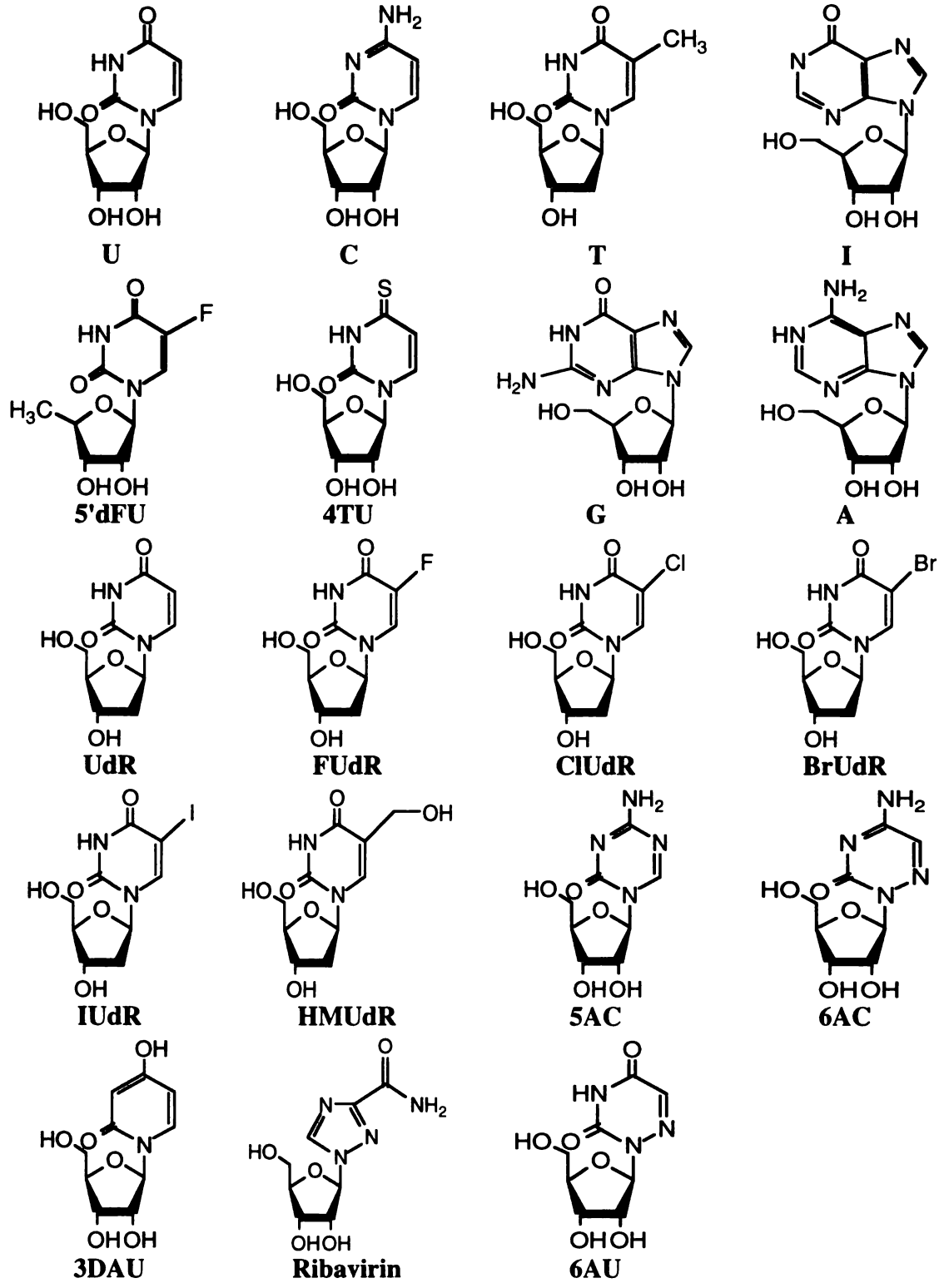


Figure 1. Structures of the nucleosides and nucleoside analogs used in this study.

Radiotracer transport assays. Transport of ^{3}H-uridine (11 μM) (37.8 Ci/mmol, Moravek, Brea, CA) in oocytes was measured 3 – 4 days after cRNA injection, as described previously (18). The composition of the sodium buffer was: 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris; pH 7.4. In the sodium-free buffer, NaCl was replaced with choline chloride. For the inhibition study (Table 1), unlabeled nucleosides and nucleoside analogs (Sigma Chemical, St. Louis, MO or Aldrich, Milwaukee, WI) were added to the reaction solutions as needed.

Electrophysiology. Three to eight days after injection, two-electrode voltage clamp recordings were performed at room temperature (GeneClamp 500B; Axon Instruments, Foster City, CA) (19). Oocytes were voltage clamped at –50 mV and were perfused with sodium buffer prior to substrate perfusions (30 – 60 s). The substrates were removed by washing in sodium-free choline chloride buffer. Recordings were obtained in a recording chamber at flow rates of ~ 3 ml/min; the functional volume of the recording chamber is ~ 25 μl. The steady-state substrate-induced currents were taken as the difference between the average current measured in the presence of substrate and the holding current recorded in sodium buffer before substrate perfusion. (When data were expressed as the difference between the peak current in the presence of substrate and the holding current similar results were obtained). Voltage pulse protocols were applied from a holding potential of –50 mV using 100-ms voltage pulse steps ranging from –150 to +50 mV in 20 mV increments, with 1-s interpulse intervals. Signals were filtered using a 4-pole low-pass Bessel filter set at a 100 Hz cutoff prior to sampling at 2 kHz. To obtain current-voltage curves, currents were averaged during the last 25 msec of the 100-ms voltage pulses and then plotted as a

function of V_m. Uninjected oocytes were used as controls, undergoing the same treatments as transcript-injected oocytes.

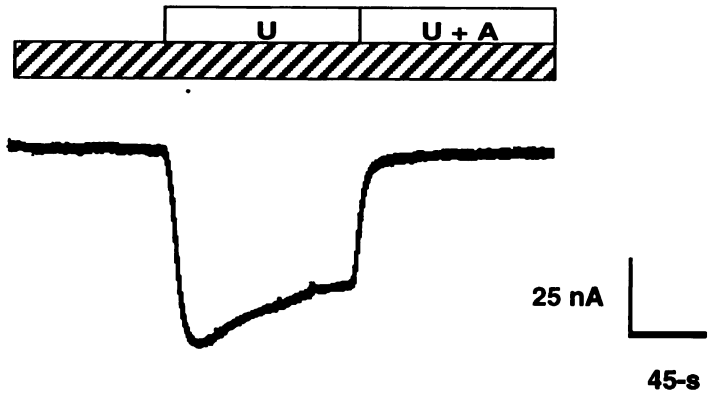
Data analysis. Values are expressed as mean ± standard deviation (SD) or mean ± standard error (SE), as indicated in the figure legends. The inhibition experiments were performed at least twice on different batches of oocytes. For the electrophysiology experiments, the number of oocytes (n) assayed for each experimental condition are reported in the figure legends and were from more than one donor frog, unless otherwise noted.

Results

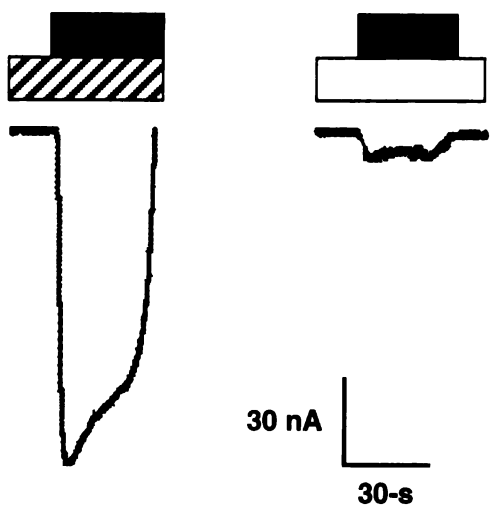
Sodium-, voltage-, and concentration-dependence of uridine-induced currents. Nucleoside transport mediated by rCNT1 is sodium-dependent and chloride-independent (11). Uridine (50 μM) induced an inward current in sodium uptake buffer in rCNT1-expressing oocytes; 500 μM adenosine reduced the uridine-induced current almost entirely (Figure 2A). This current is largely sodium dependent (Figure 2B). However, very small uridine-induced currents were measured in sodium-free choline chloride buffer in several oocytes. The uridine-induced currents are voltage-dependent and increased ~ 5-fold upon hyperpolarization of V_m from −10 to −150 mV (Figure 2C). This suggests that transport of substrate by rCNT1 is voltage-dependent. Uridine displayed saturation kinetics (Figure 3) with an estimated $K_{0.5}$ of 21 ± 2 μM.

Selectivity of rCNT1 for endogenous nucleosides. Previous radiotracer uptake and inhibition studies have shown that rCNT1 is a pyrimidine-selective nucleoside

A.


U
U + A
25 nA
45-s

B.


30 nA
30-s

C.

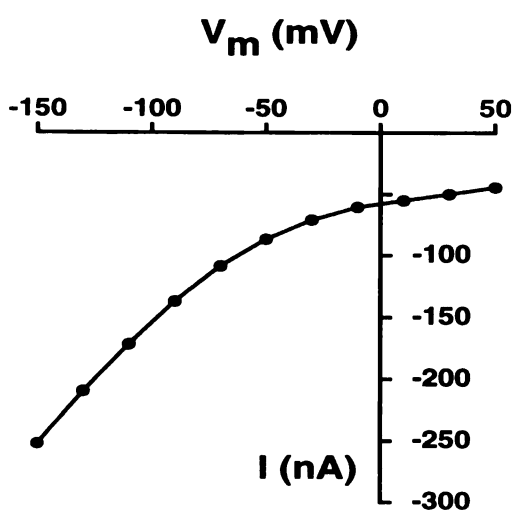
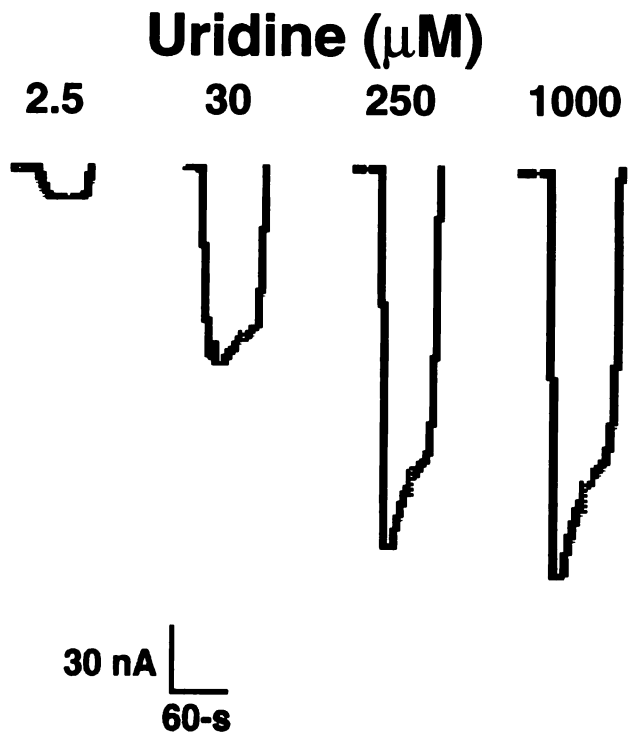

Vm (mV)
-150
-100
-50
0
50
-100
-150
-200
-250
-300
I (nA)

Figure 2. (A) Electrogenicity of rCNT1. Representative tracing from a single rCNT1-expressing oocyte voltage-clamped at -50 mV. The oocyte was perfused with the solutions indicated in the upper bars. Addition of 50 μM uridine induced an inward current. Adenosine (500 μM) abolished the uridine-induced inward current. (B) Sodium-dependence of uridine-induced currents. Representative tracing from a single rCNT1-expressing oocyte voltage-clamped at -50 mV. Striped bar indicates sodium buffer, open bar indicates sodium-free (choline) buffer and dark bars indicate uridine applications. For clarity, the current tracings are not shown between washes. Similar results were obtained in other rCNT1-expressing oocytes (n = 4). Uridine-induced currents were not observed in control (uninjected) oocytes (n = 2). (C) Representative steady-state current-voltage relationship of the current induced by uridine (500 μM) in a single oocyte expressing rCNT1. Voltage pulse protocols were applied from a holding potential of -50 mV using 100-ms pulse steps ranging from -150 to +50 mV in 20 mV increments, with 1-s interpulse intervals. Net uridine-induced currents were obtained by subtracting the current generated in the absence and presence of uridine. Similar results were observed from replica experiments (n = 3).

A.


Uridine (μM)
2.5
30
250
1000
30 nA
60-s

B.

