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Genetic Variation in Drug Transporters in Ethnic Populations

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DISSERTATION

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ABSTRACT

Genetic Variation In Drug Transporters In Ethnic Populations

Cheryl D. Cropp

Drug metabolizing enzymes and membrane transporters work together in the absorption and disposition of drugs. Although the effects of ethnicity on drug metabolism are well studied, less is known about the ethnic differences in disposition of drugs that are substrates of membrane transporters. Organic anion transporters (OATs) are members of the SLC22 drug transporter family. OATs typically transport a wide variety of endogenous and xenobiotic organic anions. The goals of this dissertation were to determine the ethnic differences in allele frequencies of OAT2 (*SLC22A7*) and OAT3 (*SLC22A8*) variants and to determine if genetic variants of OAT2 and OAT3 impact the ability of these transporters to interact with drugs and endogenous substances. We showed that OAT2 is a novel, bidirectional, facilitative plasma membrane transporter for cGMP and other guanine nucleotides. cGMP exhibited a 50- to 100-fold enhanced uptake in OAT2 transfected cells over empty vector transfected cells. We observed that acyclovir, a guanine-like antiviral agent, was also an excellent substrate of OAT2. We sequenced the coding region of OAT2 in a collection of 272 DNA samples from an ethnically diverse cohort. Six rare non-synonymous variants were identified in the 272 DNA samples, suggesting that OAT2 is under strong negative selective pressure. Only Thr110Ile was polymorphic, with an allele frequency of 2.3% in African Americans. Acyclovir exhibited a significantly reduced uptake and V_{max} in cells expressing Thr110Ile, in comparison to cells expressing the reference OAT2. OAT3, highly expressed in the basolateral membrane of renal proximal tubules, plays a significant role

in the clearance of methotrexate, an anti-cancer and anti-inflammatory drug. We characterized the impact of OAT3 and its genetic variants on the transport of methotrexate and its metabolite, 7-hydroxymethotrexate. We showed that several OAT3 protein-altering variants exhibit significant differences in methotrexate transport compared to OAT3 reference in cellular assays. Genetic variants of OAT3 may contribute to inter-individual variation in methotrexate elimination and toxicity. These studies have led to an enhanced understanding of the role of OAT2, OAT3, and their genetic variants. The knowledge gained in this dissertation research will inform future clinical studies aimed at optimizing drug therapies in ethnically diverse populations.

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CHAPTER 1

GENETIC VARIATION IN DRUG TRANSPORTERS IN ETHNIC POPULATIONS

INTRODUCTION

Drug metabolizing enzymes and membrane transporters work in concert to play crucial roles in drug absorption, distribution, and elimination. It is well recognized that genetic variation in drug metabolizing enzymes contributes substantially to inter-individual differences in drug response. Genes encoding cytochrome P450s (CYP450s), which metabolize greater than 80% of all clinically used drugs are highly polymorphic (1,2), with the notable exceptions of CYP1A1 and CYP2E1. Interethnic variation in the distribution and frequency of variant alleles in drug metabolizing enzymes is known to alter the rate of drug metabolism *in vivo*, resulting in interethnic variation in drug disposition and response.

In contrast to our understanding of the effects of ethnicity on drug metabolism, much less is known about ethnic differences in the disposition and effects of drugs that are substrates of membrane transporters. In this dissertation I summarize findings from recent pharmacogenomic studies of *SLC22A7* (OAT2) and *SLC22A8* (OAT3), two transporters in the major Solute Carrier superfamily (SLC) that are responsible for the influx and efflux of numerous clinically important drugs. This dissertation focuses on these transporters that are present in the liver and kidney (see Table 1) and work together with drug metabolizing enzymes to mediate drug absorption and disposition. Ethnic differences in allele frequencies and functional activities of non-synonymous variants in these two transporters are described.

Table 1. Human liver and kidney transporters important in drug disposition

Gene Symbol*	Protein Name	Full Protein Name	Representative Substrates	References#
Influx Transporters				
SLC22A1	OCT1	Organic Cation Transporter 1	metformin, oxaliplatin	3 , 4
SLC22A2	OCT2	Organic Cation Transporter 2	metformin, amantidine	3 , 4
SLC22A4	OCTN1	Novel Organic Cation Transporter 1	gabapentin	4
SLC22A5	OCTN2	Novel Organic Cation Transporter 2	carnitine	4
SLC22A6	OAT1	Organic Anion Transporter 1	adefovir, tenofovir	5 , 6
SLC22A7	OAT2	Organic Anion Transporter 2	ganciclovir, allopurinol	5 , 6
SLC22A8	OAT3	Organic Anion Transporter 3	cimetidine, cefotaxime	5 , 6
SLC22A11	OAT4	Organic Anion Transporter 4	bumetanide, ketoprofen	5 , 6
SLC22A12	URAT1	Urate Anion Exchanger 1	uric acid, oxypurinol	7 , 8
SLCO1A2	OATP1A2	Organic Anion Transporting Polypeptide A	methotrexate, fexofenadine	9 , 10
SLCO1B1	OATP1B1	Organic Anion Transporting Polypeptide C	pravastatin, repaglinide	9 – 11
SLCO1B3	OATP1B3	Organic Anion Transporting Polypeptide 8	digoxin, paclitaxel	9 , 10
SLCO2B1	OATP2B1	Organic Anion Transporting Polypeptide B	atorvastatin, benzylpenicillin	9, 12
SLC47A1	MATE1	Multidrug and Toxin Extrusion 1	cimetidine, metformin	13 , 14
SLC47A2	MATE2-K	Multidrug and Toxin Extrusion 2	cimetidine, metformin	14 , 15
Efflux Transporters				
ABCB1	P-gp	P-glycoprotein	etoposide, imantinib	16 – 19
ABCB11	BSEP	Bile Salt Export Pump	paclitaxel	11, 17, 20
ABCC1	MRP1	Multidrug Resistance-associated Protein 1	methotrexate	16 – 18
ABCC2	MRP2	Multidrug Resistance-associated Protein 2	doxorubicin, cisplatin	11, 16 – 18
ABCC3	MRP3	Multidrug Resistance-associated Protein 3	etoposide, methotrexate	17
ABCC4	MRP4	Multidrug Resistance-associated Protein 4	methotrexate	17
ABCC6	MRP6	Multidrug Resistance-associated Protein 6	anthracyclines	17
ABCG2	BCRP	Breast Cancer Resistance Protein	mitoxantrone, doxorubicin	16, 19

* SLC = Solute Carrier; ABC = ATP-Binding Cassette.

These are the review articles describing the pharmacological, physiological and/or pharmacogenetic characteristics of the specific membrane transporters.

In the liver, transporters on the sinusoidal membrane of the hepatocyte (*e.g.* OCT1, OAT2, OATP1B1 and OATP1A2) play key roles in hepatic uptake of drugs and thereby control access to hepatic drug metabolizing enzymes (Fig. 1A). These influx transporters also work in concert with efflux transporters on the canalicular membrane (*e.g.* MRP2, BSEP, BCRP and P-gp) to excrete drugs and their metabolites into the bile (Fig. 1A). Because of their critical roles in drug absorption and disposition, identification of genetic variants in hepatic influx and efflux transporters has been the subject of many studies. Such studies have centered primarily on genetic variation in the coding region of the transporter genes, with a particular focus on non-synonymous SNPs, which have a high propensity to produce functional changes in the encoded transporter proteins. In Table 2, for each hepatic and kidney drug transporter, we show the most common non-synonymous variant in each of three populations (African American, AA; European American, EA; and Asian, AS). Frequencies of the non-synonymous variants vary widely among the different transporter proteins. Noteworthy are the low allele frequencies of non-synonymous variants in the organic anion transporter, OAT2 (2% or less), suggesting a high degree of purifying selection on the gene, *SLC22A7*. In contrast, OCT1 has a common non-synonymous variant with an allele frequency greater than 20% in the three populations. Also noteworthy is that there may be large inter-ethnic differences in allele frequencies of particular transporter variants. For example, the MRP4 variant, Lys304Asn, has an allele frequency ranging from 8.7% in European Americans to 22.5 % in Asians. If this allele were associated with functional changes in MRP4, the frequency differences could result in inter-ethnic differences in drug disposition and response.

Figure 1. Common non-synonymous transporter variants in ethnic populations.

Distribution of influx and efflux drug transporters in the liver **(A)** and kidney **(B)**.

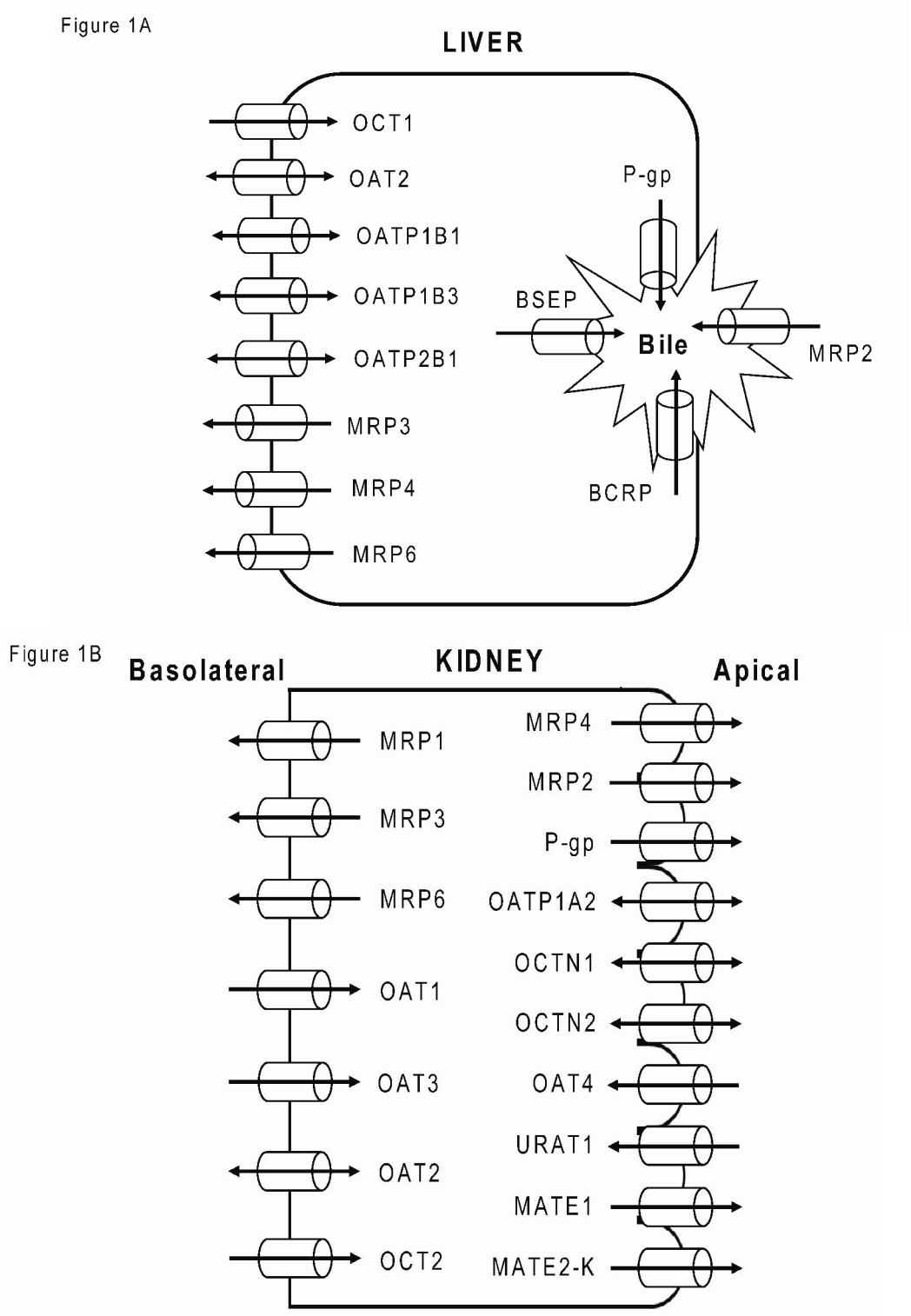

Table 2. The allele frequency of the most common non-synonymous variants for African Americans, European Americans and Asians.

Protein Name	African Americans (%)	European Americans (%)	Asians (%)
Influx Transporters			
OCT1	Val408Met (26.5)	Val408Met (40.2)	Val408Met (23.8)
OCT2	Ala270Ser (11.0)	Ala270Ser (15.8)	Ala270Ser (8.6)
OCTN1	Thr306Ile (26.9)	Leu503Phe (41.2)	Ile306Thr (35.8)
OCTN2	Pro549Ser (10.0)	Val481Phe (0.6)	Phe17Leu (1.7)
OAT1 [a]	Arg50His (3.2)	Ile226Thr (0.6)	
OAT2 [a]	Thr110Ile (2.3)	Arg227His (0.8)	
OAT3	Val281Ala (6.0)	Val448Ile (1.3)	Ile305Phe (3.5)
OAT4 [a]	Arg121Cys (2.3)	Arg48STOP (2.3)	
URAT1 [b]	Thr542LysfsX13 (1.5)		Arg342His (0.8)
OATP1A2 [c]	Thr668Ser (4.4)	Ile13Thr (16.3)	Ile281Val (0.8)
OATP1B1	Asp130Asn (27.2)	Asn130Asp (44.1)	Asp130Asn (19.9)
OATP1B3 [d]	Met233Ile (42.0)	Ala112Ser (19.0)	Ala112Ser (32.0)
		Ile233Met (19.0)	Ile233Met (32.0)
OATP2B1 [e]	Ser486Phe (40.5)	Arg312Gln (10.8)	Arg312Gln (38.9)
MATE1 [b]	Val338Ile (5.1)		Val480Met (0.8)
MATE2-K [a]	Pro162Leu (5.6)	Gly429Arg (0.9)	
Efflux Transporters			
P-gp	Ser1141Thr (11.1)	Ala893Ser (43.8)	Ala893Ser (45.0)
BSEP	Ala444Val (47.0)	Ala444Val (42.9)	Ala444Val (33.3)
MRP1 [a]	Cys1047Ser (4.5)	Val353Met (0.5)	
MRP2	Cys1515Tyr (19.6)	Val417Ile (17.0)	Val417Ile (11.7)
MRP3	Pro920Ser (11.3)	Ser346Phe (2.5)	Gly11Asp (0.8)
MRP4	Lys304Asn (18.1)	Lys304Asn (8.7)	Lys304Asn (22.5)
MRP6	Val614Ala (41.2)	Val614Ala (41.9)	Val614Ala (14.2)
BCRP	Val12Met (7.7)	Gln141Lys (8.1)	Gln141Lys (40.8)

[a] No non-synonymous variants in Asians.
[b] No non-synonymous variants in European Americans
[c] Variant positions were determined relative to the ATG start site and were based on the reference complementary DNA sequence of OATP1A2 (accession number NM_021094)
[d] Allele frequency obtained from dbSNP build 129 (http://www.ncbi.nlm.nih/gov/projects/SNP) using the submission number (ss#) ss38964433 or ss38884356
[e] Allele frequency obtained from dbSNP build 129 (http://www.ncbi.nlm.nih/gov/projects/SNP) using the submission number (ss#) ss19226212 or ss48424042

In the kidney, the basolateral uptake transporters (*e.g.* OAT1, OAT3 and OCT2) are responsible for transporting drugs from blood into renal proximal tubule cells, whereas apical efflux transporters (*e.g.* MRP2, MRP4 and BCRP) transport drugs into the urine for excretion (Fig. 1B). In addition, the uptake transporters on the apical membrane of proximal tubules (*e.g.* OCTN1 and OCTN2), may reabsorb xenobiotics filtered through glomeruli back into tubule cells, whereas basolateral efflux transporters (*e.g.* MRP1, MRP3 and MRP6) transport these reabsorbed chemicals back into the blood. Some transporters, *e.g.*, OCTN1 and OCTN2, have a bifunctional role, transporting selected xenobiotics in the secretory direction into the tubule lumen and particular endogenous compounds (and xenobiotics) in the reabsorptive direction.

Non-synonymous variants of renal anionic transporters (OATs) appear to have low allele frequencies (OAT1, OAT2, OAT3 and OAT4, Fig. 1B and Table 2), consistent with purifying selection on these transporters, and perhaps consistent with a low redundancy in their functions. As in the liver, ethnic differences in allele frequencies are apparent in renal transporters. For example, the most common non-synonymous variant of the novel organic cation transporter, OCTN2, occurs at an allele frequency of 10 % in African Americans and < 1 % in European Americans. For more information on these transporter variants see PharmGKB (www.pharmgkb.org), dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/) and Pharmacogenomics of Membrane Transporters (www.pharmacogenetics.ucsf.edu).

CELLULAR ANALYSIS OF VARIANT TRANSPORTERS

Ethnic differences in allele frequencies of non-synonymous variants in membrane transporters may not translate to ethnic differences in drug disposition or response if the particular transporter variant is not functionally significant. To understand whether such variants may be functionally significant, cellular studies characterizing the functional activity of variant transporters are important. In cellular studies, variants are constructed by site-directed mutagenesis, expressed in cellular systems and functionally characterized. Cellular systems for studies of drug transport include stably and transiently transfected mammalian cells (e.g., HEK-293 and MDCK), and oocytes from *Xenopus laevis*. Low or absent expression of endogenous transporters, and the ease of transfection, make these cell systems optimal for drug transport studies. Use of site-directed mutagenesis in transfected cell lines also allows experimental control of the sequence of the entire cDNA, as opposed to use of actual cell lines derived from patients in which other purportedly silent mutations may confound the results. A drawback of these systems is that transfected cells may not reflect the actual *in vivo* situation.

Typical cellular analyses of the function of transporter variants include a functional screen followed by kinetic studies of interesting variants. However, additional criteria for performing clinical studies examining interethnic difference would include the knowledge that the transporter plays a considerable role in the drug's response and/or disposition. The fact that a drug is a substrate of a transporter does not imply that the transporter will be important *in vivo*. Diffusional processes or other transporters with redundant functions should be considered.

Urban *et al*. (21) conducted an analysis of cellular studies of 88 protein-altering variants (including non-synonymous variants and insertions and deletions) in 11 membrane transporters identified in DNA samples from ethnically diverse populations including African Americans, European Americans, Asians and Mexican Americans. Twenty-two of the variants had reduced function and of these, 5 were shared by two or more populations, whereas 17 were unique to a single ethnic group. Most of these reduced-function variants were found at low allele frequencies; however, some achieved frequencies greater than 5%. Assuming that reduced-function variants associate with variation in drug disposition and response, the population differences in the allele frequencies of reduced-function variants suggest that there will be important ethnic differences in drug disposition and response of substrates of these transporters.

In this dissertation, we address two questions. First, in transporters that are important in drug disposition in the liver and kidney (particularly OAT2 and OAT3), are there ethnic differences in allele frequencies of variants? Second, do variants in OAT2 and OAT3 alter function of the transporters with respect to their interaction with drugs and with endogenous substances? Our results show that there are meaningful ethnic differences in the allele frequencies of variants in OAT2 and OAT3, which could potentially cause variation in disposition and effects of drugs that are substrates of these two transporters. We also show that transporter variants exhibiting effects on function in cellular assays have notable interethnic differences in allele frequencies, suggesting that these reduced-function variants may be a source of variation in drug disposition among ethnic groups. Clearly, to understand the sources and mechanisms responsible for inter-ethnic differences in the disposition and effects of transported drugs, studies that directly

compare the pharmacokinetics and pharmacodynamics of transported drugs among ethnic groups are needed.

SUMMARY OF DISSERTATION CHAPTERS

The studies in this dissertation focus on the functional characterization of OAT2 (*SLC22A7*) and OAT3 (*SLC22A8*). This dissertation unveils novel endogenous substrates (cGMP for OAT2), and drugs used in the treatment of herpes simplex virus (acyclovir for OAT2) and cancer/inflammatory disease (methotrexate for OAT3). A brief description of each of the chapters in this dissertation is presented below.

Chapter 2. Organic Anionic Transporter 2 (*SLC22A7*) is a Facilitative Transporter of cGMP

The second messenger, cGMP, mediates a host of cellular responses to various stimuli, resulting in the regulation of many critical physiologic functions. The existence of specific cGMP transporters on the plasma membrane that participate in the regulation of cGMP levels has been suggested in a large number of studies. In this study, we identified a novel plasma membrane transporter for cGMP. In particular, we show that OAT2 (*SLC22A7*), a member of the solute carrier (SLC) superfamily, is a facilitative transporter for cGMP and other guanine nucleotides.

Chapter 3. The Role of Human Organic Anion Transporter 2 (*SLC22A7*) and its Genetic Variants in the Transport of Acyclovir

Acyclovir, an antiviral agent, is the pharmacologic treatment of choice for Herpes Simplex Virus (HSV) hepatitis, a potentially fatal disease. In Chapter 2, we report that organic anion transporter 2 (OAT2; *SLC22A7*) demonstrates a robust preference for the transport of guanine-based analogs, including cGMP. We further report that acyclovir, a guanine analog antiviral, is also an excellent substrate for OAT2. With the knowledge that OAT2 is abundantly expressed in the liver and has a preference for the transport of acyclovir, we identified genetic variants of OAT2 in an ethnically diverse population and characterized the function of the variants in cellular studies as a first step towards understanding whether genetic variants in OAT2 contribute to the efficacy and drug disposition of acyclovir.

Chapter 4. Interaction of Methotrexate with the Organic Anion Transporter 3 (OAT3; *SLC22A8*) and its Genetic Variants

Methotrexate (MTX) is clinically used in the treatment of cancer and inflammatory diseases. This drug is actively secreted in the proximal tubule of the kidney by organic anion transporters (OATs). At the high doses used in the treatment of leukemia, MTX exhibits nephrotoxicity. Thus, it is important to determine the mechanisms by which it is transported in the kidney and whether genetic variants in relevant transporters affect its transport. In this study we analyzed genetic variation in OAT3 (*SLC22A8*) and characterized the interaction of MTX and its major metabolite, 7-hydroxymethotrexate (7-OH-MTX), with OAT3 and its genetic variants.

Chapter 5. Conclusions

In this chapter, we summarize the results of the studies in this dissertation, highlighting major findings. We describe future studies needed to determine whether genetic variants in membrane transporters may play a role in clinical drug response. We also describe basic and cellular mechanistic studies that will be needed to determine whether variation in noncoding regions of transporters alters the expression level or activity of the transporters. The focus of Chapter 5 is on OAT2 and OAT3.

REFERENCES

1. Ingelman-Sundberg M. Polymorphisms of cytochrome P450 and xenobiotic toxicity. *Toxicology* **181:**447-452 (2002).
2. Jonker JW and Schinkel AH. Pharmacological and physiological functions of the polyspecific organic cation transporters: OCT1, 2, and 3 (SLC22A1-3). *J Pharmacol Exp Ther* **308:**2-9 (2004).
3. Koepsell H, Lips K and Volk C. Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharm Res* **24:**1227-1251 (2007).
4. Rizwan AN and Burckhardt G. Organic anion transporters of the SLC22 family: biopharmaceutical, physiological, and pathological roles. *Pharm Res* **24:**450-470 (2007).
5. Sekine T, Miyazaki H and Endou H. Molecular physiology of renal organic anion transporters. *Am J Physiol Renal Physiol* **290:**F251-261 (2006).
6. Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, Goto A, Sakamoto A, Niwa T, Kanai Y, Anders MW and Endou H. Molecular identification of a renal urate-anion exchanger that regulates blood urate levels. *Nature* **417:**447-452 (2002).
7. Iwanga T, Kobayashi D, Hirayama M, Maeda T and Tamai I. Involvement of uric acid transporter in increased renal clearance of the xanthine oxidase inhibitor oxypurinol induced by uricosuric agent, benzbromarone. *Drug Metab Dispos* **33:**1791-1795 (2005).

8. Niemi M. Role of OATP transporters in the disposition of drugs. *Pharmacogenomics* **8:**787-802 (2007).

9. Tirona RG and Kim RB. Pharmacogenomics of organic anion-transporting polypeptides (OATP). *Adv. Drug Deliv Rev* **54:**1343-1352 (2002).

10. Ito K, Suzuki H, Horie T and Sugiyama Y. Apical/basolateral surface expression of drug transporters and its role in vectorial drug transport. *Pharm Re*s **22:**1559-1577 (2005).

11. Grube M, Kock K, Oswald S, Draber K, Meissner K, Eckel L, Bohm M, Felix SB, Vogelgesang S, Jedlitschky G, Siegmund W, Warzok R, and Kroemer HK. Organic anion transporting polypeptide 2B1 is a high affinity transporter for atorvastatin and is expressed in the human heart. *Clin Pharmacol Ther* **80:**607-620 (2006).

12. Otsuka M, Matsumoto T, Morimoto R, Arioka S, Omote H and Moriyama Y. A human transporter protein that mediates the final excretion step for toxic organic cations. *Proc Natl Acad Sci USA* **102:**17923-17928 (2005).

13. Tanihara Y, Masuda S, Sato T, Katsura T, Ogawa O and Inui K-I. Substrate specificity of MATE1 and MATE2-K, human multidrug and toxin extrusions/H+-organic cation antiporters. *Biochem. Pharmacol* **74:**359-371 (2007).

14. Masuda S, Terada T, Yonezawa A, Tanihara Y, Kishimoto K, Katsura T, Ogawa O and Inui K. Identification and functional characterization of a new human kidney-specific H+/organic cation antiporter, kidney-specific multidrug and toxin extrusion 2. *J Am Soc Nephrol* **17:**2127-2135 (2006).

15. Cascorbi I. Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs. *Pharmacol. Ther* **112:**457-473 (2006).

16. Huang Y. Pharmacogenetics/genomics of membrane transporters in cancer chemotherapy. *Cancer Metastasis Rev* **26:**183-201 (2007).

17. Kerb R, Hoffmeyer S and Brinkmann U. ABC transporters: hereditary polymorphisms and pharmacological impact in MDR1, MRP1 and MRP2. *Pharmacogenomics* **2:**51-64 (2001).

18. Lepper ER, Nooter K, Verweij J, Acharya MR, Figg WD and Sparreboom A. Mechanisms of resistance to anticancer drugs: the role of the polymorphic ABC transporters ABCB1 and ABCG2. *Pharmacogenomics* **6:**115-138 (2005).

19. Stieger B, Meier Y. and Meier PJ. The bile salt export pump. *Pflugers Arch* **453:**611-620 (2007).

20. Badagnani I, Castro RA, Taylor TR, Brett CM, Huang CC, Stryke D, Kawamoto M, Johns SJ, Ferrin TE, Carlson EJ, Burchard EG and Giacomini KM. Interaction of methotrexate with organic-anion transporting polypeptide 1A2 and its genetic variants. *J Pharmacol Exp Ther* **318:**521-529 (2006).

21. Urban TJ, Sebro R, Hurowitz EH, Leabman MK, Badagnani I, Lagpacan LL, Risch N and Giacomini KM. Functional genomics of membrane transporters in human populations. *Genome Res* **16:**223-230 (2006).

CHAPTER 2

ORGANIC ANION TRANSPORTER 2 (*SLC22A7*) IS A FACILITATIVE TRANSPORTER OF cGMP*

INTRODUCTION

The cyclic nucleotide cGMP is a second messenger involved in mediating cellular response to various stimuli in numerous cell types. cGMP signaling through the activation of cGMP-dependent protein kinases regulates a wide variety of intracellular functions, and the potential involvement of extracellular cGMP in a number of biological processes has been suggested (1,2). Because of its critical roles, proteins that regulate intracellular cGMP levels have been the subject of many studies. Such proteins include guanylate cyclases (GC), which play key roles in cGMP production, and phosphodiesterases (PDEs), involved in cGMP degradation.

The existence of plasma membrane transporters capable of transporting cGMP into as well as out of cells has been suggested in a large number of studies in a variety of cell types (2), although the molecular identities of such transporters have remained unclear. Recently, three members of the multidrug resistance protein (MRP) family (part of the ATP binding cassette (ABC) superfamily of transporters), MRP4, MRP5, and MRP8 have been shown to transport cGMP (3-5). These transporters may play a role in the extrusion of cGMP from cells; however, these transporters do not participate in the transmembrane influx of cGMP since they are exclusively efflux pumps.

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The solute carrier (SLC) superfamily is a major class of transporters responsible for the cellular influx and efflux of a great variety of endogenous substances including amino acids, peptides, sugars, organic ions, and nucleosides as well as a multitude of xenobiotic drugs. Although nucleoside transporter families exist within the SLC superfamily, (i.e., the concentrative nucleoside transporter (CNT) family (*SLC28A*) and the equilibrative nucleoside transporter (ENT) family (*SLC29A*), none of these have been shown to transport cGMP (6,7).

In humans, four organic anion transporters, OAT1 (*SLC22A6*) (8,9), OAT2 (*SLC22A7*) (10), OAT3 (*SLC22A8*) (11), and OAT4 (*SLC22A11*) (12), have been functionally characterized and have been shown to mediate the facilitative transport of various structurally diverse organic anions with partly overlapping substrate specificities (13). Among the OAT family, only hOAT2 has ubiquitous expression pattern with abundant expression in many tissues, whereas the other OATs are primarily expressed in the kidney. The specificity of hOAT2 has not been studied in great detail, although it has been thought to be an organic anion-preferring transporter with substrate specificity overlapping that of the other OATs.

