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Physiological and Pharmacological Roles of Novel Organic Cation Transporters

Author
Urban, Thomas

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Physiological and Pharmacological Roles of Novel Organic Cation Transporters

by

Thomas J. Urban

DISSERTATION

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As of this writing, I've been in school for twenty six years, which is almost ninety percent of my lifetime. Despite having now achieved the highest degree in my field, I still feel that I have so much to learn. Fortunately, in addition to the information detailed in the following two hundred pages, my course of study has taught me important lessons in *how* to learn. By this, I mean the scientific method: how to learn things that no one else has yet to discover.

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ABSTRACT

Physiological and Pharmacological Roles of Novel Organic Cation Transporters

Thomas J. Urban

The novel organic cation transporters (OCTNs) are bifunctional and bidirectional tranporters that are highly expressed at the apical membrane in renal tubular epithelia, and are thought to contribute to both the renal salvage of nutrients and to the active tubular secretion of xenobiotics. We used a genotype-to-phenotype approach to study the importance of OCTN1 (encoded by *SLC22A4*) and OCTN2 (encoded by *SLC22A5*) in the disposition of drugs and endobiotics. Resequencing of the coding region of these genes in a large ethnically-diverse sample of human DNA revealed six amino acid-altering nucleotide substitutions in *SLC22A4* and eight in *SLC22A5*. When expressed heterologously, several rare variants of OCTN1 (D165G, R282X) were found to result in complete loss of transport function. Rare OCTN2 variants showed functional differences from the reference protein, including reduced function (V481F) and substrate selectivity differences (Y449D). Functionally significant single-nucleotide polymorphisms (SNPs) in OCTN1 (L503F) and OCTN2 (-207G>C and F17L) were also discovered. These common SNPs were tested for functional significance *in vivo* by recruiting subjects based on genotype at these positions, and testing for quantitative differences between genotype groups in the pharmacokinetics of gabapentin (an OCTN1 substrate) and carnitine (a preferred substrate of OCTN2). OCTN1 genotype was found to be a significant predictor of gabapentin renal clearance, with OCTN1 reference homozygotes showing net active secretion of gabapentin, but L503F homozygotes showing almost no active secretion of

this drug. This finding adds the OCTN1-L503F polymorphism to a small list of SNPs known to influence drug disposition. OCTN genotype did not affect carnitine disposition or lipid metabolism. A meta-analysis of protein-altering variants of membrane transporters revealed that, as exemplified by the OCTNs, rare variants are more likely to affect global biochemical function; however, variants with substrate-specific effects (such as the OCTN1-L503F variant) may be relatively common. The results of this research support the hypothesis that OCTN transporters are important determinants of active drug secretion in the kidney, and that prediction of renal clearance of drugs may be improved by renal transporter genotype information. This research may help to inform future pharmacogenetic studies of the OCTN transporters, and renal drug transporters generally.

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CHAPTER 1

PHYSIOLOGICAL AND PHARMACOLOGICAL ROLES OF THE NOVEL ORGANIC CATION (OCTN) TRANSPORTERS

Introduction

Solute carrier (SLC) transporters maintain cellular and total body homeostasis by importing nutrients and exporting cellular waste products and toxic compounds. These transporters also play a critical role in drug response, serving as drug targets and facilitating drug absorption, metabolism, and elimination. The SLC superfamily is comprised of transporters from a wide range of functional classes, including neurotransmitter, nutrient, heavy metal, and xenobiotic transporters. SLC transporters have been well studied with regard to their influence on drug disposition, and in particular, in the renal elimination of drugs and other xenobiotics.

Renal elimination, including both glomerular filtration and active tubular secretion, is a major route of clearance for many drugs and/or drug metabolites. Active secretion of drugs by the kidney occurs primarily in the renal proximal tubule, where drug transporters facilitate the flux of drug molecules from the blood to the tubular lumen for excretion. Although many transporters are known to be expressed in kidney, the transport systems most important for renal drug elimination are in the SLC22A family: the classical organic cation transporters (OCTs, *SLC22A1-3*), organic anion transporters (OATs, *SLC22A6-8*, *SLC22A11-12*), and the novel organic cation transporters (OCTNs,

SLC22A4-5). Several *SLC22A* family members, including OCTN2, have been shown to influence tissue distribution and/or elimination of their substrates *in vivo* [1-5].

The genes of the novel organic cation transporter (OCTN) family, OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*), encode plasma membrane transporters that are bifunctional and bidirectional, facilitating the pH-sensitive transport of organic cations from cell to lumen and the sodium-dependent transport of small zwitterions from lumen to cell. The two transport modes of the OCTNs suggest two distinct roles of these transporters: OCTN1 and OCTN2 are highly expressed in kidney, where they are implicated in both active secretion of organic cations, and salvage (through active reabsorption) of nutrients such as the zwitterion L-carnitine. Thus, in addition to their potential importance to drug disposition, the OCTNs play a critical role in normal human physiology.

Here, we review our current understanding of the biochemical and physiological functions of OCTNs, including their transport mechanisms, tissue distribution, localization, and substrate specificity, highlighting the distinguishing characteristics of each isoform. We then discuss the importance of OCTN transporters in human disease, including both rare and common genetic disorders. Finally, we describe the pharmacological roles of OCTNs, and the current knowledge of common genetic variation in the *SLC22A4* and *SLC22A5* genes. We hypothesize that a systematic study of human genetic variation in the OCTN genes, using a sequence-based (or reverse-genetics) approach, may aid in the elucidation of the roles of these transporters in both drug disposition and risk for common diseases in humans.

Expression, Localization and Transport Mechanisms of OCTNs

Two human OCTN genes (*SLC22A4* and *SLC22A5*) have been cloned and are found in tandem at the same chromosomal locus (5q31). The relatively small physical distance between these genes and their high degree of sequence homology (78% identity at the mRNA level) suggests that they originated by duplication from a common ancestral gene. OCTN1 and OCTN2 also share a similar pattern of tissue distribution in humans, with high expression in kidney and moderate expression in heart, liver, skeletal muscle, lung, spleen, small intestine, and brain [6-8]. Notably, OCTN1 is most highly expressed in erythroid-lineage cells, whereas OCTN2 expression is low in this cell type [9]. This distinct distribution pattern suggests a unique role for OCTN1 in erythrocytes.

OCTN1

Mouse Octn1 has been localized to the apical membrane of renal proximal tubules by immunohistochemistry [10]. Although the regional and subcellular localization of OCTN1 in human kidney have not yet been examined, the functional characteristics of OCTN1 are consistent with an apical localization in renal tubules; that is, organic cation transport via OCTN1 has been shown to be driven by an organic cation-proton exchange mechanism. OCTN1-mediated influx of TEA was enhanced at neutral and alkaline pH, while efflux of TEA was increased by an inward H^+ gradient [6,11]. OCTN1 contains a nucleotide binding sequence motif and a dependence of its transport activity on cellular ATP content, particularly at acidic pH, was observed in OCTN1-transfected human

embryonic kidney (HEK-293) cells [6]. However, it is unclear whether this reflects a direct reliance on ATP utilization for OCTN1 transport. Additional studies have demonstrated that OCTN1 can also function as an organic cation/organic cation exchanger: for example, extracellular TEA enhanced the efflux of [^{14}C]TEA in OCTN1-transfected HEK-293 cells [11]. Thus, although the subcellular localization of OCTN1 has not been clearly demonstrated, these studies suggest that OCTN1 functions as a bidirectional, pH-dependent (and possibly ATP-dependent) transporter at the brush border membrane in renal tubular epithelial cells.

In addition to its organic cation-H^+ antiport activity, OCTN1 was recently shown to act as a Na^+-dependent uptake transporter for ergothioneine, an antioxidant whose precise physiological role is unclear (discussed below) [12]. When stably expressed in HEK-293 cells, OCTN1 enhanced the uptake of L-ergothioneine ~600-fold compared with control (mock-transfected) cells, and this activity was inhibited by replacement of extracellular Na^+ with *N*-methyl-D-glucosamine. OCTN1 has also been shown to transport L-carnitine in a Na^+-dependent manner [13]. Carnitine transport by mouse Octn1 was sodium-dependent, though this activity was significantly lower than that of mouse Octn2. Two reports of Na^+-dependent carnitine transport by human OCTN1 have been published [11,14]; however, we and others [12] were not able to reproduce this result. It is likely that L-carnitine is a weak substrate of OCTN1, but that L-carnitine binds with such poor affinity, or is transported with such poor efficiency, that this activity is difficult to reliably measure in heterologous expression systems.

OCTN2

The regional and subcellular localization of human OCTN2 in the kidney have not been studied extensively. However, *in situ* hybridization using rat kidney revealed that rat OCTN2 mRNA is expressed predominantly in the cortex with very little expression in the medulla; expression of OCTN2 was detectable in the proximal and distal tubules as well as in the glomeruli [15]. Additionally, immunohistochemistry studies in mouse and rat kidney have shown that OCTN2 is localized to the apical membrane of renal tubular cells [16].

Studies in OCTN2-transfected cells, plasma membrane vesicles and OCTN2-expressing *Xenopus laevis* oocytes have demonstrated that OCTN2 transports L-carnitine with high affinity in a Na^+-dependent manner [8,16,17]. Studies suggest that the stoichiometry of L-carnitine and Na^+ co-transport is 1:1 [16,18]. Additionally, Na^+ enhances the ability of L-carnitine to compete with [^{14}C]TEA, suggesting that Na^+ may enhance the affinity of L-carnitine for OCTN2 [15]. In contrast, Na^+ does not influence OCTN2-mediated transport of organic cations such as TEA [15]. Thus, OCTN2 is thought to function as both a Na^+-dependent carnitine transporter and a Na^+-independent organic cation transporter.

Electrogenicity and pH-sensitivity of OCTN2 organic cation and carnitine transport have also been studied. Similar to OCTN1, OCTN2 transport of TEA and L-carnitine is sensitive to pH, as demonstrated by increased uptake in OCTN2-expressing cells at alkaline pH [7,17,18]. However, the pH-sensitive transport of L-carnitine is unlikely to

be mediated by a H^+ antiport mechanism, as internal acidification of OCTN2-expressing oocytes does not increase the L-carnitine-induced current [17]. Studies also suggest that Na^+-dependent transport of L-carnitine by OCTN2 is electrogenic. One study, using plasma membrane vesicles from OCTN2-expressing human embryonic kidney cells, showed that the initial rate of uptake of L-carnitine increased in the presence of an outwardly directed K^+ gradient, a relatively negative potential, caused by treatment with valinomycin [16]. Another study reported that L-carnitine-induced currents decreased with depolarization of OCTN2-expressing oocytes [17]. Therefore, transport of L-carnitine by OCTN2 is a Na^+-dependent, electrogenic process suggestive of Na^+-carnitine symport.