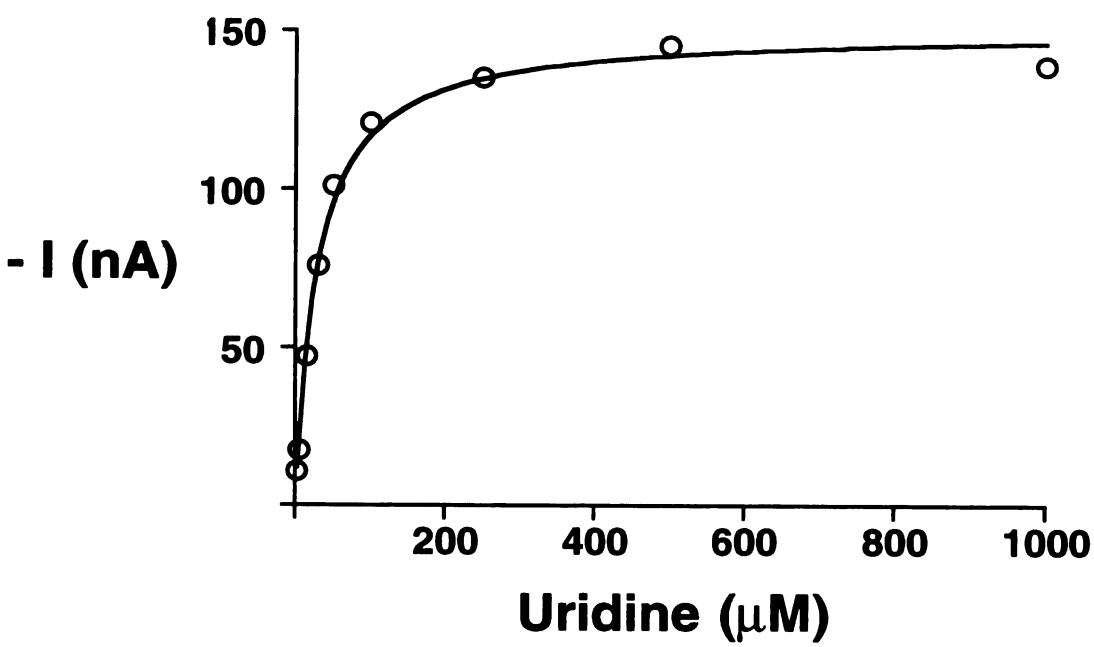

150
100
50
- I (nA)
200
400
600
800
1000
Uridine (μM)

Figure 3. Concentration-dependence of uridine-induced sodium currents. (A) representative recordings from a single rCNT1-expressing oocyte exposed to increasing uridine concentrations (for clarity, only four of the nine concentrations are shown). Between applications, the substrate was removed by washing in sodium-free buffer. After washing, the original sodium baseline current was restored before application of a new substrate solution. (B) data from a single experiment using one rCNT1-expressing oocyte was fit using the Michaelis-Menten equation ($I = (I_{max} * S)/(K_{0.5} + S)$ where I is equal to the current, I_{max} is the maximal current, $K_{0.5}$ is the concentration at the half-maximal current and S is the substrate concentration. Parameters calculated from these data were: $K_{0.5} = 30 \pm 3$ μM; - $I_{max} = 153 \pm 3$ nA. Similar $K_{0.5}$ values were determined from replica experiments (n = 7) (average ± SE: 21 ± 2 μM). The $K_{0.5}$ is comparable to values obtained by tracer uptake measurements in oocytes (i.e., 37 and 21 μM) (11, 20). Uridine-induced currents were not detected in control (uninjected) oocytes (n = 2) (data not shown).

transporter; it can also translocate adenosine at slow rates (11, 20). It has also been suggested that the sodium/nucleoside coupling stoichiometry of rCNT1 is 1:1 (21). Based on this information, one would predict that 1) uridine, thymidine and cytidine should induce sodium currents in rCNT1-expressing oocytes; 2) guanosine and inosine should not induce currents; and 3) adenosine may or may not induce currents due to its slow uptake rate. Figure 4 shows that thymidine, uridine and cytidine induced currents in rCNT1-expressing oocytes, whereas guanosine, inosine and adenosine did not.

Selectivity of rCNT1 for nucleosides analogs. The selectivity of rCNT1 for nucleoside analogs (Fig. 1) was investigated by substrate inhibition and two-electrode voltage clamp studies. ^{3}H-uridine uptake mediated by rCNT1 was potently inhibited by U, C, T, A, 4TU, UdR, FUdR, ClUdR, BrUdR, IUdR, 3DAU and 5AC, and was moderately inhibited by HMUdR, 5'dFU, and ribavirin. rCNT1 activity was weakly inhibited by I and G (Table 1).

The results of the two-electrode voltage clamp studies are summarized in Table 1. Five deoxyuridine analogs, modified at the 5-position of the pyrimidine ring (i.e., FUdR, ClUdR, BrUdR, IUdR, HMUdR), were transported by rCNT1; the rank order of their $I_{substrate}$ / $I_{uridine}$ values is HMUdR > FUdR > IUdR > ClUdR ~ BrUdR. The moderately potent inhibitors HMUdR and 5'dFU were also translocated by rCNT1; their $I_{substrate}/I_{uridine}$ ratios were 1.17 ± 0.2 and 1.61 ± 0.18, respectively. Three uridine analogs, 4TU, 3DAU, and 6AU, and two cytidine analogs, 5AC and 6AC, which contain base modifications at the 3, 4, 5, or 6 position were studied. Analogs with modifications at the 3, 4, or 5 position were all transported by rCNT1, whereas the analogs with modifications at the 6 position were not (Table 1).

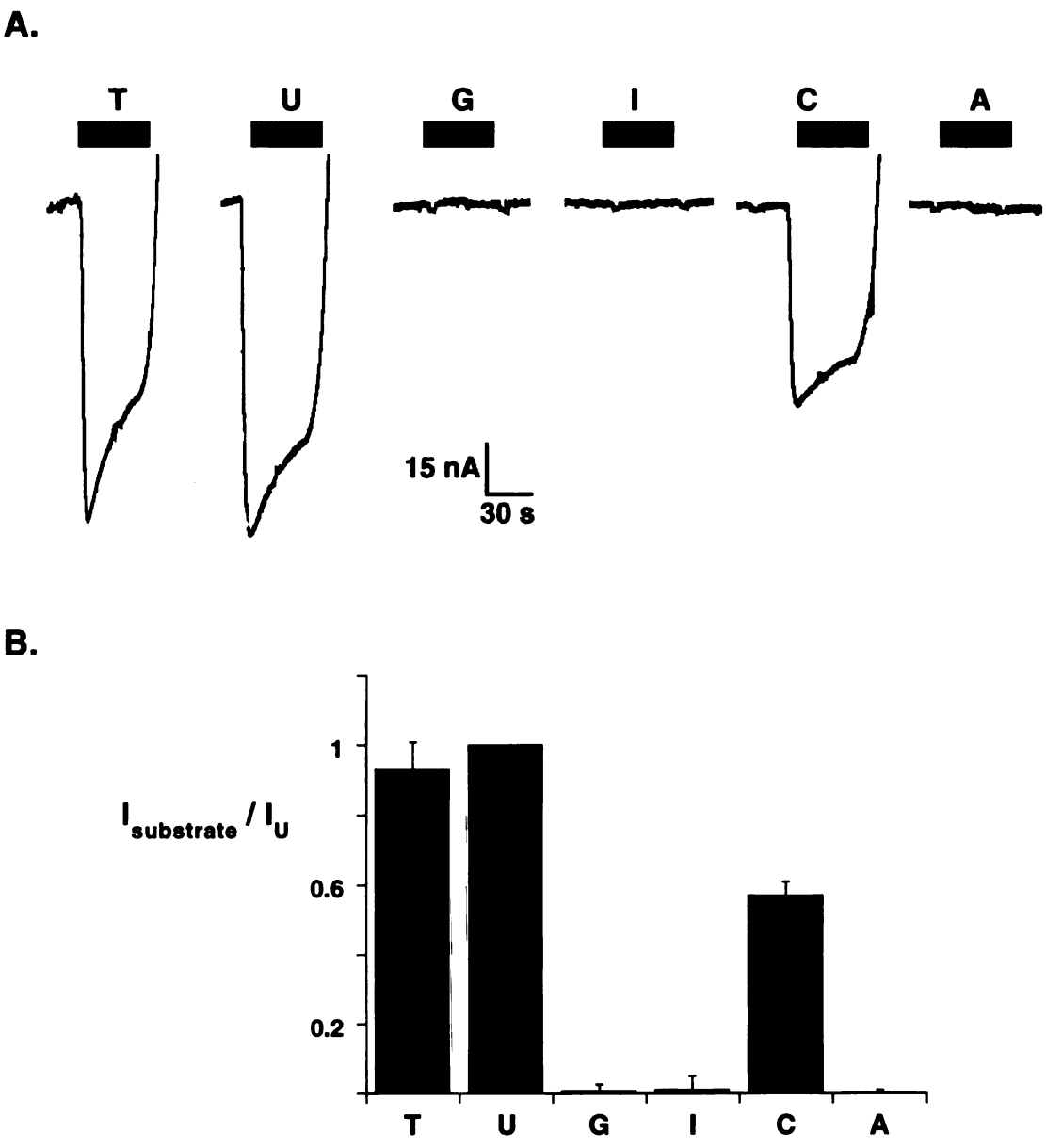


Figure 4. (A) Representative sodium currents in the presence of different endogenous nucleosides (the concentrations used and substrate names are listed in Table 1). An rCNT1-expressing oocyte was voltage-clamped at -50 mV in sodium buffer. 30-s application of nucleoside is indicated by the dark bars above the traces. Between applications, the substrates were removed by washing in sodium-free buffer. After washing, the original sodium baseline current was restored. (B) Nucleoside-induced

currents under voltage clamp conditions in rCNT1-expressing oocytes. Currents were individually normalized to the uridine-induced currents within one set of recordings. Data represent mean ± SD (n = 9). The nucleosides did not induce currents in control (uninjected) oocytes (n = 3) (data not shown).

Table 1. Summary of inhibition and substrate-induced current data for nucleosides and nucleoside analogs.

Compound	Abbreviation	Concentration (μM) [a]	% inhibition [b]	$I_{substrate} / I_U$ [c]
Adenosine	A	500	78 ± 10	0.01 ± 0.01
5-Azacytidine	5AC	1000	90 ± 3	0.47 ± 0.03
6-Azacytidine	6AC	1000	1.8 ± 1.1	0.05 ± 0.04
6-Azauridine	6AU	1000	5.9 ± 2.4	0.01 ± 0.01
5-Bromo-2'-deoxyuridine	BrUdR	500	88 ± 2	0.61 ± 0.08
5-Chloro-2'-deoxyuridine	ClUdR	500	89 ± 3	0.62 ± 0.06
Cytidine	C	500	85 ± 4	0.58 ± 0.05
3-Deazauridine	3DAU	1000	73 ± 2	0.39 ± 0.01
2'-Deoxyuridine	UdR	500	74 ± 7	0.97 ± 0.14
5-Fluoro-2'-deoxyuridine	FUdR	500	83 ± 4	0.90 ± 0.08
5-Fluoro-5'-deoxyuridine	5'dFU	500	53 ± 6	1.61 ± 0.18
Guanosine	G	1000	23 ± 10	0.01 ± 0.02
5-Hydroxymethyl-2'-deoxyuridine	HMUdR	500	65 ± 1	1.17 ± 0.20
Inosine	I	500	10 ± 7	0.01 ± 0.04
5-Iodo-2'-deoxyuridine	IUdR	500	78 ± 4	0.76 ± 0.13
Ribavirin	R	1000	51 ± 7	0.06 ± 0.01
4-Thiouridine	4TU	500	92 ± 1	0.57 ± 0.03
Thymidine	T	250	76 ± 5	0.93 ± 0.08
Uridine	U	500	85 ± 8	NA

[a] Concentrations used for both inhibition and current-measurement experiments.

[b] % inhibition of ^{3}H-uridine uptake in rCNT1-expressing oocytes by unlabeled compound. The data represent mean ± SD.

[c] Values were calculated as described in the Figure 4 legend. Data represent mean ± SD (n = 3 - 7). None of the compounds induced currents in control oocytes (n = 2) (data not shown).

Discussion

In order to reach their cellular targets, therapeutic nucleosides must pass a number of membrane barriers, such as the intestinal epithelium and the blood-brain barrier. Membrane transporters play an important role in the cellular uptake and release of these agents (2, 4). The sodium-coupled nucleoside cotransporter studied in this investigation, rCNT1, is expressed in the intestine, kidney, liver, and brain and is likely to play a primary role in the intestinal uptake of nucleoside analogs (and thereby influence rate and extent of absorption) and in their central nervous system distribution (and thereby influence drug targeting) (11-13). Knowledge of the substrate selectivity of rCNT1 will greatly enhance our understanding of tissue-specific distribution of therapeutic nucleoside analogs and may facilitate the design of selectively targeted, orally available nucleoside analogs. Our knowledge of the selectivity of rCNT1 has been greatly limited by the lack of robust assays to investigate the structural features of nucleoside analogs essential for translocation by rCNT1. In this report, we used the two-electrode voltage clamp technique to investigate the substrate selectivity and electrogenic properties of rCNT1. Electrophysiology methods can be used to differentiate between transporter substrates and inhibitors (15).

rCNT1 has been classified as a pyrimidine-selective nucleoside transporter (11). Consistent with this classification, we found that pyrimidine nucleosides induced currents in rCNT1-expressing oocytes, whereas purine nucleosides did not. In addition, ribavirin, an antiviral agent that is structurally related to guanosine (Fig. 1), did not induce a current. This is in agreement with a report that showed that ribavirin is a substrate of the purine-selective transport system in human intestine (22). Adenosine, a potent inhibitor

of rCNT1 activity, has been shown to be transported by rCNT1 at very slow rates (20). At the rCNT1 expression levels achieved in this study, one would predict adenosine-induced currents < 2.4 nA, which are too small to be accurately measured with our two-electrode voltage clamp system. Adenosine did, however, block uridine-induced currents.

Nucleoside transport mediated by rCNT1 is voltage-dependent and also largely dependent on cotransport with sodium. However, in the absence of sodium, small uridine-induced currents were observed, which may represent proton- or choline-coupled uridine currents. It has been shown that other cations, in the absence of sodium, can drive substrate transport for several sodium-coupled cotransporters (23, 24). The voltage-dependence of the uridine-induced currents suggests that substrate movement via rCNT1 across the cell membrane is sensitive to membrane potential in native cells. rCNT1 modulation by membrane potential may be important in neural tissue, where rCNT1 transcripts have been detected (13).

The substrate-induced currents of the analogs were found to depend on both the position and the nature of their modifications. Uridine, deoxyuridine, and cytidine analogs with modifications at the 3, 4, or 5 positions were all transported by rCNT1. Uridine and cytidine analogs with modifications at the 6 position were not transported. More specifically, six deoxyuridine analogs containing a halogen atom or methoxy functional group at the 5-position were all substrates of rCNT1. Replacement of carbon with nitrogen at the 5-position of cytidine slightly affected the inhibition potency and $I_{substrate} / I_{uridine}$ value of 5AC compared with cytidine, whereas replacement of carbon with nitrogen at the 6-position of cytidine dramatically reduced the inhibition potency and $I_{substrate} / I_{uridine}$ value of 6AC. Similar results were found for 6AU. The seemingly small

change in moving the nitrogen from the 5-position to the 6-position of azapyrimidines has been shown to dramatically alter their conformations, which may explain why the 6AC and 6AU are not substrates of rCNT1 (25, 26). Two uridine analogs, 4TU and 3DAU, were studied. The oxygen at the 4-position is replaced with sulfur in 4TU, resulting in the loss of a good hydrogen-bond acceptor. The nitrogen at the 3-postion is replaced with a carbon in 3DAU, resulting in an altered nucleoside structure. Despite these changes, both 4TU and 3DAU were found to be substrates of rCNT1. Finally, 5'dFU was a substrate, which demonstrates that a 5'-hydroxyl group on the sugar is not essential for transport by rCNT1.

