High Performance Liquid Chromatography Analysis of Nucleosides and Nucleobases in Mouse Plasma and *Xenopus* Oocytes

by

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Abstract

In humans, there are three members of the cation-coupled concentrative nucleoside transporter CNT (SLC28) family, hCNT1-3: hCNT1 is selective for pyrimidine nucleosides but also transports adenosine, hCNT2 transports purine nucleosides and uridine, and hCNT3 transports both pyrimidine and purine nucleosides. hCNT1/2 transport nucleosides using the transmembrane Na^+ electrochemical gradient, while hCNT3 is both Na^+- and H^+-coupled. By producing recombinant hCNT3 in *Xenopus laevis* oocytes, I used radiochemical high performance liquid chromatography (HPLC) analysis to investigate the metabolic fate of transported radiolabelled [^{3}H] and [^{14}C] pyrimidine and purine nucleosides inside cells. My findings suggest that transported nucleosides in oocytes after 30 minute of incubation are generally subject to minimal intracellular metabolism (uridine 7%, cytidine 0%, thymidine 0%, inosine 16% and guanosine 0%). The exception was adenosine, for which only 49% remained unmetabolized. I also used radiochemical HPLC analysis to study mechanisms by which adenosine functions as an atypical low K_m, low V_{max} permeant of pyrimidine nucleoside-selective hCNT1. Oocytes producing recombinant hCNT1 were pre-loaded with [^{3}H]uridine, after which efflux of accumulated radioactivity was measured in transport medium alone, or in the presence of extracellular non-radiolabelled adenosine or uridine. The results found that hCNT1-mediated [^{3}H]-efflux was stimulated by extracellular uridine, but inhibited by extracellular adenosine, with > 95% of the radioactivity exiting cells being unmetabolized radiolabelled uridine. This suggests that the low V_{max} for adenosine transport by hCNT1 is a consequence of low transmembrane mobility of the hCNT1/adenosine complex.

Humans also possess four members of the equilibrative nucleoside transporter ENT (SLC29) family, hENT1-4. hENT1 and hENT2 function to transport both nucleosides and nucleobases into and out of cells, but their relative contributions to nucleoside and nucleobase homeostasis and, in particular, to adenosine signaling via purinoreceptors, are not known. My second project addressed this question by using HPLC to compare plasma nucleoside and nucleobase concentrations in wild-type, mENT1-, mENT2- and mENT1/mENT2-knockout (KO) mice. Results from these studies demonstrated the importance of ENT1 relative to ENT2 in determining plasma adenosine concentrations, indicating a modest role of both transporters in inosine homeostasis, and suggested that neither is a major participant in handling of nucleobases.

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Contributions

Functional expression of hCNT3 and hCNT1 in *Xenopus* oocytes was performed by Dr. Sylvia Yao with my assistance.Preparation of oocyte extracts and subsequentHPLC analysis were undertaken by me. Preparation of mouse plasma extracts and subsequent HPLC analysis were performed by me with Dr. Sylvia Yao's assistance.

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List of Nomenclature and Abbreviations

2-CdA	2-chlorodeoxyadenosine
AZT	azidothymidine
cif	concentrative, insensitive to NBMPR, formycin B-transporting
cit	concentrative, insensitive to NBMPR, thymidine-transporting
cib	concentrative, NBMPR-insensitive, broadly selective
CNT	Concentrative Nucleoside Transporter
ddC	2', 3'-dideoxycytidine
ddI	2', 3'-dideoxyinosine
dFdC	2', 2'-diflurodeoxycytidine
ENT	Equilibrative Nucleoside Transporter
F-ara-A	9-β-D-arabinosyl-2-fluoroadenine
HIV	human immunodeficiency virus
HPLC	High Performance Liquid Chromatography
KO	knock out
MPP^+	1-methy-4-phenylpyridinium
NBMPR	nitrobenzylmercaptopurine ribonucleoside
NT	nucleoside transporter
PNP	purine nucleoside phosphorylase
SEM	standard error of mean
SLC	solute carriers
RNA	ribonucleic acid
RT-PCR	reverse transcription polymerase chain reaction
TLC	thin layer chromatography

TM	transmembrane segments
UMP	uridine monophosphate
UDP	uridine diphosphate
UTP	uridine triphosphate
WT	wild-type

Chapter 1:

General Introduction

Nucleoside Transport

Physiological and Pharmacological Importance:

Nucleosides and nucleobases are important physiological molecules that play many essential physiological roles, including in lipolysis, neurotransmission, platelet aggregation and coronary vasodilation (Parkinson et al., 2011; Young et al., 2013). Naturally occurring nucleosides include the purine nucleosides adenosine, guanosine and inosine,as well as the pyrimidine nucleosides uridine, cytidine and thymidine (Figure 1-1). Naturally occurring nucleobases include uracil, adenine, cytosine, thymine and hypoxanthine(Figure 1-2). Nucleosides and nucleobases serve as precursorsof DNA and RNA and high-energy compounds such as ATP(Young & Jarvis, 1983; Baldwin et al., 1999). Moreover, adenosine is a ubiquitous physiological signaling molecule. For example, in the cardiovascular system,adenosine regulates myocardial O_2 supply-demand via coronary vasodilation, and cardiac pacemaking and contractility. It also functions as a cardioprotective agent in ischemic/reperfused myocardium (Mubagwa et al., 1996; Cass et al., 1999;Mubagwa et al., 2001).Adenosine has other physiologicroles on other systems, including the nervous, cardiovascular, gastrointestinal, renal, muscular, and immune systems (Cass et al., 1999; Ishikawa et al., 2013; Masino & Boison 2013). Furthermore, adenosine is also considered to be a valuable pharmacological agent due to its broad range of physiological effects in mammalian tissues, with particularly profound effects on the cardiovascular system (Belardinelli et al., 1989; Thorn & Jarvis, 1996; Baldwin et al., 1999;).

Since most nucleosides are hydrophilic in nature, they require specialized nucleoside transporter proteins for passage across cell membranes (Griffith & Jarvis, 1996; Baldwin et al., 1999). These nucleoside transporter proteins are responsible for determining levels of free nucleosides in the general circulation and thus regulate their biochemical and physiological effects and functions(Cass et al., 1998; Cass et al., 1999). Metabolically, nucleoside transporters are central elements of purine and pyrimidine nucleoside salvage pathways (Young et al., 2013), which are energetically more favorable than *de novo* purine and pyrimidine biosynthesis, and thus have key roles in nucleoside homeostasis

(Young et al., 2013). In cells that are deficient in purine *de novo* biosynthesis, nucleoside and nucleobases transport is crucial for purine nucleotide synthesis (Griffith et al., 1997; Baldwin et al, 2004).Such cells include bone marrow, erythrocytes, enterocytes, leukocytes and certain brain cells (Murray et al., 1971;Baldwin et al., 2004). The major sources of nucleosides are from the diet, reabsorption from the kidney and production by the liver (Pritchard et al., 1975;Baldwin et al., 2004). Nucleoside transporters also have a role in preventing excess accumulation of intracellular nucleotide breakdown products by releasing them from cells. Breakdown occurs in lysosomes, which are responsible for degradation of nucleic acids into nucleosides (Pisoni & Thoene, 1989).

The uptake of anticancer and antiviral nucleoside analogs isalso mediated through nucleoside transportprocesses(Zhang et al., 2007; Young et al., 2013). These compounds are used in the treatment of neoplastic diseases including hematological and solid malignancies, as well as viral infections including human immunodeficiency virus (HIV) and herpes virus infections (Mackey et al., 1998; Pastor-Anglada et al., 1998, Zhang et al., 2007;Ishikawa et al., 2013). To exert their pharmacological actions, these drugs must initially enter cellsthrough nucleoside transporters found in plasma membranes, and thenbe metabolized. Therefore, cell surface expression levels of these transporters influence the pharmacokinetics and cytotoxic actions of nucleoside drugs (Ishikawa et al., 2013). Nucleoside analogs exert their cytotoxic actionby undergoing phosphorylation to their 5'-triphosphate derivatives by nucleoside and nucleotide kinases (Zhang et al., 2007).Phosphorylated nucleoside analogs interfere with nucleotide metabolism and RNA and DNA replication events, resulting in antiproliferative effects and resistance to virus replication.

Purine nucleoside derivatives that are extensively used to treat hematological malignanciesinclude cladribine (2-CdA; 2-chlorodeoxyadenosine) and fludarabine (F-ara-A; 9-β-D-arabinosyl-2-fluoroadenine), while the pyrimidine nucleoside analog gemcitabine (dFdC; 2',2'-diflurodeoxycytidine) is used in treatment of solid tumors (Ishikawa et al, 2013). Antiviral nucleoside analogs include the pyrimidine nucleoside analogs zidovudine (AZT; azidothymidine), zalcitabine (ddC; 2', 3'-dideoxycytidine),

and the purine nucleoside derivative didanosine (ddI; 2', 3'-dideoxyinosine) (Figure 1-3)(Baldwin et al., 1999;Ishikawa et al., 2013).

Nucleoside Transport Proteins:

Two structurally and evolutionarily unrelated gene families have been identified as responsible for cellular uptake of nucleosides (Young et al, 2013), the Concentrative Nucleoside Transporter (CNT) family and the EquilibrativeNucleoside Transporter (ENT) family. A summary of these nucleoside transporters is presented in Tables 1-1 and 1-2. Only those that are involved in the experimentation of this thesis are discussed.

Equilibrative Nucleoside Transporter (ENT) Family:

This family of transporters belongs to the human SLC29 family of proteins, and human and mousemembers of this family are listed in Table 1-1.In humans and other mammals, theENT protein family consists of four isoforms, ENT1-4. Members are ubiquitously expressed across tissues and transport nucleosides and nucleoside analogsby Na^+-independent mechanisms. ENT1 and ENT2 transport nucleosides passively across cell membranes, while ENT3 and ENT4 may be proton-coupled. Movements of nucleosides by ENT1/2 occur in a bidirectional matter mediating both influx and efflux (Young et al., 2013), and arelikely the main participants in the transport of adenosine. Furthermore, these ENTs contribute tothe salvage of nucleosides for the synthesis of nucleotides and nucleic acids in cell types which lack synthesis of nucleosides *de novo*.

The facilitated diffusion of nucleosides mediated by ENTs in mammalian cells was initially studied and characterized in human erythrocytes(Oliver & Paterson, 1971)..These were ideal for functional studies due to their possession of a single nucleoside transport process, now known as hENT1, and to the absence of metabolism of uridine and thymidine.

ENTs have been differentiated by their sensitivityto inhibition to the S^6-substituted 6-thiol-purine nucleoside derivative nitrobenzylmercaptopurine ribonucleoside (NBMPR) Figure 1-4(Paterson & Oliver, 1971; Baldwin et al., 1999). The processes mediated by ENT1 and ENT2 were initially termed *es*and *ei* respectively, due to the *e*quilibrative nature of transport and their *s*ensitivity or *i*nsensitivity to nanomolar concentrations of NBMPR (Figure 1-4) (Baldwin et al., 2004). In addition to NBMPR, most *es* and *ei* transport processes are also inhibited by the non-nucleoside coronary vasodilators dipyridamole and dilazep (Figure 1-4) (Baldwin et al., 2004). ENT1 and ENT2 were the first two members of this family to be identified and characterizedby molecular cloning of their cDNAs (Baldwin et al., 2004).Similar to ENT1 and ENT2, ENT3 is also widely distributed and has board permeant selectivity, but functions predominantly intracellularly in lysosomes (Figure 1-5) (Baldwin et al., 2005; Govindarajan et al., 2009). ENT4 is found in plasma membrane and transports adenosine and monoamines in the brain and heart (Figure 1-5) (Barnes et al., 2006; Zhou et al., 2007; Zhou et al., 2010).

ENTs have 11 predicted transmembrane segment (TMs), with a topology that includes a cytoplasmic N-terminus, an extracellular C-terminus and a large cytoplasmic loop linking TMs 6 and 7(Figure 1-6)(Baldwin et al., 2004; Young et al., 2013). Crystal structuresfor ENTs are not currently available.

ENT1 (*es* subtype):

In humans,the *es* nucleoside transporter(i.e., hENT1) corresponds to a protein of 456 amino acids with a predicted size of 50kDa (Griffiths et al, 1997a, Jeffery et al, 2000). Comparing mouse(m) ENT1 (containing 458 residues) to human(h) ENT1, the two amino acid sequences are78% identical (Yao et al., 1997, Cass et al., 1998; Kiss et al., 2000; Handa et al., 2001).TheseENTs are reversibly inhibited by NBMPR. The inhibitory effect of NBMPR on nucleoside influx and efflux occurs at nanomolar concentrations (Baldwin et al, 2004, Young et al., 2013). The hENT1 gene chromosomal location is 6p21.1(Coe et al, 1997; Sankar et al., 2002). There are two isoforms of mENT1-designated mENT1.1 with 460 residues and mENT1.2 with 458 residues (Choi et al., 2000; Baldwin et al.,

2004).mENT1.2 is widely distributedand is functionally identical to mENT1.1, except for altered NBMPR binding affinity (Young et al., 2008). Several single nucleotide polymorphisms (SNPS) resulting in non-synonymous variant hENT1 transporters have been identified. Those that have been characterized functionally show normal nucleoside and nucleoside drug uptake kinetics. mENT1 knockout (KO) mice are viable and fertile (Chen et al., 2007; Young et al., 2008).

In addition to transporting physiological nucleosides and nucleobases (except cytosine), hENT1 is responsible for the transport of several anticancer and antiviral nucleoside drugs (Yao et al., 2011).ENT1 is ubiquitously distributed in human and rodent tissues, although its abundance varies between tissues (Baldwin et al., 2004; Young et al., 2008). Transcripts for this transporter are found in fetal brain, liver and spleen and in adult adipose tissue, aortic endothelial cells, brain, breast, colon, heart, lung, ovary, placenta, prostate and uterus, as well as in malignant cells (Cass et al., 1998).

ENT2 (*ei* subtype):

In humans,the *ei* nucleoside transporter (i.e., hENT2) is predicted to have a size of 50kDa with 456 residues (Griffiths et al., 1997b; Crawford et al., 1998; Jeffrey et al, 2000;Baldwin et al., 2004). hENT2 to hENT1 are 46% identical in amino acid sequence (Yao et al., 1997; Young et al., 2008). hENT2is 88% identical in amino acid sequence to mENT2 (Young et al., 2008). As indicated by its name *ei*, this subtype is insensitive to inhibition by nanomolar concentrations of NBMPR (Yao et al., 1997). The hENT2 gene chromosomal location is 11q13 (Sankar et al., 2002).

Similar to human and mouse ENT1, human and mouse ENT2 mediate the transport of a wide range of purine and pyrimidine nucleosides but with lower affinitiesexcept for the nucleoside inosine (Figure 1-5) (Ward et al., 2000;Baldwin et al., 2004). hENT2 also transports nucleobases (Yao et al., 2002). Although apparent affinities of hENT2 for nucleobases are lower than those for the corresponding nucleosides, the turnover numbers for transport are higher, such thattransport of nucleosides and nucleobases are similar at

physiological concentrations (Yao et al., 2002). Compared to hENT1, hENT2 exhibits a much greater capacity for transporting antiviral drugs such as AZT, ddC and ddI (Yao et al., 2002).

Like hENT1, hENT2 is widely expressed in a broad range of tissues. Transcriptsare present in brain, heart, placenta, thymus, pancreas, prostate, lung and kidney,andare particularly abundant in skeletal muscle (Pennycooke et al., 2001; Young et al., 2008).Its predominance in skeletal muscle suggests that it has an important role during muscular activity (Ward et al., 2000).mENT2 KO mice are viable and fertile(Grenz et al., 2012).

