Title
Genetic variation and specificity of membrane transporters

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Publication Date
2006

Peer reviewed|Thesis/dissertation

Genetic Variation and Specificity of Membrane Transporters

by

Ryan P. Owen

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Pharmaceutical Sciences and Pharmacogenomics

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

ACKNOWLEDGEMENTS

Graduate school is unlike any previous academic experience that I have had. Yes, it is about science, learning new techniques, reading the literature, and late night experiments, but it also teaches you how to think independently, to start asking the unknown, and then how to move the unknown into the realm of the known. It is that possibility that drove me to graduate school to begin with, but it was the help and support of those around me that enabled me to finish.

I would first of all like to thank my advisor and mentor Kathy Giacomini. Kathy took me into her lab as a first year graduate student, and has shown faith in me both when I had data, and when experiments were not working. She has helped me to improve my presentation skills, and has taught me an enormous amount about how to be an effective science writer. She has spent countless hours poring over work that I have given her: seminars, abstracts, posters, papers, job talks, thesis chapters, etc. In all the time I have been in graduate school, nothing I have sent her has ever been given back to me without careful suggestions and comments, which just tells me that there is always more to learn. I would also like to thank my thesis committee members Deanna Kroetz and Patsy Babbitt for their helpful suggestions and input during my graduate career.

Whether you like it or not, graduate school is not a solitary venture. You share it with the people in the lab, and we all know the ups and downs that come with the job description. Fortunately, I have had the chance to work with a group of wonderful people during my time in Kathy's lab. They have made it a fun place to work, and it is a lot easier to

swallow another failed experiment when you can do so with good friends who are there with you. When I first started, there was an excellent group of scientists that were there to help me learn the ropes and answer all my questions. I would like to thank my initial mentor, Dr. Guangqing Xiao for his patience and confidence in me, Dr. Bo Feng and Dr. Yan Shu for always making me laugh, Dr. Karin Gerstin and Dr. Lara Mangravite for knowing the answer to virtually every question, and Dr. Maya Leabman for her patience and help in teaching me the frog surgery – a staple of my graduate career. Several other lab members also made lab a great place to work: Dr. Jennifer Gray with her always entertaining off-the-wall conversations, the human alarm clock Ilaria Badagnani, the energetic Chesi Ho and Leah Lagpacan, the great Northwest's own Jim Shima, and my close friend and drinking buddy Tom Urban who can talk baseball, politics, science, philosophy, and everything in between.

I have made some other great friends outside of the lab: my partner in crime Jason Gow, Kim Fife, Eric Peters, and my movie buddies Jeff and Molly Kraft. I would also like to thank my parents and my brother Tim, who have always supported me. Whenever I needed help or encouragement, my family was always there for me, even though they lived 2,000 miles away.

Finally, there is one more person that I have to thank, who has been the most helpful of all, my wonderful wife Leslie. She has been the sympathetic ear, the voice of encouragement, the tech support, the editor, the audience, and the reason to smile through it all. I couldn't have done it without you Les...thank you.

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ABSTRACT

Genetic Variation and Specificity of Membrane Transporters

Ryan Owen

Variation in response to a therapeutic agent is a common problem in clinical drug use. One potential source of variation in drug response is interindividual genetic variation in transporters which interact with drugs.

This dissertation focuses on nucleoside transporters, but also includes characterization of genetic variants of organic cation transporter 1 (OCT1). Nucleoside transporters, including concentrative (CNT1-3) and equilibrative (ENT1-2) mediate the uptake of nucleosides, and nucleoside analog drugs, which are used as anti-cancer and anti-viral agents. In this dissertation, we focus on genetic variants of CNT2 and ENT2. We observed that all of the nonsynonymous variants of CNT2 were able to take up inosine, and one variant, CNT2-F355S, had an altered ratio of inosine:uridine uptake. Three protein-altering variants of ENT2 caused either a reduction or a loss in function.

We further examined the concept of substrate specificity in CNT2 by comparing the differing activities of the human and rat CNT2 orthologs (rCNT2>>>hCNT2 for the anti-leukemia drug 2CdA). To determine the residues, and protein domains responsible for this difference in activity, we constructed chimeric proteins and single mutants of CNT2. We found that the C-terminal region of CNT2 contained the determinants for substrate

specificity, and that the amino acid at position 345 in hCNT2 had a strong influence on uptake.

OCT1 (SLC22A1) is found in abundance in human liver, and mediates the transport of endogenous compounds (e.g., dopamine), and xenobiotics including cationic drugs (e.g., metformin). In this dissertation, we characterized the interaction of OCT1 variants with the anti-diabetic agent metformin, and the platinum-based anti-cancer agent oxaliplatin. We found several reduced functioning OCT1 variants for metformin and oxaliplatin. One of the variants that showed a reduction in function, OCT1-420 del, was found at an allele frequency of 19% in the European-American population. This finding is of particular importance due to the frequency of the variant, and the widely-prescribed nature of both metformin and oxaliplatin.

Collectively, our studies demonstrate functional variation in coding region variants of nucleoside and organic cation transporters. Further studies should focus on determining the relevance of variation in these transporters to clinical drug response and disposition.

Kathleen M. Giacomini

October 3, 2006

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Chapter 1

Introduction to Nucleoside Transporters

Nucleoside transporters in the **S**olute **C**arrier **S**uperfamily (SLC) mediate the transport of nucleosides and nucleoside analog drugs across the plasma membrane of most cell types. There are two major families of nucleoside transporters within the SLC superfamly: concentrative and equilibrative. Concentrative nucleoside transporters (CNTs) are secondary active, influx transporters that couple the transmembrane flux of nucleosides to the Na^+ gradient, and have a high affinity for naturally occurring nucleosides. Equilibrative nucleoside transporters (ENTs) facilitate the bidirectional transport of nucleosides according to the nucleoside concentration gradient, and have a lower affinity for nucleosides than the CNTs. Together, the CNTs and ENTs are thought to maintain nucleoside homeostasis in the body, and to be key players in salvage pathways, in which circulating nucleosides are transported into cells for use as nucleic acid precursors. Transporters in the two families are of therapeutic interest because of their potential to take up anti-cancer and anti-viral nucleoside analog drugs. This introductory chapter summarizes nucleoside transporter research from the early characterization of transport activity, to more recent studies of genetic variation, regulation, and analysis of molecular transport mechanisms.

History

The initial work characterizing the intracellular flux of nucleosides suggested the presence of specific, saturable and inhibitable nucleoside transporters in various tissues and cell lines. Prior to molecular identification, the number of nucleoside transporters

was unknown, and nothing was known about their molecular mechanisms. In 1976, Strauss *et al.* observed that data on the concentration dependence of adenosine transport in murine lymphocytes was best interpreted as coming from two different uptake systems, a low affinity system, and a high affinity system (Strauss et al. 1976). Two years later, Ungemach et al also characterized a high affinity and a low affinity system for thymidine uptake into isolated rat hepatocytes. These authors suggested that the high affinity system was active and served to concentrate nucleosides intracellularly (Ungemach and Hegner 1978).

In early studies, distinct types of equilibrative transporter activities were characterized: one that was sensitive to inhibition by the thioguanosine nucleoside nitrobenzylthioinosine (NBMPR), and one that was insensitive to NBMPR inhibition. These transport activities were later termed: es and ei, for equilibrative sensitive, and equilibrative insensitive. Later experiments would identify ENT1 as the es transporter, and ENT2 as the ei transporter (Table 1.1).

Our understanding of concentrative nucleoside transport systems developed more slowly primarily due to overlapping substrate specificities among the systems and lack of specific inhibitors. In 1984, it was determined by several groups that the high affinity transport system was Na^+-dependent, which explained the driving force for cellular accumulation against the concentration gradient (Le Hir and Dubach 1984; Le Hir et al. 1984; Schwenk et al. 1984; Spector and Huntoon 1984). Four years later Vijayalakshmi and Belt published a critical paper classifying concentrative nucleoside transport

Common Name	HGNC name	Activity	Specificity	Cloned
ENT1	*SLC29A1*	*es*	Broad	Human (Griffiths et al 1997) Rat (Yao et al 1997)
ENT2	*SLC29A2*	*ei*	Broad, nucleobase	Human (Crawford et al 1998) Rat (Yao et al 1997)
ENT3	*SLC29A3*		Broad	Human (Hyde et al 2001) Mouse (Hyde et al 2001)
ENT4, PMAT	*SLC29A4*		Organic cations	Human (Engel et al 2004)

Table 1.1. Substrate specificity of equilibrative nucleoside transporters in the SLC29A family.

activities (Vijayalakshmi and Belt 1988). Through a series of elegant experiments, they were able to show that there were at least two putative Na^+-dependent, concentrative nucleoside transporters, one of them preferentially transporting pyrimidines and adenosine, and another transporting purines and uridine; these transport activities were named cit and cif respectively. Cit indicated that the transporter was Concentrative, NBMPR-Insensitive, and Thymidine served as a model substrate. Similarly, cif implied that the transporter was Concentrative, NBMPR-Insensitive, and Formycin B (a non-metabolized form of the purine nucleoside inosine) served as the model substrate (Table 1.2). This paper also resolved several seemingly contradictory results in the literature due to the overlapping substrate specificities of some nucleoside transporters i.e. both cit and cif were able to transport uridine and adenosine.

A third class of Na^+-dependent concentrative transport was later characterized in rabbit choroid plexus (Wu et al. 1992). This transporter exhibited broad substrate selectivity, was able to take up both purine and pyrimidine nucleosides, and had a Na^+: nucleoside stoichiometry of 2:1 instead of the previously observed 1:1 for cit and cif (Plagemann and Aran 1990). This activity was later termed cib (Concentrative NBMPR-Insensitive, Broadly selective).

Molecular Identification of Nucleoside Transporters

Rat CNT1 was the first mammalian concentrative nucleoside transporter to be cloned and charactrerized (Huang et al. 1994). rCNT1 was pyrimidine preferring, but also weakly transported adenosine, characteristics that explained the previously described cit activity

Common Name	HGNC name	Activity	Specificity	Cloned
CNT1	*SLC28A1*	N2, *cit*	adenosine cytosine thymidine uridine	Human (Ritzel et al 1997) Rat (Huang et al 1994)
CNT2, SPNT	*SLC28A2*	N1, *cif*	adenosine guanosine inosine uridine	Human (Wang et al 1997) Rat (Che et al 1995)
CNT3	*SLC28A3*	N3, *cib*	adenosine cytosine guanosine inosine thymidine uridine	Human (Ritzel et al 2001) Rat (Ritzel et al 2001)

Table 1.2. Substrate specificities for naturally occurring nucleosides of concentrative nucleoside transporters in the SLC28A family.

(see Figure 1.1 for structures of endogenous nucleosides). SPNT (sodium-dependent purine nucleoside transporter), was cloned shortly after rCNT1, and was later termed rCNT2 (Che et al. 1995). This transporter was shown to be purine preferring, but was also able to transport uridine, characteristics that were consistent with cif activity. The human orthologs of CNT1 and CNT2 were cloned shortly after the rat isoforms, and found to have similar transport characteristics for the naturally occurring nucleosides (Ritzel et al. 1997; Wang et al. 1997). A few years later, human and mouse CNT3 were cloned and characterized (Ritzel et al. 2001). The sequence of CNT3 is not as similar to those of CNT1 and CNT2 as they are to each other. However, CNT3 exhibits a high degree of sequence homology to hagfish CNT (hfCNT), which was shown to be a broadly selective concentrative nucleoside transporter (Yao et al. 2002). hCNT3 and mCNT3 were shown to have broad specificities for purine and pyrimidine nucleosides, reflecting the previously described cib like activity (Table 1.2).

Another broadly selective transporter was cloned from the nematode *C. elegans*, and it was designated CeCNT3 due to its broadly selective phenotype (Xiao et al. 2001). The transport profiles of hfCNT and CeCNT3 are similar, although CeCNT3 was not able to transport cytosine. Interestingly, CeCNT3 was not found to be Na^+-dependent, but instead used the H^+ gradient as the driving force for nucleoside transport, suggesting that the Na^+ dependent nature of concentrative nucleoside transporters evolved somewhere between *C. elegans* and hagfish. CeCNT3 did not exhibit a high sequence identity to any of the cloned concentrative nucleoside transporters (23.6% identity with CNT3), but the percent identity was higher in regions of the CNTs that have been found to be relevant for

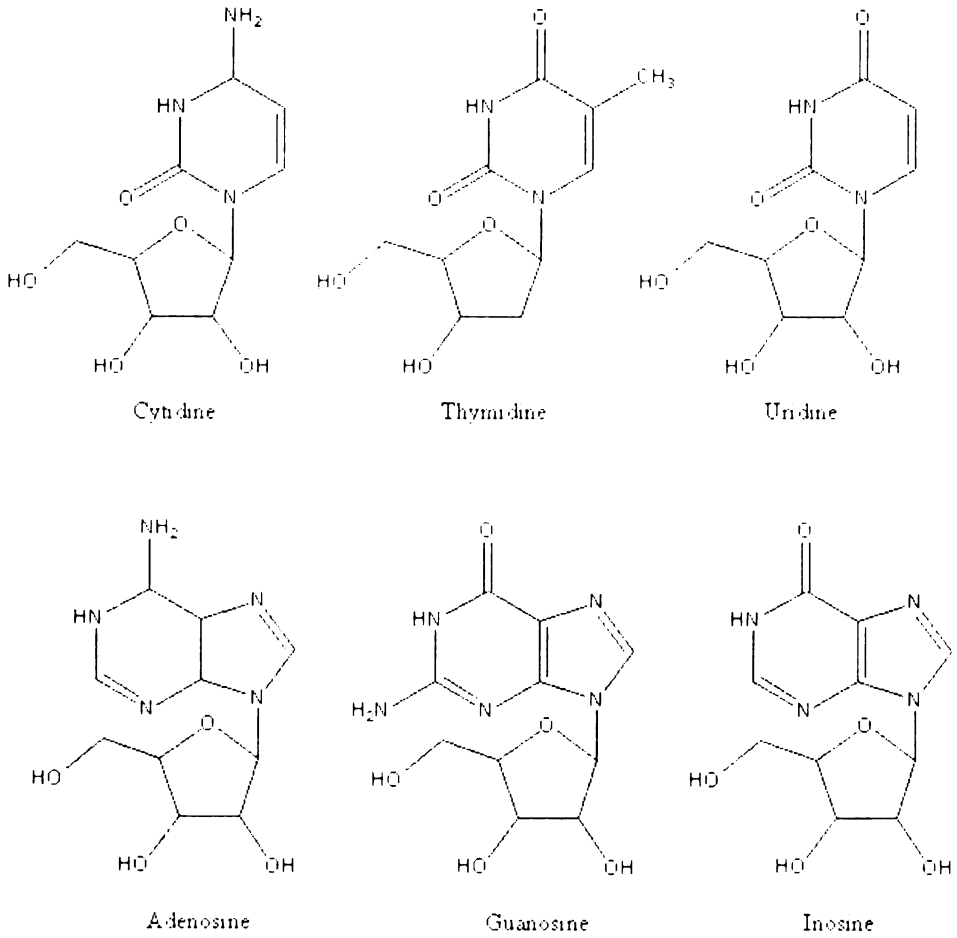


Figure 1.1. Structures of the endogenous nucleosides. The top row consists of the chemical structures of the pyrimidines cytidine, thymidine, and uridine. Thymidine is shown in its deoxyform because it is incorporated exclusively into DNA. The bottom row shows the chemical structures of the endogenous purine molecules adenosine, guanosine, and inosine.

substrate specificity (44% identity to CNT2 and 33% to CNT1 in this region) (Xiao et al. 2001).

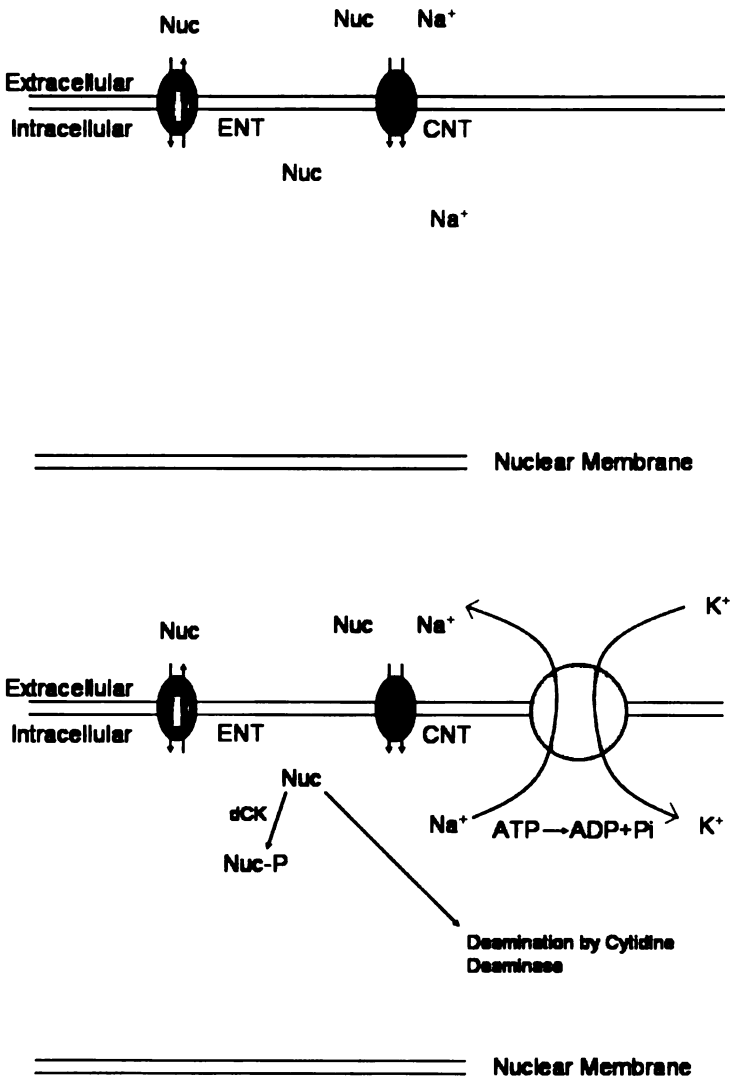
The human and rat orthologs of the ENT family were also cloned and characterized. As expected, two transporters were identified: ENT1 had characteristics of es transport activity whereas ENT2 was similar in function to ei transport (Griffiths et al. 1997; Yao et al. 1997; Crawford et al. 1998). The substrate specificities of ENT1 and ENT2 are similar, but ENT2 is unique in its ability to transport certain nucleobases. In addition to ENT1 and ENT2, two other nucleoside transporters in the ENT family have been identified and characterized. ENT3 is a nucleoside transporter, but is expressed intracellularly, and is believed to be localized to endosomes and lysosomes (Hyde et al. 2001; Baldwin et al. 2005). This activity was not detected in earlier studies, which focused largely on plasma membrane uptake processes. ENT3 was characterized by artificially targeting it to the plasma membrane by mutating its dileucine endosomal/lysosomal targeting motif, and then characterizing its activity (Baldwin et al. 2005). ENT4 was identified by sequence homology to the other ENTs, but it showed only a weak transport of adenosine, and has been shown to more closely resemble a polyspecific organic cation transporter in its transport profile (Table 1.1, (Engel et al. 2004; Engel and Wang 2005)).

Endogenous roles of nucleoside transporters

Nucleoside transporters have a high degree of overlap in their substrate specificities, particularly with respect to the naturally occurring nucleosides *e.g.* every nucleoside transporter can take up uridine (Baldwin et al. 2004; Gray et al. 2004). The presence of multiple nucleoside transporters suggests that each has a distinct endogenous role. It has been well established that expression of nucleoside transporters is highly regulated by several factors, and that the tissue distribution of nucleoside transporters varies greatly (Pastor-Anglada et al. 2001; Pennycooke et al. 2001).

Nucleoside transporters play an important role in nucleotide salvage pathways, in which circulating nucleosides are converted into intracellular nucleotides: the building blocks of nucleic acids (see Figure 1.2) (Cass et al. 1998; Cass et al. 1999; Kong et al. 2004). Salvage pathways are crucial because it alleviates the need for the more energy consuming process of *de novo* nucleoside synthesis. Nucleoside transporters mediate the first step in the salvage of nucleotides by transporting circulating nucleosides from the blood into the cell. Once inside the cell, nucleosides may be modified by a series of cytosolic enzymes (which catalyze reactions such as the removal of a hydroxyl group, and phosphorylation), until they become the tri-phosphorylated nucleotides, which are incorporated into an elongating nucleic acid chain (Figure 1.2).

Another role for nucleoside transporters is in the absorption of dietary nucleosides from the intestine (Pennycooke et al. 2001; Podgorska et al. 2005). One study involving fasting rats supports this contention. The authors analyzed CNT1 expression level before


Nuc
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Na+
Extracellular
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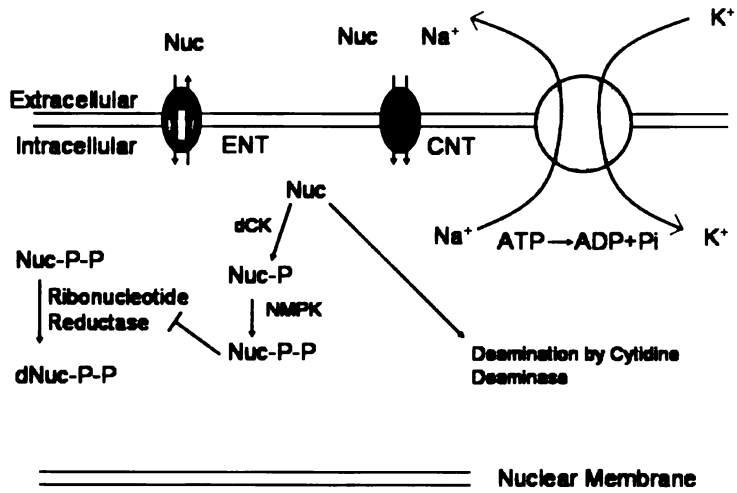
Nuc-P
Na+
ATP→ADP+Pi
K+
Deamination by Cytidine Deaminase
Nuclear Membrane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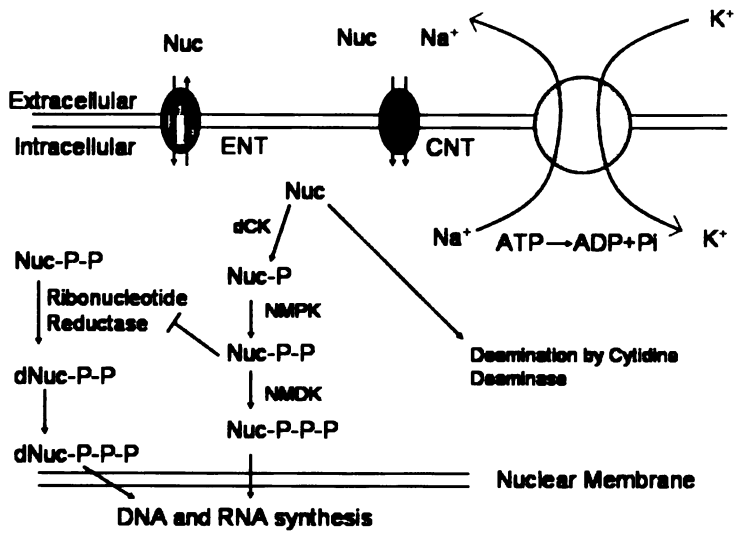
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Nuc-P
Ribonucleotide
Reductase
NMPK
Nuc-P-P
Deamination by Cytidine
Deaminase
dNuc-P-P
NMDK
Nuc-P-P-P
dNuc-P-P-P
Nuclear Membrane
DNA and RNA synthesis

Figure 1.2. Role of nucleoside transporters in salvage pathways. Top page 10: Nucleosides enter the cell via ENT or CNT family members. The ENT transporters move nucleosides across the plasma membrane according to the nucleoside gradient, whereas the CNT transporters couple the influx of nucleosides to the Na^+ gradient. Nucleosides can not exit the cell via CNT transporters. Bottom page 10: The Na^+ gradient is restored through the action of the Na^+/K^+ pump, or Na^+, K^+-ATPase; this reaction is powered by the hydrolysis of ATP. Once inside the cell, nucleosides are phosphorylated to their monophosphate form by cellular enzymes (such as deoxycytidine kinase (dCK). An alternative metabolism pathway is the deamination of nucleosides by adenosine deaminase. Top page 11: Monophosphorylated nucleosides are then phosphorylated again to the diphosphorylated form by intracellular kinases. The di- and tri-phosphorylated forms of some nucleosides can inhibit the activity of ribonucleotide reductase, which catalyzes the reduction of diphosphorylated nucleosides into their deoxy form. Bottom page 11: The diphosphorylated nucleoside is phosphorylated once more to its triphosphorylated species. The NTPs or dNTPs then enter the nucleus where they are ready for incorporation into RNA or DNA, respectively.

and after fasting in rat intestine, and found that the expression of CNT1 in the fasting rats was higher. These data suggest a possible mechanism for sensing nutrient abundance and availability, and that intestinal nucleoside transporters contribute to the dietary absorption of nucleosides (Valdes et al. 2000).

Another intriguing endogenous role for nucleoside transporters is in the regulation of extracellular concentrations of adenosine (Thorn and Jarvis 1996; Olah and Stiles 2000; Dunwiddie and Masino 2001; Mubagwa and Flameng 2001; Rosales et al. 2004). In addition to its role as a nucleic acid precursor, adenosine is also a signaling molecule that regulates many physiological processes including blood flow, myocardial slow action potentials, neurotransmission and glucose metabolism (Podgorska et al. 2005). There are four adenosine receptors that are able to bind adenosine and thus initiate signaling: A1, A2a, A2b, and A3 (Mubagwa and Flameng 2001). It has been shown that metabolism and transport across the plasma membrane are the main factors influencing extracellular adenosine concentrations, and thus exposure to adenosine receptors (Podgorska et al. 2005). By reducing extracellular nucleoside concentrations, nucleoside transporters play a role in adenosine signaling.

Therapeutic roles of nucleoside transporters

In addition to the plethora of endogenous roles, nucleoside transporters also play a role in the transport of clinically used nucleoside analog drugs (Baldwin et al. 2004; Gray et al. 2004). There is a large body of literature that has examined the specificity of nucleoside transporters for synthetic nucleoside analogs (Table 1.3). Nucleoside analogs are used

Substrate	CNT1	CNT2	CNT3	ENT1	ENT2	Use
Adenosine	x	x	x	x	x	Endogenous nucleoside
Uridine	x	x	x	x	x	Endogenous nucleoside
Cytidine	x	n	x	i	x	Endogenous nucleoside
Thymidine	x	n	x	x	x	Endogenous nucleoside
Inosine	n	x	x	i	x	Endogenous nucleoside
Guanosine	n	x	x	i	x	Endogenous nucleoside
Formycin B	n	i	x			Inosine analog
Hypoxanthine	n	n	n	n	x	Endogenous nucleobase
NBMPR	n			i	i	Classic ENT inhibitor
AZT, Zidovudine	x	n	x	i	i	Anti-viral
ddC, Zalcitabine	x	n	x	n		Anti-viral
ddI, Didanosine		x	x	n		Anti-viral
ddA		i				Anti-viral
ddU				n		Anti-viral
Idoxuridine, IudR		n				Anti-viral
Floxidine, FUdR		n	x			Anti-viral
Lamivudine, 3TC	x			n		Anti-viral
Ribavirin		x	x	i		Anti-viral
6-Azauridine				n		Anti-viral
Gancyclovir				n		Anti-viral
Stavudine, D4T				n		Anti-viral
Trifluridine				i		Anti-viral
Brivudine						Anti-viral
Emtricitabine						Anti-viral
Entecavir						Anti-viral

Penciclovir						Anti-viral
Cidofovir						Anti-viral
Cytarabine	x			i	n	Anti-cancer
Gemcitabine	x	n	x	x	x	Anti-cancer
Cladribine, 2CdA		x	x	i	x	Anti-cancer
Tubercidin				x	x	Anti-cancer
5-fluorouridine	x	x	x	i		Anti-cancer
5-Fluorouracil				x		Anti-cancer
dFU	x		x	i		Anti-cancer
Capecitabine	n			x		Anti-cancer
Fludarabine	n	n	x	i	x	Anti-cancer
Ara-A		n		i		Anti-cancer
Troxacitabine	n	n	n	n	n	Anti-cancer
Zebularine			x			Anti-cancer
Clofarabine	n	x	x	x	x	Anti-cancer
Nelarabine						Anti-cancer
TaraC	n	n	x	x	x	Anti-cancer

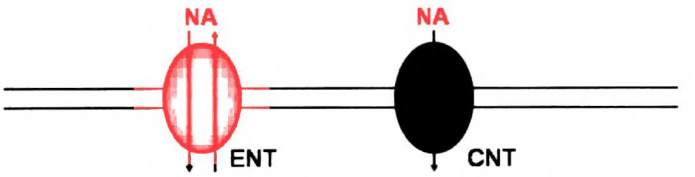
Table 1.3. Substrate specificity of endogenous nucleosides and nucleoside analog drugs.[1] The far left column lists the name of the substrate, and the five middle columns list the major nucleoside transporters. An "x" indicates that the substrate has been directly tested via radiolabled or electrophysiological studies, and found to be a substrate of the transporter. An "i" indicates that the substrate has been found to be an inhibitor of the transporter. An "n" indicates that the substrate has been tested with the transporter, and was found to be neither a substrate nor an inhibitor. A blank square means that particular substrate-transporter interaction has not yet been investigated. The far left column indicates the therapeutic usage of the substrate.

[1] This table was taken in part from the thesis of Dr. Lara Mangravite, and represents an updated version of much of the same information.

extensively in the treatment of leukemias, solid tumors, and viral infections including HIV (Clarke et al. 2002; Galmarini et al. 2003; Mangravite et al. 2003; Kong et al. 2004; Pastor-Anglada et al. 2004). In general, nucleoside analog drugs are hydrophilic molecules, and require some type of transporter mediated process in order to cross the plasma membrane; therefore, most nucleoside analog drugs are substrates of at least one of the nucleoside transporters (most commonly ENT1 or ENT2) (Pastor-Anglada et al. 2005). Figure 1.3 shows a typical pathway for a nucleoside analog drug. Non-response or lack of response to nucleoside analog drugs is a common problem, and it could be related to genetic variation in nucleoside transporters, a subject of this dissertation (Damaraju et al. 2003; Leabman et al. 2003).

Tissue Distribution

Tissue specific expression of nucleoside transporters has been examined in several studies. The method most commonly used to measure nucleoside transporter expression is quantification of transporter mRNAs (see Table 1.4 for nucleoside transporter expression in select tissues). ENT1 has been identified in virtually every tissue type that has been studied, and though expressed at low levels is believed to represent the major nucleoside transporter activity in many cell types (Griffith and Jarvis 1996; Lum et al. 2000; Pennycooke et al. 2001; Molina-Arcas et al. 2003). Immortalized cell lines commonly retain some ENT1 expression, although it can be lost in some cell lines that derive from cancers. ENT2 is also found in most tissues, but is often expressed at lower levels than ENT1. However, ENT2 is expressed at high levels in skeletal muscle, and is considered to be the predominant equilibrative nucleoside transporter in that tissue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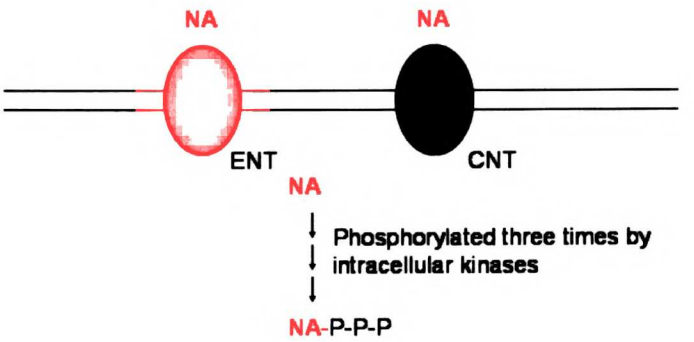
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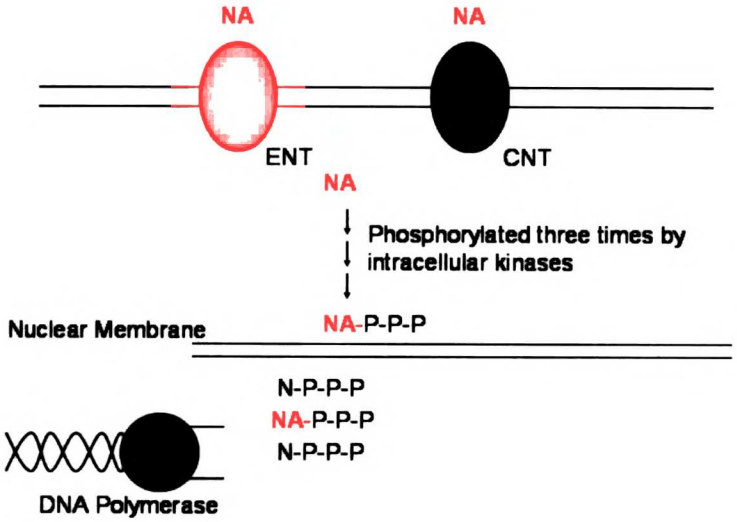
ENT
CNT
NA
Phosphorylated three times by intracellular kinases
NA-P-P-P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Phosphorylated three times by intracellular kinases
Nuclear Membrane
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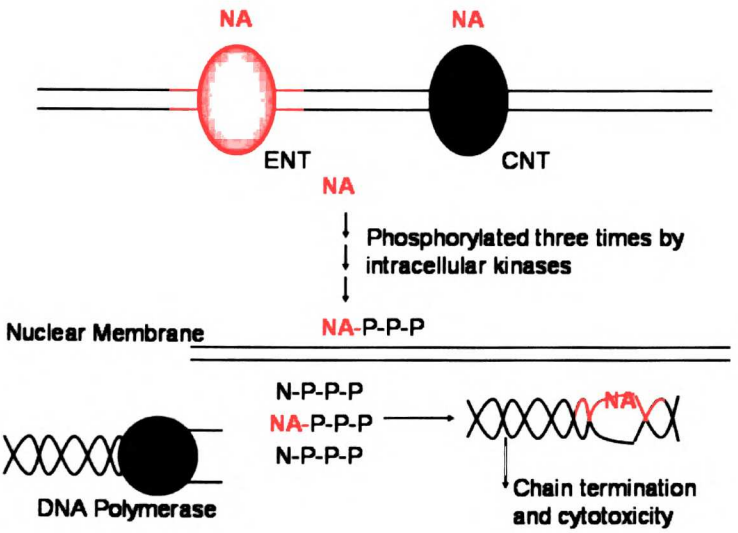
N-P-P-P
DNA Polymerase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Phosphorylated three times by intracellular kinases
Nuclear Membrane
NA-P-P-P
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NA
DNA Polymerase
Chain termination and cytotoxicity

Figure 1.3. Typical cytotoxic pathway of nucleoside analog drugs. Nucleoside analogs (NA) are transported into the cell in the same way as the naturally occurring nucleosides described in Figure 1.2 (top page 17). Similar to the endogenous nucleosides, the NA are phosphorylated three times by intracellular kinases (bottom page 17). The triphosphorylated nucleoside analogs then enter the nucleus where they compete with the natural pools of dNTPs for incorporation into the elongating DNA strand by DNA polymerase (top page 18). At high concentrations, the triphosphorylated nucleoside analogs are occasionally incorporated into the DNA strand by DNA polymerase (bottom page 18). The introduction of a non-natural base leads to a mismatch in base pairing that is difficult to repair. The exact mechanism of nucleoside analog drugs vary at this point, but a typical result is that the misincorporation leads to the termination of the elongating strand, and activation of a cytotoxic pathway that will eventually lead to cell death.

Gene Name	Protein	Tissues
SLC28A1	CNT1	Intestine, liver, kidney, jejunum
SLC28A2	CNT2	Kidney, liver, heart, brain, intestine, jejunum, colon, pancreas, spleen, skeletal muscle
SLC29A3	CNT3	Pancreas, mammary gland, trachea, bone marrow, placenta, intestine, liver kidney, prostate
SLC29A1	ENT1	Ubiquitous: erythrocytes, leukocytes, placenta, heart, liver, spleen, kidney, lung, brain, colon
SLC29A2	ENT2	Ubiquitous: highly expressed in skeletal muscle

Table 1.4. Tissue distribution of nucleoside transporters.

(Griffiths et al. 1997; Crawford et al. 1998; Lum et al. 2000; Pennycooke et al. 2001; Molina-Arcas et al. 2003). The higher expression of ENT2 in skeletal muscle may be related to its unique ability among the nucleoside transporters to transport nucleobases.

The expression of CNTs is in general more specialized: they are found at higher levels in epithelial tissues. hCNT1 is found in high abundance in the liver, but is also found in other epithelial tissues in the kidney, intestine, and choroid plexus (Huang et al. 1993; Anderson et al. 1996; Ritzel et al. 1997; Pennycooke et al. 2001). CNT2 is mainly expressed in liver, kidney, and intestine, and is also expressed at lower levels in other tissues, such as brain and skeletal muscle (Che et al. 1995; Wang et al. 1997; Pennycooke et al. 2001; Molina-Arcas et al. 2003). CNT3 is predominantly expressed in pancreas, trachea, bone marrow, and mammary gland (Pennycooke et al. 2001; Ritzel et al. 2001; Molina-Arcas et al. 2003).