While performing a routine screen to identify substrates of hOAT2, we discovered that the guanine nucleoside analog, acyclovir, vigorously interacted with hOAT2 (unpublished data). This finding led us to explore the interaction of other guanine nucleotides with hOAT2. The data presented in this study suggest that an important function of hOAT2 is to transport naturally occurring nucleobases, nucleosides and nucleotides, with a particular preference for guanine analogs. The transport of cGMP is the most avid of the substrates yet identified for hOAT2 and our data indicate that hOAT2 functions as a bidirectional facilitative cGMP transporter.

MATERIALS AND METHODS

Materials

All standard chemicals were purchased from Sigma. [^{3}H]-PAH and [^{3}H]-estrone sulfate (ES) were obtained from American Radiolabeled Chemicals, and all radiolabeled nucleobases, nucleosides, and nucleotides were from Moravek. Cell culture materials were supplied from UCSF Cell Culture Facility.

Human total RNA and cDNA were purchased from Clontech. All primers were obtained from Invitrogen and probes for TaqMan assays were from Applied Biosystems.

Bioinformatics

Full length reference sequences for all proteins (OAT1-7, ENT 1-4, and CNT1-3) were obtained from NCBI. A multiple sequence alignment and neighbor-joining tree were created using ClustalX 1.83 using the default parameters (14). The resulting dendrogram was created using TreeView 1.6.6 (15).

Cloning and transient and stable expression of human OATs

cDNAs coding human OAT2 (hOAT2 or hOAT2-546aa, GenBank accession number NM_006672) and its splice variant (hOAT2-548aa, GenBank accession number NM_153320) were cloned by RT-PCR from human liver and kidney cDNA (Clontech, CA), respectively. Primers used were 5'-CCAGAGTCCAAGGGTCTATGT-3' (sense) and 5'-ATCAAGGATGGATGAGCAGAG-3' (antisense). Human OAT4 cDNA (GenBank accession number NM_018484) was cloned from kidney. cDNA clones for human OAT1 (GenBank accession number NM_004790) and OAT3 (GenBank accession number NM_004254) were obtained similarly as described above. Each cDNA was subcloned into pcDNA5/FRT (Invitrogen) and used to generate transient or stable HEK293 cell lines using the Flp-In® system (Invitrogen) as described previously (16). To generate stable cell lines, Flp-In-293 cells were plated at a density of 6 x 10^5 cells per well in six-well tissue culture treated plates using antibiotic-free media and incubated overnight. Cells reached 95% confluence at 24 hours after seeding, at which point cells were transfected with 0.4μg of hOAT2 cDNA or pcDNA5/FRT (empty vector), 3.6 μg of pOG44 DNA, and 20 μg of Lipofectamine 2000. Two days after transfection, cells were trypsinized and split 1:4 into new six-well plates and selected for stable transfectants by addition of hygromycin B (75 μg/mL) to the growth media (DMEM containing 10% fetal bovine serum, 100 U/mL penicillin and 100μg/mL streptomycin). After 10 to 14 days under selection, colonies were pooled and expanded in 25-cm^2 flasks.

Functional assays

Human embryonic kidney (HEK293) cells were cultured in the growth media described above at

37ºC, 95% humidity, and 5% CO_2. Cells were transiently transfected with 1 μg of hOAT2 or other transporter and 3 μg of Lipofectamine 2000 (Invitrogen) in each well according to the manufacturer's protocol. Cells were seeded at a density of 4 x 10^5 cells per well in poly-D-lysine-coated 24-well plates (Becton Dickenson, MA) and were grown overnight. Uptake assays with transiently transfected cells were conducted 18-24h after transfection. For uptake studies, the cells were rinsed with pre-warmed uptake buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM HEPES, pH 7.4), and then incubated in 0.3 mL of pre-warmed buffer containing radiolabeled test compounds in the presence or absence of 10 μM nitrobenzylthioinosine (NBMPR), which was added to inhibit background ENT-mediated uptake of nucleosides and nucleobases. At the indicated time points, the reaction was terminated by washing with ice-cold buffer. Test substrates were quantified by liquid scintillation counting and the uptake amounts were normalized to total protein in each well. cGMP levels were measured radiometrically (Figs. 1, 2A, 3, and 5A) or enzymatically (Figure 4). For the enzymatic measurement of cGMP, stably transfected empty vector and hOAT2 cells were rinsed with 0.5 mL uptake buffer and then incubated with 0.25 mL of test solution. After a 1 hour incubation, an aliquot of an extracellular sample was immediately aspirated. The remaining portion was subsequently washed twice with ice-cold buffer. Afterward, 0.5 mL of lysis reagent was added and shaken for 1 hour to retrieve the intracellular cGMP sample according to the manufacturer's protocol (cGMP Enzymeimmunoassay Biotrak (EIA) System; Amersham Biosiences). The determined concentrations of intracellular and extracellular cGMP were corrected as cGMP level per cells.

RNA extraction, reverse transcription, and real-time PCR assay

The expression of the organic anion tranpsorters (OAT1, OAT2, OAT3 and OAT4) was quantified by real-time reverse transcription-PCR, using TaqMan® Gene Expression Assays and an ABI 7500 Fast sequence detection system (Applied Biosystems, Foster City). Human GAPDH was used as an endogenous control to normalize expression.

Total RNA was extracted 48 h after transfection using the RNAqueous® system (Ambion Inc.,

Texas). One microgram of each RNA preparation was reverse-transcribed by random priming using High Capacity cDNA synthesis kit (Applied Biosystems, Foster City). Real-time PCR was performed using a 4.5 μL aliquot of the total cDNA sample using the TaqMan® Gene Expression Assays Hs00537914_m1 for human OAT1, Hs00198527_m1 for human OAT2, Hs00188599_m1 for human OAT3 and Hs00218486_m1 for human OAT4. Human GAPDH endogenous control (Hs99999905_m1) was used as an internal standard for sample normalization. Relative levels of the human OAT1, OAT2, OAT3 and OAT4 mRNAs were calculated using the $\Delta\Delta C_T$ (comparative threshold cycle) method. Each test was performed as a duplicate and all experiments were repeated three times. The levels of the human OAT1, OAT2, OAT3 and OAT4 mRNAs were expressed relative to the HEK293 Flp-In cell lines transfected with pcDNA5FRT vector only, which was normalized to 1.

Tissue distribution of an alternative splice form of hOAT2 was detected using a TaqMan® real-time PCR custom assay was performed with an ABI Prism 7700 Sequence Detector system.

Thin Layer Chromatography (TLC)

After allowing uptake to proceed for 1.5 minutes in OAT2-transfected and corresponding untransfected HEK cell lines in a 24-well plate, cells were lysed with 1% pentadecafluorooctanoic acid/ammonium salt buffer for an hour. The lysate was diluted to twice its volume with acetonitrile to precipitate all protein content. An aliquot of supernatant was evaporated and redissolved in 10 μL of water. Silica gel TLC of each uptake sample processed as described above was carried out with $CHCl_3$/MeOH/H_2O (6:6:1) as the mobile phase and compared with a standard sample of cGMP. The identity of the compound was confirmed by analysis using a PerSeptive Mariner ESI-TOF mass spectrometer. In addition, the amount of cGMP present in uptake samples was determined by measuring the radioactivity of the cGMP spot on the TLC plate and comparing it with the measured uptake.

Construction for GFP-tagged proteins and microscopic studies

Plasmids containing GFP-fused proteins were constructed as described previously (16). The resulting fusion constructs were used to generate stable HEK293 cell lines using the Flp-In System, and

analyzed by confocal microscopy as described previously (17).

RESULTS

hOAT2 transports nucleobases and nucleosides

OAT2 was capable of transporting a wide range of purine and pyrimidine nucleobases, nucleosides and nucleotides (Fig. 1, Table 1). Among the nucleobases and the nucleosides, cGMP was the preferred hOAT2 substrate (approximately 60-fold uptake over empty vector (EV) transfected cell lines in the absence of NBMPR; 4.11 $\pm$ 1.65 pmol/mg protein/minute for hOAT2 vs. 0.0710 $\pm$ 0.0315 pmol/mg protein/minute for EV). When ENT-mediated background transport was inhibited with 10 μM NBMPR, hOAT2 mediated transport of cGMP was approximately 86-fold uptake over EV transfected cell lines; 3.26 ± 0.819 pmol/mg protein/minute for hOAT2 vs. 0.0380 ± 0.0196 pmol/mg protein/minute for EV. 2'-Deoxyguanosine was also shown to be a hOAT2 substrate in the presence of NBMPR (approximately 24-fold uptake over EV; 74.0 ± 26.2 fmol/mg protein/minute for hOAT2 vs. 3.13 ± 0.745 fmol/mg protein/minute for EV). To determine whether the compounds tested were also substrates of hENT1 and hENT2, we compared their relative uptake in empty vector transfected HEK cells in the presence and absence of NBMPR (Table 1). Gemcitabine, a known substrate of ENTs, served as a positive control. As shown, cGMP was not transported by ENTs, whereas some of the nucleobases, nucleosides, and nucleotides were. Additional experiments with TLC and mass spectrometry confirmed that >90% of the intracellular [^{3}H]-cGMP in hOAT2 stably transfected cells at 1.5 minutes was intact (data not shown).

Figure 1. Uptake of naturally-occurring nucleotides, nucleobases, and nucleosides by hOAT2
hOAT2 (solid bars)-transiently transfected HEK293 cells were incubated for 1.5 minute with each radiolabeled compound (2 μM) in the presence of 10 μM NBMPR. Uptake of p-aminohippurate (PAH), a typical substrate of organic anion transporters, is shown for comparison. Each value represents the mean uptake via hOAT2 divided by the mean respective uptake in empty vector transfected cells. Data are from three independent experiments performed on separate days. The uptake of each compound was tested in triplicate samples in each independent experiment.

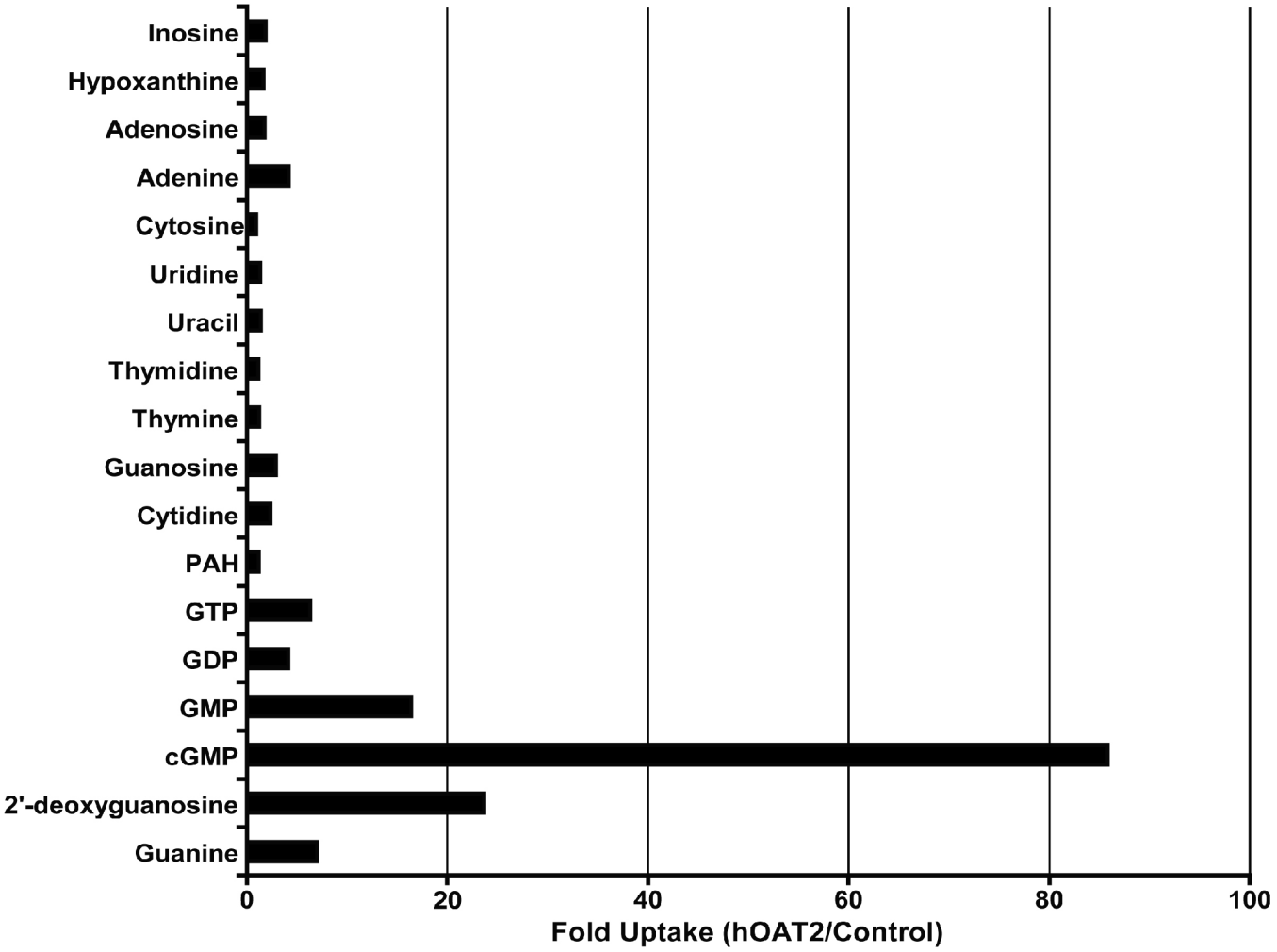


Table 1. Uptake of naturally-occurring nucleotides, nucleobases and nucleosides in empty vector and hOAT2 transfected cells

	Uptake Rate in the Absence of NBMPR (pmol/mg protein/min)		Uptake Rate in the Presence of NBMPR (pmol/mg protein/min)		
Compound	**EV**	**hOAT2**	**EV**	**hOAT2**	**ENT Transport Fold**
cGMP	**0.0710 ± 0.0315**	**4.11 ± 1.65**	**0.0380 ± 0.0196**	**3.26 ± 0.819**	**1.87**
Guanine	**0.263 ± 0.119**	**0.804 ± 0.513**	**0.0859 ± 0.0292**	**0.604 ± 0.313**	**3.06**
Adenine	**0.412 ± 0.0578**	**1.30 ± 0.239**	**0.262 ± 0.0530**	**1.10 ± 0.212**	**1.57**
Guanosine	**0.109 ± 0.0575**	**0.226 ± 0.169**	**0.0775 ± 0.0110**	**0.225 ± 0.163**	**1.41**
GMP	**0.00935 ± 0.00813**	**0.0196 ± 0.0190**	**0.00113 ± 0.000545**	**0.0185 ± 0.0185**	**8.27**
GDP	**0.00638 ± 0.000838**	**0.0110 ± 0.00328**	**0.00218±0.0000522**	**0.00892 ± 0.00271**	**2.93**
GTP	**0.00724 ± 0.000970**	**0.0113 ± 0.00135**	**0.00143 ± 0.000502**	**0.00901 ± 0.00216**	**5.06**
PAH	**0.0228 ± 0.00326**	**0.0344 ± 0.00699**	**0.0265 ± 0.00684**	**0.0312 ± 0.00568**	**0.860**
2'-deoxyguanosine	**0.0551 ± 0.0234**	**0.0790 ± 0.0314**	**0.00313 ± 0.000745**	**0.0740 ± 0.0262**	**17.6**
Uracil	**0.0280 ± 0.00870**	**0.0411 ± 0.00825**	**0.0198 ± 0.00725**	**0.0258 ± 0.00333**	**1.41**
Uridine	**1.14 ± 0.242**	**1.34 ± 0.102**	**0.115 ± 0.0305**	**0.151 ± 0.0224**	**9.91**
Hypoxanthine	**0.351 ± 0.0523**	**0.403 ± 0.0351**	**0.123 ± 0.0171**	**0.203 ± 0.0304**	**2.85**
Thymine	**0.00774 ± 0.00451**	**0.00845± 0.00544**	**0.00585 ± 0.00296**	**0.00707 ± 0.00394**	**1.32**
Adenosine	**0.108 ± 0.0353**	**0.126 ± 0.0133**	**0.0295 ± 0.00581**	**0.0521 ± 0.0132**	**3.66**
Thymidine	**0.306 ± 0.0694**	**0.331 ± 0.0306**	**0.0338 ± 0.00415**	**0.0391 ± 0.00254**	**9.05**
Cytidine	**0.0548 ± 0.0177**	**0.0571 ± 0.00903**	**0.00194 ± 0.000239**	**0.00463 ± 0.00145**	**28.2**
Cytosine	**0.0101 ± 0.00552**	**0.0112 ± 0.00124**	**0.00768 ± 0.00512**	**0.00704 ± 0.00176**	**1.32**
Inosine	**0.306 ± 0.0904**	**0.282 ± 0.0174**	**0.0193 ± 0.00159**	**0.0354 ± 0.00524**	**15.8**
Gemcitabine	**0.699 ± 0.0958**	**N/A**	**0.0998 ± 0.0343**	**N/A**	**7.00**

A table displaying the mean uptake values of nucleotides, nucleobases, and nuclesides in the absence and presence of NBMPR (10 μM). The uptake of PAH (a known hOAT2 substrate) and gemcitabine (a known ENT substrate) are shown for comparison. Each value represents the mean ± S.D. ENT fold transport was calculated by dividing empty vector in the presence of NBMPR.

hOAT2 is distinct from other hOATs in substrate selectivity and structure

Although the other hOAT transporters showed efficient uptake of model substrates (61-fold uptake of adefovir by hOAT1, 25-fold uptake of estrone sulfate by hOAT3, and 21-fold uptake of estrone sulfate by hOAT4), they exhibited less uptake of cGMP compared to hOAT2 (Fig. 2B). In contrast, hOAT2 exhibited a great ability to transport cGMP (50-fold enhanced uptake) compared to its model substrate PAH (Fig. 2B). However, surface expression of these proteins is not known, and differences in surface expression may have influenced the comparative uptake values of cGMP. Multiple sequence analysis indicated that hOAT2 is distinct from other organic anion transporters and in spite of its functional similarities compared to nucleobase transporters, it does not appear to be related to ENT and CNT transporters at the amino acid level to any greater extent than the other members of the organic anion transporter family (Fig. 2C).

hOAT2 facilitates the transport of cGMP and 2'-deoxyguanosine

Multiple time course studies of cGMP and 2'-deoxyguanosine confirmed that 1.5 minutes was in the linear range of transport (data not shown); therefore, 1.5 minutes was used for cGMP and 2'-deoxyguanosine kinetics studies. The transport kinetics of cGMP and 2'-deoxyguanosine were saturable, with K_m estimates of 88 ± 11 μM for cGMP and 128 ± 17 μM for 2'-deoxyguanosine, and V_{max} values of 3373 ± 126 pmol/mg protein/1.5minutes for cGMP and 7225 ± 332 pmol/mg protein/1.5minutes for 2'-deoxyguanosine (Fig. 3A and 3B). cGMP was shown to have the lowest K_m among the guanine derivatives tested.

hOAT2-mediated transport of cGMP was inhibited by 2'-deoxyguanosine with a K_i value of 93 ± 12 μM, which was approximately equal to the K_m value for hOAT2-mediated 2'-deoxyguanosine transport in the presence of NBMPR (Fig. 3C).

Figure 2. Specificity of cGMP and model substrate transport among hOAT family members (A, B) and dendrogram of the human OAT, ENT, and CNT transporter families (C)

(A) HEK cells transiently transfected with each hOAT isoform were incubated for 1.5 minutes with 2 μM cGMP. Only hOAT2-transfected cells showed substantially increased rate of uptake of cGMP above empty vector (EV)-transfected controls. cGMP transport by hOAT1 and hOAT3 was 6-fold and 5-fold greater than control, respectively, and was not transported by hOAT4 (< 2 fold). hOAT2 was the most selective for cGMP (50-fold enhanced uptake). Each bar represents the mean ± S.D. from triplicate samples in a representative experiment. **(B)** A 1.5 minute uptake following a transient transfection of each hOATs' respective model substrate (black bars) tested simultaneously with EV (white bars). The model substrates are as follows: adefovir for hOAT1, PAH for hOAT2, and estrone sulfate for hOAT3 and hOAT4. **(C)** A multiple sequence alignment using reference sequences for each transporter was performed using ClustalX; the dendrogram was generated from the ClustalX alignment output.

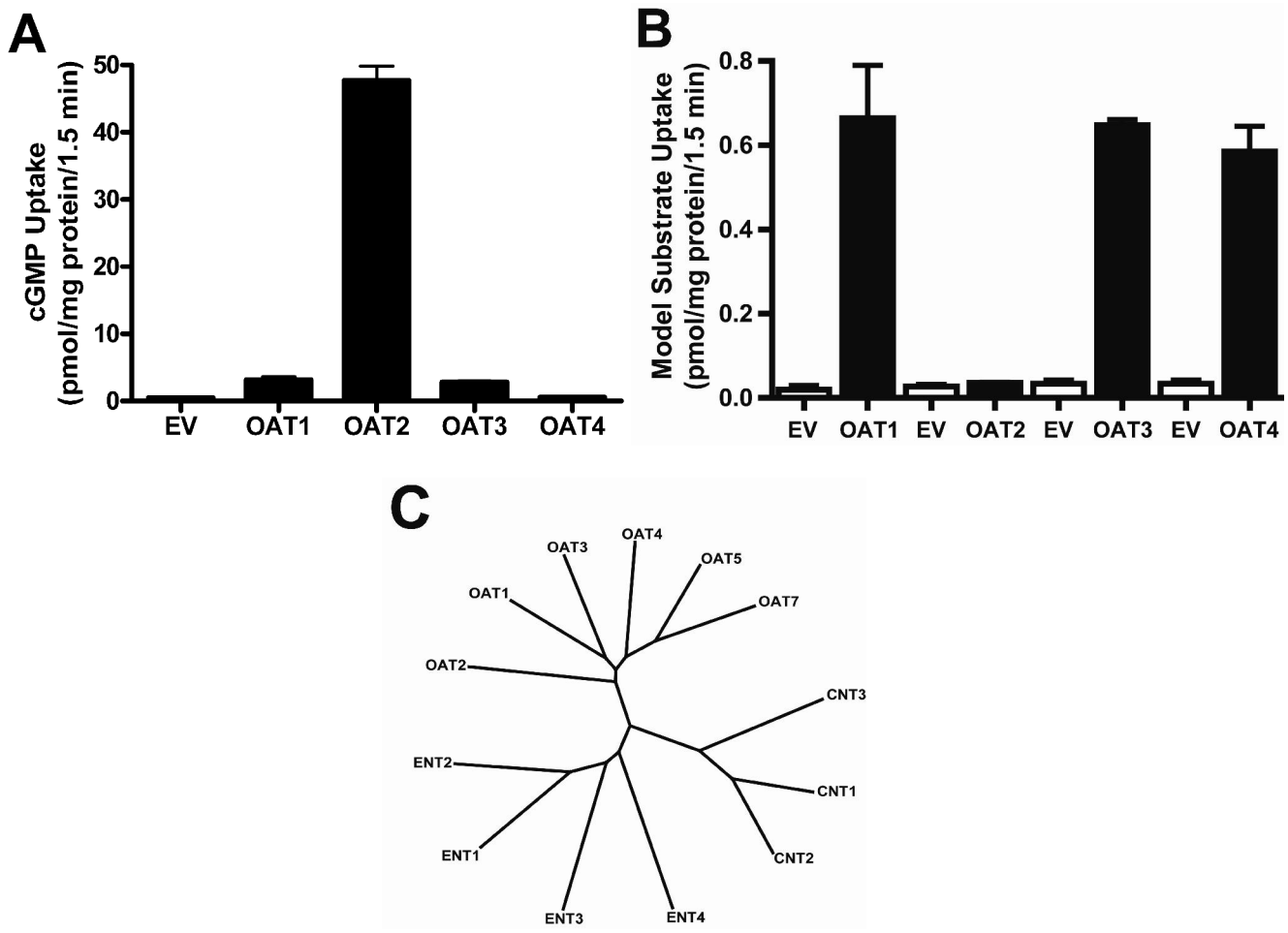
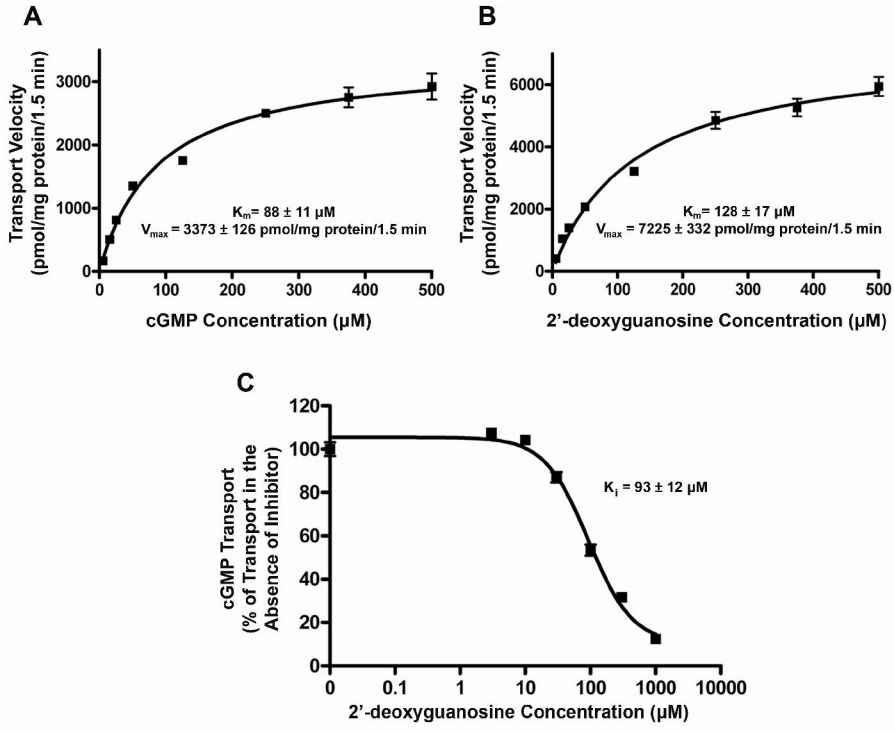


Figure 3. Concentration-dependent transport of (A) cGMP and (B) 2'-deoxyguanosine by hOAT2 and (C) 2'-deoxyguanosine inhibition of hOAT2-mediated cGMP transport

Empty vector-stably transfected and hOAT2-stably transfected HEK293 cells were incubated for 1.5 minutes with varying concentrations (5, 15, 25, 50, 125, 250, 375, and 500 μM) of cGMP **(A)**, or 2'-deoxyguanosine **(B)**. 2'-deoxyguanosine transport rate was measured in the presence of 10 μM NBMPR to inhibit background uptake. hOAT2-specific uptake was determined by subtracting uptake in empty vector-transfected cells from that in hOAT2-transfected cells after correcting for total protein. Kinetic parameters were estimated by fitting hOAT2-specific uptake rates to a Michaelis-Menten equation by nonlinear regression. Data are shown as the mean ± S.D. from triplicate samples in a representative experiment. **(C)**, Empty vector-transfected and hOAT2-transfected HEK293 cells were incubated for 1.5 minutes with 2 μM radiolabeled cGMP and varying concentrations (0, 3, 30, 100, 300, and 1000 μM) of 2'-deoxyguanosine. NBMPR (10 μM) was added to all of the 2'-deoxyguanosine reaction mixes to inhibit ENT1 transport. hOAT2-specific uptake was determined by subtracting uptake by EV-transfected cells from that in hOAT2-transfected cells after correcting for total protein. IC_{50} and K_i parameters were estimated by nonlinear regression. Uptake results shown in the graphs are from a representative experiment. Each point represents the mean ± S.D. of triplicate samples.