ENT3:

hENT3 consists of 475 residues, and its chromosomal location is 10q22.1 (Hyde et al., 2001; Sankar et al., 2002; Baldwin et al., 2005; Young et al., 2008). hENT3 is 73% identical to mENT3 and 31-33% identical in sequence to hENT1 and mENT2 (Hyde et al., 2001; Baldwin et al., 2005). Compared to h/mENT1 and h/mENT2, h/mENT3 have very long (51 amino acid residue) N-terminal regions (Young et al., 2008). These N-terminal regions contain the sequence (DE)*XXXL*(L1) typical of endosomal/lysosomal targeting motifs (Baldwin et al., 2005), suggesting that ENT3 functions predominantly in intracellular membranes where it is likely involved in nucleoside and nucleobase transport into the cytoplasm following nucleic acid breakdown in lysosomes (Pisoni et al., 1989; Baldwin et al., 2005;Govindarajan et al., 2009). Transport mediated by hENT3 exhibits proton dependence, suggesting H^+-coupling. The topology of ENT3 is similar to other ENTs with 11TMs, a cytoplasmic-N-terminus and a putative N-glycosylation site in the extracellular loop between TMs 1 and 2 (Baldwin et al., 2005). Furthermore, hENT3 exhibits broad purine and pyrimidine nucleoside selectivity (Figure 1-5) and is widely distributed, with particular abundance in the placenta, heart and liver (Baldwin et al., 2004; Baldwin et al., 2005). High levels of transporter transcriptswere also detected in uterus, ovary, spleen, lymph node and bone marrow (Baldwin et al., 2005).

ENT4:

hENT4contains 530 amino acid residues and shares asequence identity of 18% with hENT1. mENT4 is 528 residues in length and is 86% identical to hENT4 (Barnes et al., 2004). ENT4 is alsoreferred to as the plasma membrane monoamine transporter (PMAT) and is alow affinity transporter which is selective for adenosine and the nucleobase adenine under acidic conditions and, in addition,also for monoamines and 1-methy-4-phenylpyridinium (MPP^+) (Figure 1-5) (Barnes et al., 2004; Zhou et al., 2007; Zhou et al., 2010).Like ENT3, ENT4 exhibits proton dependence, suggesting H^+-coupling.The hENT4 chromosomal location is 7p22.1(Acimovic & Coe, 2002). ENT4is predicted to have 11 TMs and contains glycosylation sites in the C-terminal tail at Asn^{523} and Asn^{521} (Acimovic & Coe, 2002; Barnes et al., 2006). hENT4 is especially abundant in the central nervous system and in cardiac tissue (Vialou et al., 2007). Substantial levels of hENT4 mRNA were also found in regions of heart and intestine, as well as in pancreas, kidney, liver, bone marrow, and lymph node. (Barnes et al., 2006;Xia et al., 2006;Zhou et al., 2007; Zhou et al., 2010).

ENT Physiological Implications

ENTs have many important physiological implications through their bidirectional transport of different nucleosides and nucleobases. ENT1 and ENT2 are widely distributed, often coexisting in many cell and tissue types. They play a key role in the provision of nucleosides derived from the diet or produced by the liver for salvage pathways of nucleotide synthesis in cells that are deficient in *de novo* biosynthetic pathways (Baldwin et al., 2004).Although both ENT1 and ENT2 have similar nucleoside specificities, hENT2 has a high affinity for the uptake (and efflux) of hypoxanthine and inosine. As mentioned previously, and because of its abundance in skeletal muscle, this may relateto a role during muscular exercise and recovery(Baldwin et al., 2004; Young et al., 2013).

ENT3 has a key role in the transport of nucleosides across lysosomal membranes and is important for the release of nucleosides produced by nucleic acid breakdown in the lysosomal interior (Pisoni et al., 1991; Baldwin et al., 2005). ENT3 may also have a role

in basal and amino acid deprivation-induced cytoplasmic RNA degradation that occurs in the liver (Heydrick et al., 1991). Predominant expression of ENT4 in brain and cardiac tissue suggests that ENT4 may be involved in the regulation of extracellular adenosine levels in the heart, particularly during ischemia (Tattersall et al., 1983). ENT4 also has a role in organic cation reabsorption in the kidney and in monoamine uptake in the brain (Zhou et al., 2007).

ENTs have important roles in regulating the concentration of adenosine in cardiovascular, neural and pulmonary tissues. This is important because adenosine modulates coronary blood flow, myocardial O_2 supply, inflammation and neurotransmission. Both ENT1 and ENT4 are highly expressed in heart tissue and therefore are pharmaceutical targets for cardiac disorders such as ischemia associated with coronary artery disease and during cardiac surgery.The potential benefits of ENT inhibition during ischemia-reperfusion in the heart also extend to neuronal injury. In the brain the bidirectional activity of ENTs is important for maintaining adenosine concentrations (Gu et al., 1995, Parkinson et al., 2011). The regulation of adenosine concentrations by hENT1 and hENT2 in human airway epithelial cells influences adenosine-activated A_1 receptor-mediated control of K^+ channels (Szkotak et al., 2001).

mENT3 KO mice have a significantly shorter life span than wild-type mice. They develop spontaneous and progressive macrophage-dominated histiocytosis, lymphadenopathy and splenomegaly which may be associated with the developmentoffamilial Rosai-Dorfman disease, Faisalabad histiocytosis, H syndrome, and pigmented hypertrichosis with insulin-dependent diabetes (Morgan et al., 2010; Hsu et al., 2012)

Concentrative Nucleoside Transporter (CNT) Family

CNTs transport nucleosides against their concentration gradients by coupling to cations movingdown their electrochemical gradients. The human concentrative nucleoside transporter family (SLC28) consists of three isoforms, hCNT1-3, which belong to two distinct CNT phylogenetic subfamilies.hCNT1 and 2 belong to one subfamily, while hCNT3 belongs to the other.

CNTsare NBMPR-insensitive and Na^+-dependent (Williams et al., 1989;Baldwin et al, 1999). The CNT family is also responsible for uptake of several antiviral and anticancer nucleoside analog drugs (Young et al., 2013). To mediate nucleoside transport, CNTs undergo conformational changes between two states that expose the permeant binding site to either the intracellular or extracellular environment, but with additional conformational transitions in regions associated with cation binding (Smith et al, 2005). While hCNTs exhibit greater permeant affinities (lower K_m values) than hENTs, their transport turnover numbers areseveral orders of magnitude lower (Smith et al., 2005). CNTs differ from each other in nucleoside selectivity and Na^+/nucleoside coupling stoichiometry.

Using radiolabeled nucleosides to characterize nucleoside transport in mouse intestinal epithelial cells, it was demonstrated that uptake of formycin B, a non-metabolized inosine analog, was not inhibited by the presence of thymidine, while the corresponding uptake of thymidine was not inhibited by the presence of formycin B(Vijayalakshmi & Belt, 1988).These two transport processes were designated N1 or *cif* (*c*oncentrative, *i*nsensitive to NBMPR, *f*ormycin B-transporting) and N2 or *cit* (*c*oncentrative, *i*nsensitive to NBMPR, *t*hymidine-transporting); additional permeants for *cif*are the purine nucleosides guanosine and inosine and,for *cit*,the pyrimidine nucleoside cytidine (Vijayalakshmi & Belt, 1988; Jarvis & Griffith, 1991). Both processes(*cit* and *cif*)displayed Na^+-dependence and a 1:1 Na^+: nucleoside stoichiometry (Lee et al., 1988; Griffith & Jarvis, 1996). A broadly selective concentrative nucleoside transport process, transporting both pyrimidine and purine nucleosides, was also found and is designated N3 or *cib* (*c*oncentrative, NBMPR-*i*nsensitive, *b*roadly selective). In comparison to *cif* and *cit*, the *cib* Na^+: nucleoside stoichiometry is 2:1 (Wu et al., 1992).

The proteins corresponding to *cit*, *cif* and *cib* are designated CNT1 (*cit*), CNT2 (*cif*) and CNT3 (*cib*). In humans these belong to the SLC28 family of proteins.hCNT1/2 are located primarily in the intestinal and renal epithelia, as well as other specialized cells(Baldwin et al., 1999; Smith et al., 2007). In contrast, hCNT3 is more widely distributed in human tissues, such as the pancreas, bone marrow, trachea, mammary gland, liver, prostate, and regions of the intestine, brain and heart (Ritzel et al., 2001). CNTs and ENTs are co-expressed in epithelial cells and in some nonpolarized cells (e.g. leukemic cells), whereas other nonpolarized cells (e.g.,erythrocytes) exhibit only ENTs (Oliver & Paterson, 1971).

CNTs are Na^+-dependent, transporting both physiologic nucleosides and antiviral and anticancer nucleoside analog drugs. hCNT1 is pyrimidine nucleoside selective in that it transports pyrimidine nucleosides only (with the exception of low activity adenosine transport), whereas hCNT2 is purine selective, although it also transports the pyrimidine nucleoside uridine. hCNT3 is broadly selective and mediates the uptake of both purine and pyrimidine nucleosides (Figure 1-5).

CNTs wereinitially predicted to have 13 TMs with an intracellular N terminus and an extracellular C terminus(Hamilton et al., 2001). However, the crystal structure of the bacterial Na^+-linked CNT from *Vibrio cholera* (vcCNT) with bound uridine and sodium has beendetermined at a resolution of 2.4Å (Johnson et al., 2012). vcCNT shows 39% amino acid sequence identity with hCNT3. By analogy with vcCNT, hCNT3 topology can be subdivided into an outer or “scaffold” domain comprising TM4, TM5, IH1, TM6 and TM9, which surrounds an inner or “transport” domain comprising IH2, HP1, TM7, TM8, IH3, HP2, TM10 and TM11 (Figure 1-7).

The research in this thesis is focused on hCNT3, for which*Xenopus laevis* oocytes were used as a heterologous expression system. *Xenopus laevis* oocytes were used because of their large size (1.0 mm) and their lack of endogenous nucleoside transport activity (Huang et al., 1994;Miller & Zhou, 2000).

CNT1

The first nucleoside transporter to be identified at the molecular level was obtained by expression screening of a rat jejunum cDNA library and was designated rCNT1. rCNT1mediates nucleoside transport of *cit*-type and consists of 648 amino acids (Huang et al., 1994). hCNT1was later identified in human kidney by hybridization/RT-PCR cloning, consists of 650 amino acids, and is 83% identical to rCNT1 in amino acid sequence (Ritzel et al., 1997).The hCNT1 chromosomal location is 15q25.3 (Ritzel et al., 1997).

Upon production in *Xenopus laevis* oocytes, recombinant rCNT1 and hCNT1 show*cit*/N2-type Na^+-dependent pyrimidine nucleoside-selective transport activities(Figure 1-5) (Huang et al., 1994; Ritzel et al., 1997). Electrophysiological studies using *Xenopus* oocytes found r/hCNT1-mediated transport to be pyrimidine nucleoside-selective andboth Na^+- and voltage-dependent (Dresser et al., 2000; Larrayoz et al., 2004;Smith et al., 2004). The purine nucleoside adenosine is a permeant for both hCNT1 and rCNT1. This transport occurs in a high-affinity, low-capacity manner (Yao et al., 1996).CNT1 also mediates the uptake of several antiviral and antineoplastic analogs and has an important role in both their pharmacokinetics and pharmacodynamics (Ritzel et al., 1997;Damaraju et al., 2003). hCNT1and rCNT1 are 83% identical in amino acid sequence (Ritzel et al., 1997).

CNT2

CNT2 is a Na^+-dependentpurine nucleoside selectivetransporter. hCNT2 and rCNT2 are 81% identicalin amino acid sequence, but differ in tissue distribution, permeant specificity, and regulation (Ritzel et al., 1998). rCNT2 is 659 amino acids in length and was identified byexpression cloning in rat jejunum, while hCNT2 was identified from the intestine through the use of RT-PCR homology cloning strategies (Wang et al., 1997; Ritzel et al., 1998). The human gene mapped to chromosome 15q15 (Ritzel et al., 1998). Ar/hCNT2 homolog from mouse, designated mCNT2, was also identified (Patel et al.,

1997), and functionally correspondsto a *cif*/N1-type Na^+-dependent nucleoside transporter. It transports the purine nucleosides adenosine, inosine and guanosine, as well as the pyrimidine nucleoside uridine (Figure1-5) (Yao et al., 1996; Ritzel et al., 1998).Transcripts for CNT2 are present in kidney, liver, heart, brain, placenta, pancreas, skeletal muscle, colon, rectum, duodenum, jejunum, and ileum (Ritzel et al., 1998).

CNT3

hCNT3 has a size of 77kDa and is 691 amino acids in length,and is 48% identical in amino acid sequence to hCNT1 and 47% identical to hCNT2 (Ritzel et al., 2001). Unlike hCNT1/2, the hCNT3 transporter can utilize both sodium and proton electrochemical gradients, with 2:1 and 1:1 (sodium: nucleoside and proton: nucleoside) coupling ratios, respectively. Na^+- and H^+- bound hCNT3 have different permeant selectivity profiles, suggesting different cation-induced conformational changes in the binding pocket(Ritzel et al., 2001; Smith et al., 2005). Na^+-coupled transport is broadly selective, while H^+-coupled transport is uridine-preferring(Figure 1-5) (Smith et al., 2005; Smith et al., 2009). mCNT3 KO mice are viable and fertile(unpublished data).

Nucleoside transport by hCNT3-producing *Xenopus* oocytes is concentrative, NBMPR-insensitive and Na^+-dependent, as seen by the two-electrode voltage-clamp technique (Ritzel et al., 2001). Radiolabeled pyrimidine and purine nucleosides, but not the nucleobases uracil and hypoxanthine,are transported by hCNT3 (Ritzel et al., 2001). hCNT3also transportsseveral nucleoside analogdrugs (Smith et al., 2005). High stringency hybridization studies found hCNT3 transcripts to be more ubiquitously expressed than those of hCNT1/2,with greatest abundance in mammary gland, pancreas, bone marrow, trachea, duodenum and lowest abundance in kidney, liver, lung, placenta, prostate, testis, brain and heart (Ritzel et al., 2001; Smith et al., 2005).

CNT Physiological Implications

The abundance of CNT1/2/3 in specialized epithelial cells of the intestine, kidney, and liver, suggests that these transporters may have a rolein systemic absorption, distribution

and excretion of physiologic nucleosides and nucleoside analogs (Young et al., 2013). For example, CNT3 is found in the kidney, and its 2:1 Na^+: nucleosidestoichiometry, allows it to have a greater ability to concentrate nucleosides in comparison to CNT1 and CNT2.The ability of CNT3 to mediate H^+- nucleoside cotransport may also be important in the acidic environment of the kidney (Elwi et al., 2006;Damaraju et al., 2007). CNT nucleoside transport activities in cancer cellsdiffer depending on the stage of development of neoplastic transformation and cell type (Damaraju et al., 2003). hCNT3,in comparison to other CNTs,is transcriptionally regulated and changes in expression levels may have roles in both physiological and pathophysiological conditions (Ritzel et al., 2001).

Adenosine is a universal permeant of CNT1/2/3, indicating that CNTs may havea role in regulating extracellular concentrations of adenosine available to purinergic receptors (Young et al., 2013). In so doing, CNTs may also play a key role in the intracellular metabolism of adenosine and adenosine analogs. Interactions of adenosine, adenosine analogs and adenosine receptor ligands with CNTs are kinetically specific to each transporter type and may be clinically relevant with respect to therapeutic manipulation of adenosine concentrations and metabolism. (Lang et al., 2004, Young et al., 2013).

ENT and CNT Mechanism(s) of Transport

Based upon the alternating-access model of transport (Widdas 1952), it is suggested that both ENTs and CNTs mediate transport of permeant by alternating between two conformations during the transport cycle, *i.e.*, an outward, extracellular-facing orientation or an inward, intracellular-facing orientation. Permeant binding to the transporters cause conformational changes which allow the release of the permeant to the opposite side of the membrane. In the absence of crystal structures for both inward facing and outward facing conformations, little is known about the mechanistic processes of transport for human and other mammalian ENT and CNT family members.Currently, the crystal structure of abacterial member of the CNT family, vcCNT, is available onlyin its inward-facing conformation (Johnson et al., 2012; Johnson et al, 2014). 3-D homology models

for hENT1 and hCNT3 are presented in Figures 1-6 and 1-7, the formerbased on the structure of the*Escherichia coli* glycerol 3-phosphate/phosphate exchange transporter GlpT (Lemieux et al., 2003), and the latter based upon vcCNT(Johnson et al., 2012; Johnson et al, 2014). CNTs and some ENTs transport nucleosides across cell membranes by coupling with a cation. This cation in ENTs is H^+, whereas it can be Na^+ and/or H^+ in CNTs (Young et al.,2013).