Most of the compounds that inhibited rCNT1 activity were substrates of the transporter, but there are notable exceptions. For example, ribavirin and 5'dFU both inhibited ^{3}H-urdidine uptake by ~ 50%; however, while the $I_{substrate}$ / $I_{uridine}$ value for 5'dFU was 1.6 (the highest of the nineteen compounds studied), ribavirin did not induce a current. Likewise, adenosine, a potent inhibitor of rCNT1 activity, failed to induce a current (Fig. 2A). These results suggest that inhibition data are not good predictors of the substrate selectivity of rCNT1.

In summary, we have used an electrophysiological assay to investigate the substrate selectivity of rCNT1. rCNT1 was able to transport pyrimidine nucleoside analogs modified at the 3, 4, or 5 position of the base and an analog lacking the 5'-hydroxyl group of the sugar. rCNT1 was not able to transport analogs with modifications at the 6 position. Substrate-induced currents in rCNT1-expressing oocytes are substrate-, sodium-, concentration- and voltage-dependent. The two-electrode voltage clamp assay could be further utilized to screen for rCNT1 substrates and inhibitors.

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CHAPTER 4

MOLECULAR DETERMINANTS OF THE PYRIMIDINE-SELECTIVITY OF A NUCLEOSIDE TRANSPORTER (rCNT1)

Introduction

Concentrative nucleoside transporters, which reside in the epithelial cells of tissues such as the intestine and kidney, are involved in the salvage of endogenous nucleosides as well as the absorption or reabsorption of nucleoside analog drugs. These transporters are secondary active transporters that employ the inwardly-directed sodium gradient to transport nucleosides into the cell, against their concentration gradient. Several concentrative processes have been characterized functionally and have distinct substrate selectivities. N1, a purine-selective nucleoside transporter transports purine nucleosides and uridine; N2, a pyrimidine-selective nucleoside transporter, transports pyrimidine nucleosides and adenosine; N3, N4 and N5 are termed broadly-selective transporters and transport selected purine and pyrimidine nucleosides (1).

Two isoforms of concentrative nucleoside transporters, N1 and N2 (termed SPNT and CNT1, respectively), were cloned from rat and human tissues (2-5). Subsequently, additional species homologs have been isolated including the mouse homolog of CNT1 and the pig and rabbit homologs of SPNT (6-8). These clones were found primarily in the epithelia of tissues such as the kidney, intestine and liver. Hydropathy analysis of the deduced amino acid sequences suggests 11-14 membrane-spanning segments. Recent

experimental evidence suggesting that the C-terminus is extracellular, supports a 13 transmembrane domain model (9).

The cloning of these transporters has allowed investigators to begin to study the domains and specific amino acids involved in substrate recognition and binding. In the first of these studies, the substrate binding and discrimination region was determined through the construction of chimeric transporters composed of rat SPNT (rSPNT) and CNT1 (rCNT1) (10). During these studies, a novel transporter was created when a transmembrane (TM) domain of rCNT1 (TM 8 in a 14 transmembrane model and TM 7 in a 13 transmembrane model) was substituted with the corresponding TM of rSPNT. This chimeric transporter accepted both pyrimidine and purine nucleosides. Site-directed mutagenesis studies revealed that a single substitution, serine to glycine at position 318, was sufficient to broaden the selectivity of rCNT1 so that it accepted purine as well as pyrimidine nucleosides (11). Substitutions at other positions within this transmembrane domain did not alter the pyrimidine-selectivity of rCNT1 (11). These findings were subsequently confirmed with the human clones and extended to include the identification of amino acids involved in purine nucleoside discrimination. In studies with the human clones, mutation of serine-353 and leucine-354 to threonine and valine, respectively (the corresponding amino acid residues of SPNT) converted a broadly selective mutant into a purine-selective transporter (12). With the recent cloning of several species orthologs of SPNT and CNT1, multiple sequence alignments of SPNT and CNT1 have shown that the serine residue at positions equivalent to 318 of rCNT1 is conserved among all species orthologs of CNT1 and that the corresponding glycine residue of rSPNT is conserved among all SPNT species orthologs (Figure 1). Interestingly, two nucleoside transporters

in the CNT family with broader substrate selectivities, cloned recently from *C. elegans* (CeCNT3), Pacific hagfish (hfCNT3) mouse (mCNT3) and human (hCNT3), contain a glycine residue at this position (12-14). These findings further underscore the importance of this residue in determining substrate selectivity.

The goal of the present study was to determine the mechanism by which a serine to glycine substitution at position 318 broadens the selectivity of CNT1. Our hypotheses are that 1) the smaller side chain of glycine at position 318 permits bulkier purine nucleosides to access the recognition site and permeation pathway or that 2) the hydroxyl group of serine forms a hydrogen bond which selectively recognizes pyrimidine nucleosides but restricts the interaction with purine nucleosides. This hydrogen bond could be between this residue at position 318 and another amino acid within the transporter, with the substrate or with a constituent of the membrane. A combination of the two hypotheses is also possible. To investigate the mechanism of selectivity change incurred by the serine to glycine substitution, we generated a series of mutants in which the residue at position 318 of rCNT1 was changed to residues of increasing size as well as to residues that can form hydrogen bonds. Our data suggest that the size of the side chain at position 318 is important for conferring pyrimidine-selectivity. That is, the smaller glycine side chain allowed both pyrimidine and purine nucleosides as substrates whereas the larger side chains of alanine, threonine and serine (in wildtype rCNT1) (Table 1) allowed only pyrimidine nucleosides and adenosine. The hydroxyl of the serine residue may be critical in the transport of adenosine by rCNT1.

Transporter	Sequence	Substrates					
		Pyrimidine Nucleosides			Purine Nucleosides		
aa #	303 322	Urd	Thd	Cyd	Ado	Guo	Ino
rSPNT	TLAVAGNIFV **G**MTEAPLLIR	+	-	-	+	+	+
hSPNT1	TLAVAGNIFV **G**MTEAPLLIR	+	-	-	+	+	+
mCNT2	TLAVAGNIFV **G**MTEAPLLIR	+[a]	-[b]	-[b]	+[a]	ND	+[a]
rbSPNT1	TLAVAGNIFV **G**MTEAPLLIR	+	-	-	+	+	+
aa #	308 327						
rCNT1	TLSVAGNIFV **S**QTEAPLLIR	+	+	+	+	-	-
hCNT1	TLSVAGNIFV **S**QTEAPLLIR	+	+	+	+	-	-
pkCNT1	TLSVAGNIFV **S**QTEAPLLIR	+	+	+	+	-	-
CeCNT3	SVNAWACTFL **G**MSEAPLMVP	+	+	-	+	+	+
rCNT1.S318G	TLSVAGNIFV **G**QTEAPLLIR	+	+	+	+	+	+
T8 chimera	TLAVAGNIFV **G**MTEAPLLIR	+	+	+	+	+	+
hfCNT	SMVSAGNIFV **G**QTESPLLIS	NR	NR	NR	NR	NR	NR
hCNT3	SVVASGNIFV **G**QTESPLLVR	+	+	+	+	+	+
mCNT3	SVVAAGNIFI **G**QTESPLLVQ	+	+	+	+	+	+

[a] Inhibits
[b] Does not inhibit
ND not determined
NR not reported

Figure 1. Multiple sequence alignments of the putative transmembrane domain 7 region. Alignment of the putative transmembrane domain 7 region of all of the mammalian cloned CNT family members and the recently cloned transporters from the nematode, CeCNT3, Pacific hagfish, hfCNT, human (hCNT3) and mouse (mCNT3). Also shown are two bioengineered transporters, rCNT1.S318G and the rCNT1/rSPNT chimera, T8. Shown in bold is the residue corresponding to serine-318 of rCNT1. Substrates of each of the cloned transporters are indicated (+ for a substrate; - for a non-substrate) and are abbreviated as follows: Urd, uridine; Thd, thymidine; Cyd, cytidine; Ado, adenosine; Guo, guanosine; Ino, inosine. The substrate-selectivity of mCNT2 is represented as an inhibition profile (6) rather than a direct uptake; however, this profile is consistent with that of a purine-selective transporter. The report of the cloning of hfCNT classified it as broadly-selective, accepting both purine and pyrimidine nucleosides; however, the specific substrates were not reported (12).

Table 1. Side chains of wildtype and mutant transporters

Mutant	Residue at Position 318	Side Chain
rCNT1 (wildtype)	Serine	$-CH_2OH$
S318G	Glycine	-H
S318A	Alanine	$-CH_3$
S318T	Threonine	$-CHCH_3$ | OH

Materials and Methods

Reagents. Chemicals were from Sigma Chemical (St. Louis, MO). Labelled nucleosides were from Moravek (Brea, CA).

Site-Directed Mutagenesis. Wildtype rCNT1 cDNA *(10)* was subcloned into the pOX vector *(15)*, an amphibian expression vector. Sense and antisense oligonucleotide primers, incorporating the mutated amino acid codon, were synthesized by the Biomolecular Resource Center (UCSF). Site-directed mutagenesis was carried out using the Quick-Change kit (Stratagene) per the manufacturer's protocol. Mutant sequences were verified by sequencing (Biomolecular Resource Center, UCSF).

Expression in *Xenopus laevis* Oocytes. Plasmids containing the mutant or wildtype cDNA were linearized downstream of the cDNA insert with *NotI*. cRNA was synthesized with T3 RNA polymerase from the linearized template using the mCAP RNA Capping Kit (Stratagene). Fifty nanoliters (approximately 30-40 ng) of cRNA was injected into healthy stage V and VI defolliculated *Xenopus laevis* oocytes. Oocytes were maintained at 18°C in Barth's medium (16). Uninjected oocytes were used as negative controls.

Uptake Assays. Uptake assays were performed as described previously on oocytes 2-3 days after cRNA injection (5). Briefly, 6-9 oocytes per data point were washed 3 times with room temperature, Na^+-free choline buffer, (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris; pH 7.4). Reaction mixture (100μL) containing tritiated substrate, with or without inhibitor in Na^+ buffer (100 mM NaCl substituted for choline chloride) or choline buffer was added to the oocytes and the oocytes were incubated at 25°C for 30 minutes. The oocytes were then

washed 5 times with 3 mL of ice-cold choline buffer. Individual oocytes were solubilized in 0.1 mL 10% SDS and the radioactivity associated with each oocyte was determined using a liquid scintillation counter.

Data Analysis. Uptake values are expressed as the mean ± S.E. of data obtained from 6-9 oocytes. Concentration-dependence data were fit to the Michaelis-Menten equation $V=(V_{max}*S)/(K_m+S)$ where V is the uptake rate; V_{max} is the maximal uptake rate; S is the substrate concentration and K_m is the concentration at the half-maximal rate. Statistical analysis was carried out by comparing uptake in oocytes expressing mutant transporters to control oocytes using a Student's unpaired t-test. Results were considered statistically significant with a probability of $p<0.05$.

Results

Substitution of the serine residue at position 318 with alanine or threonine (mutants S318A and S318T, respectively) resulted in functional, although somewhat less active transporters than the wildtype transporter (Figure 2). Uptake of ^{3}H-uridine (11.5 μM) by oocytes expressing the mutant transporters was 4.0 ± 2.2 pmol/oocyte/30 min and 14 ± 3.2 pmol/oocyte/30 min for S318A and S318T, respectively as compared to the uptake by oocytes expressing the wildtype transporter, rCNT1 (28.2 ± 10.9 pmol/oocyte/ 30 min). Uptake of ^{3}H-uridine by uninjected control oocytes was 0.04 ± 0.002 pmol/oocyte/30 min. This represents an enhancement in uptake of 100- and 350-fold over uninjected oocytes by oocytes expressing mutants S318A and S318T, respectively. Other substitutions (phenylalanine, leucine, valine and cysteine) at this position resulted in non-functional mutants (data not shown).

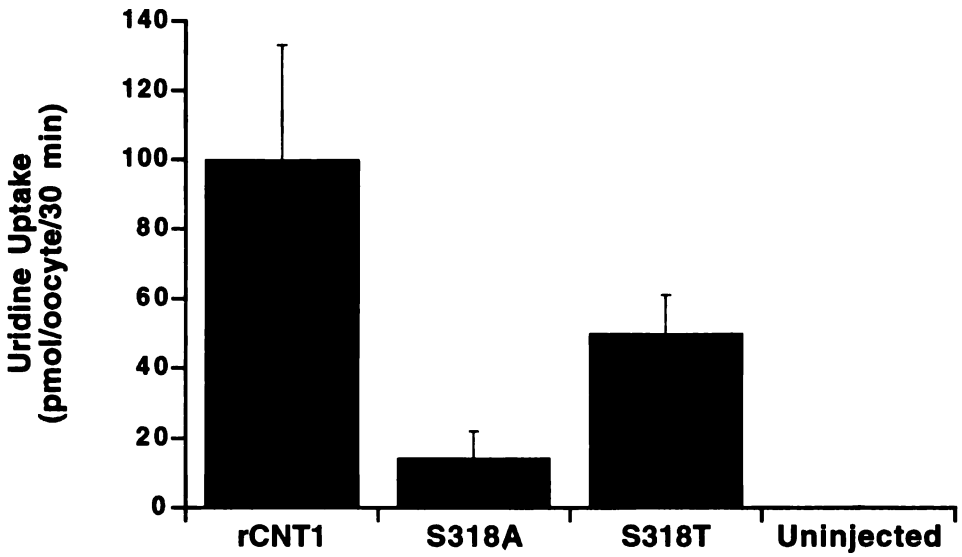
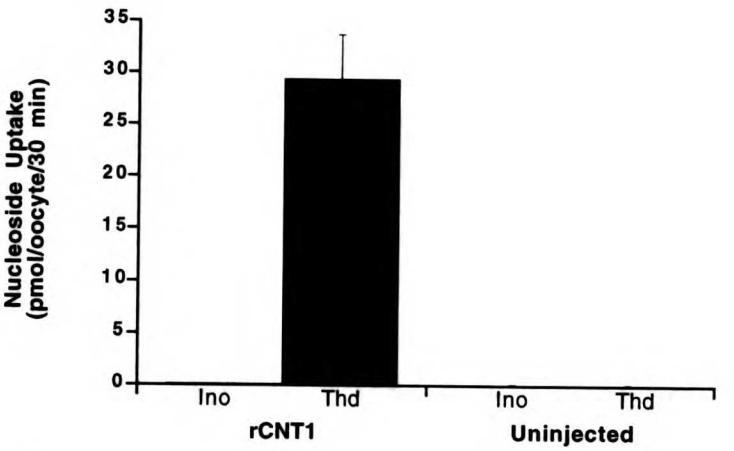


Figure 2. Uridine uptake by wildtype and mutant transporters. *Xenopus laevis* oocytes were injected with 30-40 ng of cRNA; uninjected oocytes served as a negative control. Uptake of ^{3}H-uridine (11.5 μM) was determined approximately 2 days after cRNA injection. Values represent the mean ± S.E. of data obtained in 7-8 oocytes.