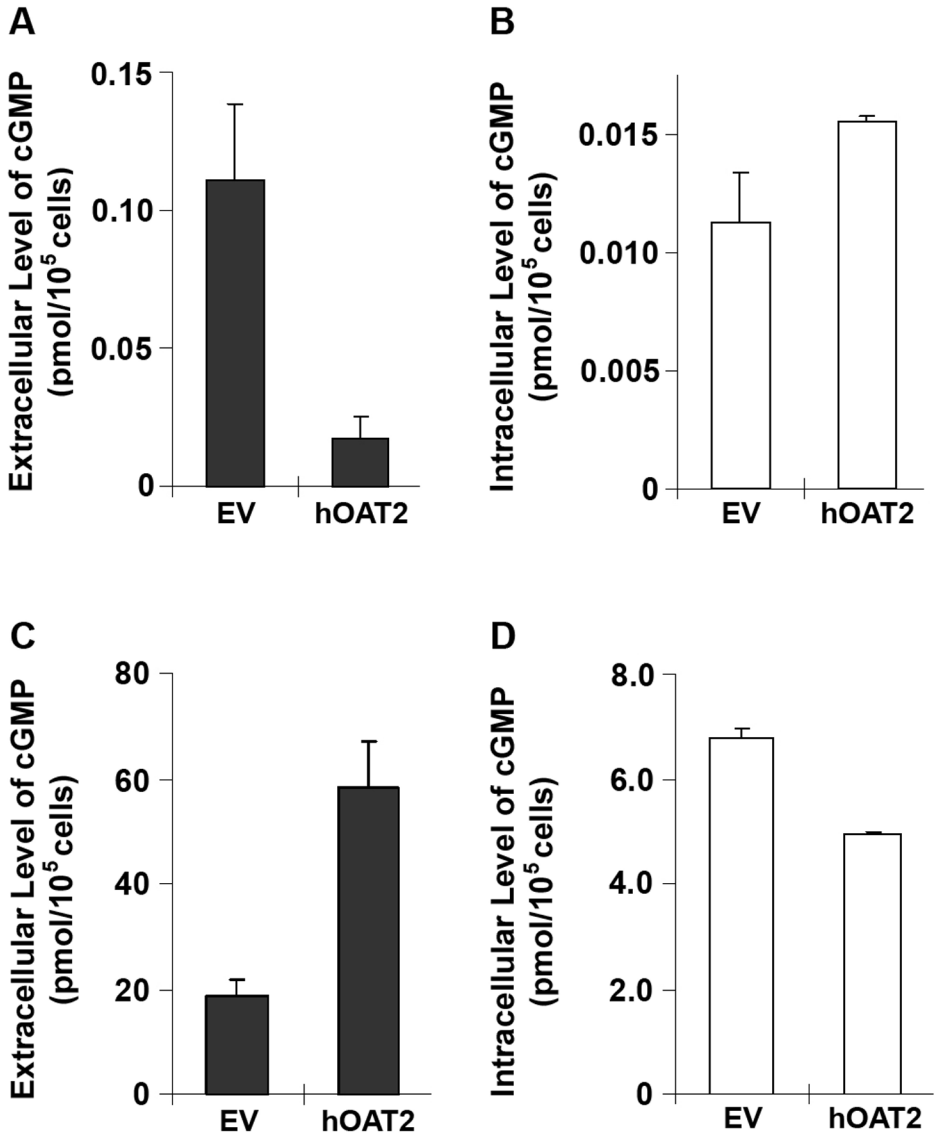
Intracellular and extracellular levels of cGMP are modulated by hOAT2

Since our preliminary data indicated that hOAT2 can mediate either uptake or efflux, we were interested in whether the transporter served primarily in the influx or efflux of cGMP. We determined the extracellular levels of endogenously produced cGMP extruded from EV- and hOAT2-transfected HEK293 cells treated with or without 750 μM sodium nitroprusside (SNP, a guanylate cyclase stimulator) plus 750 μM 3-isobutyl-1-methylxanthine (IBMX, a phosphodiesterase inhibitor). Fig. 4A shows that basal extracellular levels of cGMP in hOAT2-transfected cells were 6-fold lower than those in EV-transfected cells, suggesting that hOAT2 mediates cGMP influx under these conditions. That is, the net effect of hOAT2 under basal conditions is in the influx direction. In contrast, when intracellular cGMP levels were increased by 1 hour-treatment with SNP and IBMX, extracellular cGMP levels were 3-fold higher in hOAT2- transfected cells in comparison to EV-transfected cells (Fig. 4C). In this case, when a steep outwardly-directed cGMP concentration gradient was generated, the net effect of hOAT2 was in the efflux direction. In both EV and hOAT2 expressing cells, the intracellular pools of cGMP were a small fraction of the total, suggesting endogenous cGMP efflux activity in both cell lines. However, the intracellular cGMP amounts qualitatively and inversely reflected extracellular cGMP levels (Fig. 4B and 4D). This is consistent with hOAT2 acting in the influx of cGMP (in opposition to the efflux transporters) under basal conditions, and in the efflux of cGMP when its concentration gradient was directed outwardly by SNP and IBMX. Taken together, the data suggest that hOAT2, as a bidirectional facilitative transporter, can modulate cellular levels of cGMP.

Additionally, timed efflux studies in hOAT2 and EV transiently transfected HEK cells preloaded with [^{3}H]-cGMP (2 μM) demonstrated that the extracellular concentrations of [^{3}H]-cGMP increased with time in the hOAT2 expressing cells (16.4 ± 3.20 pmol/mg protein at 10 minutes versus 21.8 ± 0.988 pmol/mg protein at 30 minutes) indicating that the transporter mediated [^{3}H]-cGMP efflux. Concentrations of [^{3}H]-cGMP in the extracellular media of empty vector transfected cells did not increase with time (data not shown). Mass spectrometric analysis of the extracellular media, indicated that cGMP was transported in the efflux direction by hOAT2, i.e., we detected substantial [^{3}H]-cGMP concentrations in the extracellular media at 10 minutes.

Figure 4. Extracellular and intracellular cGMP levels in HEK293 cells stably transfected with hOAT2

Extracellular (**A, C**; solid bar) and intracellular (**B, D**; open bar) cGMP levels in cells stably transfected with empty vector (EV) or hOAT2 were determined after 1 hour incubation in the absence (**A, B**) or presence (**C, D**) of sodium nitroprusside (750 μM) and IBMX (750 μM). Data are the mean ± S.D. of three independent experiments. Each experiment was conducted in duplicate samples.



hOAT2 has two splice variants that differ in terms of two amino acids (hOAT2-546 and hOAT2-548)

The hOAT2 cDNA used in the previous experiments (hOAT2-546aa) contained a 1638 bp open reading frame encoding a 546 amino acid protein, which corresponds to the NCBI-registered reference sequence for *SLC22A7* (NM_06672). During our initial procedures, we cloned another hOAT2 cDNA (hOAT2-548aa), which has been listed as a related sequence to *SLC22A7* in the NCBI data base (NM_153320) from a human kidney cDNA library. The sequence analysis revealed that the only difference between the two clones is a 6 bp-insertion (TCCCAG) between exon 1 and exon 2 of the reference *SLC22A7* gene. This additional nucleotide sequence is found in intron 1 of the reference *SLC22A7* gene, and is flanked by the consensus sequence for intron/exon boundaries, suggesting that the two cDNAs of hOAT2 are alternatively spliced variants.

Our studies indicated that hOAT2-546aa was capable of transporting cGMP, whereas the hOAT2-548aa isoform exhibited a complete loss of cGMP uptake (Fig. 5A). Mutagenesis analysis demonstrated that deletion of two amino acid residues from hOAT2-548aa restored function, whereas insertion of two amino acid residues into hOAT2-546aa caused a complete loss of function (data not shown). Additionally, cellular localization via fluorescence and confocal microscopy of GFP (control), hOAT2-546aa-GFP (functional isoform), and hOAT2-548aa-GFP (non-functional isoform) revealed that hOAT2-546aa-GFP was localized to the plasma membrane. In contrast, hOAT2-548aa-GFP remained exclusively in the intracellular compartment with markedly lower fluorescence (Fig. 5B). The lack of surface expression of the hOAT2-548aa isoform most likely results in the observed loss of its transport function. Quantitative RT-PCR assays specific for each isoform showed that each hOAT2-expressing tissue contained approximately equal levels of the mRNA species of the two splice forms (Fig. 6).

Figure 5. cGMP uptake (A) and intracellular localization in HEK293 cells expressing two alternatively spliced variants of hOAT2 (B)

(A) HEK293 cells were transiently transfected with empty vector, hOAT2-546aa-GFP, or hOAT2-548aa-GFP. Cells were incubated for 1.5 minutes with 2 μM cGMP. Each bar represents the mean ± S.D. from triplicate samples in a representative experiment. Results were confirmed in two independent experiments. **(B)** Cells stably expressing GFP, hOAT2-546aa-GFP, or hOAT2-548aa-GFP were visualized by confocal microscopy. Plasma membrane was stained using AlexaFluor594 wheat germ agglutinin. Cells transfected with GFP alone show diffuse expression throughout the intracellular space. hOAT2-546aa-GFP localized specifically to the plasma membrane, whereas hOAT2-548aa-GFP showed diffuse localization throughout the cytoplasm with lower fluorescence intensity.

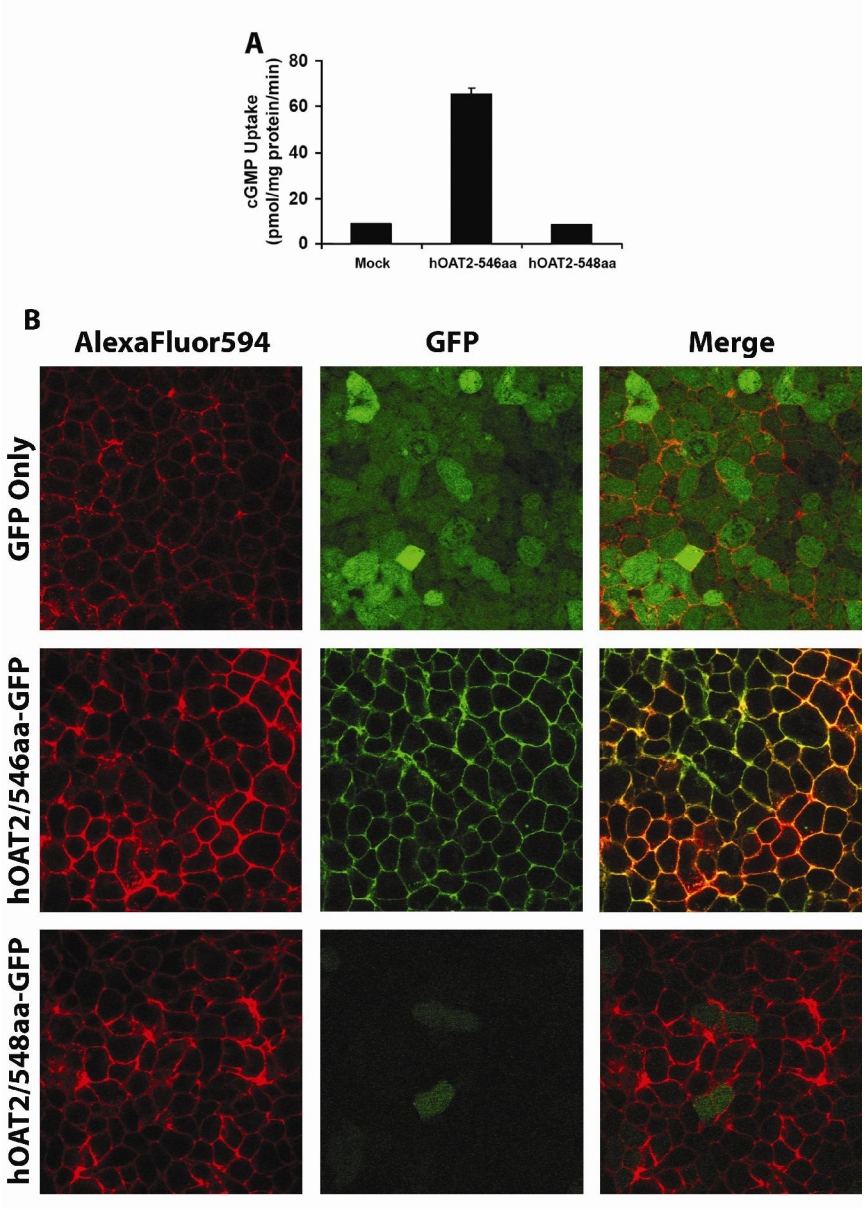
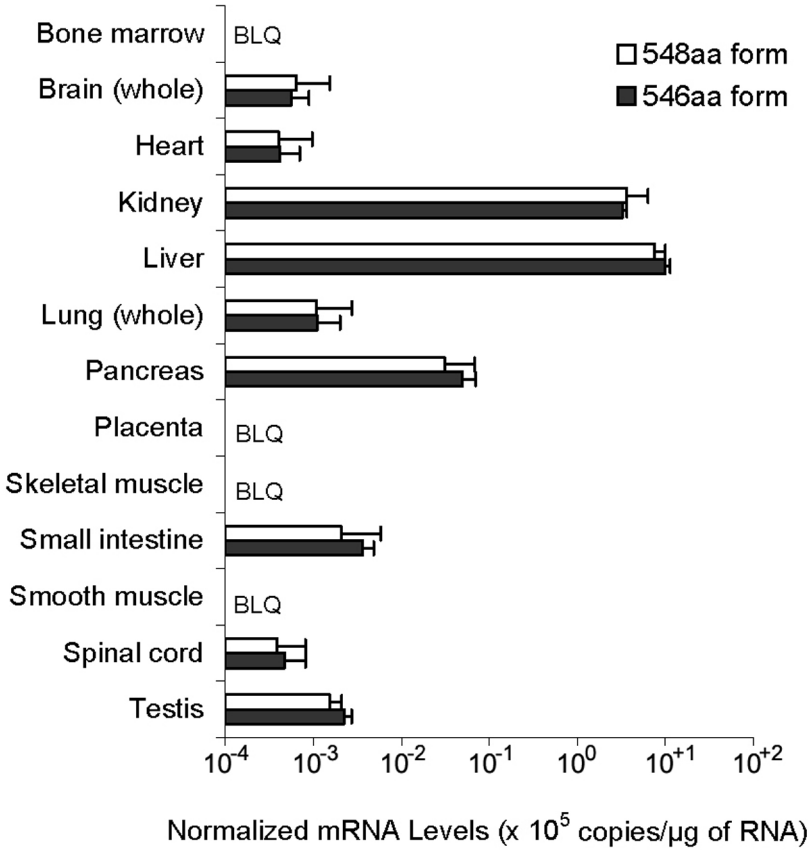


Figure 6. Tissue distribution of alternative splice forms of hOAT2

mRNA expression of hOAT2-546aa (solid bars) and hOAT2-548aa (open bars) was determined by real-time PCR using TaqMan primers and probes specific to each isoform. Sensitivity of each assay for its target sequence was >10,000-fold greater than for the alternative target. Expression level of each isoform was normalized to GAPDH expression. Each bar represents the mean ± S.D. from quadruplicate assays of two independent cDNA sources. BLQ = below the limit of quantification.



DISCUSSION

Our studies indicate that hOAT2 exhibits a robust transport function for a wide array of naturally occurring nucleobases, nucleosides, and nucleotides (Fig. 1 and Table 1) with a particularly, unique role for cGMP.

cGMP is a second messenger involved in the intracellular signal transduction of a variety of extracellular stimuli in many tissues. Intracellular levels of cGMP are thought to be determined primarily by synthesizing and catabolizing enzymes, in the GC and PDE families. cGMP signaling can be modulated pharmacologically by nitric oxide (GC activator) and sildenafil (PDE5-specific inhibitor) (18,19). Our data suggest that hOAT2 works in concert with these enzymes in regulating intracellular cGMP levels. The interaction of cGMP with PDE5 has a lower K_m (2-5 μM) in comparison to its interaction with hOAT2 determined in this study (K_m = 88 μM). These interaction kinetics support the notion that both hOAT2 and PDE5 are determinants of intracellular levels of cGMP. Although speculative, it is possible that hOAT2 acts to eliminate excess intracellular cGMP that is not enzymatically inactivated by PDE5. Intracellular cGMP levels vary widely depending upon the expression levels and activities of transporters and enzymes involved in its production and breakdown. Some reports include values as low as 1 μM and as high as 300 μM (20). The K_m for OAT2 obtained in our study is consistent with reported intracellular concentrations of cGMP. In particular, we observed that under control conditions, hOAT2 mediates the influx of cGMP, whereas under conditions in which GC is stimulated and PDE is inhibited, hOAT2 functions in the efflux of cGMP from cells (Fig. 4A-4D). Additional experiments in our lab reveal that ENTs do not transport cGMP. Also, CNTs have not been demonstrated to accept nucleotide monophosphates of any kind. Sequence comparisons indicate that OAT2 is about 40% identical to other OATs and only 15 to 20% identical to the ENTs and CNTs. Such sequence comparisons may not provide much information on the substrate specificities of various transporters since small differences in sequence can result in noticeable differences in protein structure.

To our knowledge, only one other influx transporter has been shown to transport cGMP (21).

This transporter, OAT1, is almost exclusively expressed in the kidney and takes up cGMP much less avidly than hOAT2 (Fig. 2A). Because hOAT2 is expressed at high levels in many tissues, our data suggest that hOAT2 plays a critical role in transmembrane influx of cGMP in many cell types.

Three efflux transporters for cGMP in the ABC superfamily have been identified and are members of the multidrug resistance protein family with K_m values between 2-10 μM (MRP4, MRP5, and MRP8) (3-5,20). Because hOAT2 is a facilitative transporter, it will have a distinct role from the MRPs, which, as efflux transporters, will function to actively eliminate cGMP from cells in tissues in which these transporters are expressed. In contrast, hOAT2 may facilitate both the uptake and efflux of cGMP in hOAT2-expressing tissues. Based on its wide tissue distribution, we speculate that hOAT2 is a key transporter for modulating intracellular cGMP levels in many tissues. In addition, there are several studies suggesting a role for cGMP as an extracellular effector molecule (1), particularly in brain (22-24) and kidney (25,26), tissues in which hOAT2 is abundantly expressed. hOAT2 may be involved in modulating the effects of extracellular cGMP in these tissues.

Structure activity studies indicated that the addition of a monophosphate, rather than a cyclic monophosphate, at the 5'-position reduced both the affinity and the capacity of hOAT2-mediated transport (Fig. 1B). Addition of one (GDP) or two (GTP) phosphate groups caused a further reduction in hOAT2 transport activity. These results suggest that cyclic 5', 3' monophosphate increases substrate-hOAT2 interaction and further suggest that the primary substrate of hOAT2 is cGMP.

To date, alternatively spliced variants in the OAT family have been identified for hOAT1 (27). Two of four hOAT1 isoforms are non-functional due to deletion of transmembrane domain 11 and a portion of transmembrane 12. The OAT2 splice variant, hOAT2-548aa, is unusual because it contains such a small exon, and does not result in a frameshift. Nevertheless, the small difference between the two isoforms of hOAT2 produced a clear distinction in the activity of hOAT2, with hOAT2-548aa resulting in complete loss of transport function. Furthermore, mRNA expression studies demonstrating that the 6 bp insertion did not affect mRNA stability suggest that alternative splicing of this small exon is a stochastic

event.

We determined that the mechanism for the loss of function of hOAT2-548aa was related to a reduced expression level of the protein on the plasma membrane (Fig. 5A). Similar to other OATs, the predicted secondary structure of hOAT2 consists of 12 TMDs with a large hydrophilic loop between TMD1 and TMD2. hOAT2-548aa contains a two-amino acid insertion, Ser and Gln, between Glu-131 and Trp-132 in the large extracellular loop 1 of hOAT2-546aa. It is likely that addition of these two amino acids results in reduced stability of the 548 form. In particular, hOAT2-548aa-GFP-transfected cells displayed a much lower GFP-derived signal in comparison with those transfected with hOAT2-546aa-GFP (Fig. 5B). This suggests that the lower levels of hOAT2-548aa protein on the plasma membrane are the result of a generalized increased rate of degradation of the 548 aa form. In fact, the 548 aa form was not present in Western blots using a GFP-specific antibody; whereas the 546 form was present in abundance (data not shown). It is also possible that these two amino acids disrupt a motif responsible for proper trafficking to the plasma membrane. It is noteworthy that the amino acid sequence in the vicinity of Glu131 is highly conserved among members of the OAT family suggesting that this region may contain a motif(s) that is/are essential for stability or trafficking of the protein.

In summary, our study revealed a novel functionality of hOAT2 to transport guanine nucleosides and in particular, cGMP in a bidirectional manner. This activity was present in one splice form, hOAT2-546aa, but not in an alternatively spliced form, hOAT2-548aa, which appeared to exhibit reduced stability in cells. By regulating intracellular as well as extracellular levels of cGMP, hOAT2, together with guanylate cyclases and phosphodiesterases, may play a key role in cGMP mediated signaling. Small molecule inhibitors may be used as tools to understand the role of hOAT2 in the context of regulation of intracellular and extracellular cGMP levels.

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REFERENCES

1. Poulopoulou C and Nowak LM. Extracellular 3',5' cyclic guanosine monophosphate inhibits kainate-activated responses in cultured mouse cerebellar neurons. *J Pharmacol Exp Ther* **286:**99-109 (1998).
2. Sager G. Cyclic GMP transporters. *Neurochem Int* **45:**865-873 (2004).
3. Chen ZS, Lee K and Kruh GD. Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *J Biol Chem* **276:**33747-33754 (2001).
4. Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG and Kruh GD. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrmidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *J Biol Chem* **278:**29509-29514 (2003).
5. Jedlitschky G, Burchell B and Keppler D. The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275:**30069-30074 (2000).
6. Baldwin SA, Beal PR, Yao SY, King AE, Cass CE and Young JD. The equilibrative nucleoside transporter family, SLC29. *Pflugers Arch* **447:**735-743 (2004).
7. Gray JH, Owen RP and Giacomini KM. The concentrative nucleoside transporter family, SLC28. *Pflugers Arch* **447:**728-734 (2004).
8. Cihlar T, Lin DC, Pritchard JB, Fuller MD, Mendel DB and Sweet DH. The antiviral nucleotide analogs cidofovir and adefovir are novel substrates for human and rat renal organic anion transporter 1. *Mol Pharmacol* **56:**570-580 (1999).
9. Koepsell H and Endou H. The SLC22 drug transporter family. *Pflugers Arch* **447:**666-676 (2004).
10. Sun W, Wu RR, van Poelje PD and Erion MD. Isolation of a family of organic anion transporters from human liver and kidney. *Biochem Biophys Res Commun* **283:**417-422 (2001).
11. Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T and Endou H. Identification and

characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59:**1277-1286 (2001).

12. Cha SH, Sekine T, Kusuhara H, Yu E, Kim JY, Kim DK, Sugiyama Y, Kanai Y and Endou H. Molecular cloning and characterization of multispecific organic anion transporter 4 expressed in the placenta. *J Biol Chem* **275:**4507-4512 (2000).
13. Burckhardt BC and Burckhardt G. Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev Physiol Biochem and Pharmacol* **146:**95-158 (2003).
14. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F and Higgins DG. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25:**4876-4882 (1997).
15. Page RD. TreeView: an application to display phylogenetic trees on personal computers. *Comput Appl Biosci* **12:**357-358 (1996).
16. Erdman AR, Mangravite LM, Urban TJ, Lagpacan LL, Castro RA, de la Cruz M, Chan W, Huang CC, Johns SJ, Kawamoto M, Stryke D, Taylor TR, Carlson EJ, Ferrin TE, Brett CM, Burchard EG and Giacomini KM. The human organic anion transporter 3 (OAT3; SLC22A8): genetic variation and functional genomics. *Am J Renal Physiol* **290:**F905-F912 (2006).
17. Urban TJ, Gallagher RC, Brown C, Castro RA, Lagpacan LL, Brett CM, Taylor TR, Carlson EJ, Ferrin TE, Burchard EG, Packman S and Giacomini KM. Functional genetic diversity in the high-affinity carnitine transporter OCTN2 (SLC22A5). *Mol Pharmacol* **70:**1602-1611 (2006).
18. Dousa TP. Cyclic-3',5'-nucleotide phosphodiesterase isoenzymes in cell biology and pathophysiology of the kidney. *Kidney Int* **55:**29-62 (1999).
19. Friebe A and Koesling D. Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* **93:** 96-105 (2003).
20. Andric SA, Kostic TS and Stojilkovic SS. Contribution of multidrug resistance protein MRP5 in control of cyclic guanosine 5'-monophosphate intracellular signaling in anterior pituitary cells.

Endocrinology **147:**3435-3445 (2006).

21. Sekine T, Watanabe N, Hosoyamada M, Kanai Y and Endou H. Expression cloning and characterization of a novel multispecific organic anion transporter. *J Biol Chem* **272:**18526-18529 (1997).

22. Erceg S, Monfort P, Cauli O, Montoliu C, Llansola M, Piedrafita B and Felipo V. Role of extracellular cGMP and of hyperammonemia in the impairment of learning in rats with chronic hepatic failure. Therapeutic implications. *Neurochem Int* **48:**441-446 (2006).

23. Montoliu C, Llansola M, Kosenko E, Corbalan R and Felipo V. Role of cyclic GMP in glutamate neurotoxicity in primary cultures of cerebellar neurons. *Neuropharmacology* **38:**1883-1891 (1999).

24. Wang M, Urenjak J, Fedele E and Obrenovitch TP. Effects of phosphodiesterase inhibition on cortical spreading depression and associated in extracellular cyclic GMP. *Biochem Pharmacol* **67:**1619-1627 (2004).

25. Chevalier RL, Fang GD and Garmey M. Extracellular cGMP inhibits transepithelial sodium transport by LLC-PK1 renal tubular cells. *Am J Physiol* **270:**F283-F288 (1996).

26. Neant F and Bailly C. Luminal and intracellular cGMP inhibit the mTAL reabsorptive capacity through different pathways. *Kidney Int* **44:**741-746 (1993).

27. Bahn A, Ebbinghaus C, Ebbinghaus D, Ponimaskin EG, Fuzesi L, Burckhardt G and Hagos Y. Expression studies and functional characterization of renal human organic anion transporter 1 isoforms. *Drug Metab Dispos* **32:**424-430 (2004).

CHAPTER 3

THE ROLE OF HUMAN ORGANIC ANION TRANSPORTER 2 (*SLC22A7*) AND ITS GENETIC VARIANTS IN THE TRANSPORT OF ACYCLOVIR

INTRODUCTION

Herpes simplex virus (HSV) infection is globally endemic (1, 2). The seropositivity of herpes simplex infections increases with age (1, 2) and in the United States, this seropositivity markedly increases in early adulthood. In 2003, it was estimated that 536 million people worldwide between the ages of 15 to 49 years-old were living with HSV-II, the genital form of the virus (3). In the immunocompetent patient, HSV infections may cause a significant degree of morbidity without a high degree of mortality. However, if HSV infections are not appropriately treated, complications may arise (4-6) . These complications arise due to disseminated spread of HSV to other organs leading to more serious, life-threatening infection including HSV hepatitis.

Acyclovir is the cornerstone for the treatment of HSV infections and is the pharmacologic treatment of choice for HSV hepatitis. The drug inhibits herpes viral replication by competing with and inhibiting deoxyguanosine triphosphate for viral DNA polymerases resulting in slowed viral DNA replication and chain termination. Acyclovir is an anionic, polar molecule with a small molecular weight. These physiochemical properties present a challenge for systemic drug delivery and for delivery to target tissues where it exerts its antiviral activity, including the liver. Large inter-individual variation in systemic blood levels of acyclovir has been reported after therapeutic oral doses and it is likely that similarly large variations in target tissue levels of the drug also exist.

Recently, we discovered that OAT2 (*SLC22A7*) prefers guanine-based analogs as substrates, including cGMP (7) as described in Chapter 2. OAT2 has a wide tissue distribution with abundant levels in the liver and kidney. Because of its tissue distribution and interaction with guanine analogs, we hypothesized that OAT2 may play a role in the absorption, disposition and tissue specific distribution of acyclovir. The goals of this study were to characterize the interaction of acyclovir with OAT2 and to identify and functionally characterize genetic variants of OAT2. We conducted a sequence analysis of OAT2 in an ethnically diverse population sample and evaluated the function of its genetic variants to gain a greater understanding of the factors that influence the efficacy and disposition of acyclovir.

METHODS

Materials

^{3}H-Acyclovir (25.4 Ci/mmol), ^{3}H-cGMP (7.7 Ci/mmol), ^{3}H-Estrone sulfate (57.3 Ci/mmol), ^{3}H-Zalcitabine (30.2 Ci/mmol), ^{3}H-Ribivirin (8.1 Ci/mmol), ^{3}H-Tenofovir (8.8 Ci/mmol), ^{3}H-Adefovir (11.1 Ci/mmol), ^{3}H-Didanosine (39.5 Ci/mmol), ^{3}H-Azidothymidine (5 Ci/mmol), ^{3}H-Vidarabine (13 Ci/mmol), and ^{3}H-Abacavir (0.7 Ci/mmol) were purchased from PerkinElmer Life Sciences (Wellesley, MA) and Moravek (Brea, CA). ^{3}H-PAH (5 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All unlabeled chemicals were obtained from Sigma (St. Louis, MO). Human embryonic kidney cells (Flp-In™-HEK293) and the mammalian expression vector, pcDNA5/FRT were purchased from Invitrogen (Carlsbad, CA).

Identification of *SLC22A7* variants

Using automated sequencing, we screened the coding region of OAT2 in a collection of 272 genomic DNA samples from ethnically diverse, unrelated, healthy human populations living in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. The SOPHIE project was reviewed and approved by the UCSF Committee on Human Research, and informed consent was obtained from all subjects enrolled. The cohort consisted of 68 African Americans, 68 European Americans, 68 Asian Americans and 68 Mexican Americans. *SLC22A7* (OAT2) variants were identified by direct and pooled DNA-sequencing approaches as previously described (8, 9). The primers for these sequences are listed at http://www.pharmgkb.org.