Though a high-resolution crystal structure is not available, functional domains of OCTN2 that are important in substrate recognition or sodium stimulation have been identified using chimeric proteins and mutational analysis. Studies using chimeras of human and rat OCTN2 showed that the carnitine and organic cation recognition sites are distinct, based on the differences in relative activity of the native proteins for carnitine *vs*. TEA [19]. Similarly, studies of chimeric human OCTN1/OCTN2 constructs allowed the observation that the 214 residues at the C-terminus of OCTN2 are necessary for carnitine transport, and residues 341-454 are involved in carnitine recognition [20]. Specifically, Arg-341, Leu-409, and Thr-429 were shown through mutational analysis to be involved in carnitine binding. Studies using a S467C mutant OCTN2 showed that the carnitine and TEA recognition sites are overlapping, but not identical: S467C had decreased affinity for carnitine, but increased affinity for TEA, and the mutual inhibition kinetics of

these two compounds were not completely competitive [21]. Chimeras mouse Octn3 (which transports carnitine in a sodium-independent manner, and is a poor transporter of organic cations) and human OCTN2 demonstrated that transmembrane domains 1 through 7 are responsible for organic cation transport and sodium stimulation of carnitine transport by OCTN2 [22], and that Gln-180 and Gln-207, located in TMD3 and TMD4, respectively, are essential for sodium dependence of OCTN2-mediated carnitine transport. Several tyrosine residues in the C-terminus of OCTN2 (Tyr-447 and Tyr-449), located in an intracellular loop between TMD10 and TMD11, have also been shown to affect sodium stimulation of carnitine transport [23].

OCTN3

In the mouse, there exists an additional paralog of the OCTNs, termed Octn3. Mouse Octn3 is distinct from the other Octn transporters in its tissue distribution and transport activity [13]. Whereas Octn1 and Octn2 have a broad tissue distribution, with highest expression in kidney and energetic tissues such as skeletal muscle and heart, Octn3 expression is restricted to the testes of male mice. In terms of its biochemical function, mouse Octn3 has been shown to transport L-carnitine with high affinity; however, unlike Octn1 or Octn2, L-carnitine transport by Octn3 is Na^+-independent. Octn3 also transports the organic cation TEA in a Na^+-independent manner, although with much lower efficiency than the other Octn transporters. The transport efficiency of the mouse Octns for L-carnitine *vs*. TEA was compared *via* the ratio of carnitine to TEA transport. Octn3 was shown to be the most carnitine-selective transporter in the Octn family, with a carnitine:TEA transport ratio of 746:1 (*vs*. 11.3:1 for Octn2 and 1.78 for Octn1). Thus,

mouse Octn3 is a testes-specific, Na^+-independent, highly selective L-carnitine transporter.

There is some controversy regarding the possible existence of a human OCTN3 ortholog. Orthologous mRNA sequences have been amplified from intestine of rat and chicken, and localized to the basolateral membrane of enterocytes [24]. The transport of L-carnitine by intestinal basolateral membrane vesicles was shown to be an equilibrative process that is independent of Na^+ or pH, consistent with the activity of mouse Octn3. Two reports from the same laboratory of a human OCTN3 ortholog, identified by Western blotting and immunohistochemistry using an anti-mouse Octn3 antibody, and localized to the peroxisomal membrane of hepatocytes and skin fibroblasts, have been published [25,26]. The putative human ortholog is thought to be a carnitine carrier in peroxisomes, where it facilitates the peroxisomal uptake of free carnitine or the transport of chain-shortened products of peroxisomal fatty acid oxidation out of peroxisomes as acylcarnitine esters. The human OCTN3 mRNA, however, has been resistant to cloning efforts and has not been characterized directly. Based on synteny with mouse chromosome 11q, the gene encoding the human OCTN3 would be expected to lie on chromosome 5q31, between *SLC22A4* and *SLC22A5*; however, this gene does not appear in the public draft sequence of the human genome. Thus, it remains unclear whether such an ortholog exists in humans, and whether the reactivity of the anti-mOctn3 antibody with the peroxisomal membrane indicates specific recognition of human OCTN3, rather than reactivity with an unrelated protein with a common epitope.

Substrate Specificity of the OCTNs

Table 1.1 alphabetically lists prescribed drugs and various dietary supplements which interact with OCTN transporters. Studies in various heterologous expression systems have shown that L-carnitine and acetyl-L-carnitine are substrates of OCTN2 [8,15,17]; transport of L-carnitine by OCTN1 has also been demonstrated, although this activity appears to be much weaker compared with OCTN2-mediated carnitine transport [11,14]. The antioxidant ergothioneine appears to be the preferred substrate of OCTN1, but is not a substrate of OCTN2 [12]. There appears to be considerable overlap in substrate specificity between the two transporters, with a wider variety of substrates having been identified for OCTN2 than for OCTN1. Substrates of OCTN2 include organic anions such as valproic acid and zwitterions such as the antibiotic cephaloridine in addition to the OCTN1 substrates quinidine, verapamil and pyrilamine [11,17,18,27]. However, the broader range of identified substrates for OCTN2 is likely the result of bias toward investigation of this transporter (*i.e.*, these compounds may also be substrates of OCTN1).

In addition to these known substrates, the interactions of many organic cation drugs (*e.g.*, cimetidine, levofloxacin, nicotine, and procainamide) and nutritional/dietary supplements (*e.g.*, betaine, choline, L-lysine and L-methionine) with OCTN transporters have been studied and many appear to be inhibitors [6-8,11,12,15,17,18,27,28].

Table 1.1. Subtrates and inhibitors of human OCTN transporters.

	OCTN1	OCTN2	Reference
Preferred Endogenous Substrates			
L-Carnitine	S	S (K_m ~2-10 µM)	[8,11,15,17]
D-Carnitine	I	S (K_m ~100 µM)	[8,11,17]
Acetylcarnitine	I	S (K_m ~ 10 µM)	[8,11,15,17,27]
Propionylcarnitine	N.D.	S	[15,27]
L-Ergothioneine	S	N.I.	[12]
Other Endogenous Compounds			
Betaine	S	S	[17]
Butyrobetaine	N.D.	I	
Choline	I	S	[11,17]
Cysteine	N.D.	I	[17]
L-Lysine	N.I.	S	[11,17]
L-Methionine	N.D.	S	[17]
Drugs and Xenobiotics			
Aldosterone	N.D.	I	[18]
Cephaloridine	I	S	[11,27]
Cefepime	N.D.	I	[27]
Cefluprenam	N.D.	I	[27]
Cefoselis	N.D.	I	[27]
Cefsulodin	N.D.	I	[27]
Ceftazidime	N.D.	I	[27]
Cimetidine	I	I	[7,11,15,18]
Clonidine	I	I	[11,15]
Desipramine	N.D.	I	[15]
Emetine	N.D.	S	[17]
Levofloxacin	I		[11]
Mildronate	N.D.	S (K_i ~ 21 µM)	[28]
MPTP	N.D.	I	[7,15]
Nicotine	I	I	[7,11,18]
Ofloxacin	I		[11]
Pivalic Acid	N.D.	S	[17]
Procainamide	I	I	[7,11,18]
Pyrilamine	S	I	[11,18]
Quinidine	S	S	[11,17,18]
Quinine	I	I	[11,18]
Spironolactone	N.D.	S (K_i ~ 26 µM)	[28]
Tetraethylammonium	S	S	[6,11,15,17]
Valproic Acid	N.D.	S	[18]
Verapamil	S	S (K_i ~ 25 µM)	[11,15,17,18,28]

Abbreviations: S = substrate; I = inhibitor; N.I. = not inhibitory; N.D. = not determined

No direct comparison of the substrate selectivity of human OCTN1 and OCTN2 has been performed; however, studies in mice have provided limited information on differences between OCTN isoforms. Although all are multifunctional carnitine/organic cation transporters, differences among the murine Octn1 and Octn2 transporters have been found with respect to transport mechanisms and relative activity toward the two prototypic substrates, with Octn1 having relatively greater activity toward TEA *vs.* carnitine, and Octn2 being carnitine-preferring [13]. Cumulatively, the evidence gleaned from studies of carnitine transport by human OCTN1 and OCTN2 suggests that, at least for carnitine transport, this trend is true for human OCTNs as well (*i.e.*, L-carnitine is a much weaker substrate of human OCTN1 *vs*. OCTN2). However, it is unknown whether the human orthologs universally exhibit preferences similar to the mouse orthologs. Notably, interspecies differences in the function of orthologous genes have been found for other transporters, suggesting that the functional comparison of murine Octn transporters may not hold for their human orthologs [29,30]. Additionally, data such as these, obtained in a cellular system, may not reflect the relative contribution of each isoform in the *in vivo* situation with respect to disposition of substrates of either category.

Perusal of the available literature on human OCTN1 and OCTN2 suggests that they are both multifunctional and broadly-specific transporters of organic cations and zwitterions. The available data suggest that these transporters have overlapping, but distinct roles in the transport of carnitine and organic cations, with OCTN1 having a greater role in the disposition of organic cations, and OCTN2 contributing more significantly to carnitine status.

Role of OCTN Transporters in Human Physiology

Systemic Carnitine Deficiency

Much of the interest in the OCTN transporters has focused not on their pharmacological relevance, but on their role in carnitine transport. L-carnitine is important physiologically as an essential cofactor in mitochondrial fatty acid oxidation, where it aids in translocation of long-chain fatty acids as carnitine esters across the mitochondrial inner membrane into the mitochondrial lumen, where beta-oxidation takes place [31,32]. OCTN transporters, particularly OCTN2, are responsible for uptake of carnitine across the plasma membrane, one of the first steps in long-chain fatty acid oxidation (see Figure 1.1). Inherited deficiencies in plasmalemmal carnitine transport have been identified, and have been linked to mutations in the OCTN2 gene [33-35]. The primary result of OCTN2 deficiency is a loss of renal tubular reabsorption of filtered carnitine. In healthy individuals, this process is very efficient, with >95% of the filtered load reabsorbed; however, in patients homozygous for loss of function mutations in OCTN2, carnitine renal clearance is approximately equal to the glomerular filtration rate. The genetic defect therefore results in depletion of total carnitine stores, and is referred to as primary systemic carnitine deficiency (SCD) or primary carnitine uptake deficiency (CUD). The clinical presentation of patients with mutations in OCTN2 is variable, but common features include early-onset cardiomyopathy, skeletal myopathy, hypoketotic hypoglycemia, hyperammonemia, and a Reye's-like syndrome. The relationship between OCTN2 and systemic carnitine deficiency has been extensively reviewed [34,35].