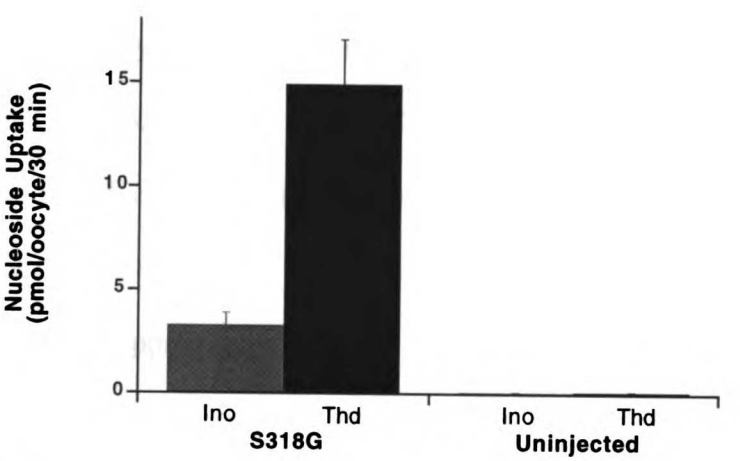
Studies using a model pyrimidine nucleoside, thymidine, and a model purine nucleoside, inosine, showed that the mutant transporters, S318A and S318T, maintained the pyrimidine-selectivity of the wildtype transporter. That is, they preferred the pyrimidine nucleoside, thymidine, as a substrate over the purine nucleoside, inosine (Figure 3). Uptake of ^{3}H-thymidine (10.8 μM) was 4.5 ± 0.7 pmol/oocyte/30 min, 5.8 ± 1.2 pmol/oocyte/30 min and 29.4 ± 4.2 pmol/oocyte/30 min in oocytes expressing S318A, S318T, and rCNT1, respectively. This represents an enhancement of uptake of thymidine of 43-, 56- and 290-fold over uninjected oocytes (0.1 ± 0.01 pmol/oocyte/30 min) for S318A, S318T, and rCNT1, respectively. ^{3}H-Inosine (12.6 μM) uptake was only slightly enhanced (0.6- to 1.2-fold over uninjected oocytes) in oocytes expressing the mutant (0.13 ± 0.02 pmol/oocyte/30 min and 0.09 ± 0.01 pmol/oocyte/30 min for S318A and S318T, respectively) or wildtype transporters (0.13 ± 0.01 pmol/oocyte/30 min) versus 0.06 ± 0.01 pmol/oocyte/30 min in uninjected oocytes. This is in contrast to the glycine mutant, S318G, which readily accepted both thymidine and inosine as substrates (Figure 3). This transporter exhibited an enhancement in uptake of thymidine of 124-fold over uninjected oocytes and of inosine of 40-fold over uninjected oocytes.

To further characterize the mutant transporters, a panel of ^{3}H-labelled pyrimidine (Figure 4A) and purine nucleosides (Figure 4B) was tested. The S318A and S318T mutants had a selectivity similar to that of the wildtype rCNT1. Both exhibited a preference for pyrimidine nucleosides (Figure 4). Adenosine, a purine nucleoside which is a substrate for all known concentrative nucleoside transporters, was not taken up as well as the pyrimidine nucleosides and was taken up less well by the mutant transporters (0.24 ± 0.02 pmol/oocyte/30 min and 0.26 ± 0.01 pmol/oocyte/30 min for S318A and

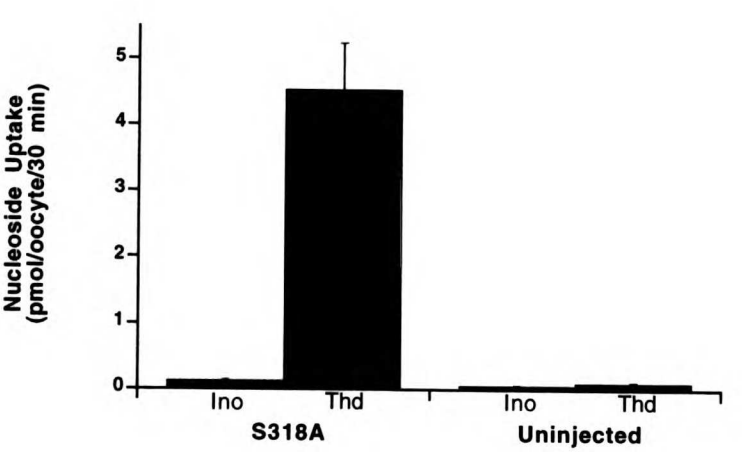
A


Nucleoside Uptake
(pmol/oocyte/30 min)
35
30
25
20
15
10
5
0
Ino
Thd
Ino
Thd
rCNT1
Uninjected

B


Nucleoside Uptake
(pmol/oocyte/30 min)
15
10
5
0
Ino
Thd
Ino
Thd
S318G
Uninjected

C


Nucleoside Uptake
(pmol/oocyte/30 min)
5
4
3
2
1
0
Ino
Thd
Ino
Thd
S318A
Uninjected

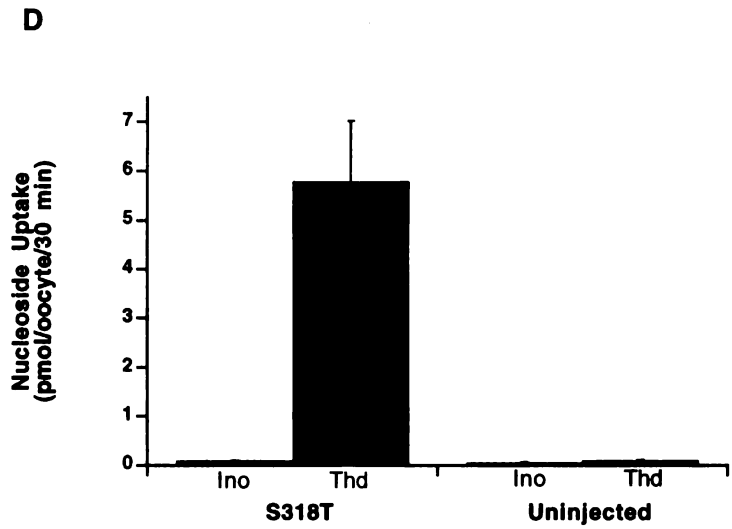
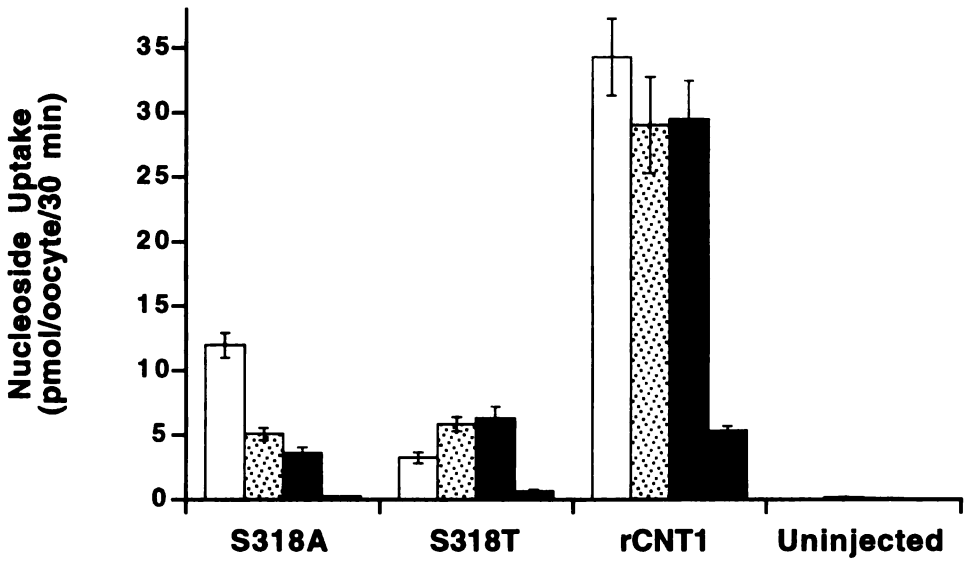


Figure 3. Thymidine and inosine uptake by wildtype rCNT1 (A) and mutant transporters, S318G (B), S318A (C) and S318T (D). *Xenopus laevis* oocytes were injected with 30-40 ng of cRNA of rCNT1 and mutants, S318A, and S318T; uninjected oocytes served as a negative control. Uptakes of ^{3}H-inosine (12.6 μM) and ^{3}H-thymidine (10.8 μM) were determined approximately 2 days after cRNA injection. Uptake values represent the mean ± S.E. of data obtained in 8-9 oocytes from a single experiment. Data for mutant S318G was adapted from previously published work (11).

A


Nucleoside Uptake
(pmol/oocyte/30 min)
35
30
25
20
15
10
5
0
S318A
S318T
rCNT1
Uninjected

B

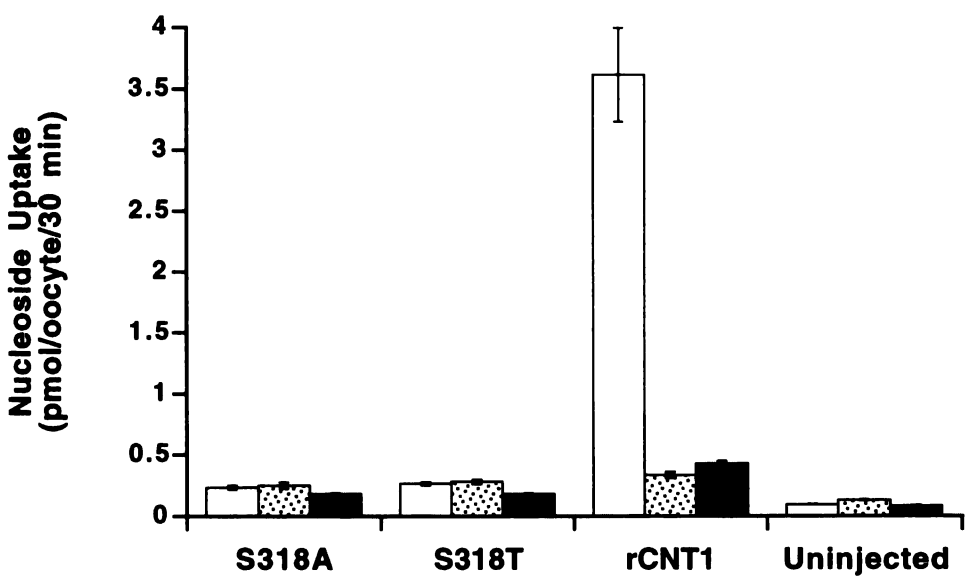

Nucleoside Uptake
(pmol/oocyte/30 min)
4
3.5
3
2.5
2
1.5
1
0.5
0
S318A
S318T
rCNT1
Uninjected

Figure 4. Uptake of pyrimidine and purine nucleosides by wildtype and mutant transporters. *Xenopus laevis* oocytes were injected with 30-40 ng of cRNA of each transporter; uninjected oocytes served as a negative control. Uptake of ^{3}H-labelled nucleosides (approximately 11μM) was determined approximately 2 days after injection of cRNA. Uptake values represent the mean ± S.E. of data obtained in 8-9 oocytes from a single experiment. (A) Uptake of pyrimidine nucleosides (in Na^{+} buffer unless specified otherwise): ^{3}H-cytidine (open bars); ^{3}H-thymidine (stippled bars); ^{3}H-uridine in Na^{+}-buffer (gray bars); ^{3}H-uridine in Na^{+}-free buffer (black bars). (B) Uptake of purine nucleosides (in Na^{+} buffer unless specified otherwise): ^{3}H-adenosine (open bars); ^{3}H-guanosine (stippled bars); ^{3}H-inosine (solid bars). Concentration of radiolabelled nucleosides ranged between 10 and 14 μM.

S318T, respectively) than the wildtype (3.61 ± 0.38 pmol/oocyte/30 min). The mutant transporters and the wildtype transporter exhibited a slight enhancement of uptake of inosine (1.2- to 4.1-fold) and guanosine (0.9- to 1.5-fold) over uninjected control oocytes. Although these values were statistically significant ($p<0.05$), they were low and only slightly enhanced compared to the uptake of the pyrimidine nucleosides (e.g. 33- to 275-fold enhancement of uridine uptake over uninjected oocytes) (Table 2). The mutant transporters did not take up adenosine very well. For example, adenosine:inosine uptake ratios for the mutant transporters were 1.3 and 1.4 for S318A and S318T, respectively whereas this ratio for the wildtype transporter was 9.0.