The subcellular localization of CNT1, CNT2, ENT1, and ENT2 in epithelial cells has also been examined. ENT1 and ENT2 localize to the basolateral membrane of epithelial cells, whereas CNT1 is found on the apical membrane. CNT2 had a unique expression pattern, with about an 80% apical localization, and 20% basolateral localization (Mangravite et al. 2001). CNT3 has also been localized to the apical membrane (Mangravite et al. 2003). This distribution of the transporters suggests a transepithelial flux model in which nucleosides would enter via a concentrative nucleoside transporter located on the apical membrane, and then exit through the equilibrative nucleoside transporter on the basolateral membrane (see Figure 1.4) (Mangravite et al. 2003). The

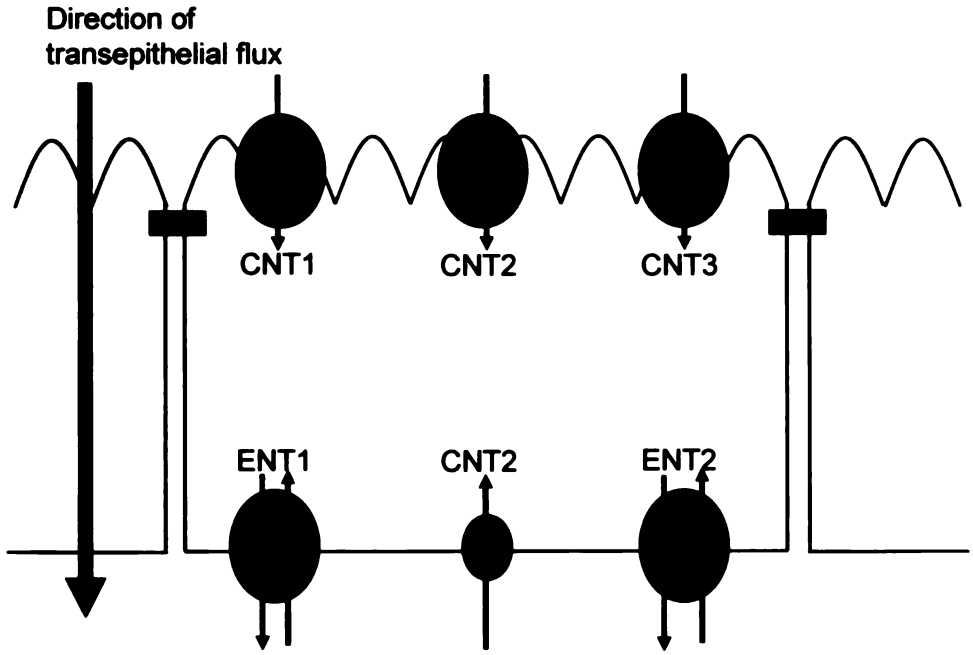

Figure 1.4. Model of epithelial transmembrane flux. The apical membrane is indicated at the top with the wavy border. The concentrative nucleoside transporters have been localized to the apical membrane of epithelial cells in MDCK cells. The equilibrative nucleoside transporters are shown on the basolateral membrane. The black rectangles between the cells represent the tight junctions that are found in epithelial cell types. The large black arrow indicates the overall direction of transport: nucleosides can enter the epithelial cells via the concentrative nucleoside transporters on the apical membrane, and then exit the cell via the equilibrative nucleoside transporters on the basolateral membrane. Nucleosides can also be reabsorbed into the epithelial cell from the basolateral membrane by the ENT transporters. The unique localization of CNT2 is indicated by the presence of a smaller amount of CNT2 on the basolateral membrane. The distribution of CNT2 is estimated to be about 80% apical and 20% basolateral.

reason for the altered localization of CNT2 as compared to the other three transporters is unclear, but may be related to a potential role in the salvage of purine nucleosides from the blood.

Regulation

Marcal Pastor-Anglada and his colleagues have taken the lead in the study of the factors influencing the regulation of expression of the CNT family. The results from select regulation studies from his laboratory and others are shown in Table 1.5. The Pastor-Anglada group first demonstrated that the uptake of ^{3}H-uridine increased after partial hepatectomy in rats suggesting a change in nucleoside transporter expression patterns (Ruiz-Montasell et al. 1993). It was later shown by the same laboratory that CNT2 mRNA levels increased after partial hepatectomy, and also that the regulation of CNT2 was likely to be cell cycle dependent (del Santo et al. 1998). Hormonal regulation of some CNTs has been observed in rat parenchymal cells following treatment with glucagons and insulin (Gomez-Angelats et al. 1996). In human lymphoblast cell lines, the B-cell activators PMA and LPS were shown to up-regulate CNTs but down regulate ENTs. This effect was mimicked by Tumor Necrosis Factor a (TNF-α) (Soler et al. 1998). CNT1 expression was shown to be sensitive to nutrient availability in the small intestine of rats, and CNT expression also increased after fasting, implying a role for the CNTs in the absorption of nucleosides from the diet (Valdes et al. 2000). The expression of CNT2 in rat liver was shown to be developmentally regulated by endocrine factors (del Santo et al. 2001). Interferon γ has also been shown to have a profound effect on the regulation of expression of CNT1 and CNT2. Interferon γ stopped proliferation in

Species/cell type	Causative agent/treatment	Effect observed	Reference
Rat liver	Partial hepatectomy	Increased uridine transport by rats in treatment group	Ruiz-Montasell et al 1993
Rat liver parenchymal cells	glucagon	Increase in Na^+-dependent uridine transport	Gomez-Angelats et al 1996
Rat liver parenchymal and hepatoma cells	Partial hepatectomy, induced proliferation	Expression of CNT2 mRNA is cell cycle dependent	del Santo et al 1998
Human B-lymphocyte cell lines	B-cell activators, TNF-α	TNF-α induces expression of CNT2, reduces expression of ENT1	Soler et al 1998
Human cultured umbilical vein endothelial cells	D-glucose	Elevated glucose concentrations resulted in a reduction of adenosine transport by ENT1	Montecinos et al 2000
Jejunal brush border membrane vesicles from fed and starved rats	Starvation	Expression of CNT1 is influenced by nutrient availability	Valdes et al 2000
Human cultured umbilical artery smooth muscle cells from diabetic and non-diabetic pregnancies	Insulin	Insulin increased ETN1-dependent adenosine transport in cells derived from non-diabetic pregnancies	Aguayo et al 2001
Rat hepatocytes isolated at different phases of development	Development	Na^+-dependent uridine transport was highest in adult hepatocytes	del Santo et al 2001
Murine bone marrow macrophages	Lipopolysaccharide (LPS)	LPS treatment upregulates expression of CNT1 and CNT2	Soler et al 2001
Rat hepatoma (FAO) cells	Cell cycle synchronization	Expression of CNT1 is cell cycle dependent	Valdes et al 2002

Tissues of diabetic rat, non-diabetic rat	Diabetes	Diabetic rats a had significant reduction in expression of ENT1 and ENT2 versus their matched controls	Pawelczyk et al 2003
Rat T-lymphocytes	Insulin and glucose	Insulin: increases ENT2 expression, decreases CNT2 expression. Glucose: lowers ENT1 expression at high concentrations	Sakowicz et al 2004
Human aortic smooth muscle cells	D-glucose	Glucose acts through the MAPK pathway to influence ENT1 expression	Leung et al 2005
Rat kidney	Microdisection of control, diabetic, and insulin treated kidney	Expression of CNT1, CNT2, and CNT3 is dependent on the section of kidney	Rodriguez -Mulero et al 2005
B-lymphocytes isolated from diabetic rat	Insulin and glucose treatment	Insulin influences ENT2 expression by PI3K signaling and CNT2 expression by MAPK signaling	Sakowicz et al 2005

Table 1.5. Summary of studies identifying factors involved in the regulation of expression of nucleoside transporters.

murine bone marrow macrophages, and also induced RNA synthesis of CNT1 and CNT2; the regulation of ENTs was also affected, but in the opposite manner (Soler et al. 2001).

Recently, it has been shown that CNT1 may also be under cell cycle-dependent regulation, at least in the rat hepatoma cell line FAO (Valdes et al. 2002). An emerging area of regulation of nucleoside transporters is the effect of insulin, glucose, and diabetes on nucleoside transporter expression. A study in rats showed that mRNA levels of rCNT1, rCNT2, rENT1, and rENT2 were significantly altered in diabetic heart, liver, and kidney compared to healthy animals (Pawelczyk et al. 2003). Another rat study focusing on the CNTs revealed that the effect of diabetes on concentrative nucleoside transporters in kidney was regiospecific. Diabetes caused a reduction in expression of all three CNTs in nearly all nephron segments, and this effect was not blocked by insulin treatment. However, diabetes increased the expression of both CNT1 and CNT3 in the glomerulous whereas insulin treatment caused a decreased expression of CNT1 and CNT3 (Rodriguez-Mulero et al. 2005). Experiments in rat lymphocytes have suggested that rENT2 and rCNT2 expression levels are highly dependent on insulin, but are independent of extracellular glucose concentrations (Sakowicz et al. 2004). Conversely, rENT1 expression was found to be sensitive to glucose concentration, but unaffected by insulin (Leung et al. 2005).

Related studies have examined the pathways by which insulin alters the expression of ENT2 and CNT2. This was found to occur by two independent mechanisms: PI3K signaling for ENT2, and MAPK signaling for CNT2 (Sakowicz et al. 2005). The MAPK

pathway also appears to be the signaling pathway for ENT1 expression as determined by glucose. This effect has been seen in different cell types including rat B lymphocytes (Sakowicz et al. 2005), and human umbilical vein endothelial cells (Montecinos et al. 2000; Aguayo et al. 2001). Our collective understanding of regulation of nucleoside transporters is poor. It is clear that the regulation of these transporters is complex, and the years to come will likely add to the growing knowledge of the mechanisms influencing nucleoside transporter expression.

Structure/function and topology

CNT1 and CNT2 are similar proteins, and share a 62.7% amino acid sequence identity; they also have some overlap in their substrate specificity, as both transporters are able to take up uridine and adenosine. However, CNT1 and CNT2 have unique substrate profiles for the other naturally occurring nucleosides. The protein domains and amino acid residues responsible for the observed specificity have been identified in a series of elegant studies using chimeric transporters and amino acid mutations. Specificity studies were first conducted with chimeric proteins between rCNT1 and rCNT2, and it was determined that transplanting two predicted TMDs from rCNT2 into rCNT1 converted the resulting protein from pyrimidine preferring to purine preferring (Wang and Giacomini 1997). The transplantation of one TMD from rCNT2 to rCNT1 was sufficient to alter the specificity from pyrimidine preferring to broadly selective. Further studies identified Ser318 in rCNT1 as the residue causing the pyrimidine preference, with the adjacent residue, Gln319 modulating this effect (see Figure 1.5a) (Wang and Giacomini 1999; Wang and Giacomini 1999). Later work on the human orthologs identified two

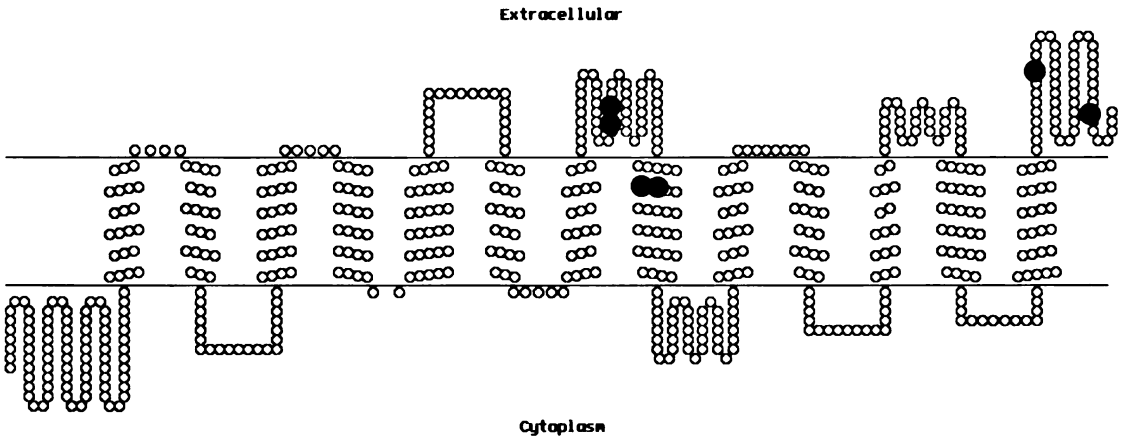

Figure 1.5a. Critical residues in CNT specificity and topology. A secondary structure prediction of CNT1 is shown, and individual residues of interest are color coded. Asn605 and Asn643 are shown in black. These two residues have been shown to be glycosylated, and therefore the loop containing them is extracellular. The red residue is Ser319; when this residue is mutated to the corresponding residue in CNT2, the resulting mutant protein becomes broadly selective. The neighboring residue, Gln320 orange, modulates this effect. The Ser319/Gln320 double mutant of CNT1 is able to transport a broad range of nucleosides at a similar rate as CNT1-reference. The blue residue, Ser353, is involved in the pyrimidine/purine preference, and the neighboring residue, Leu354 (orange) also modulates this effect. The CNT1 quadruple mutant CNT1-Ser319/Gln320/Ser353/Leu354 has its specificity switched from pyrimidine to purine preferring.

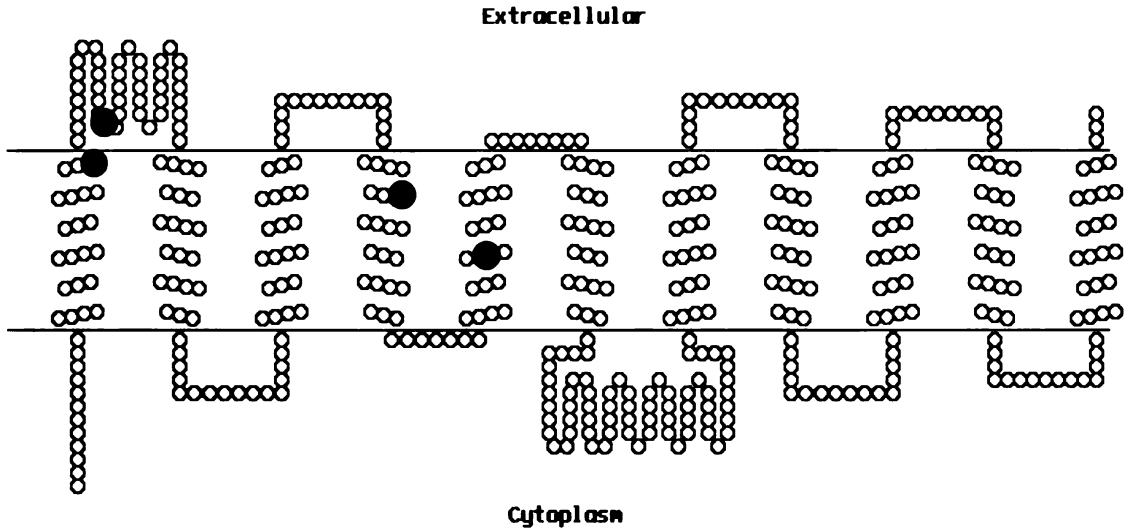

Figure 1.5b. Critical residues in ENT specificity and topology. A secondary structure of ENT1 is shown, and individual residues are highlighted. Glycosylation studies have shown that Asn48 is glycosylated, and it is shown in black. Residue 33 (red) in ENT1 and ENT2 has been shown to be responsible to the sensitivity of of these transporters to the coronary vasodilators dilazep and dipyridimole. Mutation of residue 154 (blue) eliminates the binding of NBMPR, and also decreases the affinity of ENT1 for adenosine and cytosine. Residue 184 (green) has been shown to be important in the proper folding/targeting to the plasma membrane.

sets of residues in adjacent TMDs that controlled the purine/pyrimidine preference of human CNTs. Mutation of a pair of residues from CNT1 to their equivalent residues in CNT2 caused the resulting double mutant CNT1 protein to exhibit a broadly selective phenotype, and the mutation of two additional residues caused the broadly selective quadruple mutant to become purine selective (Loewen et al. 1999). Therefore, the mutation of four residues in hCNT1 to their equivalents in hCNT2 was sufficient to transform the specificity from pyrimidine to purine preferring (Figure 1.5a). Human and rat nucleoside transporters have very similar specificities for the naturally occurring nucleosides, although this is not necessarily the case for nucleoside analogs, particularly in the case of CNT2 (Gerstin et al. 2002). Gerstin et al showed that rCNT2 transported more nucleoside analogs than did the human ortholog, hCNT2. Residues and domains responsible for species differences in interaction with nucleoside analogs are identified in this dissertation research (see Chapter 4).

Chimeric transporters have also been employed to study the ENTs. Human and rat ENT1 are both strongly inhibited by NBMPR, however, they differ in the inhibition profiles for the coronary vasoactive drugs dypyridamole and dilazep (hENT1 is inhibited by these compounds whereas rENT1 is not). Chimeric proteins between hENT1 and rENT1 revealed that the determinants for sensitivity to dipyridamole/dilazep inhibition reside in the region between TMDs 3-6, or amino acids 100-231 (Sundaram et al. 1998). Similar strategies were employed for determining the region of ENT2 that is responsible for nucleobase uptake (TMD 5-6). Various other studies have highlighted the importance of

individual residues in the ENTs (Figure 1.5b) (Hyde et al. 2001; Yao et al. 2001; Visser et al. 2002; SenGupta and Unadkat 2004).

Despite their similarities in function, the concentrative nucleoside transporters and the equilibrative nucleoside transporters are not closely related protein families. The differences in predicted topology reflect the separate origins of the two families. In order to determine the orientation of the ENT family members in the plasma membrane, glycosylation studies were performed on the prototype ENT transporter, rENT1. Through the use of N-glycosidase F and mutagenesis studies, it was determined that the Asn residue at position 48 was glycosylated and therefore extracellular (Figure 1.5b) (Sundaram et al. 2001). Other putative glycosylation sites showed no difference in electrophoretic mobility following treatment with endoglycosidase F, and were therefore not glycosylated and determined to be intracellular. The resulting model for ENTs consists of 11 transmembrane domains (TMD), with an intracellular N-terminus, an extracellular C-terminus, a large extracellular loop between the first and second TMDs (including the glycosylated N48), and a large intracellular loop between TMD 6 and 7 (Figure 1.5b) (Sundaram et al. 2001).

Similar glycosylation techniques were used to determine the topology of the CNTs using rCNT1 (Hamilton et al. 2001). The glycosylation studies on rCNT1 revealed that the protein was glycosylated on two Asn residues in the C-terminal tail of the protein, Asn605 and Asn643. The potential glycosidation site in the N-terminal portion of the protein, Asn4, was not found to be glycosylated, and is thus presumed intracellular. The

resulting model is 13 TMDs, with a large intracellular N-terminus, and a large extracellular C-terminal tail (Figure 1.5a).

Genetic variation in nucleoside transporters

Nucleoside analog drugs are among the most commonly utilized therapeutic agents. Despite the frequency with which they are used, the therapeutic response to nucleoside analog drugs can vary greatly from patient to patient. It has been hypothesized that polymorphisms in nucleoside transporters could contribute to some of the observed interindividual variability that is seen in nucleoside analog therapy. In order to investigate this possibility, DNA was isolated from a large population, and was screened for polymorphisms in the genes encoding the nucleoside transporters (Leabman et al. 2003). Variants were identified in all five major nucleoside transporters, and all protein-altering variants have been constructed and characterized in vitro (Osato et al. 2003; Gray et al. 2004; Badagnani et al. 2005; Owen et al. 2005; Owen et al. 2006) (see also Chapters 2, 3, and 7). Several variants with altered function were identified, although typically at low frequencies, with the notable exception of CNT1-1153del, which was found at a 3% allele frequency in the African American sample (Gray et al. 2004). CNT1-1153del caused a frameshift mutation that severly truncated the resulting protein, causing a complete loss of function. Non-functional variants were also found in CNT3. In this dissertation research, studies are performed to determine the function of coding region variants of CNT2 and ENT2 (see Chapters 2 and 3).

Summary

Fifteen years ago, nucleoside transporters were only known by their activity in cell lines, and little was known about how many there were or what properties they might have. Since that time, many of the original questions have been answered. It is now known that there are two major nucleoside transporter families (equilibrative and the concentrative), and five major plasma membrane nucleoside transporters (ENT1, ENT2, CNT1, CNT2, and CNT3). The substrate specificity of each of the major transporters for the naturally occurring nucleosides has been examined in detail, and many more studies have determined the specificity of the transporters for nucleoside analog drugs. There have also been some studies furthering our understanding of the structural components of the nucleoside transporters, that is, which residues define the specificity and topology. In this dissertation research, we focused on the following questions: What are the functional characteristics of coding region variants of CNT2 and ENT2? What are the molecular determinants in CNTs of specificity for synthetic nucleoside analogs? Are they the same residues that control the specificity for naturally occurring nucleosides? In the chapters to follow, we address these questions, with a focus on CNT2 and ENT2.

Summary of Chapters

The goals of my dissertation were threefold: to characterize genetic variants in the nucleoside transporters CNT2 and ENT2 with model substrates and clinically used drugs, to determine the molecular determinants of substrate specificity in CNT2 for 2CdA, and to characterize genetic variants in the organic cation transporter 1 (OCT1) for the anti-diabetic drug metformin and the anti-cancer drug oxaliplatin. All of these goals are described in detail in the chapters that follow. Below is a brief summary of each chapter, and our major findings.

In Chapter 2, we identify genetic variants of CNT2 in a large, ethnically diverse population. Six non-synonymous variants were found in CNT2. Variants were constructed and characterized in *X. laevis* oocytes. All six variants were able to transport the model compound inosine. We then kinetically characterized the high frequency variants with the anti-viral agent Ribavirin. One variant, CNT2-F355S was found to have an altered preference for uridine relative to that of CNT2-reference in dual label experiments. We also include a genetic analysis of CNT2, and describe CNT2 haplotypes.

Chapter 3 continues the study of nucleoside transporter variants by characterizing the protein-altering variants of ENT2. Five protein altering variants were found in ENT2, three non-synonymous variants, and two deletion variants. All five variants were characterized with the model substrates inosine and uridine, the nucleobase hypoxanthine, and some select nucleoside analog drugs. One of the deletion variants

resulted in a frameshift mutation which caused a truncation of the protein, and a loss of function. Another variant was found to have reduced function.

Chapter 4 examines the molecular determinants of substrate specificity of adenosine analog drugs. Prior to this study, it was known that there was a large species difference between the human and rat orthologs of CNT2 in their ability to transport the nucleoside analog drug cladribine (2CdA), although both orthologs exhibited a similar uptake of the model purine nucleoside inosine. In order to investigate the structural domains and amino acid residues that were responsible for the observed difference in 2CdA specificity, we constructed chimeric proteins of human and rat CNT2. We found that the determinants of 2CdA specificity reside in the C-terminal portion of the protein, and that the amino acid at position 345 in human CNT2 strongly influenced the ability of the protein to transport 2CdA.

In chapter 5, we continue the themes of genetic variation and specificity extended to another type of transporter: organic cation transporter 1 (OCT1). We construct the genetic variants previously identified in OCT1, and construct stable human cell lines. The function of OCT1 variants has been previously examined using the model compound MPP^+ in *X. laevis* oocytes, but here we functionally characterize the OCT1 variants using the widely prescribed anti-diabetic agent metformin. We found that several variants showed a reduction in function in their ability to transport metformin, including some high frequency variants.

Using the cell lines generated in Chapter 5, studies described in Chapter 6 examine their ability to transport the platinum based anti-cancer agent oxaliplatin. Using the variant cell lines, we show the uptake, cytotoxicity, and adduct formation of oxaliplatin. The results from the oxaliplatin study are in very close agreement with the metformin study, as the reduced function variants identified in Chapter 5 for metformin were also found to be reduced for oxaliplatin. We show a correlation between the uptake of platinum by cells expressing the variants of OCT1, and the resulting cytotoxicity.

Chapter 7 is the concluding chapter. It provides a review of the pharmacogenetics of nucleoside transporters, includes information about how many variants were found in each transporter, where they are located, the functional consequences of the variants, and an in-depth analysis of the differences between the ENT and CNT families on a population genetic level.

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Chapter 2

Genetic Analysis and Functional Characterization of Polymorphisms in the Human Concentrative Nucleoside Transporter, CNT2[2]

Abstract

The concentrative nucleoside transporter CNT2 (SPNT1; *SLC28A2*) plays a role in the absorption and disposition of naturally occurring nucleosides as well as nucleoside analog drugs. The goal of this study was to characterize genetic variation in *SLC28A2*, the gene encoding CNT2, and to functionally analyze non-synonymous variants of CNT2, as a first step towards understanding whether genetic variation in this nucleoside transporter contributes to variation in response to nucleoside analogs. As part of a larger study, DNA samples from an ethnically diverse population (100 African Americans, 100 European Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders) were screened and 10 coding region variants of CNT2 were identified (Leabman et al. 2003). The non-synonymous variants were then constructed and characterized in *X. laevis* oocytes. Six non-synonymous variants were identified, and all were able to transport guanosine. The four common variants (>1% in the sample population) were further characterized with the anti-viral nucleoside analog drug ribavirin. No differences were observed among the four common variants in the uptake kinetics of ^{3}H-ribavirin (K_m in μM: 35.6

[2] This data has been previously published: Owen, R.P. et al. "Genetic analysis and functional characterization of polymorphisms in the human concentrative nucleoside transporter, CNT2." *Pharmacogenetics and Genomics* **15**(2): 83-90, 2005. Reprinted with permission from the original copyright owner Lippincott Williams & Wilkins. PMID: 15861032.

± 9.27 for CNT2-reference, 40.7 ± 6.47 for CNT2-P22L, 31.2 ± 15.8 for CNT2-S75R, 26.7 ± 6.13 for CNT2-S245T, and 49.9 ± 14.6 for CNT2-F355S). The variant CNT2-F355S exhibited a change in specificity for the naturally occurring nucleosides, inosine and uridine. All non-synonymous variants of CNT2 took up guanosine, and the four variants examined showed no significant difference in ribavirin kinetics. However, CNT2-F355S (3% allele frequency in the African American sample) was found to alter specificity for naturally occurring nucleosides, which may have implications for nucleoside homeostasis.

Introduction

Many anti-cancer and anti-viral drugs are nucleoside analogs that are structurally similar to the naturally occurring nucleosides, and thus share many of the same biological pathways, including the nucleoside salvage pathway. The drugs enter cells via nucleoside transporters, after which they are modified by cellular enzymes (typically phosphorylated), and then go on to exert a therapeutic effect, often by interfering with DNA replication.

Concentrative nucleoside transporters (CNTs) are located on the plasma membrane, and are predominantly found in epithelial tissues. CNTs function as influx transporters, coupling the transport of nucleosides to the Na^+-gradient. The concentrative nucleoside transporter family (*SLC28*, reviewed recently by Gray *et al.*(Gray et al. 2004)) has three members that have been cloned and functionally characterized: the pyrimidine preferring CNT1 (*SLC28A1*) (Ritzel et al. 1997), the broadly selective CNT3 (*SLC28A3*) (Ritzel et al. 2001), and the purine preferring CNT2 (*SLC28A2*, SPNT1) (Wang et al. 1997), which also transports uridine. These transporters are involved in the absorption, disposition and targeting of nucleosides and nucleoside analog drugs.

There is considerable interindividual variation in therapeutic and adverse drug response to anti-viral and anti-cancer nucleoside analogs. For example, only 40 to 60% of patients with Hepatitis C respond to the nucleoside analog, ribavirin, given in combination with other agents (Pearlman 2004), (Main et al. 1998). The factors that contribute to variation in response to nucleoside analogs are largely unknown. Since CNT family members

participate in the influx of many different nucleoside analog drugs, it is conceivable that genetic variation in CNT family members contribute to problems of interindivdual variation that plague nucleoside analog therapy such as resistance and non-response.

In a recent study, we functionally characterized coding region variants of CNT1 that had been identified in an ethnically diverse sample population as part of our large pharmacogenomics of membrane transporters project (Gray et al. 2004). Our studies indicated that CNT1 is genetically and functionally diverse. In particular, we identified two variants of CNT1 that had no function and one variant with a change in the affinity for the anti-cancer pyrimidine nucleoside analog, gemcitabine (Gray et al. 2004).

The goal of the current study was to functionally characterize the non-synonymous polymorphisms identified in our sample population in the purine-preferring concentrative nucleoside transporter, CNT2. In addition, we characterized the population genetic parameters and the haplotypes of CNT2 and compared these to those of CNT1 and other membrane transporters examined in the study of Leabman *et al.* (Leabman et al. 2003). In contrast to CNT1, CNT2 had no amino acid variants that exhibited a loss of function. However, we found an amino acid polymorphism of CNT2 in African Americans that showed an altered specificity with respect to inosine and uridine in comparison to the reference CNT2. This specificity change was mimicked when the anti-cancer nucleoside analog, 5-fluorouridine, was used in place of uridine.

Materials and Methods

Genetic Analysis of CNT2. CNT2 variants were identified in the study of Leabman *et al.* through a combination of direct sequencing and DHPLC from an ethnically diverse population of 247 individuals. The nucleotide diversity (π), was calculated as described by Tajima (Tajima 1989). This parameter was calculated for synonymous and non-synonymous sites for the entire population, and for each racial and ethnic group. Non-synonymous and synonymous polymorphisms were defined as described by Hartl and Clark (Hartl and Clark 1997). Haplotypes were reconstructed from variant positions using PHASE, a Bayesian statistical method (Stephens et al. 2001). Before PHASE analysis, all singletons were removed from the analysis. Of the 24 distinct haplotypes identified by PHASE, 17 occurred multiple times. The 17 haplotypes were aligned using the ClustalW program, and the cladogram was constructed by hand.

Construction of CNT2-Reference and CNT2-Variant Plasmids. Human CNT2 cDNA was subcloned into the amphibian high-expression vector pOX (Jegla and Salkoff 1997). Reference CNT2, the most common allele in the population studied, was constructed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two nucleotide changes were required to make CNT2 reference, T899G and A225C. CNT2 reference was then used as a template to create five of the non-synonymous variants identified in the study of Leabman *et al.* One variant, CNT2-S75R was cloned separately in a different version of the pOX vector (Wang et al. 1997). Reference and variant sequences were confirmed by complete DNA sequencing at the UCSF Biomolecular Resourse Center.

Functional Screening of Variants in *X. laevis* Oocytes. *X. laevis* oocytes were chosen as an expression system because they express membrane proteins very well, they are easily maintained, and can be injected individually. *X. laevis* oocytes have also been shown to have low background transport of nucleosides, and to have no significant metabolism of some model nucleosides over a 30 min time period (Jarvis and Griffith 1991). The oocytes are also quite robust, showing no signs of cytotoxicity during the course of a kinetic study, even at nucleoside concentrations of 400 μM. Healthy stage V and stage VI *X. laevis* oocytes were injected with 30-50 ng of capped cRNA transcribed *in vitro* with T3 RNA polymerase from *Pvu*I-linearized pOX plasmids containing reference or variant CNT2. Spectrophotometry was used to determine the concentration of cRNA, and an aliquot of each RNA preparation was run on a 1% agarose gel to ensure that the RNA was not degraded. Injected oocytes were stored in modified Barth's solution at 18°C (changed 1-2 times daily) for 2-3 days of expression before uptake studies (Gray et al. 2004). Seven to nine oocytes were incubated in Na^+ buffer containing 0.1 μM ^{3}H-guanosine (Moravek, Brea, CA) and 1 μM unlabeled guanosine for 30 min at room temperature. Uptake was terminated by the removal of buffer containing the radioligand and the oocytes were washed five times in ice-cold choline buffer (in which choline is substituted for Na^+). Oocytes were then individually lysed by the addition of 100 μL of 10% SDS, and the radioactivity associated with each oocyte was determined by scintillation counting. Uptake of guanosine in oocytes expressing each variant was determined in 8-9 oocytes from a single frog. The functional studies were repeated in oocytes from at least one other frog. Data are presented as pmol guanosine/oocyte/30

minute uptake, and the error bars indicated are +/- SE. Uninjected oocytes incubated with the same reaction mix were used as a control. Statistics were done with ANOVA.

^{3}H-Ribavirin Kinetics in Oocytes Expressing Variants of CNT2. Ribavirin is a nucleoside analog drug that is used in the treatment of Hepatitis C and other diseases and is a known substrate of CNT2 (Patil et al. 1998). The interaction of ribavirin with variants that occurred at a frequency of greater than 1% in the sample population was examined. Reaction mixes contained 0.1 μM ^{3}H-ribavirin (Moravek Brea, CA), and eight different concentrations (0, 1, 5, 10, 50, 100, 250, and 400 μM) of unlabeled ribavirin. Uptake was measured according to the procedures described above. Michaelis-Menten analysis and curve fits were done with the graphing program Kaleidagraph. This experiment was repeated in oocytes harvested from two different frogs, and the mean and standard deviations of the resultant K_m values are reported.

IC_{50} Studies with Inosine and Ribavirin. CNT2-F355S and CNT2-reference were further examined in IC_{50} studies. The uptake of ^{3}H-inosine (Moravek, Brea, CA) (0.1 μM) was determined in the presence of various concentrations of unlabeled ribavirin (0, 1, 5, 10, 50, 100, 250, and 400 μM) in *X. laevis* oocytes. Studies were repeated three times. The curve fit and IC_{50} values were generated using Kaleidagraph.

Dual Label Studies with ^{3}H-Uridine and ^{14}C-Inosine. Groups of 8-9 oocytes were incubated with a reaction mix containing 1 μM ^{3}H-uridine (Moravek) and 1 μM ^{14}C-inosine (Moravek, Brea, CA) in Na^{+} buffer. The oocytes were incubated in this reaction

mix for 30 min as described above. The reaction mix was then removed by choline washes, the oocytes were lysed with 100 μL of 10% SDS, and then the radioactivity was assayed by liquid scintillation counting. The pmol/oocyte/30 min uptake values were then calculated for the two individual isotopes. The ratio of pmol inosine:pmol uridine was calculated and compared for CNT2-reference and CNT2-F355S. These experiments were repeated four times.

Results

Genetic Variation of SLC28A2

Genetic variation in *SLC28A2* was examined as part of a larger project, the goal of which was to identify polymorphisms in membrane transporter proteins. All 17 exons of *SLC28A2* were screened, as were the flanking intronic regions. Twenty-three total polymorphic sites were identified: 13 intronic sites, and 10 coding region sites. Four of the coding SNPs resulted in synonymous changes, and six involved non-synonymous changes. These data are available at www.pharmgkb.org. Table 2.1 summarizes the frequencies at which each polymorphic site was found in the total population sample as well as in the samples from the three ethnic groups with 30 or more subjects. Interestingly, we identified only two synonymous polymorphisms (allele frequencies greater than 1%), in our sample, both in African Americans; no synonymous variants were found in either the European American or Asian ethnic groups. To compare the variation of *SLC28A2* to that in other genes, we calculated nucleotide diversity, π. *SLC28A2* had a similar total nucleotide diversity, π_T, (6.37×10^{-4} vs. 5.09×10^{-4}) and a somewhat higher amino acid diversity, π_{NS} (5.1×10^{-4} vs.2.2×10^{-4}) compared to the average for the transporter genes studied by Leabman *et al.* (Leabman et al. 2003). The usual measure of selective pressure, i.e., π_{NS}/π_S, could not be calculated in the European American and Asian samples since π_S in these samples was 0; however, in the African American sample, the ratio was 0.55 (Leabman et al. 2003). A transmembrane prediction of CNT2 is shown in Figure 2.1 with non-synonymous changes shown in red and synonymous changes in green. Four of the six non-synonymous variants were found in the putative transmembrane domains.

Exon	Nucleotide Position[3]	Nucleotide Change	Amino Acid Position	Amino Acid Change[4]
1	**65**	**C→ T**	**22**	**Pro→ Leu**
3	**225**	**C→ A**	**75**	**Ser→ Arg**
5	488	T→ G	163	Leu→ Trp
5	531	T→ C	177	syn
7	**734**	**G→ C**	**245**	**Ser→ Thr**
10	993	T→ A	331	syn
10	**1064**	**T→ C**	**355**	**Phe→ Ser**
11	1140	G→ A	380	syn
12	1311	C→ T	437	syn
13	1384	C→ T	462	Leu→ Phe

Amino Acid Position	Total Population Frequency[5]	African American Frequency	European American Frequency	Asian Frequency
22	**0.399**	**0.182**	**0.629**	**0.448**
75	**0.374**	**0.157**	**0.67**	**0.183**
163	0.008	0	0	0.033
177	0.209	0.47	0	0
245	**0.101**	**0.227**	**0**	**0.017**
331	0.008	0.015	0	0
355	**0.014**	**0.035**	**0**	**0**
380	0.002	0.005	0	0
437	0.002	0.005	0	0
462	0.004	0.01	0	0

Table 2.1. Coding region variants of CNT2 identified in an ethnically diverse population sample.