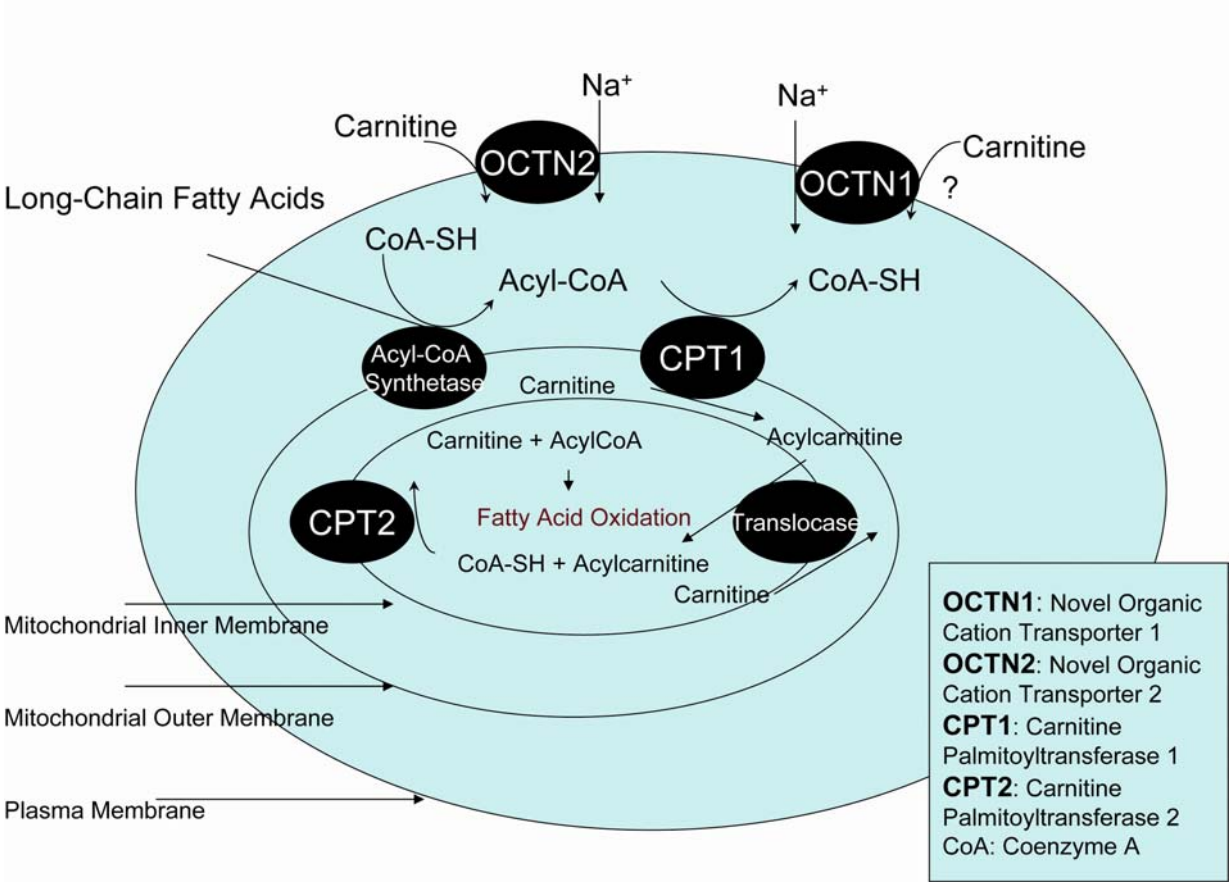

Figure 1.1. Role of carnitine in mitochondrial fatty acid oxidation.
Carnitine uptake into cells is facilitated by OCTN2, and with weaker affinity by OCTN1. Long chain fatty acids are liberated from the plasma membrane by diffusion or by the activity of lipoprotein lipase, then activated in the cytosol by condensation with CoA, a process catalyzed by acyl-CoA synthetase. The acyl moiety is then transferred to the beta-hydroxyl group of carnitine by the mitochondrial outer membrane-bound CPT1. Acylcarnitine esters are then transported across the mitochondrial inner membrane in exchange for free carnitine, a process facilitated by the carnitine-acylcarnitine translocase. The acyl moiety is then transferred to CoA by CPT2, and the acylCoA product serves as a substrate for beta-oxidation in the mitochondrial lumen. Adapted from [32].

The process through which OCTN2 was identified as the causative gene in systemic carnitine deficiency is interesting for several reasons. Initially, the genomic region that includes the SCD susceptibility allele was localized by a linkage study in a single large (26-member) pedigree containing only one affected individual [36]. It was observed that roughly half of the subjects in the pedigree exhibited mild asymptomatic carnitine deficiency; thus, although the affected individual (who had almost undetectable plasma carnitine) presumably inherited the disease in a recessive mode, the disease locus could be found through linkage by treating the heterozygotes as "affected" individuals, and assuming a dominant mode of inheritance. This allowed the mapping of the SCD locus to human chromosome 5q31, with the nearest marker D5S436. In parallel with this, studies of the juvenile visceral steatosis (*jvs*) mouse, a murine model of systemic carnitine deficiency, had shown that the *jvs* phenotype was linked to mouse chromosome 11q [37], in a region syntenic to human 5q31. The *jvs* gene was then identified by fine mapping in mice, and discovered to be the high-affinity carnitine transporter, Octn2 [33,38]. The human OCTN2 gene was then quickly proven to be the causative gene in SCD in human patients [33,39]. This story highlights 1) the importance of mouse models of human disease, which can allow more rapid fine mapping through experimental crosses, 2) the power of using heterozygote phenotypes to aid in linkage mapping of rare diseases, and 3) the fact that moderate deficiency in OCTN2 activity (*i.e.*, as seen in heterozygotes for OCTN2 mutations) produces an observable phenotype that may have implications for common genetic variation in OCTN genes.

Nonsense Mutations and Insertions/Deletions

Twenty four nonsense or insertion/deletion mutations that have been shown to produce systemic carnitine deficiency are listed in Table 1.2 [23,33,39-58]. Many of these mutations were shown to cause deficiencies in saturable carnitine transport when measured using cultured fibroblasts or heterologously expressed mutant cDNA. The Phe23del mutation has been found in one SCD patient as a compound heterozygote with a splice donor site mutation (g.14344G>A, or IVS3+1G>A), and carnitine uptake in fibroblasts from this patient was reduced to 2% of controls [42]. In the same report, another SCD patient was found to be homozygous for a single nucleotide deletion in exon 5 (c.844delC), which leads to an early stop codon at amino acid position 295 (295X), and this patient had fibroblast carnitine uptake of only 1% of controls. c.1450G>A or IVS8-1 G>A, an acceptor splice site mutation, was among the earliest OCTN2 mutations identified in SCD, and most likely results in the joining of exon 8 with exon 10, resulting in a premature stop codon at position 485. Although most of the protein would thus be expected to be intact, a patient homozygous for this mutation had reduced carnitine uptake at approximately 10% of controls in fibroblasts [33]. The same report described the c.4-5insC mutation, which leads to a frameshift just after the start codon and an early stop at codon position 137. This mutation was found in an SCD patient as a compound heterozygote with the Trp132X mutation. One additional mutation, a 113-bp deletion of the 5' end of the coding region, results in the loss of the start codon, with the next in-frame start codon at amino acid position 177 [33]. This mutation was carried in two SCD siblings as homozygotes.

Table 1.2. Known pathological mutations in OCTN2.

Mutation	Function*	Recurrent	# Alleles	Ethnicity	Clinical History	References
Nonsense/Insertions/Deletions						
-91 to -22 del	N.D.	Yes	4	Not specified		[33,41]
g.4-5ins C	N.D.	No	1	Not specified		[33,41]
1-176 del	0%	No		Not specified	Cardiomyopathy	[33]
g.254-264 ins/dup	N.D.	Yes	1	Not specified	Coma	[42,43]
g.255-1649del/ c.34-1428del	N.D.	Yes	1	Not specified		[47]
g.874ins19bp/ c.653-654 ins19	N.D.	No	1	Not specified		[47]
g.875-1046 del/ c.654-825del	N.D.	No	1	Not specified		[47]
226 ins C	0%	No		Not specified	Metabolic abnormalities	[33]
Y4X	N.D.	No		Indian	Metabolic abnormalities; hepatomegaly	[48,49]
133X	N.D.	No		Not specified		[43]
W132X	0%	Yes	5	Not specified	Reye's syndrome	[33,40,41]

Mutation	Function	Recurrent	# Alleles	Ethnicity	Clinical History	References
IVS3+1G>A	2%	No	1	Irish	Cardiomyopathy	[42]
IVS7 del 21 nt	<5%	No	1	Vietnamese	Cardiomyopathy	[48]
IVS8-1G>A	10%	No	2	Not specified	Reye's syndrome	[33]
V153fsX193	<5%	No	1	Anglo-Celtic	Sibling with cardiomyopathy; death	[48]
R254X	<1%	Yes	14	Chinese; Saudi; Lebanese	Cardiomyopathy (4 cases); Metabolic crisis w/o cardiomyopathy (1 case)	[44,48,50]
W275X	<5%	No	1	Vietnamese	Cardiomyopathy	[48]
R282X	5%-10%	Yes	8	German; East Indian; Chinese	Hypoglycemia, myopathy, Reye's syndrome; cardiomyopathy	[39,45,46,48]
294X/R282D	1%	No	2	Croatian	Hypotonia, hypertrophic cardiomyopathy	[42]
295X/1027delT	<2% in lymphoblasts; splicing abnormality	Yes	4	Mexican; Roma gypsies	Hepatic encephalopathy, hypertrophic cardiomyopathy; SIDS	[51,52]
19015delA;347X		No	3	Turkish	Hypertrophic cardiomyopathy, hepatic encephalopathy	[42]
Y387X		No	1	Chinese	Metabolic crisis	[44]
Y401X	0%-4.8%	Yes	3	Caucasian	Hypoglycemia; dilated cardiomyopathy, hepatic encephalopathy	[39,42]
458X	0%	No	1	Not specified	Hypoglycemia	[39]