Transport of acyclovir by OAT2 and other OATs

Original human OAT1, OAT2 and its variants, OAT3, and OAT4 (*SLC22A6-8* and *SLC22A11)* (GenBank accession numbers: NM_004790, NM_006672, NM_004254, and NM_01484, respectively), were obtained by homology cloning and subcloned into the mammalian expression vector pcDNA5/FRT as previously described (10) . HEK293 cells were propagated according to the manufacturer's instructions. Cells were grown at 37°C and seeded on Biocoat® poly-d-lysine-coated 24-well plates (Becton Dickenson, Bedford, MA) 24 hours before transfection, as previously described (10). Cells were transfected with 1 μg of vector per well using either pcDNA5/FRT (empty vector) or OAT2-pcDNA5/FRT (OAT2) and incubated overnight as previously described. Uptake studies were performed 48 hours after transfection as described previously. Briefly, Na^+ uptake buffer containing ^{3}H-acyclovir (25 nM) or ^{3}H-PAH (70 nM) in the absence or

presence of acyclovir (2 to 600 μM), probenecid (1000 μM), prostaglandin F2α (150 μM), guanosine (150 μM), or cGMP (2 or 150 μM) was incubated for specified times with the transfected cells. Uptake was terminated by washing and the cells were lysed using 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS). Radioactivity in an aliquot of the resultant mixture was measured using a Beckman Coulter 6500 liquid scintillation counter. Normalization for protein content was performed to correct for differences in cell number from well to well. A spectrophotometric plate reader and the BCA™ Protein Assay Kit (Pierce, Rockford, IL) were used to assess protein concentrations. Unless otherwise specified, the uptake and inhibition data shown are representative of two independent experiments. Statistical evaluations compared acyclovir uptake in the presence of inhibitors to OAT2-mediated acyclovir uptake in the absence of inhibitors using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

Saturation kinetics of acyclovir

HEK293 cells stably transfected with empty vector or OAT2 were pre-incubated and treated as previously described and were exposed for 2 minutes at 37°C in Na^+ uptake buffer containing ^{3}H-acyclovir (2 μM) in the presence of various concentrations of unlabeled acyclovir (25 μM to 600 μM). All kinetic data on acyclovir uptake by OAT2 and its genetic variant, Thr110Ile, were fitted to a Michaelis-Menten curve by non-linear regression, using GraphPad Prism® 4 Software (La Jolla, CA).

Functional analysis of variants in *SLC22A7*

The sequence of OAT2 designated as "reference" corresponded to the OAT2 coding sequence that occurred at the highest frequency in all ethnic groups. Each of the 6

non-synonymous OAT2 variants was constructed by site-directed mutagenesis of the OAT2 reference sequence using the QuickChange mutagenesis protocol (Stratagene, La Jolla, CA), as previously described (10). HEK293 cells transiently transfected with OAT2 or each of the non-synonymous variants in OAT2 were pre-incubated and treated in the same manner as previously described. These cells were exposed for 2 minutes at 37°C in Na^+ uptake buffer containing ^{3}H-cGMP (2 μM) or ^{3}H-acyclovir (2 μM). The data are presented as percentage of cGMP or acyclovir uptake by OAT2 reference. Each value is expressed as mean ± SD from a representative experiment out of three independent experiments. Statistical significance was determined by comparing variant uptake to OAT2 reference uptake for each substrate using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance. Kinetic studies of acyclovir's interaction with OAT2 and the polymorphic non-synonymous variant OAT2-Thr110Ile were performed in transiently transfected HEK293 cells. Cells transiently transfected with empty vector, OAT2, or Thr110Ile were pre-incubated and treated as described above. The cells were exposed for 2 minutes at 37°C in Na^+ uptake buffer containing ^{3}H-acyclovir (2 μM) in the presence of various concentrations of unlabeled acyclovir (ranging 25 μM to 600 μM). A summary of the kinetic parameters of acyclovir uptake by OAT2 reference and Thr110Ile is given as mean ± SD from three independent experiments.

RNA extraction, reverse transcription, and real-time PCR

Total RNA from human brain, colon, kidney, liver, and placenta was purchased from Clontech (Mountain View, CA). One microgram of RNA from these tissues was reverse transcribed using the Superscript III First Strand Synthesis kit from Invitrogen.

The reactions were diluted 5 fold with TE buffer and 2 microliters from each sample was used to quantify gene expression of OAT1-4 in the aforementioned tissues using TaqMan® Gene Expression Assays and an ABI 7900 HT Fast Real-Time PCR sequence detection system (Applied Biosystems) following the default protocol from Applied Biosysytems. GAPDH was used as an endogenous control for normalization purposes. Fold differences were calculated using the $\Delta\Delta C_T$ (comparative threshold cycle) method and were normalized to expression in the brain. The following TaqMan® Gene Expression Assays were used for OAT1-4 and GAPDH respectively: Hs00537914_m1, Hs00198527_m1, Hs00188599_m1, Hs00218486_m1, and Hs99999905_m1. Each measurement was performed in triplicate.

The expression of OAT2 and their respective variants was quantified by real-time reverse transcription-PCR, using TaqMan® Gene Expression Assays and an ABI 7500 Fast Real-Time PCR sequence detection system (Applied Biosystems) as described previously (7). The TaqMan® Gene Expression Assay Hs00198527_m1 was used for human OAT2. Human GAPDH endogenous control (Hs99999905_m1) was used as an internal standard for sample normalization.

Membrane topology of OAT2 and location of its non-synonymous variants

The TMRPres2D program was downloaded from the TMRPres2D website (http://biophysics.biol.uoa.gr/TMRPres2D/) as a Java Archive file and used to generate the OAT2 topology image (11). The OAT2 sequence and previously determined transmembrane segments were entered using the "User Defined Entry" option of the program to generate a draft image. The image was amended with red circles at the positions of the 6 protein-altering variants and a yellow halo to denote the Thr110Ile

single nucleotide polymorphism (SNP) of interest.

RESULTS

OAT2 is more highly expressed in the liver than other OATs

Consistent with a previous report (12), we found OAT2 to be expressed at its highest level in the liver. In the liver, its expression was ~5000-fold higher than in the brain and slightly more than 3-fold higher than in the kidney (Fig. 1A). Likewise, expression of OAT2 in the liver was more than 4500-fold higher than expression of OAT1, OAT3, or OAT4 in the liver (Fig. 1B).

OAT2 transports acyclovir

As our previous Chapter 2 studies demonstrated that OAT2 exhibits a preferred transport for guanine analogs, including cGMP (7), we screened other nucleotide analogs for transport by OAT2. We observed that acyclovir, a guanine-based antiviral, was also a model substrate for OAT2 (Fig. 2A and 2B). As shown in Fig. 2A, HEK293 cells stably transfected with OAT2 displayed a 40-fold mean change over cells similarly transfected with EV. This fold change was much greater than that seen with PAH, a previously identified model substrate for OAT2. The actual uptake of acyclovir in EV- and OAT2-stably transfected HEK293 cells was 0.073 ± 0.005 pmol/mg protein/5 minutes and 3.062 ± 0.114 pmol/mg protein/5 minutes, respectively (Fig. 2B).

Comparison of acyclovir transport among OATs

We compared the selectivity of acyclovir transport in selected OAT family members by using HEK293 cells stably expressing transporters OAT1-4. The functionality of each transporter expressed in HEK293 cells was confirmed with each respective transporter's model substrates (170-fold uptake of PAH by OAT1, 4-fold

Figure 1. mRNA expression of OAT2 in human tissues (A) and OAT mRNA expression in the liver (B)

Real-time PCR was performed to evaluate mRNA expression of OAT1-4 using TaqMan® Gene Expression Assays in various human tissues using commercially available total RNA. Fold differences were calculated using the ΔΔCt method and were normalized to expression in the brain. Each measurement was performed in triplicate.

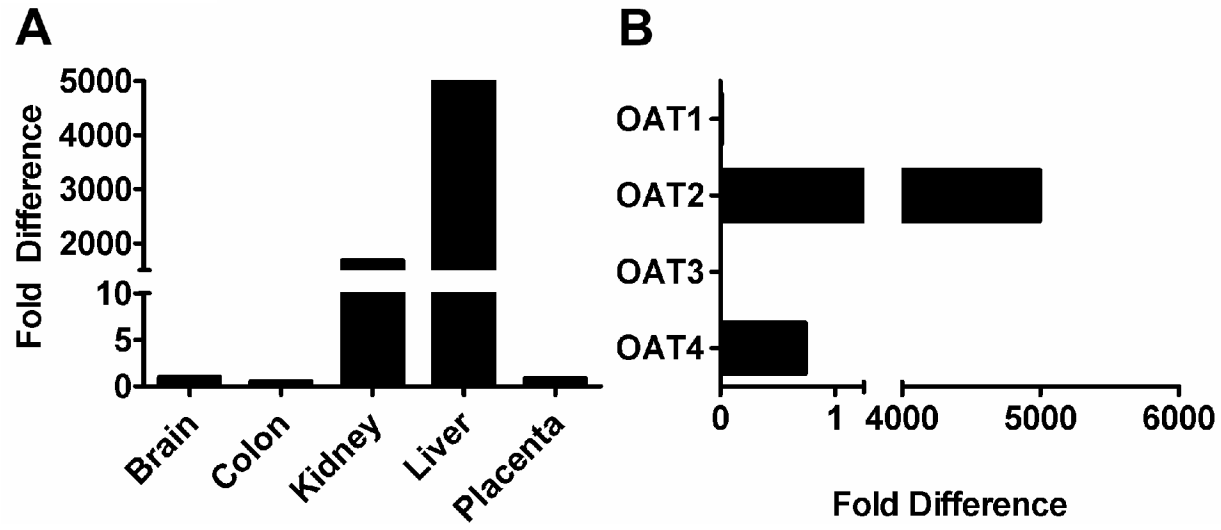
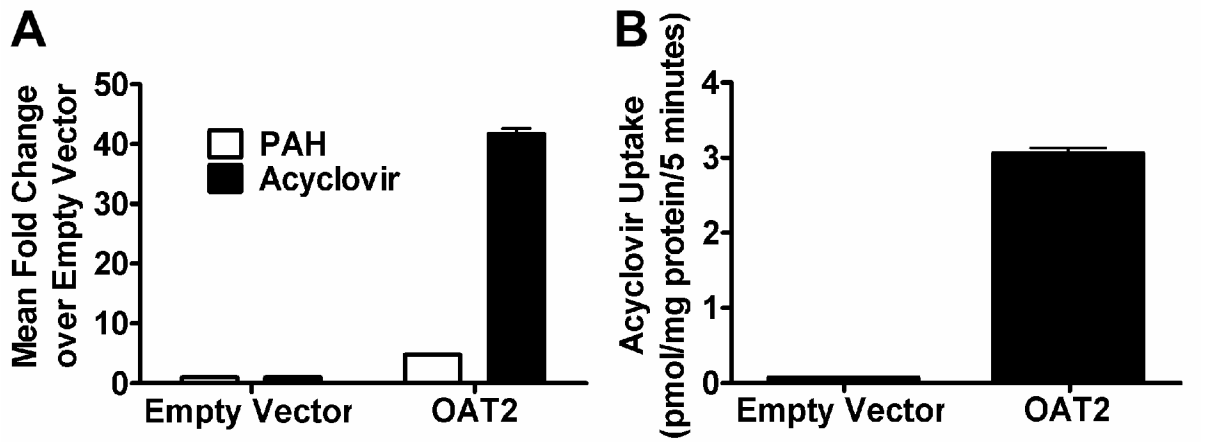


Figure 2. Acyclovir is a novel OAT2 substrate

Empty vector of pcDNA5/FRT and pcDNA5/FRT containing OAT2 cDNA were transiently transfected into HEK293 cells. Uptake was studied with a 5 minute incubation of 1:1000 reaction mixes of ^{3}H-PAH (70 ηM) or ^{3}H-acyclovir (25 ηM). The mean fold change of OAT2-mediated uptake of PAH and acyclovir over empty vector **(A)** and the uptake of acyclovir via empty vector and OAT2 **(B)** are shown. Each bar represents the mean ± SD (n = 3). Results were confirmed in two independent experiments. Representative results are shown.



uptake of PAH by OAT2, 61-fold uptake of estrone sulfate by OAT3, and 35-fold uptake of estrone sulfate by OAT4, data not shown). OAT1 and OAT3 detectably transported acyclovir but OAT4 did not. However, cellular uptake of acyclovir by OAT1 or OAT3 increased by only 2-fold over empty vector control whereas uptake via OAT2 was more than 21-fold over empty vector (Fig. 3). Furthermore, the relative ratio of transport efficiency (acyclovir uptake divided by PAH uptake) exhibited values of 0.055 for OAT1 and 29 for OAT2, suggesting a greater preference of OAT2 for acyclovir compared to OAT1.

Transport of nucleoside analog antiviral drugs by OAT2

In addition to acyclovir, eight other nucleoside analog antiviral drugs 1-ß-D-arabinofuranosyladenine (vidarabine), 9-(2-phosphonylmethoxypropyl) adenine (tenofovir), 9-(2-phosphonylmethoxyethyl) adenine (adefovir), 2',3'-dideoxyinosine (didanosine), 1-β-D-ribofuranosyl-1H-1,2,4-triazole-3-carboxamide (ribavirin), (1S,cis)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]-2-cyclopentene-1-methanol (abacavir), 2',3'-dideoxycytidine (zalcitabine), 3'-azido-2',3'-dideoxythymidine (azidothymidine) were studied. As shown in Fig. 4, OAT2 was capable of transporting a wide array of purine and pyrimidine analogs. A more than 2-fold increase in uptake by OAT2 over EV was observed for all of drugs tested except for abacavir (purine mimic) and azidothymidine (thymidine analog). Among the nucleoside analogs, acyclovir exhibited the largest differential rate of OAT2-specific transport, followed by didanosine (inosine analog). A significant, but moderate transport of adenosine analogs (vidarabine; 2-fold), acyclic adenosine phosphonate analogs (tenofovir, adefovir; 5-fold each), cytidine analog (zalcitabine; 3-fold), and purine mimic (ribavirin; 2-fold) was observed.

Figure 3. Acyclovir transport is selective for OAT2 among other OAT transporters
Cells stably transfected with each vector construct were incubated for 5 minutes with ^{3}H-acyclovir (2 μM). Each value represents the mean ± SD (n=3).

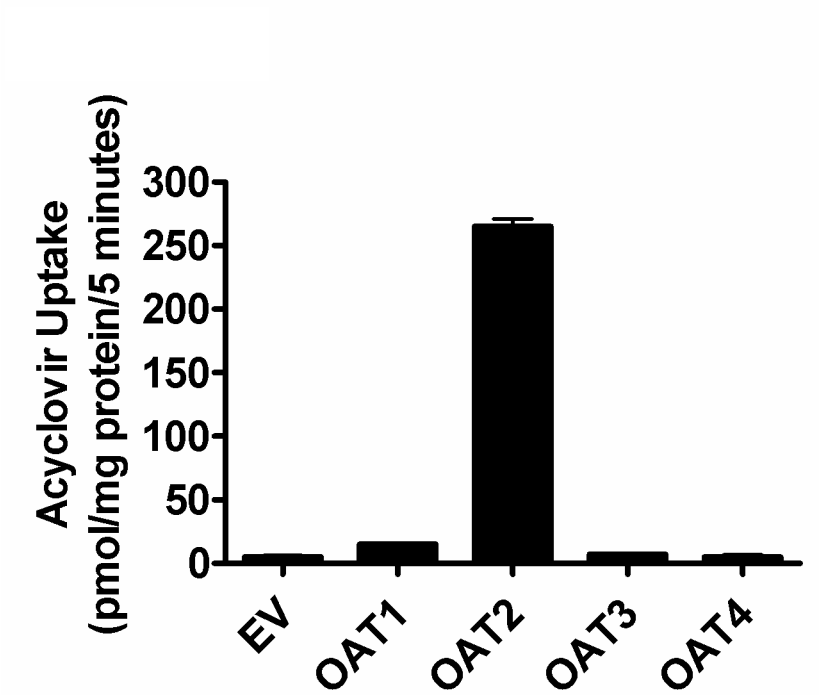
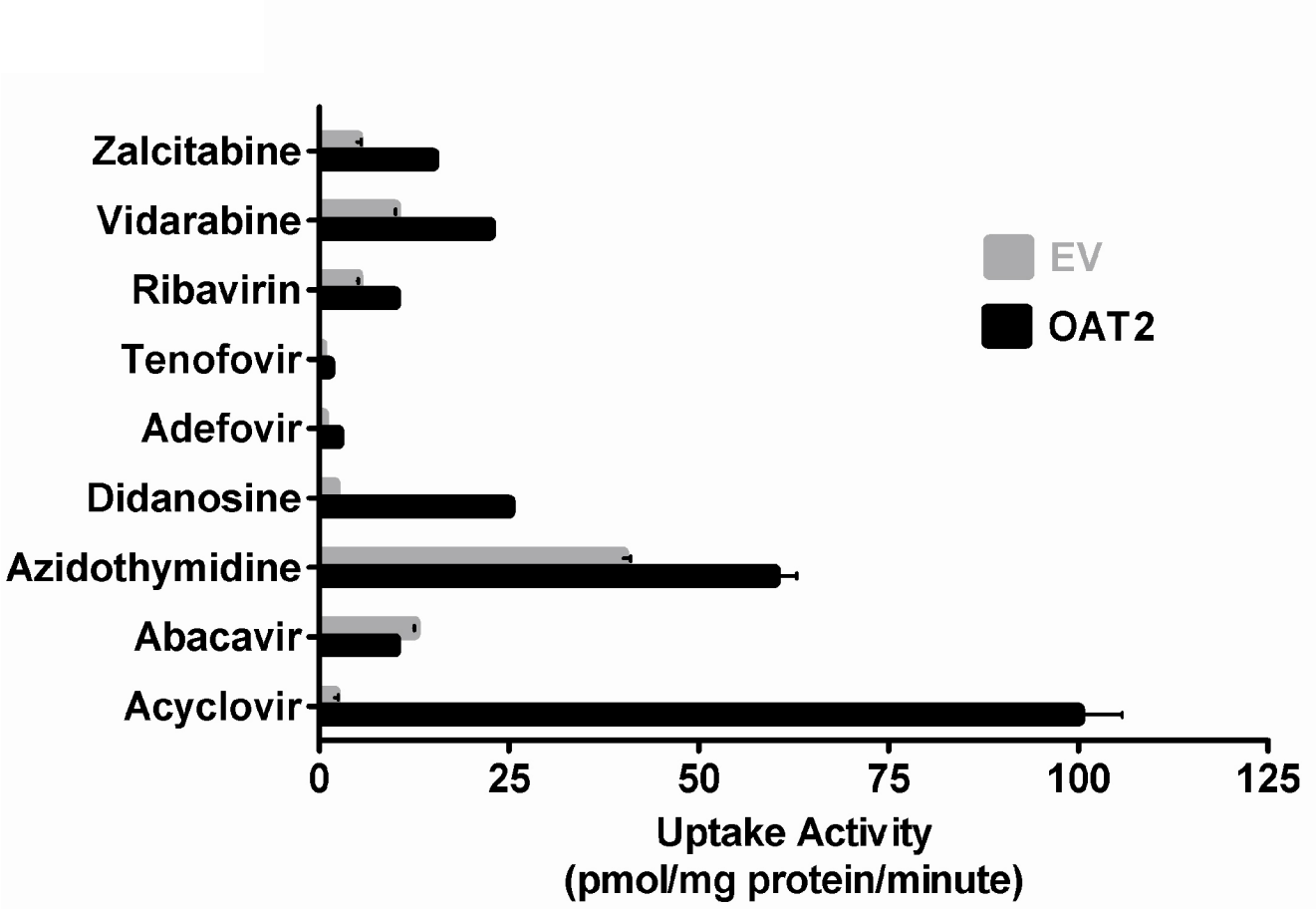


Figure 4. Characterization of the transportability of nucleoside analog antiviral drugs by OAT2

Empty vector- and OAT2-transfected HEK293 cells were incubated for 1.5 minutes with 2 μM concentrations of each antiviral compound. Each value represents the means ± SD of three independent experiments. Each experiment was conducted in duplicate or triplicate samples.



Cis-inhibition of OAT2-mediated transport of acyclovir

To further define the OAT2-mediated transport of acyclovir, we examined the inhibition of acyclovir uptake in OAT2 transfected HEK cells (Fig. 5). Both probenecid (1000 μM), a low affinity inhibitor of OAT2, and prostaglandin $F_{2\alpha}$ (150 μM), a high affinity substrate for OAT2 inhibited acyclovir uptake via OAT2. Also, guanosine (150 μM) and cGMP (150 μM), identified as OAT2 substrates in our early studies (7), inhibited the OAT2-mediated transport of acyclovir by 60% and 80% respectively, compared to control.

Kinetics studies

To characterize acyclovir transport mediated by OAT2, we first determined the time course of cellular uptake of acyclovir. Acyclovir uptake was significantly higher in OAT2/HEK cells than EV/HEK cells at all time points through 30 minutes and acyclovir uptake by OAT2 increased linearly with time for up to 4 minutes (data not shown). With this knowledge, uptake kinetics studies were conducted using a 2 minute incubation time to assess the initial rate of uptake. The initial rate of cellular uptake mediated by OAT2 was concentration-dependent and saturable with an apparent K_m of 142 ± 50 μM and a maximal transport rate (V_{max}) of 3442 ± 439 pmol/mg protein/2 minutes (Fig. 6).

Membrane topology diagram of OAT2 and location of its non-synonymous variants

We screened the coding region of OAT2 in a collection of 272 DNA samples from ethnically diverse human populations, and identified six non-synonymous variants all with frequencies of < 5% (Table 1). A single non-synonymous variant, C329T (Thr110Ile), was polymorphic with an allele frequency of 2.3% in African Americans. The other five non-synonymous variants were found on only one chromosome each in the

Figure 5. *Cis*-inhibition of OAT2-mediated acyclovir uptake by various compounds
OAT2-transfected HEK293 cells were incubated for 1.5minutes with ^{3}H-acyclovir (2 μM) in the absence or presence of probenecid (1000 μM), prostaglandin F2α (150 μM), guanosine (150 μM), or cGMP (150 μM). The values were expressed as percentage of ^{3}H-acyclovir uptake by OAT2-transfected cells without any compounds. Data are mean ± SD (n = 3). Results were confirmed in two independent experiments. Representative results were shown here.

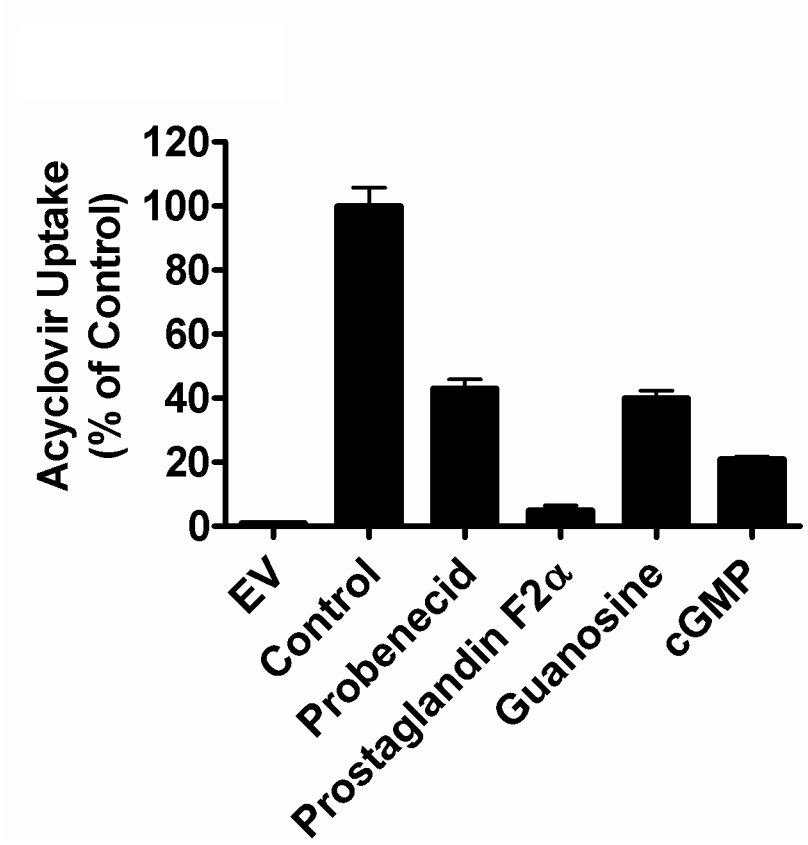


Figure 6. Concentration-dependent uptake of acyclovir by EV- and OAT2-transfected HEK293 cells

EV/HEK and OAT2/HEK (solid squares) cells were incubated for 2 minutes with ^{3}H-acyclovir (2 μM) and acyclovir concentrations ranging from 25 μM to 600 μM. The OAT2-specific uptake was determined by subtracting the uptake in EV from that in OAT2 HEK293 transfected cells. Kinetic parameters obtained by fitting each value to the Michaelis-Menten equation are shown as mean ± SD of three independent experiments. Representative results are shown here. Each point represents the mean ± SD of triplicate samples.

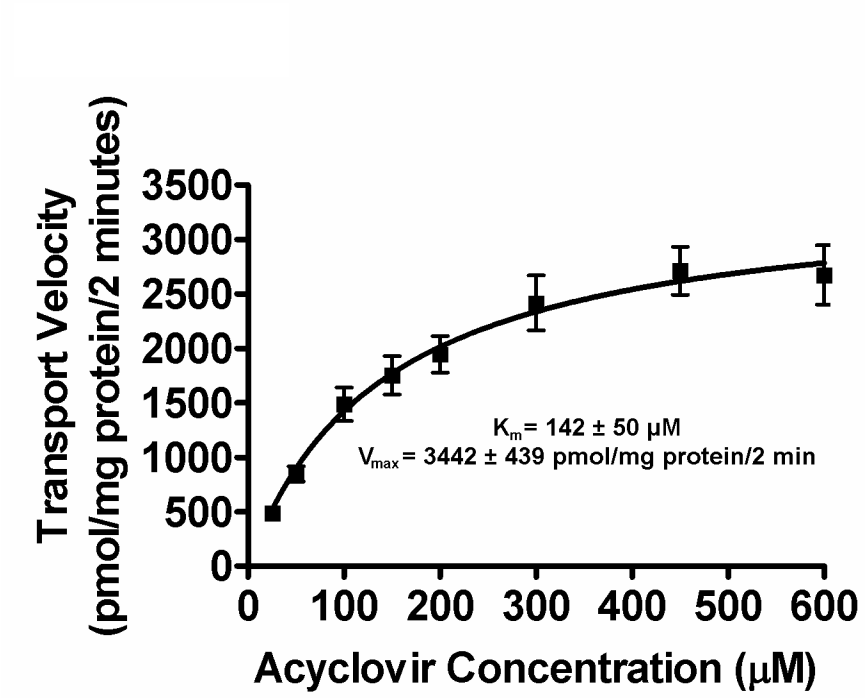


Table 1. Summary of variants in the coding region of OAT2 identified in ethnically diverse populations

Exon	SNP ID	dbSNP ID	CDS Position[1]	Nucl. Change	Amino Acid Position[1]	Amino Acid Change	Allele Frequency (%)[2]			
							AA (n=136)	EA (n=136)	AS (n=136)	ME (n=136)
1	1.1	rs35263175	222	T→C	74	syn	5.3 (o=132)	0	0	0
1	1.2		244	C→T	82	*Arg→Cys*	0	0	0	0.8 (o=132)
1	1.3		301	C→T	101	*Arg→Cys*	0.8 (o=132)	0	0	0
1	**1.4**		**329**	**C→T**	**110**	***Thr→Ile***	**2.3 (o=132)**	**0**	**0**	**0**
2	2.1		474	G→A	158	syn	0.7 (o=136)	0	0	0
4	4.1		680	G→A	227	*Arg→His*	0	0.8 (o=124)	0	0
6	6.1		961	G→A	321	Ala→Thr	0	0	0	0.8 (o=132)
6	6.2	rs36040909	973	C→T	325	*Arg→Trp*	0.7 (o=136)	0	0	0
7	7.1	rs2651184	1215	T→C	405	syn	11.7 (o=128)	0	0	0
7	7.2	rs2270860	1269	C→T	423	syn	68.8 (o=128)	29.8 (o=124)	33.1 (o=124)	32.1 (o=112)

Table 1. Summary of variants in the coding region of OAT2 identified in ethnically diverse populations (continued)

Data are available at http://www.pharmgkb.org. The common non-synonymous variant (≥1% in any ethnic group) is shown in bold. The variants that show reduced transport of cGMP and/or acyclovir indicated in italics.
[1]Positions are relative to the ATG start site and are based on the cDNA sequence from GenBank accession number NM_006672.
[2]Some samples contained amplicons that could not be sequenced. The total number of chromosomes analyzed is shown below the ethnic group. Allele frequencies were calculated from actual DNA samples sequenced. Total, entire sample; AA, African American; EA, European American; AS, Asian American; ME, Mexican American; n, number of chromosomes

272 DNA samples. The location of each *SLC22A7* variant and its population frequency information has been deposited in the online databases dbSNP (http://www.ncbi.nlm.nih.gov/SNP) and PharmGKB (http://www.pharmgkb.org). The location of the non-synonymous SNPs of *SLC22A7* is displayed in the proposed membrane protein topology shown in Fig. 7.