Adenosine and thymidine (1 mM) both inhibited uridine uptake by the wildtype transporter (approximately 71% and 80% inhibition, respectively) and the S318A mutant transporter (approximately 93% and 89%, respectively). In contrast, adenosine inhibited uridine uptake by S318T by only approximately 25% whereas thymidine inhibited uridine uptake by approximately 90% (Figure 5).

Concentration-dependence studies were performed to determine the kinetics of interaction of uridine, thymidine and adenosine with the mutant and wildtype transporters. The apparent K_m values are shown in Table 3. The apparent K_ms of uridine were 64.7 ± 29.6 μM, 19.9 ± 9.9 μM 72.5 ± 26.5 μM for S318A, S318T and wildtype, respectively. The mutant transporters had similar K_ms for thymidine (32.5 ± 7.4 μM and 34.2 ± 4.4 μM for S318A and S318T, respectively) compared to the published apparent K_m of 4.9 ± 1.7 μM for the wildtype rCNT1 (10). Substantial differences ($p<0.01$) in K_m values were observed for the interaction of adenosine with the mutant transporters in

Table 2. Enhancement of nucleoside uptake in oocytes expressing wildtype (rCNT1) and mutant transporters.

Transporter	Adenosine	Inosine	Guanosine	Uridine	Cytidine	Thymidine
rCNT1	37	4.1	1.5	275	1270	180
S318A	1.5	1.2	0.9	33	443	31
S318T	1.7	1.2	1.2	58	120	36

Values indicate the enhancement in uptake over that in uninjected oocytes. Values represent the average enhancement obtained in at least 8 oocytes.

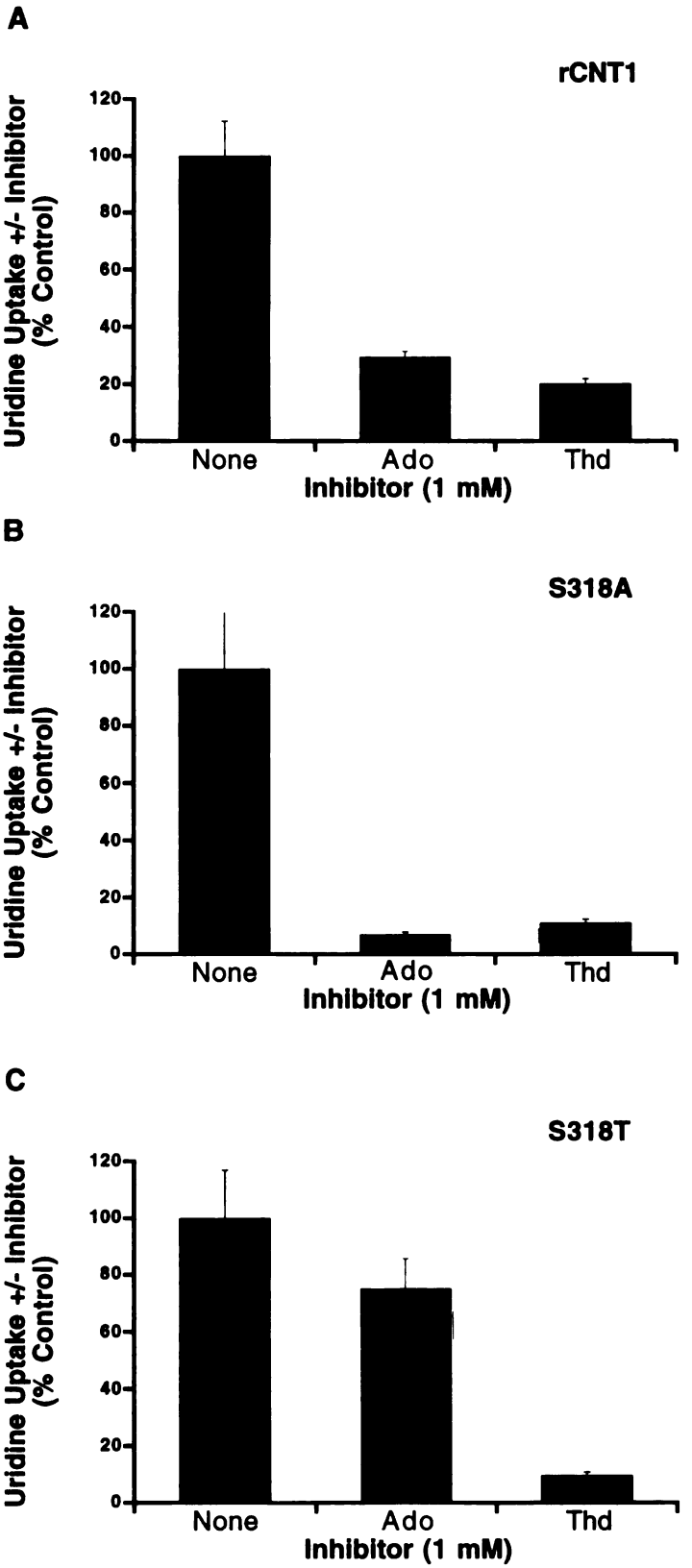

A
rCNT1
Uridine Uptake +/- Inhibitor (% Control)
120
100
80
60
40
20
0
None
Ado
Thd
Inhibitor (1 mM)
B
S318A
Uridine Uptake +/- Inhibitor (% Control)
120
100
80
60
40
20
0
None
Ado
Thd
Inhibitor (1 mM)
C
S318T
Uridine Uptake +/- Inhibitor (% Control)
120
100
80
60
40
20
0
None
Ado
Thd
Inhibitor (1 mM)

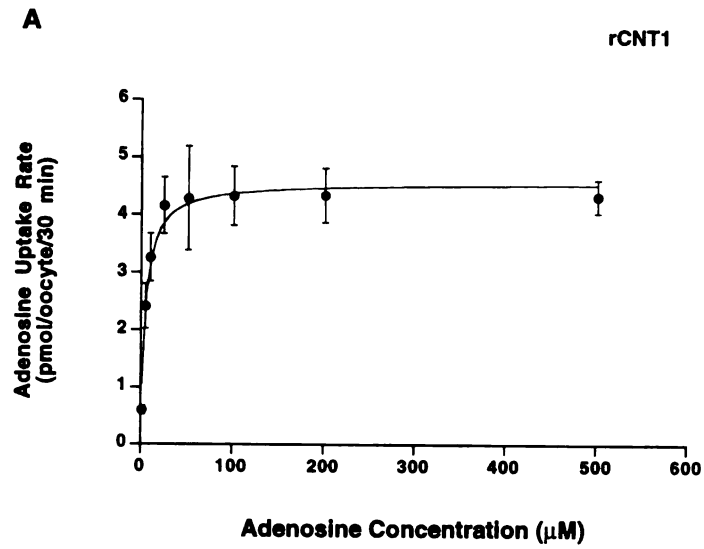
Figure 5. Inhibition of uridine uptake by wildtype rCNT1 (A), mutant S318A (B) and mutant S318T (C). *Xenopus laevis* oocytes were injected with 30-40 ng of cRNA of each transporter; uninjected oocytes served as a negative control (data not shown). Uptake of ^{3}H-uridine (11.5 μM) with or without unlabelled adenosine (1 mM) or thymidine (1 mM) was determined approximately 2 days after cRNA injection. Values are the mean ± S.E. of data obtained in 6-9 oocytes expressed as % control (uridine uptake in the absence of unlabeled nucleoside). Uptake of ^{3}H-uridine (11.5 μM) by oocytes expressing S318A, S318T and rCNT1 was 4.6 ± 1.5, 11.8 ± 2.0 and 28.7 ± 3.5 pmol/oocyte/30 min, respectively. Uptake of ^{3}H-uridine (11.5 μM) by uninjected oocytes in the absence of unlabeled nucleoside was 0.17 ± 0.05 pmol/oocyte/30 min and 0.07 ± 0.01 pmol/oocyte/30 min and 0.08 ± 0.01 pmol/oocyte/30 min in the presence of 1 mM adenosine and 1 mM thymidine, respectively.

Table 3. Kinetic parameters of wildtype and mutant transporters.

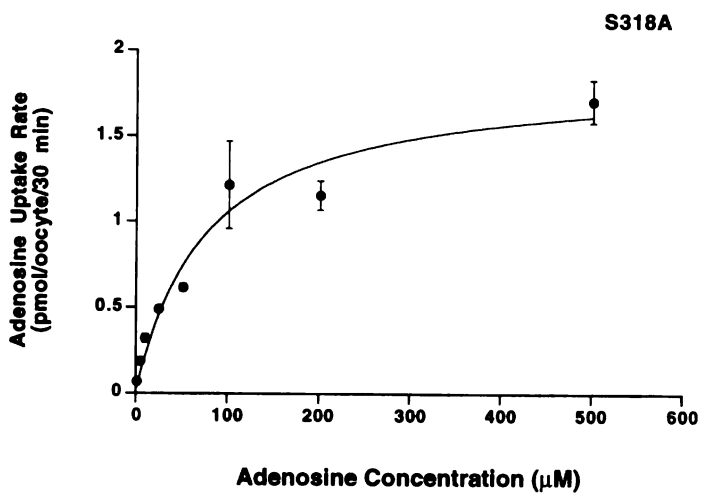
Residue at Position 318	Uridine K_m (μM)	Thymidine K_m (μM)	Adenosine K_m (μM)
Serine (rCNT1)	72.5 ± 26.5	4.9 ± 1.7 [a]	4.6 ± 0.8
Alanine	64.7 ± 29.6	32.5 ± 7.4	76.4 ± 22.3
Threonine	19.9 ± 9.9	34.2 ± 4.4	251.2 ± 46.9

[a] Published value (10)

A


rCNT1
Adenosine Uptake Rate (pmol/oocyte/30 min)
0
1
2
3
4
5
6
0
100
200
300
400
500
600
Adenosine Concentration (μM)

B


S318A
Adenosine Uptake Rate (pmol/oocyte/30 min)
0
0.5
1
1.5
2
0
100
200
300
400
500
600
Adenosine Concentration (μM)

C

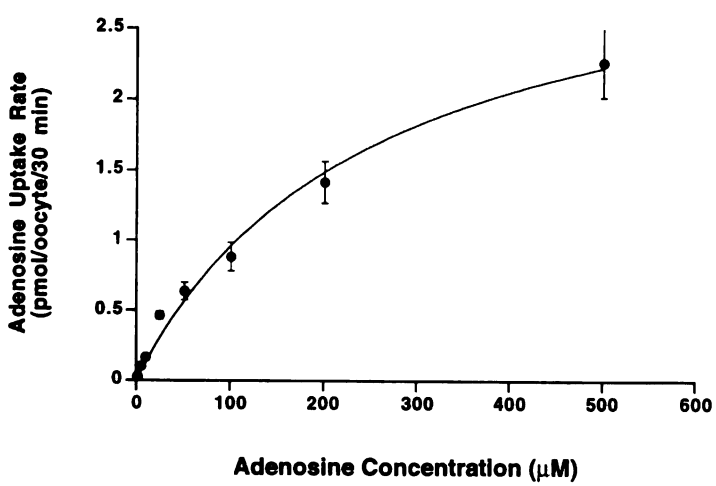

S318T
Adenosine Uptake Rate (pmol/oocyte/30 min)
0
0.5
1
1.5
2
2.5
0
100
200
300
400
500
600
Adenosine Concentration (μM)

Figure 6. Adenosine concentration-dependence studies in oocytes expressing rCNT1 (A), S318A (B) or S318T (C). *Xenopus laevis* oocytes were injected with 30-40 ng of cRNA; uninjected oocytes served as a negative control. Initial rate of uptake of ^{3}H-labeled adenosine (1.4 – 500 μM) was determined approximately 2 days after cRNA injection. Values represent the mean ± S.E. of data obtained in 6-8 oocytes.

comparison to the wildtype rCNT1 (76 ± 22.3 μM, 251 ± 47 μM and 4.6 ± 0.8 μM for S318A, S318T and rCNT1, respectively) (Figure 6).

Discussion

Multiple sequence alignments of SPNT and CNT1 have shown that the serine residue at positions equivalent to 318 of rCNT1 is conserved among all species orthologs of CNT1 (Figure 1). Furthermore, the corresponding residue of the SPNT sequences is a glycine residue that is conserved among all SPNT species orthologs. Previous work has shown that mutation of the serine residue at position 318 of rCNT1 (or position 319 in hCNT1) to glycine results in a transporter with a broadened substrate-selectivity, that is, it accepts purine nucleosides as substrates (11, 12). Glycine residues are present at corresponding positions of broadly-selective transporters cloned recently from *C. elegans*, the Pacific hagfish, mouse and human (12-14). To explain why glycine broadens the selectivity of rCNT1 and serine narrows it, we tested two mechanisms. 1) The smaller side chain of glycine at position 318 permits bulkier purine nucleosides to access the recognition site and permeation pathway. 2) The hydroxyl group of serine forms a hydrogen bond that restricts purine nucleosides.

A series of mutants were constructed in which serine-318 of rCNT1 was mutated to several hydrophobic residues of increasing size: alanine, valine, leucine, and phenylalanine or to residues that formed hydrogen bonds: cysteine and threonine. Only the alanine (S318A) and threonine (S318T) mutants were functional. Trafficking problems, defects in overall protein folding, or a decrease in RNA or protein stability, to name a few, possibly contribute to the inactivity of the non-functional mutants (17-19).

The two functional mutants, S318A and S318T, were characterized further with respect to substrate-selectivity, inhibition and kinetics.

Uptake of model purine and pyrimidine nucleosides (inosine and thymidine, respectively) revealed that both S318A and S318T functioned like the wildtype rCNT1. That is, they exhibited a preference for the pyrimidine nucleoside, thymidine, over the purine nucleoside, inosine. This suggests that the presence of a hydroxyl group on residue 318 is not necessary to confer pyrimidine-selectivity. That is, the hydroxyl can be present (e.g., in the wildtype and S318T mutant) or absent (e.g., in the S318A mutant) and the transporter retains its pyrimidine-selectivity. Further studies of the substrate selectivity of the mutant transporters showed that they took up the same substrates as the wildtype (thymidine, cytidine, uridine and adenosine) but not to the same extent. In particular, adenosine was a much poorer substrate of the mutant transporters (uptake approximately 2-fold over uninjected control oocytes) than the wildtype transporter (uptake approximately 37-fold over uninjected control oocytes). Consistent with these data, we observed that the K_m of adenosine was lowest for the wildtype (4.6 ± 0.8 μM) and increased for the mutant transporters (76.4 ± 22.3 μM for S318A and 251.2 ± 46.9 μM for S318T). These data suggest that the serine residue is optimal for translocation of adenosine.