Research Objectives

Project 1:

Over the past 25 years, there has accumulated a large body of work studying properties of recombinant nucleoside transporters produced in oocytes of *Xenopuslaevis*. Oocytes lack endogenous nucleoside transport activity. To date, however, there is essentially no information on the metabolism of nucleosides in oocytes, and how this might potentially impact findings of influx or efflux nucleoside transport experiments. The objective of Project 1 was to fill in this knowledge gap by determining the extent of intracellular nucleoside metabolism under conditions typical of nucleoside transport assays. Similar to human erythrocytes, our hypothesis was that nucleosides are subject to only limited metabolism in oocytes.

In humans, there are three members of the ubiquitous cation-coupled concentrative nucleoside transporter (CNT) family, hCNT1, hCNT2 and hCNT3. hCNT1 is selective for pyrimidine nucleosides but also transports adenosine, hCNT2 transports purine nucleosides and uridine, and hCNT3 transports both purine and pyrimidine nucleosides. By producing recombinant hCNT3 in *Xenopus* oocytes, we used radiochemical HPLC analysis to investigate the metabolic fate of transported radiolabeled [^{3}H] and [^{14}C] purine and pyrimidine nucleosides once inside the cell. We also used radiochemical HPLC analysis to investigate the efflux of internalized [^{3}H]uridine from oocytes producing hCNT1, and effects extracellular nonradioactive adenosine and uridine upon that efflux.

Project 2:

mENT1, mENT2 are murine members of the equilibrative nucleoside transporter family (ENT). Knockout mice have been produced with homozygous (-/-) disruption of the genes mENT1, mENT2, and mENT1 plus mENT2. Project 2 had the objective of using these miceto assess the contributions of mENT1 and mENT2 to nucleoside and nucleobase homeostasis.

In our experiments we used high performance liquid chromatography (HPLC) analysis to investigate differences in plasma levels of nucleosides and nucleobases in wild-type (WT) and in mENT1, mENT2 and in mENT1 plus mENT2 knockout (KO) mice. Our hypothesis was that the plasma levels of different nucleosides and nucleobases in these KO mice differ in comparison with normal wild-type (WT) animals.

Table 1-1. Human and mouseequilibrative nucleoside transporter (ENT) family members.

Species	Protein	Amino Acids	Permeant Selectivity	Transport Mode	Reference
Homo sapiens	hENT1	456	nucleosides	facilitative	Griffiths et al., 1997a
	hENT2	456	nucleosides; nucleobases	facilitative	Griffiths et al., 1997b
	hENT3	475	nucleosides; adenine	H^+-dependent	Baldwin et al., 2005
	hENT4	530	adenosine; monoamines; MPP^+	H^+-dependent	Barnes et al., 2006
Mus musculus	mENT1	458	nucleosides	facilitative	Choi et al, 2000
	mENT1.1	460	nucleosides	facilitative	Handa et al., 2001
	mENT1.2	458	nucleosides	facilitative	Bone et al., 2007
	mENT2	456	nucleosides; nucleobases	facilitative	Kiss et al, 2000
	mENT3	475	nd	nd	Baldwin et al, 2005
	mENT4	528	adenosine; adenine; monoamines	H^+-dependent	Barnes et al, 2006

nd, not determined.

Table 1-2. Human and mouse concentrative nucleoside transporter (CNT) family members.

Species	Protein	Amino Acids	Permeant Selectivity	Transport Mode	Reference
Homo sapiens	hCNT1	649	pyrimidine nucleosides; adenosine	Na^+-dependent	Ritzel et al., 1997
	hCNT2	658	purine nucleosides; uridine	Na^+-dependent	Ritzel et al., 1998
	hCNT3	691	nucleosides	Na^+/H^+-dependent	Ritzel et al., 2001
Mus musculus	mCNT1	648	pyrimidine nucleosides	Na^+-dependent	Niitani et al., 2010
	mCNT2	660	purine nucleosides; uridine	Na^+-dependent	Patel et al., 1997
	mCNT3	703	nucleosides	Na^+/H^+-dependent	Ritzel et al., 2001

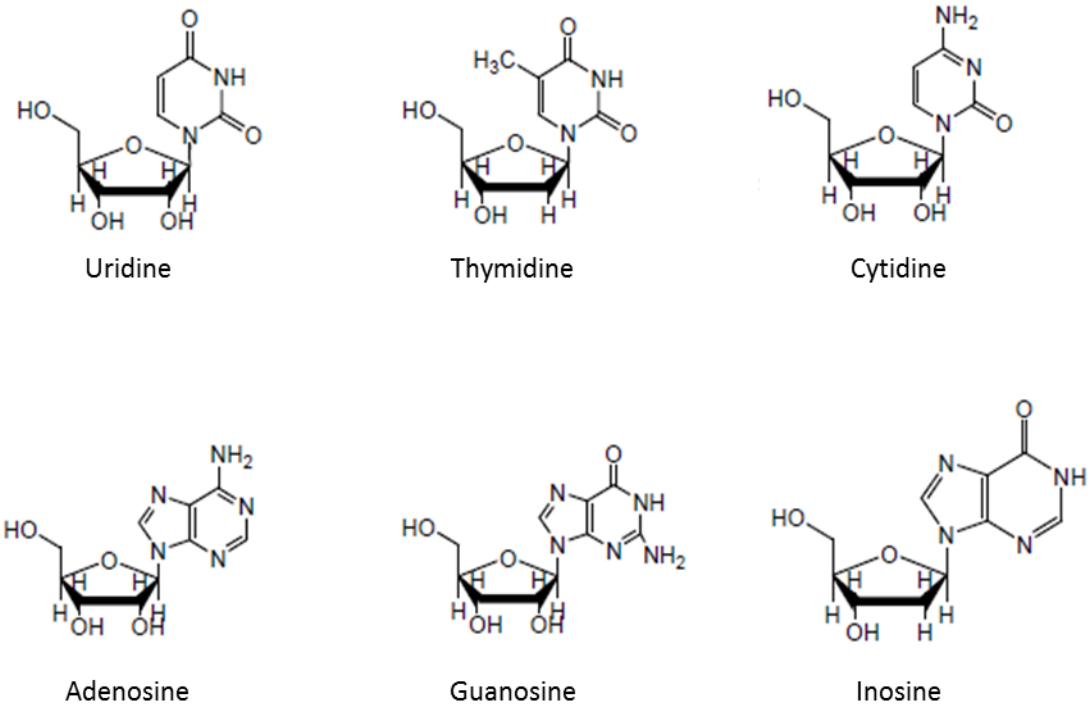


Figure 1-1. Chemical structures of physiological pyrimidine and purine nucleosides.

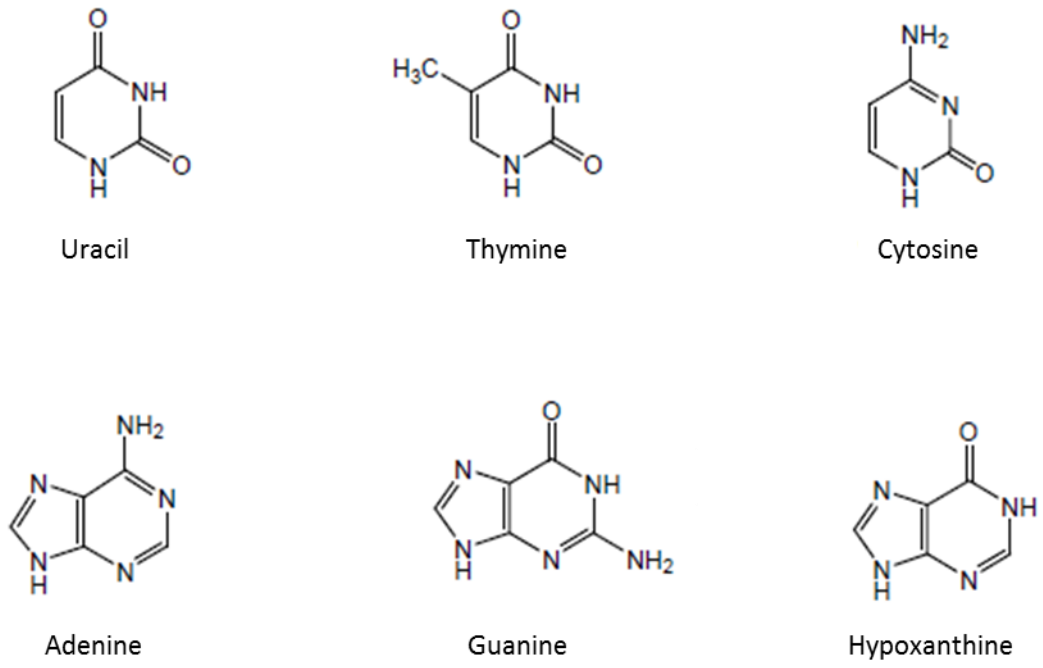


Figure 1-2. Chemical structures of physiological pyrimidine and purine nucleobases.

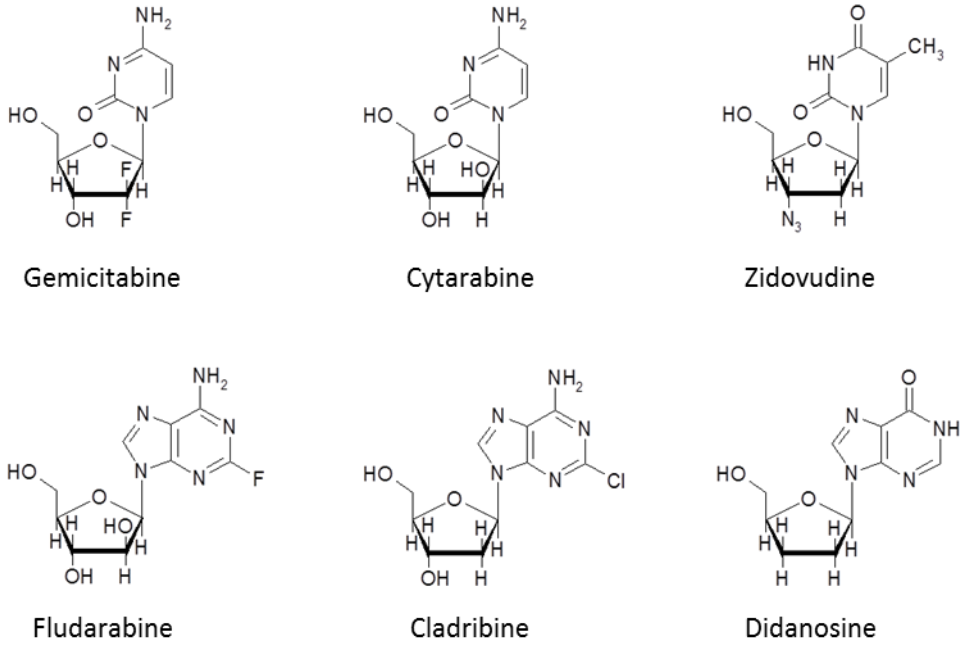
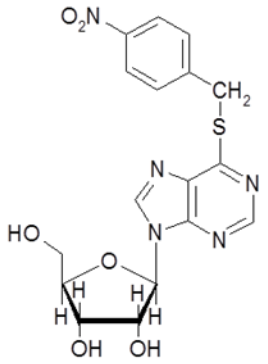
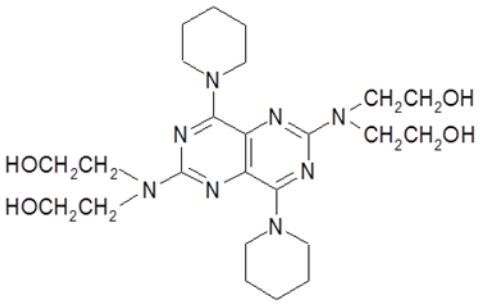


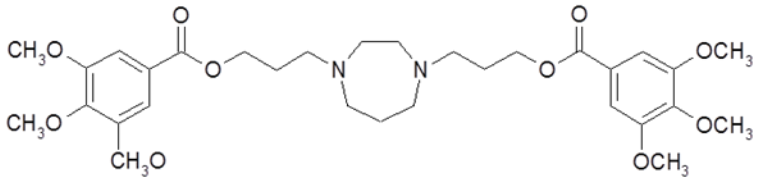
Figure 1-3. Chemical structures of therapeutic nucleoside analogs.



NBMPR



Dipyridamole



Dilazep

Figure 1-4. Chemical structures of ENT inhibitors

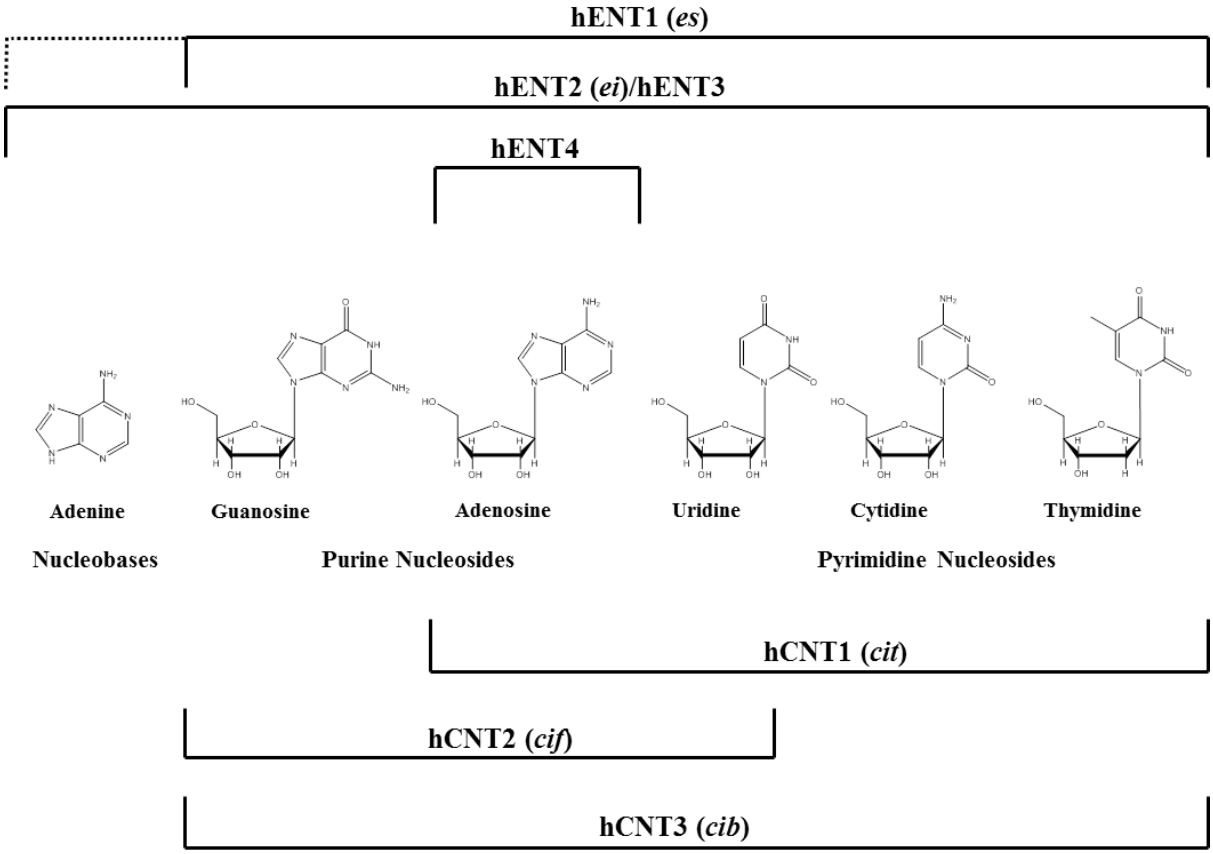


Figure 1-5. Permeant selectivities of human CNT and ENT family members. Modified from(Young et al., 2013)