Mutation	Function	Recurrent	# Alleles	Ethnicity	Clinical History	References
Missense						
M1I	<5%; normal surface expression	No	1	Chinese	Cardiomyopathy	[48,49]
R19P	<5%; cytosolic retention			Not specified		[43,49]
del F23	<2%; cytosolic retention	No	1	East Indian	Dilated cardiomyopathy	[42,49]
S28I	>10%	No	2	Saudi	Neonatal seizures	[53]
N32S	20.6%	Yes	2	Danish; Asian	Hepatic encephalopathy	[42]
R83L	<1% (fibroblasts); cytosolic retention	No	4	Not specified	Motor delay, peripheral neuropathy	[49,54]
A142S/R488H	cytosolic retention	No	1	Caucasian	Cardiomyopathy	[49]
R169Q		No	1	Not specified	Cardiomyopathy	[45]
R169W	0% (20% in cis with T440M); normal surface expression	Yes	?	Italian	Metabolic abnormalities; hypertrophic cardiomyopathy	[42,49,55]
M179L	74%	No	1 (Carrier)	Japanese	Reduced plasma carnitine in asymptomatic heterozygote	[41]
Y211C	N.D.	Yes	4	Moroccan/Cape Verde	Cardiomyopathy	[46]
T232M	>5% (fibroblasts), <1% (heterologous expression); normal surface expression	No	1	Anglo-Celtic	Diagnosed by newborn screening; asymptomatic	[48,49]

Mutation	Function	Recurrent	# Alleles	Ethnicity	Clinical History	References
G242V	0%; GFP-fusion protein had normal surface expression	No		Not specified	Cardiomyopathy	[49,55]
S280F	cytosolic retention	No	1	Not specified	Cardiomyopathy and ischemic heart disease	[56]
R282Q	normal surface expression	No	2	Hispanic	Metabolic abnormalities, hypotonia	[49]
W283C	N.D.	No	1 (carrier)	Japanese	Reduced plasma carnitine in asymptomatic heterozygote	[41]
W283R	normal surface expression		1	Caucasian	Unknown	[49,57]
R289X	<5%	No	1	Anglo-Celtic	Sibling with cardiomyopathy and death	[48]
A301D	2-3%; normal surface expression	No		Not specified	Hypoketotic hypoglycemia	[49,55]
W351R	normal surface expression	No		Not specified	Metabolic abnormalities	[49,55]
P398L	cytosolic retention	No	1	Caucasian	Diagnosed by newborn screening; asymptomatic	[49]
R399Q	<5%; normal surface expression			Not specified		[43,49]
T440M	0.5% - 20.5%; normal surface expression		6	Croatian/Caucasian/Turkish	Hypertrophic cardiomyopathy, anemia, hepatic encephalopathy, hypotonia	[42,49]
V446F	N.D.	No	1	Not specified	Unknown	[57]
Y447C	<1% - >10%; cytosolic retention	Yes	4	Saudi, Saudi	Cardiomyopathy; left ventricular hypertrophy	[23,49,53]

Mutation	Function	Recurrent	# Alleles	Ethnicity	Clinical History	References
Y449D	50% (fibroblasts of symptomatic heterozygote); 18% (heterologous expression in CHO cells); increased K_{Na}+	No	1	Not specified	Cardiac arrest	[23]
E452K	2-4%, increased K_{Na}+; normal surface expression	No	2	Not specified	Cardiomyopathy	[49,97]
S467C	11% (normal activity toward TEA)	Yes	4 (carrier)	Japanese	Reduced plasma carnitine in asymptomatic heterozygotes	[41]
T468R	<1% - 5.9%; normal surface expression	Yes	2	Anglo-Celtic	Coma; hypertrophic cardiomyopathy; newborn screening	[42,48,49]
S470F	7.1%	No	2	Asian	Coma	[42]
R471H	<10%	Yes	7	Turkish	Hepatic encephalopathy	[58]
P478L	0% (increased activity toward TEA)	No	1	Not specified	Metabolic abnormalities	[19,40]

* "Function" refers to carnitine transport activity as a percent of control (normal) activity unless otherwise noted.

Missense Mutations

A total of 33 nucleotide substitutions resulting in amino acid changes have been identified in patients with SCD [23,40-43,45,46,48,49,53-58]. The functional consequences of these mutations are variable, with some causing complete or near-complete loss of protein function, and some retaining residual carnitine transport activity. The range of residual carnitine transport by the mutant OCTN2 proteins varied from 0% to >20% of control when measured in heterologous expression systems, in cultured fibroblasts or mutant cell lines derived from affected patients. An exception is the M179L mutation, which was identified in a screen of subjects with below-normal serum carnitine levels ("candidate carriers" of OCTN2 loss-of-function mutations) [41]. M179L was shown to only minimally reduce carnitine transport by OCTN2 (to 74% of controls when expressed heterologously in HEK293 cells), and is therefore not likely to be the cause of the observed phenotype. Interestingly, the P478L mutation, which was the first missense mutation found in an SCD patient [40], was later shown to have differential effects on carnitine *vs*. organic cation transport function, *i.e*., P478L abolished carnitine transport, but significantly stimulated organic cation transport by OCTN2 [19]. This observation supported studies using human/rat chimeric OCTN2 proteins, which suggested distinct sites of interaction of OCTN2 with carnitine *vs*. organic cations [19]. There are, to date, no examples of natural mutations in OCTN2 that confer the inverse effect (*i.e.*, reduce organic cation transport but retain or stimulate carnitine transport),

which should be expected owing to the fact that the known OCTN2 mutations have been identified almost entirely in screens of patients with systemic carnitine deficiency.

Mouse Models of OCTN Deficiency

Two independently-derived mouse strains have provided additional information on the function of the OCTN transporters. As mentioned above, the juvenile visceral steatosis (*jvs*) mouse, a model of systemic carnitine deficiency, was discovered to be a spontaneous Octn2-null mutant, and aided in fine mapping of the human SCD gene [38,59]. These Octn2-deficient mice exhibit a phenotype similar to human SCD, including depletion of plasma carnitine and carnitine esters, fatty infiltration of the heart and visceral organs, and increased mortality. Importantly, this phenotype, like human SCD, is reversible with carnitine supplementation. The primary mechanism of carnitine depletion is through decreased renal tubular reabsorption of carnitine (seen as a decrease in net renal clearance), suggesting that the major role of OCTN2 is in the active reabsorption of carnitine in the kidney [60,61]. However, *jvs* mice also show reduced uptake of carnitine into several tissues, including brain, lung, heart, liver, kidney, spleen, and skeletal muscle, and reduced bioavailability of carnitine administered orally [2].

The del^{11}/del^{11} mouse, which carries a 450kb deletion on chromosome 11 including the OCTN locus, has a phenotype similar to the *jvs* mouse (cardiomyopathy, fatty infiltration of the liver, reduced life span), and complementation with a human OCTN2 transgene was able to correct these abnormalities. This work allowed for the observation that

carnitine transport influences hepatic triglyceride secretion and plasma triglycerides, and may influence risk for cardiovascular disease [62].

Mutations in OCTN2, but not in OCTN1, have been shown to cause systemic carnitine deficiency, suggesting that OCTN2 may play a more vital role in maintenance of total body carnitine levels (primarily through active tubular reabsorption of filtered carnitine). However, OCTN1 may play a complementary role in carnitine transport.
Studies on the *jvs* and del^{11}/del^{11} mice provide several lines of evidence that OCTN1 plays an additive or complementary role to OCTN2. First, *jvs* mice do not show a complete loss of OCTN-like activity: administration of supplemental carnitine was able to correct the abnormalities due to systemic carnitine deficiency, suggesting that some residual carnitine uptake activity exists in these animals; this may be due to compensatory activity of Octn1 (or other transporters). The del^{11}/del^{11} phenotype shared some similarities with the *jvs* mouse; however, unlike *jvs* mice, the abnormalities in the del^{11}/del^{11} mice were not improved with carnitine supplementation. These facts suggest that the residual carnitine transport capacity of Octn1 likely serves as a compensatory mechanism to maintain normal carnitine status in the *jvs* mice, and that these transporters play additive roles in carnitine homeostasis in healthy animals. It has also been shown in mice that Octn2 is at least partially responsible for the uptake of carnitine across the blood-brain barrier [63]; however, sodium-dependent uptake of carnitine and acetylcarnitine was only slightly reduced in *jvs* mice as measured by tissue-to-plasma concentration ratios, suggesting a role for OCTN1 in distribution of carnitine (and perhaps other substrates) into the CNS.

Importantly, heterozygous *jvs* or *del*^{11}/+ mice provide some insight into the possible phenotypic consequences of partial OCTN deficiency. Heterozygous *del*^{11}/+ mice showed two-fold elevations in hepatic triglyceride content and hepatic VLDL-triglyceride secretion. Heterozygous *jvs* mice display hepatic steatosis, cardiomyopathy, and increased mortality, in what may be considered a delayed or attenuated version of the *jvs* phenotype. As mentioned above, the mapping of the SCD locus in humans was made possible in part by the fact that heterozygous subjects exhibited a significant reduction in plasma carnitine levels and increased carnitine renal clearance. Also, similar to the *jvs* heterozygotes, human carriers of loss-of-function mutations in OCTN2 are at increased risk of late onset benign cardiac hypertrophy. This suggests that common genetic variants of the OCTN transporters, which result in partial defects in transport function, may produce pathological consequences of carnitine insufficiency.

Inflammatory Diseases

Both OCTN1 and OCTN2 have been implicated through genetic association studies as contributors to common inflammatory diseases, including rheumatoid arthritis, Crohn's disease, and ulcerative colitis. However, there remains a lack of consensus on the validity of these associations.

In a Japanese population, a SNP in the first intron of *SLC22A4* was reported to be associated with risk for rheumatoid arthritis, with an odds ratio of 1.98 under a recessive

model [64]. This SNP introduced a binding site for the transcription factor RUNX1 and led to suppression of OCTN1 transcription. The authors showed that OCTN1 is highly expressed in immunological and hematological tissues, and that this expression is enhanced by proinflammatory stimuli such as TNFα. An additional SNP in the *RUNX1* gene itself was also shown to be associated with rheumatoid arthritis in the same population. It was suggested that *RUNX1* acts to suppress OCTN1 expression in response to inflammation, but that excessive suppression of OCTN1 causes susceptibility to rheumatoid arthritis.