Our data suggest that the size of the side chain at position 318 is a determinant of substrate selectivity of rCNT1, i.e., the smaller side chain of glycine allowed larger purine nucleosides to be recognized and translocated whereas larger side chains of alanine, serine and threonine did not. Hydrogen bond formation at position 318 is not critical for the pyrimidine-selectivity of rCNT1. Residue 318 appears to be important in

determining the kinetics of interaction of adenosine with rCNT1 with the wildtype serine residue being optimal for adenosine recognition and translocation.

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CHAPTER 5

SPECIES DIFFERENCES IN THE SUBSTRATE SELECTIVITY OF THE HUMAN AND RAT ORTHOLOGS OF THE CONCENTRATIVE NUCLEOSIDE TRANSPORTER, SPNT

Introduction

Nucleoside analogs are clinically important in the treatment of various neoplasms and viral infections. Many of these compounds are hydrophilic and thus exploit endogenous nucleoside transporters to cross cell membranes. In particular, concentrative or Na^+-dependent nucleoside transporters present in intestinal, hepatic and renal epithelia may play a role in the absorption and elimination of nucleoside analogs. Several subtypes of concentrative nucleoside transport activity have been characterized in intestine and kidney. The N1 subtype prefers purine nucleosides as substrates but also transports uridine; N2 prefers pyrimidine nucleosides but also transports adenosine; N3 is broadly selective and transports both purine and pyrimidine nucleosides (1). Transporters with activities corresponding to the N1, N2 and N3 subtypes have been cloned from various species and include CNT1 (N1 subtype), CNT2 or SPNT (N2 subtype) and CNT3 (N3 subtype) (2-11).

To understand the role of the individual concentrative nucleoside transporters in drug absorption, elimination and tissue-specific distribution, it is necessary to determine the substrate specificity of each transporter. Because these transporters are electrogenic, the two-electrode voltage clamp method can be used to assess specificity (12, 13). This

method has two major advantages over conventional isotopic uptake methods. First, the method clearly distinguishes between inhibitors of transport and actual substrates (14). Second, radiolabeled compounds are not required; therefore, specificity can be assessed for a wide array of compounds.

The goal of this study was to determine the substrate specificity of rat and human orthologs of the purine-selective transporter, SPNT. This transporter is particularly interesting because it is located in the intestine, liver and kidney as well as in a variety of other tissues suggesting that it may play a role in the absorption, elimination and tissue-specific targeting of nucleoside analogs (2, 3). Furthermore, many nucleoside analogs are purine nucleoside derivatives and may interact with the transporter. For these studies we focused on three aspects of specificity. First, we determined the specificity of the SPNT orthologs for naturally occurring nucleosides and nucleobases. Second, we assessed specificity for base-modified nucleoside analogs and finally, we evaluated the specificity for ribose-modified analogs. Our findings suggest that there are profound interspecies differences in the specificities of the transporters for both naturally occurring nucleosides and synthetic analogs. In general, the rat ortholog tolerated a variety of ribose-modified analogs whereas the human ortholog was less tolerant.

Materials and Methods

Expression in *Xenopus laevis* Oocytes . Oocytes were isolated from oocyte positive *Xenopus laevis*. The oocytes were dissected and treated with collagenase D (Boehringer Manheim) in Ca^{++}-free ORII medium to remove the follicular layer. Healthy, defolliculated Stage V and VI oocytes were maintained at 18°C in Barth's medium. The

cDNA coding for rSPNT or hSPNT1 were subcloned into an amphibian high expression vector, pOX, (15) and linearized using NotI. cRNA was transcribed and capped *in vitro* with T3 RNA polymerase using the mCAP RNA Capping Kit (Stratagene). Fifty nanoliters of cRNA (approximately 1μg/μl) was injected into healthy Stage V or VI oocytes (16).

Two-Electrode Voltage Clamp. Electrophysiological analysis using a two-electrode voltage clamp was carried out as described previously (17). Briefly, healthy oocytes injected with cRNA for rSPNT or hSPNT1 or uninjected were voltage clamped (GeneClamp 500B) at a membrane potential of –50mV unless noted otherwise. The oocyte was perfused with Na^+ buffer until a stable baseline current was achieved. The oocyte was then perfused with Na^+ buffer containing a test compound for approximately 30-60 seconds. Following exposure to a compound the oocyte was washed with Na^+-free buffer (NaCl replaced with choline chloride) followed by Na^+ buffer until a stable baseline was obtained.

Data Analysis. Currents from a given oocyte were normalized to the guanosine current induced in that same oocyte. Normalized data from at least two oocytes from 2-3 donor frogs were pooled and plotted. Values are expressed as mean ± S.D. Data at varying concentrations of substrate from individual oocytes were fit to the Michaelis-Menten equation ($I = (I_{max} * S)/(K_{0.5} + S)$ where I is equal to the current, I_{max} is the maximal current, $K_{0.5}$ is the concentration at the half-maximal current and S is the substrate concentration. Statistical comparisons were made using a Student's unpaired t-test. Results were considered statistically significant with a probability of $p<0.05$.

Results

Figure 1 shows representative currents induced in an oocyte expressing either rSPNT or hSPNT1 or an uninjected control oocyte. Significant currents were induced by saturating concentrations (500 μM) of guanosine, inosine, adenosine and uridine but not cytidine (500 μM) or thymidine (125 μM) in both the human and rat SPNT-expressing oocytes. No currents were induced by any of the nucleosides in uninjected oocytes. The currents observed in each oocyte were normalized to the guanosine currents in that oocyte. Data pooled from approximately 6-8 oocytes are shown in Figure 2 and are consistent with the currents shown for the single oocyte in Figure 1.

A series of naturally occurring adenosine analogs including adenine, AMP, ADP and ATP as well as 2'-deoxyadenosine were studied (Figure 3). Adenine, a nucleobase, did not induce a current in oocytes expressing either rSPNT or hSPNT1, suggesting that it is not a substrate of either of these transporters. 2'-Deoxyadenosine induced a current similar in magnitude to that of adenosine in oocytes expressing hSPNT1 and greater in magnitude than adenosine in oocytes expressing rSPNT. Interestingly, 5'-AMP was found to induce significant currents in oocytes expressing rSPNT but not hSPNT1. Only very small currents were induced by 5'-ADP and 5'-ATP. No currents were induced by any of these compounds in uninjected oocytes.

A series of base-modified and ribose-modified nucleoside analogs were studied. Uridine, which is a substrate of SPNT, and thymidine, which is not a substrate, are structural analogs of each other; thymidine is deoxyuridine methylated at the 5-position of the base. We therefore tested whether any moieties other than hydrogen were

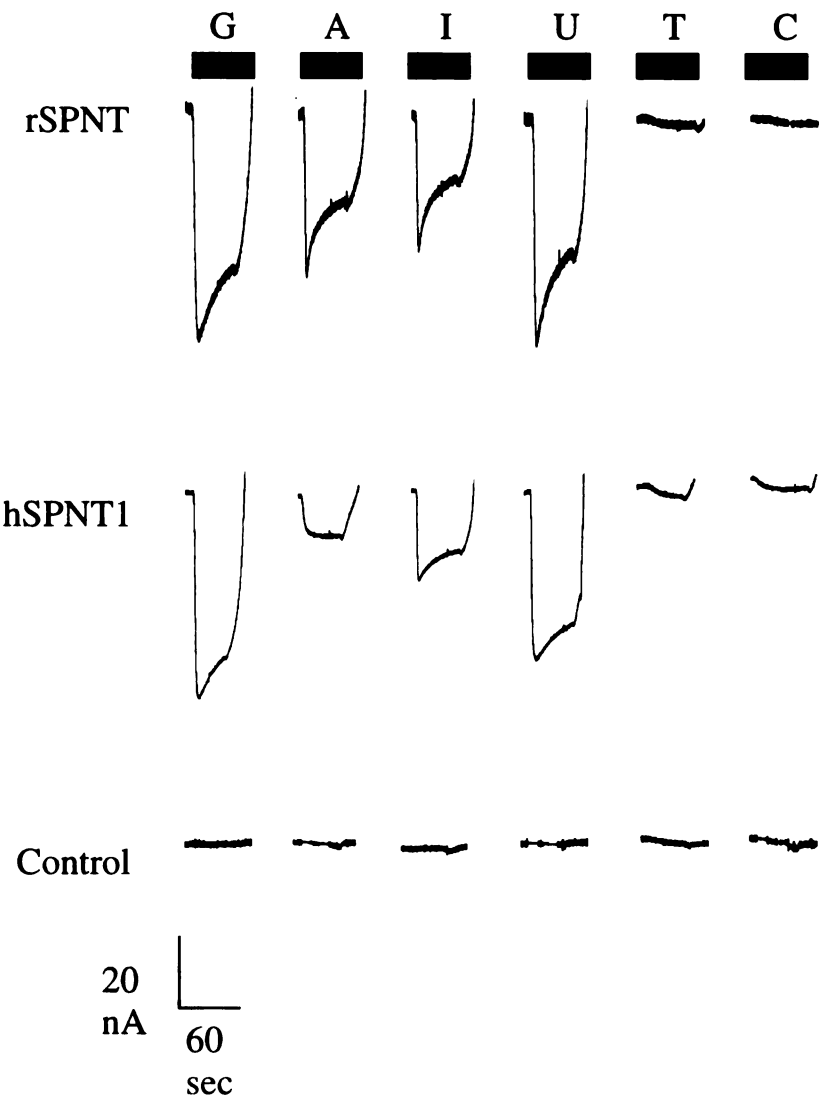

Figure 1. Individual currents induced by naturally occurring nucleosides. Representative currents from a single oocyte expressing either rSPNT, hSPNT1 or uninjected control. Oocytes were voltage-clamped at –50 mV and perfused with Na^+ -buffer until a baseline was reached for at least 30 seconds. A nucleoside (0.5 mM for all nucleosides except thymidine which was 0.125 mM) in Na^+-buffer solution was applied for 30-60 seconds (indicated by black bars). Next, the oocytes were washed with Na^+-free buffer then equilibrated with Na^+-buffer before the next nucleoside solution was applied. Abbreviations are as follows: G, guanosine; A, adenosine; I, inosine; U, uridine; T, thymidine; C, cytidine.

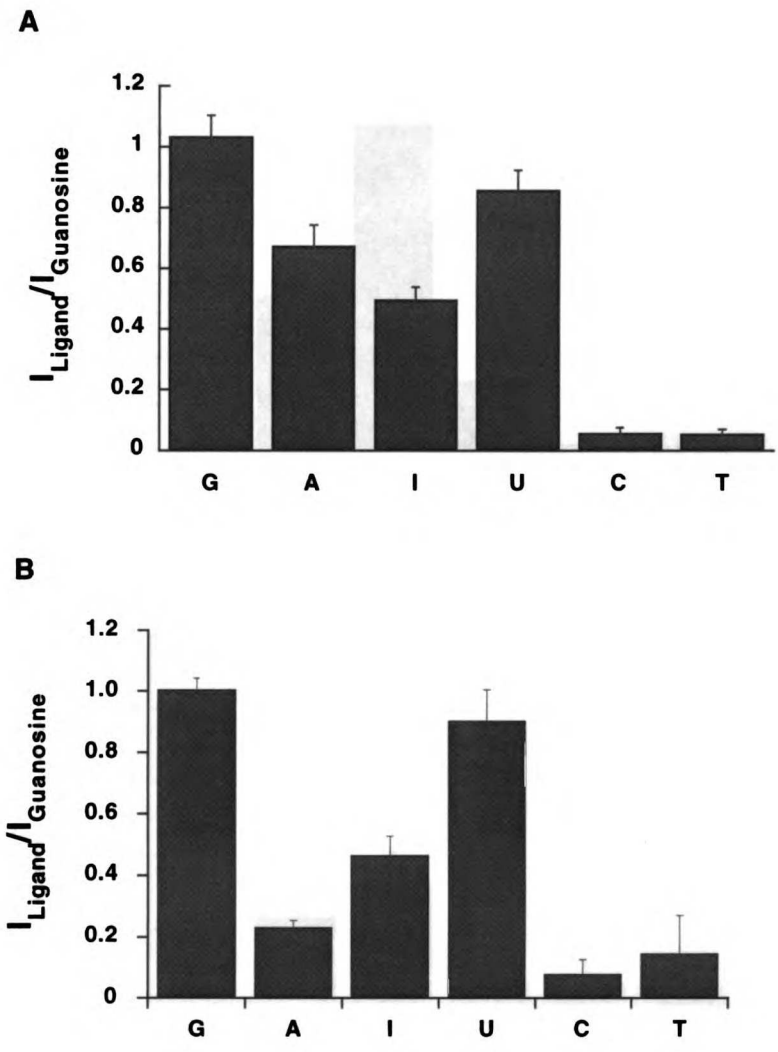

Figure 2. Currents induced by naturally occurring nucleosides. Experiments were conducted 5-7 days after injection of cRNA. Currents were determined by taking the difference between the baseline current in Na^+-buffer and the average current during the application of the ligand (average current in 30 seconds) in nA. Currents for each ligand are normalized to the current induced by guanosine in a single set of recordings for that oocyte, then pooled with replicate data from other oocytes. Bars represent the mean ± S.D. of data from 6-8 oocytes (from 3-4 donor frogs) expressing rSPNT (A) or hSPNT1 (B).