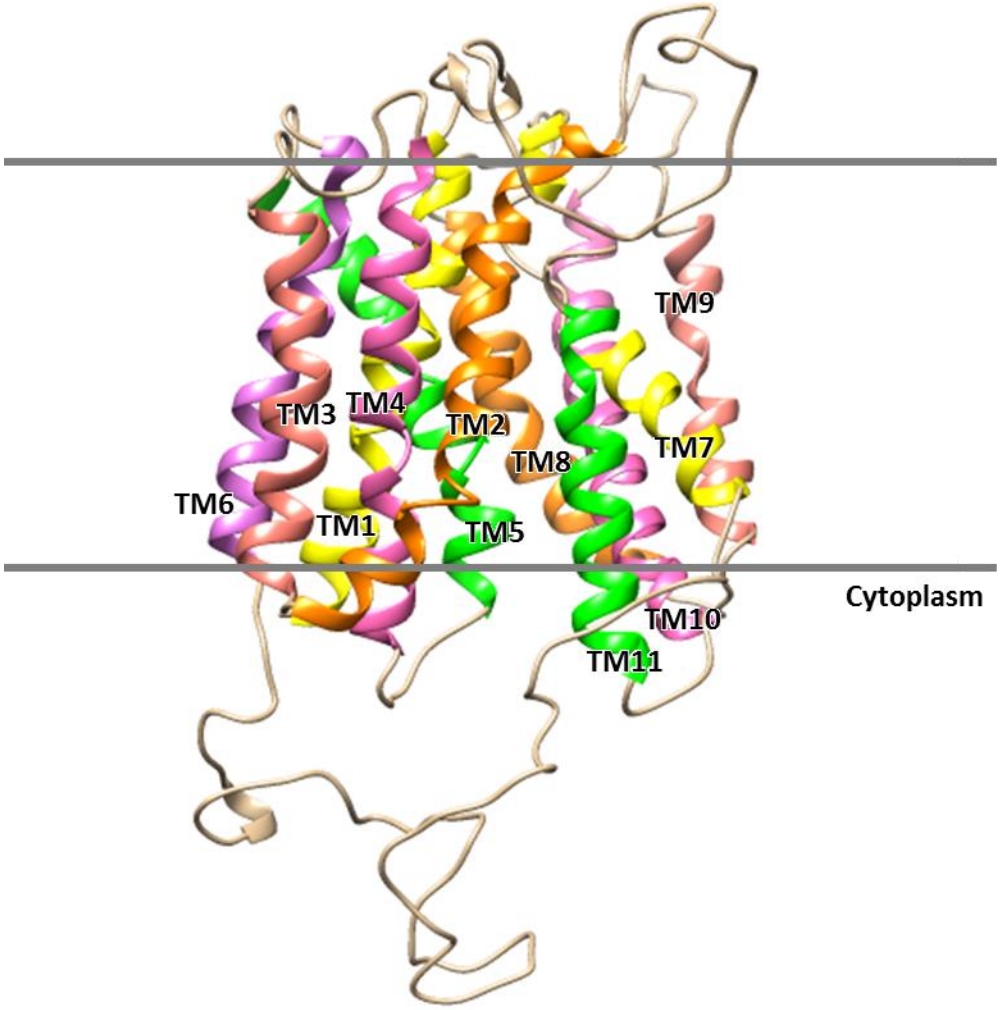


Figure 1-6. Homology model of hENT1. Predicted structural topology of hENT1 based on the structure of *Escherichia coli* glycerol 3-phosphate/phosphate exchange transporter GlpT (Lemieux et al., 2003),viewed from within the plane of the lipid bilayer.

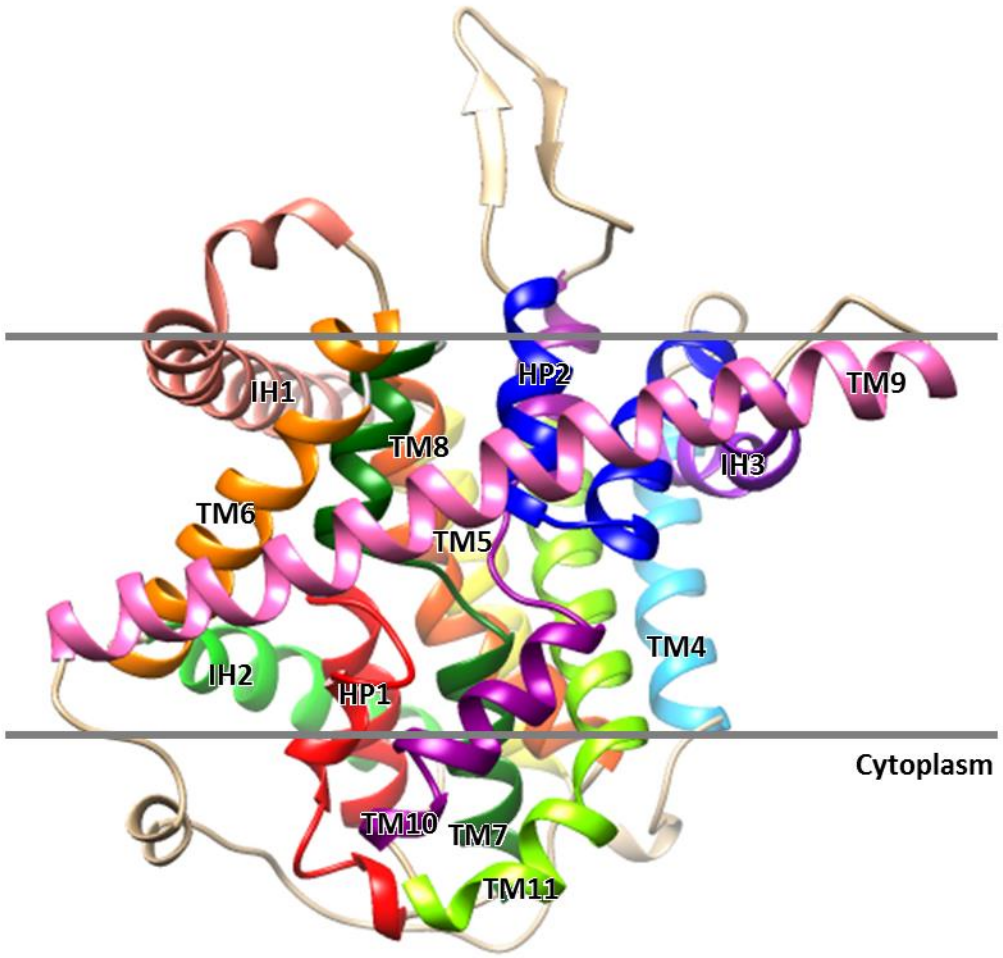


Figure 1-7. Homology model of hCNT3. Predicted structural toplogy of hCNT3 based upon that of its counterpart from *Vibrio cholera* (Johnson et al., 2012), viewed from within the plane of the lipid bilayer.

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Chapter 2:

Materials and Methods

Project 1:

*Xenopus laevis*Oocytes as a Heterologous Expression System:

The *Xenopus laevis* oocyte heterologous expression system was fundamental to the initial discovery and subsequent characterization of human CNT proteins. The key advantage of *Xenopus* oocytes over other heterologous expression systems is that they lack, or have insignificant, endogenous nucleoside transport activity (Jarvis & Griffith, 1991; Huang et al., 1994; Wang et al., 1997; Sigel & Minier, 2005). Oocytes therefore provide a powerful experimental vehicle to produce and functionally characterize recombinant nucleoside transport proteins in the absence of other competing transport processes with potentially overlapping permeant preferences (Yao et al., 2000). In radiolabeled flux experiments, the slow non-mediated uptake of nucleosides that occurs in oocytes by simple diffusion across the lipid bilayer is readily quantified by measuring uptake in control water-injected oocytes. The cDNAs encoding human and rodent CNT1, CNT2 and CNT3 were all initially cloned and characterized in *Xenopus* oocytes (Huang et al., 1994; Che et al., 1995; Yao et al., 1996a; Yao et a., 1996b; Ritzel et al., 1997; Ritzel et al., 1998; Ritzel et al., 2001). In our experiments, we used oocytes producing recombinant hCNT3 to investigate oocyte metabolism of transported nucleosides.

In vitro Transcription and Expression in *Xenopus* Oocytes:

Plasmid cDNAs of hCNT1 and hCNT3 (GenBank™ accession numbers U62968, AF305210) were linearized with *Nhe1*. Following linearization, cDNA was transcribed with T7 polymerase using the mMESSAGE mMACHINE^{TM} (Ambion, USA) *in vitro* transcription system. Template DNA was removed by digestion with RNase-free DNase1. Healthy stage V - VI oocytes were isolated by collagenase treatment (2 mg/ml for 2 hours) of ovarian lobes from female *Xenopus laevis* (Biological Sciences Vivarium, University of Alberta, Canada) that had been anaesthetized by immersion in 0.3% (w/v) tricaine methanesulfonate (pH 7.4). Frogs were humanely sacrificed following collection of oocytes in compliance with guidelines approved by the Canadian Council on Animal Care. The remaining follicular layers were removed by phosphate treatment (100 mM

K_2PO_4) and manual defolliculation. Twenty-four hours after defolliculation, oocytes were injected with either 10 nl of water containing 1 ng/nl of capped RNA transcript or the same volume of water alone. Injected oocytes were then incubated for 4 days at 18°C in modified Barth's solution (changed daily) (88 mM NaCl, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 10 mM Hepes, 2.5 mM sodium pyruvate, 0.1 mg/ml penicillin and 0.05 mg/ml gentamycin sulfate, pH 7.5) prior to the assay of nucleoside transport activity and oocyte extraction.

Radioisotope Flux Studies:

Radioisotope transport assays were performed in groups of 20 oocytes at 20°C using ^{3}H-or^{14}C-labeled nucleosides (2.5 μCi/ml or 1 μCi/ml, respectively) in 200 μl transport medium containing 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES, pH 7.5. Nucleoside uptake was determined at a concentration of 20 μM. Radioactive nucleosides were obtained from Moravek Biochemicals (Brea, California). Transport medium for adenosine uptake experiments also contained 1 μM deoxycoformycin to inhibit adenosine deaminase activity. Following either 1-min or 30-min incubations, extracellular radioactivity was removed by six rapid washes in ice-cold Na^+-free choline chloride transport medium (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES pH 7.5). Individual oocytes were then dissolved in 1% (w/v) SDS solution for quantification of oocyte-associated radioactivity by liquid scintillation counting (LS 6000IC, Beckman Canada Inc., Canada). In adenosine uptake experiments, oocytes were pretreated (30 min at 20°C) with 1 μM deoxycoformycin to inhibit adenosine deaminase activity. The deoxycoformycin was a generous gift from Dr. W. Gati, Department of Pharmacology, University of Alberta.

For efflux studies, groups of 20 hCNT RNA-injected oocytes were preloaded with 10 μM [^{3}H] uridine (2.5 μCi/ml) in 0.2 ml of NaCl transport medium at 20° for 30min, followed by six rapid ice-cold washes in NaCl transport medium to remove extracellular ^{3}H. One group of 20 oocytes was processed to determine the time-zero [^{3}H] uridine content (typically 50-60 pmol/oocyte). Other groups of 20 oocytes were resuspended in 1 mM of NaCl transport medium (± 1 mM nonradioactive nucleoside) at 20° to initiate

efflux. At predetermined time intervals, duplicate 10-µl samples of incubation medium were removed and counted for ^{3}H.

Preparation of Oocyte Extracts for HPLC:

Twentyoocytes were collected after radioisotope flux studies and homogenized together in 500µl of ice-cold hypotonic buffer (7.5 mM HPO_4, 1 mM EDTA, pH 7.4) and subsequently deproteinized by adding 30µl ice-cold 70% w/w perchloric acid, prior to centrifugation for 15 min at 1500 *g*, 4°C (Chantin et al., 1996). The resulting supernatant was extracted with an equal volume of ice-cold chloroform. The supernatant was then neutralized to a pH of 6 to 7 with 5 M NaOH solution and stored at -80°C until HPLC analysis (Agilent 1260 Infinity system, Agilent Technologies Canada Inc., Canada) and radioactivity detection using a Beta-RAM radio HPLC detector (Primer Biotect, Canada).

High Performance Liquid Chromatography (HPLC):

Analysis of radiolabelled purine and pyrimidine nucleosides was performed on an HPLC Agilent 1260 Infinity series system equipped with a quaternary pump with a built-in 4-channel degassing unit, an autosampler, a column temperature controller and a variable wavelength detector connected to an Agilent Open Lab running Chemstation software. Eleven nucleosides and nucleobases (adenine, thymidine, guanosine, inosine, adenine, thymine, uridine, hypoxanthine, cytidine, uracil, and cytosine) were separated and analyzed by using an Agilent Eclipse Plus C18 column (250mm × 4.6mm, 5µm) and an Eclipse Plus C18 guard column (12.5mm × 4.6 mm, 5µm) at 22°C. The mobile phase was composed of water (A) and acetonitrile (B) with a gradient program as follows: 0-5min, isocratic 1% B; 5-15min, linear gradient 1-3% B; 15-25min, linear gradient 3-6% B; 25-30min, linear gradient 6-15% B; 30-35min, linear gradient 15-1% B; 35-50min, isocratic 1% B (Cao et al., 2010). The flow rate was kept constant at 0.8ml/min and the injection volume was 30µl. The variable wavelength detector was set at 254nm. All injections were repeated three times. Identification of nucleosides and nucleobases was determined by comparing their retention times and UV spectra against known of known standards.

Radioactivity detection was performed on a Beta-RAM Model 5 radio HPLC detector equipped with a 500µl cell volume radiochemical liquid cell at a scintillant flow rate of 1-2 ml/min.

Figure 2-1 shows a representative HPLC chromatogram for separation of nucleoside and nucleobase standards with a 20 min pre-equilibration time resulting in retention times of 4.3 min (cytosine), 5.2 min (uracil), 6.4min (cytidine), 7.2 min (hypoxanthine), 8.9min (uridine), 10.9min (thymine), 14.6 min (adenine) 18.0min (inosine), 19.3 min (guanosine), 24.4min (thymidine), and 29.2min (adenosine). The limit of detection was about 0.1µg/ml for both the nucleosides and nucleobases tested.

Project 2:

Animal Models:

Mice that were heterozygous for a disruption in either the mENT1 or mENT2 genes were purchased from Lexicon Pharmaceuticals, Inc. (The Woodlands, Texas). The heterozygotes were backcrossed for 6 generations into FVB/N mice (Taconic, Hudson, New York), and the resulting FVB/N mENT1 and FVB/N mENT2 heterozygous mice were then crossed to derive the mENT1homozygous knockout strain and the mENT2 homozygous knockout strain. mENT1 homozygous knockout mice were then crossed with mENT2 homozygous knockout mice to generate an mENT1/mENT2 double homozygous knockout strain. These fully backcrossed mENT1 knockout mice, mENT2 knockout mice, and mENT1/mENT2 double knockout mice,which were backcrossed again into FVB/N every 30 generations to maintain genetic homogeneity, were used throughout this study (Graham et al., 2011). FVB/N mice, also from Taconic, were used as wild-type controls. Mice of 4–12 months in age were used. Animals were housed in ventilated cages in a barrier-maintained facility at the Cross Cancer Institute. Animal work in this study was approved by the Animal Care Committee of the Cross Cancer Institute, a facility of Alberta Health Services, Cancer Care, and all animals were handled in accordance with the regulations of the Canadian Council on Animal Care.