Polymorphisms in *SLC22A4* and *SLC22A5* have been associated with risk for inflammatory bowel disease, including both Crohn's disease and ulcerative colitis [14,65-71]. The region on chromosome 5q31 that includes the OCTN genes has been termed the *IBD5* locus after its initial linkage with Crohn's disease in a Canadian population of European descent [72]. The first follow-up association study identified 11 haplotype tagging SNPs in the region that were equally associated with Crohn's disease [73,74]. Numerous studies have replicated the association of *IBD5* tagging SNPs with both Crohn's disease and ulcerative colitis, making *IBD5* one of the few established true positive associations in human disease genetics [75-79]. Peltekova *et al*. proposed that a two-point haplotype, defined by a SNP in exon 9 of *SLC22A4* (c.1672C>T, which leads to the amino acid substitution L503F) and a SNP in the core promoter of *SLC22A5* (-207G>C, which disrupts a heat shock element), were the causative mutations at the *IBD5* locus, based on biochemical and statistical evidence [14]. In cellular assays, the OCTN1 L503F variant was shown to affect the substrate selectivity of the transporter,

causing an increase in transport activity toward some substrates (such as tetralkylammonium compounds) but decreased activity toward the endogenous substrate L-carnitine. The OCTN2 -207G>C polymorphism was shown to cause reduced transcriptional activity in response to heat shock and reduced binding to nuclear extracts. In their association study, which included 203 cases and 200 controls of European descent, this two-point OCTN haplotype was associated with Crohn's disease independently of the other SNPs in the *IBD5* risk haplotype, and was more significantly associated with disease risk than any of the markers that were previously used to define the *IBD5* risk haplotype. These findings were replicated in 300 cases and 190 controls in the same report.

The conclusion that these polymorphisms in the OCTN genes are causative for Crohn's disease has not been supported consistently through replication. Although some reports have replicated the association of these OCTN variants with Crohn's disease, most have investigated only these two variants, and did not include other *IBD5* risk markers [65,68-71]. Other investigators have shown poor association or lack of independence of these variants from other SNPs in the risk haplotype background (*i.e.*, weaker association of the OCTN variants, *vs*. other *IBD5* tagging SNPs, with disease risk) [66,67]. Recently, in the largest study to date of *IBD5* association with Crohn's disease, Silverberg *et al*. showed that variants in several other candidate genes in the *IBD5* region exhibited equivalent statistical evidence to that for the OCTN SNPs in association with Crohn's, and specifically rejected the -207G>C polymorphism in *SLC22A5* as a causal variant [80].

The mechanism by which the OCTN transporters might contribute to risk for inflammatory diseases has been inadequately addressed. Peltekova *et al*. proposed that the carnitine transport activity of the OCTNs may be important for intestinal integrity by contributing to fatty acid oxidation in intestinal epithelium or to oxygen burst-mediated pathogen killing; alternatively, it was suggested that the substrate selectivity variant L503F may cause reduced uptake of physiological compounds but increased uptake of xenobiotic toxins, such as bacterial waste products [14]. After the discovery that OCTN1 is a high-affinity transporter of the antioxidant ergothioneine [12], Tauber *et al*. showed that OCTN1-L503F was more efficient in ergothioneine transport compared with the reference OCTN1 and suggested that this variant may contribute to disease risk by increasing uptake of ergothioneine into immunological tissues such as $CD14^+$ macrophages, which play a key role in the pathogenesis of Crohn's disease [81]. Lamhonwah *et al*. observed that the OCTN1-L503F variant protein shares a 9-amino acid epitope with *Campylobacter jejuni* and *Mycobacterium paratuberculosis*, bacteria that have been implicated in the development of Crohn's disease, and suggested that cross-reactivity with this epitope may lead to development of an autoimmune response to OCTN1-L503F in the intestine [82].

The *IBD5* locus, in addition to the OCTN genes, includes a host of strong candidate genes for inflammatory diseases: interferon regulatory factor 1 (*IRF1*), prolyl 4-hydroxylase (*P4HA2*) and a cytokine-rich gene cluster (*IL3*, *IL4*, *IL5*, and *IL13*). Given that the putative mechanism(s) for the role of OCTNs in inflammatory disease risk are currently speculative, and the statistical evidence for the OCTNs having a causal role in

Crohn's disease has been mostly discredited, it would be prudent to continue to consider these other candidate genes as potentially containing the causative variants at the *IBD5* locus, or as co-contributors to disease risk.

Pharmacological Significance of OCTNs

Secondary Systemic Carnitine Deficiency

As listed in Table 1.1, emetine, cephalosporins, pivalic acid and valproic acid have been shown to inhibit L-carnitine transport via OCTN1 and OCTN2 in heterologous expression systems [17,18,27]. These drugs have been implicated in a carnitine deficiency syndrome, termed secondary carnitine deficiency, which resembles the primary SCD syndrome in many respects [83-88]. This adverse effect is likely due to the inhibition of renal tubular reabsorption of L-carnitine and short-chain acylcarnitines by the OCTN transporters, though other mechanisms have been proposed, such as depletion of carnitine due to formation of acyl esters with these drugs [34].

Role of OCTNs in Drug Disposition

Studies of organic cation transport in the kidney have been investigated using a variety of methods including isolated renal tubules and plasma membrane vesicles. These studies have demonstrated that there are multiple organic cation transporters on the basolateral

membrane (BLM) and brush border membrane (BBM) of renal tubules that work in concert to drive the net secretion and/or reabsorption of organic cations (Figure 1.2). These organic cation transport mechanisms have been found primarily in the proximal tubules and to a lesser extent in the distal tubules. Transport of organic cations across the BLM of renal tubules occurs by a facilitated transport mechanism driven by the transmembrane potential difference of the renal tubule epithelium. The classical organic cation transporters (OCTs) are believed to be the molecular entities responsible for this activity, with OCT2 being the predominant basolateral organic cation uptake transporter in human kidney. Efflux of organic cations across the apical membrane is thought to occur by a separate transporter-mediated process, either by the primary active efflux pump, P-glycoprotein (which generally transports bulky hydrophobic cationic compounds) or by an organic cation-H^+ exchange mechanism for small, hydrophilic organic cations (see Figure 1.2) [3,89-94]. The molecular identity of the organic cation-H^+ exchanger is currently unclear; the novel organic cation transporters (OCTN1 and OCTN2) and the more recently-discovered multidrug and toxin exclusion transporters (MATE1 and MATE2) are likely candidates, as all of these are highly expressed in kidney and have been shown to transport the typical cation TEA in a manner consistent with H^+ exchange. It is possible that all of these transporters participate in apical organic cation efflux, with differential importance depending on the substrate under question. Among the apical organic cation transporters, only OCTN2 has been shown to influence renal organic cation (TEA) secretion *in vivo* [3]; however, appropriate animal models to study the *in vivo* role of OCTN1, MATE1 and MATE2 in renal organic cation secretion do not yet exist. The functional characteristics of the OCTNs suggest that they (perhaps

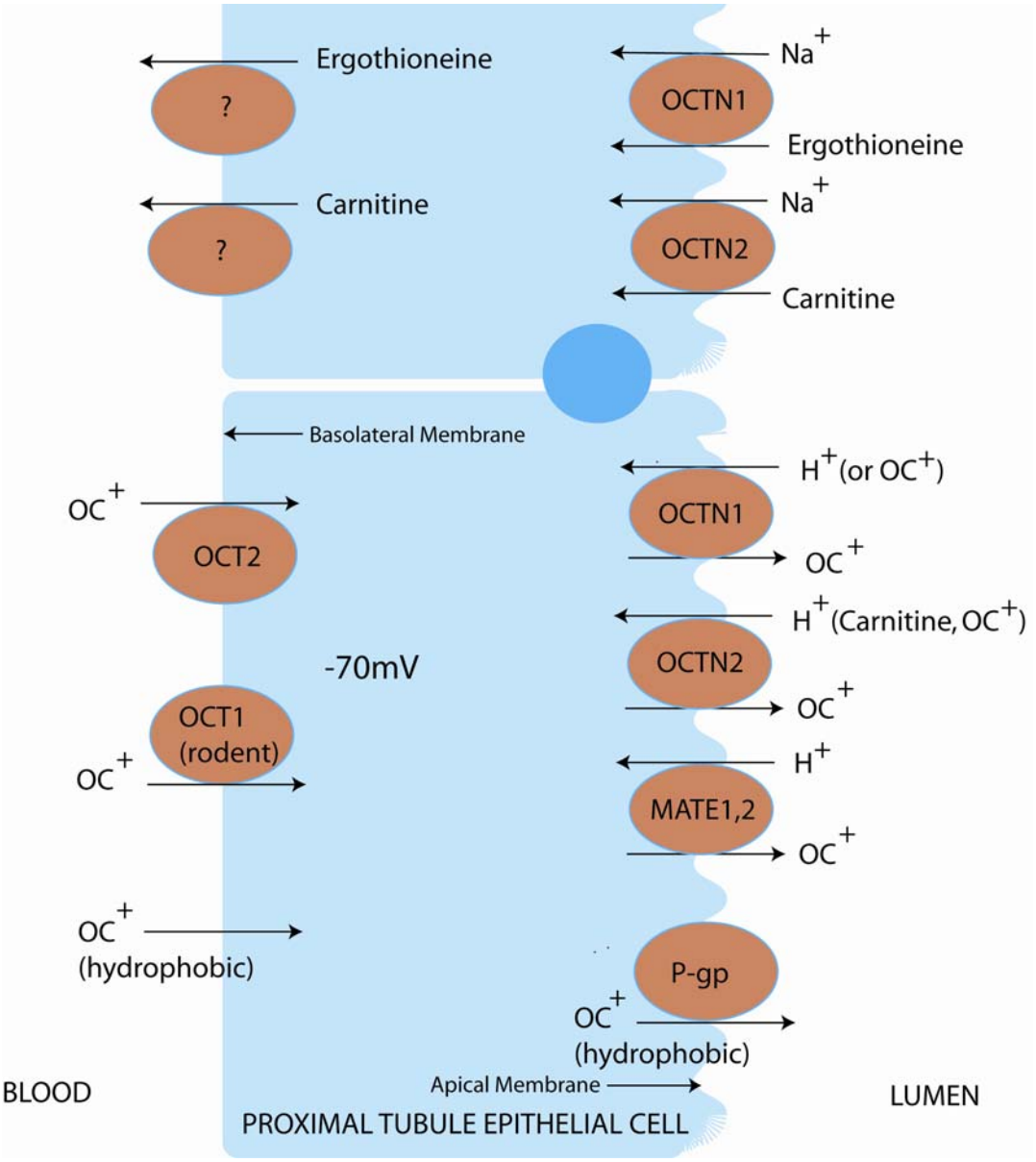

Figure 1.2. Function of OCTNs in renal transport of nutrients and xenobiotics. OCTN transporters are expressed at the brush border membrane in renal proximal tubules, where they act as Na^+-dependent reabsorptive transporters for nutrients such as ergothioneine (OCTN1) and carnitine (OCTN2). OCTNs may also facilitate the efflux of xenobiotic organic cations across the brush border membrane. In humans, OCT2 is the primary transporter responsible for uptake of hydrophilic organic cations (OC^+) across the basolateral membrane, in a process driven by the inside-negative membrane potential. In rodents, OCT1 and OCT2 are expressed to a similar degree in kidney. Efflux of organic cations across the luminal (apical) membrane for excretion occurs by an organic cation-H^+ exchange mechanism and several transporters are suspected to be involved in apical OC^+ transport, including OCTN1 and OCTN2 in addition to the recently cloned MATE transporters. Membrane-permeable organic cations may not require facilitative transport across the basolateral membrane, and these hydrophobic cations may be substrates for the apical efflux pump P-glycoprotein (P-gp).

along with other transporters such as the MATE transporters) contribute to the apical organic cation-proton antiport activity in the kidney.