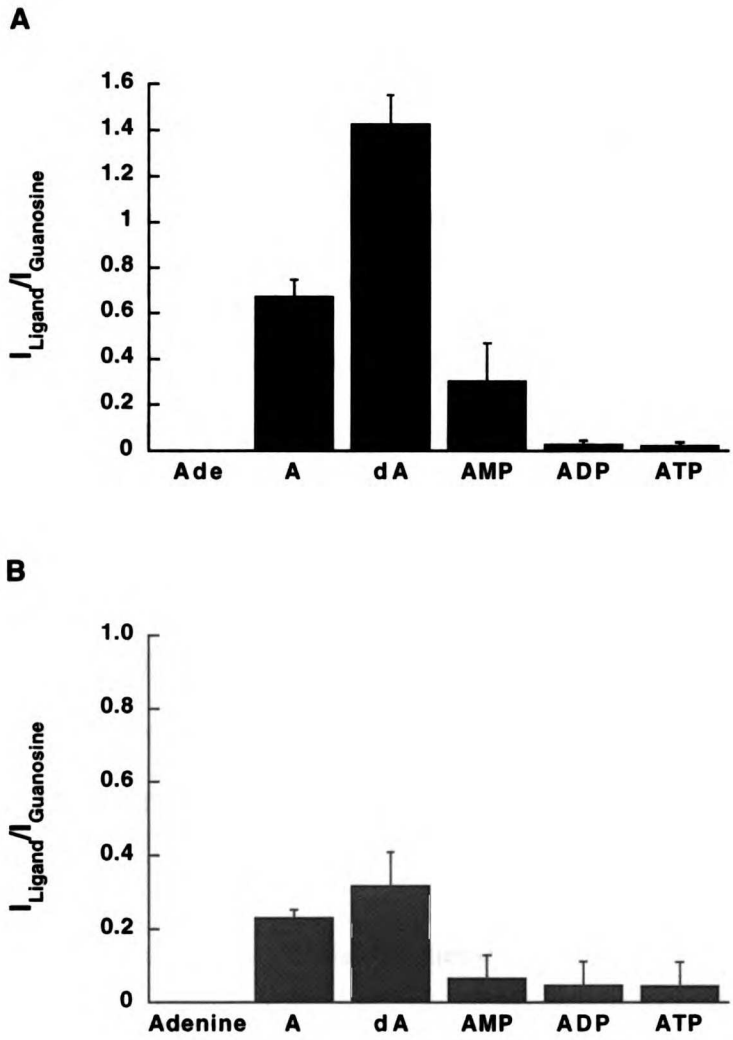

Figure 3. Currents induced by adenosine analogs. Currents were determined by taking the difference between the baseline current in Na^+-buffer and the average current during the application of the ligand (average current in 30 seconds) in nA. Currents for each ligand are normalized to the current induced by guanosine in a single set of recordings for that oocyte, then pooled with replicate data from other oocytes. Bars represent the mean ± S.D. of data from 6-8 oocytes (from 3-4 donor frogs) expressing rSPNT (A) or hSPNT1 (B). No currents were induced by any of these compounds in uninjected oocytes. Abbreviations are as follows: Ade, adenine; A, adenosine; dA, 2'-deoxyadenosine.

allowable at this position by measuring the currents induced by a series of analogs with different halogen groups or a hydroxy-methyl at this position (Figure 4). 5-Chloro-deoxyuridine (CldU) and 5-bromo-deoxyuridine (BrdU) induced small currents relative to the currents induced by uridine suggesting that they are only poorly transported by both rSPNT and hSPNT1. 5-Fluoro-deoxyuridine (FdU), 5-iodo-deoxyuridine (IdU) and 5-hydroxymethyl-deoxyuridine induced no detectable current in oocytes expressing either rSPNT or hSPNT1 suggesting that none of these compounds are substrates. The base-modified adenosine analog, 2-chloro-2'-deoxyadenosine (2CdA), induced large currents in rSPNT-expressing oocytes. In contrast, only very small currents were induced by 2CdA in oocytes expressing hSPNT1. A study of the concentration dependence of the current induced by 2CdA was then carried out in oocytes expressing rSPNT to determine the affinity of 2CdA for rSPNT. The $K_{0.5}$ of 2CdA in interacting with rSPNT was 57 ± 12 μM (Figure 5).

Several ribose-modified nucleoside analogs were studied to determine whether modifications to the ribose were tolerated (Figure 6). 2',3'-Dideoxyinosine (ddI) and 2',3'-dideoxyadenosine (ddA) induced modest currents (approximately 15% and 30%, respectively, of the current induced by guanosine) in oocytes expressing rSPNT. In contrast, ddA induced no detectable current in oocytes expressing hSPNT1 and only small currents were induced by ddI (approximately 5% of the current induced by guanosine). Interestingly, 2'-deoxyadenosine (dA) induced substantial currents in both rSPNT- and hSPNT1-expressing oocytes. The currents induced by dA in rSPNT-

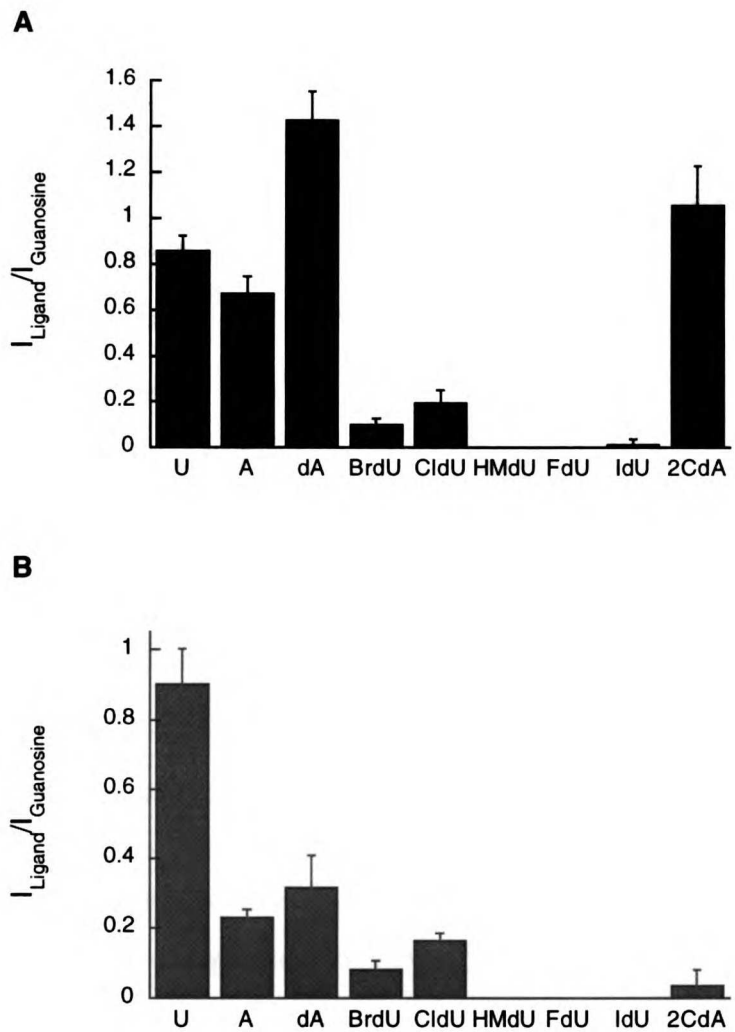

Figure 4. Currents induced by base-modified nucleoside analogs. Test compounds are either uridine or adenosine analogs. Experiments were conducted 5-7 days after injection of cRNA. Currents were determined by taking the difference between the baseline current in Na^+-buffer and the average current during the application of the ligand (average current in 30 seconds) in nA. Currents for each ligand are normalized to the current induced by guanosine in a single set of recordings for that oocyte, then pooled with replicate data from other oocytes. Bars represent the mean ± S.D. of data from 6-8 oocytes (from 3-4 donor frogs) expressing rSPNT (A) or hSPNT1 (B). No currents were induced by any of these compounds in uninjected control oocytes. Abbreviations are as follows: U, uridine; A, adenosine; dA, 2'-deoxyadenosine; BrdU, 5-bromo-2'-deoxyuridine; CldU, 5-chloro-2'-deoxyuridine; HMdU, 5-hydroxymethyl-2'-deoxyuridine; FdU, 5-fluoro-2'-deoxyuridine; IdU, 5-iodo-2'-deoxyuridine; 2CdA, 2-chloro-2'-deoxyadenosine.

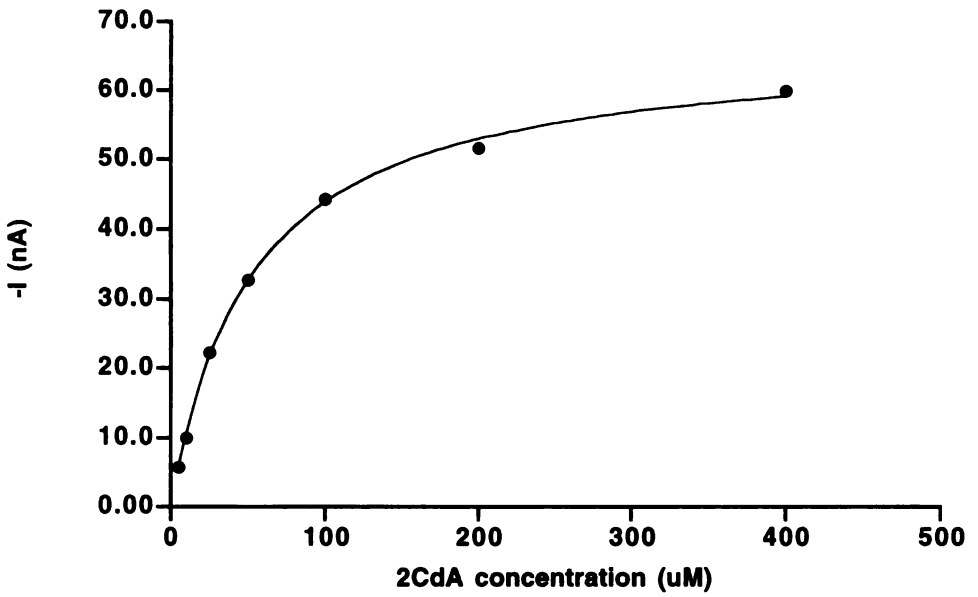

Figure 5. Concentration-dependence of the current induced by 2CdA in rSPNT expressing oocytes. Representative plot of the data from a single oocyte. These data were fit to the Michaelis-Menten equation. $K_{0.5}$was determined to be 52 ± 2.6 μM. This experiment was repeated in 5 oocytes from 3 different batches of oocytes with similar results (mean $K_{0.5}$ 57 ± 12 μM).

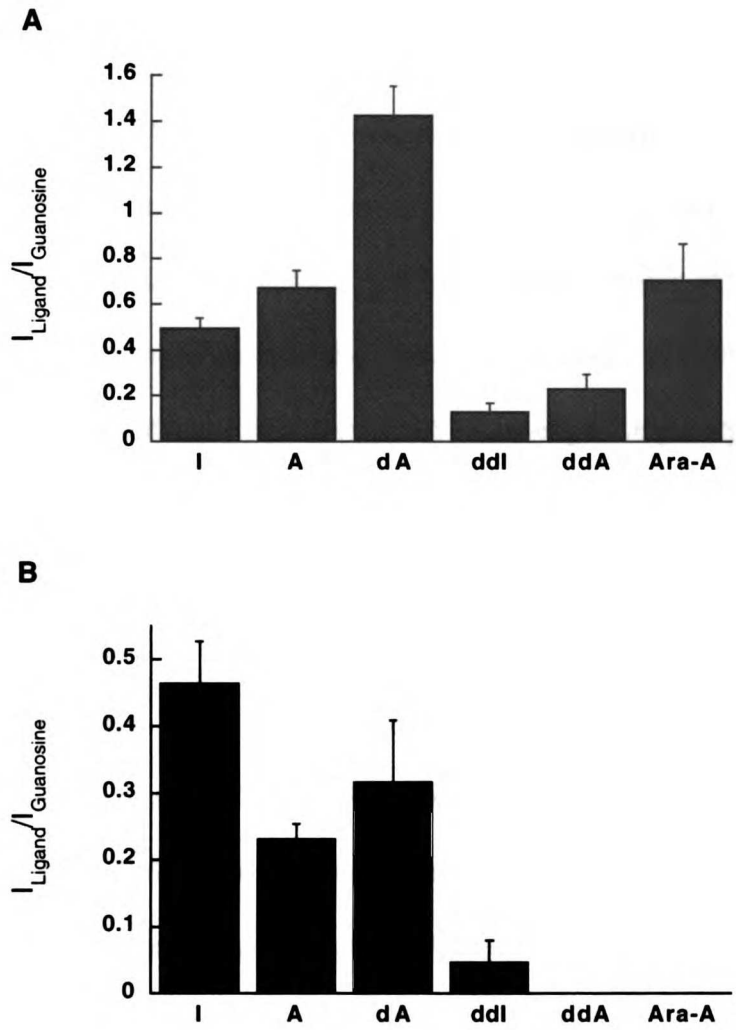

Figure 6. Currents induced by ribose-modified nucleoside analogs. Test compounds are inosine or adenosine analogs. Experiments were conducted 5-7 days after injection of cRNA. Currents were determined by taking the difference between the baseline current in Na^+-buffer and the average current during the application of the ligand (average current in 30 seconds) in nA. Currents for each ligand are normalized to the current induced by guanosine in a single set of recordings for that oocyte, then pooled with replicate data from other oocytes. Bars represent the mean ± S.D. of data from 6-8 oocytes (from 3-4 donor frogs) expressing rSPNT (A) or hSPNT1 (B). No currents were induced by any of these compounds in uninjected control oocytes. Abbreviations are as follows: I, inosine; A, adenosine; dA, 2'-deoxyadenosine; ddI, 2',3'-dideoxyinosine; ddA, 2',3'-dideoxyadenosine; Ara-A, adenine arabinoside.

expressing oocytes were larger (approximately double) than those induced by adenosine but similar to those induced by adenosine in hSPNT1-expressing oocytes. Adenine arabinoside (Ara-A), another ribose-modified adenosine analog, differs from adenosine by virtue of its 2' and 3' hydroxyl groups being in a *trans* configuration rather than a *cis* configuration. In our studies, Ara-A produced a current of approximately 70% of that induced by guanosine and about equal to adenosine in rSPNT-expressing oocytes but produced no detectable current in hSPNT1-expressing oocytes or in uninjected control oocytes.