[3] The nucleotide position is numbered such that 1 corresponds to the A in the initial ATG start codon.
[4] Synonymous changes are marked syn.
[5] The allele frequency of each variant in the total sample population is shown. Boldface type is used to highlight information on variants that are found at allele frequencies greater than 1% in the total population sample.

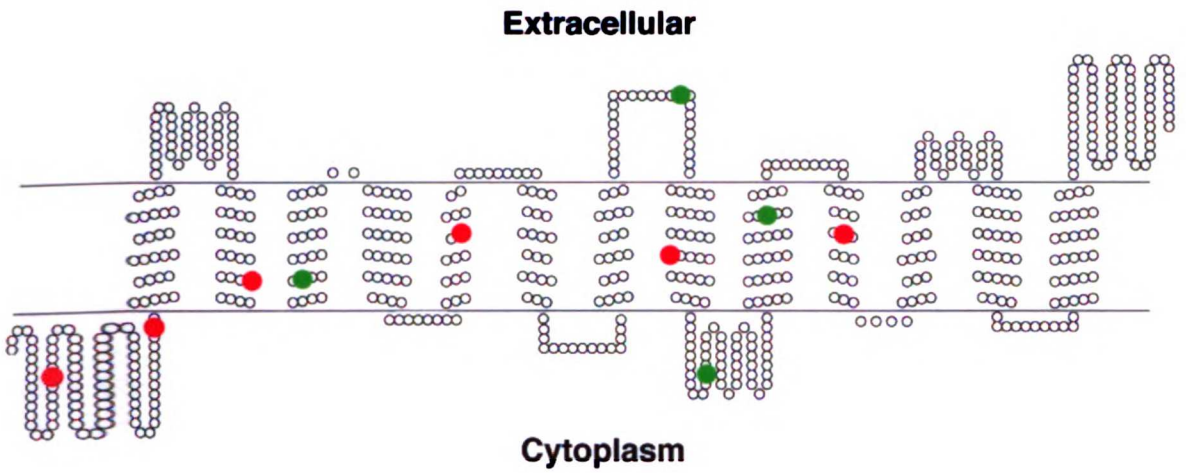


Figure 2.1. Transmembrane domain prediction of the secondary structure of CNT2. The variants identified as part of the study by Leabman *et al.* are shown. The positions of the non-synonymous variants are shown in red, and the synonymous variants are shown in green.

Haplotype analysis of SLC28A2

Although 23 total variants were identified during the screening process, nine were singletons (found on only one chromosome). After removing the singletons, haplotype analysis was performed. Twenty-four distinct haplotypes were estimated from the 14 variant positions by the haplotype estimation algorithm PHASE. Seventeen of these 24 haplotypes occurred more than once and are thus included in our further haplotype analysis. We named the haplotypes according to the conventions described by Nebert (Nebert 2002). The haplotype names and cladogram are shown in Figure 2.2. From the figure, it is clear that certain changes may have arisen multiple times in evolutionary history or that recombination events occurred. The most common haplotype in our sample was found in each ethnic group examined, and is also the predominant haplotype in the African American population. This haplotype was designated *1. The chimp sequence is the same at all the variant positions as *5A, which differs from *1 at only one synonymous position, suggesting either *5A or *1 is the ancestral haplotype. The second most common haplotype (*4A) is evolutionarily very distant from *1, and is the most common haplotype in the European American population.

Functional and Kinetic Characterization of CNT2 Variants

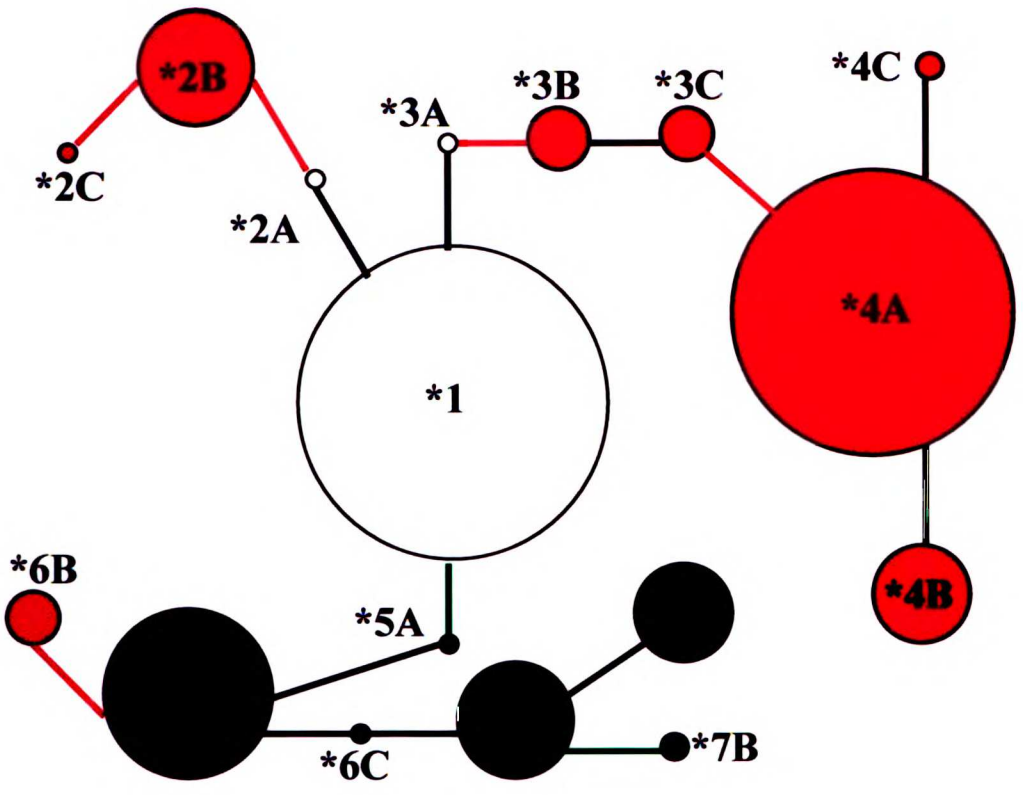
X. laevis oocytes were injected with cRNA from each of the six non-synonymous CNT2 variants. The oocytes were then incubated in Na^+ buffer with ^{3}H-guanosine for 30 min. Figure 2.3 is a representative graph of the guanosine uptake in a typical experiment. All of the variants were able to take up guanosine. None of the variants had significantly different guanosine uptake values with respect to the reference CNT2 ($p > 0.13$ by

Haplotype Name	*1	*2A	*2B	*2C	*3A	*3B	*3C	*4A	*4B
(-32) exon 1	C	-	-	-	C	C	-	-	-
65	C	C	T	T	C	C	C	T	T
225	C	C	C	C	C	A	A	A	A
488	T	T	T	G	T	T	T	T	T
531	T	T	T	T	T	T	T	T	T
(-24) exon 6	A	A	A	A	G	G	G	G	G
(+153) exon 6	A	A	A	A	A	A	A	A	A
(-135) exon 7	C	C	C	C	C	C	C	C	C
734	G	G	G	G	G	G	G	G	G
993	T	T	T	T	T	T	T	T	T
1064	T	T	T	T	T	T	T	T	T
(+38) exon 10	G	G	G	G	G	G	G	G	G
1384	C	C	C	C	C	C	C	C	C
(-26) exon 17	T	T	T	T	T	T	T	T	C
Frequency	35.3	1.4	4.3	0.4	0.4	1.2	1.2	30.4	3.7

Haplotype Name Continued	*4C	*5A	*6A	*6B	*6C	*7A	*7B	*7C
(-32) exon 1	-	C	C	C	C	C	C	-
65	T	C	C	C	C	C	C	C
225	A	C	C	C	C	C	C	C
488	T	T	T	T	T	T	T	T

531	T	C	C	C	C	C	C	C
(-24) exon 6	G	A	G	G	G	G	G	G
(+153) exon 6	G	A	A	A	A	A	A	A
(-135) exon 7	C	C	G	G	G	G	G	G
734	G	G	G	G	C	C	C	C
993	T	T	T	T	T	T	A	T
1064	T	T	T	C	T	T	T	T
(+38) exon 10	G	G	G	G	G	A	A	A
1384	C	C	C	C	C	C	C	C
(-26) exon 17	T	T	T	T	T	T	T	T
Frequency	0.6	0.4	9.8	1.4	0.4	4.7	0.6	3.3

Figure 2.2. a) Haplotypes of *SLC28A2*. The 17 haplotypes that were found more than once are shown. The variant positions are indicated in the column to the left, and are color coded with red indicating a non-synonymous change, green a synonymous change, and black an intronic change. The numbering for the coding region variant sites corresponds to the A in the initial ATG being 1. The intronic variant sites are numbered relative to the closest exon, with a negative number indicating nucleotides before the start of the exon, and a positive number indicating nucleotides after the end of the exon. The *1 haplotype was the most common in the population sample and was considered to be CNT2-reference. At the bottom of each column is the overall frequency of the haplotype in the population sample. These haplotypes account for 99.5% of the overall haplotypes found.



2.2 b) Cladogram of the common haplotypes of *SLC28A2*. The circumference of the circles is proportional to the overall frequency at which the haplotypes were found. White circles indicate no change in the coding region from *1, green circles have only synonymous changes, and red circles have non-synonymous changes. The lines connecting the haplotypes are also color coded to indicate the type of change the nucleotide substitution causes. Every line on the cladogram indicates one nucleotide change except the line between *5A and *6A in which there are two intronic changes.

The nucleotide change(s) corresponding to each line can be identified by comparison with Figure 2a. The *1 haplotype is the most common haplotype overall, and in the African American sample. It was also found in all 5 populations. The second most common haplotype, *4A was the predominant haplotype in the European American sample.

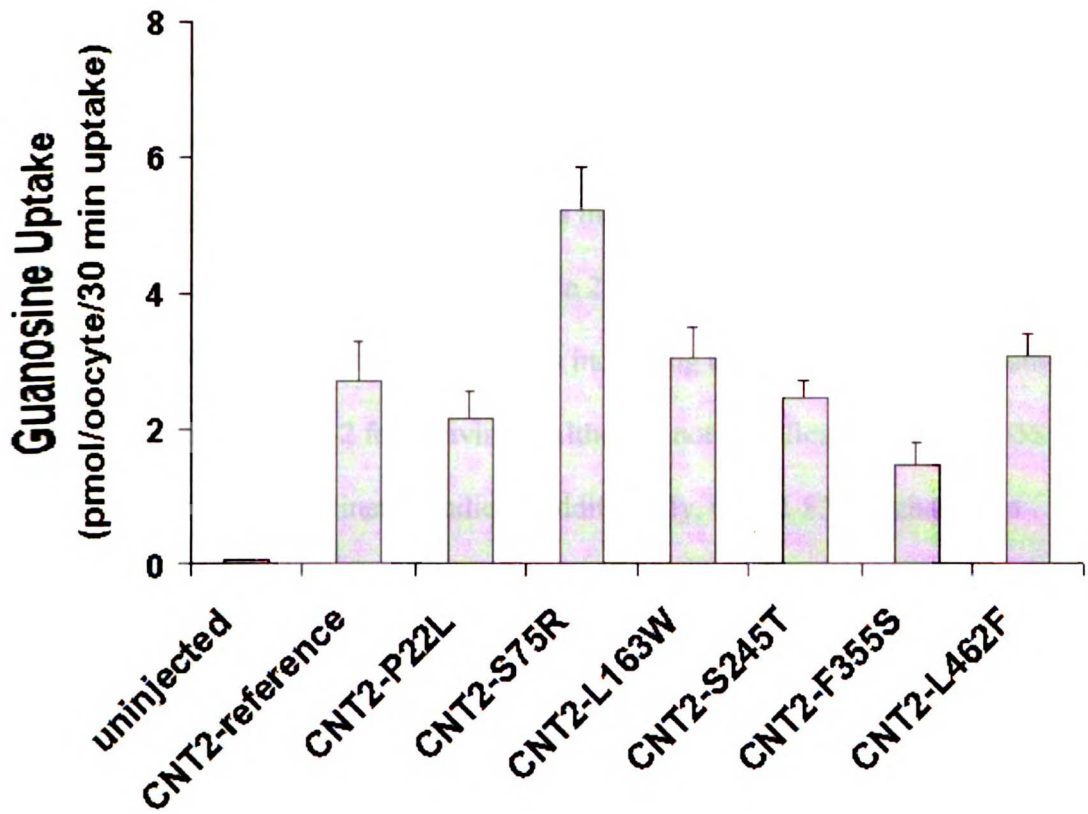


Figure 2.3. Uptake of guanosine in oocytes expressing the non-synonymous variants of CNT2. The bars represent the average pmol guanosine/30 min of all the oocytes in the group (8-9), and the error bars are the standard error of the mean. Guanosine uptake was not significantly different between the variants and the reference CNT2 with the exception of CNT2-S75R. This variant was present in a different version of the pOX plasmid, and therefore direct comparisons to the other variants should not be made. All non-synonymous variants of CNT2 were found to be functional with respect to guanosine.

ANOVA) with the exception of CNT2-S75R. Because CNT2-S75R was cloned separately, and was ligated into a different cloning vector, direct comparisons between the function of this variant and the reference CNT2 cannot be made. The kinetics of the guanosine analog, ribavirin was determined in the four protein-altering variants that occurred at frequencies greater than 1% (Table 2.2). No significant differences were observed in the K_m values of the four variants indicating that these common variants do not alter the affinity of CNT2 for ribavirin. Although not significant, CNT2-F355S had the highest ribavirin K_m in kinetic studies. Additionally, CNT2-F355S changed a conserved residue in a region of the protein that has previously been shown to be relevant for specificity (Loewen et al. 1999). Therefore, this variant was further examined in IC_{50} studies with ^{3}H-inosine and increasing concentrations of unlabeled ribavirin. No significant difference was found between the IC_{50} values of CNT2-reference and CNT2-F355S. A representative IC_{50} curve is shown (Figure 2.4). In light of our specificity results for CNT2-F355S, we also performed an IC_{50} study with ^{3}H-uridine and increasing concentrations of unlabeled ribavirin. The IC_{50} curves from this experiment were similar to those that we obtained using ^{3}H-inosine (data not shown). Again, there did not appear to be any significant difference in the IC_{50} of ribavirin between CNT2-reference and CNT2-F355S.

Specificity of CNT2-F355S

Preliminary electrophysiological analysis suggested that CNT2-F355S exhibited an altered preference for the naturally occurring nucleosides, inosine and uridine (data not shown). To explore this possibility, we incubated oocytes in a reaction mix containing

Variant	Average K_m[1] (μM)	Standard Deviation (μM)
CNT2-reference	35.5	9.27
CNT2-P22L	40.8	6.47
CNT2-S75R	31.2	15.8
CNT2-S245T	26.7	6.13
CNT2-F355S	49.9	14.6

[1] Values are the mean of two trials in each case.

Table 2.2. K_m values (μM) of CNT2-reference and the four non-synonymous variants found at a frequency of greater than 1% for the nucleoside analog drug, ribavirin.

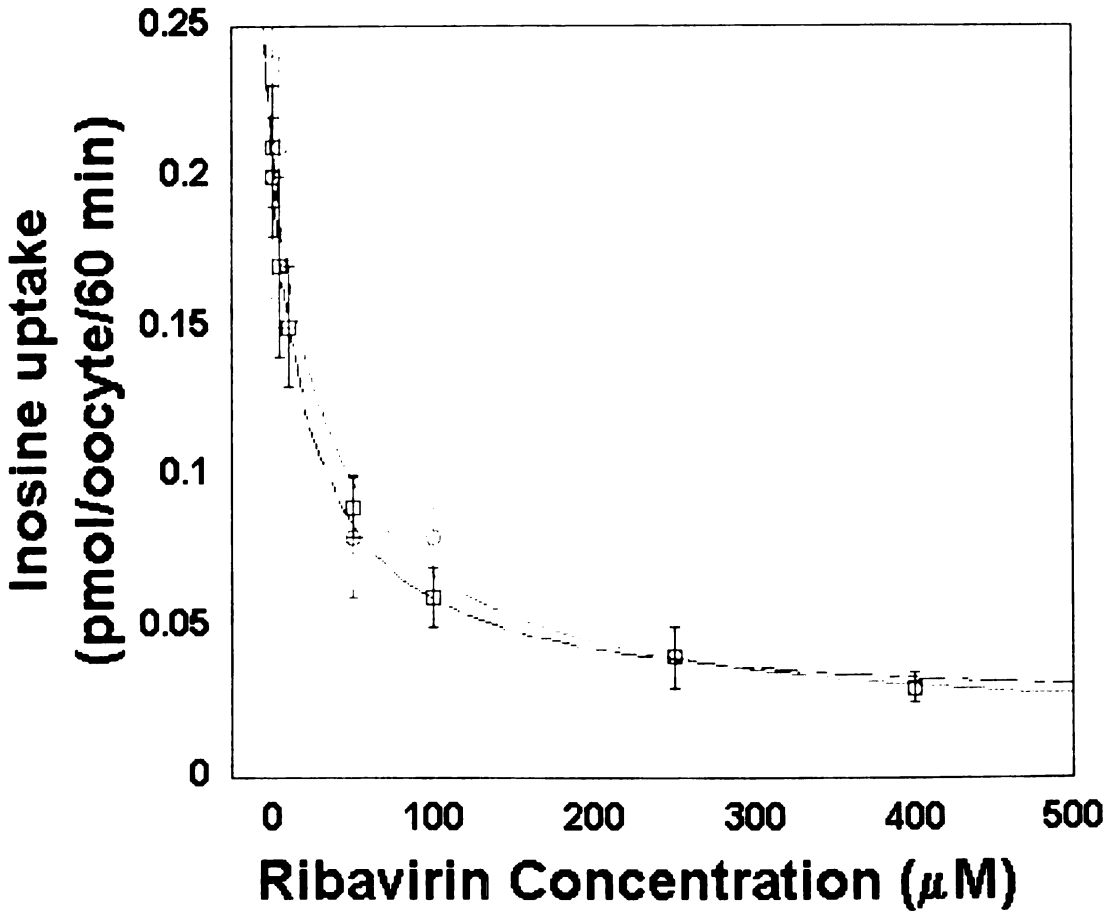


Figure 2.4. Potency of ribavirin in interacting with CNT2-reference and CNT2-F355S. Uptake of labeled inosine in oocytes expressing CNT2-reference is shown with the solid circles, and in oocytes expressing CNT2-F355S is shown with the open squares. The concentration of unlabeled ribavirin is shown on the x-axis, and the inosine uptake is shown on the y-axis. In this representative experiment, the IC_{50} values of ribavirin plus or minus standard deviations were 38.1 μM ± 14.9 μM for CNT2 reference and 25.8 μM ± 4.85 μM for CNT2-F355S.

equal concentrations (1 μM) of ^{14}C-inosine and ^{3}H-uridine. A low concentration was selected so that the total nucleoside concentration would be below the K_m for either nucleoside, and thus would be unlikely to cause any inhibitory effects. The ratio of inosine (pmol): uridine (pmol) was consistently higher for CNT2-F355S than for CNT2-reference (Figure 2.5). Possible reasons for this include an increase in inosine preference or a decrease in uridine preference or some combination of both. When the ratio of inosine uptake between the reference and variant transporters was examined it was unchanged, whereas the uridine uptake ratio between the reference and variant transporter shifted dramatically suggesting that CNT2-F355S causes a decrease in uridine selectivity. A specificity change in this region of the protein is consistent with earlier findings that indicate that residues in this vicinity are involved in purine and pyrimidine specificity (Loewen et al. 1999).

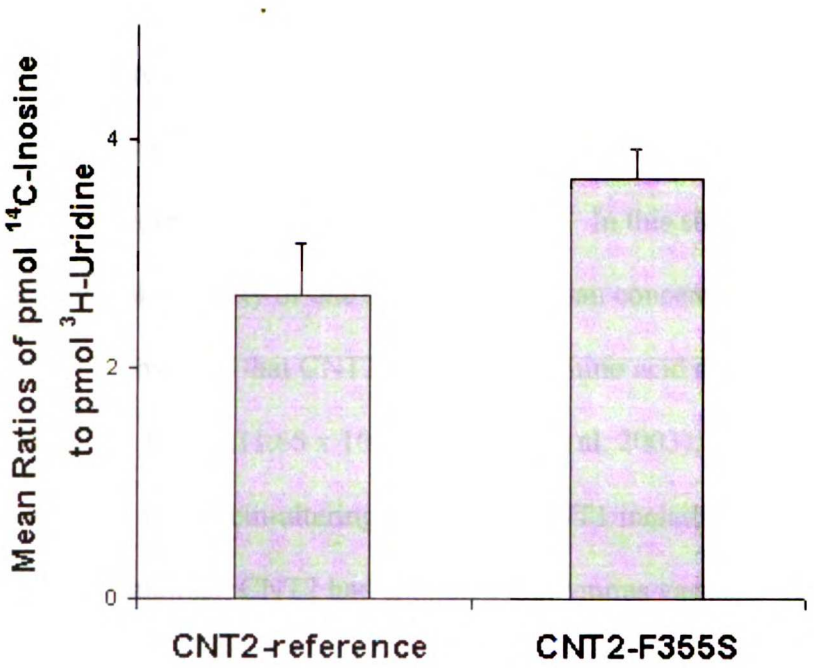


Figure 2.5. Specificity of CNT2-reference and CNT2-F355S for inosine and uridine. Inosine to Uridine uptake ratios for CNT2-reference and CNT2-F355S from oocytes injected with CNT2 reference or CNT2-F355S and exposed to 1μM ^{3}H-Uridine and 1μM ^{14}C-Inosine are shown. CNT2-F355S has a consistently higher Inosine:Uridine ratio than reference CNT2. P-value for the observed difference is <0.01.

Discussion

Although there are several genes that are involved in the disposition and metabolism of nucleosides and nucleoside analogs in the nucleoside salvage pathway, transporters such as CNT2 represent the first step in the pathway. In this study, we examined the genetic and functional diversity of one of the three human concentrative nucleoside transporters, CNT2. We observed that CNT2 had a lower amino acid diversity than that of its paralog, CNT1, (5.1×10^{-4} vs.11.65×10^{-4}) (Leabman et al. 2003), (Gray et al. 2004). Previously, we identified 14 protein-altering variants of CNT1 including 12 non-synonymous variants. In contrast, CNT2 had six non-synonymous variants. Both CNT1 and CNT2 have a large amino acid diversity in comparison to other transporter genes (11.65×10^{-4} and 5.1×10^{-4} versus 2.2×10^{-4} for average) suggesting that these proteins are less constrained than the average membrane transporter. Variation may be tolerated in CNT1 and CNT2 because these transporters along with CNT3 have overlapping functional characteristics and tissue distributions. A higher degree of amino acid variation is tolerated in non-essential and/or functionally redundant genes. Consistent with its lower nucleotide diversity, *SLC28A2* had fewer haplotypes in comparison to *SLC28A1*. In particular, *SLC28A2* had 17 non-singleton haplotypes whereas *SLC28A1* had 82 in the same population sample. The number of non-singleton haplotypes in *SLC28A2* is in agreement with the average number of haplotypes per gene (14) in the study of Stephens *et al.*, who estimated haplotypes in 313 different genes in 82 unrelated, ethnically diverse individuals (Stephens et al. 2001).

Four of the six non-synonymous changes are predicted to lie within transmembrane domains (see Figure 1), and three of these occur at evolutionarily conserved positions (CNT2-L163W, CNT2-S245T, CNT2-F355S). Previously, we and others have shown that substitutions at evolutionarily conserved positions are more likely to alter function than changes at residues that are not conserved (Leabman et al. 2003). However, our results indicate that all six amino acid variants of CNT2 including the three variants that alter evolutionarily conserved residues, retain function. Detailed kinetic analysis of four of the variants indicate that the variants do not alter the affinity for ribavirin. These data suggest that amino acid variants of CNT2 are not likely to contribute to variation in response to ribavirin. However, three points should be made. First, the kinetic analysis focused on only one substrate, ribavirin; it is possible that other drug substrates may exhibit different interaction kinetics with these CNT2 variants. Secondly, we cannot compare V_{max} values among the variants because expression level differences in the oocytes may be due to differences in experimental conditions that may alter mRNA stability or protein trafficking. Finally, it is important to note that functional characteristics in overexpression assays such as those used in our studies may not reflect *in vivo* function.

Whereas all of the non-synonymous CNT2 variants characterized in our study retained function, CNT2-F355S influenced the relative specificity of the transporter for the nucleosides, inosine and uridine. Variants influencing specificity are of particular interest because they can offer unique insights into the molecular determinants of the interactions between substrates and protein. In the case of CNT2-F355S, the relative

uptake of inosine and uridine in individuals carrying the variant may be altered. The Phe to Ser amino acid substitution represents a substantial chemical change, and has the highest Grantham value, a measure of the degree of chemical change, of any of the amino acid substitutions identified in CNT2 (Grantham 1974).

Previous specificity studies of both human and rat CNT family members have shown that the alteration of even one residue is enough to cause specificity changes (Loewen et al. 1999), (Wang and Giacomini 1999), (Wang et al. 1997). Amino acid residues 347 and 348 of human CNT2 have previously been shown to be relevant for purine and pyrimidine specificity (Loewen et al. 1999); it is therefore not surprising that residue 355 would also have specificity implications given its proximity to the previously identified residues. The presence of a variant that causes a specificity change among naturally occurring nucleosides could mean that individuals with that variant may have an alteration in purine and pyrimidine homeostasis. For example, CNT2-F355S may cause a relative decrease in uridine preference, which in turn could affect the purine/pyrimidine concentrations in tissues in which it is expressed. Because CNT2 is expressed in the intestine and kidney, total body purine homeostasis may be altered in individuals with CNT2-F355S. Finally, it is also possible that individuals carrying this variant may exhibit changes in the disposition of nucleoside analogs. For example, we showed that the ratio of inosine to 5-fluorouridine uptake was altered in oocytes expressing CNT2-F355S in comparison to those expressing the reference CNT2 (data not shown). Further studies are needed to elucidate the biological implications of the altered specificity of CNT2-F355S.

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Chapter 3

Functional Characterization and Haplotype Analysis of Polymorphisms in the Human Equilibrative Nucleoside Transporter, ENT2[6]

Abstract

The equilibrative nucleoside transporter 2 ENT2 (*SLC29A2*), is a bidirectional transporter that is involved in the disposition of naturally occurring nucleosides as well as a variety of anticancer and antiviral nucleoside analogs. The goal of the current study was twofold: to evaluate the function of genetic variants in ENT2 in cellular assays, and to determine the haplotype structure of the coding and flanking intronic region of the gene. As part of a large study focused on genetic variation in membrane transporters (Leabman et al. 2003), DNA samples from ethnically diverse populations (100 African-Americans, 100 European-Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders) were screened for variants in membrane transporters, including *SLC29A2*. Fourteen polymorphic sites in *SLC29A2* were found, including 11 in the coding region. Five protein-altering variants were identified: three non-synonymous variants, and two deletions. Each of the protein-altering variants was found at a very low frequency, occurring only once in the sample population. The non-synonymous variants and the deletions were constructed via site-directed mutagenesis, and were subsequently

[6] This data has been previously published: Owen *et al.* "Functional characterization and haplotype analysis of polymorphisms in the human equilibrative nucleoside transporter 2, ENT2." *Drug Metabolism and Disposition* 34(1): 12-15, 2005. Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved. Copyright © 2006 by the American Society for Pharmacology and Experimental Therapeutics. PMID: 16214850.

characterized in *Xenopus laevis* oocytes. All variants were able to take up inosine with the exception of ENT2-Δ845-846, which resulted in a frameshift mutation that prematurely truncated the protein. ENT2 showed very infrequent variation compared with most other transporter proteins studied, and it was found that five haplotypes were sufficient to describe the entire sample set. The low overall genetic diversity in *SLC29A2* makes it unlikely that variation in the coding region contributes significantly to clinically observed differences in drug response.

Introduction

Synthetic nucleoside analogs are widely used to treat a variety of diseases, including various types of cancer, HIV, Hepatitis C, and other illnesses (Barreiro et al. 2004; Byrd et al. 2004; Li et al. 2004; Pearlman 2004). Although nucleoside analogs are often the best available therapy, some common problems with nucleoside analog therapies occur, including lack of an initial response, or the development of resistance to therapy. One potential hypothesis for the ineffectiveness of some nucleoside analogs is genetic variation in nucleoside transporters, which function in the uptake of these compounds into cells. Genetic variation could lead to reduced function, or non-functional transporter proteins, which in turn could reduce the amount of drug able to enter the cell and therefore, the intracellular levels of the drug. Genetic variation in both concentrative and equilibrative nucleoside transporter members has been previously examined including CNT1 (Gray et al. 2004), CNT2 (Owen et al. 2005), CNT3 (Badagnani et al. 2005), and ENT1 (Osato et al. 2003).

In this report, we describe the functional characteristics of genetic variants of ENT2 that were identified previously in a large DNA sample set. ENT2 is thought to play a role in nucleoside analog therapy, as it has broad substrate specificity, and is able to transport many of the currently used nucleoside analogs (Baldwin et al. 2004), including the pancreatic cancer drug gemcitabine (Garcia-Manteiga et al. 2003), and fludarabine (Molina-Arcas et al. 2003), used in the treatment of chronic lymphocytic leukemia (CLL). In a recent study, it was reported that expression levels of ENT2, but not ENT1,

as measured by a Western Blot, correlated with cytotoxicity of fludarabine in cells isolated from patients with CLL (Molina-Arcas et al. 2005). This makes studies of genetic variation in ENT2 of particular interest for fludarabine therapy. The aim of this study was to evaluate the functional characteristics of amino acid variants of ENT2, and to describe its haplotype structure.

Materials and Methods

Genetic Analysis of ENT2. ENT2 variants were identified in the study of Leabman *et al.* (Leabman et al. 2003) through direct sequencing of its exons and flanking intronic regions in an ethnically diverse population sample of 247 individuals. The nucleotide diversity (π), was calculated as described by Tajima (Tajima 1989). Synonymous and non-synonymous polymorphisms were defined as described by Hartl and Clark (Hartl and Clark 1997). Haplotypes were reconstructed from variant positions using PHASE, a Bayesian statistical method (Stephens et al. 2001). Before PHASE analysis, all singletons were removed from the analysis. Five haplotypes were estimated through the PHASE analysis. The cladogram describing the ENT2 haplotypes was constructed by hand.

Construction of ENT2-Reference and ENT2-Variant Plasmids. Human ENT2 cDNA was subcloned into the amphibian high-expression vector pOX (Jegla and Salkoff 1997). ENT2 reference was used as a template to create the three non-synonymous variants, and two deletions of ENT2 identified in the study of Leabman *et al* (Leabman et al. 2003). Reference and variant sequences were confirmed by complete DNA sequencing at the UCSF Biomolecular Resourse Center.

Functional Screening and Kinetic Studies of Variants in *X. laevis* Oocytes. Healthy stage V and stage VI *X. laevis* oocytes were injected with 30-50 ng of capped cRNA transcribed *in vitro* with T3 RNA polymerase (mCAP^{TM} RNA Capping Kit, Stratagene, La Jolla, CA) from *Not*I-linearized pOX plasmids containing reference or variant ENT2

(*NotI* from New England Biolabs, Beverly, MA). Spectrophotometry was used to determine the concentration of cRNA, and an aliquot of each RNA preparation was run on a 1% agarose gel to ensure that the RNA was not degraded. Injected oocytes were stored in modified Barth's solution at 18°C (changed 1-2 times daily) for 2-3 days of expression before uptake studies. Seven to nine oocytes were incubated in Na^+ buffer containing 1 μM ^3H substrate. Several different substrates were used including inosine, guanosine, uridine, hypoxanthine, fludarabine, and gemcitabine. All radiolabeled compounds were purchased from Moravek (Brea, CA). The injected oocytes were incubated with ^3H substrate (1 μM) for 20 min; ENT2 transport using *X. laevis* oocytes has been previously reported at 30 min (Yao et al. 2001). In the inhibition study, unlabeled inosine (2 mM) was used to inhibit the uptake of ^3H-guanosine (1 μM). For the kinetic studies, 0.25 μM of ^3H-inosine was incubated with the unlabeled concentrations of inosine (1, 10, 50, 100, 500, 1000, 2000, 4000 μM), and the uptake was examined for 30 min. The V_{max} values are reported as pmol inosine/30 min uptake ± SE. For all the studies with oocytes, uptake was terminated by the removal of buffer containing the radioligand and the oocytes were washed five times in ice-cold choline buffer. Oocytes were then individually lysed by the addition of 10% SDS (100 μL), and the radioactivity associated with each oocyte was determined by scintillation counting. Uptake of all substrates in oocytes expressing each variant was determined in 8-9 oocytes from a single frog. The functional studies were repeated in oocytes from at least one other frog. Data are presented as pmol substrate/oocyte/20 minute uptake, and the error bars indicated are ± SE. Uninjected oocytes incubated with the same reaction mix were used as a control.

Results and Discussion

The variable sites in *SLC29A2* were identified through direct sequencing of all 11 *SLC29A2* exons, and some flanking intronic regions. A summary of all of the total variants identified, as well as the frequency in which they were found in the sample population is shown in Table 3.1 and can also be found at http://www.pharmgkb.org. Of the 14 variable sites, only five resulted in protein-altering variants, and were chosen for further study. All of the protein-altering variants were singletons, or variants that were found on only one chromosome in one individual; three of the five protein-altering variants were found in the African American sample, and the other two were found in the European American sample. ENT2 was unique among the 24 transporters studied by Leabman *et al.* in that it contained two deletion variants in the coding region. In total, 680 polymorphic sites were identified across the 24 transporter genes, but only five of these sites were deletions found in the coding region of the gene, two of which were found in *SLC29A2*. The two deletions likely arose from separate mechanisms because one is near the middle of an exon whereas the other is at the intron-exon boundary. The overall variation in *SLC29A2* was much lower than the average found in the other genes in the studies of Leabman *et al.* (Leabman et al. 2003); the π_T of *SLC29A2*, a measure of nucleotide diversity, is 1.64×10^{-4}, versus the average π_T of the genes 5.09×10^{-4}. However, the variation in *SLC29A2* was similar to that observed for *SLC29A1* (Osato et al. 2003). The low overall variation in the equilibrative nucleoside transporter family suggests that these two transporter genes are under high selective pressure, with non-synonymous variation being highly selected against. This contention is also supported by

Exon	Nucleotide Position	Nucleotide Change	Amino Acid Position	Amino Acid Change	Frequency
1	13	G->T	5	D5Y	.002
3	30	C->G	synonymous		.006
3	66	G->A	synonymous		.002
3	93	C->A	68	N68K	.002
4	281	C->T	94	P94L	.002
4	288	G->A	synonymous		.002
5	551-556	deletion	184-186	S184M, deletes G185 and V186	.002
8	-50	G->A	intronic		.193
8	795	G->A	synonymous		.002
8	845-846	deletion	282	frameshift	.002
9	927	C->T	synonymous		.019
10	+27	G->A	intronic		.002
11	-11	T->C	intronic		.029
11	1233	G->A	synonymous		.002

Table 3.1. Genetic variants in ENT2 identified in DNA samples from 247 ethnically diverse subjects. The exon in which the change occurred, as well as the position of the nucleotide in the exon or flanking intronic region is also shown. The amino acid residue affected by the nucleotide change is indicated where appropriate, and the nature of the change is described. The frequency at which each variant occurred in the sample population is also indicated; a frequency of .002 indicates that the variant was found on one chromosome in one individual.

the greater frequency of synonymous variants in both genes, which would not result in a change in the encoded protein. Although there are no reports of an ENT2 knockout mouse, an ENT1 knockout mouse is viable and fertile (Choi et al. 2004). Consistent with its low variability, ENT2 has few haplotypes (in the coding and flanking intronic region), which are shown in the cladogram in Figure 3.1. The *1 and *2 haplotypes account for nearly 95% of the overall haplotypes found, with *1 comprising about 75% of the population sampled. This haplotype profile is similar to that observed for ENT1, but showed considerably lower variability than the haplotype profiles of CNT family members (Gray et al. 2004), (Owen et al. 2005), (Badagnani et al. 2005).

Functional studies in oocytes revealed that ENT2-Δ845-846 was not able to take up inosine (or guanosine). In contrast, the other variants (as well as ENT2-reference) were able to transport both substrates (Figures 3.2a and 3.2b). ENT2-Δ845-846 was unable to transport inosine, because the deletion of two base pairs produced a change in the reading frame, which results in a severe truncation of the protein, and a subsequent loss of function. The variants ENT2-D5Y and ENT2-Δ551-556 showed reduced inosine uptake when compared to ENT2-reference, with ENT2-D5Y reaching statistical significance: $p=0.048$ for ENT2-D5Y and $p=0.061$ for ENTΔ551-556 (Figure 3.2a). The loss of six base pairs in ENT2-Δ551-556 results in a two amino acid deletion, and a non-synonymous change of a third residue.