OAT2 variants demonstrate reduced uptake of acyclovir and cGMP

Acyclovir and cGMP (a known model substrate for OAT2) uptake was assayed in OAT2 reference and OAT2 variant transiently transfected cells (Fig. 8). The single polymorphic variant, Thr110Ile, demonstrated approximately 30% reduced uptake of acyclovir compared to reference OAT2 ($p < 0.01$). Of note, Arg82Cys and Arg227His exhibited approximately 60% and 85% reduced uptake, respectively ($p < 0.001$). Among the remaining singleton variants, Arg101Cys and Arg325Trp exhibited a decrease of approximately 25 to 30% of acyclovir and cGMP uptake compared to reference OAT2.

OAT2 variant mediated transport of acyclovir is saturable and uptake is reduced

Given that Thr110Ile was the only polymorphic variant found and it exhibited a reduced function compared to OAT2 reference on our initial screen, we performed detailed kinetic analyses of EV, OAT2 and Thr110Ile (Fig. 9). Thr110Ile exhibited a reduced V_{max} compared to OAT2 reference (V_{max} values were 2630 ± 209 pmol/mg protein/2 minutes for OAT2 versus 1361 ± 124 pmol/mg protein/2 minutes for Thr110Ile ($p < 0.001$)). The difference in K_m values between OAT2 and Thr110Ile was not statistically significant ($p = 0.09$).

Figure 7. Membrane topology diagram of OAT2 and location of its non-synonymous variants

Predicted secondary structure of OAT2 showing the location of its 6 non-synonymous variants. The TMRPres2D program was downloaded from the TMRPres2D website (http://biophysics.biol.uoa.gr/TMRPres2D/) as a Java Archive file and used to generate this image. Red circles represent the positions of the 6 non synonymous variants and a yellow halo denotes the Thr110Ile SNP of interest.

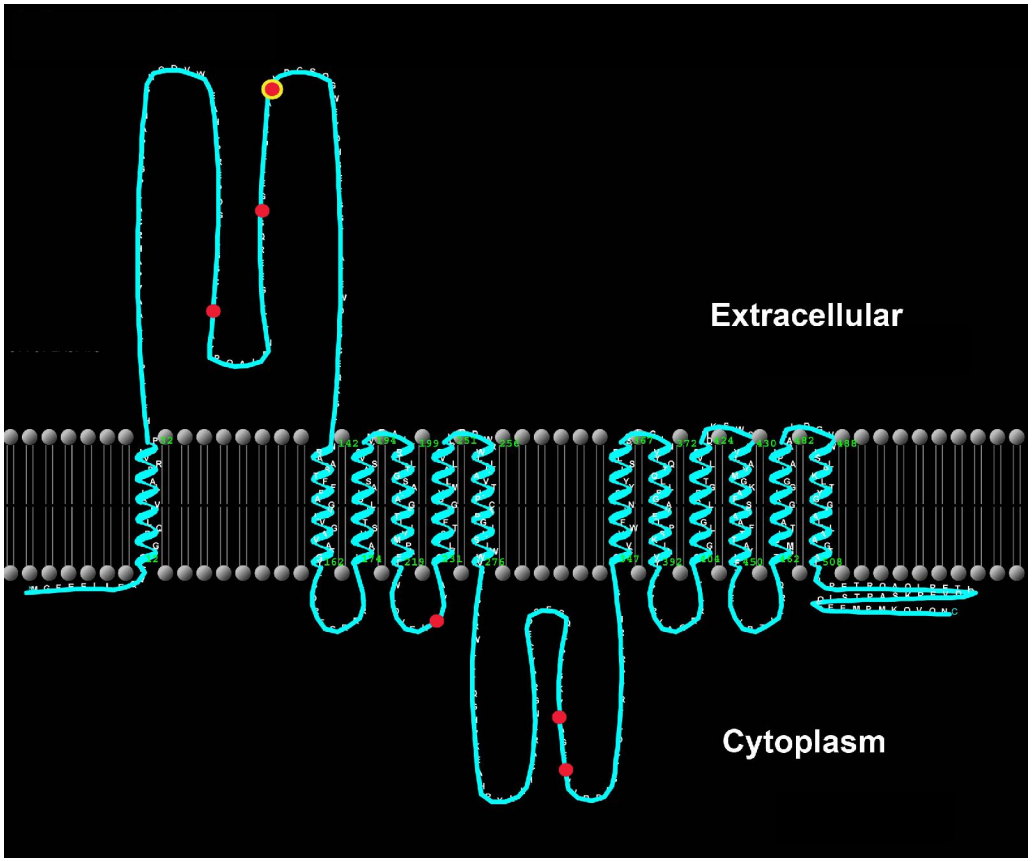


Figure 8. OAT2 protein-altering variants transport cGMP and acyclovir

HEK293 cells transiently transfected with each vector (EV, OAT2, and each OAT2 non-synonymous variant) construct were incubated for 2 minutes with ^{3}H-cGMP (2 μM) or ^{3}H-acyclovir (2 μM). Each value is expressed as a percentage of OAT2 reference and represents the mean ± SD (n=3). P values were determined using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

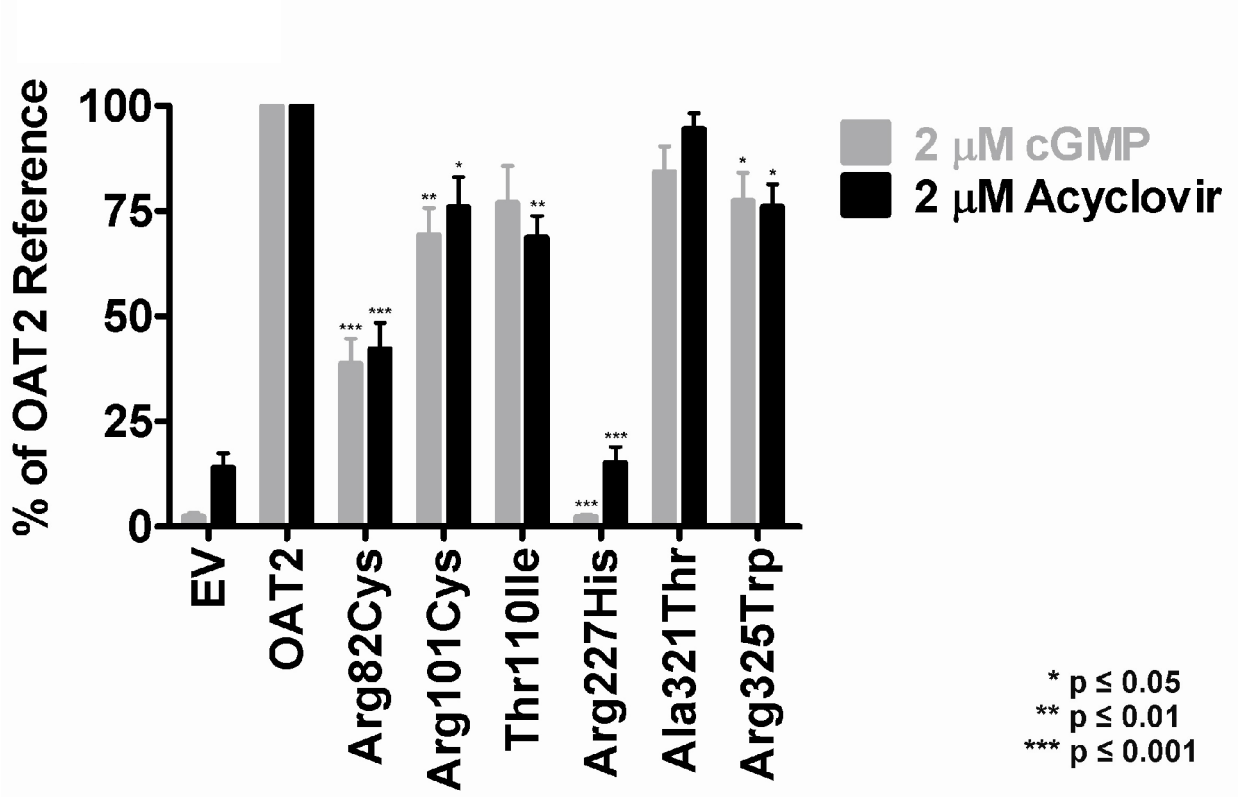
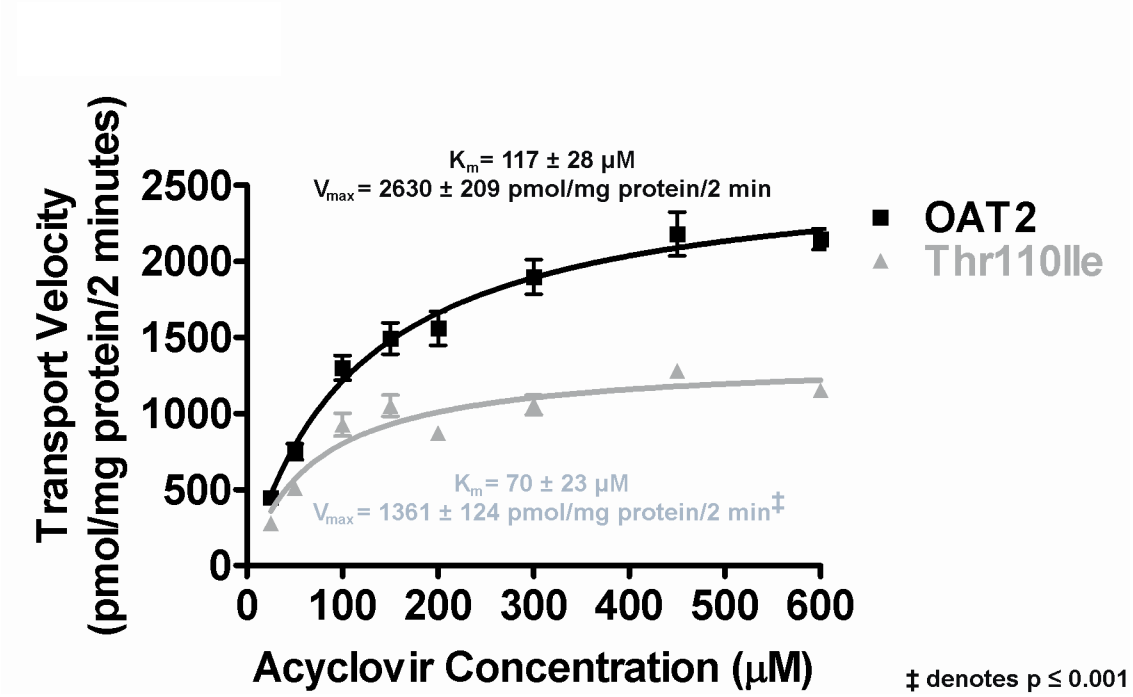


Figure 9. Acyclovir uptake via OAT2 variant Thr110Ile is saturable and exhibits reduced uptake

EV, OAT2, and variant Thr110Ile stably transfected HEK293 cells were incubated for 2 minutes with radiolabeled tracer acyclovir and acyclovir concentrations ranging from 25 µM to 600 µM. OAT2 reference and variant-specific uptake was determined by subtracting the uptake in EV from OAT2 reference and its variants. Kinetic parameters obtained by fitting each value to the Michaelis-Menten equation are shown as mean ± SD of three independent experiments. Representative results were shown here. Each point represents the mean ± SD of triplicate samples. P values were determined by comparing kinetic parameters for the variant to that of OAT2 reference using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.



DISCUSSION

Viral infections caused by the herpes virus are prevalent in the human population (2, 3). For immunocompetent individuals, HSV infection may be self-limiting. However, in individuals with compromised immune function, complications due to HSV infection may be more detrimental, affecting organs such as the eye, brain or liver. Acyclovir is used in the treatment of herpes infections including herpes hepatitis. In Chapter 2, we reported that the organic anion transporter, OAT2 (*SLC22A7*) is expressed abundantly in the liver and interacts potently with cGMP (guanosine based structure) (7). In light of this observation, we screened other xenobiotics with nucleotide-like structures, including antiviral drugs used in the treatment of HSV and HIV infections. The results of this screen revealed that among the antiviral agents evaluated, acyclovir (guanine-like structure) had an avid interaction with OAT2 (Fig. 4).

In the present study, we characterized the interaction of OAT2 with acyclovir. Our data revealed that acyclovir was an excellent substrate of OAT2 (Fig. 2). Kinetic analysis indicated that acyclovir uptake by OAT2 was saturable with an apparent K_m of 142 μM (Fig. 6). This concentration is close to therapeutic maximum plasma concentrations of acyclovir (100 μM) suggesting that OAT2 is a relevant transporter for acyclovir at therapeutic doses (13).

Among other evaluated members of the OAT family, OAT2 exhibited the greatest ability (21-mean fold change) to transport acyclovir compared to other *SLC22* transporters specifically OAT1, OAT3, and OAT4 (Fig. 3). These results contrast to the results by Takeda et al. who found that acyclovir was best transported by OAT1 and not OAT2 (14). In Chapter 2, we demonstrated that OAT2 has a non-functional splice variant

that exists at the same tissue levels as the functional splice variant (7). Thus, it is possible that the non-functional splice form of OAT2 was primarily expressed in OAT2 transfected cell lines used by Takeda *et al*. The finding that both probenecid and prostaglandin $F_{2\alpha}$ inhibited acyclovir uptake via OAT2 (Fig. 5) is consistent with previous studies in the literature demonstrating that prostaglandin is an excellent substrate of OAT2 and that the transporter can be inhibited by probenecid, a general inhibitor of organic anion transport (15-18).

As a first step towards determining whether coding region variants of OAT2 may contribute to the large variation in pharmacokinetics of the drug observed clinically, we screened 272 DNA samples from ethnically diverse U.S. populations. The six non-synonymous variants all had allele frequencies of < 5% (Table 1, Fig. 7). These data suggest that non-synonymous variants of OAT2, because of the rarity, would not contribute substantially to the population variation observed in the pharmacokinetics of the drug. The data also argue that OAT2 is under strong purifying selection and thus plays a critical biological role in humans.

To determine the functional significance of the variants, we first conducted a PolyPhen analysis followed by experimental studies (19, 20). The results of the PolyPhen analysis revealed that three of the six non-synonymous mutations of OAT2 were considered “benign” (Arg101Cys, Arg227His, and Ala321Thr) whereas the remaining 3 were predicted to be “probably damaging” (Arg82Cys and Thr110Ile) or “possibly damaging” (Arg325Trp).

Our experimental results demonstrate that the uptakes of cGMP and acyclovir are significantly reduced in cells expressing Arg82Cys, Thr110Ile, Arg227His, and

Arg325Thr as compared to cells expressing OAT2 reference. With the exception of the Arg227His (allele frequency < 1%) in which the "benign" label assigned by PolyPhen did not agree with the experimental results, the experiments were in general agreement with the prediction of PolyPhen. We note that the change from arginine to histidine at coding amino acid position 227 results in a change in an evolutionarily conserved amino acid residue of OAT2 among 5 mammalian orthologs (orangutan, cow, rabbit, mouse, and rat). It is unclear why PolyPhen predicted the change to be apparently benign since evolutionarily conserved residues are known to affect function (21-24).

Kinetic studies were performed on the single polymorphism of OAT2 that we identified. Thr110Ile exhibited a significantly reduced V_{max} for acyclovir transport compared to the V_{max} of OAT2 reference with no significant change in K_m (Fig. 9). The mechanism for this reduced V_{max} may be related to a slower turnover rate of the transporter or to reduced expression on the plasma membrane. Our studies with GFP-tagged chimeras were inconclusive as to alterations in expression levels of the variant transporters on the plasma membrane. The studies did show that all of the variants including Thr110Ile were present at least to some extent on the plasma membrane. Since methods of determining levels of protein expression on the cell surface, including GFP tagged chimeras and biotinylation with immunoblotting, are semi-quantitative at best, it is difficult to decipher the exact mechanism for the reduced V_{max} of the Thre110Ile.

In conclusion, our studies suggest that OAT2 plays a primary role in the transport of acyclovir. Additionally, genomic analysis of OAT2 and its coding region in 272 human DNA samples revealed a low degree of genetic variation with only one polymorphism, which was exclusive to the African American cohort. Our results suggest

that rare non-synonymous variants of OAT2 may result in reduced intracellular levels of OAT2 particularly in organs such as the liver in which the transporter is highly expressed.

REFERENCES

1. Smith JS and Robinson NJ. Age-specific prevalence of infection with herpes simplex virus types 2 and 1: a global review. *Journal Infect Dis* **186:**S3-S28 (2002).
2. Whitley RJ and Roizman B. Herpes simplex virus infections. *Lancet* **357:**1513-1518 (2001).
3. Looker KJ, Garnett GP and Schmid GP. An estimate of the global prevalence and incidence of herpes simplex virus type 2 infection. *Bull World Health Organ* **86:**805-812, A (2008).
4. Colgan R, Michocki R, Greisman L and Moore TA. Antiviral drugs in the immunocompetent host: part II. Treatment of influenza and respiratory syncytial virus infections. *Am Fam Physician* **67:**763-766, 675 (2003).
5. Goodman ZD, Ishak KG and Sesterhenn IA. Herpes simplex hepatitis in apparently immunocompetent adults. *Am J Clin Pathol* **85:**694-699 (1986).
6. Velasco M, Llamas E, Guijarro-Rojas M and Ruiz-Yague M. Fulminant herpes hepatitis in a healthy adult: a treatable disorder? *J Clin Gastroenterol* **28:**386-389 (1999).
7. Cropp CD, Komori T, Shima JE, Urban TJ, Yee SW, More SS and Giacomini KM. Organic anion transporter 2 (SLC22A7) is a facilitative transporter of cGMP. *Mol Pharmacol* **73:**1151-1158 (2008).
8. Leabman MK, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T, Clark AG, Herskowitz I and Giacomini KM. Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered

function. *Pharmacogenetics* **12:**395-405 (2002).

9. Taillon-Miller P, Piernot EE and Kwok PY. Efficient approach to unique single-nucleotide polymorphism discovery. *Genome Res* **9:**499-505 (1999).

10. Erdman AR, Mangravite LM, Urban TJ, Lagpacan LL, Castro RA, de la Cruz M, Chan W, Huang CC, Johns SJ, Kawamoto M, Stryke D, Taylor TR, Carlson EJ, Ferrin TE, Brett CM, Burchard EG and Giacomini KM . The human organic anion transporter 3 (OAT3; SLC22A8): genetic variation and functional genomics. *Am J Physiol Renal Physiol* **290:**F905-F912 (2006).

11. Spyropoulos IC, Liakopoulos TD, Bagos PG and Hamodrakas SJ. TMRPres2D: high quality visual representation of transmembrane protein models. *Bioinformatics* **20:**3258-3260 (2004).

12. Sekine T, Cha SH, Tsuda M, Apiwattanakul N, Nakajima N, Kanai Y and Endou H. Identification of multispecific organic anion transporter 2 expressed predominantly in the liver. *FEBS Lett* **429:**179-182 (1998).

13. Boulieu R, Gallant C and Silberstein N. Determination of acyclovir in human plasma by high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl* **693:**233-236 (1997).

14. Takeda M, Khamdang S, Narikawa S, Kimura H, Kobayashi Y, Yamamoto T, Cha SH, Sekine T and Endou H. Human organic anion transporters and human organic cation transporters mediate renal antiviral transport. *J Pharmacol Exp Ther* **300:**918-924 (2002).

15. Burckhardt BC and Burckhardt G. Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev Physiol Biochem Pharmacol*

146:95-158 (2003).

16. Enomoto A, Takeda M, Shimoda M, Narikawa S, Kobayashi Y, Kobayashi Y, Yamamoto T, Sekine T, Cha SH, Niwa T and Endou H. Interaction of human organic anion transporters 2 and 4 with organic anion transport inhibitors. *J Pharmacol Exp Ther* **301:**797-802 (2002).
17. Kimura H, Takeda M, Narikawa S, Enomoto A, Ichida K and Endou H. Human organic anion transporters and human organic cation transporters mediate renal transport of prostaglandins. *J Pharmacol Exp Ther* **301:**293-298 (2002).
18. Uwai Y, Ida H, Tsuji Y, Katsura T and Inui K. Renal transport of adefovir, cidofovir, and tenofovir by SLC22A family members (hOAT1, hOAT3, and hOCT2). *Pharm Res* **24:**811-815 (2007).
19. Sunyaev S, Ramensky V, Koch I, Lathe W 3rd, Kondrashov AS and Bork P. Prediction of deleterious human alleles. *Hum Mol Genet* **10:**591-597 (2001).
20. Xi T, Jones IM and Mohrenweiser HW. Many amino acid substitution variants identified in DNA repair genes during human population screenings are predicted to impact protein function. *Genomics* **83:**970-979 (2004).
21. Leabman MK, Huang CC, DeYoung J, Carlson EJ, Taylor TR, de la Cruz M, Johns SJ, Stryke D, Kawamoto M, Urban TJ, Kroetz DL, Ferrin TE, Clark AG, Risch N, Herskowitz I, Giacomini KM and Pharmacogenetics of Membrane Transporters Investigators. Natural variation in human membrane transporter genes reveals evolutionary and functional constraints. *Proc Natl Acad Sci USA* **100:**5896-5901 (2003).
22. Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, Kawamoto

M, Johns SJ, DeYoung J, Carlson E, Ferrin TE, Herskowitz I, Giacomini KM and Pharmacogenetics of Membrane Transporters Investigators. Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc Natl Acad Sci USA* **100:**5902-5907 (2003).

23. Urban TJ, Giacomini KM and Risch N. Haplotype structure and ethnic-specific allele frequencies at the OCTN locus: implications for the genetics of Crohn's disease. *Inflamm Bowel Dis* **11:**78-79 (2005).

24. Wolff NA, Thies K, Kuhnke N, Reid G, Friedrich B, Lang F, and Burckhardt G. Protein kinase C activation downregulates human organic anion transporter 1-mediated transport through carrier internalization. *J Am Soc Nephrol* 2003 **14:**1959-1968 (2003).

CHAPTER 4

INTERACTION OF METHOTREXATE WITH THE ORGANIC ANION TRANSPORTER 3 (OAT3; *SLC22A8*) AND ITS GENETIC VARIANTS

INTRODUCTION

The antiproliferative folate antagonist methotrexate (MTX) has been widely used at high doses in the treatment of a variety of malignancies and at low doses in the treatment of several chronic inflammatory disorders. Despite being highly efficacious, methotrexate therapy can result in serious complications, including renal dysfunction, neurotoxicity, myelosuppression, and mucositis (1). It has been determined that 50-90% of each MTX dose is excreted unchanged in the urine and net renal elimination occurs via active secretory and reabsorptive processe (2-4). MTX and its metabolite, 7-hydroxymethotrexate (7-OH-MTX) are poorly soluble and have been shown to precipitate in the lumen of distal tubules resulting in nephrotoxicity and delayed elimination (5-8). Accumulation of MTX in blood due to renal dysfunction and delayed clearance can then lead to severe multi-organ toxicity.

Xenobiotic membrane transporters are important determinants of systemic blood levels and tissue specific levels of drugs, such as MTX, thereby affecting their efficacy and toxicity. *SLC19A1*, also known as Reduced Folate Carrier (RFC), is a membrane transporter deemed important in determining systemic and tissue levels of reduced folate and its analogs, including MTX (9). Another important uptake family in humans is the *SLC22* transporter family, whose members interact with a broad variety of organic solutes and xenobiotics (10). In particular, the organic anion transporter family (OAT;

SLC22A6-8 and *SLC22A10-11*) has been shown to be a critical determinant of MTX renal tubular secretion and elimination (2, 3, 11). Furthermore, it was determined that blocking organic anion transport activity with the non-specific inhibitor, probenecid, led to a significant increase in the half-life, exposure, and plasma concentrations of MTX, which are associated with MTX-induced toxicity (3, 12). Of all the human OAT transporters that have been identified and characterized, OAT3 (*SLC22A8*) is the most abundant in the kidney where it is located on the basolateral surface of renal proximal tubules (10, 13). This transporter is responsible for the cellular uptake of a structurally diverse set of endogenous organic anions and commonly used drugs, including MTX (10, 14-17). VanWert and Sweet demonstrated that the clearance of MTX was significantly decreased in Oat3 knockout mice compared to wildtype mice when MTX clearance was normalized to inulin clearance (18). Recently, Erdman *et al.* identified naturally occurring variants in human OAT3 and determined that these variants exhibit significant functional differences with respect to the model substrates estrone sulfate (ES) and cimetidine (19).

Given that OAT3 plays an important role in the renal tubular secretion of MTX, we undertook a comprehensive genetic and functional analysis of OAT3 in ethnically diverse populations to gain useful insights about the genetic factors that influence inter-individual variability in MTX disposition, elimination, and toxicity.

METHODS

Materials

^{3}H-ES (57.3 Ci/mmol) and ^{3}H-MTX (49.6 Ci/mmol) were purchased from PerkinElmer Life Sciences (Wellesley, MA) and Moravek (Brea, CA), respectively. All unlabeled chemicals were obtained from Sigma (St. Louis, MO), except for 7-hydroxymethotrexate (7-OH-MTX), which was purchased from Schircks Laboratories (Jona, Switzerland). Human embryonic kidney cells (Flp-In™-HEK293) and the mammalian expression vector, pcDNA5/FRT were from Invitrogen (Carlsbad, CA).

Transport of folates and folate antagonists by OAT3

The original human *SLC22A8* (GenBank accession number NM_004254) was obtained by homology cloning and subcloned into the mammalian expression vector pcDNA5/FRT as previously described (19). HEK293 cells were propagated according to the manufacturer's instructions. They were grown at 37°C and 5% CO2 in Dulbecco's Modified Eagle's media (DME H-21; UCSF Cell Culture Facility, San Francisco, CA) containing 10% fetal bovine serum (UCSF Cell Culture Facility), 100 μg/mL streptomycin (UCSF Cell Culture Facility), 100 units/mL penicillin "G" (UCSF Cell Culture Facility), and 100 μg/mL Zeocin™ (Invitrogen). Cells were seeded at 3 x 10^{5} cells/ well on Biocoat® poly-d-lysine-coated 24-well plates (Becton Dickenson, Bedford, MA) 24 hours before transfection. Cells were transfected with 1 μg/well of pcDNA5/FRT (empty vector) or OAT3-pcDNA5/FRT (OAT3) and 3 μL of the lipid vehicle, Lipofectamine™ 2000 (Invitrogen). Incubation with the transfection mixture was carried out overnight, which was then replaced with fresh media. Uptake studies were performed 48 hours after transfection by pre-incubating transfected cells in 5 mM glutaric

acid in PBS (UCSF Cell Culture Facility) for 30 minutes at 37°C. This solution was then replaced with Na^+ buffer (128 mM NaCl, 4.73 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 5 mM Hepes or Tris, in H_2O, pH 7.4) and cells were incubated for an additional 5 minutes. The solution was then removed and cells were incubated for 3 minutes at 37°C in Na^+ buffer containing 20 nM ^{3}H-ES in the absence or presence of folate, leucovorin, or the folate antagonists, MTX and 7-OH-MTX at a final concentration of 1 or 2 mM. Uptake was terminated by washing wells three times with ice-cold Na^+ free buffer in which Na^+ was replaced by choline chloride. The cells were then lysed using 0.1 N NaOH/0.1% sodium dodecyl sulfate (SDS) and incubated for at least 2 hours on an agitating platform. Radioactivity in an aliquot of the resultant mixture was measured using a scintillation counter. Normalization for protein content was performed to correct for differences in cell number from well to well. A spectrophotometric plate reader and the BCA™ Protein Assay Kit (Pierce, Rockford IL) were used to assess protein concentrations. The inhibition data shown are representative of two independent experiments. P values were determined by comparing ES uptake in the presence of inhibitors to OAT3-mediated ES uptake in the absence of inhibitors using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

Saturation kinetics of ^{3}H-MTX

HEK293 cells transiently transfected with empty vector or OAT3 were pre-incubated and treated as previously described and were exposed for 3 minutes at 37°C in Na^+ buffer containing 20 nM ^{3}H-MTX in the presence of various concentrations of unlabeled MTX. The curve of MTX uptake by OAT3 was obtained by fitting the data to a

Michaelis-Menten curve by non-linear regression, using GraphPad Prism® 4 Software. A summary of the kinetic parameters of MTX transport by OAT3 is shown as mean ± SE from a representative experiment.

Genetic analysis of *SLC22A8*

Genetic variants in *SLC22A8* were previously identified by Erdman *et al*. (19). The neutral parameter (θ), nucleotide diversity (π), and Tajima's D statistic were determined using the methods described by Tajima (20). Each parameter was calculated for non-coding and coding sites within *SLC22A8* (e.g. synonymous and non-synonymous sites) for the entire population and for each ethnic group. The definition of synonymous and non-synonymous sites was adapted from Hartl and Clark (21). Haplotypes were inferred from variant sites using the Bayesian statistical method of PHASE (22). Before PHASE analysis, all singleton samples were removed and only haplotypes found in at least 7 of 10 PHASE runs were reported.