Preparation of Mouse Plasma:

Mouse blood samples were collected into 1.5-ml Eppendorf micro-centrifuge tubes containing heparin (10,000 USP units/ml), NBMPR (500 nM), and 2'-deoxycoformycin (500 nM) in 50 µl sterile distilled H_2O (Young et al., 1986). The tubes were gently mixed and 600-800µl of blood immediately transferred to another set of 1.5-ml Eppendorf micro-centrifuge tubes on ice. This was immediately followed by centrifugation at 15600x*g*for 2min at 4°C. Plasma was then removed and rapidly deproteinized with 70% w/w perchloric acid to reach a final concentration of 4%. The deproteinized samples were centrifuged at 15600x*g* for 2 min at 4°C. The resulting supernatants were removed and neutralized with 5M sodium hydroxide to a pH of 6-7. The extracts were stored at -80°C until HPLC analysis.

High Performance Liquid Chromatography (HPLC):

The analysis of mouse plasma for the detection of purine and pyrimidine nucleosides was performed on the same HPLC Agilent 1260 infinity series system described for Project 1. The flow rate was kept constant at 0.8ml/min with an injection volume of 15µl. All analyses were performed in triplicate.

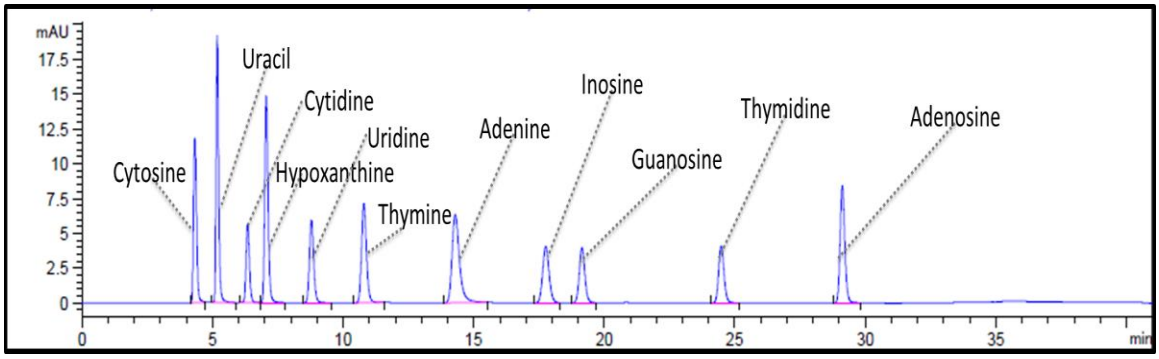

Figure 2-1. A representativeHPLC chromatogram of mixed nucleoside and nucleobase standards. The *y*-axis is UV absorbance (in mAU) and the *x*-axis is retention time (in min). Individual retention times and other experimental details are provided in the text.

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Chapter 3:

Metabolism of Radiolabelled Nucleosides in *Xenopus laevis* Oocytes

Introduction

Nucleosides and nucleobases are important physiological molecules that play essential roles in intermediary metabolism, biosynthesis of macromolecules and cell signaling through interaction with purinergic receptors (Murray, 1977; Berne et al., 1983; Mubagwa & Flameng, 2001; Masino& Boison, 2013). Naturally occurring nucleosides include the purine nucleosides adenosine, guanosine and inosine, and the pyrimidine nucleosides uridine, cytidine and thymidine. Naturally occurring nucleobases include uracil, adenine, cytosine, thymine and hypoxanthine.*Xenopus*oocytes are known to contain the following enzymes by which nucleosides and their corresponding nucleotides are metabolized; adenosine deaminase, nucleoside phophorylase, cytidine deaminase, adenosine kinase, PNP (purine nucleoside phophorylase), cytidine kinase, thymidine kinase, thymidine phophorylase, nucleoside monophosphate kinase, nucleoside diphosphate kinase. (Woodland & Pestell, 1971; Buczynski & Potter, 1990; Saccomanno & Bass, 1994; Riegelhaupt et al., 2010). The nucleoside adenosine is deaminated by the enzyme adenosine deaminase into inosine (Figure 3-1). The ribose from inosine, and guanosine is removed by the enzyme purine nucleoside phosphorylase (PNP), yielding ribose-1-phosphate and the nucleobases hypoxanthine and guanine, respectively (Figure 3-1). Both hypoxanthine and guanine can be phosphoribosylized back to inosine and guanosine, respectively, by the enzyme purine nucleoside phosphorylase (Figure 3-1). Salvage pathway rescue *via* hypoxanthine-guanine phosphoribosyltransferase is another possibility. For pyrimidine nucleosides, cytidine is first deaminated to uridine by the enzyme cytidine deaminase (Figure 3-2). Uridine and thymidine are then converted to uracil and thymine, respectively, by pyrimidine nucleoside phosphorylase (Figure 3-2). Subsequent reactions convert uracil and thymine into β-alanine andβ-aminoisobutyrate, respectively (Figure 3-2). Purine and pyrimidine nucleosides can also be phosphorylated by specific kinases in the cell to produce nucleotides.

Since most nucleosides are hydrophilic in nature, they require specialized nucleoside transporter proteins for passage across cell membranes. Humans possess three members of the concentrative nucleoside transporter family, hCNT1, hCNT2, hCNT3. hCNT1 transports pyrimidine nucleosides and adenosine, hCNT2 transports purine

nucleosides and uridine, and hCNT3 transports both purine and pyrimidine nucleosides (Young et al., 2013). hCNT1/2 transport nucleosides using the transmembrane Na^+ electrochemical gradient, while hCNT3 is both Na^+- and H^+-coupled (Young et al., 2013).

Xenopus laevis oocytesrepresent a heterologous expression system used to functionally characterize human and other mammalian CNT (and ENT) nucleoside transport proteins (Young et al, 2013). It was the expression system used in my first project.*Xenopus* oocytes are effective in translating exogenous mRNA and are capable of post-translational protein modifications(Yao et al., 2000). The cDNAs encoding human and rodent CNT1, CNT2 and CNT3 were all initially cloned and characterized in *Xenopus* oocytes (Young et al., 2013 and Young, 2016). The main advantage of *Xenopus* oocytes is that they lack, or have insignificant, endogenous nucleoside transport activities (Yao et al., 2000; Parker et al., 2000; Yao et al., 1996). Their large size (1.0-1.3mm in diameter) and correspondingly large cytoplasmic volume (~1µl) is also of an advantage, allowing transport equilibria to occur at longer time intervals compared to smaller cells (Yao et al., 2000). These spherical oocytes consist of a dark brown-colored animal pole, beneath which lies the nucleus, and a light beige-colored vegetal pole (Yao et al., 2000).

To study the metabolic fate of transported nucleosides in hCNT3-producing oocytes, and how this might potentially impact the findings of influx and efflux nucleoside transport experiments, we used radiochemical HPLC analysis to investigate the intracellular metabolism of transported radiolabelled [^{3}H] and [^{14}C] purine and pyrimidine nucleosides in oocytes.

In addition to the hCNTs, the *Xenopus laevis* oocyte expression system was also used to clone a cDNA encoding the pyrimidine-selective nucleoside transporter rCNT1 from rat jejunum (Huang et al., 1994). In a previous study, it was found that rCNT1 mediates the efflux of [^{3}H]uridine from preloaded oocytes, demonstrating a capacity for bidirectional transport of nucleoside permeants (Yao et al., 1996). Uridine efflux was also stimulated by extracellular uridine but inhibited by extracellular adenosine, suggesting that the rate of conversion of rCNT1 from outward-facing conformation to its inward-

facing conformation was increased when the transporter was complexed with uridine, but decreased when it was complexed with adenosine. Thus, although rCNT1 binds adenosine and uridine with similar affinities, it kinetically favors the transport of uridine (Yao et al., 1996). Therefore, we also used radiochemical HPLC analysis to study the mechanism by which adenosine functions as an atypical low K_m, low V_{max} permeant of the corresponding human pyrimidine nucleoside-selective transporter, hCNT1. Oocytes producing hCNT1 were preloaded with [^{3}H]uridine, after which efflux of accumulated radioactivity was measured in transport medium alone, or in the presence of extracellular non-radiolabelled adenosine or uridine.

Results

We used radiochemical HPLC analysis to investigate the intracellular metabolic fate of transported radiolabelled [^{3}H] or [^{14}C] pyrimidine and purine nucleosides inside *Xenopus* oocytes producing recombinant hCNT3 after 1-min or 30-min incubation periods with medium containing individual 20 μM pyrimidine or purine nucleosides.

Radiochemical HPLC Analysis of IntracellularTransported Nucleosides in hCNT3-Producing Oocytes.

Uridine – Figure 3-3 presents a representative HPLC chromatogram of radiochemical analysis of intracellular [^{3}H]uridine after 1-min or 30-min incubations with 20 μM [^{3}H]uridine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min, 100% of radioactivity was uridine. After 30 min, 93% of radioactivity was uridine, and 3% of radioactivity was nucleotides (values calculated as percentages of the total radioactivity detected in the sample).

Adenosine – Figure 3-4 presents a representative HPLC chromatograms of radiochemical analysis of intracellular [^{14}C]adenosine after 1-min or 30-min incubations with 20 μM [^{14}C]adenosine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min,

100% of radioactivity was adenosine. After 30 min, 49% of radioactivity was adenosine, 38% of radioactivity was nucleotides, 8% of radioactivity was adenine and 5% of radioactivity was inosine.

Cytidine – Figure 3-5 presents a representative HPLC chromatograms of radiochemical analysis of intracellular [^{14}C]cytidine after 1-min or 30-min incubation with 20 µM [^{14}C]cytidine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min, 100% of radioactivity was cytidine. After 30 min, 100% of radioactivity was cytidine.

Inosine – Figure 3-6 presents a representative HPLC chromatograms of radiochemical analysis of intracellular [^{14}C]inosine after 1-min or 30-min incubation with 20 µM [^{14}C]inosine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min, 100% of radioactivity was inosine. After 30 min, 84% of radioactivity was inosine; 9% of radioactivity was nucleotides and 6% of radioactivity was hypoxanthine.

Thymidine – Figure 3-7 presents a representative HPLC chromatograms of radiochemical analysis of intracellular [^{14}C]thymidine after 1-min or 30-min incubation with 20 µM [^{14}C]thymidine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min, 100% of radioactivity was thymidine. After 30 min, 100% of radioactivity was thymidine.

Guanosine – Figure 3-8 presents a representative HPLC chromatograms of radiochemical analysis of intracellular [^{14}C]guanosine after 1-min or 30-min incubation with 20 µM [^{14}C]guanosine in hCNT3-producing *Xenopus* oocytes. Results showed that after 1 min, 100% of radioactivity was guanosine. After 30 min, 100% of radioactivity was guanosine.

Our findings suggest that transported nucleosides by hCNT3 in oocytes after 1-min incubation were not subject to significant metabolism. After 30-min, transported nucleosides were generally subject to minimal intracellular metabolism (uridine 7%, cytidine 0%, thymidine 0%, inosine 16% and guanosine 0%),with the exception being

adenosine, for which only 49% remained unmetabolized after 30-min incubation (Figures 3-3 – 3-8).

Radiochemical HPLCAnalysis of IntracellularTransported Nucleosides in hCNT1-Producing Oocytes.

We used radiochemical HPLC analysis to study the mechanism by which adenosine functions as an atypical low K_m, low V_{max} permeant of pyrimidine nucleoside-selective hCNT1. Oocytes producing recombinant hCNT1 were pre-loaded with 10 μM [^{3}H]uridine, after which efflux of accumulated radioactivity was measured in transport medium alone, or in the presence of extracellular non-radiolabelled adenosine or uridine (Figure 3-9). Efflux of intracellular [^{3}H]uridine against the Na^+ electrochemical gradient in transport medium alone was relatively slow (a 23% decrease in 60 min). The addition of 1 mM non-radiolabelled uridine to the extracellular medium resulted in a large increase in uridine efflux (a 63% decrease in 60 min). In marked contrast to the *trans*-stimulation seen with uridine, 1 mM extracellular adenosine (1 mM) caused a substantial *trans*-inhibition of uridine efflux (a 9% decrease in 60 min).

Wealso used radiochemical HPLC analysis to investigate both the intracellular and extracellular metabolic fate of preloaded radiolabelled [^{3}H]uridine after 60 min efflux in transport medium alone, or in the presence of extracellular non-radiolabelled adenosine or uridine (Figure 3-10). Our results again found that hCNT1-mediated [^{3}H]-efflux was stimulated by extracellular uridine, but inhibited by extracellular adenosine (extracellular radioactivity in transport medium alone, or in the presence of either extracellular non-radiolabelled adenosine or uridinewere 3958 dpm, 12068 dpm and 1200 dpm, respectively, in a sample volume of 50 μl) (Figures 3-9 and 3-10; Table 3-1). Almost all (97%)of the radioactivity exiting cells when stimulated by extracellular non-radiolabelled uridine wasunmetabolized radiolabelled uridine (Table 3-1).

Discussion

A previous study used thin layer chromatography (TLC) to show that in *Xenopus* oocytes producing rat Na^+-dependent nucleoside transporters preloaded by 30 min incubation with 10 μM [^{3}H]uridine, the majority of the accumulated intracellular radioactivity remained as unmetabolized uridine (uridine, 77%; UTP/UDP, 21%; UMP, 1% and uracil, 1%) (Huang et al., 1993). In the present HPLC study, we have confirmed that transported uridine and other radiolabelled purine and pyrimidine nucleosides by hCNT3 in oocytes after 1 min incubation were not subject to significant metabolism. This is the incubation period used in most hCNT kinetic experiments. After 30 min, however, there was detectable metabolism of uridine (7%) and inosine (16%), and substantial metabolism of adenosine (51%). There was little if any metabolism of cytidine, thymidine or guanosine. In most of our human and other nucleoside transport studies using *Xenopus* oocytes as the heterologous expression system, initial rates of transport are determined for CNTs and ENTs using 1-min and 5-min uptake intervals, respectively. Thus our HPLC analysis of nucleoside metabolism in *Xenopus* oocytes confirmed that minimal nucleoside metabolism occurs within the incubation periods typically used in initial rate determinations for both ENTs and CNTs.

Our results also showed that recombinant hCNT1-mediated efflux of [^{3}H]uridine from preloaded oocytes was stimulated by extracellular uridine and inhibited by extracellular adenosine, with > 95% of the radioactivity exiting cells being unmetabolized radiolabelled uridine. Thus, the rate of conversion of hCNT1 from its outward-facing conformation to its inward-facing conformation was increased when the transporter was complexed with uridine and decreased when it was complexed with adenosine, providing confirmationthat adenosine kinetically functions as a low K_m, low V_{max} hCNT1 permeant.

In the efflux study by hCNT1-producing oocytes, we not only confirmed previous findings with rCNT1 that extracellular adenosine inhibited uridine efflux (Yao et al., 1996) but, in addition, established that the radioactivity exiting the cells wasstill present chemicallyasuridine, either in the absence or presence of extracellular uridine or adenosine.

Efflux condition	Extracellular	Radioactivity (dpm)	% of total radioactivity
NaCl transport buffer	Uridine	3958 ± 151	93
	Nucleotides	320 ± 33	7
1 mM uridine	Uridine	12068 ± 200	97
	Nucleotides	388 ± 36	3
1 mM adenosine	Uridine	1200 ± 68	74
	Nucleotides	412 ± 55	26

Table 3-1. **Summary of radiochemical HPLC analysis of extracellular [^{3}H]uridine in hCNT1-producing oocytes.** Sample volume was 30 µl, and values are means ± SEM (*n* = 3)following60-minefflux from groups of 20 [^{3}H]uridine-containingoocyteseither in the absence or in the presence of extracellular nonradioactive uridine or adenosine.Please see text for other experimental details.

Efflux condition	Intracellular	Radioactivity (dpm)	% of total radioactivity
NaCl transport buffer	Uridine	5300 ± 179	77
	Nucleotides	1562 ± 70	23
1 mM uridine	Uridine	496 ± 45	34
	Nucleotides	954 ± 101	66
1 mM adenosine	Uridine	6446 ± 176	76
	Nucleotides	2034 ± 125	24

Table 3-2. **Summary of radiochemical HPLC analysis of intracellular [^{3}H]uridine in hCNT1-producing oocytes.**Sample volume was 30 µl, and values are means ± SEM (*n* = 3) following 60-min efflux of groups of 20 [^{3}H]uridine-containing oocytes either in the absence or in the presence of extracellular nonradioactive uridine or adenosine.Please see text for other experimental details.