In addition to its effects on carnitine status (described above), Octn2 deficiency also causes alterations in the pharmacokinetics of the prototypical organic cation, TEA, leading to decreased renal secretory clearance and decreased uptake of TEA into brain, lung, liver and spleen [3]. Thus, OCTN2 likely participates in the active tubular secretion of organic cations, as well as their distribution into tissues.

Although a decrease in renal secretory clearance of TEA was found in *jvs* mice, some residual active secretion was still observed, indicating that other transporters (including Octn1) likely compensate for the loss of Octn2 at the apical membrane of renal tubular epithelia. Deconvolution of the phenotypes of *jvs* and del^{11}/del^{11} mice to estimate the effect of Octn1 on organic cation disposition is not possible at this time, as the del^{11}/del^{11} mice have not been studied with respect to the pharmacokinetics of TEA or other xenobiotics. Again, the lack of a specific OCTN1-knockout mouse model has limited our understanding of the physiological and pharmacological roles of this transporter.

Common Genetic Variants of OCTNs

OCTN1

There is a small number of reports describing functional genetic variation in the *SLC22A4* gene. As described above, the nonsynonymous polymorphism OCTN1-L503F has been shown to influence the substrate selectivity of the transporter, leading to increased affinity for tetraalkylammonium compounds, but decreased affinity for other substrates, such as L-carnitine and several cationic drugs [14]. This variant was also shown to transport L-ergothioneine with increased efficiency compared with the reference OCTN1 [81]. Two additional nonsynonymous variants of OCTN1, found in a Japanese population, have been functionally characterized: the common polymorphism T306I was shown to have no effect on the transport of TEA, while the variant G462E showed complete loss of TEA transport activity [95]. Both the T306I and G462E variants were found to have similar expression at the plasma membrane compared with the reference OCTN1, suggesting that the effects of the G462E substitution on OCTN1 activity occur at the level of mature protein (*e.g.*, through loss of substrate recognition or protein-level regulation of the transporter). The allele frequency of the G462E variant is unknown, but is expected to be rare or absent outside of the Japanese population, as this variant has not been reported elsewhere.

Additional information on sequence variation in the OCTN1 gene is available. Resequencing of the genomic region of *SLC22A4* in 48 unrelated Japanese individuals revealed 54 SNPs and four deletion or tandem repeat polymorphisms in the OCTN1 gene [96]. Of these polymorphisms, thirty had not been previously identified by the SNP Consortium (NCBI SNP). Two nonsynonymous SNPs (T306I and G462E) and one synonymous SNP (c.1182G>C, Thr394Thr) were detected in the coding region, two of

which (Thr306Ile and c.1182G>C/Thr394Thr) had been previously submitted to the NCBI SNP Database (NCBI SNP ID rs272893 and rs272879, respectively). The remaining SNPs and deletion polymorphisms appeared in 3' and 5' flanking regions and introns of *SLC22A4*. The NCBI SNP database includes 29 entries in the *SLC22A4* gene, including 16 SNPs in the coding region: seven synonymous SNPs and nine nonsynonymous SNPs. Six of the protein sequence-altering variants on NCBI SNP were discovered by our group; further detail on the allele frequencies and functional characteristics of these variants is given in Chapter 3.

OCTN2

A polymorphism in the promoter of *SLC22A5* was discovered as part of a fine-mapping effort to identify the causative variants in the IBD5 haplotype, which contributes to risk for Crohn's disease [14]. This variant disrupts a consensus sequence for a heat shock element, and was shown in cellular assays to influence transcriptional activity in response to heat shock or treatment with arachidonic acid *via* reporter assays. The significance of this variant with respect to gene function *in vivo* is unclear.

The genomic region of *SLC22A5* has been screened by direct sequencing in a sample of 48 Japanese volunteers to identify more common genetic variants in this gene [96]. These authors identified thirty eight SNPs and two insertion/deletion (indel) polymorphisms in *SLC22A5*. Two synonymous SNPs (c.285C>T Leu95Leu and

c.807G>A Leu269Leu) were detected in the coding region, which had been reported previously on a public SNP database (NCBI SNP, ID rs2631365 and rs274558). The remaining SNPs and indel polymorphisms appeared in introns, 3'-untranslated regions and 5'-flanking regions of the *SLC22A5* gene. No nonsynonymous SNPs were identified in this report. The allele frequencies of these SNPs have not been estimated, and functional effects of these polymorphisms, if any, have not been investigated. A total of 27 variants, including 15 coding region variants, have been reported on NCBI SNP. Three missense mutations (G484V, R488H, and Q523R) are reported in this database; however, the allele frequencies and functional characteristics of these variants are unknown. Apart from the -207G>C promoter polymorphism, there have been no published reports of more common genetic polymorphisms in the coding region of *SLC22A5*. Unpublished data from our laboratory demonstrate very low amino acid variation in OCTN2 in an ethnically diverse American population, suggesting that this gene is under a high degree of selective pressure. Our group has identified eight nonsynonymous variants of *SLC22A5*; the population-specific allele frequencies and functional characteristics of these variants are described in Chapter 4.

Conclusions

The OCTNs (esp. OCTN2) are critical for maintenance of systemic carnitine levels *via* active tubular reabsorption in the kidney, and the two transporters likely comprise the cellular carnitine uptake system. The apical localization of OCTNs allows for the

sodium-dependent uptake of filtered L-carnitine and short-chain acylcarnitine esters from the tubular lumen into renal tubular epithelia. Mutations in OCTN2 have been shown to be responsible for primary systemic carnitine deficiency, a rare Mendelian disorder. However, transient or moderate deficiencies in expression or function of OCTN transporters may lead to mild reductions in systemic or cellular carnitine levels. Insufficient L-carnitine levels may in turn result in elevations in triglycerides, an independent risk factor for heart disease, as well as other sequelae of lipid metabolism disorders, such as hepatic steatosis and cardiomyopathy. Several commonly used drugs have been shown to cause a secondary carnitine deficiency, which may be explained by interaction of these drugs with OCTN transporters. Genetic variation in OCTNs could lead to increased susceptibility to this toxicity, as well as to variability in disposition of drugs that are substrates of these transporters. While both OCTN1 and OCTN2 are multifunctional transporters of carnitine and organic cations, data suggest that OCTN2 plays a greater role in carnitine transport. Recent data have suggested that OCTN1 may play an analogous role in the disposition of ergothioneine.

Significant progress has been made over the past decade in defining the substrate specificities, transport mechanisms, and localization of organic cation transporters in the kidney. While all of the cloned human organic cation transporters are expressed to some degree in the kidney, studies in knockout mice suggest that OCT1 and OCT3 do not significantly contribute to renal elimination of model organic cations. Studies in *jvs* mice have revealed that OCTN2 contributes to renal secretion and reabsorption of organic

cations (*e.g.*, TEA) and carnitine, respectively. The tissue distribution and transport mechanisms of OCTs and OCTNs suggest that they work in concert to drive the net secretion of organic cations across the renal tubule epithelium, with OCT2 mediating the first step (uptake of organic cations across the basolateral membrane) and OCTN1 and OCTN2 (in addition to the MATE transporters) mediating the second step (efflux of organic cations across the brush border membrane into the tubular lumen). Future studies of OCTN1 knockout mice will help to define the role of this transporter in the renal handling of organic cations. In addition, further examination of substrate specificities and identification of isoform-specific inhibitors will aid in dissecting the relative role of each transporter in renal drug disposition.

Genetic variants of OCTN1 and OCTN2 have been identified in human populations. Studies in heterologous expression systems have directly examined the effects of many of these variants on transporter function and provide valuable information on structure-function relationships. In the case of OCTN2, rare nonsense and missense mutations have been associated with primary carnitine deficiency, but information on more common genetic variants that may influence transport function is scarce. In contrast, a common variant of OCTN1, L503F, which has altered interactions with inhibitors and substrates has been identified. This variant and other variants of the OCTN transporters may alter protein function and contribute to interindividual differences in the renal handling of organic cation drugs. Further studies examining the pharmacokinetic phenotypes of individuals harboring genetic variants that change transport function may

help to define the roles of each transporter in renal elimination of drugs. Furthermore, such studies may help identify particular genetic variants that may lead to susceptibility to drug toxicities resulting from drug-drug interactions.

Summary of Chapters

The overall goal of this dissertation research was to explore the roles of the OCTN transporters in human physiology and drug disposition, using a sequence-based (or reverse-genetics) approach in humans, with the hope of identifying common genetic polymorphisms in these genes that are predictive of carnitine homeostasis or drug disposition in man.

In *Chapter 2*, we describe the results of our resequencing effort, in which we discovered both common and rare genetic variants in the OCTN genes in a large, ethnically diverse population. We also describe the high degree of linkage disequilibrium across these two genes, and estimate the structure and allele frequencies of the common haplotypes at the OCTN locus in each ethnic group.

In *Chapters 3 and 4*, we investigate the functional significance of the protein-altering genetic variants of OCTN1 and OCTN2. Using heterologous expression systems, we interrogate common and rare variants of the OCTN transporters in terms of their transport function and subcellular localization, and show that several rare variants and some relatively common variants have significantly altered function compared with the reference proteins.

Following this, we apply the knowledge gained from our biochemical assays of OCTN genetic variants to guide clinical studies of OCTN genetic variation.

Traditionally, genetic association studies have begun with a known phenotype (e.g., aberrant response to an anti-hypertensive drug) followed by discovery of the variant protein and gene responsible for the phenotype. With the sequence of the human genome, it is now possible to carry out genetic studies in the reverse manner, i.e., genotype-to-phenotype studies. By identifying genetic variants with functional significance in cellular assays, and then phenotyping individuals who carry these interesting variants, we determine a) whether there is a genetic basis for variation in the phenotype, and b) learn specifically about the influence of individual variants or haplotypes of particular genes on the phenotype. In *Chapter 5*, we use a genotype-to-phenotype strategy to study the role of OCTN1 in the disposition of the anticonvulsant drug, gabapentin, and in *Chapter 6*, we use a similar approach to study the roles of the OCTNs in carnitine homeostasis and lipid metabolism.