Functional analysis of variants in *SLC22A8* in HEK293 cells

The sequence of OAT3 reference corresponds to the OAT3 coding sequence that occurred at the highest frequency in all ethnic groups. Each of the 10 non-synonymous OAT3 variants was constructed by site-directed mutagenesis of the OAT3 reference using the QuickChange mutagenesis protocol (Stratagene, La Jolla, CA, USA), as previously described (19). HEK293 cells transiently transfected with OAT3 or each of the non-synonymous variants in OAT3 were pre-incubated and treated in the same manner as previously described. These cells were exposed for 3 minutes at 37°C in Na^+ buffer containing 20 nM ^{3}H-ES or 20 nM ^{3}H-MTX. The data are presented as percentage of ES or MTX uptake by OAT3 reference. Each value is expressed as mean ± SE for

three wells from an experiment representative of three independent experiments. P values were determined by comparing variant uptake to OAT3 reference for each substrate using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance. Kinetics studies of MTX interaction with OAT3 and the three common protein-altering variants, Val281Ala, Ile305Phe, and Val448Ile, were performed in transiently transfected HEK293 cells. Cells transiently transfected with empty vector, OAT3, or each of the three common OAT3 protein-altering variants were pre-incubated and treated as described above. The cells were exposed for 3 minutes at 37°C in Na^+ buffer containing 20 nM ^{3}H-MTX in the presence of various concentrations of unlabeled MTX. The kinetic curves were generated by fitting the data to a Michaelis-Menten curve fit, using GraphPad Prism® 4 Software. A summary of the kinetic parameters of MTX uptake by OAT3 reference and variants is given as mean ± SE from three independent experiments. P values were determined by comparing K_m and V_{max} values for each variant to OAT3 using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

7-OH-MTX inhibition of OAT3 and Val448Ile mediated ES and MTX transport

HEK293 cells transfected with empty vector, OAT3, or Val448Ile were pre-incubated and treated as described above and then incubated for 3 minutes with 20 nM ^{3}H-ES or 20 nM ^{3}H-MTX and varying concentrations (0, 0.2, 2, 5, 25, 50, 100, and 300 μM) of 7-OH-MTX. OAT3 and Val448Ile-specific uptake was determined by subtracting uptake by empty vector-transfected cells from that in OAT3 and Val448Ile-transfected cells after correcting for total protein. IC_{50} and K_i parameters were estimated by nonlinear regression, using GraphPad Prism® 4 Software, fit to the equation

Y = Bottom + (Top – Bottom)/1 + 10 ^ ((log IC_{50} – X)*Hill Slope)); where X is the log concentration and Y is the response. Uptake results shown in the graphs are from a representative experiment. Each point represents the mean ± S.E. of triplicate samples.

Construction for GFP-tagged proteins and microscopic studies

Plasmids containing GFP-fused proteins were constructed to determine their subcellular localization. The GFP coding sequence was ligated to the 5' end of OAT3 and selected variant cDNA in the expression vector pcDNA5/FRT. The resulting fusion constructs were used to generate transient HEK293 cell lines and analyzed by confocal microscopy as described previously (23).

RNA extraction, reverse transcription, and real-time PCR assay

The expression of the OAT3 and its variants was quantified by real-time reverse transcription-PCR, using TaqMan® Gene Expression Assays and an ABI 7500 Fast sequence detection system (Applied Biosystems, Foster City). Human GAPDH was used as an endogenous control to normalize expression.

Total RNA was extracted 48 h after transfection using the RNAqueous® system (Ambion Inc., Texas). One microgram of each RNA preparation was reverse-transcribed by random priming using High Capacity cDNA synthesis kit (Applied Biosystems, Foster City). Real-time PCR was performed using a 4.5 µL aliquot of the total cDNA sample using the TaqMan® Gene Expression Assays. Hs00188599_m1 was used for human OAT3. Human GAPDH endogenous control (Hs99999905_m1) was used as an internal standard for sample normalization. Relative levels of the human OAT3 mRNAs were calculated using the $\Delta\Delta C_T$ (comparative threshold cycle) method. Each test was performed in triplicate and all experiments were repeated three times. The levels of the

human OAT3 mRNAs were expressed relative to the HEK293 Flp-In cell lines transfected with pcDNA5FRT vector only, which was normalized to 1.

RESULTS

Interaction of folates and folate antagonists with OAT3

Folic acid and reduced folates are essential cofactors for the synthesis of nucleotides during DNA synthesis in dividing cells. This requirement has been successfully exploited in cancer therapy by the development of folic acid analogs, such as MTX, as chemotherapy agents. Since folates and folate antagonists undergo active tubular secretion in the kidney, their interaction with OAT3 was examined in transiently transfected HEK293 cells. As shown in Fig. 1, both MTX and 7-OH-MTX significantly inhibited ES transport by approximately 90%. On the other hand, folate and leucovorin, which is a reduced folate drug that rescues normal cells from the cytotoxic effects of MTX, only weakly inhibited ES uptake even at concentrations of 2 mM, despite being structurally similar to MTX. Further characterization of MTX transport revealed that its initial rate of uptake by OAT3 was saturable (Fig. 2), with a mean K_m of 75 ± 12 μM and V_{max} of 2090 ± 140 pmol/mg of protein/3 minutes.

Analysis of genetic variation in *SLC22A8*

Variants in *SLC22A8* were identified by direct automated sequencing of all 10 *SLC22A8* exons and 50-200 bp of flanking intronic sequences by Erdman *et al.* (19) as

Figure 1. Folates and folate antagonists are transported by OAT3

Inhibition of ES uptake by folates (leucovorin and folate) and folate antagonists (MTX and 7-OH-MTX) in HEK293 cells transiently transfected with empty vector (white bars) or OAT3 (black bars). Cells were incubated for 3 minutes with ^{3}H-ES (20 nM) in the absence or presence of various concentrations of unlabeled compounds. Uptake is expressed as percentage of ES uptake by OAT3. Each value represents the mean ± SE from three wells in a representative experiment of two independent experiments. P values were determined by comparing ES uptake in the presence of inhibitors to OAT3-mediated ES uptake in the absence of inhibitors using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

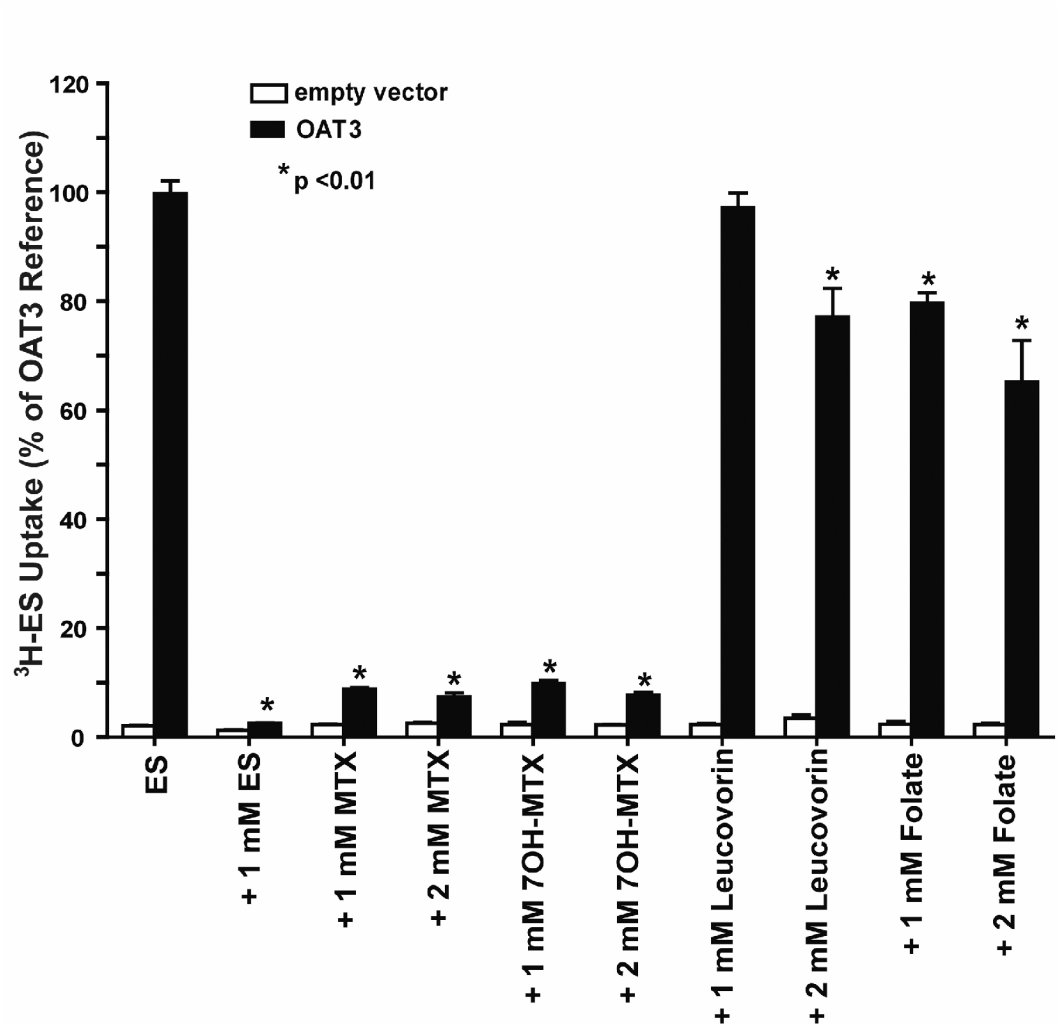
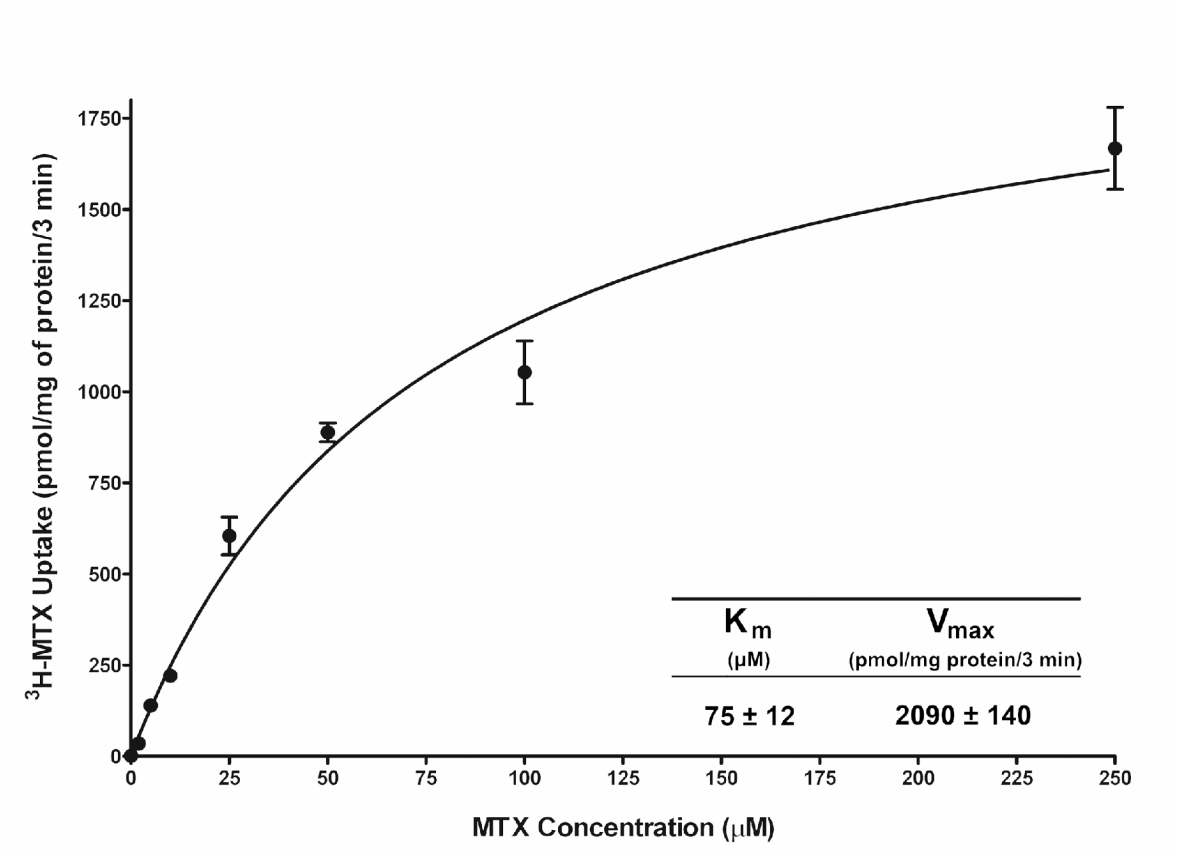


Figure 2. Transport kinetics of MTX in HEK293 cells expressing OAT3

Uptake of ^{3}H-MTX (20 nM) was measured in the presence of various concentrations of unlabeled ES in Na^{+} buffer over 3 minutes. Each value represents the mean ± SE from three wells in a representative experiment. Transport data were fitted using nonlinear regression analysis.



part of a large-scale project with the goal of examining the effects of natural variation in membrane transporter genes in drug efficacy and toxicity. The variant data from 270 ethnically diverse and healthy individuals are deposited in the Pharmacogenetics and Pharmacogenomics Knowledge Base at http://www.pharmgkb.org. A total of 26 single nucleotide polymorphisms (SNPs) were found in the 3706 bp sequenced, resulting in a frequency of approximately 7 SNPs per kb. This is in line with the average frequency of SNPs/kb obtained in the analysis of 24 membrane transporter genes (24). Eleven SNPs were found in the non-coding intronic regions of *SLC22A8*. Of the remaining 15 variable sites, 10 altered the protein sequence and five were synonymous (Table 1). Three of the 5 synonymous variants had an allele frequency of 4% or higher in an ethnic group. Of the 10 non-synonymous variants, three (Val281Ala, Ile305Phe, and Val448Ile) were polymorphic occurring at a frequency of $\geq 1\%$ in an ethnic group, and 6 (Phe129Leu, Gln239Stop, Ile260Arg, Arg277Trp, Ala310Val, and Ala399Ser) were singletons, meaning that they were found only once in 540 chromosomes. The protein-altering variant, Val281Ala was found only in African-Americans. The position of each protein-altering variant in the putative membrane topology of OAT3 is shown in Fig. 3. The majority of nonsynonymous variants were found in intracellular loops (six of ten), while synonymous SNPs were equally distributed between transmembrane domains (TMDs) and intracellular or extracellular loops.

Table 2 shows a summary of the estimated neutral parameter (θ), nucleotide diversity (π), and Tajima's D statistic for the total population and for each of the four

Table 1. Summary of variants in the coding region of OAT3 identified in ethnically diverse populations

Exon	SNP #	CDS Position[1]	Nucleotide Change	Amino Acid Position[1]	Amino Acid Change	Grantham[2]	EC/EU[3]	Allele Frequency (%)[4]				
								Total (n=540)	AA (n=160)	EA (n=160)	AS (n=120)	ME (n=100)
1	1.2	51	G→A	17	syn	-	EC	0.2	0	0	0.8	0
	1.3	153	G→A	51	syn	-	EU	15.7	40.0	5.1	8.5	3.1
2	*2.1*	*387*	*C→A*	*129*	*Phe→Leu*	*22*	*EC*	*0.2*	*0*	*0*	*0*	*1.0*
3	*3.1*	*445*	*C→A*	*149*	*Arg→Ser*	*110*	*EC*	*0.4*	*0*	*0.6*	*0.8*	*0*
4	4.1	714	G→T	238	syn	-	EC	0.2	0	0	0.8	0
	4.2	*715*	*C→T*	*239*	*Gln→Stop*	-	*EC*	*0.2*	*0*	*0*	*0.8*	*0*
	4.3	723	T→A	241	syn	-	EU	19.9	9.0	24.4	18.3	29.0
5	*5.1*	*779*	*T→G*	*260*	*Ile→Arg*	*97*	*EC*	*0.2*	*0*	*0*	*0.8*	*0*
	5.2	*829*	*C→T*	*277*	*Arg→Trp*	*101*	*EU*	*0.2*	*0.7*	*0*	*0*	*0*
	5.3	**842**	**T→C**	**281**	**Val→Ala**	**64**	**EU**	**1.7**	**6.0**	**0**	**0**	**0**
6	***6.1***	***913***	***A→T***	***305***	***Ile→Phe***	***21***	***EC***	***0.9***	***0***	***0***	***3.5***	***1.1***
	6.2	929	C→T	310	Ala→Val	64	EU	0.2	0	0.6	0	0
7	7.1	1195	G→T	399	Ala→Ser	99	EU	0.2	0	0	0	1.0
9	***9.3***	***1342***	***G→A***	***448***	***Val→Ile***	***29***	***EU***	***0.7***	***0.6***	***1.3***	***0***	***1.0***
10	10.5	1590	C→A	530	syn	-	EC	1.3	4.4	0	0	0

Data are available at http://www.pharmgkb.org. The three common non-synonymous variants (≥1% in any ethnic group) are shown in bold. The variants that show reduced transport of ES and/or MTX are indicated in italics.

[1]Positions are relative to the ATG start site and are based on the cDNA sequence from GenBank accession number NM_004254.

[2]Grantham values range from 5 to 215, where low values indicate chemical similarity and high values indicate radical differences.

[3]EC/EU classifies coding variants as being evolutionarily conserved (EC) or evolutionarily unconserved (EU) based on sequence alignments with 7 mammalian orthologs.

[4]Some samples contained amplicons that could not be sequenced. Allele frequencies were calculated from actual DNA samples sequenced. Total, entire sample; AA, African-American; EA, European-American; AS, Asian-American; ME, Mexican-American; n, number of chromosomes.

Figure 3. Secondary structure prediction of OAT3 showing the location of the 10 protein-altering variants

The transmembrane topology diagram was obtained using TOPO [S. J. Johns (UCSF, San Francisco) and R. C. Speth (Washington State University, Pullman), transmembrane protein display software available at the UCSF Sequence Analysis Consulting Group website, http://www.sacs.ucsf.edu/TOPO/topo.html. Non-synonymous variants are labeled with red circles, the deletion variant (Gln239Stop) is identified by the blue circle, and putative N-glycosylation sites are shown by the black circles. The three most common non-synonymous variants of OAT3, which were kinetically characterized, are shown in bold and boxed in green and the highest allele frequency in a given ethnic group is shown in parenthesis (AA, African-American; EA, European-American; AS, Asian-American; ME, Mexican-American).

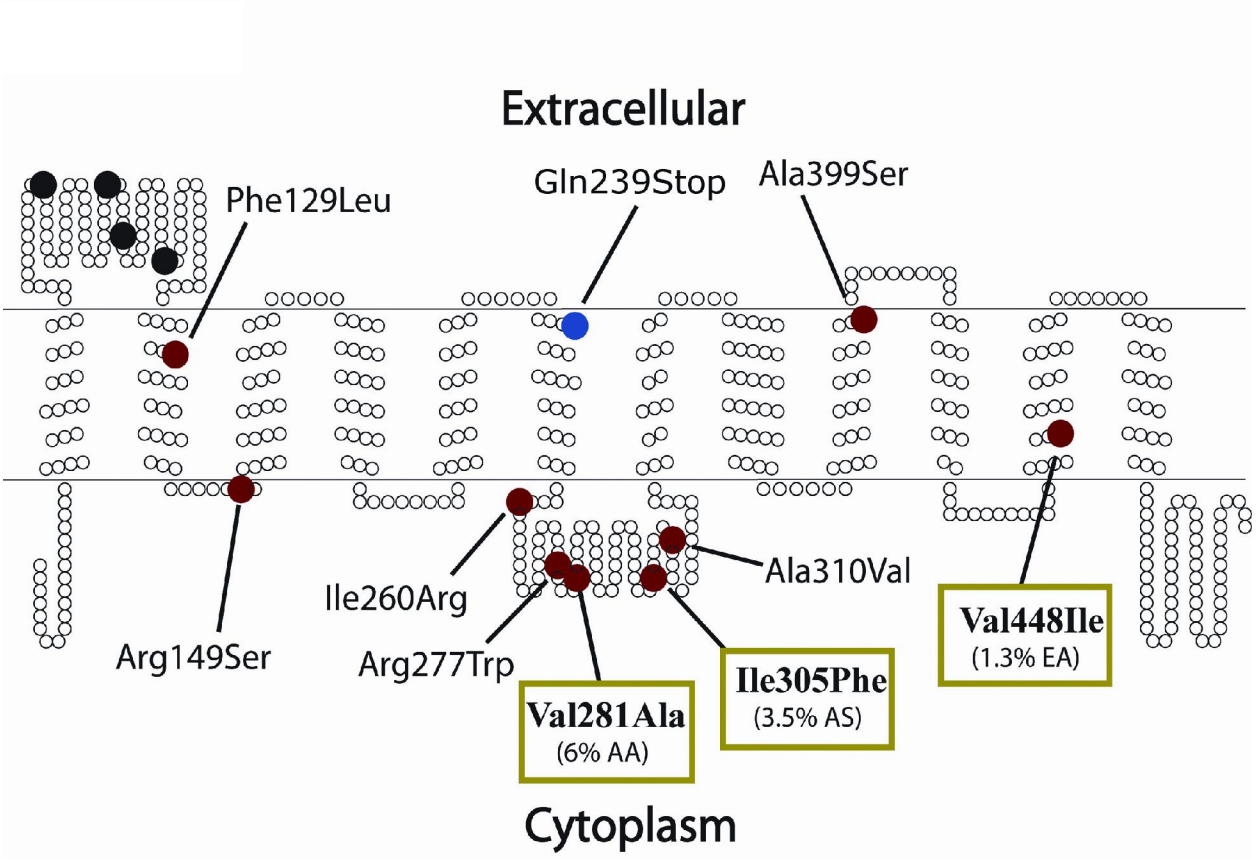


Table 2. Estimates of the neutral parameter (θ), nucleotide diversity (π), and Tajima's D for *SLC22A8*[1]

Population	SNP type	Base pairs screened	n[2]	No. variable sites	θ (x 10^4)	π (x 10^4)	D
Total[3]	All	3706	540	26	10.1 ± 2.8	5.2 ± 3.3	-1.53
	Non-coding	2077		11	7.7 ± 2.7	5.9 ± 4.1	-0.44
	Coding	1629		15	13.4 ± 4.3	4.3 ± 3.6	-1.53
	Synonymous	395		5	18.4 ± 8.9	15.2 ± 13.4	-0.19
	Non-synonymous	1234		10	11.8 ± 4.3	0.74 ± 1.5	-1.63
AA	All	3706	160	12	5.7 ± 2.1	5.4 ± 3.4	-0.15
	Non-coding	2077		6	5.1 ± 2.4	5.5 ± 4.0	0.11
	Coding	1629		6	6.5 ± 3.0	5.2 ± 4.1	-0.3
	Synonymous	395		3	13.4 ± 8.3	18.3 ± 15.2	0.34
	Non-synonymous	1234		3	4.3 ± 2.7	1.0 ± 1.7	-0.72
EA	All	3706	160	11	5.3 ± 2.0	3.9 ± 2.6	-0.58
	Non-coding	2077		6	5.1 ± 2.4	4.4 ± 3.4	-0.21
	Coding	1629		5	5.4 ± 2.7	3.2 ± 3.0	-0.56
	Synonymous	395		2	9.0 ± 6.7	11.7 ± 11.5	0.22
	Non-synonymous	1234		3	4.3 ± 2.7	0.4 ± 1.1	-0.85
AS	All	3706	120	14	7.1 ± 2.5	5.9 ± 3.7	-0.43
	Non-coding	2077		6	5.4 ± 2.6	7.7 ± 5.1	0.66
	Coding	1629		8	9.2 ± 3.9	3.7 ± 3.3	-1.13
	Synonymous	395		4	18.9 ± 10.5	12.3 ± 11.8	-0.42
	Non-synonymous	1234		4	6.1 ± 3.4	0.9 ± 1.6	-1.02
ME	All	3706	100	13	6.8 ± 2.5	4.5 ± 3.0	-0.87
	Non-coding	2077		7	6.5 ± 2.9	5.4 ± 3.9	-0.30
	Coding	1629		6	7.1 ± 3.4	3.4 ± 3.1	-0.85
	Synonymous	395		2	9.8 ± 7.3	12.0 ± 11.6	0.17
	Non-synonymous	1234		4	6.3 ± 3.5	0.63 ± 1.4	-1.11

[1]θ, π, and Tajima's D were calculated as described in Methods
[2] n, number of chromosomes
[3]Total, entire sample; AA, African-American; EA, European-American; AS, Asian-American; ME, Mexican-American. Data are listed as mean x 10^4 ± SD.

ethnic groups. Overall the genetic variation in *SLC22A8* was similar to the average previously reported for 24 transporter genes, since π (x 10^4) for *SLC22A8* is 5.2 ± 3.3 compared to the average of 5.1 ± 2.4 (24). However, the ratio of π for non-synonymous sites to π for synonymous sites (π_{NS}/π_S) was considerably lower than the one previously reported, 0.05 for *SLC22A8* versus 0.23 for the average of 24 transporter genes (24), suggesting that OAT3 is under significant purifying selection acting against amino-acid changes. In addition, since most values of θ were greater than π, Tajima's D statistics were mostly negative, again consistent with negative selection acting on OAT3. Nucleotide diversity at non-synonymous sites in loops ($\pi_{NS\text{-}loop\text{-}OAT3}$ = 0.97 ± 2.2 versus 3.6 ± 1.9) and TMDs ($\pi_{NS\text{-}TMD\text{-}OAT3}$ = 0.45 ± 1.7 versus 1.6 ± 1.1) of OAT3 was considerably lower than the average for 24 transporter genes (24) and OAT3 π_{NS} for TMDs was much lower than OAT3 π_{NS} for loops, indicating that there are evolutionary constraints on both regions of the protein, but especially in TMDs.

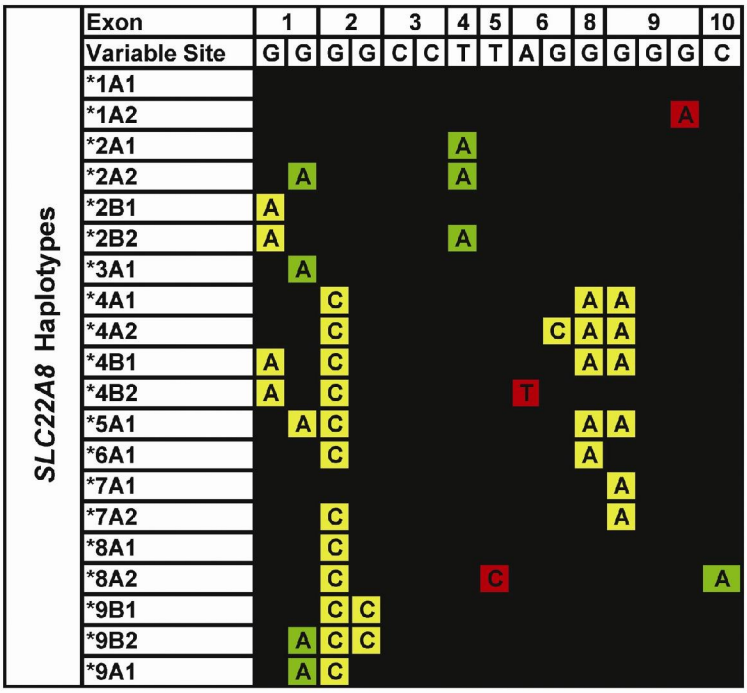
Haplotype analysis of *SLC22A8*

A total of 30 haplotypes in *SLC22A8* were identified from 15 variable sites using the Bayesian statistical method, PHASE (22). Twenty of the 30 haplotypes occurred on at least two chromosomes and, since their combined frequency was 98%, further analyses were conducted using this smaller haplotype set. Naming of the haplotypes was done based on the modified convention of Nebert *et al.* according to two main criteria: evolutionary divergence and frequency in the sample (25).

As shown in Fig. 4, haplotype *1A1 occurred with the highest frequency in all 4 ethnic groups. Haplotype *2A1 was the second most common haplotype, except in African-Americans where *3A1 was instead more common after *1A1. Among the 20

Figure 4. Structure and population frequency of 20 haplotypes of *SLC22A8*

Left panel, sequences of the haplotypes at each of the 15 variable sites. The first row shows the exons of *SLC22A8*; the second row contains the sequence of the reference allele at each variable site. Each of the subsequent rows shows the 20 haplotypes clustered and named based on their evolutionary divergence and population frequencies using a modified convention of Nebert (25). A black filled square is used to indicate that there is no change from the reference allele; a green filled square shows a synonymous change in the coding region; a red filled square indicates a non-synonymous change; changes occurring at non-coding sites are denoted by yellow filled squares. Letters in the squares indicate the base pair change and a "-" means a deletion occurred. Haplotypes were predicted using PHASE software version 1.0.1 as previously described (22). Right panel, frequencies of the 20 haplotype in the total population and in each ethnic group.