The uptake of a diverse array of substrates by ENT2 and its variants was examined (Figure 3.3). Included in the analysis was a model purine (inosine), a model pyrimidine

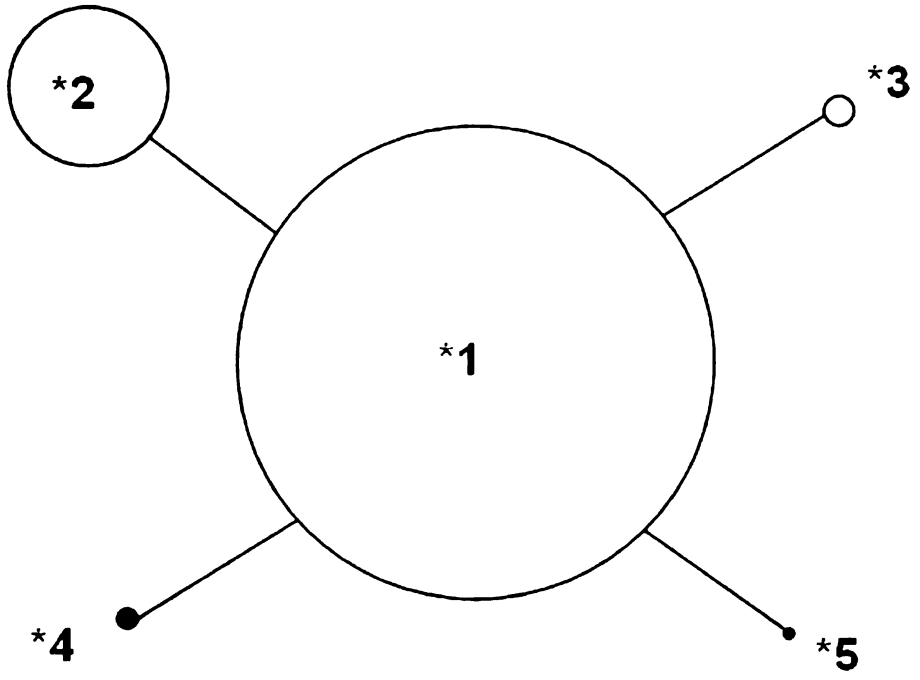


Figure 3.1. Cladogram of ENT2. PHASE analysis of the ENT2 haplotypes estimated only five distinct haplotypes. *1 accounted for the largest fraction of the population sample, and is considered to be reference ENT2. The *2 and *3 haplotypes have a base pair substitution in the flanking intronic regions. The *4 and *5 haplotype each encode a synonymous change, and are found at much lower frequency. The circumference of the circle is proportional to the frequency with which the haplotype was found within the sample population. The *1 and *2 haplotypes were found in every ethnic group, but *3, *4, and *5 were found in the African American sample only. None of the protein-altering variants that we examined are included in the haplotype structure because all singletons were removed prior to haplotype analysis.

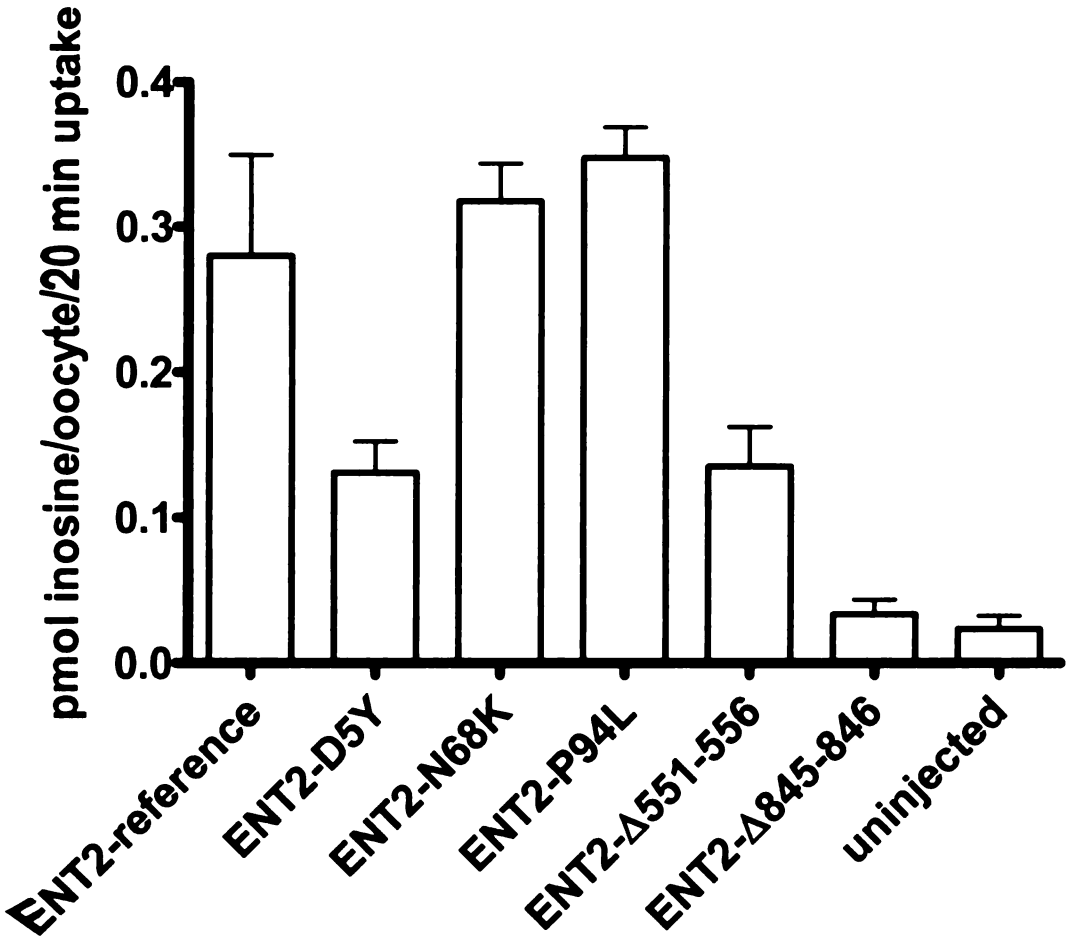


Figure 3.2. a) Uptake of inosine in oocytes expressing the reference ENT2 and five protein altering variants. Oocytes injected with cRNA encoding ENT2-reference, all five protein-altering variants of ENT2, and uninjected oocytes were incubated with ^{3}H-Inosine (1 μM). ENT2-Δ845-846 was not able to transport inosine.

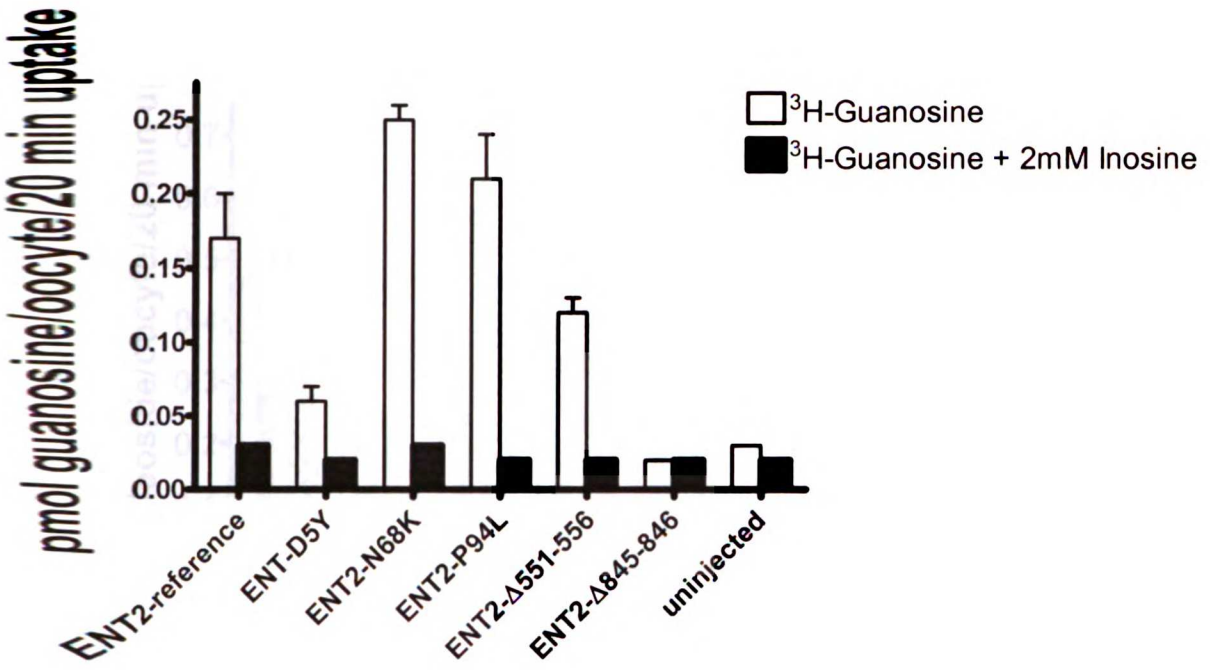


Figure 3.2 b)Transport of guanosine by ENT2 and its variants is inhibited by inosine. The uptake of ^{3}H-guanosine (1 μM) by ENT2-reference and its variants was inhibited by the addition of inosine (2 mM). Open bars represent the uptake of radiolabeled guanosine alone, and dark bars indicate the uptake of radiolabeled guanosine inhibited by insoine.

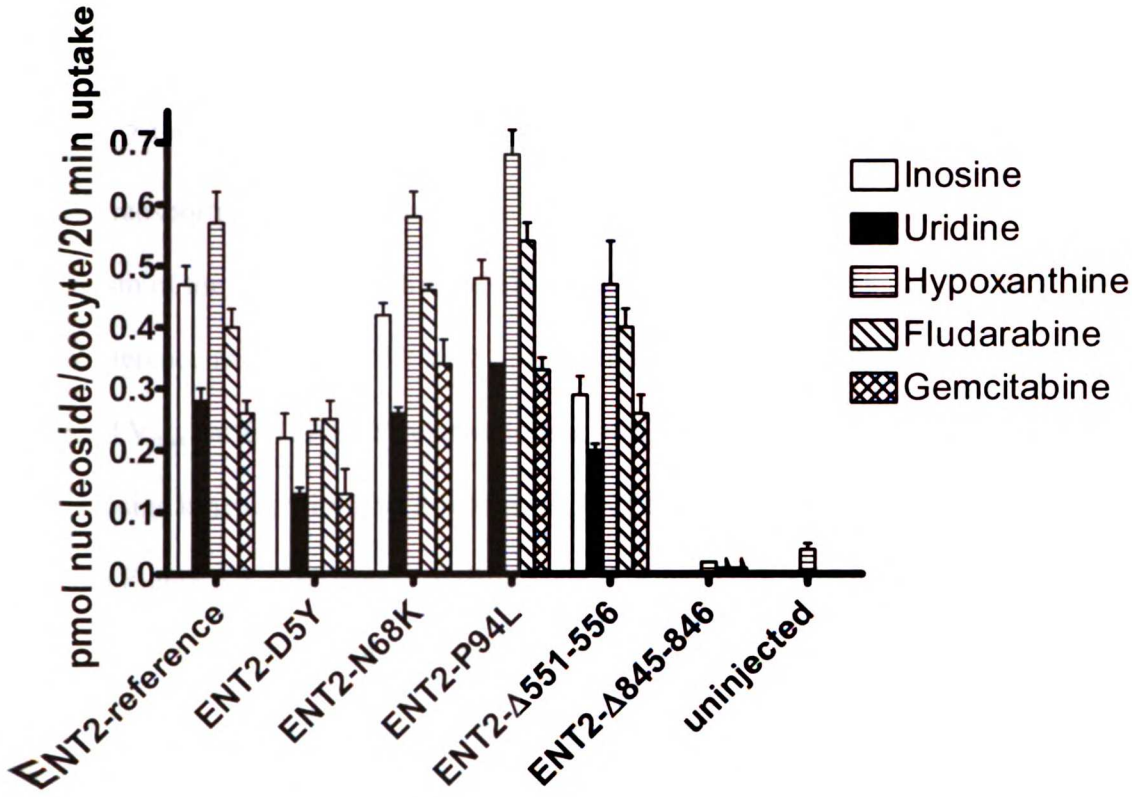


Figure 3.3. Uptake of a panel of substrates by ENT2-reference and its coding region variants. ENT2-reference and its variants are able to transport a variety of different compounds. The bars are coded according to the legend to the right of the figure. ENT2-Δ845-846 is unable to transport any of the substrates, whereas all the other variants can. ENT2-D5Y appears to have globally reduced function, and ENT2-Δ551-556 may have a reduced affinity for inosine relative to the other substrates.

(uridine), the nucleobase hypoxanthine, as well as the nucleoside analog drugs gemcitabine and fludarabine. Transport profiles were similar regardless of the substrate, with ENT2-D5Y exhibiting reduced function for all tested substrates, and ENT2-Δ845-846 not transporting any of the substrate panel. In order to gain insight into the mechanism of the reduced function of ENT2-D5Y, we performed kinetic studies with ENT2-reference and ENT2-D5Y with inosine and fludarabine. Both compounds showed a reduced V_{max} for ENT2-D5Y relative to that of ENT2-reference. Representative curves of the inosine kinetics with ENT2-reference, ENT2-D5Y, and uninjected oocytes are shown in Figure 3.4. The observed difference in Vmax (958 ± 53.6 pmol inosine/oocyte/30 min uptake vs. 706 ± 68.1 pmol inosine/oocyte/30 min uptake for ENT2-reference and ENT2-D5Y respectively) is statistically significant (p=0.03), while the respective K_m values were not statistically significant (p=0.45). These data support that the mechanism of reduction in activity of ENT2-D5Y is due to a reduced V_{max}, possibly reflecting a reduced turnover rate constant or a reduction in the number of functional transporters expressed on the plasma membrane.

In summary, the low genetic and functional variation observed in ENT2 suggests a critical physiological role, similar to its homolog ENT1. Variants with altered function were observed in ENT2; however, because of their low frequency, these variants are unlikely to be a major source of variability in drug response. Our data do not explain previous studies in which enhanced ENT2 expression has been associated with response to anticancer drugs (Molina-Arcas et al. 2005). It is possible that polymorphisms in noncoding regions of ENT2 may explain variation in the expression levels of this gene.

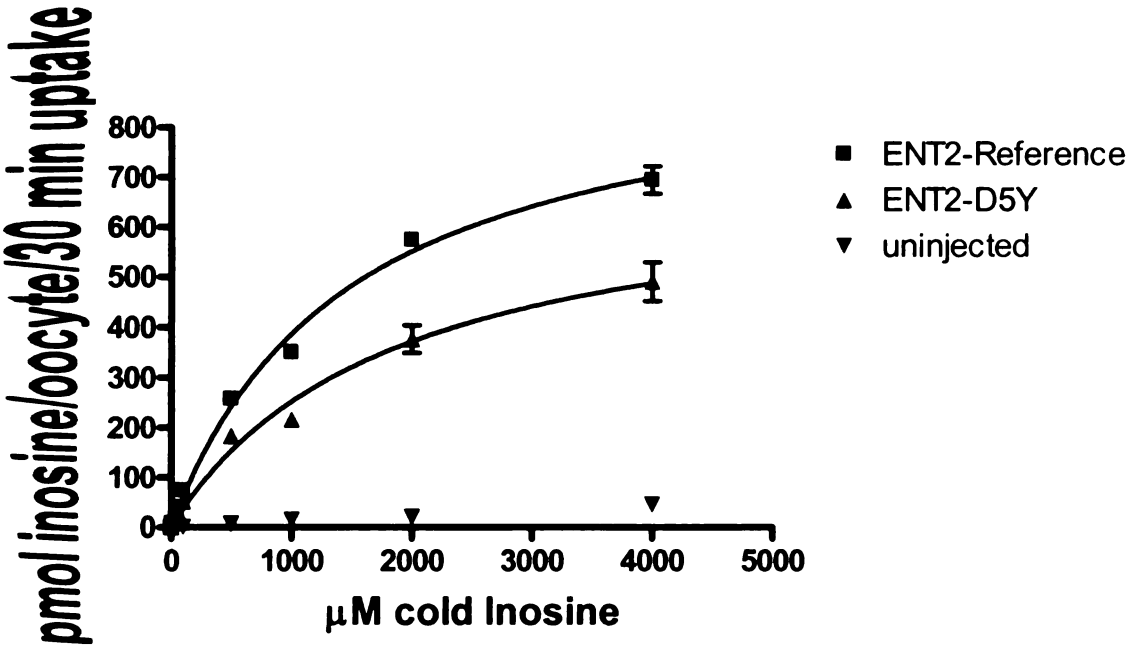
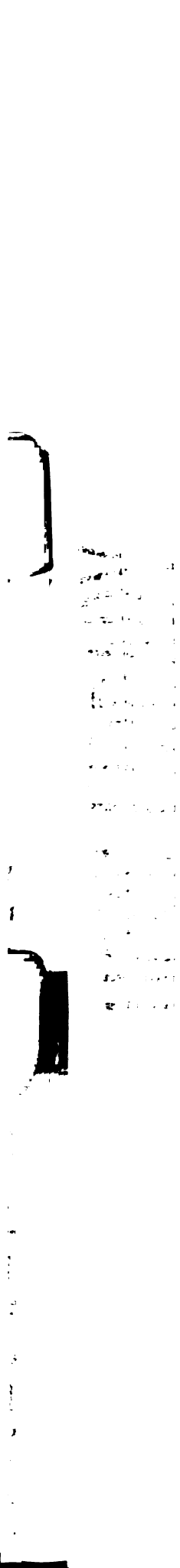


Figure 3.4. Inosine kinetics by ENT2-reference and ENT2-D5Y. Oocytes injected with either ENT2-reference or ENT2-D5Y were incubated with 0.25 μM ^{3}H-inosine with eight different concentrations of unlabeled inosine (1, 10, 50, 100, 500, 1000, 2000, and 4000 μM) for 30 min, and the counts associated with each concentration were plotted. Uninjected oocytes were used as a control to show the low background transport of the oocytes. Error bars represent standard error for each condition. The V_{max} of ENT2-reference is significantly higher than the V_{max} of ENT2-D5Y ($p=0.03$).



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Chapter 4

Molecular Determinants of Specificity for Synthetic Nucleoside Analogs in the Concentrative Nucleoside Transporter, CNT2[7]

Abstract

Members of the concentrative nucleoside transporter (CNT) family (*SLC28*) mediate the transport of naturally-occurring nucleosides, and nucleoside analog drugs across the plasma membrane of epithelial cells. Each of the three CNT family members has a distinct specificity for naturally-occurring nucleosides and residues that contribute to the specificity of each transporter have been identified. In contrast, the molecular determinants of specificity for synthetic nucleoside analogs are not known. In this study, we take advantage of the large species difference that exists between human and rat CNT2 (hCNT2 and rCNT2) in their ability to transport the nucleoside analog drug cladribine, 2CdA, (rCNT2 >>>hCNT2) to identify the critical domains and amino acid residues that contribute to the observed difference in specificity between CNT2 orthologs. Using chimeric proteins of human and rat CNT2, we determined that the C-terminal half of CNT2 contained the determinants of 2CdA selectivity. We replaced key residues in the C-terminus of hCNT2 with the equivalent residue in rCNT2. One residue in the C-terminal portion of CNT2 was found to significantly contribute to 2CdA selectivity: hCNT2-S354A. This mutant caused an increase in 2CdA uptake of 5-6 fold over hCNT2. The 2-chloro pharmacophore, rather than the 2'deoxyribose was

[7] This data has been previously published: Owen, R.P., et al. "Molecular determinants of specificity for synthetic nucleoside analogs in the concentrative nucleoside transporter, CNT2." *Journal of Biological Chemistry* **281** (36): 26675-82, 2006. Reprinted with permission of the American Society for Biochemistry and Molecular Biology. Copyright © 2006 by the American Society for Biochemistry and Molecular Biology. PMID: 16840788

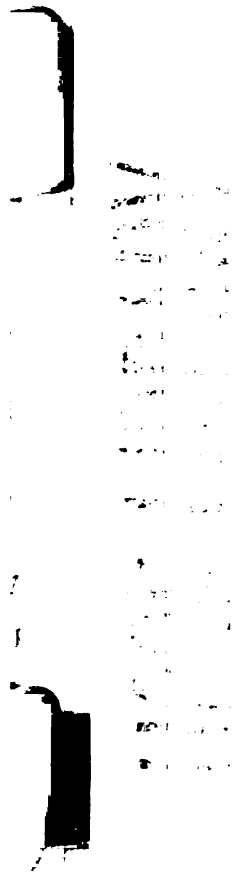
responsible for the reduced 2CdA uptake by hCNT2. Our data are consistent with a model in which an increased capability for hydrogen bonding in critical amino acids that reside in the C-terminus of rCNT2 contributes to its enhanced selectivity for 2CdA.

Introduction

The concentrative nucleoside transporter (CNT, *SLC28*) family consists of Na^+-dependent influx transporters located on the plasma membrane of many different epithelial cell types (Pennycooke et al. 2001; Gray et al. 2004). There are three members of the CNT family, and each has a distinct specificity for naturally occurring nucleosides: CNT1 prefers pyrimidine nucleosides, CNT2 prefers purine nucleosides, but also transports uridine, and CNT3 transports both purine and pyrimidine nucleosides (Huang et al. 1994; Wang et al. 1997; Ritzel et al. 2001). Because of their unique specificity and their reasonably high sequence identity (e.g. 62.7% for CNT1 and CNT2) the molecular determinants of substrate specificity has been a subject of great interest. Studies using chimeric transporters and site-directed mutagenesis have revealed large structural domains and key residues responsible for specificity of the naturally occurring nucleosides (Wang and Giacomini 1997; Wang and Giacomini 1999; Wang and Giacomini 1999). These studies have mapped critical amino acid residues responsible for purine and pyrimidine specificity. Notably, switching two sets of amino acid residues in CNT1 to their equivalent residues in CNT2 is sufficient to change the specificity of CNT1 to that of CNT2 (Loewen et al. 1999; Wang and Giacomini 1999).

In addition to the large specificity differences among paralogs of concentrative nucleoside transporters, there are striking specificity differences among species orthologs particularly with respect to synthetic nucleoside analogs. Evolutionary pressures exist on orthologs to maintain their specificity for naturally occurring nucleosides, however, such pressures do not exist for synthetic nucleoside analogs. Thus, striking species differences may exist among orthologs in their specificity for drugs. For example, whereas human and rat CNT2 (hCNT2 and rCNT2, respectively) have very similar specificities for naturally occurring nucleosides, there are notable specificity differences for nucleoside analogs (Gerstin et al. 2002). As shown in Figure 4.1a, hCNT2 and rCNT2 exhibit comparable rates of transport for the naturally occurring nucleoside, inosine, whereas the orthologs differ dramatically in their ability to transport the nucleoside analog, cladribine (2CdA), a drug used in the treatment of various leukemias and lymphomas (Gerstin et al. 2002; Byrd et al. 2004). In particular, rCNT2 transports 2CdA at a substantially greater rate than does hCNT2 (Figure 4.1a and b). Similar species differences in transport rates between human and rat CNT2 have been previously observed for the anti-viral nucleoside analog adenosine arabinoside (Ara-A) (Gerstin et al. 2002).

The goal of this study was to identify the molecular determinants of substrate specificity for the nucleoside analog drug 2CdA by taking advantage of the observed difference in rate of transport of 2CdA between human and rat CNT2 orthologs. Since the two CNT2 orthologs are ~80% identical at the amino acid level, identifying key residues and domains that are responsible for the specificity differences is experimentally tractable



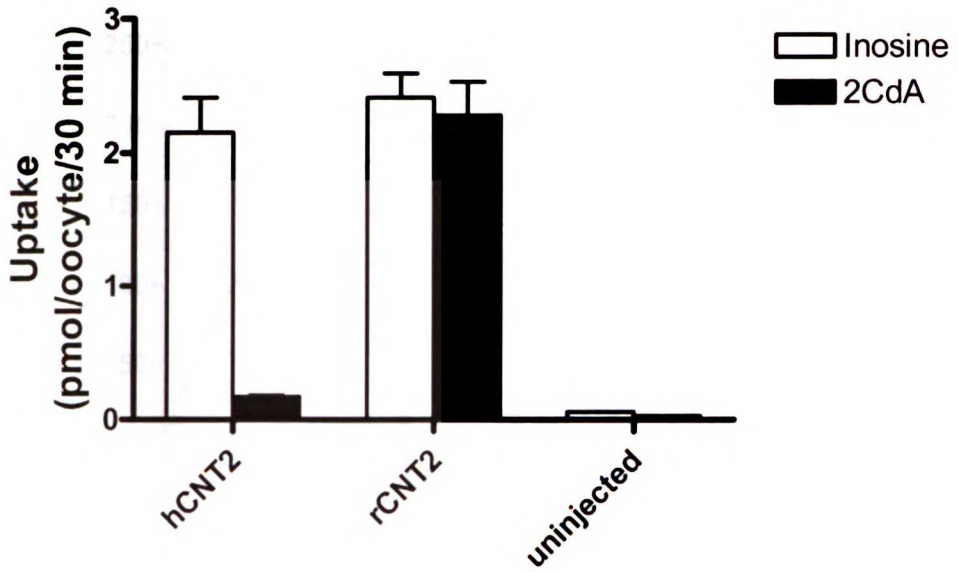


Figure 4.1. rCNT2 transports 2CdA at a higher rate than hCNT2. A) Oocytes injected with hCNT2 or rCNT2 cRNA were incubated with reaction mixtures containing unlabeled compound (1 μM) and either ^{3}H-inosine (0.1 μM) (white bars) or 0.1 μM ^{3}H-2CdA (0.1 μM) (black bars). Uninjected oocytes were also incubated with the same reaction mixes, and show negligible background transport of both inosine and 2CdA. Error bars are standard error of the mean, and each bar represents the mean uptake values of 7-9 oocytes.

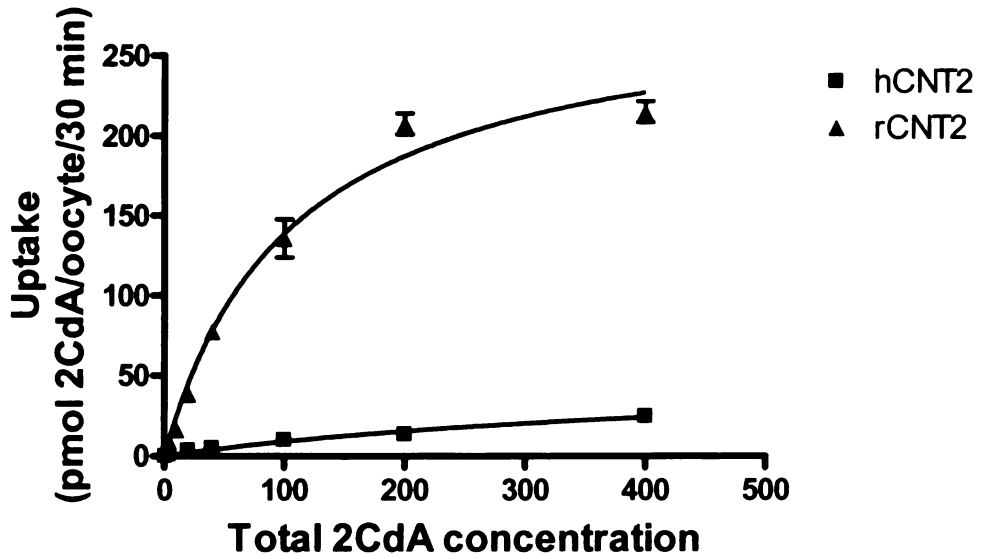
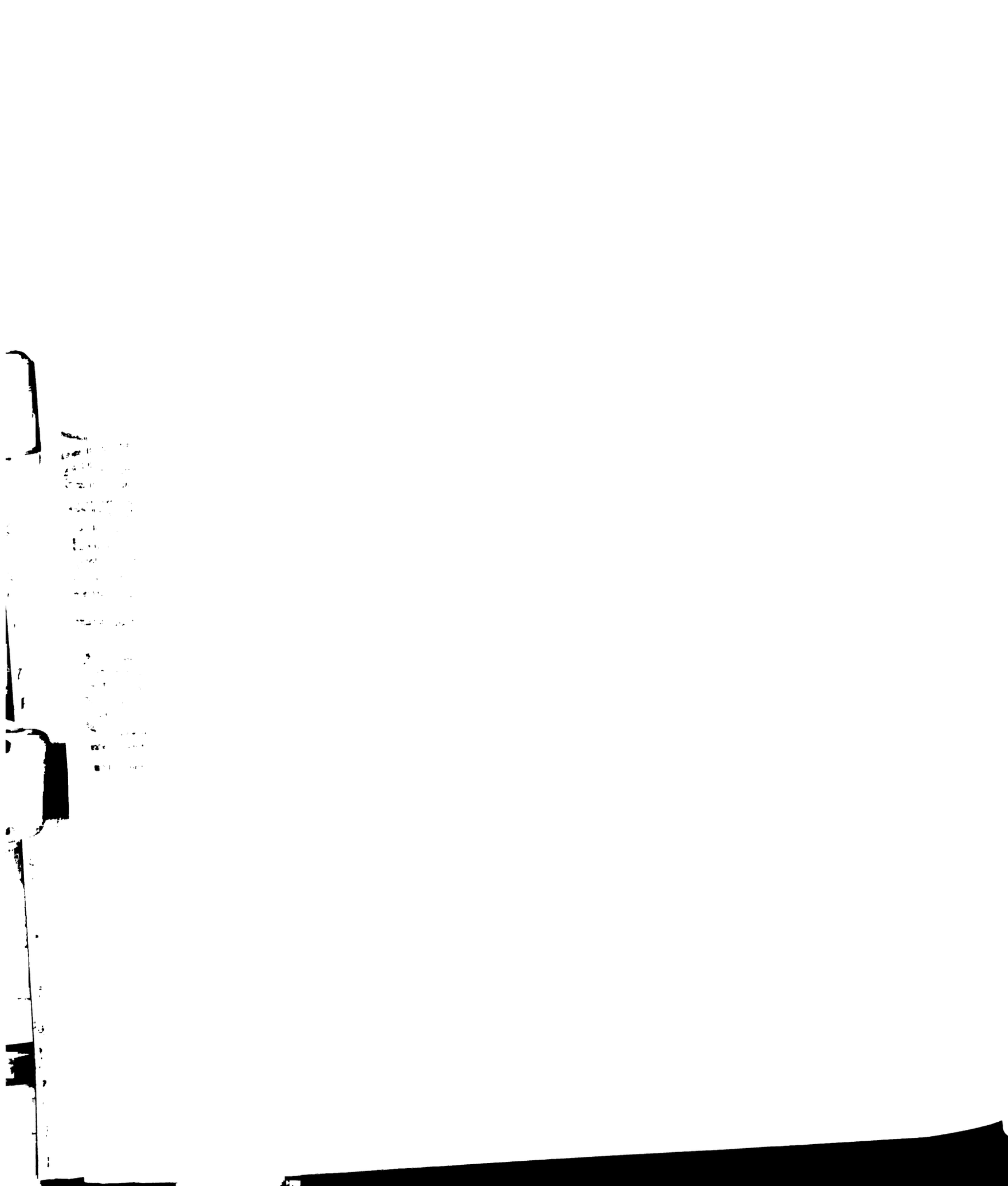


Figure 4.1 B) 2CdA kinetics in human and rat CNT2. Oocytes expressing hCNT2 (squares) or rCNT2 (triangles) were incubated with 0.1 μM ^{3}H-2CdA (0.1 μM) and eight different concentrations of unlabeled 2CdA. The uptake associated with each concentration was determined by scintillation counting and plotted. The error bars represent the standard error for the 7-9 oocytes used per condition. Kinetic parameters were determined by a Michaelis-Menton curve fit as described in the Experimental Procedures Section. The estimated K_m values for 2CdA are 187 ± 20.3 μM for hCNT2 and 56.1 ± 3.5 μM for rCNT2 (mean ± SE)

using molecular biology methods. Such information will provide an understanding of the critical structural regions responsible for specificity for nucleoside analogs in CNT2. In this study, we identified the region of the protein most likely involved in the selectivity of 2CdA (as well as Ara-A and fludarabine). We also identified a key amino acid residue that contributes to the overall observed specificity of CNT2 for 2CdA.



Materials and Methods

Construction of human and rat CNT2 single mutants. Human and rat CNT2 cDNA was subcloned into the amphibian high-expression vector pOX (Jegla and Salkoff 1997). Human and rat CNT2 were used as templates to create the mutations described in the Results section. Mutants were created via site-directed mutagenesis, with the following PCR conditions: 94°C for 2 min (1 cycle); 94°C for 1 min, 55°C for 1 min, 68°C for 8 min (16 cycles); 72°C for 8 min, 4°C ∞(1 cycle). Primers were custom designed according to the mutation, and were synthesized by Invitrogen. The mutation sequences were confirmed by DNA sequencing at the UCSF Biomolecular Recourse Center.

Construction of Chimeric Proteins. In order to create the chimeric proteins HHRR and RRHH, a *NarI* site was introduced via site-directed mutagenesis at amino acid position 318 of hCNT2 and 319 of rCNT2. The introduction of the *NarI* site was confirmed by sequencing. The human and rat CNT2 containing the *NarI* mutation were digested with *NarI* and *SpeI* at 37°C for two hours, and then the digest was loaded into a 1% agarose gel, and the bands were run until separated. The bands were then excised from the gel, and the DNA was isolated using the Qiagen gel extraction kit. The gel extracted bands were then ligated to the appropriate fragments in a ligation reaction using T4 DNA ligase enzyme (New England Biolabs). The ligation reaction was then transformed into DH5α *E. Coli* cells, and colonies were picked and sent for sequence confirmation. Creation of the chimeric proteins RRRH and HHHR involved the introduction of an *AatII* site (instead of *NarI*) at positions 529 and 530 of hCNT2 and rCNT2, respectively. The *AatII* site (which caused an I529T substitution in hCNT2) initially rendered all the chimeric

proteins nonfunctional, but after the chimeras were generated, the *AatII* site was reversed back to the native sequence via site-directed mutagenesis, and function was restored. *AatII*, *NarI*, and *SpeI* were all obtained from New England Biolabs

Functional Screening of Variants and Chimeras in *X. laevis* Oocytes. *X. laevis* oocytes were chosen as an expression system because they have previously been used to characterize the specificity of naturally occurring nucleosides (Loewen et al. 1999). Oocytes are also easily maintained, can be injected individually, and express membrane proteins very well. pOX plasmids containing CNT2 were linearized with *PvuI* (New England Biolabs), and cRNA was synthesized *in vitro* from the linearized plasmids by RNA transcription kits from either the mCAP RNA capping kit (Stratagene) or mMESSAGE mMACHINE T3 kit (Ambion). Healthy stage V and stage VI *X. laevis* oocytes were injected with 30-50 ng of capped cRNA transcribed *in vitro* with T3 RNA polymerase. Spectrophotometry was used to determine the concentration of cRNA, and an aliquot of each RNA preparation was run on a 1% agarose gel to ensure that the RNA was not degraded. Injected oocytes were stored in modified Barth's solution at 18°C (changed one or two times daily) for 2-3 days of expression before uptake studies. Seven to nine oocytes were incubated in Na^+ buffer containing 0.1 μM ^3H substrate, and 1μM unlabeled substrate. The uptake of radiolabeled inosine, cladribine, 2-chloroadenosine, 2'-deoxyadenosine, adenosine arabinoside, and fludarabine was measured (all radiolabeled compounds were purchased from Moravek). Oocytes were incubated in radiolabeled substrate for 30-40 minutes. Uptake was terminated by the removal of buffer containing the radioligand and the oocytes were washed five times in ice-cold

choline buffer. Oocytes were then individually lysed by the addition of 100 μL of 10% SDS, and the radioactivity associated with each oocyte was determined by scintillation counting. Uptake of all substrates in oocytes expressing each construct was determined in 7-9 oocytes from a single frog. The functional studies were repeated in oocytes from at least one other frog. Data are presented as pmol substrate/oocyte/30 minute uptake, and the error bars indicated are +/- SE. Uninjected oocytes incubated with the same reaction mix were used as a control.

Curve Fitting and Statistics. The 2CdA kinetic curves for human and rat CNT2 were generated by fitting the data to a Michaelis-Menton curve, which was done by GraphPad Prism 4 Software. For the statistical tests, the mean value of the mutant in question was compared to the mean value of the reference CNT2, and not any of the other mutants. The p-values for the single mutants were also calculated by GraphPad Prism 4 software using an unpaired two-tailed t-test.

Transmembrane Domain Modeling and Alignment. The models of the individual transmembrane domains (TMDs) were created using the program HelicalWheel, and the alignment of human, rat, and rabbit CNT2 was generated using ClustalW. Both programs are available as part of the SACS software package for UCSF (http://www.sacs.ucsf.edu).