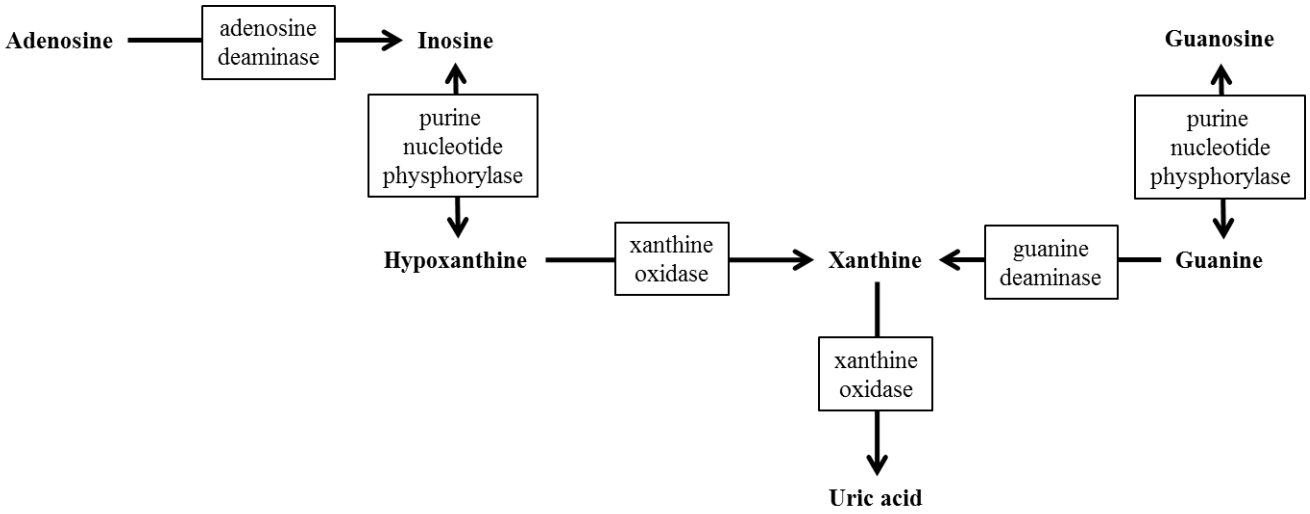


Figure 3-1. Pathways for the catabolism of purine nucleosides.

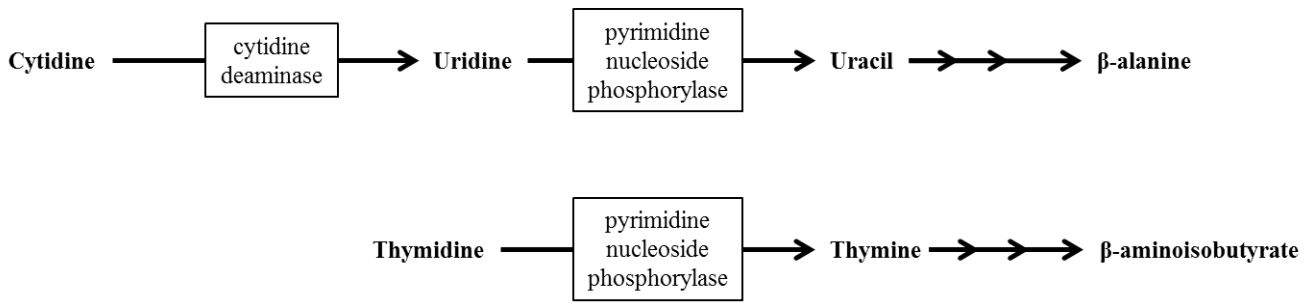
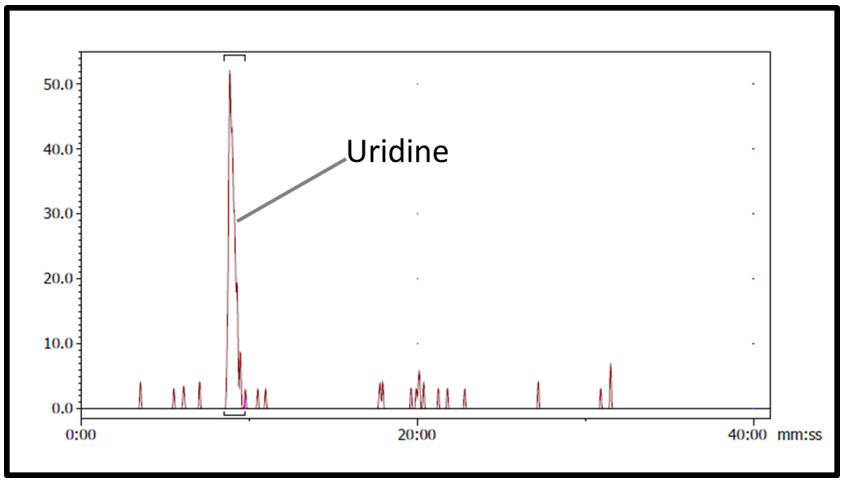


Figure 3-2. Pathways for the catabolism of pyrimidine nucleosides.

(A)



(B)

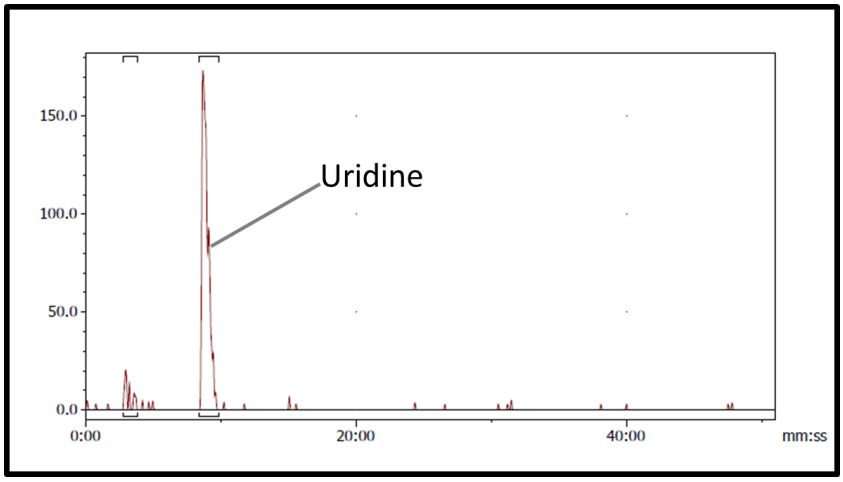
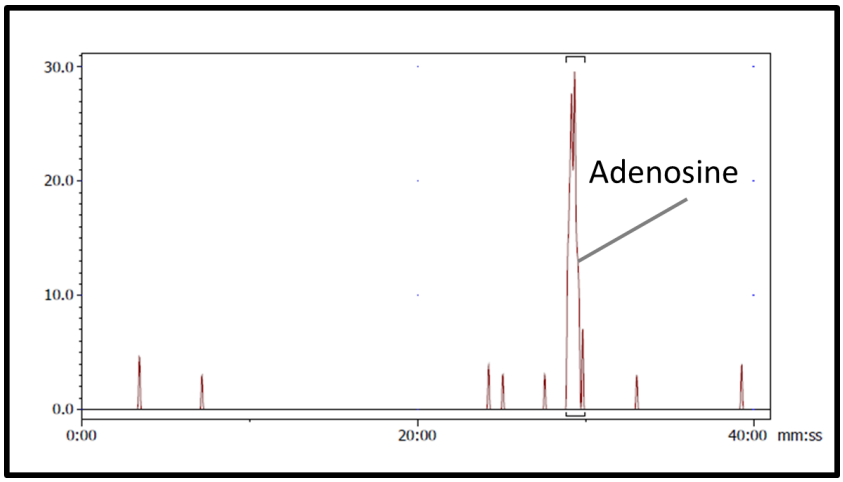


Figure 3-3.Radiochemical HPLC analysis of intracellular [^{3}H]uridine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivityin hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 µM [^{14}C]uridine.Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

(A)



(B)

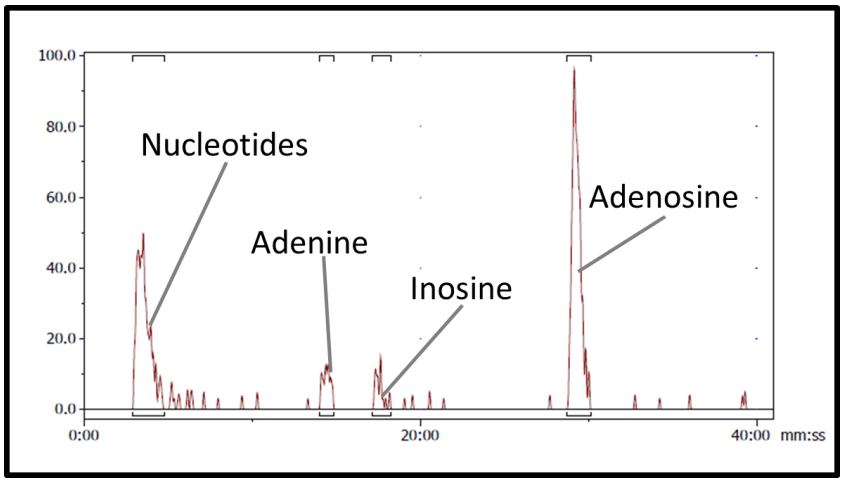
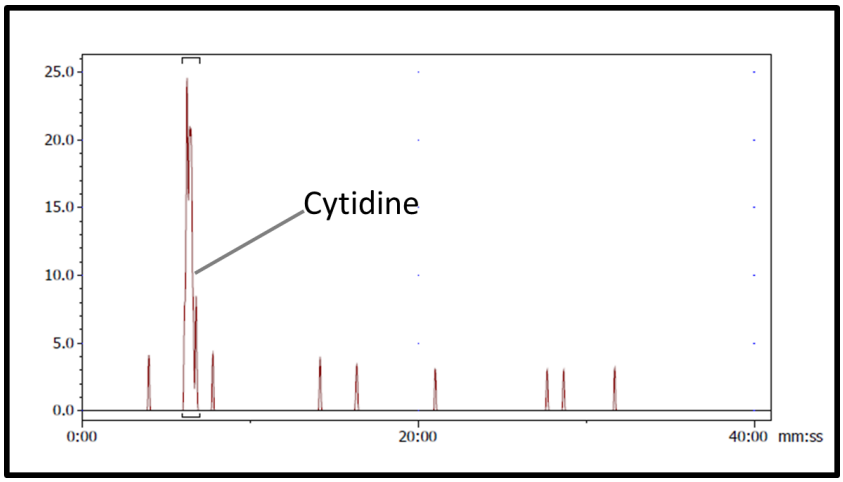


Figure3-4.Radiochemical HPLC analysis of intracellular [^{14}C]adenosine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivity in hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 µM [^{14}C]adenosine. Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

(A)



(B)

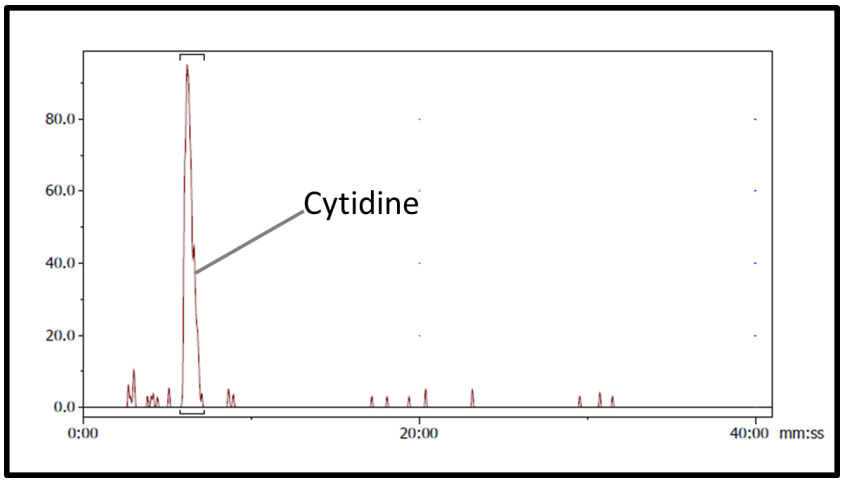
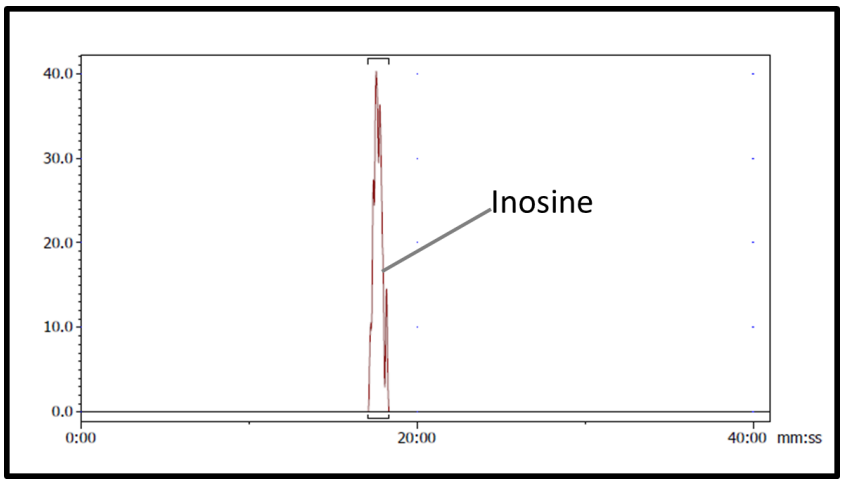


Figure3-5.Radiochemical HPLC analysis of intracellular [^{14}C]cytidine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivity in hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 µM [^{14}C]cytidine. Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

(A)



(B)

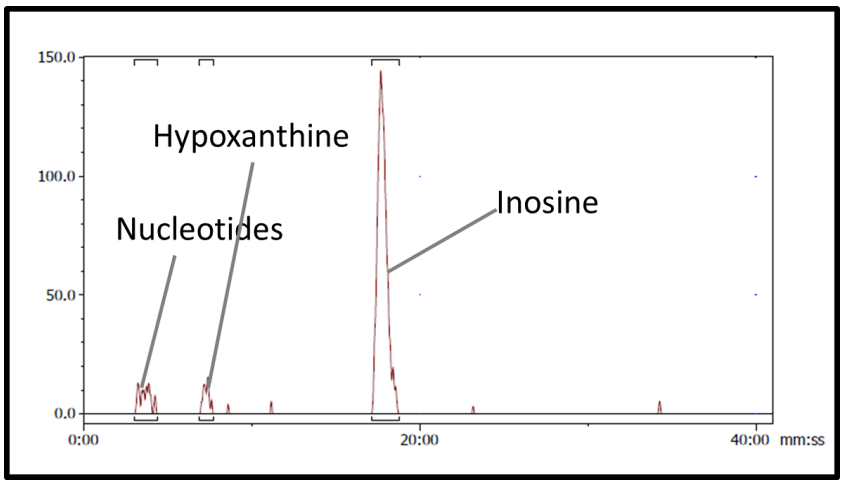
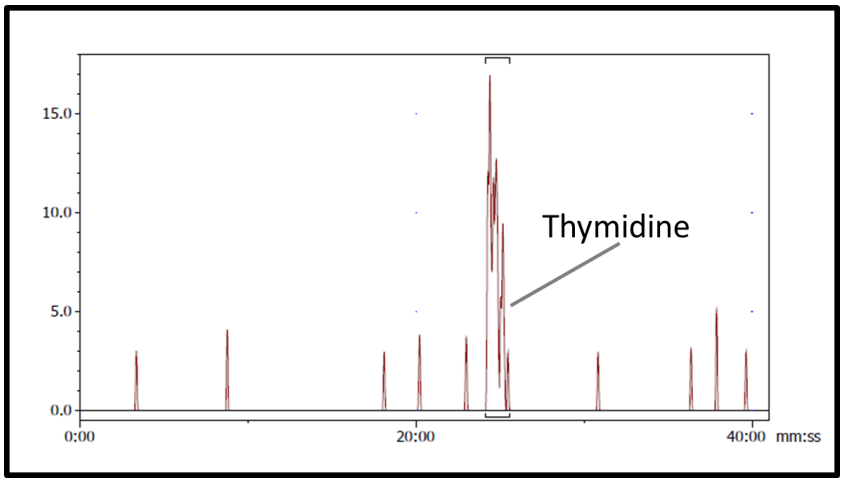