Frequency of *SLC22A8* Haplotypes (%)				
Total	EA	AA	AS	ME
34.0	47.5	25.9	25	37.5
0.4	1.3	0.6	0	1
18.4	23.4	7	17.5	27.1
0.7	0	1.9	0.8	0
8.8	4.4	5.1	15.8	14.6
0.4	0	0	0	2.1
10.1	5.1	24.1	5.8	2.1
10.5	15.2	1.3	15	10.4
0.4	0	0	0	2.1
0.9	0	0	4.2	0
0.6	0	0	2.5	0
2.2	0	6.3	1.7	0
0.4	1.3	0	0	0
0.6	0.6	0	1.7	0
0.4	0	0	0.8	1.0
2.2	0	2.5	5.8	0
1.3	0	4.4	0	0
3.7	0	12.7	0	0
1.1	0	3.2	0	1.0
0.9	0	3.2	0	0

haplotypes, 5 (*1A1, *2A1, *2B1, *3A1, and *4A1) had total frequencies of ≥ 5% and occurred in all ethnic groups. On the other hand, 8 out of 20 haplotypes (40%) were found in only one ethnic group: *6A1 in European-Americans; *8A2, *9A1, and *9B1 in African-Americans; *4B1 and *4B2 in Asian-Americans; and *2B2 and *4A2 in Mexican-Americans. African-Americans (13 out of 20) and Asian-Americans (13 out of 20) had the greatest number of haplotypes, while African-Americans had the largest number of ethnic specific haplotypes (3 out of 8). The common protein-altering variants Val281Ala (*8A2) and Ile305Phe (*4B2) were each found in an ethnic specific haplotype, while Val448Ile was found in haplotype *1A2.

The cladogram for OAT3 examining the relationship among the 20 haplotypes is shown in Fig. 5. Several variants (i.e. SNP # 1.1, 1.3, 2.2, 2.3, 4.3, and 9.1) appear more than once, suggesting that either substitutions have arisen multiple times in evolutionary history or that recombination events have occurred.

Functional characterization of MTX transport by protein-altering variants in OAT3

We characterized the interaction of the 10 naturally occurring protein-altering variants in OAT3 with MTX to determine their role in inter-individual variation in MTX disposition and toxicity. As shown in Fig. 6, six out of 10 protein-altering variants in OAT3 exhibited altered uptake of MTX and/or the model organic anion compound, ES. Specifically, the rare variants Arg149Ser, Gln239Stop, and Ile260Arg were non-functional. Gln239Stop occurs in the beginning of TMD 6 and results in the truncation of the last 7 TMDs.

Figure 5. Cladogram of the 20 common haplotypes of *SLC22A8*

The circles for each haplotype were drawn proportional to the overall frequency at which the haplotypes were found. The circles are subdivided in four differently colored slices according to the proportion of chromosomes carrying that particular haplotype in each ethnic group: European-Americans are identified in yellow, African-Americans in red, Asian-Americans in black, and Mexican-Americans in green. The *1A1 haplotype is the most common haplotype overall and in each ethnic group.

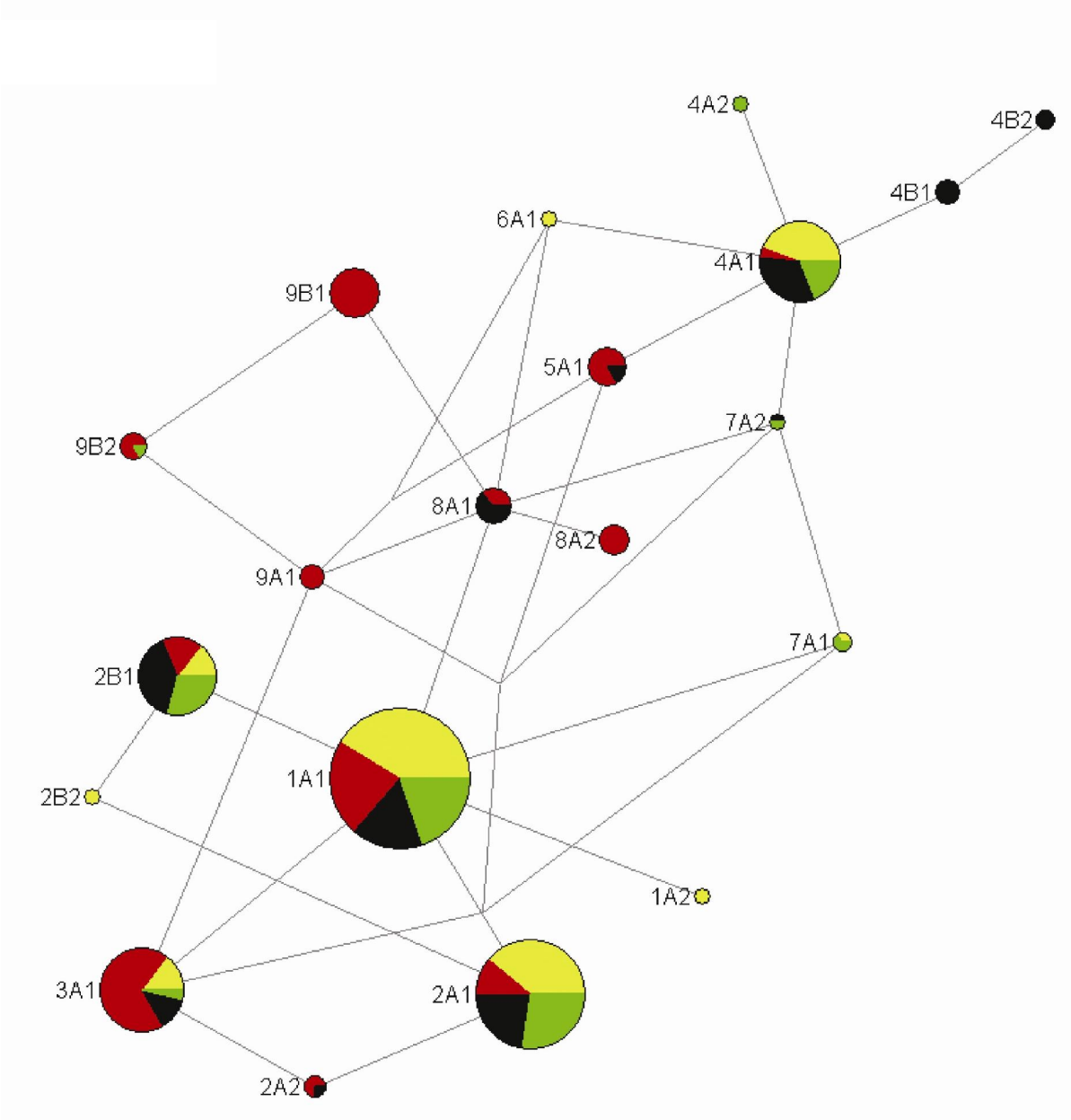
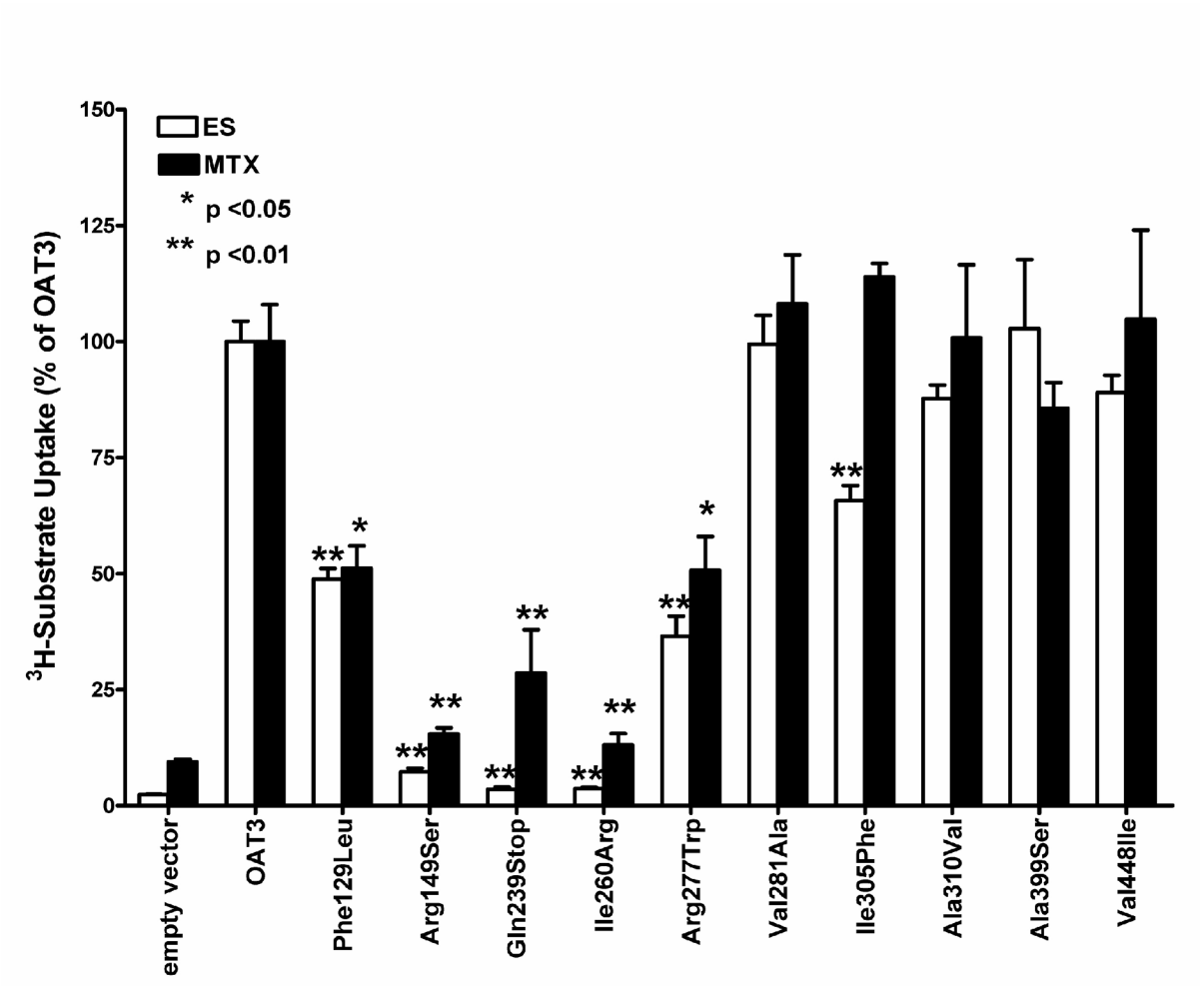


Figure 6. Uptake of ES and MTX in HEK293 cells expressing OAT3 and its 10 protein-altering variants

Uptake of 20 nM ^{3}H-ES (white bars) or 20 nM ^{3}H-MTX (black bars) in cells transiently transfected with empty vector, OAT3, or the 10 protein-altering variants. Transport was measured in Na^{+} buffer at pH 7.4 over 3 minutes. Uptake is expressed as percentage of ES and MTX uptake, respectively, by OAT3 expressing cells. Each value is expressed as mean ± SE for three wells from a representative experiment out of three independent experiments. P values were determined by comparing variant uptake to OAT3 reference for each substrate using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.



On the other hand, the singleton variants Phe129Leu (51.2 ± 4.8 %) and Arg277Trp (50.7 ± 7.3 %) showed reduced MTX transport in comparison to OAT3 reference (100 ± 8.0 %). In addition, Ile305Phe exhibited substrate specificity changes as previously reported (19), whereby it transported MTX similarly to OAT3 reference, but showed significantly reduced uptake of ES ($p < 0.01$). It is interesting to note that, except for Arg277Trp, all of the protein-altering variants that exhibited altered MTX transport occurred at evolutionarily conserved (EC) sites. On the other hand, the four protein-altering variants that showed MTX uptakes similar to OAT3 reference were instead found at evolutionarily unconserved (EU) sites. Amino acid changes were classified as EC or EU based on multiple sequence alignments with 7 mammalian orthologs (chimp, pongo, monkey, pig, rabbit, mouse, and rat).

Following these initial screens for function, the interaction kinetics of MTX with the three most common protein-altering variants in OAT3, Val281Ala, Ile305Phe, and Val448Ile, was examined in detail in HEK293 cells (Fig. 7). As shown in Table 3, cells expressing Val448Ile exhibited an approximately 2-fold increase ($p < 0.05$) in the K_m (147 ± 19 μM) for MTX, while the V_{max} (1980 ± 190 pmol/mg of protein/3 minutes) was not significantly different compared to cells expressing OAT3 (K_m = 84 ± 14 μM; V_{max} = 1510 ± 141 pmol/mg of protein/3 minutes). There were no statistically significant differences in the interaction affinity and maximal transport rate (V_{max}) of MTX between OAT3 and Val281Ala or Ile305Phe (Table 3).

Figure 7. Kinetics of interaction of MTX with OAT3 and its three common protein-altering variants

Interaction kinetics of ^{3}H-MTX in HEK293 cells transiently transfected with OAT3 reference (black circle) and the common protein-altering variants, Val281Ala (light gray triangle), Ile305Phe (gray square), and Val448Ile (black diamond). Uptake of ^{3}H-MTX (20 nM) was measured in the presence of various concentrations of unlabeled MTX in Na^{+} buffer at pH 7.4 over 3 minutes. Data are representative of three independent experiments and each value represents the mean ± SE from three wells. Transport data were fitted using nonlinear regression analysis. P values were determined by comparing kinetic parameters for each variant to that of OAT3 reference using one-way ANOVA and Tukey's multi-comparison test with $p < 0.05$ as the criterion of significance.

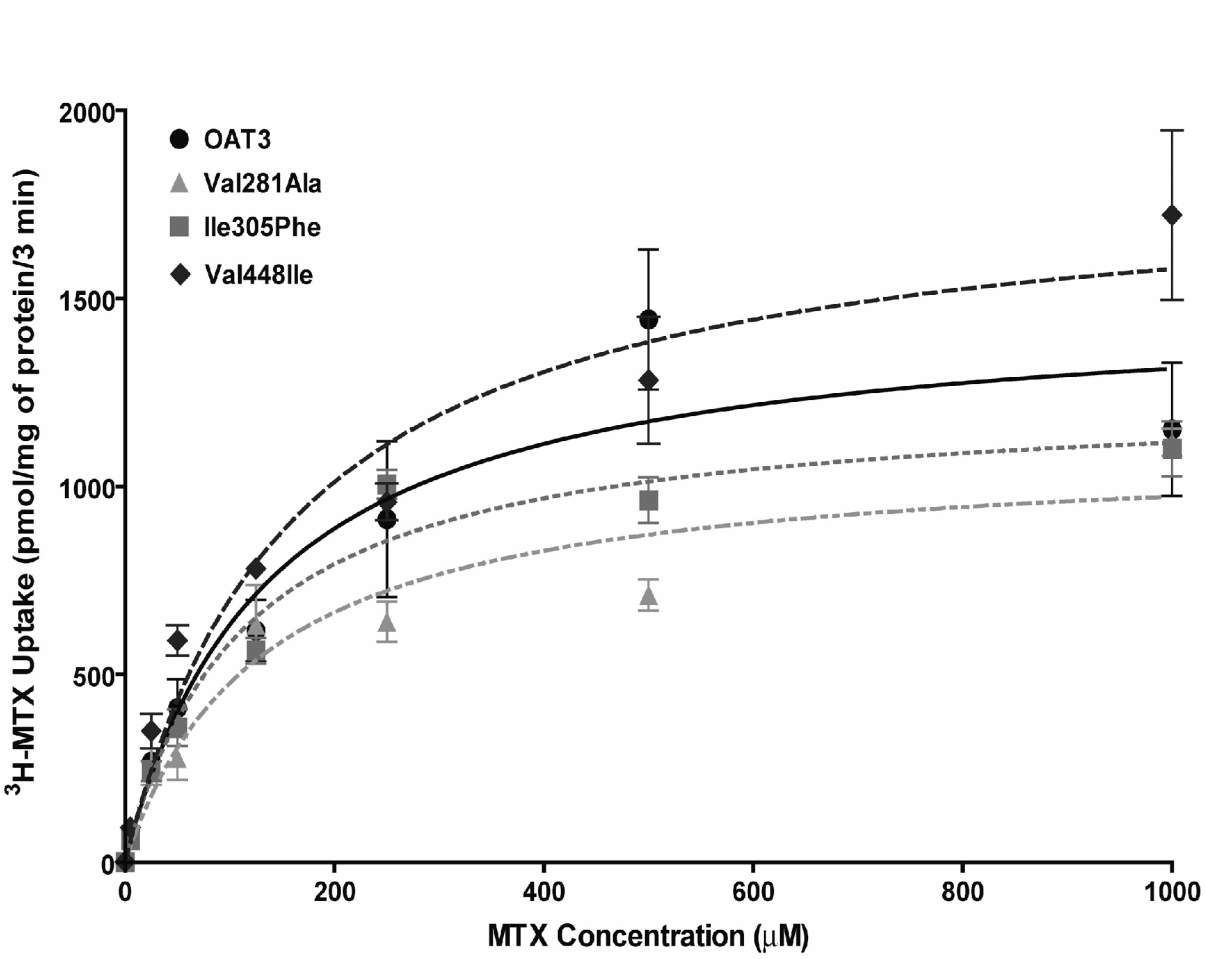


Table 3. Summary of transport kinetics of MTX with OAT3 and the three common protein-altering variants, Val281Ala, Ile305Phe and Val448Ile

	OAT3	Val281Ala	Ile305Phe	Val448Ile
K_m (μM)	84 ± 14	113 ± 12	102 ± 12	**147 ± 19***
V_{max} (pmol/mg protein/3 min)	1510 ± 141	1920 ± 415	1470 ± 262	1980 ± 190
V_{max}/K_m	17.9 ± 3.6	18.1 ± 5.4	14.5 ± 1.9	13.8 ± 1.4

Uptake of ^{3}H-MTX (20 nM) was measured in the presence of various concentrations of unlabeled MTX in Na^+ buffer at pH 7.4 over 3 minutes. Each value represents the mean ± SE from three independent experiments. Transport data was fitted using nonlinear regression analysis. Significant differences in the kinetics parameters of the variant proteins compared to OAT3 reference are shown in bold. P values were determined by comparing K_m, V_{max}, and V_{max} / K_m values for each variant to OAT3 using one-way ANOVA and Dunnett's multi-comparison test with $^*p < 0.05$ as the criterion of significance.

Characterization of 7-OH-MTX inhibition of ES and MTX in OAT3 and Val448Ile

Due to the significant 2-fold increase in the K_m for MTX via Val448Ile compared to OAT3 wild-type, we examined to impact of 7-OH-MTX on the transport of ES or MTX in HEK293 cells transiently transfected with OAT3 and Val448Ile. OAT3-mediated transport of ES and MTX was inhibited by 7-OH-MTX with a K_i values of approximately 20 μM. The K_i values of 7-OH-MTX for Val448Ile-mediated transport of ES and MTX were also approximately 20 μM for ES and MTX (Fig. 8 and Fig. 9). These results further establish that 7-OH-MTX is a potent inhibitor of ES and MTX via OAT3 and its variant Val448Ile.

Additionally, cellular localization via fluorescence and confocal microscopy of GFP (control), OAT3-GFP, Gln239Stop-GFP, Val281Ala-GFP, Ile305Phe-GFP, and Val448Ile-GFP was performed. The results revealed that OAT3-GFP, Val281Ala-GFP, Ile305Phe-GFP, and Val448Ile-GFP were localized to the plasma membrane and the cytosol. In contrast, Gln239Stop-GFP remained exclusively in the intracellular compartment without localization to the plasma membrane (Fig. 10). The lack of surface expression of Gln239Stop-GFP likely results in the observed loss of its transport function. Quantitative RT-PCR assays specific for OAT3 showed that each OAT3-expressing HEK293 expressing cell line contained approximately equal levels of OAT3 mRNA (data not shown).

Figure 8. Inhibition of ES in OAT3 and Val448Ile with 7-OH-MTX

7-OH-MTX inhibition of ^{3}H-ES in HEK293 cells transiently transfected with OAT3 reference (black circle) and the common protein-altering variant Val4448Ile (black diamond). Uptake of ^{3}H-ES (20 nM) was measured in the presence of increasing concentrations of unlabeled 7-OH-MTX in Na^{+} buffer at pH 7.4 at 3 minutes. Data are representative of three independent experiments and each value represents the mean $\pm$ SE from three wells. IC_{50} and K_i values were estimated using nonlinear regression.

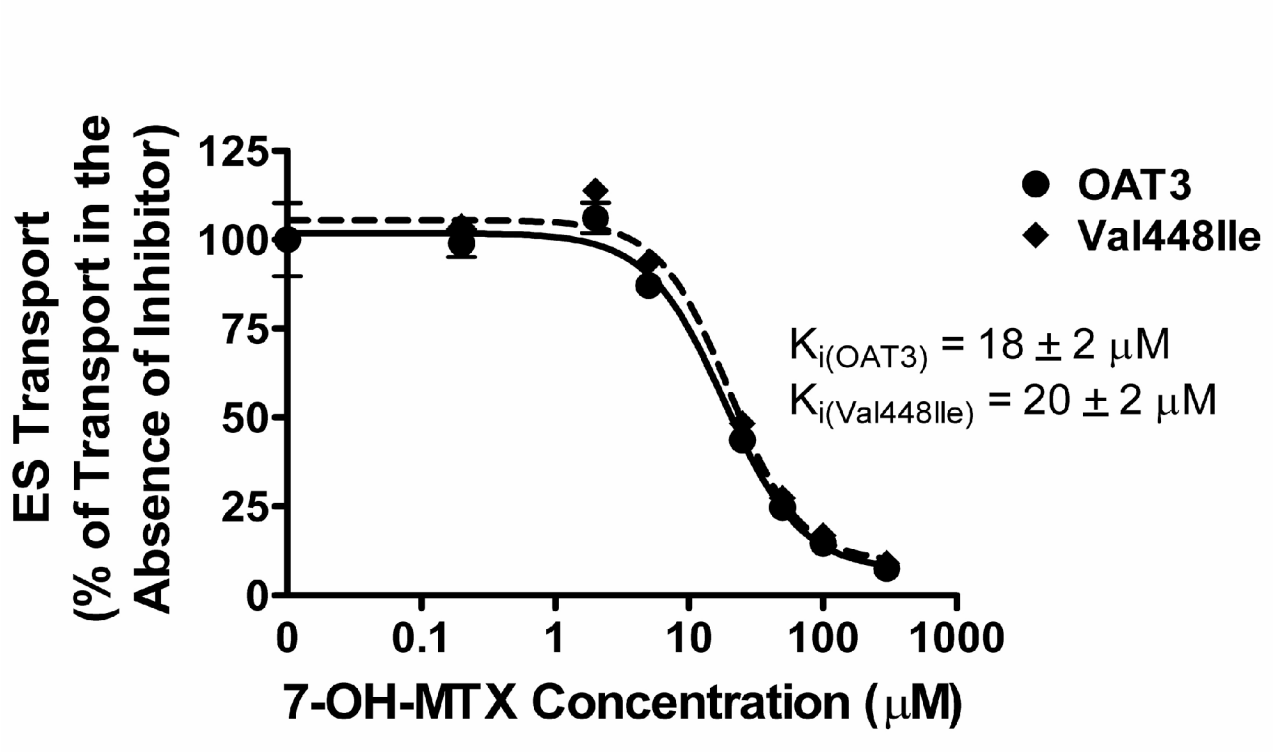


Figure 9. Inhibition of MTX in OAT3 and Val448Ile with 7-OH-MTX

7-OH-MTX inhibition of ^{3}H-MTX in HEK293 cells transiently transfected with OAT3 reference (black circle) and the common protein-altering variant Val4448Ile (black diamond). Uptake of ^{3}H-MTX (20 nM) was measured in the presence of increasing concentrations of unlabeled 7-OH-MTX in Na^{+} buffer at pH 7.4 at 3 minutes. Data are representative of three independent experiments and each value represents the mean $\pm$ SE from three wells. IC_{50} and K_i values were estimated using nonlinear regression.

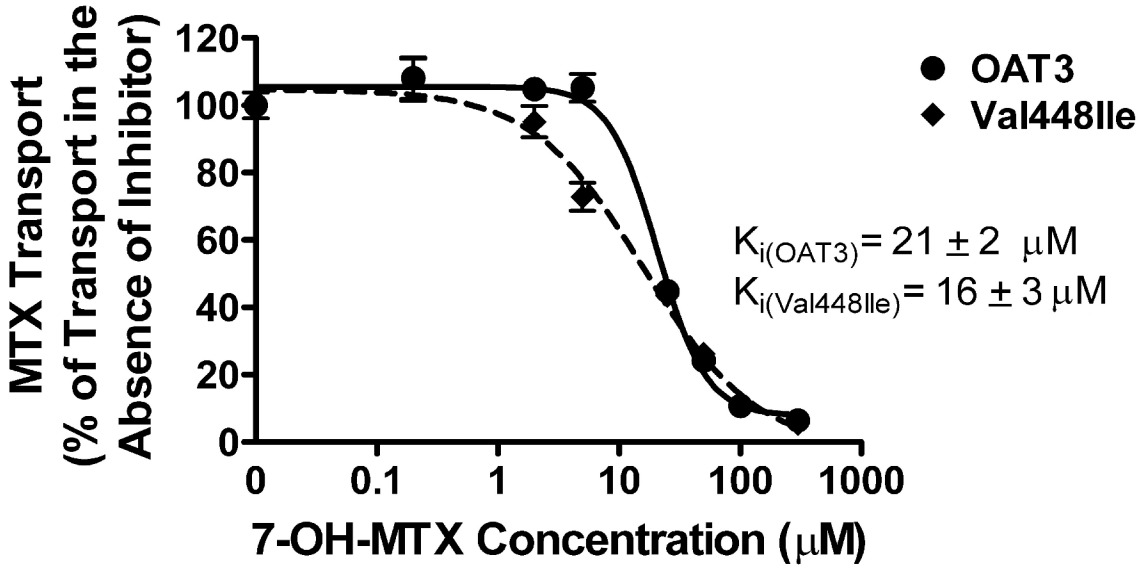
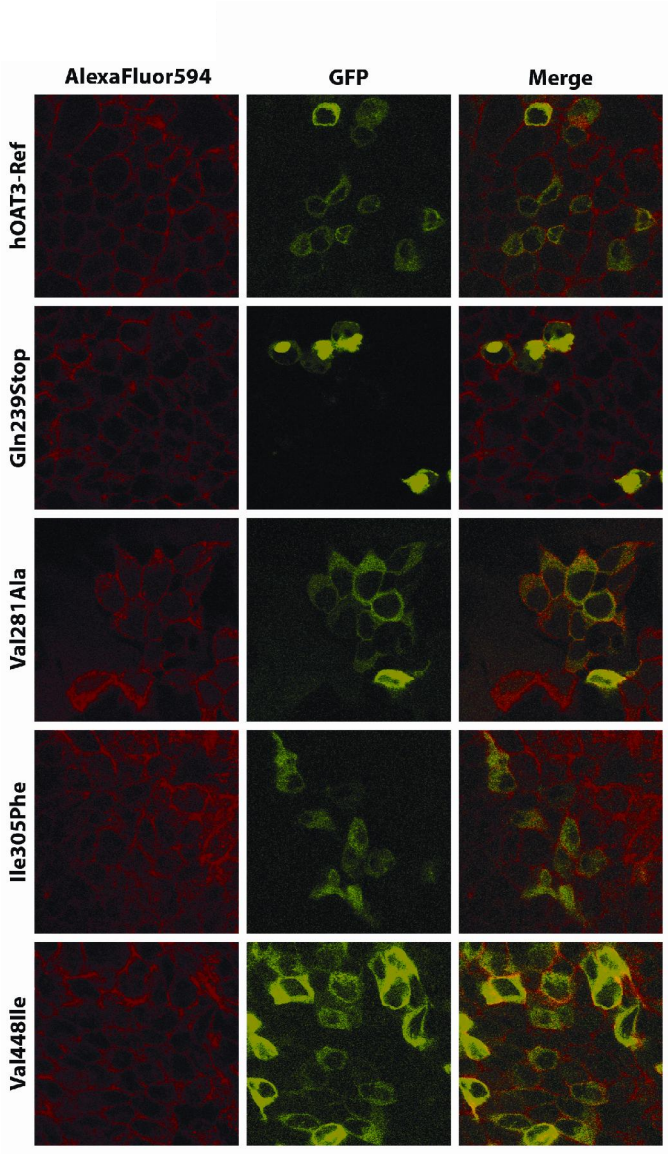


Figure 10. Intracellular localization of HEK293 transiently transfected cells expressing OAT3 and its variants

Cells transiently expressing OAT3-GFP-Reference **(A)**, Gln239Stop-GFP **(B)**, Val281Ala-GFP **(C)**, Ile305Phe-GFP **(D)**, and Val448Ile-GFP **(E)** were visualized by confocal microscopy. Plasma membrane was stained using AlexaFluor594 wheat germ agglutinin. Cells transfected with OAT3, Val281Ala, Ile305Phe, and Val448Ile localized specifically to the plasma membrane with partial distribution in the cytosol, whereas Gln239Stop showed improper trafficking resulting in lost sorting to the plasma membrane.



DISCUSSION

MTX therapy has been extensively employed to treat a variety of malignancies and inflammatory diseases (1). However, there is considerable inter-individual variability in response, pharmacokinetics parameters, as well as development of severe organ toxicity during MTX use. Specifically, it was found that co-administration of an organic anion, probenecid, with MTX resulted in the doubling of plasma MTX levels, an increase in the MTX serum half-life, and delayed elimination, which has been associated with severe systemic toxicity (3, 26). In addition co-administration of NSAIDs with MTX leads to increased MTX blood levels, decreased MTX renal clearance, and acute renal failure, primarily via inhibition of MTX secretion by NSAIDs in the proximal tubule of the kidney (2, 26-29). Since NSAIDs have been shown to be substrates of OATs, all these factors strongly suggest that OATs likely have important roles in MTX disposition and toxicity. In particular, among the OATs, human OAT3 is the most abundantly expressed organic anion transporter on the basolateral side of renal proximal tubule cells (10, 13) and, therefore, is likely to be the predominant OAT responsible for MTX secretion in the kidney lumen.