The work presented in Chapters 2 through 6, describing the functional genomics of the OCTN transporters, was performed as part of the Pharmacogenetics of Membrane Transporters project. This ongoing project has investigated genetic variation in more than 50 membrane transporter genes in the SLC and ABC superfamilies. Our research group functionally characterized 88 protein-altering variants in 11 transporter genes in the *SLC22A*, *SLC28A*, and *SLC29A* families. Although most variants have no effect on transporter function, a large fraction (25%) result in reduced function or complete loss of function, and several common variants result in differences in substrate selectivity. In

Chapter 7, we perform a meta-analysis of the characteristic features of functionally significant genetic variants in membrane transporter genes, and discuss the implications of these findings for pharmacogenetics, population genetics, and the genetics of complex disease.

Chapter 8 concludes the dissertation with a discussion of our findings, and some suggestions for future directions in this field.

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CHAPTER 2

HAPLOTYPE STRUCTURE AND ETHNIC-SPECIFIC ALLELE FREQUENCIES AT THE OCTN LOCUS[1]

Introduction

The novel organic cation transporters (OCTNs) are multispecific transporters of organic cations and zwitterions [1-13]. A unique property of the OCTNs is that they are bifunctional, transporting some substrates (such as the zwitterionic nutrients, carnitine and ergothioneine) in a Na^+-dependent manner, and others (such as the typical organic cation, tetraethylammonium) in a pH-dependent, Na^+-independent manner [1-3,11,14,15]. The OCTNs are expressed ubiquitously, with high expression in kidney [3,8,9]. Expression of OCTN1 (*SLC22A4*) and OCTN2 (*SLC22A5*) at the apical membrane of renal tubular epithelial cells is thought to contribute to the active reabsorption of nutrients, such as carnitine and ergothioneine [5,15-17]; in contrast, transport of organic cations by the OCTNs is thought to occur in the secretory direction *in vivo*. OCTN2 has been shown to contribute to active secretion of organic cations in mouse models [11]; however, knockout mouse models of OCTN1 do not currently exist. In humans, OCTN2 has been shown to be essential for maintenance of systemic carnitine levels through its role in tubular reabsorption of carnitine [5,16,17]. The influence of OCTN1 and OCTN2 in the disposition of xenobiotics has not been adequately addressed in humans.

[1] Elements of this chapter have been published previously: Urban, T.J., N. Risch, and K.M. Giacomini. "Haplotype structure and ethnic-specific allele frequencies at the OCTN locus: implications for the genetics of Crohn disease." *Inflamm Bowel Dis J* 11(1): 78-9, 2005. Reprinted with permission.

In addition to their putative roles in nutrient and drug disposition, in the past several years, genetic variants in the OCTN transporter genes have been implicated as contributing to risk for Crohn's disease. The identification of disease-associated loci by genome-wide screening and subsequent fine-mapping efforts have provided us with a number of promising candidate genes for this disorder. Among them, the *IBD5* locus, a cytokine-rich gene cluster on chromosome 5q31, has consistently shown to be associated with risk for Crohn's disease [14,18-22]; however, attempts at identifying the causative gene(s) in this region have been fruitless until recently. In a pivotal paper, Peltekova *et al.* presented evidence that two SNPs in the *SLC22A4* and *SLC22A5* genes are associated with Crohn's disease [14]. They suggested that these SNPs (one an amino acid substitution (L503F) in OCTN1, the other (-207G>C) disrupting a heat-shock element in the promoter region of OCTN2) are not only associated with risk for Crohn's disease, but are causative mutations at the *IBD5* locus conferring increased risk in patients of European descent. However, our knowledge of the nature of genetic variation at the OCTN locus, and the degree of linkage disequilibrium (LD) in this region, is currently of only crude detail. Thus, it is possible that the variants identified by Peltekova *et al.* are in LD with other potentially functional variants in these or other nearby genes, and that these linked polymorphisms may contribute to disease risk in addition to, or to the exclusion of, the L503F/-207G>C polymorphisms.

Recently, as part of a large pharmacogenomics project focused on membrane transporters [23], we identified SNPs in *SLC22A4* and *SLC22A5* in 276 DNA samples from ethnically diverse human populations (see www.PharmGKB.org). This deep resequencing effort

provides a rich source of information on the structure of genetic variation at the OCTN locus, and identifies polymorphisms in the coding region that may have significant functional consequences. The results of this screen have implications for pharmacogenetics studies of the influence of OCTN transporters on drug and endobiotic disposition, as well as for the genetics of Crohn's disease.

Methods

Variant Identification

Genomic DNA samples were collected from unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. *SLC22A4* and *SLC22A5* variants were identified by direct sequencing of genomic DNA as previously described [23] from an ethnically diverse population of 270 individuals: 80 African-Americans, 80 European-Americans, 60 Asian-Americans (50 Chinese-Americans and 10 Japanese-Americans), and 50 Mexican-Americans. The reference cDNA sequences of *SLC22A4* and *SLC22A5* were obtained from GenBank (http://www.ncbi.nlm.nih.gov, accession numbers NM_003059 and NM_003060, respectively). Primers were designed manually to span the exons and 50–200 bp of flanking intronic sequence per exon. The primer sequences can be found at http://www.pharmgkb.org. Variant positions are relative to the ATG start site and are based on the reference cDNA sequence of *SLC22A4* or *SLC22A5*.

Haplotype Estimation

Haplotypes were estimated using the PHASE algorithm [24] after excluding singleton variant positions (*i.e.*, positions that were polymorphic on only one chromosome in our sample). Analysis was performed individually on each ethnic subpopulation of our sample to reduce error in haplotype estimation resulting from allele frequency differences among ethnic groups. For the purpose of summarizing haplotype data, intronic or synonymous SNPs were not included in the table if they were redundant with (*i.e.*, perfectly or near-perfectly correlated with) others more likely to influence gene function.

Results

In total, we discovered 22 variants in *SLC22A4* and 20 variants in *SLC22A5*. The majority of these variants were extremely rare: 22 of the variants were singletons (*i.e.*, were found on only one of 276 chromosomes in our sample) or doubletons (*i.e.*, were found on only two chromosomes). Thus, obtaining precise estimates of allele frequency for these variants is not possible, and they provide little value for linkage disequilibrium mapping of the region. Further, most of the variants identified were in noncoding (intronic) regions in which a prior hypothesis for functional significance was relatively weak. For LD mapping and haplotype estimation, we focused on more common variants or variants with suggestive evidence for functional significance.

We discovered several SNPs with particularly high frequencies in African American or Chinese populations that could potentially reduce OCTN function (Table 2.1). These SNPs result in nonconservative amino acid substitutions at evolutionarily conserved sites in the protein (OCTN2-L144F and OCTN2-P549S), or disruption of a consensus splice

Table 2.1. Summary of genetic variation in the coding region of OCTN1 and OCTN2.

			Allele Frequency				
	Nucleotide Change	Location	AA	EA	AS	ME	PA
OCTN1			n=80	n=80	n=60	n=50	n=12
	C917T	**T306I**	**0.269**	**0.338**	**0.642**	**0.240**	**0.417**
	IVS6+5G>A	**Intron (splice donor)**	**0.050**	**0.056**	**0.283**	**0.010**	**0.083**
	C1507T	**L503F**	**0.088**	**0.412**	**0.000**	**0.230**	**0.000**
OCTN2							
	C430T	**L144F**	**0.075**	**0.000**	**0.000**	**0.010**	**0.000**
	C1645T	**P549S**	**0.100**	**0.000**	**0.000**	**0.000**	**0.000**
	C1721T	**3' UTR**	**0.013**	**0.094**	**0.000**	**0.030**	**0.000**

Allele frequencies within each ethnic group are indicated. AA, African American; EA, European American; AS, Asian (Chinese-American); ME, Mexican; PA, Pacific Islander. n = number of individuals in each ethnic group. Additional SNPs less likely to be functionally significant are not shown, but are available at www.PharmGKB.org.

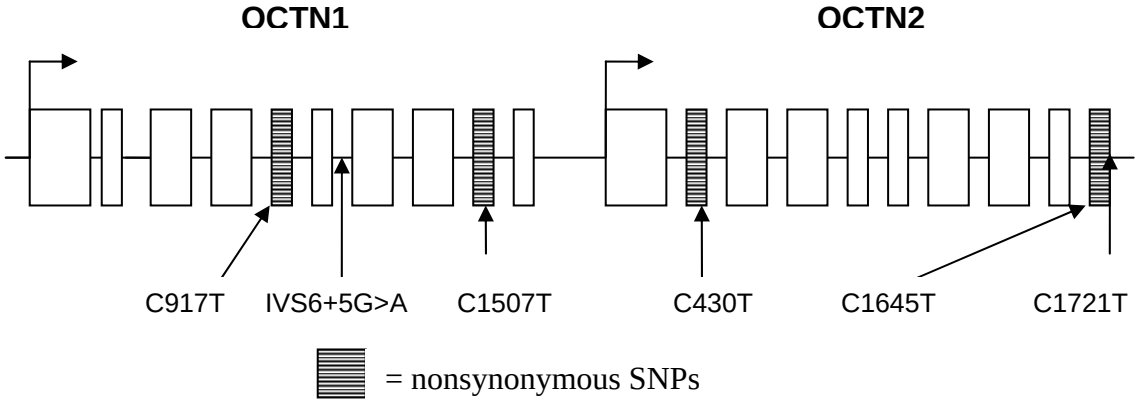

Figure 2.1. Genomic structure and genetic polymorphism at the OCTN locus. OCTN1 and OCTN2 are located in a head-to-tail arrangement on human chromosome 5q31. The intergenic region is approximately 30 kb, and the two genes span a total of 100 kb. Common SNPs that are predicted to be deleterious are indicated by arrows.

donor sequence (OCTN1-IVS6+5 G>A) [25]. In addition, we found a SNP in the 3'-untranslated region of OCTN2 (C1721T) that was uniquely common in the European population.