Results

Human and Rat Chimeric Transporters of CNT2. To narrow down the region of CNT2 that was responsible for 2CdA selectivity, we constructed a series of chimeric proteins of human and rat CNT2. We constructed two sets of chimeric proteins, and named them with four letters consisting of Hs (hCNT2 sequence) and Rs (rCNT2 sequence), with each letter representing approximately one quarter of the protein. To construct the first chimeric pair, a *NarI* site was introduced approximately halfway through the sequence of human and rat CNT2, and the subsequent chimeras HHRR and RRHH were generated. Both chimeras form functional proteins, and are able to transport inosine (Figure 4.2). HHRR shows a 2CdA uptake very similar to that of rCNT2, whereas RRHH takes up 2CdA very poorly, similar to hCNT2. These results suggest that the determinants of 2CdA selectivity are located within the C-terminal half of the protein. A second set of chimeras was generated following the introduction of a second site (*AatII*), HHHR and RRRH. HHHR and RRRH also formed functional proteins, as they were able to take up the model substrate inosine similar to human and rat CNT2. However, HHHR and RRRH both had low uptakes of 2CdA suggesting that there were determinants of 2CdA selectivity on either side of the *AatII* splice site, or in the third and fourth "quarters" of CNT2.

Examination of Pharmacophores in 2CdA Responsible for Specificity Differences Between rCNT2 and hCNT2. There are two modifications between the parent compound adenosine, and the nucleoside analog drug 2CdA: a chloro modification on the 2- position

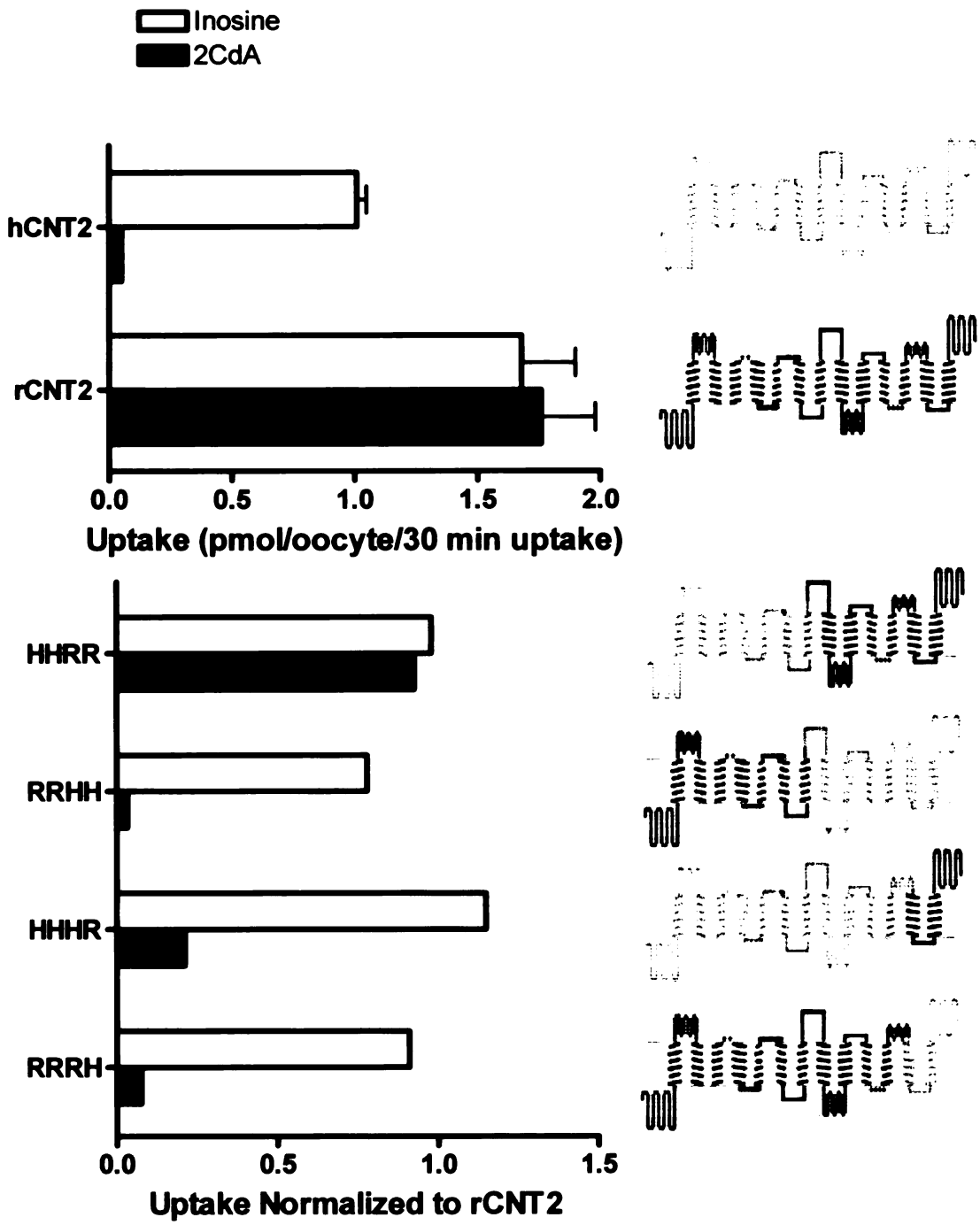

Inosine
2CdA
hCNT2
rCNT2
0.0
0.5
1.0
1.5
2.0
Uptake (pmol/oocyte/30 min uptake)
HHRR
RRHH
HHHR
RRRH
0.0
0.5
1.0
1.5
Uptake Normalized to rCNT2

Figure 4.2. The determinants of 2CdA selectivity in CNT2 are located in the C-terminal half of the protein. Top panel: Representative experiment of oocytes injected with hCNT2 or rCNT2 and incubated with ^{3}H-Inosine (white) or ^{3}H-2CdA (black). Error bars represent SE of nine oocytes in each group. A secondary structure prediction of human (white) and rat (red) CNT2 is depicted to the right of the bars representing the uptake of inosine and 2CdA. Bottom Panel: Oocytes expressing the CNT2 chimeras HHRR, RRHH, HHHR, and RRRH were incubated with either ^{3}H-inosine (white) or ^{3}H-2CdA (black), and were then normalized to the uptake of ^{3}H-inosine or ^{3}H-2CdA by rCNT2 in each experiment. Inosine and 2CdA concentrations for all experiments were 1 μM unlabeled compound, and 0.1 μM ^{3}H compound. All CNT2 chimeras were able to take up the model substrate inosine, indicating that they form functional proteins. Secondary structure predictions of all four chimeric proteins are included to the right of the normalized uptake bars. The structures are color coded to represent human (white) or rat (red) sequence.

of the base, and a 2' deoxy modification on the sugar (Table 4.1). The two intermediate compounds are 2-chloroadenosine and 2'-deoxyadenosine. To determine the pharmacophore (2'deoxyribose or 2-chloroadenine) responsible for the specificity differences of hCNT2 and rCNT2, we studied the uptake of 2-chloroadenosine and 2'-deoxyadenosine in oocytes expressing each CNT2 ortholog as well as the chimeras, HHRR and RRHH. Whereas the transport rate of 2'-deoxyadenosine was comparable to that of inosine in both human and rat CNT2 orthologs, the rate of transport of 2-chloroadenosine was substantially reduced in oocytes expressing hCNT2 and the chimera, RRHH (Figure 4.3). In contrast, the rate of transport of 2-chloroadenosine was not reduced in oocytes expressing rCNT2 and the chimera, HHRR. These data suggest that the 2-chloro group of 2CdA, rather than the 2'deoxyribose, is the pharmacophore responsible for the reduced preference of hCNT2 for 2CdA.

Ara-A and Fludarabine determinants are also located in the C-terminal half of CNT2. Ara-A is an antiviral nucleoside analog that is transported by rCNT2, but only very poorly by hCNT2, similar to the species difference that exists with 2CdA (Gerstin et al. 2002). We tested Ara-A, and the structurally similar anti-leukemia drug fludarabine (Table 4.1), with our chimeric proteins HHRR and RRHH. We found that both compounds showed the same selectivity pattern as 2CdA, with rCNT2 and HHRR transporting both compounds much better than hCNT2 and RRHH (Figure 4.4). These results are consistent with the determinants of selectivity of several synthetic analogs of adenosine being different between human and rat CNT2.

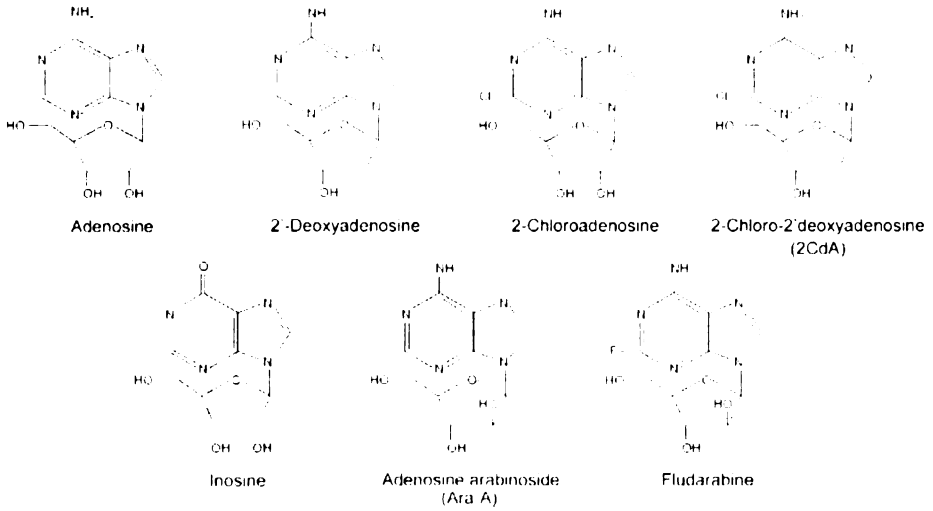


Table 4.1. Structures of nucleosides and nucleoside analogs.

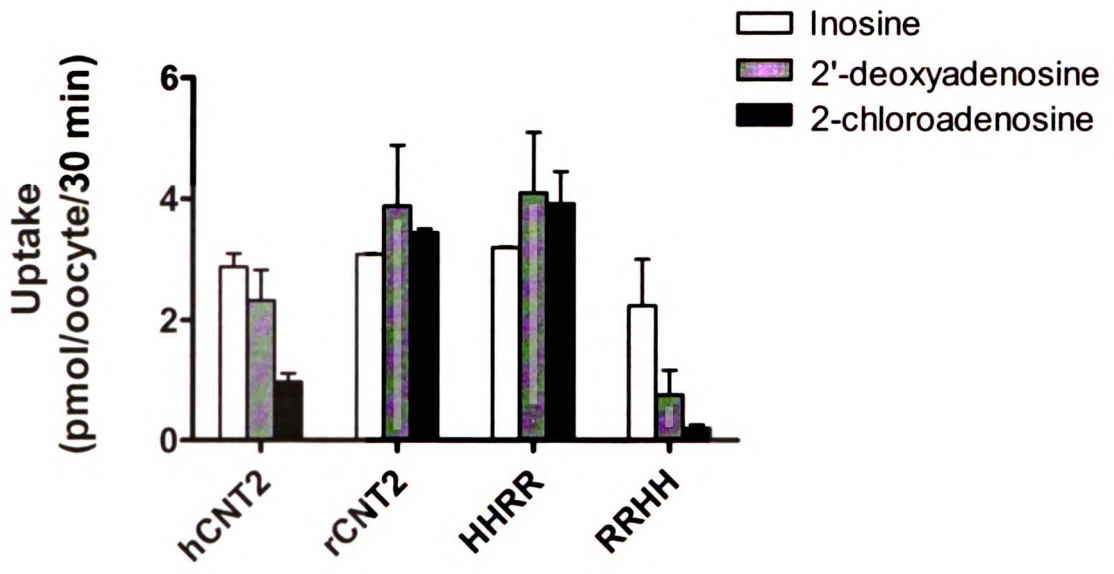


Figure 4.3. 2-Chloroadenosine is not transported as well as 2'-deoxyadenosine by hCNT2. Oocytes were injected with cRNA encoding hCNT2, rCNT2, chimera-HHRR and chimera-RRHH. The injected oocytes were incubated with ^{3}H-inosine (white bars), ^{3}H-2'-deoxyadenosine (gray bars), or ^{3}H-2-chloroadenosine (black bars). Each bar represents the mean of two experiments (each containing 7-9 oocytes), and the error bar represents the standard deviation of the mean of the two experiments.

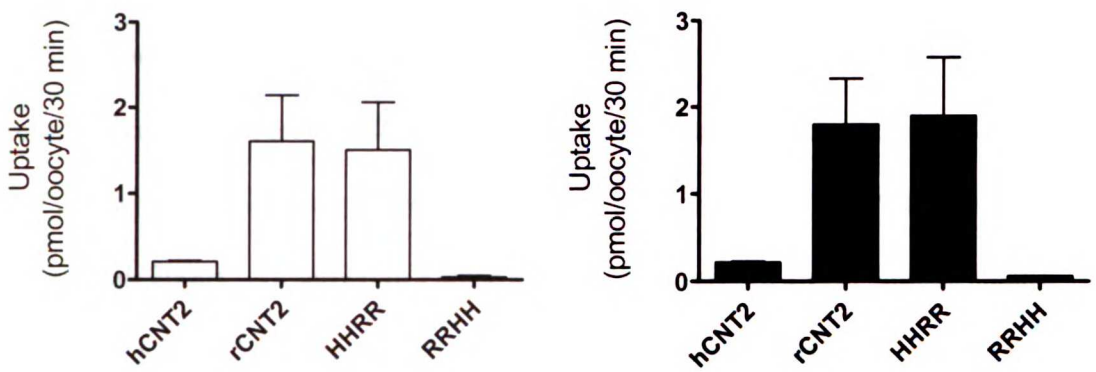


Figure 4.4. The determinants of specificity for Ara-A and Fludarabine are located in the C-terminal portion of CNT2. Oocytes injected with the either hCNT2, rCNT2, HHRR, or RRHH were incubated with ^{3}H-Ara-A (white bars) and ^{3}H-Fludarabine (black bars) (structures of Ara-A and Fludarabine are displayed in Table 4.1). Error bars represent standard error of 7-9 oocytes.

Selection of Candidate Amino Acid Residues for Site-Directed Mutagenesis. The chimera studies suggested that the critical amino acids responsible for specificity for 2CdA reside in the C-terminal region of CNT2; however, there are 51 amino acid residues which differ between the rat and human CNT2 orthologs. All of these would be potential candidates for mutagenesis studies identifying key amino acid residues. To refine our selection of candidate residues for mutagenesis we used the rabbit ortholog of CNT2 (rbCNT2), which exhibits sequence similarity to both human and rat CNT2 in this region (Gerstin et al. 2000). As shown in Figure 4.5a, rbCNT2 shows a very similar uptake profile to hCNT2 with respect to the uptake of 2CdA relative to inosine. It differs in its preference for 2CdA from rCNT2. Based on these findings, we chose residues for site-directed mutagenesis that were the same in human and rabbit CNT2, but were different in rCNT2 (Figure 4.5b). In addition, we mutated residue A354 (Figure 4.5b), which was chosen based on its proximity to residues that have previously been shown to be relevant for specificity for naturally occurring nucleosides (Loewen et al. 1999; Owen et al. 2005). Residues in the C-terminal tail were not considered good candidates for mutation because they would not likely be close to any substrate recognition portion of the protein.

Functional Analysis of Mutant CNT2 Transporters. After selecting the candidate amino acid residues, we performed site-directed mutagenesis on hCNT2 to replace the human amino acid residues with the respective residue of rCNT2. The single mutants of hCNT2 listed in Table 4.2 were constructed, and along with hCNT2-reference, were incubated with 2CdA, and the uptake values were normalized to that of hCNT2-reference. The mean of 3 trials along with the standard deviation is listed in Table 4.2. Several mutant

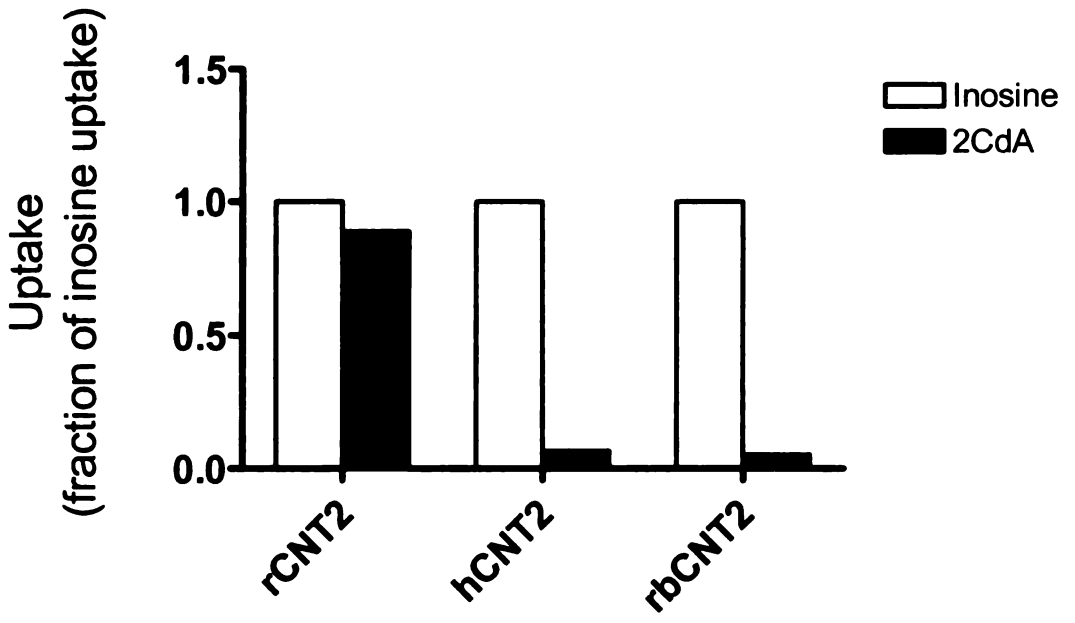


Figure 4.5. Rabbit CNT2 is similar to hCNT2 in function and structure. A) cRNA encoding rabbit CNT2 (rbCNT2), hCNT2, and rCNT2 were injected into oocytes, and then the uptake of inosine (white bars) and 2CdA (black bars) was examined. 2CdA uptake is expressed as a fraction of inosine uptake in each of the mammalian CNT2s.

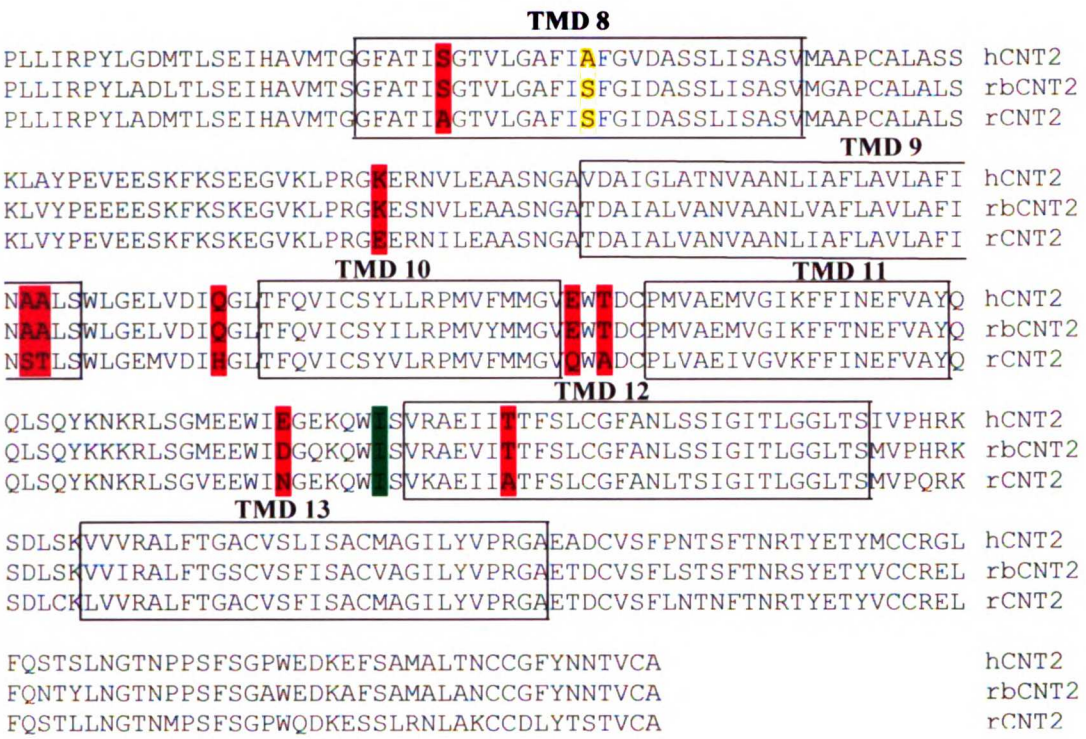


Figure 4.5 B) The amino acid sequences of human (top), rabbit (middle), and rat CNT2 (bottom) are aligned from the *NarI* splice site until the end of the proteins. Residues that were the same in hCNT2 and rbCNT2, but differed in rCNT2 were considered good candidates for site-directed mutagenesis. Mutations that met this criterion were constructed in this study and are colored in red. hCNT2-A354S was constructed due to its proximity to other residues that have previously been shown to be relevant for substrate specificity and is colored in yellow. The second splice site, *AatII*, used to generate the chimeras HHHR and RRRH is shown in green. Putative transmembrane domains are boxed and labeled based on a 13 TMD model. hCNT2 and rCNT2 are most similar in the transmembrane domains, and show the highest degree of divergence in the C-terminal tails.

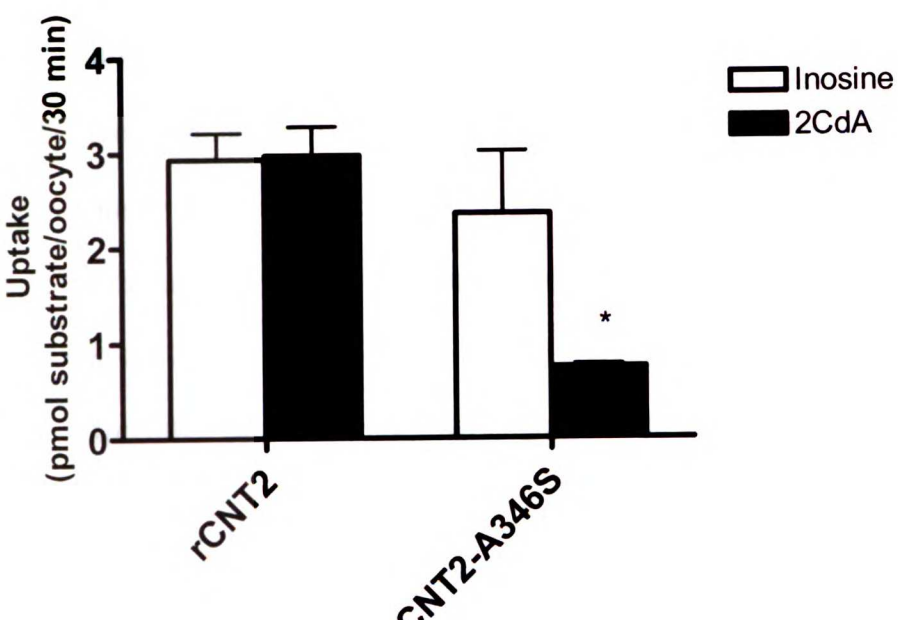
Mutants Constructed	Uptake of 2CdA (% of hCNT2-reference)
hCNT2-reference	100
hCNT2-S345A	**594 ± 13.9**
hCNT2-A354S	168 ± 39.5
hCNT2-K401E	135 ± 9.8
hCNT2-A439S	157 ± 18.3
hCNT2-A440T	129 ± 10.5
hCNT2-Q451H	175 ± 37.7
hCNT2-E473Q	112 ± 26.9
hCNT2-T475A	126 ± 7.5
hCNT2-E515N	196 ± 34.2
hCNT2-T529A	82.6 ± 3.8

Table 4.2. Uptake of 2CdA in *X. laevis* oocytes expressing synthetic mutants of hCNT2. The single mutants that were constructed are listed in the column on the left. The number on the right represents the uptake of 2CdA expressed as a percent of the hCNT2-reference. One hundred percent represents the uptake of 2CdA by hCNT2-reference on the day the experiment was performed. The values represent the mean of 3 experiments that were conducted for each variant, and the error indicates the standard error between the three repeats. hCNT2-S345A is bolded because it showed the highest increase in 2CdA uptake of any of the changes. Several other variants likely make smaller contributions.

transporters exhibited slightly enhanced or no change in 2CdA uptake relative to hCNT2, whereas hCNT2-S345A resulted in a greater than 5 fold increase in the uptake of 2CdA relative to hCNT2-reference.

We constructed a rat single mutant, rCNT2-A346S (the equivalent residue of hCNT2-S345A in rat), to examine whether the transport of 2CdA would be reduced. The single mutant rCNT2-A346S, significantly decreased the uptake of 2CdA relative to rCNT2-reference, without a significant change to inosine uptake (Figure 4.6). In each of three experiments, the difference between the uptake of rCNT2 and rCNT2-A346S was very statistically significant ($p<0.001$). A previous study reported that hydrogen bonding was critical to substrate interaction in CNT2 (Chang et al. 2004). In order to test whether a decrase in hydrogen bonding capability led to a decrease in 2CdA uptake relative to that of rCNT2, we made three additional mutants in rCNT2: rCNT2-S355A, rCNT2-S440A, and rCNT2-T441A, changing the residue back to the human equivalent, with the expectation being that these variants would lose some ability to transport 2CdA. The mutant rCNT2-S440A dramatically reduced both inosine and 2CdA transport, and the results are not shown. rCNT2-T441A specifically reduced the transport of 2CdA (Figure 4.6, $p=0.025$), and rCNT2-S355A showed a slight, but not significant decrease in 2CdA transport relative to rCNT2.

Helical Wheel of TMD 8 and 9 in Human CNT2. Using the program HelicalWheel, predictions of transmembrane domains 8 and 9 were generated for human CNT2. Residues in this region of CNT2 have previously been shown to be relevant for


Uptake
(pmol substrate/oocyte/30 min)
4
3
2
1
0
Inosine
2CdA
*
rCNT2
rCNT2-A346S

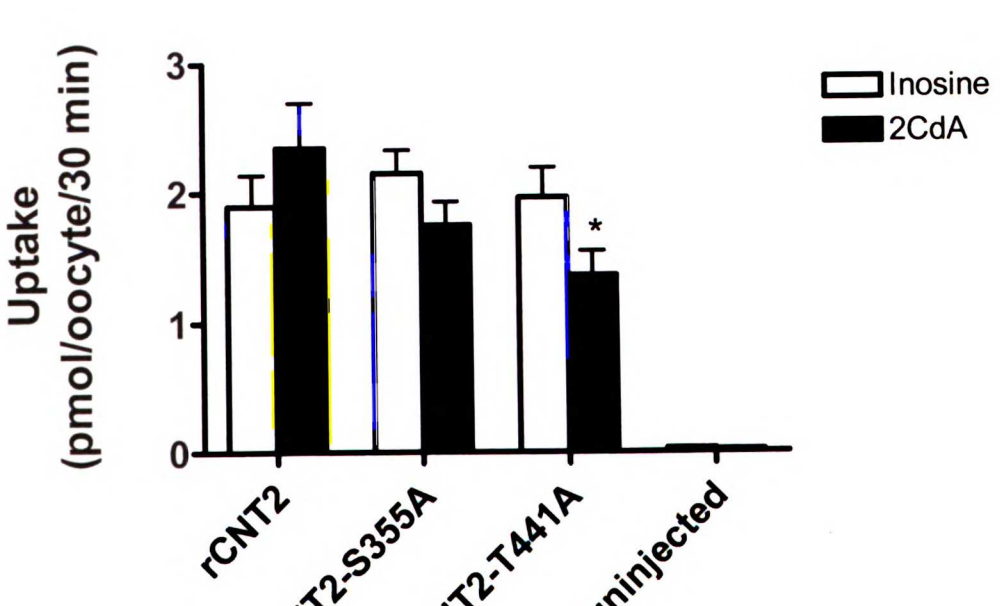

Uptake
(pmol/oocyte/30 min)
3
2
1
0
Inosine
2CdA
*
rCNT2
rCNT2-S355A
rCNT2-T441A
uninjected

Figure 4.6. Uptake of 2CdA in *X. laevis* oocytes expressing synthetic mutants of hCNT2 and rCNT2. Top Panel: rCNT2 and rCNT2-A346S were incubated with 0.1 μM radiolabeled compound and 1 μM unlabeled compound. Three trials were performed for each condition in oocytes from different frogs. The mean uptake of three experiments is plotted, and the error represents the standard error between the three trials. The difference in the 2CdA uptake between rCNT2 and rCNT2-A346S is statistically significant ($p<0.01$). Bottom Panel: Two single mutants in rCNT2 were constructed (rCNT2-S355A and rCNT2-T441A), changing the hydrophilic residues in rCNT2 to the hydrophobic equivalent residues in human. cRNA from the rat single mutants was then injected into oocytes, and incubated with either ^{3}H-inosine (white) or ^{3}H-2CdA (black). A representative experiment is shown* indicates a p-value of less than 0.05.

specificity. Two residues in TMD8 were previously determined to have implications in purine/pyrimidine base preference (Loewen et al. 1999), another is polymorphic in the human population, with the minor allele showing altered specificity for uridine and inosine (Owen et al. 2005). Residues S345A and A354S (from this study) are also indicated. The positions of hCNT2-A439S and hCNT2-A440T are shown in TMD9 (Figure 4.7). The increase in 2CdA uptake by hCNT2-S345A is consistent with a role for hydrogen bonding capability in the mutant vs. the reference, in this case a negative role. The other three hCNT2 mutants shown would all increase the hydrophilic character of their side of the transmembrane helix, which would increase the polarity of that side of the helix, and possibly facilitate 2CdA binding and or transport.

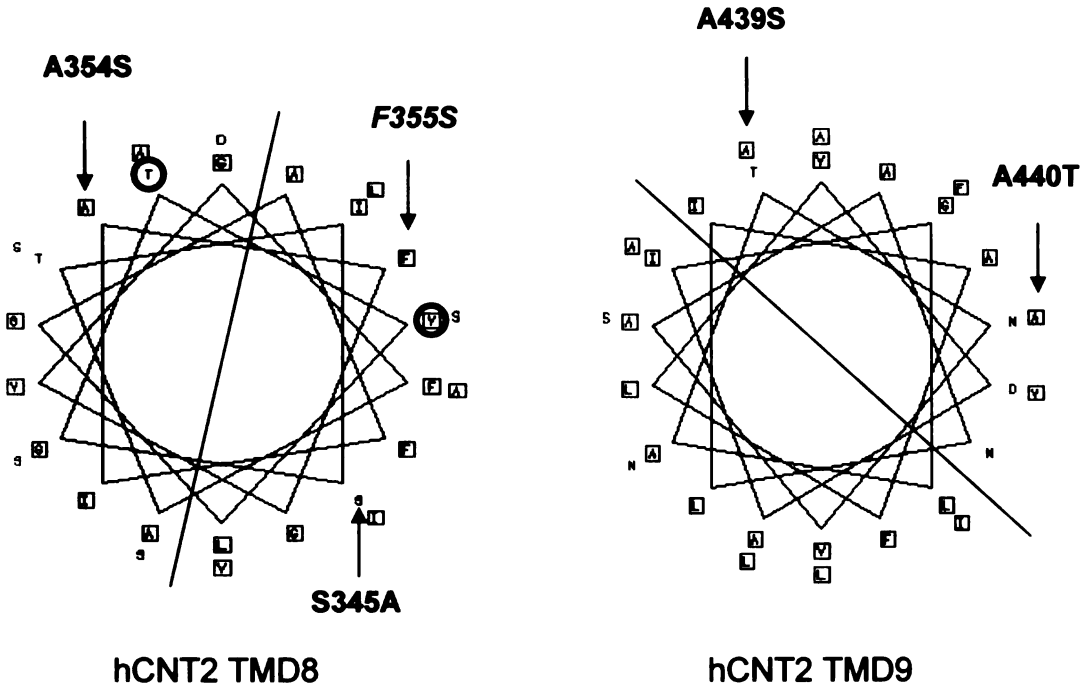


Figure 4.7. Residues important for specificity in hCNT2 transmembrane domains 8 and 9. Putative transmembrane domains 8 and 9 were generated by the program HelicalWheel. Amino acid residues of interest are indicated by arrows on both TMD 8 and 9. The positions of S345A, A354S, A439S, and A440T are all shown, as is the human polymorphism F355S (italics), and the two residues located in transmembrane domain 8 that have previously been shown to play a role in purine versus pyrimidine specificity (circled). Residues with a box are hydrophobic in character, and red residues are charged or polar. The mutation of the Ala residues in human to Ser or Thr increases the hydrophilic character of the side of the helix that faces the pore according to the model, possibly providing a mechanistic explanation for the somewhat increased 2CdA uptake observed in mutants that have hydrophilic residues in these positions.

Discussion

Human and rat CNT2 are highly similar proteins (~80% amino acid identity), and both proteins share the same substrates among the naturally occurring nucleosides (i.e. purine nucleosides and uridine) (Wang et al. 1997). Despite the similarity in sequence and specificity for naturally occurring nucleosides, hCNT2 and rCNT2 exhibit a dramatic difference in their ability to transport certain nucleoside analog drugs such as Ara-A and 2CdA (Gerstin et al. 2002). In particular, 2CdA (and Ara-A) are excellent substrates of rCNT2 and poor substrates of hCNT2. In this study, we took advantage of the large difference in specificity in the rat and human orthologs of CNT2 for 2CdA to identify structural domains and key residues that contribute to the specificity of CNT2 for the synthetic analog 2CdA.

The C-Terminal Region of CNT2 Contains the Determinants of Specificity for 2CdA. Our chimera studies indicate that the determinants of 2CdA specificity are located in the C-terminal region of CNT2. These results are consistent with the determinants of substrate specificity for the naturally occurring nucleosides, which were also found in the C-terminal region of the CNTs (Wang and Giacomini 1997; Loewen et al. 1999; Wang and Giacomini 1999). Our further studies with second generation chimeras suggested that there are determinants of 2CdA specificity in both the third and fourth quarters of the protein. That is, 2CdA uptake was low in both RRRH and HHHR suggesting that neither the third nor the fourth quarter alone of rCNT2 could reconstitute its activity for 2CdA. However, together (i.e. HHRR) the two quarters can reconstitute rCNT2 activity for 2CdA.

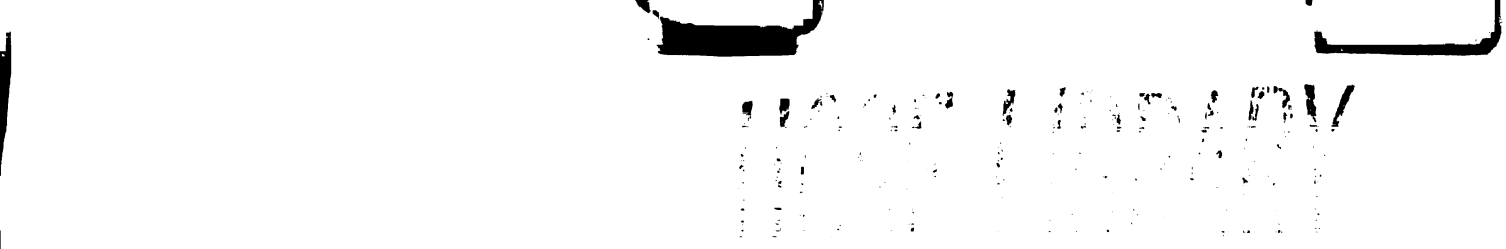
One Key Amino Acid Residue Contribute to the Recognition and Translocation of 2CdA.

Of the 51 amino acid differences in the C-terminal halves of human and rat CNT2, we constructed 14 single mutants based on criteria outlined in the results section (10 in hCNT2 and 4 in rCNT2), and information from the rabbit ortholog. One single mutant of hCNT2 showed a large increase in 2CdA uptake over hCNT2-reference: hCNT2-S345A (see Table 4.2). hCNT2-S345A is predicted to reside in a transmembrane domain near other residues that have previously been shown to be relevant for purine and pyrimidine specificity. When the equivalent residue was mutated in rCNT2, rCNT2-A346S, the uptake of 2CdA was reduced dramatically, underscoring the importance of the residue at this position for determining 2CdA specificity. Several other single mutants in hCNT2 appear to contribute towards a minor (less than two fold) increase in 2CdA uptake over hCNT2. It is possible that some combination of these minor mutants, along with the residue at position 345 would convert hCNT2 to a rCNT2 like phenotype. Chang *et al.* used computational methods to identify chemical features of nucleoside analogs required for interaction with concentrative nucleoside transporters (Chang et al. 2004). Whereas the affinity for CNT1 was found to be due to multiple factors such as electrostatic interactions and steric properties, the affinity of hCNT2 for its substrates was predicted to be dominated by hydrogen bonding interactions (Chang et al. 2004). In light of these findings, we made three additional mutations in rCNT2, all of which changed a serine or threonine residue to an alanine. rCNT2-T441A showed a statistically significant decrease in 2CdA uptake as compared to rCNT2, but this difference much less significant than the decrease seen with rCNT2-A346S, but it still is likely to have a minor contribution

towards 2CdA specificity. Many of the mutations that caused an alteration in 2CdA uptake by either hCNT2 or rCNT2 involved either the introduction or elimination of an amino acid residue containing a hydroxyl group; this finding supports the contention of Chang *et al* that hydrogen bonding could play a major role in the substrate recognition by CNT2.