Given the evidence that Oat3 knockout mice exhibited significantly decreased MTX-to-inulin clearance rates compared to wildtype OAT3 mice (18), it is reasonable to speculate that reduced function variants of human OAT3 may lead to variation in the pharmacokinetic and pharmacodynamic properties of MTX in patient populations. Therefore, in the present study we present detailed characterization of MTX interaction with OAT3 and its naturally occurring protein-altering variants previously identified by Erdman *et al.* (19). Our data show that the bicarboxylic organic anion, MTX, interacts

with OAT3 with a K_m of 75 ± 12 μM and a V_{max} of 2090 ± 140 pmol/mg of protein/3 minutes (Fig. 1 and Fig. 2). These data are consistent with previous reports using *Xenopus laevis* oocytes or mouse proximal tubule cells stably expressing human OATs, where human OAT3 and OAT4 were shown to interact with MTX with high affinity (K_m ~ 20 μM), whereas MTX was a low affinity substrate of human OAT1 (K_m = 550-750 μM) (40-41). In addition, we demonstrate for the first time that the metabolite of MTX, 7-OH-MTX, which is also predominantly eliminated by the kidney, significantly inhibits (by more than 90%) transport of the model organic anion compound, ES, by OAT3. On the other hand, despite being structurally similar to MTX, folate and leucovorin, the latter a reduced folate drug used clinically to rescue normal cells from toxic effects of MTX, only weakly interacted with OAT3. Since OAT3 is highly expressed on the basolateral surface of proximal tubule cells and interacts with MTX with 10 fold higher affinity than OAT1, our studies suggest that OAT3 plays an important and predominant role in the saturable secretion of MTX and its metabolite in the kidney proximal tubule.

We analyzed genetic variation in *SLC22A8* in an ethnically diverse population of 270 healthy individuals. Of the coding region variants, 10 altered the amino acid sequence of OAT3, while 5 were synonymous. Two of the synonymous SNPs (c. 51 G>A and c. 723 T>A) have been observed in a recent report by Xu *et al*. (30), which also identified a Japanese-specific protein-altering variant, Ala523Gly, in OAT3. This variant occurred in only one Japanese individual and was not observed in our previous re-sequencing effort most likely because the Asian-American population included in our analysis was primarily a sample of American-Chinese individuals (19, 30). Compared to the study by Xu *et al.* (30) which analyzed genetic variation of OAT3 in 12 ethnic groups

with 10 or fewer individuals, we screened a much larger sample set (between 50 and 80 individuals) in a smaller number of ethnic populations and discovered a considerably larger number of variants, especially rare variants. Nucleotide diversity at non-synonymous sites ($\pi_{NS\text{-}total}$ (x 10^4) = 0.74 ± 1.5) was much lower than in a recently reported analysis of genetic variation ($\pi_{NS\text{-}total}$ (x 10^4) = 2.2 ± 1.1) in 24 membrane transporter genes (24). In addition, the ratio of π_{NS}/π_S, which is a measure of selection for function of a particular gene, was found to be 0.05, which again was significantly lower than the previously reported average of 0.23 for a set of membrane transporters (24). Altogether, these data indicate that OAT3 is under strong negative selective pressure, which is acting to preserve its amino acid sequence, and is important for human fitness.

Consistent with a previous report by Stephens *et al.* (31), where an average of 14 haplotypes was observed in the analysis of 313 genes, a total of 30 haplotypes were identified in *SLC22A8* (Fig. 4 and Fig. 5). The five most common haplotypes (*1A1, *2A1, *2B1, *3A1, and *4A1), which were observed across all 4 ethnic populations studied, accounted for 82% of all haplotypes. A considerable number of haplotypes (8 out of 20) was found to be population-specific in African-Americans and Asian-Americans had the largest number of haplotypes (12 out of 20). Most haplotypes had only synonymous or intronic changes and only 3 of 20 haplotypes (*1A2, *4B2, and *8A2) contained one amino acid altering variant. None of the 20 haplotypes had two or more amino acid changes.

Despite showing an overall relatively low degree of genetic variation, OAT3 genetic variants exhibited significant functional variation with respect to MTX transport. Specifically, we identified three protein-altering variants (Arg149Ser, Gln239Stop, and

Ile260Arg) in OAT3 that were non-functional, two protein-altering variants (Phe129Leu and Arg277Trp) that showed reduced MTX uptake, and one specificity variant (Ile305Phe) that transported MTX similar to OAT3 reference but showed reduced transport of ES (Fig. 6). Gln239Stop results in the truncation of OAT3 after the first five TMDs (Fig. 3). Its loss of transport function of organic anions, such as MTX, is consistent with previously published findings by Feng *et al.*, which showed that residues important for substrate recognition are located in the carboxyl-terminal part of OAT3 (32). The complete loss of MTX transport observed with Ile260Arg could be related to the fact that this mutation removes a protein kinase C (PKC) phosphorylation site with the general consensus sequence of S/T-(X)-R/K. In fact, all OATs, including OAT3, have several phosphorylation sites by PKC in the large intracellular loop between TMD 6 and 7 (Fig. 3). It has been previously reported that activation of PKC leads to altered transport function and substrate selectivity of OATs (33-35).

Interestingly, we observed that of the 6 protein-altering variants, which exhibited altered transport of MTX and/or ES, five occurred at EC sites (Table 1). On the other hand, all four protein-altering variants in OAT3 with transport function similar to OAT3 reference occurred at EU sites. EC residues are those that were identical in all 7 members (chimp, pongo, monkey, pig, rabbit, mouse, and rat) of a set of OAT3 mammalian orthologs. This is consistent with previous reports on membrane transporter genes where evolutionary conservation was determined to be a strong predictor of both allele frequency and transport function, indicating that substitutions at EC sites are more deleterious than those at EU sites (24, 35-37).

Detailed kinetic characterization of MTX transport by the three common protein-altering variants of OAT3 (Val281Ala, Ile305Phe, and Val448Ile) indicate that Val448Ile, which is commonly found in European-Americans, interacts with MTX with a significantly lower affinity ($p < 0.05$) compared to OAT3 reference with no appreciable effect on the maximal transport rate (Table 3). Since Erdman *et al.* (19) previously showed that Val448Ile interacted with ES and cimetidine similarly to OAT3 reference, it can be surmised that this variant displays altered substrate specificity. Based on our kinetic findings, we can speculate that individuals carrying the Val448Ile variant may exhibit decreased secretion of MTX in the kidney and subsequently develop increased exposure and higher plasma concentrations of MTX, which could lead to MTX-induced toxicity. Altogether, the functional screen and kinetics data presented here are consistent with previous studies on functional variation in membrane transporters where it has been uniformly reported that rare variants are more likely to exhibit altered transport and interaction with substrates than common variants (19, 24, 38-43).

In addition, we have determined that 7-OH-MTX potently inhibits the transport of ES and MTX by OAT3 and Val448Ile with similar affinities (K_i ~20 μM; Fig. 8 and Fig. 9). This has particular relevance when considering the wide range of therapeutic concentrations of MTX resulting from lower doses in the treatment of rheumatoid arthritis to high doses in the treatment of cancers including acute lymphocytic leukemia and osteosarcoma. In anti-cancer regimens, MTX and 7-OH-MTX maximum plasma concentrations may be > 1000 μM (44, 45). Thus, it is feasible that physiologic concentrations of 7-OH-MTX may exceed 20 μM, inhibiting OAT3 transport of MTX. This would result in an increased risk of systemic toxicity due to accumulation of MTX

and 7-OH-MTX in blood. Since 7-OH-MTX inhibits OAT3 and its variant Val448Ile at low micromolar concentrations, it may also be a substrate for OAT3. Further studies are necessary to fully delineate this possibility.

Fluorescent microscope images of OAT3-Gln239Stop-GFP transiently transfected HEK293 cells did not localize to the plasma membrane in comparison to OAT3-GFP and its polymorphic non-synonymous variants (Fig. 10). This observation provides supporting evidence that this truncated translated protein has lost sorting capability and thus does not traffic properly to the plasma membrane, thus rendering this protein non-functional. It is also noteworthy that although OAT3-GFP distributes to the plasma membrane, there is also evidence of internal distribution in the cytosol. GFP tagged variants Val281Ala, Ile305Phe, and Val448Ile also exhibit co-localization with the plasma membrane with varying degrees of distribution to the cytosol. The possibility exists that this cytosolic distribution could be part of the normal distribution of OAT3.

In summary, we have determined that OAT3 shows reduced genetic diversity at sites that alter the amino acid sequence of the protein, but exhibits a high degree of functional variation in regards to MTX uptake. This transporter is under strong negative selection acting to preserve its amino acid sequence both in the loop and TMD regions, suggesting that OAT3 is critical for human fitness. Furthermore, our studies indicate that OAT3 plays an important role in the active secretion of MTX and, possibly, 7-OH-MTX in the kidney and that protein-altering variants in OAT3 may contribute to inter-individual variation in MTX and 7-OH-MTX disposition and toxicity. The comprehensive genetic and functional analysis of variation in OAT3 and other transporters (38-43) suggests that in many cases rare variants should be given higher

priority when selecting SNPs in membrane transporter genes to study the clinical association between genetic variants in those transporters and efficacy and toxicity of drugs, such as MTX. Since MTX therapy can result in severe toxicity in the kidney and brain, where OAT3 is expressed (1, 10) clinical studies of genetic variants in OAT3 are clearly needed to determine their significance to MTX therapy.

REFERENCES

1. Chabner BA, Amrein PC, Druker BJ, Michaelson MD, Mitsiades CS, Goss PE, Ryan DP, Ramachandra S, Richardson PG and Supko JG, "Chapter 51. Antineoplastic Agents" (Chapter). Brunton LL, Lazo JS, Parker KL: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 11th Edition: http://www.accessmedicine.com/content.aspx?aID=957513.
2. Liegler DG, Henderson ES, Hahn MA and Oliverio VT. The effect of organic acids on renal clearance of methotrexate in man. *Clin Pharmacol Ther* **10:**849-857 (1969).
3. Bourke RS, Chheda G, Bremer A, Watanabe O and Tower DB. Inhibition of renal tubular transport of methotrexate by probenecid. *Cancer Res* **35:**110-116 (1975).
4. Huang KC, Wenczak BA and Liu YK. Renal tubular transport of methotrexate in the rhesus monkey and dog. *Cancer Res* **39:**4843-4848 (1979).
5. Stoller RG, Hande KR, Jacobs SA, Rosenberg SA and Chabner BA. Use of plasma pharmacokinetics to predict and prevent methotrexate toxicity. *N Engl J Med* **297:**630-634 (1977).
6. el-Badawi MG, Abdalla MA, Bahakim HM and Fadel RA. Nephrotoxicity of low-dose methotrexate in guinea pigs: an ultrastructural study. *Nephron* **73:**462-466 (1996).
7. Perazella MA. Crystal-induced acute renal failure. *Am J Med* **106:**459-465 (1999).

8. Gronroos M, Chen M, Jahnukainen T, Capitanio A, Aizman RI and Celsi G. Methotrexate induces cell swelling and necrosis in renal tubular cells. *Pediatr Blood Cancer* **46:**624-629 (2006).

9. Ganapathy V, Smith SB and Prasad PD. SLC19: the folate/thiamine transporter family. *Pflugers Arch* **447:**641-646 (2004).

10. Koepsell H and Endou H. The SLC22 drug transporter family. *Pflugers Arch* **447:**666-676 (2004).

11. Huffman DH, Wan SH, Azarnoff DL and Hogstraten B. Pharmacokinetics of methotrexate. *Clin Pharmacol Ther* **14:**572-579 (1973).

12. McLeod HL. Clinically relevant drug-drug interactions in oncology. *Br J Clin Pharmacol* **45:**539-544 (1998).

13. Burckhardt BC and Burckhardt G. Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev Physiol Biochem Pharmacol* **146:**95-158 (2003).

14. Uwai Y, Saito H and Inui K. Interaction between methotrexate and nonsteroidal anti-inflammatory drugs in organic anion transporter. *Eur J Pharmacol* **409:**31-36 (2000).

15. Takeda M, Khamdang S, Narikawa S, Kimura H, Hosoyamada M, Cha SH, Sekine T and Endou H. Characterization of methotrexate transport and its drug interactions with human organic anion transporters. *J Pharmacol Exp Ther* **302:**666-671 (2002).

16. Nozaki Y, Kusuhara H, Endou H and Sugiyama Y. Quantitative evaluation of the drug-drug interactions between methotrexate and nonsteroidal anti-inflammatory

drugs in the renal uptake process based on the contribution of organic anion transporters and reduced folate carrier. *J Pharmacol Exp Ther* **309:**226-234 (2004).

17. Uwai Y, Taniguchi R, Motohashi H, Saito H, Okuda M and Inui K. Methotrexate-loxoprofen interaction: involvement of human organic anion transporters hOAT1 and hOAT3. *Drug Metab Pharmacokinet* **19:**369-374 (2004).

18. VanWert AL and Sweet DH. Impaired clearance of methotrexate in organic anion transporter 3 (*Slc22a8*) knockout mice: a gender specific impact of reduced folates. *Pharm Res* **25:**453-462 (2008).

19. Erdman AR, Mangravite LM, Urban TJ, Lagpacan, LL, Castro RA, de la Cruz M, Chan W, Huang CC, Johns SJ, Kawamoto M, Stryke D, Taylor TR, Carlson EJ, Ferrin TE, Brett CM, Burchard EG, and Giacomini KM. The human organic anion transporter 3 (OAT3; SLC22A8): genetic variation and functional genomics. *Am J Physiol Renal Physiol* **290:**F905-F912 (2006).

20. Tajima F. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics* **123:**585-595 (1989).

21. Hartl DL and Clark AG. *Principles of Population Genetics*. Sinauer Associates, Sunderland, MA (1997).

22. Stephens M, Smith NJ and Donnelly P. A new statistical method for haplotype reconstruction from population data. *Am J Hum Genet* **68:**978-989 (2001b).

23. Urban TJ, Gallagher RC, Brown C, Castro RA, Lagpacan LL, Brett CM, Taylor TR, Carlson EJ, Ferrin TE, Burchard EG, Packman S and Giacomini KM.

Functional genetic diversity in the high-affinity carnitine transporter OCTN2 (*SLC22A5*). *Mol Pharmacol* **70:**1602-1611 (2006).

24. Leabman MK, Huang CC, DeYoung J, Carlson EJ, Taylor TR, de la Cruz M, Johns SJ, Stryke D, Kawamoto M, Urban TJ, Kroetz DL, Ferrin TE, Clark AG, Risch N, Herskowitz I and Giacomini KM. Natural variation in human membrane transporter genes reveals evolutionary and functional constraints. *Proc Natl Acad Sci USA* **100:**5896-5901 (2003).

25. Nebert DW. Proposal for an allele nomenclature system based on the evolutionary divergence of haplotypes. *Hum Mutat* **20:**463-472 (2002).

26. Aherne GW, Piall E, Marks V, Mould G and White WF. Prolongation and enhancement of serum methotrexate concentrations by probenecid. *Br Med J* **1:**1097-1099 (1978).

27. Maiche AG. Acute renal failure due to concomitant action of methotrexate and indomethacin. *Lancet* **1:**1390 (1986).

28. Thyss A, Milano G, Kubar J, Namer M and Schneider M. Clinical and pharmacokinetic evidence of a life-threatening interaction between methotrexate and ketoprofen. *Lancet* **1:**256-258 (1986).

29. Perrin A, Milano G, Thyss A, Cambon P and Schneider M. Biochemical and pharmacological consequences of the interaction between methotrexate and ketoprofen in the rabbit. *Br J Cancer* **62:**736-741 (1990).

30. Xu G, Bhatnagar V, Wen G, Hamilton BA, Eraly SA and Nigam SK. Analyses of coding region polymorphisms in apical and basolateral human organic anion

transporter (OAT) genes [OAT1 (NKT), OAT2, OAT3, OAT4, URAT (RST)]. *Kidney Int* **68:**1491-1499 (2005).

31. Stephens JC, Schneider JA, Tanguay DA, Choi J, Acharya T, Stanley SE, Jiang R, Messer CJ, Chew A, Han JH, Duan J, Carr JL, Lee MS, Koshy B, Kumar AM, Zhang G, Newell WR, Windemuth A, Xu C, Kalbfleisch TS, Shaner SL, Arnold K, Schulz V, Drysdale CM, Nandabalan K, Judson RS, Ruano G and Vovis GF. Haplotype variation and linkage disequilibrium in 313 human genes. *Science* **293:**489-493 (2001).

32. Feng B, Dresser MJ, Shu Y, Johns SJ and Giacomini KM. Arginine 454 and lysine 370 are essential for the anion specificity of the organic anion transporter, rOAT3. *Biochemistry* **40:**5511-5520 (2001).

33. Lu R, Chan BS and Schuster VL. Cloning of the human kidney PAH transporter: narrow substrate specificity and regulation by protein kinase C. *Am J Physiol* **276:**F295-F303 (1999).

34. You G, Kuze K, Kohanski RA, Amsler K and Henderson S. Regulation of mOAT-mediated organic anion transport by okadaic acid and protein kinase C in LLC-PK(1) cells. *J Biol Chem* **275:**10278-10284 (2000).

35. Wolff NA, Thies K, Kuhnke N, Reid G, Friedrich B, Lang F, and Burckhardt G. Protein kinase C activation downregulates human organic anion transporter 1-mediated transport through carrier internalization. *J Am Soc Nephrol* **14:**1959-1968 (2003).

36. Shu Y, Leabman MK, Feng B, Mangravite LM, Huang CC, Stryke D, Kawamoto M, Johns SJ, DeYoung J, Carlson E, Ferrin TE, Herskowitz I and Giacomini KM.

Evolutionary conservation predicts function of variants of the human organic cation transporter, OCT1. *Proc Natl Acad Sci USA* **100:**5902-5907 (2003).

37. Urban TJ, Giacomini KM and Risch N. Haplotype structure and ethnic-specific allele frequencies at the OCTN locus: implications for the genetics of Crohn's disease. *Inflamm Bowel Dis* **11:**78-79 (2005).

38. Leabman MK, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, DeYoung J, Taylor T, Clark AG, Herskowitz I and Giacomini KM. Polymorphisms in a human kidney xenobiotic transporter, OCT2, exhibit altered function. *Pharmacogenetics* **12:**395-405 (2002).

39. Osato D, Huang CC, Kawamoto M, Johns SJ, Stryke D, Wang J, Ferrin TE, Herskowitz I, and Giacomini KM. Functional characterization in yeast of genetic variants in the human equilibrative nucleoside transporter, ENT1. *Pharmacogenetics* **13:**297-301 (2003).

40. Badagnani I, Chan W, Castro RA, Brett CM, Huang CC, Stryke D, Kawamoto M, Johns SJ, Ferrin TE, Carlson EJ, Burchard EG, and Giacomini KM. Functional analysis of genetic variants in the human concentrative nucleoside transporter 3 (CNT3; SLC28A3). *Pharmacogenomics J* **5:**157-165 (2005).

41. Owen RP, Gray JH, Taylor TR, Carlson EJ, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, and Giacomini KM. Genetic analysis and functional characterization of polymorphisms in the human concentrative nucleoside transporter, CNT2. *Pharmacogenet Genomics* **15:**83-90 (2005).

42. Owen RP, Lagpacan LL, Taylor TR, de la Cruz M, Huang CC, Kawamoto M, Johns SJ, Stryke D, Ferrin TE, and Giacomini KM. Functional characterization

and haplotype analysis of polymorphisms in the human equilibrative nucleoside transporter, ENT2. *Drug Metab Dispos* **34:**12-15 (2006).

43. Fujita T, Urban TJ, Leabman MK, Fujita K and Giacomini KM. Transport of drugs in the kidney by the human organic cation transporter, OCT2 and its genetic variants. *J Pharm Sci* **95:**25-36 (2006).

44. Zelcer S, Kellick M, Wexler LH, Shi W, Sankaran M, Lo S, Healey J, Huvos AG, Meyers PA, and Gorlick R. Methotrexate levels and outcome in osteosarcoma. *Pediatr Blood Cancer* **44:**638-642 (2005).

45. Erttmann R, Bielack S and Landbeck G. Kinetics of 7-hydroxy-methotrexate after high-dose methotrexate therapy. *Cancer Chemother Pharmacol* **15:**101-104 (1985).

CHAPTER 5

CONCLUSIONS

The organic anion transporters (OATs) are members of the SLC22 transporter family (1). OATs typically transport a wide variety of organic anion compounds such as antiviral drugs, ß-lactam antibiotics, diuretics, and NSAIDs (2-4). In particular, OAT2 (predominately expressed in the liver) and OAT3 (predominately expressed in the kidney) play an important role in the disposition of an array of drugs used in clinical practice. A primary goal of this dissertation was to determine the ethnic differences in allele frequencies of genetic variants of OAT2 and OAT3. A second goal was to determine if genetic variants of OAT2 and OAT3 affect the interaction of these transporters with drugs and endogenous substances.

In this thesis, we demonstrate that there are ethnic differences in the allele frequencies of OAT2 and OAT3 variants. Additionally, we demonstrate that genetic variants of OAT2 and OAT3, which exhibit effects on function in cellular assays, also show differences in allele frequencies between ethnic groups. Variants of these transporters may be a source of the heterogeneity in drug disposition within and between ethnic groups observed in the clinical setting.

The existence of plasma membrane transporters capable of transporting cGMP into, as well as out of, cells had been suggested in a large number of studies in a variety of cell types (5-8). In Chapter 2, we reported the following unexpected finding: OAT2 is a novel, bidirectional, facilitative plasma membrane transporter for cGMP and other guanine nucleotides. This finding enhances our understanding of OAT2, which was previously thought to primarily transport organic anions such as PAH. Among purine

and pyrimidine nucleobases, nucleosides and nucleotides, OAT2 preferentially transported cGMP with a K_m value of 88 $\pm$ 11 μM, and exhibited between 50- and 100-fold enhanced uptake compared to control cells. A common alternatively spliced variant of OAT2 demonstrated a complete loss of transport function as a result of a low expression level on the plasma membrane. In summary, OAT2 is a highly efficient, facilitative transporter of cGMP and may be involved in cGMP signaling in many tissues.

In Chapter 3, we expanded on the findings of Chapter 2 by further investigating the role of OAT2 in the disposition of antiviral agents with nucleotide-like structures. Acyclovir is an antiviral used in the treatment of herpes viral infections, including HSV hepatitis. Our studies demonstrate that acyclovir is an excellent substrate for OAT2. The actual uptake of acyclovir in empty vector and OAT2-stably transfected HEK293 cells was 0.073 ± 0.005 pmol/mg protein/5 minutes and 3.062 ± 0.114 pmol/mg protein/5 minutes, respectively. Additionally, the coding region of OAT2 was screened in a collection of 272 DNA samples from an ethnically diverse cohort. Six non-synonymous OAT2 variants were identified, of which only one, Thr110Ile, was polymorphic with an allele frequency of 2.3% in African Americans. Thr110Ile exhibited an approximately 30% reduced uptake of acyclovir compared to reference OAT2 ($p < 0.01$). Thr110Ile demonstrated reduced V_{max} values compared to reference OAT2 (2630 ± 209 pmol/mg protein/2 minutes for OAT2 versus 1361 ± 124 pmol/mg protein/2 minutes for Thr110Ile ($p < 0.001$)). The low level of genetic variation in OAT2 argues that it has a critical biological function. Our study suggests that rare variants of OAT2 may have important effects on the disposition and efficacy of acyclovir in the treatment of HSV hepatitis.

Among the OATs, OAT3 (*SLC22A8*) is the most abundantly expressed in the basolateral membrane of renal proximal tubules (9). OAT3 plays an important role in the clearance of clinically important drugs from the blood into the proximal tubules of the kidney (2-4). In Chapter 4, we examined the impact of OAT3 and its genetic variants on the transport of MTX, used in the treatment of cancer and inflammatory diseases. Our cellular assays found that MTX and its major metabolite, 7-OH-MTX, inhibited transport of the model substrate ES by 90%. MTX uptake by OAT3 was saturable with a K_m of 75 ± 12 μM. Ten non-synonymous variants were identified in previous sequencing studies of OAT3 in DNA samples from 270 unrelated individuals from four ethnic groups. We observed that MTX and ES uptake was significantly reduced in cells expressing five of the ten protein-altering variants: Phe129Leu, Arg149Ser, Gln239Stop, Ile260Arg, and Arg277Trp. In addition, Ile305Phe transported MTX similarly to OAT3 reference, but showed significantly reduced uptake of ES ($p < 0.01$). The common variant, Val448Ile, showed a 2-fold increase in the K_m of MTX ($p < 0.05$). OAT3 and Val448Ile-mediated transport of ES and MTX was inhibited by 7-OH-MTX ($K_i \sim 20$ μM). Our studies suggest the need for genotype-driven clinical studies examining the effects of genetic variants of OAT3 in MTX elimination and toxicity.

Clinical differences between ethnic groups in the disposition and pharmacologic effects of drugs that are substrates of transporters may be attributed to differences in genetic and environmental factors that influence all aspects of the pharmacokinetic and pharmacodynamic pathway. For example, substrates of transporters may also be substrates of various polymorphic enzymes. These complexities make it difficult to understand the effects of genetic differences in transporter activity on drug disposition

and response. Further, in contrast to the numerous clinical studies assessing the effects of polymorphisms in drug metabolizing enzymes on drug disposition, there have been comparably few clinical studies of genetic variants in membrane transporters (10). Studies of the disposition of model substrates of transporters in single ethnic groups suggest that inter-ethnic differences in drug disposition may be present (10).

Clearly, there is a need for direct comparisons between ethnic groups in the disposition and response of transported drugs. Currently, we are conducting a clinical study comparing the renal clearance of cefotaxime, a ß-lactam antibiotic, in Asian subjects homozygous for OAT3 reference compared to Asian subjects who carry the Ile305Phe polymorphism. These genotype-driven studies will test the hypothesis that genetic variants of OAT3 contribute to variation in the renal clearance of drugs.

Due to the low level of variation in OAT2, the recruitment of African American subjects who possess the Thr110Ile polymorphism for clinical pharmacokinetic studies may not be fruitful. However, the findings in this dissertation suggest that OAT2 represents a potential new drug target for regulating cGMP levels similar to pharmacologic agents currently being used clinically such as sildenafil and milrinone. Additionally, an improved understanding of the role of OAT2 in the cellular targeting of antiviral drugs such as acyclovir may lead to the development of improved antiviral agents with enhanced efficacy in the diseased target organ (e.g. the liver). A more in-depth investigation of the structure-activity relationships of endogenous and synthetic guanine-like analogs and their interactions with OAT2 may lead to the evolution of a pharmacophore platform for future development of drugs that modulate intracellular cGMP levels or target specific tissues that express OAT2.

REFERENCES

1. Koepsell H and Endou H. The SLC22 drug transporter family. *Pflugers Arch* **447:**666-676 (2004).
2. Burckhardt BC and Burckhardt G. Transport of organic anions across the basolateral membrane of proximal tubule cells. *Rev Physiol Biochem Pharmacol* **146:**95-158 (2003).
3. Sekine T, Miyazaki H and Endou H. Molecular physiology of renal organic anion transporters. *Am J Physiol Renal Physiol* **290:**F251-F261 (2006).
4. Wright SH and Dantzler WH. Molecular and cellular physiology of renal organic cation and anion transport. *Physiol Rev* **84:**987-1049 (2004).
5. Chen ZS, Lee K and Kruh GD. Transport of cyclic nucleotides and estradiol 17-beta-D-glucuronide by multidrug resistance protein 4. Resistance to 6-mercaptopurine and 6-thioguanine. *Journal Biol Chem* **276:**33747-33754 (2001).
6. Guo Y, Kotova E, Chen ZS, Lee K, Hopper-Borge E, Belinsky MG and Kruh GD. MRP8, ATP-binding cassette C11 (ABCC11), is a cyclic nucleotide efflux pump and a resistance factor for fluoropyrimidines 2',3'-dideoxycytidine and 9'-(2'-phosphonylmethoxyethyl)adenine. *J Biol Chem* **278:**29509-29514 (2003).
7. Jedlitschky G, Burchell B and Keppler D. The multidrug resistance protein 5 functions as an ATP-dependent export pump for cyclic nucleotides. *J Biol Chem* **275:**30069-30074 (2000).
8. Sager G. Cyclic GMP transporters. *Neurochem Int* **45:**865-873 (2004).

9. Cha SH, Sekine T, Fukushima JI, Kanai Y, Kobayashi Y, Goya T and Endou H. Identification and characterization of human organic anion transporter 3 expressing predominantly in the kidney. *Mol Pharmacol* **59:**1277-1286 (2001).

10. Zhang W, Yu BN, He YJ, Fan L, Li Q, Liu ZQ, Wang A, Liu YL, Tan ZR, Fen-Jiang, Huang YF and Zhou HH. Role of BCRP 421C>A polymorphism on rosuvastatin pharmacokinetics in healthy Chinese males. *Clin Chim Acta* **373:**99-103 (2006).

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