Figure3-6.Radiochemical HPLC analysis of intracellular [^{14}C]inosine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivity in hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 µM [^{14}C]inosine. Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

(A)



(B)

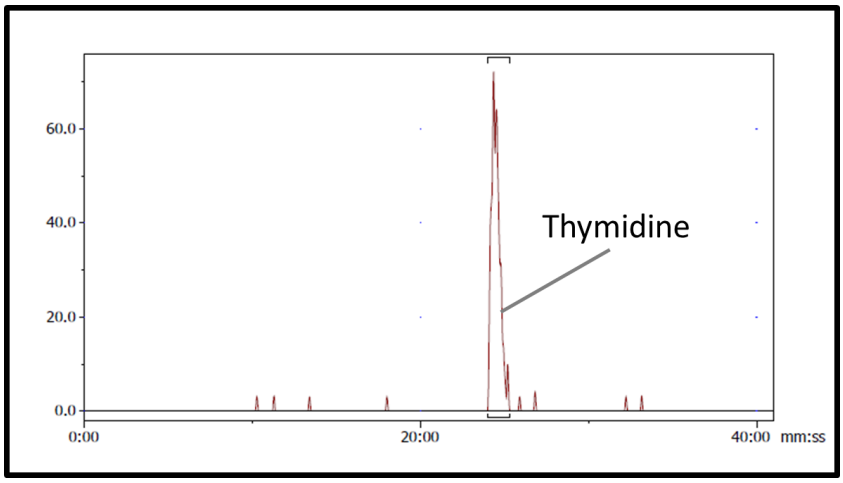
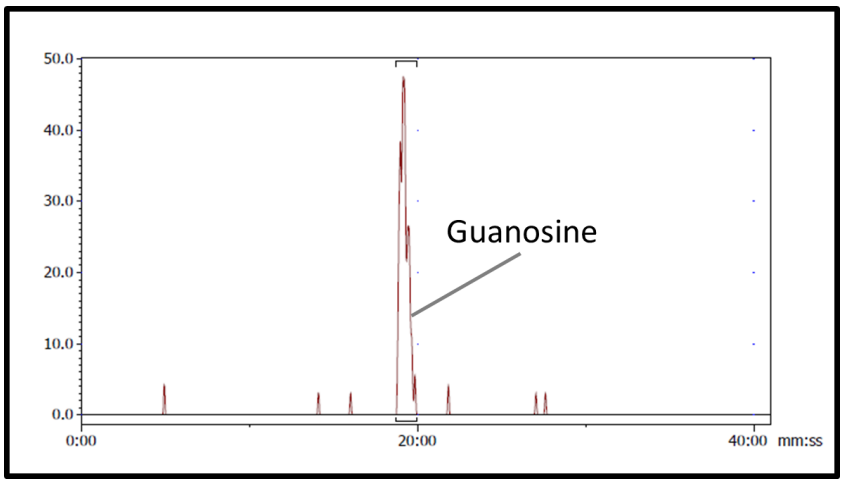


Figure3-7.Radiochemical HPLC analysis of intracellular [^{14}C]thymidine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivity in hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 μM [^{14}C]thymidine. Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

(A)



(B)

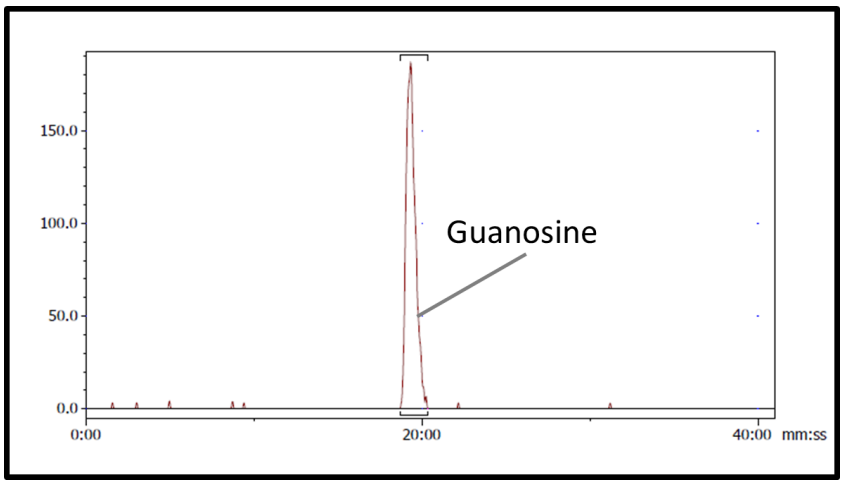


Figure3-8.Radiochemical HPLC analysis of intracellular [^{14}C]guanosine after 1-min (A) and 30-min (B) incubation periods. Representative HPLC chromatograms of intracellular radioactivity in hCNT3-producing *Xenopus* oocytes after 1-min or 30-min incubation periods with 20 μM [^{14}C]guanosine. Values are for groups of 20 oocytes. The *y*-axis is cpm and the *x*-axis is retention time (min). Please see text for other experimental details.

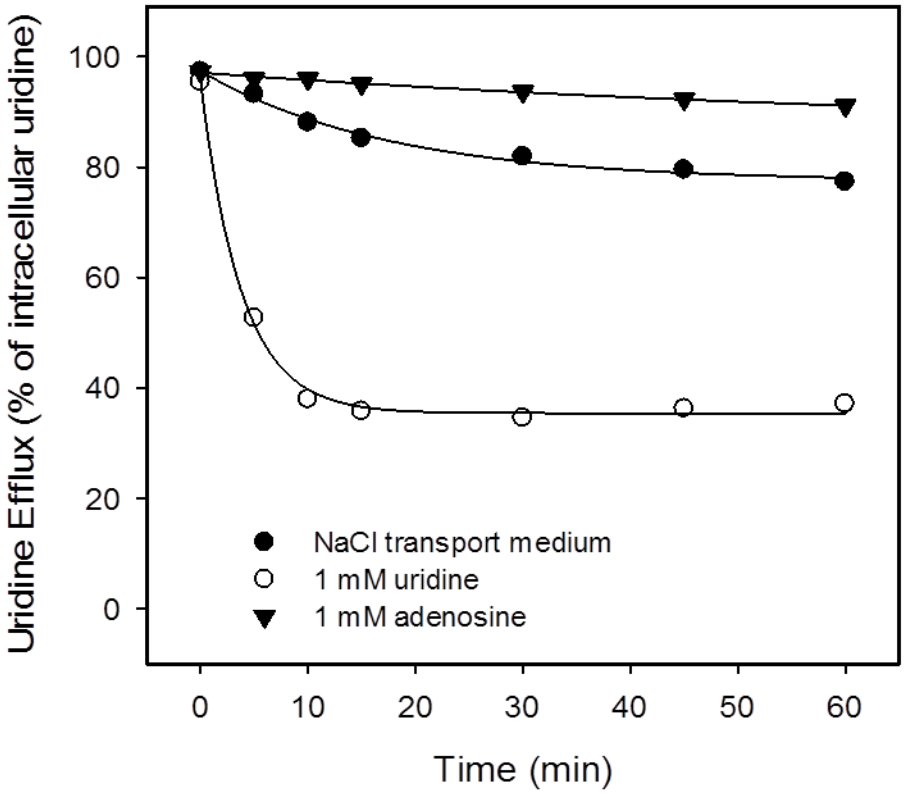


Figure 3-9.*Trans*-stimulation and *trans*-inhibition of hCNT1-mediated uridine efflux by extracellular uridine and adenosine. Efflux of [^{3}H]uridine from hCNT1-producing oocytes preloaded with 10 μM [^{3}H]uridine for 30 min at 20°C from groups of 20 oocytes suspended in (●) NaCl transport medium alone or in NaCl transport medium containing (○) 1 mM non-radioactive uridine or (▼) 1 mM non-radioactive adenosine.

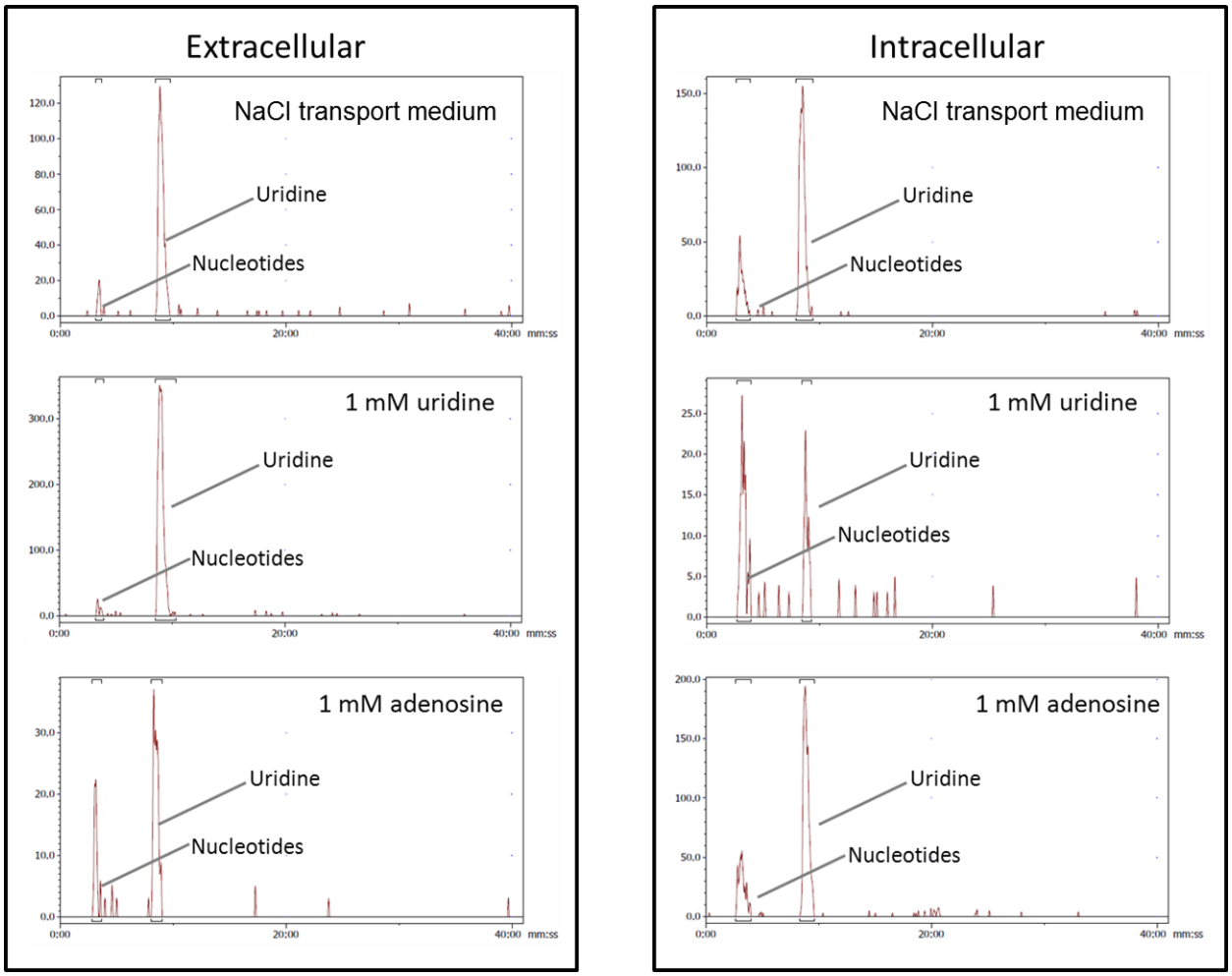


Figure 3-10.Radiochemical HPLC analysis of extracellular and intracellular [^{3}H]uridine in hCNT1-producing oocytes. Groups of 20 hCNT1-producing oocytes were pre-loaded with 20 µM [^{3}H]uridine for 30 min, washed, and resuspended in medium with or without 1 mM adenosine or uridine. HPLC analysis of extracellular and intracellular radioactivity was determined after 60 min efflux**.**Please see text for other experimental details.

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Chapter 4:

Differences in plasma levels of nucleosides and nucleobases in wild-type and in ENT1, ENT2, ENT1/2 knockout mice

Introduction

Owing to the overlapping functional characteristics of nucleoside transporter proteins, the physiological importance and roles of individual nucleoside transporters has been difficult to assess. Mice (m) possess the same two nucleoside transporter protein families (ENT and CNT) as humans, and characteristics of mENTs resemble those of the hENTs (Kiss et al., 2000; Young et al., 2013; Baldwin et al., 2005, Barnes et al., 2006; Niitani et al., 2010; Patel et al., 1997; Ritzel et al., 2001). Therefore generation of mENT knockout mice with targeted disruptions of the genes encoding mENT proteins offers a potentially valuable tool in gaining understanding of roles and importance of individual ENTs in transport and homeostasis of various nucleosides and nucleobases, as well as nucleoside analog drugs.

mENT1 and mENT2 are murine members of the ENT equilibrative nucleoside transporter protein family from mice. These transporters are integral to mammalian cell function by transporting into cells a broad range of nucleosides and nucleobases, including adenosine, by Na^+-independent mechanisms. Since ENTs are bidirectional transporters, facilitating movement of nucleosides down their concentration gradients, they also allow release of adenosine into the extracellular milieu for purinergic signaling (Rose et al., 2011). In my second project, we hypothesized that in the absence of mENT1 or mENT2 or both mENT1 and mENT2, plasma levels of different nucleosides and nucleobases might be affected compared to those of wild-type (WT) mice. To test our hypothesis, we produced mENT1 and mENT2 homozygous (-/-) knockout (KO) mice from heterozygous mice (+/-) purchased from Lexicon Pharmaceuticals, Inc. (The Woodlands, Texas) (Paproski et al., 2010; Graham et al., 2011). mENT1 homozygous KO mice were then crossed with mENT2 homozygous KO mice to generate a mENT1/mENT2 double KO knockout strain. Availability of mENT1 KO mice, mENT2 KO mice and mENT1/2 KO mice allowed us to examine changes in plasma concentrations of nucleosides and nucleobases in response to individual and combined deletion of the each of the two mouse transporter isoforms.

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Results

To produce strains of mice with a disruption mutation of the mENT1or mENT2 gene, heterozygous (+/-) mice that carried one copy of the disrupted mENT1 gene or mENT2 gene were fully backcrossed into FVB/N mice for 6 generations, and the resulting FVB/N mENT1or FVB/N mENT2 heterozygous mice were then crossed to derive the mENT1homozygous KO strain(mENT1 (-/-) and the mENT2 homozygous KO strain (mENT2 (-/-). mENT1 homozygous KO mice were then crossed with mENT2 homozygous KO mice to generate a mENT1/mENT2double homozygous KO strain (mENT1 (-/-)/mENT2 (-/-)).These knockout mice were then compared to FVB/N WT mice with a homozygous non-mutated gene (mENT1 (+/+) and mENT2 (+/+)).

Plasma samples fromWT control FVB/N mice and KO mice with a disruption mutation in either mENT1, or mENT2 or both mENT1/2 were collected and analyzed by the HPLC Agilent 1260 infinity series system as described in *Materials and Methods* (Chapter 2). Concentrations of the following nucleosides and nucleobases were quantified: adenosine, uridine, thymidine, cytidine, guanosine, cytosine, uracil, hypoxanthine, thymine and adenine (Tables 4-1 & 4-2).