The Chloride Modification of the Purine Base in 2CdA is Poorly Recognized by hCNT2. Both human and rat CNT2 are able to transport adenosine, yet there are clear species differences in the ability of the orthologs to transport the synthetic analog 2CdA. Our results indicated that the 2'deoxy pharmacophore had a relatively innocuous effect on transport. In contrast, the 2-chloroadenosine pharmacophore was poorly taken up by hCNT2, whereas rCNT2 and chimera-HHRR were able to transport it very well. Further studies are needed to determine whether these findings are applicable to other compounds that are halogenated on the purine or pyrimidine base such as 8-chloroadenosine or 5-fluorocytosine (Yin et al. 2001; Fischer et al. 2005).

There is also a large species difference between hCNT2 and rCNT2 in their ability to transport arabinoside compounds such as Ara-A and fludarabine. The only difference between Ara-A and adenosine is the configuration of the hydroxyl groups on the ribose sugar (Table 4.1), indicating that the hydroxyl configuration in arabinosides is not tolerated by hCNT2. These findings indicate that clinically used arabinosides such as Ara-G will likely not be substrates for hCNT2.



Our efforts at determining the region of CNT2 and the individual amino acids that contribute to 2CdA specificity paint a complicated picture when compared with the residues that were found to dictate purine vs. pyrimidine nucleoside specificity. The question of specificity for nucleoside analogs, while important, is likely to be more complex since the proteins that transport the nucleoside analog drugs have not been honed by millions of years of selective pressures to transport clinically used drugs. Despite the differences in complexity between nucleoside analog specificity and naturally occurring specificity, it should be noted that the determinants of both are located in similar regions of the protein. Although the region is the same, the individual amino acids that make up the determinants of substrate specificity are not the same for 2CdA as for the naturally occurring nucleosides. The large difference in specificity between human and rat CNT2 for many synthetic nucleoside analogs suggests that rat may be a poor animal model in the testing of these compounds for their applicability in human therapy. Beginning to understand the complex relationship between transporter proteins and nucleoside analog drugs could facilitate efforts at rational drug design, and pre-clinical experimentation in relevant animal models, including transgenic animals. Our chimera studies hint at interactions among residues that contribute to 2CdA specificity in both the third and the fourth quarters of the protein. For example, an interaction between amino acid residues in the third and fourth quarters of rCNT2 may lead to a difference in the folding of the protein and shape of the substrate recognition domain as compared to hCNT2. Unfortunately, interactions such as these cannot be identified without a crystal structure or three dimensional model of some kind. Our efforts at generating such a model were stymied by the lack of an available crystal structure template on which to

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Chapter 5

The Effect of Polymorphisms in the Human Organic Cation Transporter 1 (OCT1) on the Transport and Cytotoxicity of Metformin in HEK Cells

The organic cation transporter 1 (OCT1), expressed on the basolateral membrane of hepatic epithelial cells, is involved in the bidirectional transport of many clinically used drugs, including the anti-diabetic agent, metformin. Previously, many non-synonymous polymorphisms in OCT1 were identified in ethnically diverse populations and functionally characterized for the model substrate, N-methylpyridinium (MPP^+) in *X. laevis* oocytes. The goal of this study was to characterize the interaction of non-synonymous polymorphisms of OCT1 with metformin as a first-step towards determining whether genetic polymorphisms in OCT1 may play a role in variation in hepatic uptake and response to metformin. Stable cell lines expressing OCT1 variants were generated in HEK-293 cells, and the uptake of metformin was measured. Of the 12 variants tested, seven resulted in a reduction or complete loss of metformin uptake when compared with the OCT1 reference transporter. Kinetic studies with OCT1-G401S, a polymorphism found at a frequency of 1.1% in Caucasians suggested that although the K_m value was similar to the reference OCT1, the V_{max} was significantly different (6.74 ± 0.88 nmol metformin/min/mg protein for OCT1-reference, and 0.67 ± 0.09 nmol metformin/min/mg protein for OCT1-G401S, $p<0.0001$), possibly due to defects in protein expression on the membrane. These findings are of particular relevance to the disposition and response

Introduction

The organic cation transporter 1 (OCT1) is a member of the solute carrier (SLC) superfamily of membrane transporters, and more specifically a member of the *SLC22A* family. The *SLC22A* family has several cloned members in humans, and consists primarily of transporters that accept charged organic compounds (Koepsell and Endou 2004). In addition to OCT1, there are two other OCTs that have been cloned and characterized (Grundemann et al. 1994; Okuda et al. 1996; Kekuda et al. 1998). The OCT family is able to transport both endogenous molecules and xenobiotic compounds in a bidirectional manner (Koepsell et al. 2003). Although there is a high degree of overlap in the substrate specificity of the OCT family, each one has a unique pattern of distribution in human tissues. OCT1 is predominantly found in liver (Gorboulev et al. 1997; Zhang et al. 1997; Muller et al. 2005; Wright 2005); OCT2 is the major isoform in kidney (Gorboulev et al. 1997; Wright 2005), and OCT3 is ubiquitously expressed at low levels, with the highest expression in placenta (Grundemann et al. 1998; Verhaagh et al. 1999).

The OCTs have been a subject of research interest for many years because they are thought to contribute to the efficacy of many clinically used compounds, and to the toxicity of environmental chemicals. Several clinically used drugs that are substrates for OCTs, including metformin, exhibit variation in their therapeutic response (Joshi 2005). Genetic variation in OCT transporters, and specifically OCT1, may explain some of the interindividual differences in response associated with metformin therapy. OCT1 is thought to play an important role in metformin efficacy due to its high tissue expression

in the liver, an important target organ for metformin (Shu et al. 2001; Shu et al. 2003; Kimura et al. 2005). Variants of OCT1 were identified as part of a large project focused on discovery of polymorphisms in membrane transporters (Leabman et al. 2003). Several studies have characterized the function of genetic variants of OCT1 as well as its paralog, OCT2 (Kerb et al. 2002; Leabman et al. 2002; Shu et al. 2003).

Metformin, a widely prescribed drug used in the treatment of type 2 diabetes, is a known substrate for OCT1 (Shu et al. 2003; Joshi 2005). Metformin is given as an orally, and is thought to exert its biological effects primarily in the liver and skeletal muscle. In particular, metformin inhibts gluconeogenesis in the liver and stimulates hepatic and skeletal muscle uptake of the drug (Goodarzi and Bryer-Ash 2005). Metformin is charged at physiological pH, and therefore requires a transport protein to cross the plasma membrane and enter the liver (Scheen 1996; Wang et al. 2002). OCT1 is thought to be the primary metformin transporter in the liver, and is a determinant of the hepatocellular concentrations of the drug. In fact, Oct1 knockout mice have a significantly lower hepatic uptake of metformin after intravenous doses (Wang et al. 2002).

The goal of the present study was to characterize the transport of metformin by the OCT1 variants identified in the study of Leabman et al (Leabman et al. 2003). In particular, we characterized the activity of the variants with respect to metformin uptake through the construction of stable cell lines in human embryonic kidney cells. Several of the OCT1 polymorphisms occur at high frequencies in the sample population, so any differences

Materials and Methods

Cell Lines and Transfection. Variants of OCT1 were identified in a previous study (Leabman et al. 2003). OCT1-reference was subcloned into the pcDNA5/FRT vector, and site directed mutagenesis was used to construct the variants. PCR cycling conditions were used as previously described (Shu et al. 2003). The desired mutations were confirmed through DNA sequencing done at McLab laboratories (South San Francisco, CA). Following sequence confirmation, human embryonic kidney (HEK-293) cells were stably transfected using Lipofectamine™ 2000 (Invitrogen) as per the manufacturer's instructions with the full length human OCT1 cDNA (OCT1-reference), empty pcDNA5-FRT (mock), and the 12 single mutants of OCT1 which are listed in Table 1. After the initial transfection, stable clones were selected for by adding 75 μg/ml of hygromycin B to the growth media for 10 days, and then pooling the surviving cells in a fresh flask.

Cell Culture. The culture medium for stably transfected HEK293 cells is DMEM supplemented with 10% FBS and 1X pen/strep (Invitrogen), and 75 μg/mL hygromycin B. Cells were cultured in T25 flasks, and were split two to three times weekly.

Cellular Uptake of Metformin or MPP^+. HEK 293 cells were grown in 24-well plates to 100% confluence in the culture medium on poly-D-lysine coated plates. The cells were washed once with room temperature PBS, and then incubated in uptake buffer (MPP^+ in PBS or metformin in serum and antibiotic-free culture media) containing 31.4 μM ^{14}C-metformin or ^{3}H-MPP^+ diluted 1:1000. The uptake was performed at room temperature for either 20 sec or 2 min (^{3}H-MPP^+) or 10 min at 37°C (^{14}C-Metformin) and then the

cells were washed three times in ice-cold PBS. The cells were then lysed with 0.1 N NaOH, 0.1% SDS and the lysate was used for scintillation counting and for protein assay.

RNA Isolation. Cultured cells expressing OCT1-reference, mock transfected, and OCT1 variants were grown in six well poly-D-lysine coated plates in triplicate. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) according to the online protocol.

RT-PCR and Taqman. The first-strand cDNA was synthesized from 1 μg of total RNA using SuperScript™ III First-Strand Synthesis System for RT-PCR kit (Invitrogen) in a 20 μl reaction mixture, and oligo dTs were used as the primers. Primers and probes for OCT1 were purchased from ABI, and standard Taqman methods were used to amplify the OCT1 cDNA. Results were analyzed for the fold change in expression of all the variants relative to mock transfected cells. Data was graphed using GraphPad Prism software.

Statistical Analyses and Curve Fitting. All single comparisons between the uptake of OCT1 at a single concentration, or the comparison of kinetic parameters between OCT1-reference and variants of OCT1 were performed using an unpaired, two-tailed t test. For the Taqman results, comparison of all the data was done by a one way ANOVA. The kinetic parameters were generated by fitting the data to the Michaelis-Menten equation. All statistical analyses and curve fits were done using GraphPad Prism software.

Results

Position of Non-synonymous Polymorphisms in OCT1

Figure 5.1 shows a secondary structure prediction of OCT1 with the position of the variants that are included in this study. The non-synonymous variants of OCT1 are distinguished from the amino acid deletion by color coding.

MPP^+ uptake

The twelve variants chosen for this study and the frequency at which they occur in the sample population are shown in Table 5.1. MPP^+ was taken up by cells expressing the reference OCT1 and each of the variant OCT1s with the exception of OCT1-G465R (Figure 5.2).

Taqman RT-PCR

To exclude differences in mRNA expression influencing the uptake of MPP^+ in the cell lines expressing the OCT1 variants, RT-PCR studies were conducted to determine the level of OCT1 mRNA in each of the cell lines. All of the cell lines expressing OCT1 variants had similar levels of mRNA, and all had levels significantly higher than those in mock- transfected cells (Figure 5.3). We performed a one way ANOVA on the data shown in Figure 5.3, and determined that none of the values showed a statistical difference in mRNA expression ($p=0.16$), indicating that any differences in the uptake or kinetics of the compounds studied are likely to be due to post-translational effects.

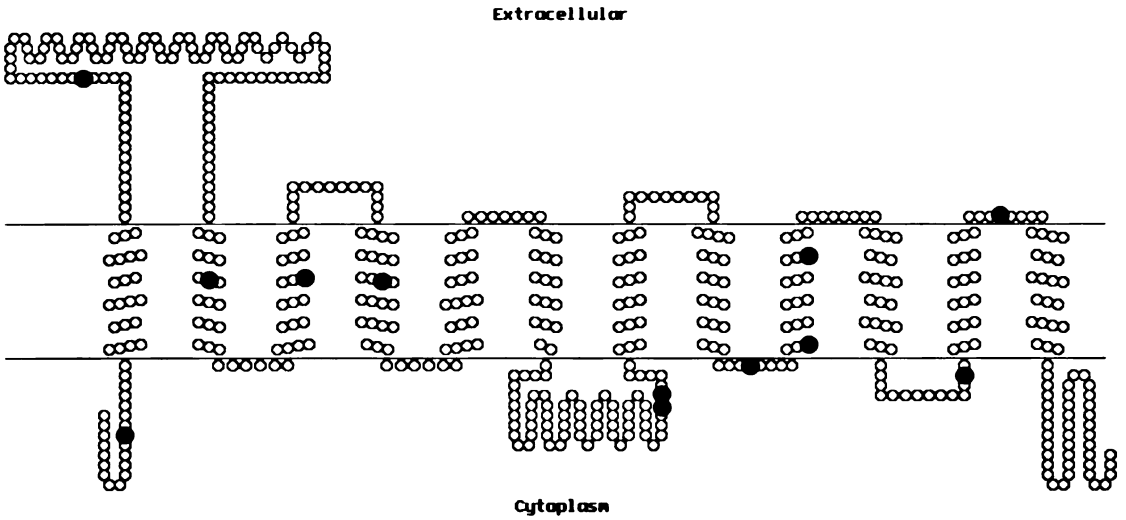

Figure 5.1. TOPO image of OCT1 and its variants. Secondary structure prediction of OCT1 showing the positions of the 12 variants in OCT1 that were characterized in this study. Non-synonymous variants are represented by the red circles, and the blue circle indicates a single amino acid deletion.

Variant	AA	CA	AS	ME	PA
S14F	.031	0	0	0	0
R61C	0	.072	0	.056	0
F160L	.005	.065	.017	.05	0
S189L	0	.005	0	0	0
G220V	.005	0	0	0	0
P341L	.082	0	.117	0	0
R342H	.031	0	0	0	0
G401S	.007	.011	0	0	0
V408M	.265	.402	.238	.214	.071
420 del	.029	.185	0	.214	0
G465R	0	.04	0	0	0
R488M	.05	0	0	0	0

Table 5.1. Variants of OCT1.

The frequency of each variant in each individual ethnic group is listed. AA: African American, CA: Caucasian American, AS: Asian, ME: Mexican, PA: Pacific Islander. The number of individual DNA samples that were sequenced for each ethnic group are as follows: 100 AA and CA, 30 AS, 10 ME, and 7 PA. The variant 420 del corresponds to the deletion of one amino acid (methionine at position 420).

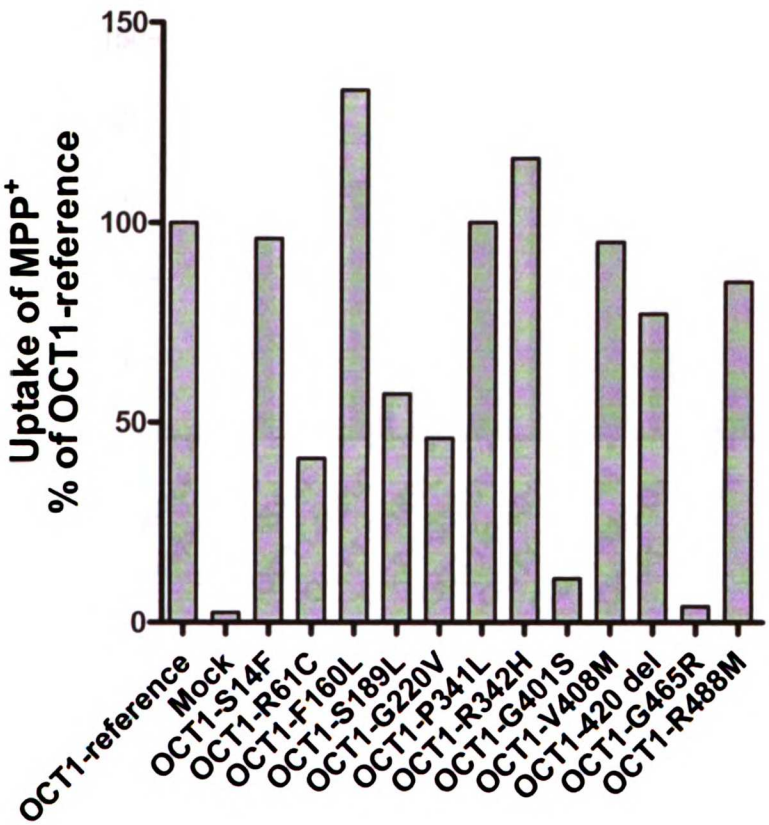

Figure 5.2. Uptake of MPP^+ in HEK cells stably expressing OCT1 variants. Stably transfected HEK cells were incubated with ^3H-MPP^+ in triplicate, and the radioactivity associated with each well was determined by scintillation counting. The bars for the variant OCT1s represent the mean uptake in three wells. Mean values are normalized to the MPP^+ uptake of OCT1-reference that was performed on the same day. Mock cells are stably transfected with empty pcDNA5-FRT.

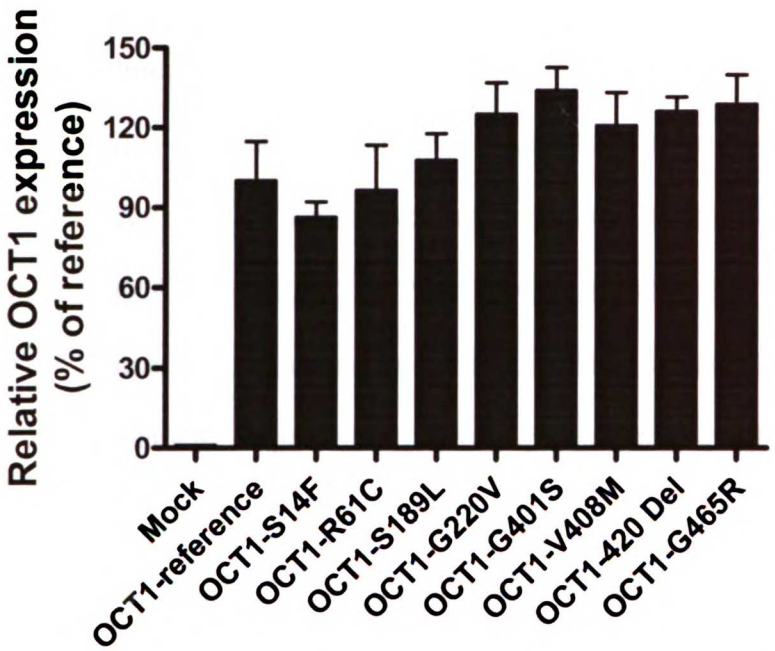

Figure 5.3. RT-PCR of cell lines expressing OCT1 variants. RNA was isolated from cells expressing OCT1 and its variants and then reverse transcribed into cDNA and amplified with primers and probes specific to OCT1. The fold change in OCT1 expression over mock transfected cells was then calculated for each cell line and plotted. The error bars represent the standard deviation of three trials.

Metformin uptake and kinetics

Stable cell lines expressing OCT1-reference, and twelve OCT1 variants were screened for their ability to transport metformin (Figure 5.4). Five of the variants tested showed no difference from OCT1-reference in the uptake of metformin at the concentration examined: OCT1-F160L, OCT1-P341L, OCT1-R342H, OCT1-V408M, and OCT1-R488M. Consistent with the results from the MPP^+ screen, OCT1-G465R was entirely non-functional with respect to metformin uptake. Interestingly, several variants had a significantly reduced metformin uptake in comparison to OCT1-reference: OCT1-S14F, OCT1-R61C, OCT1-S189L, OCT1-G220V, OCT1-G401S, and OCT1-420 del (p-values range from <0.001 for OCT1-S14F to <0.0001 for OCT1-G220V). Of the seven variants with altered metformin uptakes, four had sufficiently high activities to allow for kinetic analysis (Figure 5.5). Whereas no difference in the K_m values of metformin were observed between the OCT1-reference and any of the variants, the V_{max} values showed large differences (Table 5.2). All of the V_{max} values for the rate of metformin transport by the OCT1 variants were statistically different from the V_{max} in OCT1-reference. Notably, the V_{max} of metformin in cells expressing OCT1-G401S was one tenth that in cells expressing OCT1-reference ($p<0.0001$).

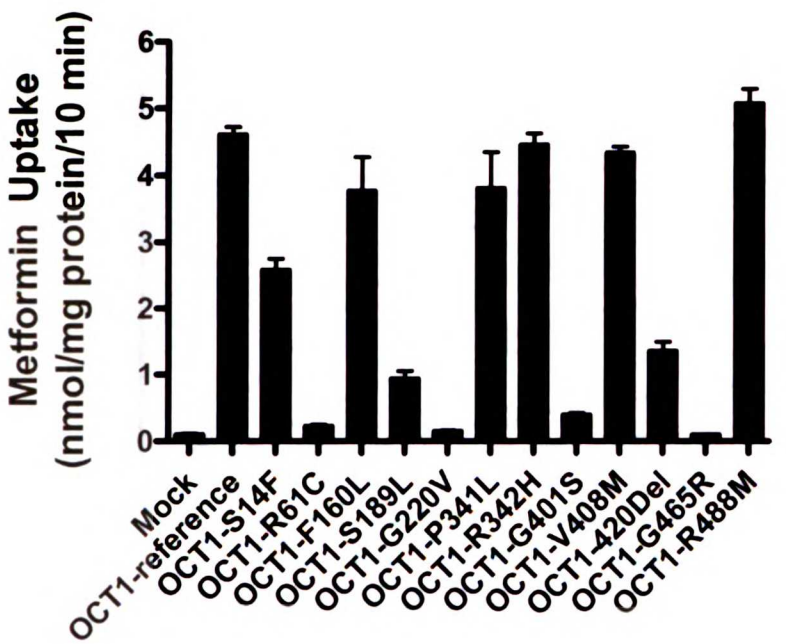

Figure 5.4. Uptake of metformin in HEK cells stably expressing OCT1 variants. Stably transfected HEK cells were incubated with ^{14}C-metformin for 10 min, and the radioactivity associated with each well was assayed by scintillation counting. The pmol of metformin taken up by the cells was then determined, normalized for protein content, and then plotted. The uptake bars respresent the mean of three wells, and the error bars are the standard deviation of the uptake values from the three wells.

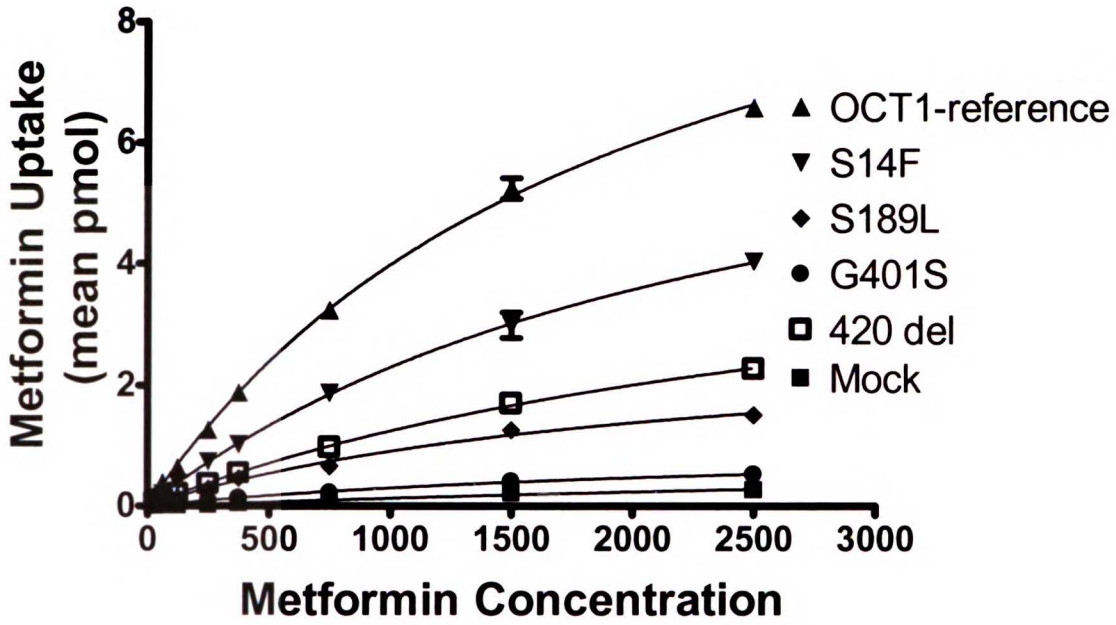

Figure 5.5. Metformin kinetics for select variants of OCT1. The four variants of OCT1 that showed reduced function, but still had sufficient activity were analyzed kinetically. The uptake of metformin at eight different concentrations was studied. The uptake values at each concentration were plotted and fit with a Michaelis-Menten equation.

	OCT1-reference	OCT1-S14F	OCT1-S189L	OCT1-G401S	OCT1-420 del
V_{max} ± SE (nmol metformin/ min/mg protein)	6.74 ± 0.88	3.77 ± 0.65	1.40 ± 0.05	0.67 ± 0.09	3.31 ± 0.56
K_m ± SE (mM)	2.42 ± 0.52	2.65 ± 0.74	2.41 ± 0.15	3.88 ± 0.75	4.56 ± 1.08
V_{max}/K_m	2.79	1.42	0.67	0.17	0.73
R^2	0.991	0.986	0.999	0.996	0.995

Table 5.2. Representative kinetics of metformin with OCT1-reference and OCT1 variant cell lines. HEK cells stably expressing OCT1-reference and four variants were incubated with eight different reaction mixtures, each containing a different concentration of metformin (Figure 5). The experiments were performed in triplicate wells, and the amount of metformin associated with each clone was determined. The data were plotted and fit to a Michaelis-Menten equation. The V_{max} and K_m parameters, and the SE of the values are shown in this table. The V_{max} values are all statistically different from reference OCT1 (p values range from 0.02 for OCT1-S14F to <0.0001 for OCT1-G401S), but none of the K_m values are statistically different from OCT1-reference. Also included are the V_{max}/K_m values, an approximation of the turnover rate for each transporter.

Discussion

The potential role of OCTs in the absorption, distribution, and elimination of organic cation drugs makes them attractive candidates for explaining variation in response, efficacy, and toxicity associated with the use of many therapeutic agents (Koepsell and Endou 2004). Our group and others have studied naturally occurring polymorphisms in OCT family members (Kerb et al. 2002; Leabman et al. 2002; Shu et al. 2003). The non-synonymous variants of OCT1 examined in this study were identified as part of a large pharmacogenomic study (Leabman et al. 2003), and were characterized with the model substrate MPP^+ in *X. laevis* oocytes (Shu et al. 2003). In this study, we determined the effects that the variants in OCT1 have on the transport characteristics of metformin.

Prior to this study, there were two previous studies that examined polymorphisms of OCT1 in *X. laevis* oocytes using MPP^+ (Kerb et al. 2002; Shu et al. 2003). The first study examined four OCT1 variants, three of which we also examined in this study (Kerb et al. 2002). Our results are in agreement with those of Kerb et al, as both studies showed a reduction in MPP^+ uptake by OCT1-R61C and OCT1-G401S, and no change in MPP^+ uptake by OCT1-420 del. Our results also largely agree with those of Shu et al, with the exception of the variants OCT1-S14F and OCT1-P341L (Shu et al. 2003). The variant OCT1-S14F was found to be hyperfunctional in the oocyte expression system used by Shu et al, but we did not observe any hyperfunctionality with the transfected mammalian cells. OCT1-P341L was previously described as hypofunctional in the uptake of MPP^+ in oocytes, whereas we observed no reduction in function in the mammalian cell lines. The disparities between the current study and that of Shu et al are likely due to differences in

expression systems. In comparison to oocytes, which are derived from amphibian, the results obtained in transfected mammalian cells may more accurately reflect the function of OCT1 in human cells. Additionally, we showed through the use of quantitative RT-PCR methods that the mRNA expression levels of all the OCT1 variants were similar to that of OCT1-reference, a control not performed in the previous studies in oocytes, in which cRNA is transcribed *in vitro* and then injected into *X. laevis* oocytes.

When the anti-diabetic drug metformin was used instead of MPP^+, the uptake profile of the variants differed somewhat. In the MPP^+ studies, five variants showed reduced function with respect to OCT1-reference; however, in the metformin study, seven variants had reduced function relative to OCT1-reference. The five variants that had reduced function for MPP^+: OCT1-R61C, OCT1-S189L, OCT1-G220V, OCT1-G401S, and OCT1-G465R, also exhibited reduced function in the metformin study. In addition to these five variants, OCT1-S14F and OCT1-420 del showed a reduction in metformin, but not MPP^+ uptake. Additional data in our laboratory (see Chapter 6) suggests that OCT1-S14F and OCT1-420 del also have a reduced ability to transport oxaliplatin relative to OCT1-reference. Interestingly, all of the reduced function variants appear to exhibit a greater reduction in transport of metformin than of MPP^+, suggesting that the transport of MPP^+ is less sensitive to mutations in OCT1.

The K_m values of metformin for OCT1-reference is similar to the K_m previously determined with hOCT1 in transiently transfected HEK cells (2.4 mM (this study) versus 1.47 mM by Kimura et al (Kimura et al. 2005)), and also similar to the IC_{50} value of 2.0

mM previously obtained in *X. laevis* oocytes (Dresser et al. 2002), but higher than the K_m value obtained for transfected rat OCT1 in CHO cells (377 μM (Wang et al. 2002)). Although the K_m values that we obtained for metformin are well above the plasma concentrations reached during metformin therapy (10-40 μM), OCT1 is believed to be the primary mediator of metformin transport into the liver based on data from knockout mice from our lab and others (unpublished data and (Wang et al. 2002)). OCT1 is expressed in abundance in liver, indicating that there would be a high V_{max} in that tissue, which means that the V_{max}/K_m ratio would be more in line with expectations. K_m is a function of the affinity of the transporter for the substrate examined, so the minimal change in K_m values across the variants suggest that the mutations have a minimal effect on the affinity of OCT1 for metformin. In contrast, V_{max} is proportional to the amount of functional proteins in the membrane, so a reduction in V_{max} may indicate that the mutated form of the protein may not fold properly, or may have trafficking defects. The reduction of metformin uptake observed in the functional screen (see Figure 5.4) was ultimately predictive of the rank order of the V_{max} values in the kinetic study. This phenomenon can be easily seen by comparison of the V_{max}/K_m values listed in Table 5.2.

These findings are of particular interest because some of the variants that have been shown to have reduced uptake of metformin are common in at least one of the ethnic groups studied. Of the seven variants with a reduction or loss of activity for metformin, four occur at a frequency of 3% or greater in the ethnic group in which they are most common. OCT1-420 del is an especially high frequency variant in Caucasians, with a 19% allele frequency; thus, a large percentage of Caucasians are homozygous for this

variant (3.6% expected by Hardy-Weinberg equilibrium). Also of interest is the OCT1-R61C variant (7.2% allele frequency in Caucasians), which has such low activity for metformin as to be almost considered non-functional. The reduced functioning OCT1 variants are not specific to Caucasians, as OCT1-S14F is an African American allele that occurs at 3% in that population, and also shows a reduced ability to transport metformin. Our study suggests that OCT1 may be under low selective pressure given the number of common reduced function alleles.

The implication of our data to in vivo drug disposition can be obtained from studies on *Oct1* knockout mice. These mice exhibit a reduced hepatic uptake of certain organic cations, including metformin. Extrapolating to humans, the data suggest that low or non-functional alleles of OCT1 may affect hepatic distribution of metformin as well (Jonker et al. 2001; Wang et al. 2002; Jonker and Schinkel 2004). Given the prevalence of some of the reduced functioning variants in the sample population, and the fact that the liver is a primary target of metformin, further studies are needed in human subjects to determine whether these variants have similar effects in vivo and may contribute to variation in drug response.

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Chapter 6

The Effect of Polymorphisms in the Human Organic Cation Transporter 1 (OCT1) on the Transport and Cytotoxicity of Oxaliplatin in HEK Cells

The platinum-based anticancer drug oxaliplatin is used in the treatment of colorectal cancer in combination with 5-FU/leucovorin. Although it is structurally similar to other platinum-based chemotherapeutics such as cisplatin and carboplatin, oxaliplatin exhibits a distinct anticancer specificity. Recently, oxaliplatin, but not cisplatin or carboplatin, was found to be a substrate of OCT1 (*SLC22A1*), an organic cation transporter localized primarily to the liver, but also found in the colon and intestine. Many polymorphisms in OCT1 have been identified and characterized for their ability to transport the model substrates MPP^+ and the anti-diabetic drug, metformin. Previous studies indicate that several of the variants in OCT1 encode a reduced or non-functional protein. In this study, we examined the cellular uptake, cytotoxicity, and DNA-adduct formation of oxaliplatin in HEK 293 cells expressing OCT1-reference, and seven of its non-synonymous variants. We observed that oxaliplatin uptake was substantially reduced in cells expressing six of the seven variants in comparison to cells expressing the OCT1-reference (*e.g.*111 ± 14.1 pmol platinum/mg protein for OCT1-reference and 39.2 ± 5.39 pmol platinum/mg protein for OCT1-G465R, $p<0.0001$). Oxaliplatin cytotoxicity and DNA-adduct formation in cells expressing the OCT1 variants paralleled the platinum uptake results. These findings suggest that genetic variation in OCT1 may contribute to the variation in response to oxaliplatin.

Introduction

Cisplatin, carboplatin, and oxaliplatin are platinum-based anticancer drugs that are effective in the treatment of various solid tumors (Weiss and Christian 1993; Wong and Giandomenico 1999). The three compounds are used to treat different types of cancer. For example, cisplatin is used in the treatment of ovarian cancer, testicular cancer and prostate cancer, whereas oxaliplatin is used primarily for colorectal cancer (Rixe et al. 1996; Raymond et al. 1998; Misset et al. 2000). Mechanisms responsible for the tumor specificity of the platinum compounds have been the subject of numerous investigations. In a recent study from this laboratory, Zhang and others demonstrated that the organic cation transporters, OCT1 and OCT2, enhance the cytotoxicity of oxaliplatin, but not of cisplatin and carboplatin, suggesting that OCTs may play a role in the tumor specificity of oxaliplatin (Zhang et al. 2006).

The OCT family is a member of the major facilitator superfamily, and more specifically the solute carrier 22A family (SLC22A) (Koepsell and Endou 2004). SLC22 is defined by sequence homology, and includes organic anion transporters (OATs) and the novel organic cation transporters (OCTNs) in addition to the OCTs (Koepsell and Endou 2004). There are three members of the organic cation family that have been cloned and characterized in humans: OCT1 (Grundemann et al. 1994), OCT2 (Okuda et al. 1996), and OCT3 (Kekuda et al. 1998; Wu et al. 1998). The OCT family members are able to transport endogenous molecules and xenobiotics including several clinically used drugs (Jonker and Schinkel 2004; Wright 2005). Although there is a high degree of overlap in

the substrate specificity of members of the OCT family, each has a unique tissue distribution in humans. OCT1 is predominantly found in liver, but also has some intestinal expression (Gorboulev et al. 1997; Zhang et al. 1997; Muller et al. 2005; Wright 2005); OCT2 is mainly expressed in the kidney (Gorboulev et al. 1997; Wright 2005), and OCT3 has the highest expression in placenta (Grundemann et al. 1998; Verhaagh et al. 1999).

Genetic variants of OCT1, identified in ethnically diverse populations (Leabman et al. 2003), were functionally characterized using the model compound MPP^+ in *X. laevis* oocytes (Shu et al. 2003). Many of the OCT1 variants exhibited a reduction in the uptake of MPP^+ as compared to OCT1-reference. Additionally, some of these reduced functioning variants occurred at high frequencies in the sample population, suggesting that some patients would be homozygous for reduced functioning OCT1 variants.

As a first step towards determining the role of genetic variation in OCT1 in the anti-tumor response of oxaliplatin, we characterized the uptake and cytotoxicity of oxaliplatin in cells expressing seven of the OCT1 variants previously identified (Leabman et al. 2003). Reduced function variants of OCT1 that occur at high frequencies are of particular interest for oxaliplatin therapy, as they may explain some of the variability in its anti-tumor efficacy.

Materials and Methods

Cell Lines and Transfection. Variants of OCT1 were identified in a previous study (Shu et al. 2003). Site directed mutagenesis was used to construct the variants from OCT1 reference in the pcDNA5/FRT vector. Cycling conditions were as previously described (Shu et al. 2003). The mutations were confirmed through DNA sequencing done at McLab laboratories (South San Francisco, CA). Following sequence confirmation, human embryonic kidney (HEK-293) cells were stably transfected with the full length human OCT1 cDNA (OCT1-reference), the empty vector (mock), and seven single mutants of OCT1 which are listed in Table 1. After the initial transfection, stable clones were selected with 75 μg/ml of hygromycin B (also see Chapter 5 methods).