Discussion

In this study, we employed electrophysiologic methods to determine the specificity of rSPNT and hSPNT1 with respect to (a) naturally-occurring nucleosides; (b) base-modified nucleoside analogs; and (c) ribose-modified nucleoside analogs. Our results demonstrating that both rSPNT and hSPNT1 preferred purine nucleosides and uridine corroborate previous studies using isotopic uptake methods (2, 3, 18, 19). Interestingly, we observed that guanosine was the preferred substrate of both orthologs of SPNT (Figure 2). Guanosine is important for the cell through its role as a precursor to GTP, which provides the driving force for signal transduction pathways via G-protein coupled receptors. Salvage of guanosine from extracellular sources for GTP synthesis is much less costly in terms of energy in comparison to de novo synthesis of GTP, which involves multi-step pathways (20). Guanosine may be preferred over inosine and adenosine since de novo GTP synthesis costs more energy than de novo synthesis of ATP or ITP. Although guanosine has the highest turnover rate for both rSPNT and hSPNT1,

adenosine has a higher turnover rate for rSPNT in comparison to inosine and the converse is true for hSPNT1.

It has been shown previously that 1 mM hypoxanthine, the nucleobase of inosine, does not inhibit hSPNT1 (3). Here we tested whether adenine, the nucleobase of adenosine, was transported by either of these two transporters. Our data show that adenine does not induce a current in oocytes expressing rSPNT or hSPNT1. Interestingly, 5'-AMP induces currents in oocytes expressing rSPNT but not hSPNT1 suggesting that it is a substrate of rSPNT but not hSPNT1. 5'-ADP and 5'-ATP are not significantly transported by either SPNT ortholog. ATP and ADP are ligands of the P2 subtype of purine receptors whereas AMP and adenosine are not (21, 22). Extracellular ecto-nucleotidases have been shown to be responsible for terminating the signal transmission of the purinergic P2 receptors (23, 24). These ecto-nucleotidases (ecto-ATPase, ecto-ADPase and 5'-nucleotidase) convert ATP to adenosine in three steps with the conversion of AMP to adenosine being rate-limiting (25). SPNT and the P2Y receptor are both expressed in several tissues including heart, liver and spleen and thus may play a role in the re-cycling of AMP and adenosine produced by ecto-nucleotidases.

Several important anti-viral agents are 2'-deoxyuridine analogs with substitutions in the 5-position of the base, 5-fluoro-deoxyuridine (floxuridine, FdU) and 5-iodo-deoxyuridine (idoxuridine, IdU). We have shown previously that these drugs as well as several other analogs with modifications at this position are substrates of the pyrimidine-selective nucleoside transporter from rat, rCNT1 (17). In contrast to the previous work with rCNT1, all of these substitutions at the 5-position result here in compounds that are poorly translocated by both rSPNT and hSPNT1. These data suggest that in tissues in

which both CNT1 and SPNT are present, CNT1 preferentially transports 5-uridine analogs whereas both transporters transport uridine.

We also tested another important base-modified nucleoside analog drug, 2-chloro-2'-deoxyadenosine (cladribine or 2CdA). Cladribine, important in the treatment of hairy cell leukemia, has been shown previously to be a potent inhibitor of rSPNT (IC_{50} of 13.8 μM in inhibition of uptake of 1 μM inosine) and a modest inhibitor of hSPNT1 (IC_{50} of 371 μM in inhibition of uptake of 1 μM inosine) (18, 19). It has also been shown in radio-labeled uptake studies that 2CdA is transported by rSPNT, but very poorly by hSPNT1. Our studies show that rSPNT transports 2CdA very well (Figure 4). However, it is transported at only very low levels by hSPNT1. In agreement with the previous work, 400 μM 2CdA inhibits the current induced by 50 μM uridine by approximately 50% (data not shown). This is consistent with the reported IC_{50} of 371 μM.

Many important anti-viral agents are ribose-modified nucleoside analogs, several of which were studied here to determine if any are substrates of rSPNT or hSPNT1. Of the ribose-modified therapeutic nucleoside analogs, Ara-A and ddA were found to be substrates of rSPNT but not hSPNT1. ddI, on the other hand, was a weak substrate of both rSPNT and hSPNT1. These data suggest that hSPNT1 may not be important in the delivery of antiviral ribose-modified nucleoside analogs to their target tissues. Our studies with the adenosine analogs provide insights into the optimal configuration of the ribose group for transport. First, a 3' hydroxyl group is necessary for transport by both SPNT orthologs. That is, no detectable currents were induced by ddA. Second, a 2' hydroxyl group is accepted by both orthologs but rSPNT exhibits a preference for a 2'-deoxyribose. Finally, the alpha position of the 2' hydroxyl is preferred by hSPNT1

(adenosine is transported whereas Ara-A is not), but not by rSPNT (both adenosine and Ara-A are transported equally well).

Our data suggest that there are differences in the substrate selectivity of rSPNT and hSPNT1. Overall our data indicate that rSPNT is more permissive of base or ribose modifications than is hSPNT1 (e.g., rSPNT transports Ara-A and 2CdA whereas hSPNT1 does not). An alignment of the transmembrane domain 7-8 region (amino acids 296 – 358 of hSPNT1), shown previously to be critical in substrate discrimination (26), shows that there are 5 differences in amino acid sequence between these two transporters (Figure 7). It is possible that one or more of these in combination may be responsible for the selectivity differences between the SPNT orthologs.

These findings of differences in selectivity for both antiviral and anticancer nucleoside analogs suggest that the rat may not be a suitable animal model for evaluating candidate nucleoside analogs in preclinical drug development.

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hSPNT1   MGTTATETLA VAGNIFVGMT EAPLLIRPYL GDMTLSEIHA
rSPNT    MGTTAAETLA VAGNIFVGMT EAPLLIRPYL ADMTLSEIHA

hSPNT1   VMTGGFATIS GTVLGAFIAF GVD
rSPNT    VMTGGFATIA GTVLGAFISF GID
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Figure 7. Alignment of the TM7-8 region of the human and rat N1 orthologs. The regions shown correspond to amino acid residues 296 – 358 of hSPNT1 and 297 – 259 of rSPNT. Shown in bold are amino acid residues that differ between the two sequences.

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CHAPTER 6

SUMMARY AND CONCLUSIONS

Nucleosides analogs are commonly used in the treatment of various viral infections and cancer. Analogs of the pyrimidine nucleoside, uridine, such as 5-iodo-2'-deoxyuridine (idoxuridine, IdU) and 5-fluoro-2'-deoxyuridine (floxuridine, FdU), are important therapeutic agents in the treatment of herpes simplex virus (HSV). Two purine nucleoside analogs of adenosine, adenine arabinoside (vidarabine, ara-A) and 2-chloro-2'-deoxyadenosine (cladribine, 2CdA) are used in the treatment of HSV or varicella-zoster and hairy cell leukemia, respectively (1, 2). In general, nucleoside analogs are hydrophilic molecules and, as such, may require transporter proteins to cross cellular membranes. It is thought that nucleoside transporters (equilibrative and concentrative) may thus play a role in the absorption, elimination and tissue-specific targeting of nucleoside analogs. In order to understand the role that these transporters may play *in vivo*, it is critical to clone the cDNAs coding for these proteins, from human as well as those from important animal models (e.g., rat, mouse and rabbit). One must then characterize in detail the function of each of these transporters in isolation. For these studies, interspecies comparisons and decisions about the appropriateness of a particular animal model for preclinical animal testing can be made. Once the specificity of a particular transporter has been determined, a rational drug design approach can be used to develop the next generation of nucleoside analogs that target these transporters in order to improve their pharmacokinetic characteristics and tissue specificity. The availability of

clones of these transporters also allows researchers to begin to study the structure of these proteins. As multi-membrane spanning proteins, determining a crystal structure of a transporter is non-trivial. Hence, researchers from our and other laboratories have used molecular biological techniques to identify, among other things, the domains involved in substrate binding and discrimination as well as specific amino acids involved in determining substrate selectivity (3).

The first step in determining the role that these transport processes play with respect to drug disposition *in vivo* is to clone the proteins responsible for the observed process. By the mid-1990's two subtypes of concentrative nucleoside transporter (N1 and N2) had been cloned from rat and human tissues. The pyrimidine-selective N2 transport system was cloned from rat intestine in 1994 and termed CNT1 (4). N1, the purine-selective system, was subsequently cloned from rat liver in 1995 and termed SPNT (or CNT2) (5). The human orthologs of both of these transporters (hCNT1 and hSPNT1 or hCNT2) were cloned from human kidney in 1997 (6, 7). The mouse and pig orthologs of CNT2 and CNT1, respectively, were the next to be cloned (8, 9). Chapter 2 of this dissertation describes the cloning of the first concentrative nucleoside transporter from rabbit, rbSPNT1. Functional analysis identified this transporter as the purine-selective nucleoside transporter. The cloning of this transporter enables its function to be characterized in isolation. Further detailed functional characterization of this and other rabbit nucleoside transporters is important to understanding the role that these transporters play in the *in vivo* disposition of nucleoside analogs in preclinical testing in the rabbit model.

Functional characterization of electrogenic transporters can be performed with the two-electrode voltage clamp technique (10). Chapters 3 and 5 addressed the specificities of the rat CNT1 and human and rat SPNT using voltage-clamped oocytes. Each of these transporters is electrogenic due to the cotransport of a Na^+ ion with a nucleoside. The transport of Na^+ generates a current that can be measured using the two-electrode voltage clamp system. The use of this method enables the researcher to perform a more comprehensive investigation of the substrate selectivity of a transporter. In chapter 3, structural analogs of uridine and cytidine, two substrates of rCNT1, were investigated to determine how well rCNT1 tolerated modifications to these substrates. In general, rCNT1 was permissive to many modifications of both cytidine and uridine. A series of deoxyuridine analogs with modifications in the 5-position of the base in which the hydrogen is substituted with a halogen (two of which, IdU and FdU are important drugs) or a hydroxymethyl group were all transported by rCNT1. In addition, modifications of either uridine or cytidine at the 3 and 4 positions were also transported by rCNT1 while a modification at the 6 position (6-azauridine and 6-azacytidine) were not.

In chapter 5 the substrate specificities of the purine-selective transporter were examined in a species comparison, i.e. rSPNT versus hSPNT1. In these studies the selectivity of these transporters for naturally occurring nucleosides, nucleobases and nucleotides as well as base-modified and ribose-modified analogs was investigated. In general, rSPNT transported a broader range of compounds than did hSPNT1. For example, 2CdA and ara-A were transported well by rSPNT but were transported not at all or only to a limited extent by hSPNT1. These data suggest that although highly homologous, amino acid differences between rat and human SPNT determine the unique

specificity of each species ortholog. Another interesting finding in these studies is that AMP, not previously thought to be a substrate of any of the nucleoside transporters, was found to induce a significant current in oocytes expressing rSPNT. ATP and ADP, which are agonists for the P2 receptor, are converted to AMP and adenosine via extracellular ecto-nucleotidases thus terminating the signal. rSPNT may participate in the signal termination pathway of the activation of P2 receptors by nucleotides through the reuptake of AMP and/or adenosine. More work is required to determine how nucleoside transporters may work together with purinergic receptors.

Chapter 4 addressed the molecular determinants of the pyrimidine-selectivity of rCNT1. In recent work from our laboratory, a single amino acid mutant of rCNT1 was constructed in which the serine at position 318 was substituted with the corresponding amino acid of rSPNT, glycine. This substitution resulted in a transporter with a broadened substrate selectivity; the pyrimidine-selective transporter now allowed purine nucleosides as substrates (11). Two hypotheses that could explain this broadened selectivity were tested. The first hypothesis is that the decrease in the size of the side chain in the S318G mutant increased the size of the permeation pathway allowing the larger purine nucleosides to be transported. The second, the loss of a potential hydrogen bond involving the hydroxyl group of the serine residue allowed the purine nucleosides access to the substrate binding site. A series of mutants, in which substitutions were made at position 318, were constructed and their phenotype determined. Only two mutants, S318T and S318A, were functional. Both were pyrimidine selective. Data from these studies suggested two things, that (a) hydrogen bonding involving residue 318 is not required to confer pyrimidine-selectivity to rCNT1; and (b) the size of the side chain may

be an important determinant in the selectivity of rCNT1, with the smallest side chain (S318G) exhibiting the broadest selectivity. More work is needed to determine which other residues of rCNT1 influence substrate selectivity. In addition, almost no work has been done with the SPNT transporters to identify amino acid residues critical to the purine-selectivity of these transporters.

There remains much work to be done in the nucleoside transporter field. Many of the relevant transporters have been cloned to date but the proteins corresponding to several of the transport processes characterized in various tissue preparations and human cell lines, remain to be identified. The molecular identity of the broadly-selective transport process, N3, eluded researchers for many years. The recent cloning of the cDNA coding for an N3 transport system, termed CNT3, from *C. elegans*, human and mouse was therefore an important milestone in nucleoside transporter research (12, 13). The expression of the human and mouse N3 transporter in a broader range of tissues than N1 or N2 and its interaction with many nucleoside analogs could indicate that the N3 transporter plays a role in the disposition of nucleoside analogs. Knockout animals of each of these transporters are therefore critical to understanding the role that each transporter plays *in vivo*. Other areas such as regulation and targeting are now beginning to be addressed. A recent report from this laboratory suggests a primarily apical localization of rSPNT and rCNT1 (14). This suggests that these transporters, present in the epithelia of intestine and kidney, play a role in the absorption of nucleosides (a basolateral localization would have suggested secretion). Further work is needed in the area of regulation of these transporters in order to begin to understand the role that these transporters may play *in vivo* during drug therapy with nucleoside analogs. The presence

of nucleoside transporters in tissues such as the heart and kidney where adenosine, via the purinergic receptors (15), plays important physiological roles may indicate that these transporters and receptors are working together. Potential interactions between transporters and receptors is an interesting but as yet unexplored area of transporter research. Finally, the implication of genetic variation in nucleoside transporters to variation in response to nucleoside analogs represents a new and exciting area of research.

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