Adenosine – The concentration of adenosine in plasma of WT mice was 0.9$\pm$ 0.1 μM. Compared to WT, mENT1 KO mice adenosine plasma levels were increased 12.3-fold, while the corresponding increase in mENT2 KO mice was 2.3-fold. The increase in mENT1/mENT2 double KO mice was 10.4-fold. The small difference between mENT1 KO and mENT1/mENT2 double KO mice was statistically significant.

Guanosine – Plasma guanosine concentrations in all samples were below the limit of detection (< 0.1 μM).

Inosine – The concentration of inosine in plasma of WT mice was 0.9 $\pm$ 0.1 μM. Compared to WT, mENT1 KO mice inosine plasma levels were increased by 2.4-fold, while the corresponding inosine plasma levels in mENT2 KO mice were increased by

1.4-fold. Inosine plasma levels in mENT1/mENT2 double KO mice were also increased by 1.4-fold.

Uridine – The concentration of uridine in plasma of WT mice was 10.0 ± 0.5 μM. Compared to WT, mENT1 KO mice uridine plasma levels were increased by 1.4-fold, while the corresponding increases in mENT2 KO and mENT1/mENT2 double KO mice were also 1.4-fold.

Cytidine – The concentration of cytidine in plasma of WT mice was 6.6 ± 1.0 μM. Compared to WT, mENT1 KO mice cytidine plasma levels were increased by 1.4-fold, while the corresponding cytidine plasma levels in mENT2 KO mice were increased by 1.5-fold. The cytidine plasma levels in mENT1/mENT2 double KO mice were increased by 2.4-fold.

Thymidine – The concentration of thymidine in plasma of WT mice was 0.4± 0.1 μM. Compared to WT, mENT1 KO mice thymidine plasma levels were increased by 1.8-fold, while the corresponding thymidine plasma levels in mENT2 KO mice were increased by 1.3-fold. The thymidine plasma levels in mENT1/mENT2 double KO mice were increased by 2.75-fold.

Uracil – The concentration of uracil in plasma of WT mice was 10.3 ± 1.0 μM. Compared to WT, mENT1 KO mice uracil plasma levels were decreased by 21%, while the corresponding uracil plasma levels in mENT2 KO mice were decreased by 30%. The uracil plasma levels in mENT1/mENT2 double KO mice were decreased by 34%.

Adenine – The concentration of adenine in plasma of WT mice was 2.3 ± 0.1 μM. Compared to WT, mENT1 KO mice adenine plasma levels were increased by ~30%, while the corresponding adenine plasma levels in mENT2 KO mice were increased by ~30%. Adenine plasma levels in mENT1/mENT2 double KO mice were increased by ~50%.

Thymine– Plasma thymine concentrations in all samples were below the limit of detection (< 0.1 μM).

Hypoxanthine – The concentration of hypoxanthine in plasma of WT mice was 10.4 $\pm$ 0.4 μM. Comparing to WT, mENT1 KO mice hypoxanthine plasma levels were decreased by 21%, while the corresponding hypoxanthine plasma levels in mENT2 KO mice were increased by 1.2-fold. Hypoxanthine plasma levels in mENT1/mENT2 double KO mice were decreased by 38%.

Discussion

Nucleoside transporters are involved in the regulation of the cellular and tissue levels of nucleosides and nucleobases, especially with respect to control of levels of the purine nucleoside adenosine at extracellular surfaces and subsequent influence on purinoreceptorintracellular signaling cascades. However, the relative roles of individual nucleoside transporters in modulating, for example, plasma concentrations are poorly understood. The research described in this Chapter used mENT1, mENT2 and mENT1/mENT2 double KO mice to assess the contributions of the broadly selective nucleoside transporters ENT1 and ENT2 to plasma nucleoside/nucleobase homeostasis.
In the absence of mENT1 in mENT1 KO mice, our studies showed that there was a significant increase (~ 12 fold) in adenosine plasma levels relative to WT controls. Such an increase was also seen in mENT2 KO mice, but to a much lesser extent (~ 2 fold). In mENT1/mENT2 KO mice, the increase was ~10 fold. Inosine levels were also elevated in mENT1 KO mice, but to lesser extent than adenosine (~2 fold), and there were corresponding smaller increases in mENT2, and mENT1/mENT2 double KO mice. The nucleosides uridine, cytidine, and thymidine were all slightly elevated in mENT1, mENT2, and mENT/mENT2double KO mice.

Nucleobase uracil levels were decreased in mENT1, mENT2and mENT1/mENT2 double KO mice, while adenine showed minor increases in all three KO strains, with the highest elevation in double KO mice. Hypoxanthine levels, in contrast, were modestly decreased in both mENT1 and mENT1/mENT2 double KO mice.The plasma concentrations of nucleosides and nucleobases detected in WT mice were similar to values reported in the literature (Darnowski & Handschumacher, 1981; Slowiaczek & Taffersall, 1981; Traut, 1994).

Previous studies of mENT1 KO mice demonstrated that mENT1 plays an important role in ethanol preference and consumption (Nam et al., 2011), anxiety-related behavior (Chen et al. 2007), cardioprotection during ischemia (Rose et al. 2010; Rose et al., 2011), soft tissues calcification (Warraich et al., 2013), as well as sleep-wake regulation (Kim et al., 2015). hENT1 transgenic mice exhibit greater response to ethanol and reduced response to caffeine (Kost et al. 2011). These changes can be explained by

altered ENT1-mediated control of adenosine levels and adenosine receptor signaling and, consistent with this, mENT1 KO mice were demonstrated to have elevated plasma levels of adenosine (Rose et al., 2011). Compared to our findings, however, the increase compared to WT mice was more modest (~ 2 fold), perhaps reflecting the care we took to minimize adenosine uptake into erythrocytes during sample preparation, *e.g* mice blood samples were collected in the presence of the ENT1 inhibitor NBMPR (500 nM) and the adenosine deaminase inhibitor 2'-deoxycoformycin (500 nM), as well as all steps were performed at 4 ºC. Furthermore, our HPLC analyses of mENT1 KO mouse plasma samples demonstratedthat the nucleoside adenosine showed the most significant change in plasma circulating levels compared to other nucleosides and nucleobases, reinforcing the unique physiological importance of the ENT1 transporter isoform in adenosine homeostasis. Changes in plasma adenosine levels in response to deletion of mENT2 were much more modest. For other nucleosides and nucleobases, deletion of mENT1 and/or mENT2 had modest and broadly similar effects.

Table 4-1.Plasma nucleosides and nucleobases levels (µM) measured by HPLC in wild-type, mENT1 KO, mENT2 KO, and mENT1/mENT2 double KO mice. Please see text for experimental details. Values are means ± SEM of *n* mice.

	Wild-type	mENT1 KO	mENT2KO	mENT1/mENT2 double KO
Number of mice	21	25	34	41
Adenosine (µM)	0.9 ± 0.1	11.1 ± 0.4*	2.1 ± 0.2*	9.4 ± 0.3*[#]
Guanosine (µM)	0.0	0.0	0.0	0.0
Inosine (µM)	0.9 ± 0.1	2.2 ± 0.2*	1.3 ± 0.1*	1.3 ± 0.1*
Uridine (µM)	10.0 ± 0.5	14.3 ± 0.5*	13.5 ± 0.4*	14.2 ± 0.5*
Cytidine (µM)	6.6 ± 1.0	9.2 ± 0.6*	9.8 ± 0.9	15.6 ± 0.8*
Thymidine (µM)	0.4 ± 0.1	0.7 ± 0.1*	0.5± 0.03*	1.1 ±0.02*
Uracil (µM)	10.3 ± 1.0	8.1 ± 0.7	7.2 ± 0.5*	6.8 ± 0.4*
Adenine (µM)	2.3 ± 0.1	2.9 ± 0.1*	2.9 ± 0.1*	3.5 ± 0.1*
Thymine (µM)	0.0	0.0	0.0	0.0
Hypoxanthine (µM)	10.4 ± 0.4	8.2 ± 0.5*	12.9 ± 0.6	6.4 ± 0.2*

*P<0.05 (compared to WT)
[#] P< 0.05 (compared to mENT1 KO)

Table 4-2.Nucleosides and nucleobases level in wild-type, mENT1 KO, mENT2 KO, and mENT1/mENT2 double KO mice blood samples expressed in % of control. Please see text for experimental details.

	Wild-type	mENT1KO	mENT2KO	mENT1/mENT2 double KO
	% of control	% of control	% of control	% of control
Adenosine	100	1233	233	1044
Guanosine	100	nd	nd	nd
Inosine	100	244	144	144
Uridine	100	143	135	142
Cytidine	100	140	149	236
Thymidine	100	175	125	275
Uracil	100	79	70	66
Adenine	100	126	126	152
Thymine	100	nd	nd	nd
Hypoxanthine	100	79	124	62

nd : not determined

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Chapter 5

General Discussion

Project 1

Nucleosides are central metabolites in mammalian cells, are precursors of nucleotides, and play an essential role in intermediary metabolism, biosynthesis of macromolecules and cell signaling through interaction with purinergic receptors. Nucleoside analogs are used to treat hematological malignancies, solid tumors as well as viral diseases. Nucleosides and nucleoside analogs are hydrophilic in nature, and therefore require nucleosides transporters to cross the lipid bilayers of cell membranes. In mammalian cells, membrane transport of nucleosides is mediated by members of the ENT and CNT protein families.

The cation-coupled concentrative nucleoside transport processes originally termed*cit*,*cif* and *cib* are mediated by isoforms of the CNT (SLC28) transporter family, designated in humans as hCNT1, hCNT2 and hCNT3 (Young et al., 2013). All three proteins transport uridine and adenosine (although hCNT1-mediated adenosine transport activity is low compared to that of uridine), but are otherwise pyrimidine nucleoside-selective (hCNT1), purine nucleoside-selective (hCNT2), or broadly selective for both pyrimidine and purine nucleosides (hCNT3). These have been functionally characterized as recombinant proteins by heterologous expression in *Xenopuslaevis* oocytes in both radioisotope flux and electrophysiological experiments.

hCNT3 is the most recently discovered and functionally versatile of the three human members of the SLC28 (CNT) protein family. It is 691 amino acids in length, and utilizes both Na^+ and H^+ electrochemical gradients to accumulate a broad range of pyrimidine and purine nucleosides and nucleoside drugs within cells (Ritzel et al., 2001). hCNT3 is more widely distributed in cells and tissues than hCNT1 or hCNT2 and has a central role in renal transepithelial nucleoside and nucleoside drug transport (Ritzel et al., 2001; Elwi et al., 2006) .

In my first project, our findings using HPLC analysis demonstrated that transported nucleosides by hCNT3 in oocytes after a 1-min incubation period were not subject to intracellular metabolism. After 30 min, and with the exception of adenosine (51%

metabolism), intracellular metabolism was also minimal (uridine 7%, cytidine 0%, thymidine 0%, inosine 16% and guanosine 0%). This finding is important because it validates use of radiolabelled nucleosides in the short (1-5 min) flux intervals used to characterize both CNT and ENT functions. Together with low endogenous nucleoside transport activity, therefore, *Xenopus* oocytes have the additional advantage as a heterologous expression system of low (except for adenosine) intracellular nucleoside metabolism. Even in the case of adenosine, the dominant metabolic fate was phosphorylation into impermeable derivatives, and thus would not interfere with determination of initial rates of transport. The adenosine deaminase inhibitor deoxycoformycin is used in radioisotope flux assays with adenosine to block possible conversion to inosine and then hypoxanthine (e.g. Yao et al., 2011). Deoxycoformycin was also included in the experiments reported here. After 30 min of uptake, only 5% of intracellular adenosine had been converted to inosine, and no intracellular hypoxanthine was detected, suggesting that deoxycoformycin had fulfilled its role, although control experiments in the absence of deoxycoformycin were not undertaken.

Adenosine has been shown to be a low K_m, low V_{max} permeant of rat (r) CNT1 (rCNT1) (Yao et al., 1996). In experiments in oocytes preloaded with radiolabelled uridine, it was found that efflux of radioactivity mediated by the transporter was enhanced by extracellular uridine, but inhibited by extracellular adenosine. The interpretation of this finding was that adenosine functions as a low K_m, low V_{max} rCNT1 permeant because the transporter complexed with adenosine reorientates its permeant binding site from out-to-in more slowly than when unoccupied, or when complexed with uridine (uridine-complexed carrier > empty carrier). The validity of this interpretation rests on the assumption that the effluxed radioactivity measured in the experiment is still in the form of uridine. In this thesis, my results similarly found that recombinant hCNT1-mediated efflux of [^{3}H] uridine from preloaded oocytes was stimulated by extracellular uridine and inhibited by extracellular adenosine. Furthermore, it was demonstrated that >95% of the radioactivity exiting cells was unmetabolized radiolabeled uridine. This confirms the suggestion that the rate of conversion of hCNT1 from its outward-facing conformation to its inward-

facing conformation was increased when the transporter was complexed with uridine and decreased when it was complexed with adenosine.

Project 2

The prototypic equilibrative nucleoside transporter hENT1 (*es*) has long been recognized as a potential target for therapeutic strategies based on modulation of transport of physiological nucleosides, principally adenosine, and anticancer nucleoside drugs (Elwi et al., 2006, Young et al, 2013). Like hENT1, hENT2 (*ei*) exhibits broad permeant selectivity, but is much less studied and understood than hENT1, largely because most cell types co-express both transporters, with hENT1 typically showing the greater activity. In addition to nucleosides, both transporters also transport nucleobases (Yao et al, 2011). hENT3 has a similarly broad permeant selectivity and functions in intracellular membranes, including lysosomes (Baldwin et al., 2005). hENT4 is selective for adenosine and is found in the CNS and cardiac tissue (Barnes et al., 2006).

Mice possess the same NTs as humans, and therefore the generation of knockout mice with targeted disruptions of the genes encoding the various mouse transporters offers a means for *in vivo* investigation of their roles in nucleoside physiology and homeostasis. In the second project of my thesis, I used HPLC to investigate plasma concentrations of nucleosides and nucleobases in mice with disruptions of the genes for mENT1, mENT2 or both mENT1 and mENT2. The objective was to test whether deficiencies in the two transporters lead to defined plasma concentration phenotypes, or whether redundancy and/or compensatory changes in other NTs with overlapping permeant selectivities potentially mitigate against such changes.

In comparison to WT animals, my experiments demonstrated the greatest changes for the purine nucleosides adenosine and inosine, with adenosine > inosine and mENT1 > mENT2. We found that the absence of mENT1 in mENT1 KO mice led to a large increase in adenosine plasma levels from the WT control value of 0.9 μM to around 11 μM, a 12-fold increase. A smaller but still significant 2.3-fold increase was also seen in

mENT2 KO mice, while concentrations in double mENT1/mENT2 mice were similar to the single mENT1 KO alone. Inosine levels in WT control mice were also 0.9 μM, and increased by 2.4-, 1.4- and 1.4-fold in mENT1, mENT2 and double mENT1/mENT2 KO mice, respectively. Similar modest increases were also seen for uridine, cytidine and thymidine, while nucleobase levels were largely unchanged.

These studies reinforce the importance of the ENT1 isoform in adenosine homeostasis, provide support for a more modest role of both transporters in inosine homeostasis, and suggest that neither transporter is a major participant in handling of nucleobases.

Further studies

The metabolic studies reported here in *Xenopus* oocytes validate previous and on-going intensive use of the *Xenopus* oocyte heterologous expression system in studies of hENT and hCNT structure and function, and open the door to kinetic studies of nucleoside efflux in addition to influx as an additional means to explore their properties. For example, kinetic studies of uridine influx, efflux and equilibrium exchange have been used to explore the mechanism by which *n*-ethyl maleimide inhibits hENT1 (manuscript in preparation).

With respect to plasma nucleoside concentrations, the experiments reported here could be extended to other transporter isoforms (for example, our laboratory has access to mCNT3 KO mice), and to studies of anticancer and antiviral nucleoside drugs.

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