Cell Culture. The culture medium for stably transfected HEK293 cells is DMEM supplemented with 10% FBS and 1X pen/strep (Invitrogen), and 75 μg/mL hygromycin. Cells were cultured in T25 flasks, and were split twice weekly.

Drug Sensitivity Assay. Cytotoxicity of oxaliplatin was measured by the MTT (thiazolyl blue tetrazolium bromide) assay. Cells were seeded in 100 μl of antibiotic-free culture medium in 96-well poly-D-lysine coated plates. After overnight incubation, oxaliplatin was then added to the culture medium to give the indicated final concentrations. After seven hours of incubation, the drug-containing medium was replaced with fresh, drug-free medium and the incubation was continued for 65 more hours, for a 72 hour total study period. The MTT assay was then performed as previously described (Alley et al. 1988). The IC_{50} values were obtained by fitting the percent of the maximal cell growth at different drug concentrations (F) with the equation, $F=100\times(1-C^{\gamma}/(IC_{50}{}^{\gamma}+C^{\gamma}))$, using

WinNonlin (Pharsight, Mountain View, CA). The maximal cell growth was considered to be the cell growth in the medium without any oxaliplatin; C is the concentration of the oxaliplatin and γ is the slope factor.

Cellular Accumulation of Platinum. The platinum uptake was determined as previously described (Holzer et al. 2004) with some modifications. The cells were grown in 100 mm × 20 mm dishes in DME H21 media supplemented with 10% FBS to over 90% confluence in poly-D-lysine coated dishes. For platinum accumulation, the cells were incubated in the culture medium containing the indicated concentrations of the oxaliplatin at 37°C in 5% CO_2 for 2 hr unless specified. After incubation, the dishes were immediately placed on ice and the cells were washed with 6 ml of ice-cold PBS three times and collected. The cell pellets were obtained by centrifugation at 400 x g at 4°C for 15 min. The cell pellets were then dissolved into 200 µl of 70% nitric acid at 65°C for at least 2.5 hours, and then distilled water containing 10 ppb of iridium (Sigma) and 0.1% Triton X-100 was added to the samples to dilute nitric acid to 7%. The platinum content of the lysate was measured by inductively plasma coupled mass spectrometry (ICP-MS) in the Analytical Facility at University of California at Santa Cruz (Santa Cruz, CA). Another aliquot of the cell lysates from a set of identical cultures were used for the BCA protein assay.

Platinum-DNA Adduct Formation. The platinum content associated with genomic DNA as a result of oxaliplatin treatment was determined as previously described (Samimi et al. 2004) with some modifications (Samimi et al. 2004). The cells were grown in 100

mm × 20 mm dishes in antibiotic-free culture media to over 90% confluence in poly-D-lysine coated dishes. The cells were then incubated in the culture medium containing the specified concentrations of oxaliplatin at 37°C in 5% CO_2 for 2 hours (or 25 min as specified). Following the incubation, the cells were washed with ice-cold PBS, scraped and pelleted. Genomic DNA was isolated and purified from the cell pellets using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer's instructions. The cells were initially lysed with Nuclei Lysis Solution (Promega); following RNA digestion and protein precipitation, the cell lysates were centrifuged and the resulting supernatant was aliquoted. The genomic DNA prepared from two separate aliquots of the supernatant was used for platinum and DNA content determination, respectively. For the determination of platinum, one aliquot of the DNA samples were treated with 70% nitric acid at 65°C and diluted in the same way as described above. The platinum content was analyzed using ICP-MS and the DNA content was measured by spectroscopy.

Results

Selection of OCT1 variants

The OCT1 variants used in this study were first identified by Leabman et al (Leabman et al. 2003), and were later characterized with MPP^+ (Shu et al. 2003), and metformin (see Chapter 5). With the exception of OCT1-V408M, the variants chosen for this study were previously shown to have reduced activity for metformin. The frequency at which each variant was identified in the sample population is shown in Table 6.1. A TOPO image of the OCT1 secondary structure showing the position of each variant used in the study is shown in Figure 6.1.

Oxaliplatin: platinum uptake

Figure 6.2 shows the results of the platinum uptake in cell lines expressing OCT1-reference, and each of the seven OCT1 genetic variants. OCT1-V408M had roughly the same platinum uptake as OCT1-reference, but all the other variants showed a significantly lower platinum uptake in comparison to OCT-reference. The uptake of oxaliplatin in cells expressing the variants OCT1-R61C, OCT1-G220V, or OCT1-G465R, was not statistically different from that in mock transfected cells, indicating very low or no platinum uptake. The uptake of platinum in cells expressing OCT1-S14F, OCT1-G401S or OCT1-420 del was statistically different from both OCT1-reference, and mock transfected cells, indicating that a reduction, but not a loss in function results from these mutations.

Variants	AA	CA	AS	ME
S14F	.031	0	0	0
R61C	0	.072	0	.056
G220V	.005	0	0	0
G401S	.007	.011	0	0
V408M	.265	.402	.238	.214
420 del	.029	.185	0	.214
G465R	0	.04	0	0

Table 6.1. Frequency of OCT1 variants used in this study. Variants in OCT1 and other transporters were identified by Leabman et al. The frequency at which these variants were found are shown according to ethnicity. AA: African American, CA: Caucasian, AS: Asian, ME: Mexican

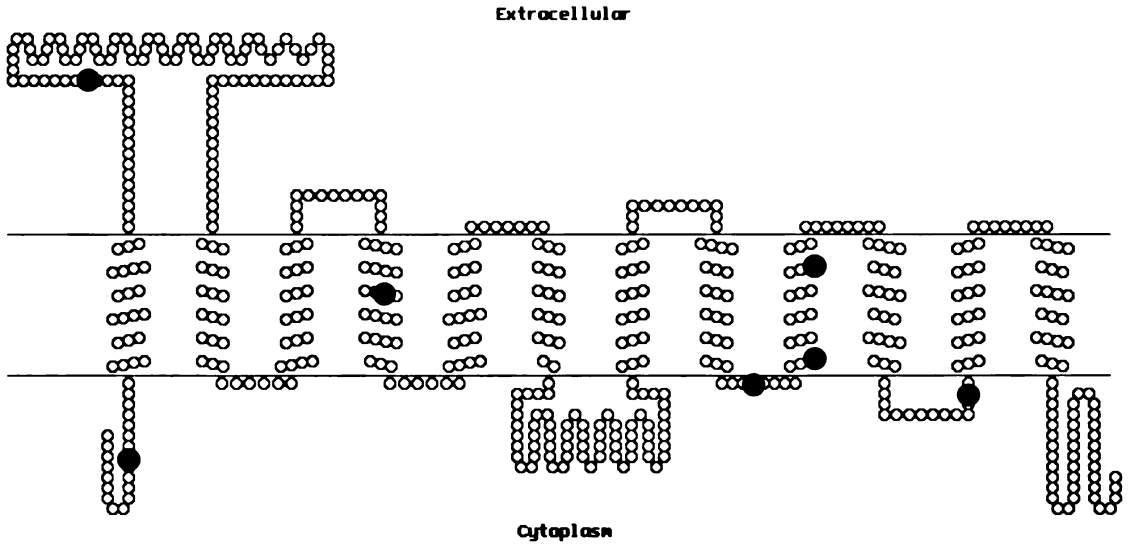

Figure 6.1. Variants of OCT1. The TOPO image is a secondary structure prediction of OCT1. OCT1 is predicted to have 12 transmembrane domains, with a large extracellular loop between the first and second TMDs, and a large intracellular loop between TMDs six and seven. The positions of all seven variants are shown. Non-synonymous variants are indicated by red circles, and the deletion variant is shown in by a blue circle.

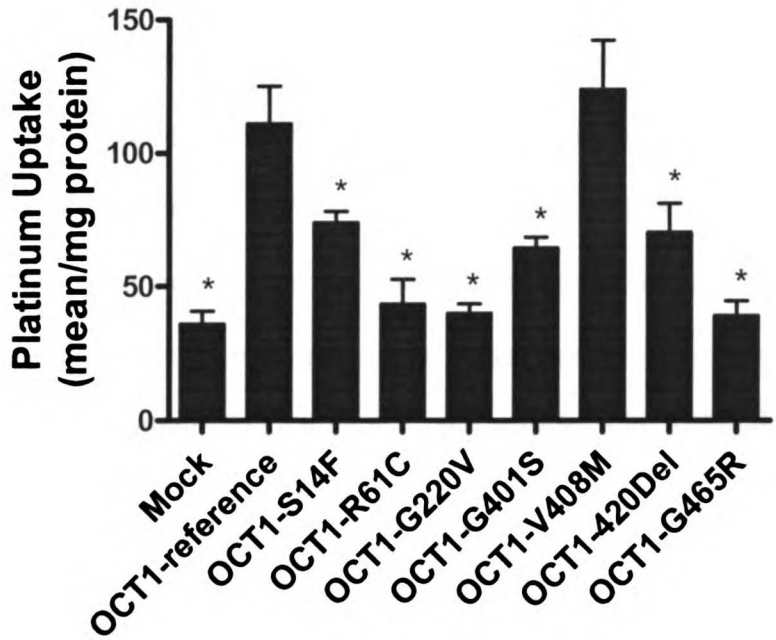

Figure 6.2. Platinum Uptake by OCT1 variants. HEK cells stably expressing OCT1-reference, and its variants were incubated with oxaliplatin, and the platinum content associated with cells expressing each variant was assayed by ICP-MS. OCT1-reference and OCT1-V408M showed the highest uptake of platinum. Three variants showed an intermediate uptake of platinum: OCT1-S14F, OCT1-G401S, OCT1-420 del, and three variants showed platinum uptake similar to that of mock transfected cells: OCT1-R61C, OCT1-G220V, and OCT1-G465R. * indicates a statistically significant difference from OCT1-reference ($p < 0.05$).

Oxaliplatin Cytotoxicity

The results of the MTT assay are displayed in Table 6.2, and representative growth inhibition curves are shown in Figure 6.3. As expected, the sensitivity of cells expressing OCT1-reference and OCT1-V408M was high (i.e. low IC_{50} values), whereas the sensitivity of cells expressing reduced function variants of OCT1 was low. The IC_{50} values mirrored the platinum uptake values shown in Figure 6.2; i.e., variants with a low uptake of platinum had a high IC_{50}, and variants with a high uptake of platinum had a lower IC_{50}.

Oxaliplatin Adduct Formation

The mechanism of oxaliplatin cytotoxicity involves the formation of DNA-platinum adducts. Reduced function variants of OCT1 would be expected to reduce the amount of oxaliplatin that enters the cell, which would in turn reduce the amount of platinum available for adduct formation. In order to investigate this possibility, cells expressing OCT1-reference and its variants were treated as described in the Methods section, and DNA-platinum adduct formation was measured (Figure 6.4). As expected, the greatest DNA adduct formation was associated with cells expressing OCT1-reference and OCT1-V408M, whereas the lowest DNA adduct formation occurred in cells expressing OCT1-G465R, and OCT1-G220V and mock transfected cells. Adduct formation in cells expressing OCT1-R61C was low, but was slightly greater than in cells expressing OCT1-G220V and OCT1-G465R. OCT1-S14F, OCT1-G401S, and OCT1-420 del showed intermediate results, with a higher degree of adduct formation when compared with mock cells, and a lower degree of adduct formation when compared with OCT1-reference.

Variant	Mean IC_{50} ± Standard Deviation (μM)
Mock	1.24 ± 0.31
OCT1-reference	0.20 ± 0.04
OCT1-S14F	0.40 ± 0.16
OCT1-R61C	0.95 ± 0.26
OCT1-G220V	1.34 ± 0.10
OCT1-G401S	0.93 ± 0.18
OCT1-V408M	0.21 ± 0.09
OCT1-420 del	0.56 ± 0.09
OCT1-G465R	1.58 ± 0.42

Table 6.2. Oxaliplatin IC_{50} values for OCT1-reference and variants. Cells expressing OCT1-reference and its variants were plated at a fixed number in 96 well plates. The cells were exposed to oxaliplatin for seven hours, and then the media was changed to normal media for another 65 hours. At 72 hours post oxaliplatin exposure, the MTT assay was conducted as described in the methods. The experiment was repeated twice, with each cell line in quadruplicate both times. The mean and standard deviation reflect the average between the two trials.

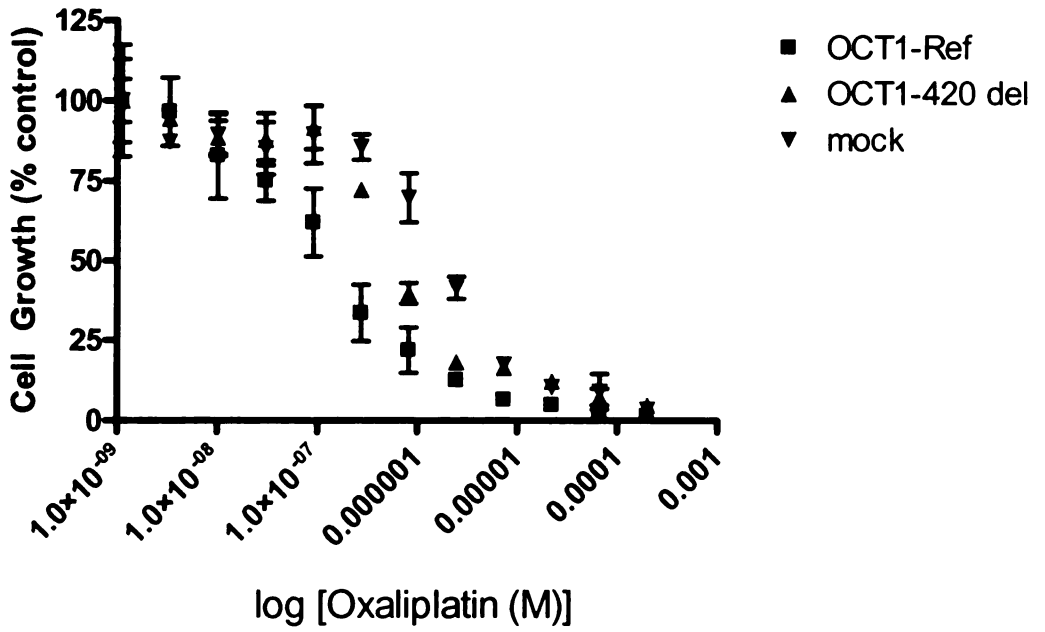

Figure 6.3. Cytotoxicity of oxaliplatin in cells expressing OCT1 variants. The IC_{50} curves for OCT1-reference, OCT1-420 del, and mock transfected cells are shown. The shift of the curves is indicative of the change in cytoxicity.

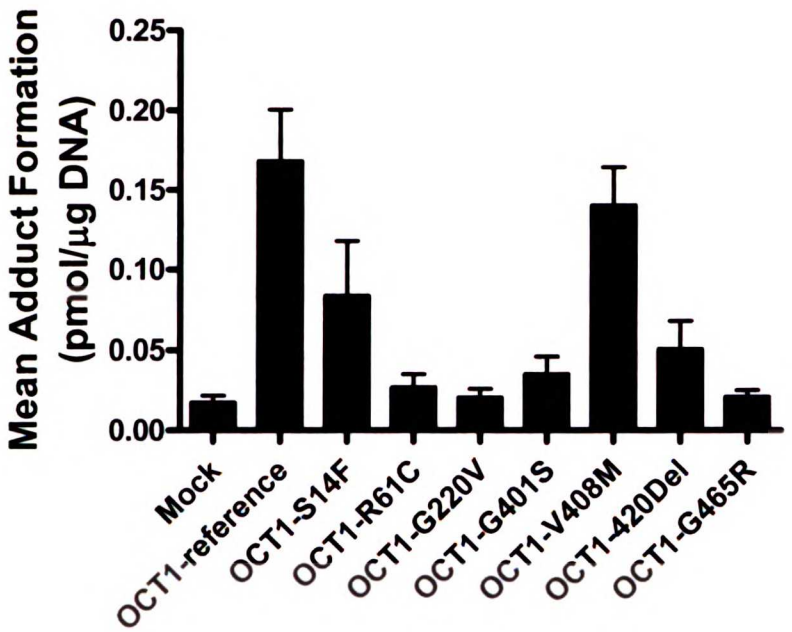

Figure 6.4. DNA Adduct formation for OCT1-reference and variants. Following an oxaliplatin incubation, genomic DNA was isolated from cells expressing OCT1 variants, and the platinum adducts were measured with ICP-MS. OCT1-reference and OCT1-V408M showed the highest adduct formation. OCT1-R61C, OCT1-G220V, and OCT1-G465R all show adduct formation similar to that of mock transfected cells, and the remaining three variants show an intermediate amount of adduct formation.

Discussion

In this study, we examined the interaction of oxaliplatin with genetic variants of OCT1 that had been previously characterized using the model substrates MPP^+ and metformin (Shu et al. 2003) (Chapter 5). Our results were similar to that observed previously for the same variants in the metformin study (see Chapter 5). The three variants with the lowest platinum uptake (OCT1-R61C, OCT1-G220V, and OCT1-G465R) exhibited the lowest metformin uptake in the previous study in mammalian cells, and the lowest MPP^+ uptake in a previous study in *X. laevis* oocytes (Shu et al. 2003). G465R appears to cause a complete loss of function irrespective of substrate whereas R61C and G220V appear to cause a partial loss of function. All of the residues, G465, R61, and G220 are conserved across multiple species. Therefore it is not surprising that changing any of these residues affects protein function. As expected, the common variant OCT1-V408M did not show any significant difference from OCT1-reference in its ability to take up platinum, consistent with previous studies of metformin and MPP^+ transport. Platinum uptake in cells expressing the remaining three variants: OCT1-S14F, OCT1-G401S, and OCT1-420 del, was statistically different from *both* OCT1-reference and mock transfected cells, thus demonstrating a distinctly intermediate phenotype. These variants also showed intermediate uptake of metformin in our previous study. However, oxaliplatin and metformin uptake by these variants differed from MPP^+ uptake by the same variants expressed in *X. laevis* oocytes (Shu et al. 2003). Some of these differences are likely due to differences in the expression system. In fact, we observed that some of the differences resolved when MPP^+ uptake was studied in the HEK cell lines stably expressing the variants (unpublished data). We obtained the same rank order uptake of metformin as

platinum by the OCT1 variants: OCT1-reference > OCT1-S14F > OCT1-420 del > OCT1-G401S. This observation suggests that the mechanism for the reduced function of the variants is not specific to oxaliplatin or metformin, and may instead relate to problems in protein folding or membrane trafficking caused by the variant. Alternatively, the interaction sites for oxaliplatin and metformin may be similar.

Oxaliplatin must gain entry to the cell in order to exert its cytotoxic effects (Andrews and Howell 1990; Gately and Howell 1993). Overall, the cytotoxicity data (expressed as IC_{50} data) correlated very well with the platinum uptake data, suggesting that the reduced function transporters are impairing the ability of oxaliplatin to gain entry into the cell, which in turn limits its ability to exert its cytotoxic effect.

We next examined the mechanism for the reduced cytotoxicity of oxaliplatin in cells expressing the six reduced function variants of OCT1. Our hypothesis was that lower levels of platinum in the cells would result in a reduction of DNA adduct formation. Our data measuring the platinum-DNA adducts parallels the uptake and cytotoxicity studies, namely that the variants cluster into three categories: no functional change (OCT1-reference and OCT1-V408M), intermediate activity (OCT1-S14F, OCT1-G401S, and OCT1-420 del), and low activity (OCT1-R61C, OCT1-G220V, and OCT1-G465R). These data are consistent with the proposed mechanism for oxaliplatin cytotoxicity (Wang and Lippard 2005), i.e. that binding of platinum to DNA results in its cytotoxicity.

Collectively, these results suggest that genetic polymorphism of OCT1 may govern its cytotoxicity and anti-tumor efficacy. Those OCT1 variants with reduced function led to a reduction in the intracellular entry, DNA adduct formation and ultimately, the cytotoxicity of oxaloplatin. The high frequency of many of the reduced function variants in OCT1 suggests that genetic polymorphisms of OCT1 may be clinically important in the treatment of many patients. Except for OCT1-G220V, all of the variants characterized in this study were found at a 1% frequency or greater in at least one ethnic group (Shu et al. 2003). Of particular interest are the severely reduced function variants OCT1-R61C, which was found at greater than a 7% frequency in the European American sample, and the apparent complete loss of function variant OCT1-G465R, found at 4% in European Americans. The intermediate phenotype variant OCT1-420 del, is also of interest because it is found at a high frequency (19%) in European Americans.

Recent data in our lab showed that OCT1 expression is maintained in all 20 human colon cancer tissue samples and in all six colon cancer cell lines that were investigated, suggesting that OCT1 expression is typically not lost in colon cancer tumors. This is in contrast to OCT2, which retained expression in 11 of the 20 tissue samples, and was not found in the colon cancer cell lines. This finding suggests that OCT1 may be the primary transporter that controls the access of oxaliplatin to colon cancer cells which would be of major clinical significance, and indicates that our findings might translate well in a clinical setting.

Our studies suggest that polymorphisms in OCT1 may associate with tumor response to oxaliplatin. Like many anti cancer drugs, oxaliplatin anti-tumor efficacy is low. That is, approximately 52% of people with colorectal cancer achieved no therapeutic benefit from the drug (Goldberg et al. 2006). It is possible that some of this variation may be explained by the level of OCT1 expressed in the tumor, as suggested by our previous study. This study builds on our previous studies, and suggests that the genetic polymorphism of OCT1 may also contribute to its anti tumor response.

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Chapter 7

Pharmacogenetics of Nucleoside Transporters

Transporters in the SLC28A and SLC29A families mediate the transport of nucleosides across the plasma membrane. There are two major families of nucleoside transporters: the Na^+-dependent, inwardly-directed concentrative nucleoside transporters (CNTs *SLC28*), and the bidirectional equilibrative nucleoside transporters (ENTs, *SLC29*). The CNTs and ENTs work in concert to maintain nucleoside homeostasis in the body. In addition to this important physiological role, nucleoside transporters are involved in the absorption and distribution of many clinically used anticancer and antiviral nucleoside analog drugs. This pharmacological role of nucleoside transporters suggests that they may have an influence in the efficacy and toxicity of nucleoside analog drugs. For example, genetic variation in nucleoside transporters may influence the outcome of nucleoside analog therapy. Recently, many nonsynonymous coding region variants in CNTs and ENTs were identified and functionally characterized. Nucleotide diversity among the nucleoside transporters dramatically differs in terms of the numbers and frequencies of polymorphisms in human populations. In this chapter, genetic variation in nucleoside transporters is described. The chapter focuses on coding region variants, and reviews the genetic and functional diversity of the transporters.

The CNTs are secondary active, Na^+-dependent transporters that are located on the plasma membrane, and are predominantly localized to epithelial cells, although they have been reported in other cell types as well (Mangravite et al. 2001; Mangravite and Giacomini 2003; Gray et al. 2004). There are three CNT family members that have been

cloned and characterized: the pyrimidine preferring CNT1 (*SLC28A1*) (Ritzel et al. 1997), the purine preferring CNT2 (*SLC28A2*) (Wang et al. 1997; Ritzel et al. 1998), and the broadly selective CNT3 (*SLC28A3*) (Ritzel et al. 2001). The CNTs are symporter proteins, coupling the influx of the substrate to the Na^+ concentration gradient. The primary physiological roles of the CNTs are to absorb dietary nucleosides from the intestine, and to function in salvage pathways by taking up circulating nucleosides from the plasma thereby reducing the need for the more energy consuming process of *de novo* nucleoside synthesis (Aymerich et al. 2005). In general, the CNTs are high affinity, low capacity transporters, with K_m values in the low micromolar range for their substrates (Casado et al. 2002). Each CNT family member has been shown to transport at least one nucleoside analog drug (see Table 1.3 in Chapter 1 for nucleoside analog drugs and their relationship with the nucleoside transporters). Studies have suggested that CNT1 may play a role in the tissue distribution of the anti-cancer drug gemcitabine, as well as other drugs (Gray et al. 2004). CNT2 transports the antiviral drug ribavirin with reasonably high affinity, but it is a very poor transporter for other purine analog drugs such as cladribine and didanosine, and does not transport most nucleoside analogs at all (Patil et al. 1998; Gerstin et al. 2002; Owen et al. 2005). The fidelity of CNT2 may be specific to the human isoform, as the rat isoform, rCNT2, is able to transport many more compounds (Gerstin et al. 2002). As expected from its broad substrate specificity for the endogenous nucleosides, CNT3 has demonstrated the ability to transport the most nucleoside analog drugs in comparison to other SLC28 family members (Ritzel et al. 2001).

The ENT family, SLC29, performs an endogenous role similar to that of the CNTs, although there are some important differences (Baldwin et al. 2004). ENT1 and ENT2 mediate the bidirectional transport of nucleosides across the plasma membrane in a Na^+-independent fashion, utilizing the concentration gradient of the nucleosides as the driving force. Therefore, these transporters can mediate both the cellular uptake and efflux of their substrates. The ENTs have a wide tissue distribution (see Table 1.3 in Chapter 1), with expression of at least one ENT family member detected in nearly every cell type tested; this is in stark contrast to the CNT family members which are located primarily in epithelial cells (Pennycooke et al. 2001; Kong et al. 2004). Although they share many common substrates, the CNTs have a much higher affinity for the nucleosides than the ENTs (Casado et al. 2002). ENTs likely assist the CNTs in the physiological roles of dietary absorption of nucleosides, and contributing to the salvage pathway. The ENT family has four members that have been reported in the literature, however only two of these appear to transport most nucleosides across the plasma membrane. ENT1 (*SLC29A1*) and ENT2 (*SLC29A2*) are the best characterized members of SLC29, and both transporters are able to transport all naturally occurring nucleosides as well as many nucleoside analog drugs (Baldwin et al. 2004). ENT1 appears to be the predominant isoform, and is expressed in virtually all tissue types examined (Yao et al. 1997; Pennycooke et al. 2001). ENT2 is more highly expressed in skeletal muscle, and has been shown to transport certain nucleobases in addition to nucleosides (Griffiths et al. 1997; Pennycooke et al. 2001; Yao et al. 2002). ENT1 and ENT2 activity can be distinguished by their interactions with the classic nucleoside inhibitor NBMPR (Baldwin et al. 2004); ENT1 is specifically inhibited at lower NBMPR concentrations. Both ENT1

and ENT2 have been shown to transport a broad array of nucleoside analogs, and absence or presence of both has been shown to influence cytotoxicity in vitro (Lu et al. 2002; Molina-Arcas et al. 2003).

A major problem in nucleoside analog drug therapy is a variable response rate to a particular therapeutic agent. Variation in the blood concentration of the agent or the tissue concentrations in which the drug acts may contribute to this variation in response. Nucleoside transporters contribute to the bioavailability, and the tissue distribution of nucleoside analog drugs. Given this important pharmacological role, nucleoside transporters are attractive targets for studies on interindividual genetic variation. Variation in the nucleoside transporter genes could lead to differences in expression or function, which in turn could alter the pharmacokinetics and pharmacodynamics of nucleoside analog drugs. Over the last several years, several papers have examined some of the protein-altering variants identified in the five major nucleoside transporters. Two of the papers resulted from work in this dissertation research (see Chapters 2 and 3) (Owen et al. 2005; Owen et al. 2006).

Genetic Variation in the SLC28A Family in Human Populations

Genetic variants in CNTs and ENTs were identified as part of a larger study (the Pharmacogenomics of Membrane Transporters, or PMT) the goal of which was to identify polymorphisms in membrane transporter genes (Leabman et al. 2003). The sample population for this study was both large and ethnically diverse, and consisted of 100 European Americans, 100 African Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders. Table 7.1 shows a summary of the variants identified in the PMT study as well as HapMap and dbSNP – two other repositories for SNPs. The analyses in this chapter deal exclusively with the PMT data; the URLs for the other databases are included in Table 7.1. The PMT variants were identified through a combination of denaturing HPLC and direct sequencing. Since variation in the coding region is of particular interest, primers were designed in the introns to amplify exons and some flanking intronic regions. Variants therefore fell into three primary categories: intronic, synonymous, and non-synonymous. A handful of insertion or deletion (indel) mutants were also identified. Of these types of variants, non-synonymous variants, and coding region indel variants were considered the most likely to contribute to an altered drug response phenotype. Non-synonymous and indel variants were constructed in vitro and analyzed for functional differences. Figure 7.1 shows the nature and position within the gene of all the nucleoside transporter variants that were identified in the study of Leabman et al.

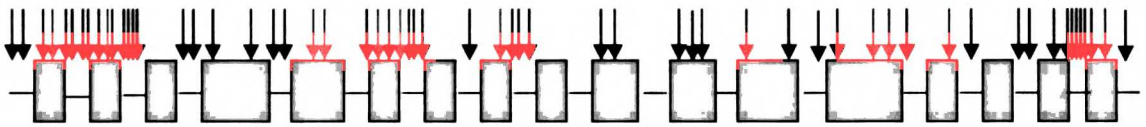
CNT1 is a highly polymorphic gene; it had the highest number of total variants among the CNT family members with 27 variants found in the coding region alone (Gray et al. 2004). Figure 7.2a shows a secondary structure prediction of CNT1 with the nature and

Database	CNT1	CNT2	CNT3	ENT1	ENT2
PMT[8]	58 27 coding	23 10 coding	56 16 coding	15 6 coding	14 11 coding
HapMap[9]	171 10 coding	45 6 coding	228 19 coding	11 19 coding	10 1 coding
dbSNP[10]	312 29 coding	69 5 coding	395 16 coding	40 7 coding	52 13 coding

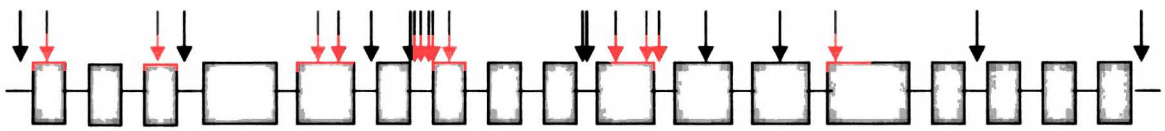
Table 7.1. Databases for SNPs in nucleoside transporters. The database column contains a footnote to the URL address for the home page of each database. Under each nucleoside transporter column is the total number of SNPs found for each gene in the different databases, as well as the number of coding SNPs. PMT is enriched for coding SNPs relative to the other databases because it focused on sequencing coding regions. There is a high degree of overlap for some SNPs, and some were found in all three databases.

[8] http://pharmacogenetics.ucsf.edu/
[9] http://www.hapmap.org/
[10] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=snp

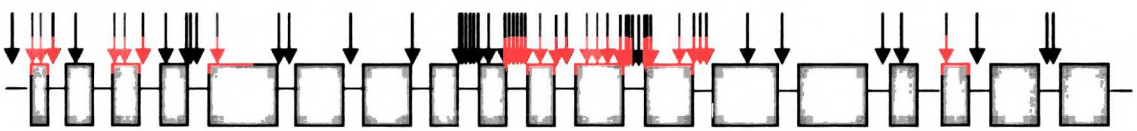
CNT1



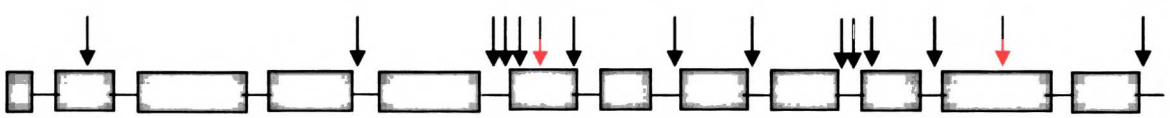
CNT2



CNT3



ENT1



ENT2

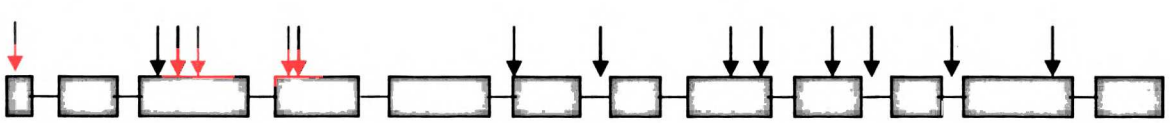
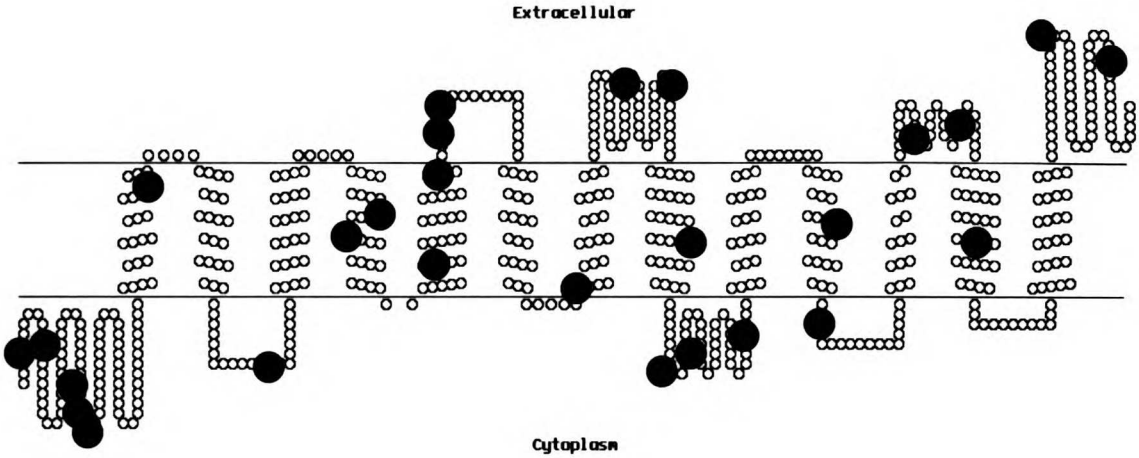


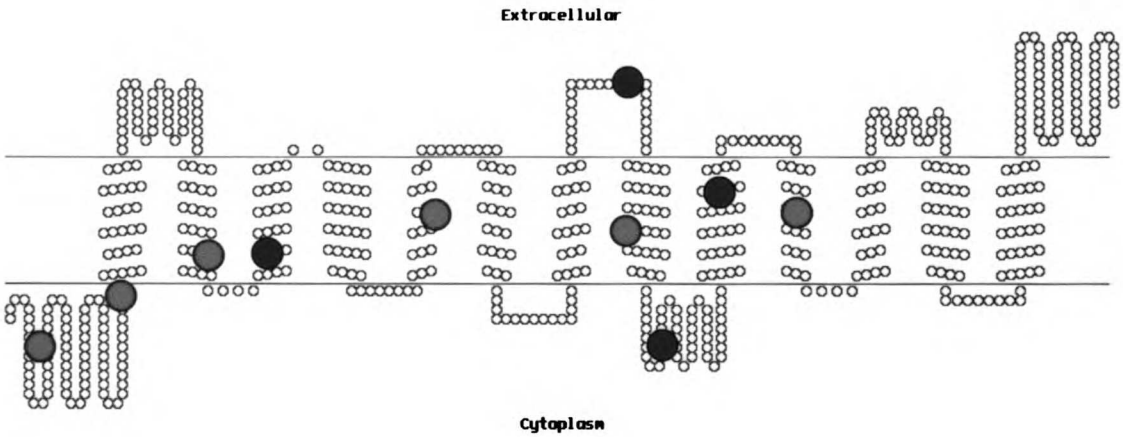
Figure 7.1. Position of the variants identified in concentrative and equilibrative nucleoside transporters. The exons of each transporter are indicated by the rectangular boxes, and the connecting introns by the horizontal black lines. The exons for each gene are to scale, but the introns are not. The complete intronic sequence between exons was not sequenced, so the pictured intronic region represents an abbreviated intronic sequence. The colored arrows represent the position of the variants that were identified in the study of Leabman et al. Black arrows: intronic variants, red arrows: non-synonymous variants, green arrows: synonymous variants, blue: insertion/deletion variants.

position of all the coding region variants identified in the study of Leabman et al. Of these 27 coding variants, 13 were synonymous changes, and were not considered for further study. Of the remaining 14 variants, 12 were non-synonymous variants, and two were indels: one insertion variant and one deletion variant. CNT2 had the fewest polymorphic sites of the CNT family, with a total of 10 coding region variants (Figure 7.2b). Six of the coding region variants in CNT2 were non-synonymous changes (four of the six of which were found commonly), and four were synonymous (Owen et al. 2005). The coding region variants of CNT3 are shown in Figure 7.2c. Ten non-synonymous variants and six synonymous variants were identified in CNT3 (Badagnani et al. 2005). The frequency at which each of the variants was identified in the concentrative nucleoside transporters can be found at http://pharmacogenetics.ucsf.edu/

Population genetics parameters within SLC28A

As part of the pharmacogenomics of membrane transporters study, many transporter proteins were screened for genetic variants in an ethnically-diverse population consisting of 100 African Americans, 100 European Americans, 30 Asians, 10 Mexicans, and 7 Pacific Islanders (Leabman et al. 2003). Following the determination of SNPs, a series of population genetic parameters were determined for each gene for the total sample set, and in each individual population. Some of the more important parameters are θ, π, and Tajima's D statistic (Tajima 1989; Hartl and Clark 1997). θ is a measure of nucleotide polymorphism, and reflects the proportion of nucleoside sites that are polymorphic in a given sample size. π is a measure of nucleotide diversity that is indicative of the proportion of total nucleoside differences between every possible pair of base sequences


Extracellular
Cytoplasm


Extracellular
Cytoplasm

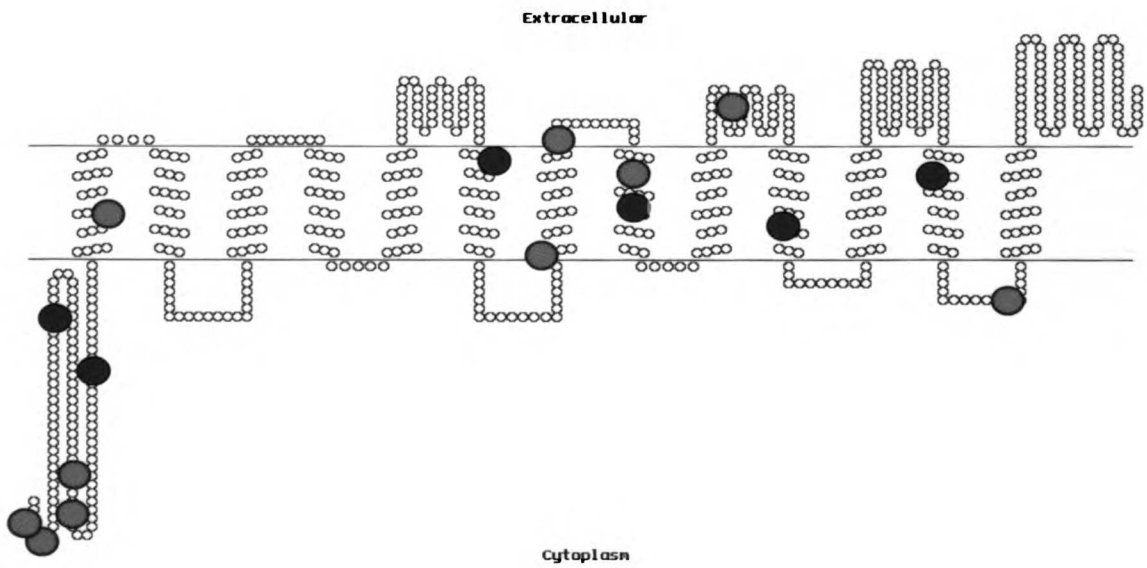
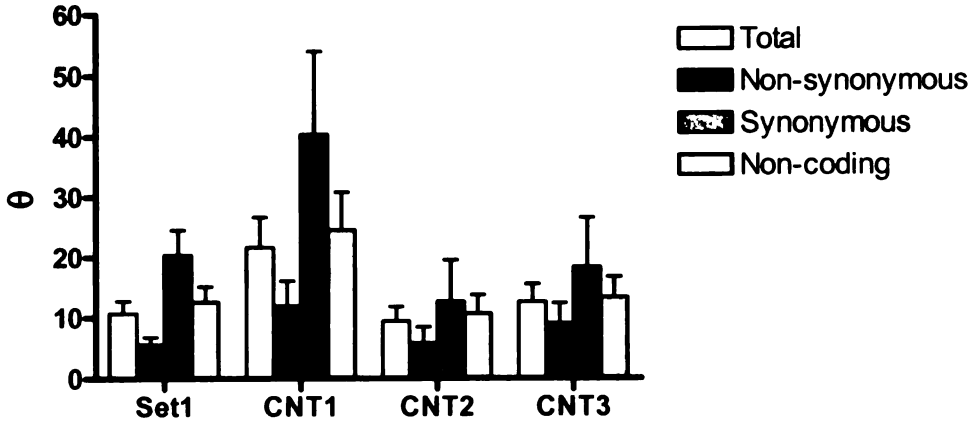

Extracellular
Cytoplasm

Figure 7.2. Position of non-synonymous and synonymous variants identified in the concentrative nucleoside transporter family by the PMT project. Secondary structure images are shown for CNT1 (A), CNT2 (B), and CNT3 (C). The coding region variants that were identified are indicated by red (non-synonymous), green (synonymous), or blue (insertion/deletion) circles at the affected amino acid.

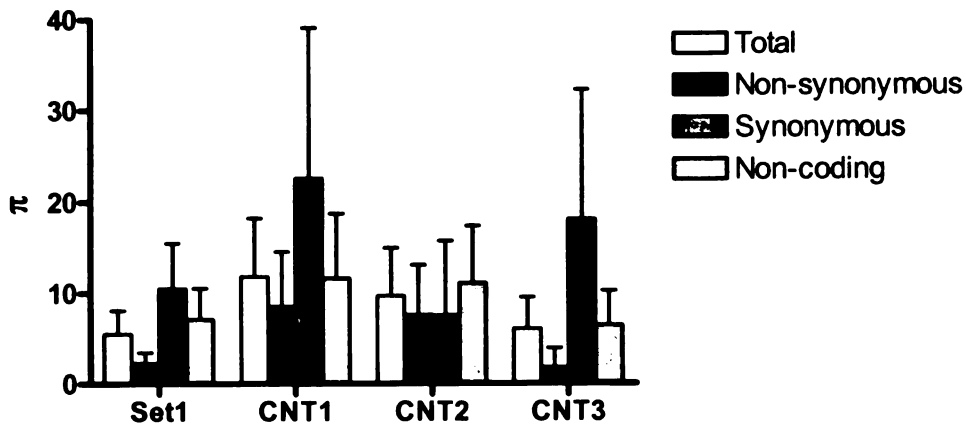
in the sample. Like θ, π can be computed for various gene regions. A subscript indicates the region of the gene the parameter has been computed for. The ratio of π_{NS}/π_S is particularly informative, because it assumes that synonymous changes are neutral, and measures the selection against non-synonymous alleles by comparing them to synonymous alleles in the same gene. Tajima's D statistic is used to detect deviations from the neutral model. The assumption in Tajima's D statistic is that a given mutation will be neutral. A negative D statistic for a gene indicates that there has been deviation from the neutral parameter, and that the gene is under selection; a D statistic of 0 would indicate that the gene is under no selection whatsoever, and a positive D statistic would indicate that the gene is under positive selection for mutations.

The θ, π, and Tajima's D parameters for the CNTs for different regions of the genes are shown in Figure 7.3. When discussing population genetic parameters in SLC28A, we will use the protein names (e.g. CNT1) instead of the gene names (e.g. SLC28A1) for clarity. CNT1 has the highest θ and π parameters, indicating a high level of variation. Despite this variation, CNT1 has a negative Tajima's D statistic, which would suggest it is under negative selection for mutations. Also, the π_{NS}/π_S ratio is less than 1 for CNT1, indicating that the synonymous variants are more tolerated. Examination of the region specific parameters for CNT1 reveals that θ and π are the lowest in the non-synonymous portion of the protein, which is also consistent with selection against non-synonymous change, or preservation of the amino acid sequence. Comparison of the π_{NS} of CNT1 with those of CNT2 and CNT3 suggest that CNT1 is under less selective pressure than the other CNTs. This contention is supported by the fact that an allele encoding a non-

θ values for CNTs in different regions



π values for CNTs in different regions



Tajima's D values for CNTs in different regions

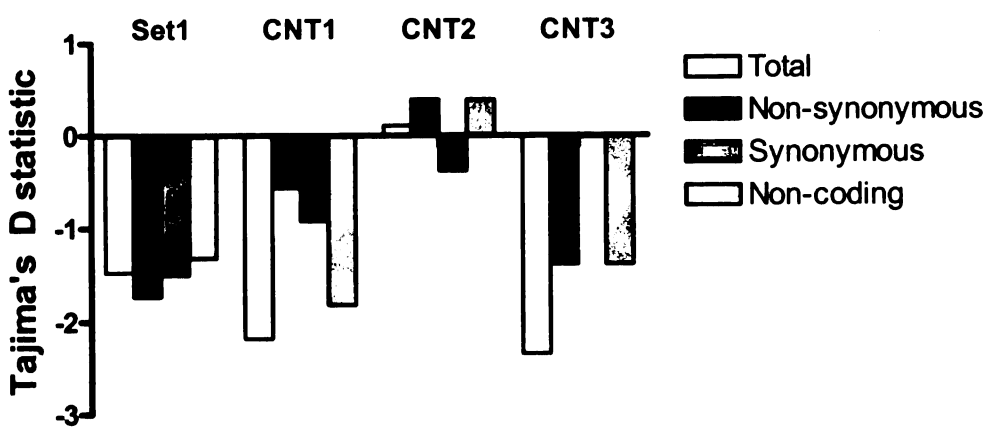


Figure 7.3. Population genetic parameters for CNT transporters in different regions. The θ, π, and Tajima's D value for CNT1, CNT2, CNT3, and the average of the Set 1 genes studied by Leabman et al were determined in four different regions. The error bars represent the standard deviation of the parameters. White bars represent the total sequence, black bars the non-synonymous changes, dark gray the coding region, and light gray the non-coding region.

functional copy of CNT1 was allowed to reach an allele frequency of 3% in African Americans (Leabman et al. 2003; Gray et al. 2004).

Of the CNTs (SLC28A), CNT2 has the lowest θ value, and an intermediate π value between the three concentrative transporters. CNT2 is the only CNT2 with a positive Tajima's D statistic (0.11). A positive D statistic was rare among the genes re-sequenced by Leabman et al., and it is potentially indicative of positive selection. However, the D statistic is close to 0, which could mean that CNT2 is not under any selective pressures. The π_{NS}/π_S is slightly higher than 1, which would also suggest that non-synonymous variants are not being selected against. CNT2 is unique among the CNTs in that it only had a few synonymous variants, all but one of which are rare (see data at http://pharmacogenetics.ucsf.edu/ for frequencies). CNT2 has only one common synonymous variant, and four common non-synonymous variants. It is not clear whether the lack of synonymous variants in CNT2 has just occurred by chance, or whether there was an evolutionary reason for positive selection. The other nucleoside transporters make CNT2 seemingly functionally redundant, but CNT2 does have some unique properties among the nucleoside transporters including differences in localization and tissue distribution (Mangravite et al. 2001).

CNT3 has an intermediate θ value, and the lowest π value among the CNTs. Unlike CNT1 and CNT2, CNT3 does not have many common variants. Only one non-synonymous variant is found at more than a 3% frequency. Although CNT3 has more non-synonymous variants than synonymous variants, the frequency of the synonymous

variants is high in comparison to the frequency of the non-synonymous variants; this is in stark contrast to CNT2 which had relatively rare synonymous variants. The Tajima's D statistic of CNT3 is the most negative of the three, indicating that mutations are more strongly selected against in CNT3 than in the other two CNTs. CNT3 is clearly an important SLC28A family member since it can transport all naturally occurring nucleosides, and it can do so more efficiently than CNT1 or CNT2 because of its 2:1 Na^+:nucleoside coupling ratio. These advantages are plausible explanations for why CNT3 might be under more apparent selective pressure than the other two CNTs.

Despite the similarities in function and tissue distribution of the CNTs, the population genetics of the three transporters are quite different. CNT1 has the most variant positions, but the parameters indicate that it is still under evolutionary constraints. CNT3 has the lowest frequency non-synonymous variants, and the population genetic parameters indicate that it is under the most constraints. CNT2 is arguably the most intriguing of the three family members; the parameters indicate that it may be undergoing positive or neutral selection. The extremely high frequency of some of the non-synonymous variants in CNT2 indicate that those variants are either ancestral, occurring very early in human evolution, or were selected for because they conferred an advantage to humans carrying those mutations. Two of the most common non-synonymous polymorphisms, CNT2-P22L, and CNT2-S75R are in partial linkage, and are the majority allele in Caucasians, although the alleles were found in other populations as well.

Functional Genomics of the SLC28A Family

CNT1: Two of the 15 variants identified in CNT1 caused the resulting variant protein to lose function entirely as indicated by its ability to take up thymidine (1153 del and S546P). The 1153 del polymorphism caused a frameshift mutation that resulted in a severe truncation of the protein (Gray et al. 2004). Somewhat surprisingly, this variant was found at a 3% frequency in the African American population sample. The other non-functional variant, S546P was found on one chromosome in one person (a singleton). One common variant, CNT1-V189I was found to have a statistically different IC_{50} value than CNT1-reference for gemcitabine although its function appeared normal (Gray et al. 2004).

CNT2: All six of the non-synonymous variants of CNT2 retained function (Owen et al. 2005). Although all of the CNT2 variants were able to transport the model compound inosine, one variant, CNT2-F355S, had an altered preference for the nucleosides inosine and uridine versus CNT2-reference. Dual label uptake studies with ^{14}C-inosine and ^{3}H-uridine in oocytes expressing either CNT2-reference or CNT2-F355S showed that the ratio of inosine:uridine uptake was significantly different between CNT2-reference and CNT2-F355S (see Chapter 2). The altered ratio in inosine to uridine uptake could be explained by a reduction in uridine transport, or an increase in inosine transport resulting from CNT2-F355S.

CNT3: CNT3 had a total of 10 non-synonymous variants that were identified and characterized; however, only one of these variants caused a severe reduction in function

(CNT3-G367R) (Badagnani et al. 2005). The uptake of both inosine and thymidine was reduced by this variant, suggesting that the mutation does not specifically alter the uptake of either purine or pyrimidine nucleosides. Another study found two additional variants in hCNT3 in a different population, but neither of the two additional variants had any functional differences from hCNT3-reference (Damaraju et al. 2005).

Genetic Variation in the SLC29A Family in Human Populations

ENT1 had the fewest variant sites of any of the nucleoside transporters, which could be reflective of its role as the predominant nucleoside transporter in many tissues (Osato et al. 2003). A total of six variants were found in the coding region of ENT1: four synonymous variants, and two non-synonymous variants. A secondary structure prediction of ENT1 showing the variants that were identified by Leabman et al. is shown in Figure 7.4a. ENT2 had more polymorphic sites than ENT1, but the protein-altering variants found were all singletons. There were 11 variants identified in the coding region of ENT2: six synonymous variants, 3 non-synonymous variants, and two deletions (Owen et al. 2006). The TOPO image of ENT2 and its variants are shown in Figure 7.4b. The variants identified, and the frequencies at which they occurred in SLC29 can be found at http://pharmacogenetics.ucsf.edu/.

Population genetics parameters within SLC29A

The population genetic parameters of the ENTs in different region are shown in Figure 7.5. The overall variation in ENT1 is very low; only two non-synonymous variants were found, and both were at low frequencies. Interestingly, the synonymous variation of

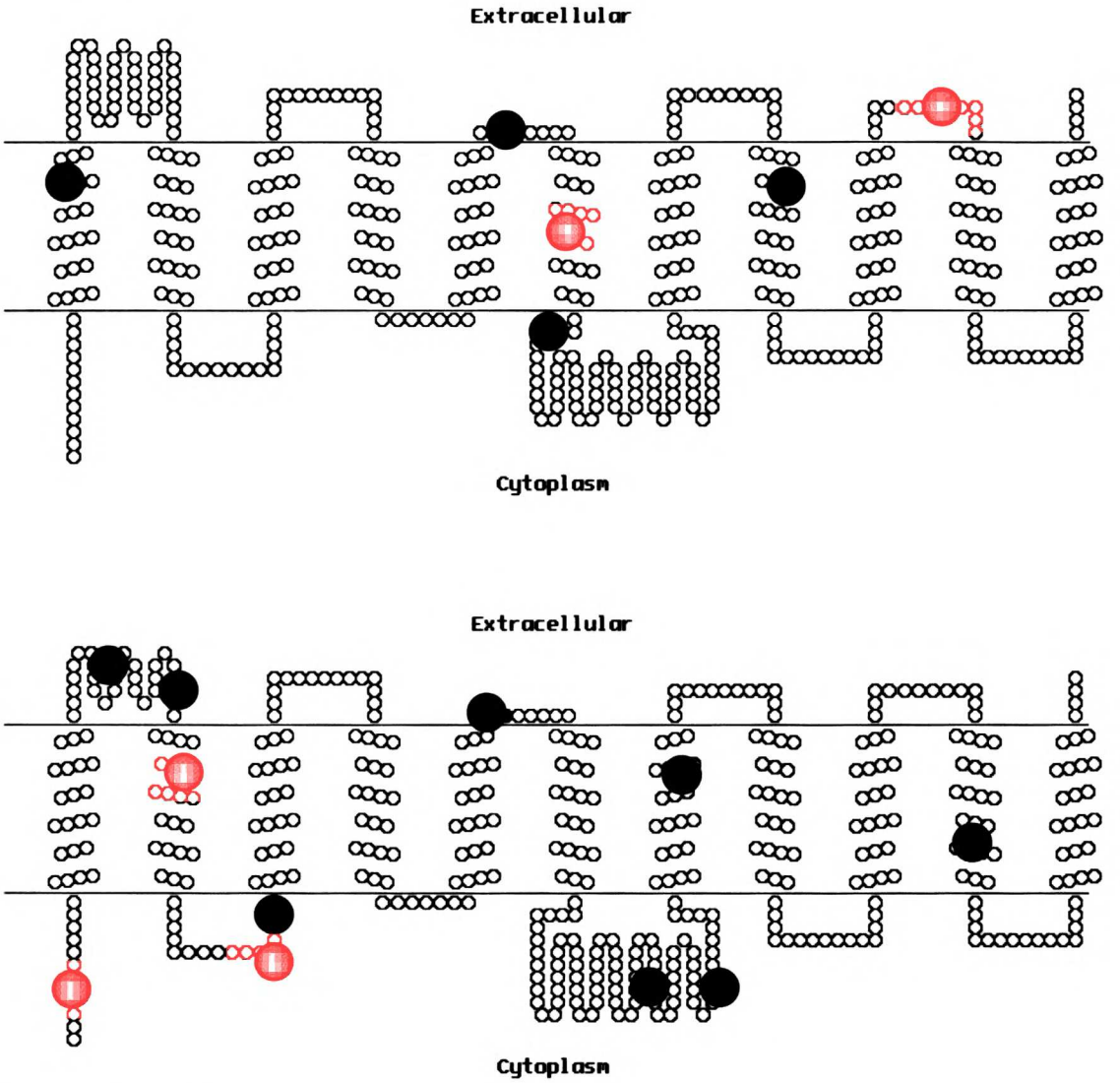
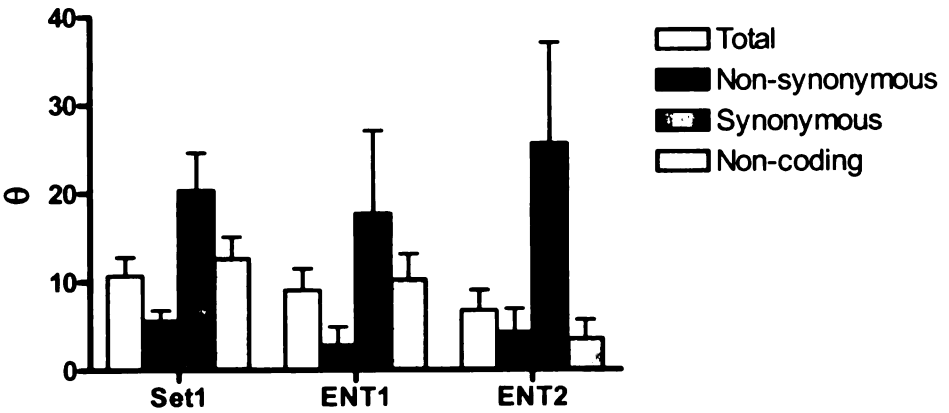
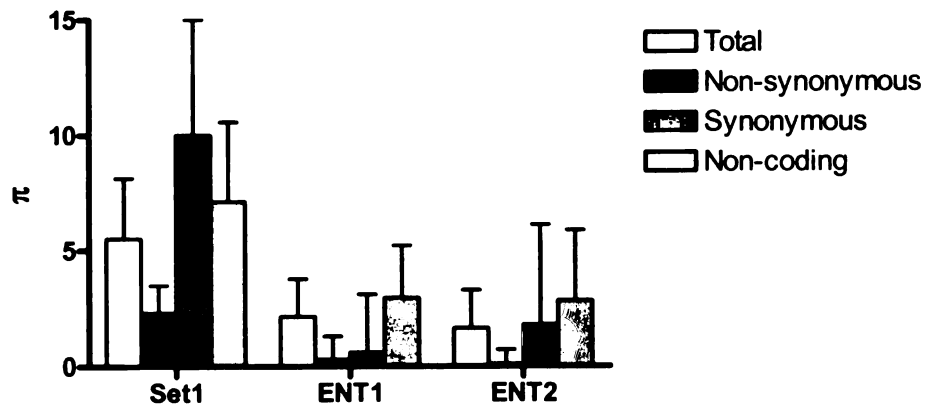


Figure 7.4. Position of non-synonymous and synonymous variants identified in the equilibrative nucleoside transporter family by the PMT project. Secondary structure images are shown for ENT1 (A), and ENT2 (B). The coding region variants that were identified are indicated by red (non-synonymous), green (synonymous), or blue (insertion/deletion) circles at the affected amino acid.

θ values of ENTs in different regions



π values for ENTs in different regions



Tajima's D statistic for ENTs in different regions

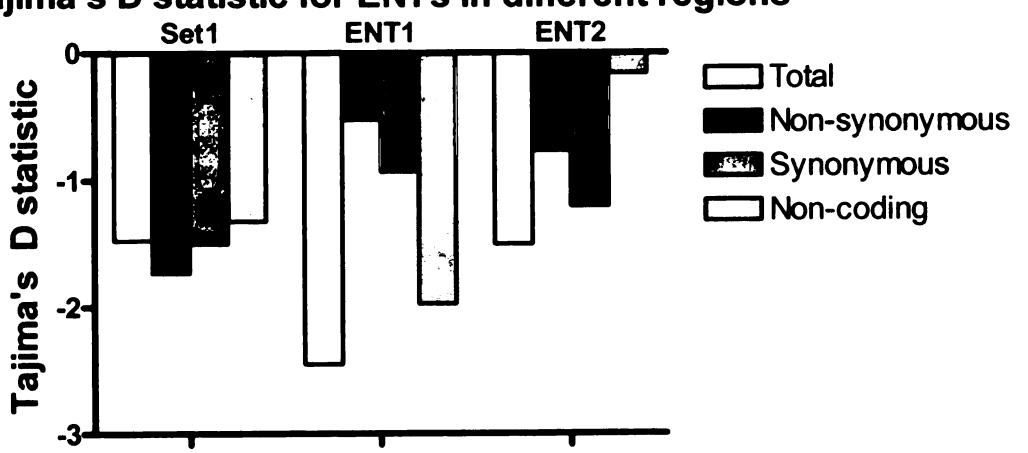


Figure 7.5. Population genetic parameters for ENT transporters in different regions. The θ, π, and Tajima's D value for ENT1, ENT2, and the average of the Set 1 genes studied by Leabman et al were determined in four different regions. The error bars represent the standard deviation of the parameters. White bars represent the total sequence, black bars the non-synonymous changes, dark gray the coding region, and light gray the non-coding region.

ENT1 was also low, indicating selection against change in the entire coding region. ENT1 has low θ and π values, as one might expect given the lack of overall variation (Figure 7.5). The values of π are higher in the non-coding region which suggests that more variation is tolerated in the intronic regions of ENT1, but even the non-coding regions show low variability among individuals. Predictably, the Tajima's D value of ENT1 is negative and the π_{NS}/π_S ratio is less than 1, consistent with a strong negative selection against coding changes.

The overall population genetics of ENT2 are similar to that of ENT1, with very few variants and low θ and π values. One interesting difference is that although ENT2 has more protein altering variants (five) than ENT1 (two), each ENT2 protein altering variant was found on only one of 494 total chromosomes sequenced, whereas ENT1 had a non-synonymous change that reached a 2.1% frequency in European Americans resulting in a π_{NS} value of close to zero (Osato et al. 2003). Another interesting point is that while the non-synonymous variants of ENT1 had a neutral effect on transport function, three of the five variants found in ENT2 had some defect in transport (Owen et al. 2006). Despite the conservation observed in *SLC29A*, there is an ENT1 knockout mouse model which is viable and fertile (Choi et al. 2004). Consistent with their low variation, ENTs have lower π values in coding and non-coding regions in comparison to the π values in the Set 1 genes.

Functional Genomics of the SLC29A Family

ENT1: The two non-synonymous variants in ENT1 were constructed and characterized. However, both variants were found to be no different from ENT1-reference in cytotoxicity and inhibition kinetic assays (Osato et al. 2003).

ENT2: One of the deletion variants in ENT2, ENT2-Δ845-846, caused a frameshift mutation which resulted in a severe truncation of the protein, and a complete loss of function. ENT2-D5Y showed reduced function in the uptake of several substrates when compared to ENT2-reference. The other deletion variant, ENT2- Δ551-556, caused the deletion of two amino acids, and the non-synonymous change of a third residue. This variant was able to take up all of the substrates tested, although it did not take up inosine as well as ENT2-reference (Owen et al. 2006).

Haplotype Analysis

The members of SLC28 and SLC29 share a similar physiological role; they share the same substrates, some of the same tissue distribution, and the same purpose of providing a cell with the building blocks of nucleic acid synthesis (Baldwin et al. 2004; Gray et al. 2004). Given these similarities, the differences in population genetics are all the more striking. ENT1 and ENT2 were among the most conserved genes that were examined in the study of Leabman et al., whereas CNT1 was the most variable gene. Up to this point, we have discussed variation of single sites, but haplotypes may represent a more accurate measure of variation. A haplotype is a block of DNA that is inherited together, over an uncertain length (Nebert 2002). The Bayesian statistical method PHASE (Stephens et al. 2001) was applied to the PMT data in order to estimate the haplotypes for individual genes. PHASE had advantages over other haplotype determination methods in that it is able to incorporate prior assumptions about population genetics and coalescent theory that previous methods did not. Haplotypes determined from PHASE are estimated and are not determined directly.

A single haplotype can be just the reference sequence, or carry one or more deviations from it that are inherited as a unit. For the initial analysis of the haplotypes of the membrane transporters, all information obtained from the study was used to estimate the haplotypes, including the variation found in intronic regions. However, the picture is more simplified, and perhaps more accurate if only "coding haplotypes" are considered. A coding haplotype would consist only of the sites found in the coding region: synonymous, non-synonymous, and indel mutations. All variants that were found on

only one chromosome in one individual were discarded prior to haplotype analysis, as they were considered to be mutations within that individual, and not likely reflective of the population as a whole.

If coding haplotypes are applied to the data for ENT1, only four distinct haplotypes emerge: one containing the non-synonymous amino acid substitution ENT1-E391K which was found on two chromosomes; one containing a synonymous variant in amino acid 28 which was also found on two chromosomes; one encoding the non-synonymous variant ENT1-I216T, which was found on six chromosomes; and the reference ENT1 sequence which was found on 484 of the 494 chromosomes sequenced. The coding haplotype picture for ENT2 is similar, all of the protein-altering variants in ENT2 were singletons, and so were removed prior to haplotype analysis. ENT2 had three coding haplotypes, two containing synonymous variants – one found on nine chromosomes and the other on three - and 482 chromosomes containing reference ENT2. If the synonymous variants are assumed to be neutral, and we only examine the protein-altering haplotypes, the ENTs become less variable still, with a combined 980 out of 988 chromosomes or 99.2% of the chromosomes containing the same coding sequence.

In general, the CNTs have much greater variation than the ENTs. CNT3, the most conserved of the CNT family, has numerous coding haplotypes when synonymous and non-synonymous changes are both considered. Only three of CNT3's 10 non-synonymous changes were found on more than one chromosome in one individual.

Those three variants were found in independent haplotypes, and ranged from fairly rare (CNT3-I328V, 9 chromosomes) to quite common (CNT3-Y113C, 60 chromosomes).

CNT2 was the only nucleoside transporter examined for which a non-synonymous singleton was not found. Therefore, all of CNT2's six non-synonymous variants were included in its haplotypic analysis. CNT2's two most common non-synonymous changes, CNT2-P22L, and CNT2-S75R were in linkage disequilibrium. The haplotype containing both CNT2-P22L and CNT2-S75R was found to be the majority haplotype in the European-American population, although it was found in every ethnic group examined. CNT2 also has other coding haplotypes that include the non-synonymous variants CNT2-L163W, CNT2-S245T, CNT2-F355S, and CNT2-L462F.

CNT1 has many coding haplotypes. In essence, CNT1 is so variable that there is no true reference sequence, and it is so diverse that there is no one, or even a small set of haplotypes that can be used to describe the majority of the sample set. If variation in non-coding regions is also considered, three haplotypes account for more than 50% of the total variation observed in our sample population for both CNT2 and CNT3, whereas 30 haplotypes are needed to account for 50% of the variation in CNT1.

Comparative variation in nucleoside transporters

The CNTs and ENTs perform similar physiological functions; that is, the transport of nucleosides across the plasma membrane. Despite this redundancy in function, the population genetic parameters between the two families are strikingly different. The rank

order of the π_{NS} parameters for the five major nucleoside transporters illustrates this point: CNT1 (8.56), CNT2 (7.64), CNT3 (1.81), ENT1 (0.3), and ENT2 (0.11). CNT1 and CNT2 are highly polymorphic genes, with several non-synonymous variants, some of which are found at high frequencies in our sample population, and thus have high π_{NS} values. On the other hand, ENT1 and ENT2 are extremely conserved with few non-synonymous variants, which results in low π_{NS} values as compared to CNT1 and CNT2. CNT3 is an interesting case because it clearly has the intermediate π_{NS} value of the five nucleoside transporters. The rank orders of π_{NS} / π_S ratios provide a slightly different result: CNT2 (1.04), ENT1 (0.5), CNT1 (0.38), CNT3 (0.1), and ENT2 (0.06). The ratio of π_{NS} / π_S is a comparison of non-synonymous variation versus synonymous variation. CNT2 has a ratio that is slightly greater than 1, which suggests that mutations that cause non-synonymous changes are not selected against any more than those that cause a synonymous change; a very unusual result. This ratio is primarily due to the relative lack of common synonymous variants in CNT2, as only one synonymous variant was found at a greater than 1% frequency. ENT1 has the second highest ratio of π_{NS} / π_S because it also has very few synonymous variants that are of low frequency. This is in contrast to ENT2, which has one common synonymous variant, but only singleton non-synonymous variants, and therefore has the lowest ratio. The evolutionary pressures on ENT1 appear to be selecting against any type of change, both synonymous and non-synonymous, as is evident for the lower π values for both terms in comparison to the π values for the noncoding region. However, large errors in these parameters make it difficult to make comparisons. CNT1, CNT3, and ENT2 all show the expected pattern of non-synonymous variation being selected against more than synonymous variation. The

general pattern is that the ENTs are more intolerant to change in all gene regions examined than the CNTs suggesting that as a family, they may be more important for human fitness.

Comparative variation of nucleoside transporters versus other SLC transporters

We have discussed how CNTs and ENTs related to each other both within and between families, but with the large amount of data generated by the studies of Leabman et al (Leabman et al. 2003), we can put the variation in nucleoside transporters in the broader context of other transporter families which were included in the study. The average π_{NS} value for all 24 genes included in the study of Leabman et al. is 2.31. As compared to all the genes included in the study, CNT1 and CNT2 were well above average in their variation whereas ENT1 and ENT2 are below the average. CNT3 has a π_{NS} value (1.81) that is near that of the PMT average of 2.31. The average ratio of π_{NS} / π_S for all the PMT transporters is 0.23. Again, the π_{NS} / π_S ratios for CNT1 and CNT2 are above this value, although CNT2 is well above it, and CNT1 is near it. ENT2 and CNT3 show a greater than average selection against non-synonymous change, whereas the value of π_{NS} / π_S for ENT1 is above the average for the genes in the study of Leabman et al. for the reasons discussed above.

Concluding Remarks and Future Directions

The genetic variation in the coding region of the five major nucleoside transporters has been identified and characterized. The overall variability was higher in the CNTs as

compared with the ENTs, but both families are less variable in the coding region than in the flanking intronic sequences that were also sequenced. The CNTs also had a greater number of total variants, although there were not many altered function variants identified with the notable exception of CNT1-1153 del (Gray et al. 2004). Some of the variants in the CNTs were found at very high frequencies, but these variants were not found to have radically different functions suggesting that they may be very ancient mutations that have accumulated over time. The variation in the coding region of the ENTs was virtually non-existent, showing the importance of preserving the coding sequence of the ENTs.

The characterization of the variants in the coding region of the nucleoside transporters does not explain the variation seen with nucleoside analog therapy. Future studies on genetic variation in nucleoside transporters will likely focus on non-coding regions that help to determine expression levels, such as the promoter regions of the nucleoside transporters. Another area of study would be genetic variation in other proteins that could influence the expression levels of nucleoside transporters such as transcription factors. The CNTs and ENTs are also under hormonal regulation, so there are many other proteins which could contribute to the overall variation observed in nucleoside analog therapy.

Research in this dissertation has set the stage for further clinical studies examining the effect of genetic variation in nucleoside transporters, particularly with CNT2 and ENT2, on the therapeutic response for nucleoside analog drugs. Additionally, work in this

dissertation has suggested future directions for studying variation in the non-coding region of nucleoside transporters. This work was among the first to characterize the effect of genetic variation in nucleoside transporters with endogenous nucleosides, and nucleoside analog drugs.

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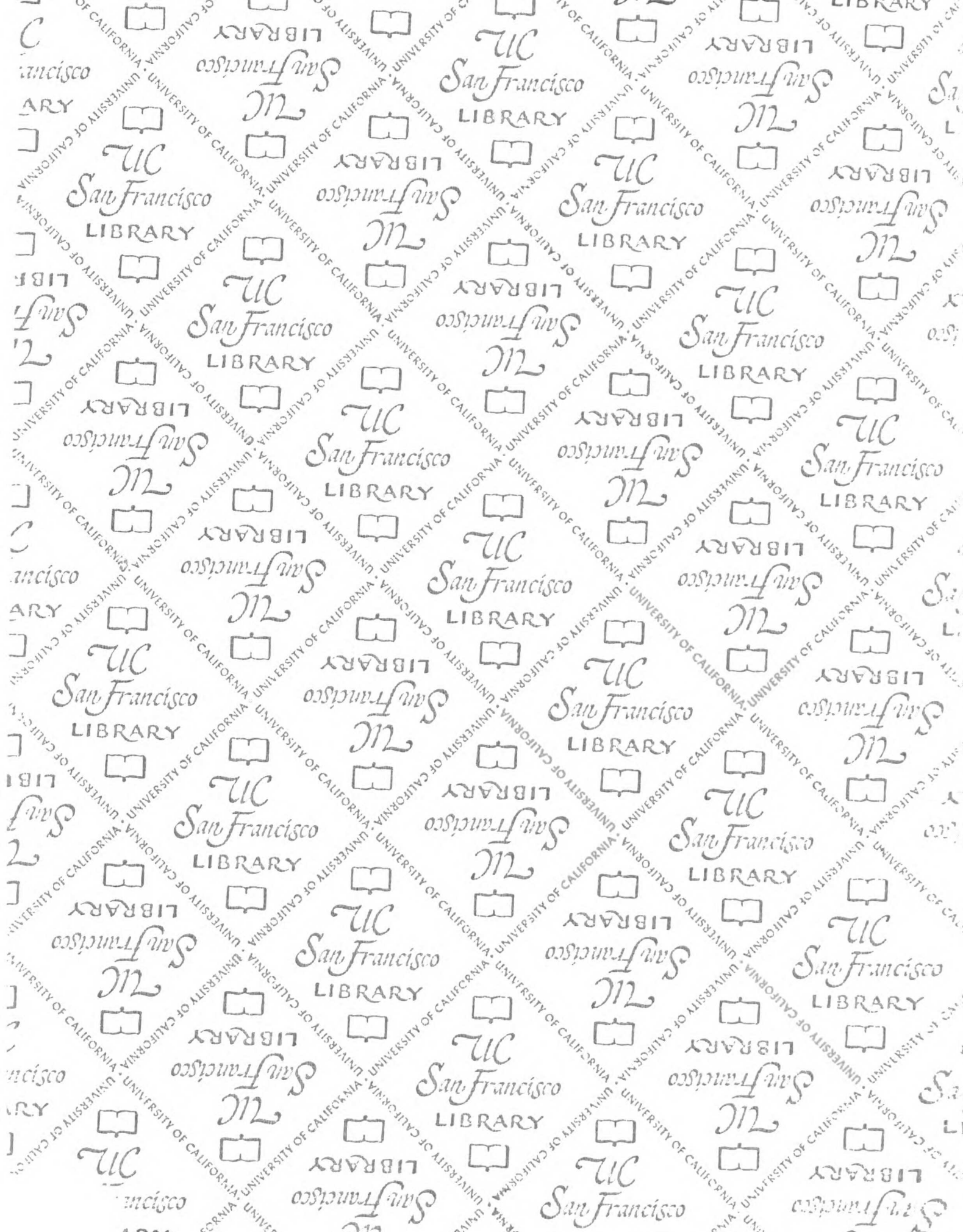
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