We also identified common nonsynonymous polymorphisms in the OCTN1 coding region. One amino acid substitution, OCTN1-T306I, had been previously published on a public database (dbSNP); however, ethnic-specific allele frequencies for this variant were not available. We found that this variant is pan-ethnic, with varying allele frequencies among the major ethnic groups studied, and highest allele frequency (64.2%) in the Asian American subset of our sample (Table 2.1). In contrast, we found that the OCTN1-L503F variant described by Peltekova et al. [14] was more population-specific, with high (41.2%) allele frequency in the European American sample, and lower frequency in the Mexican American (23.0%) and African American (8.8%) samples, consistent with European admixture in these ethnic groups.

The *SLC22A4* and *SLC22A5* genes are in close physical proximity on human chromosome 5q31, with the two genes spanning a total distance of approximately 100 kb (Figure 2.1). We found a correspondingly high degree of linkage disequilibrium among polymorphisms across these two genes, with four haplotypes accounting for approximately 80% of haplotype diversity across the OCTN locus (Table 2.2). Given the high degree of LD between *SLC22A4* and *SLC22A5*, and the apparent structural and functional redundancy between the two genes, it is likely that functional polymorphisms in one of these genes may be either suppressed or enhanced by other functional

Table 2.2. Selected OCTN haplotypes.

	Haplotype						Haplotype Frequency (%)					Significant SNPs
							AA	EA	AS	ME	PA	
OCTN*1	C	G	C	C	C	C	36.1	16.2	25.8	47.9	41.7	OCTN1-306T
OCTN*2	**T**	G	C	C	C	C	19.6	20.6	33.3	21.3	41.7	OCTN1-306I
OCTN*3	C	G	**T**	C	C	C	8.2	37.5	0.0	23.4	0.0	OCTN1-503F
OCTN*1B	C	G	C	C	**T**	C	10.1	0.0	0.0	0.0	0.0	OCTN2-549S
OCTN*1C	C	G	C	**T**	C	C	7.6	0.0	0.0	0.0	0.0	OCTN2-144F
OCTN*2B	**T**	*A*	C	C	C	C	5.1	5.0	25.0	1.1	0.0	OCTN1-306I IVS6+5G>A
OCTN*3B	C	G	**T**	C	C	*T*	0.0	2.5	0.0	1.1	0.0	OCTN1-503F OCTN2 C1721T (3'UTR)

SNP positions, from left to right, correspond to SNPs indicated in Figure 2.1. Four major haplotypes (OCTN*1-OCTN*3, OCTN*2B) account for ~80% of haplotype diversity at the OCTN locus. OCTN*3 corresponds to the common Crohn disease-associated *IBD5* haplotype. The rarer haplotype (OCTN*3B) contains the risk-associated L503F substitution in OCTN1 as well as a SNP in the 3' UTR of OCTN2. OCTN*1B and OCTN*1C contain nonconservative (*i.e.*, chemically distant) amino acid substitutions (P549S and L144F) that occur at evolutionarily conserved sites in OCTN2. OCTN*2B contains the IVS6+5G>A transition, which disrupts a consensus splice donor sequence at an intron-exon boundary.

polymorphisms in the other gene. We should therefore consider the possibility that other SNPs in LD with the common OCTN1-L503F and OCTN2(-207)G>C polymorphisms may complement these disease-associated SNPs. Among these are both high-frequency SNPs (such as the amino acid substitution T306I in OCTN1) as well as rarer SNPs (such as C1721T in the 3'-UTR of OCTN2) found on the risk-associated haplotype background. Of course, the possible contribution of SNPs at unassayed sites should be recognized; however, our thorough survey of the coding region of these genes gives us confidence that we have identified the most likely functional polymorphisms in the OCTN genes.

Discussion

Discovery of genetic variants that predispose to risk for disease or drug response phenotype is frequently achieved through genetic association studies. However, prior to the availability of HapMap data, these associations were often performed in the absence of adequate knowledge of the structure of genetic variation in the genes or regions under study, making it difficult to conclude a causative or functional role of disease-associated SNPs (*i.e.*, independent of other potential causative mutations in linkage with the associated SNPs). Heterogeneity in allele frequencies across ethnic populations also complicates genetic association studies as variants that associate with a phenotype in one ethnic group may not occur in other ethnic populations, or may differ in the degree of LD with unassayed causative polymorphisms in other populations.

The *SLC22A4* and *SLC22A5* genes, which are located at the IBD5 locus, have been suggested to contribute to risk for Crohn's disease based on genetic association and biochemical studies of two disease-associated variants in these genes. These SNPs (one an amino acid substitution (L503F) in *SLC22A4*, the other (-207G>C) disrupting a heat-shock element in the promoter region of *SLC22A5*) were not only associated with risk for Crohn's disease, but were proposed as causative mutations conferring increased risk in patients of European descent [14]. It was found that these two SNPs constitute a two-point risk haplotype for Crohn's disease that shows strong association regardless of genotype at other SNPs in the *IBD5* haplotype background.

In addition to statistical evidence from their association analysis, Peltekova *et al.* demonstrate that both SNPs cause reduced function in OCTN activity *in vitro* [14]. While functional assays are a powerful complement to genetic association studies, additional information is needed to establish that reduced function variants in a particular gene are responsible for a clinical phenotype. Without such information, it is possible that the clinical syndrome may be explained by unassayed polymorphisms in nearby genes that are in linkage disequilibrium with the disease-associated SNPs. Independent studies suggesting the involvement of the gene in the pathophysiology of the disease are also helpful in assigning a causal role to a gene. In the case of the OCTNs, there was no prior hypothesis for a role in Crohn's disease. In fact, genetic mutations in OCTN2 (in both humans and mice) cause systemic carnitine deficiency, a disorder characterized by skeletal muscle, cardiac muscle and liver disease, but not inflammatory or intestinal

disease [16,26]. Thus, we believe there is reason to accumulate additional evidence that variants in the OCTN genes are causative for Crohn's disease.

The best case for establishing the role of OCTN genes in risk for Crohn's disease would be to demonstrate that other SNPs or mutations in OCTN genes are positively associated with Crohn's disease in other ethnic groups [27]. We discovered several SNPs with particularly high frequencies in African American or Chinese populations that could potentially reduce OCTN function (Table 2.1). Association of these SNPs with Crohn's disease in African American and Chinese populations would provide strong evidence that genetic mutations in OCTN contribute to susceptibility to Crohn's disease.

Our data suggest that OCTN1-L503F, which was associated with risk for Crohn's disease [14], is a Caucasian-specific allele (Table 2.1). That is, the 503F allele is found at high prevalence in Caucasians and at reduced prevalence in African Americans and Mexicans, consistent with Caucasian admixture in those populations. Our data also indicate that there are other SNPs in linkage disequilibrium with OCTN1-L503F, which may have effects on gene function (see Figure 2.1 and Table 2.2). Among these is the high-frequency SNP, OCTN1-T306I (C917T), as well as the rarer SNP, C1721T in the 3' UTR of *SLC22A5*, found on the risk-associated haplotype background. Although neither of these SNPs was reported by Peltekova *et al*. [14], both could contribute to the Crohn's disease phenotype.

In addition to their potential involvement in inflammatory diseases, the OCTN transporters are known to be important for carnitine transport, particularly in the kidney, where efficient reabsorption of carnitine by the OCTNs (particularly OCTN2) is required to maintain adequate levels of carnitine in the body [5]. Although the severe carnitine transport defect present in patients with systemic carnitine deficiency is likely to be very rare, common genetic variants may produce partial defects in carnitine transport and thus may produce pathological consequences of carnitine insufficiency. The OCTNs are also thought to be involved in drug disposition, particularly in the active tubular secretion of drugs by the kidney [13]. The common variants identified in this screen may therefore be useful candidate SNPs in genetic association studies of a variety of phenotypes related to nutrient and drug disposition. In addition to the common SNPs described here, several rare protein sequence-altering mutations in *SLC22A4* and *SLC22A5* were discovered in this screen. The identities and functional characteristics of these mutations will be discussed in Chapters 3 and 4.

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CHAPTER 3

FUNCTIONAL EFFECTS OF PROTEIN SEQUENCE POLYMORPHISMS IN THE ORGANIC CATION/ERGOTHIONEINE TRANSPORTER OCTN1 (*SLC22A4*)

Introduction

The novel organic cation transporter OCTN1 (*SLC22A4*) was originally characterized as a multispecific pH-dependent transporter of organic cations, including the prototypical cation tetraethylammonium (TEA) as well as several clinically used drugs, such as quinidine, verapamil, and pyrilamine [1,2]. Other members of the SLC22 family include the close homolog OCTN2, as well as the organic cation transporters (OCTs) and organic anion transporters (OATs), many of which have been shown to be critical determinants of drug and nutrient disposition *in vivo* [3-9]. OCTN1 has a broad tissue distribution, with highest expression in kidney and erythrocytes, and lower expression in heart, skeletal muscle, intestine, and other tissues [2,10]. The high expression of OCTN1 in renal proximal tubules suggests that it is primarily important in the active secretion of substrate drugs [11]. In addition, OCTN1 expression in intestine may contribute to intestinal drug absorption, and distribution of substrate drugs into various tissues (heart, skeletal muscle, erythrocytes) may also depend on functional OCTN1 expression.

Recently, Grundemann *et al.* showed that the natural substrate of OCTN1 is L-ergothioneine, an antioxidant whose physiological significance is poorly understood [10].

In contrast to organic cation transport, L-ergothioneine transport by OCTN1 is Na^+-dependent. Ergothioneine is concentrated in erythrocytes, which is consistent with high OCTN1 expression in these cells, and suggests a role for ergothioneine in RBC function (e.g., as a free radical scavenger in erythrocytes, as has been suggested) [10]. Identification of human genetic variants of OCTN1 with partial or complete loss of ergothioneine transport function may aid in elucidation of the physiological importance of this compound.

Because of the potential importance of OCTN1 in the disposition of xeniobiotics, including drugs and ergothioneine, it is reasonable to inquire whether genetic variation in OCTN1 may influence its function, expression and subcellular localization. Genetic variants in other members of the *SLC22A* family have been identified and are thought to contribute to interindividual variability in the disposition of their substrates [12-19].

Two functionally significant genetic variants of OCTN1 have been identified. Peltekova *et al.* showed that a high-frequency amino acid substitution, L503F, resulted in altered substrate specificity, with increased affinity for tetraalkylammonium compounds, but decreased affinity for L-carnitine and other substrates [20]. These authors were primarily interested in OCTN1 variants as susceptibility alleles for Crohn's disease, and found that OCTN1-L503F, in combination with a polymorphism in the promoter of OCTN2 (OCTN2-207G>C), comprised a haplotype that was associated with risk for Crohn's disease. However, attempts to replicate this association have yielded conflicting results [21,22]. An amino acid substitution, G462E, that resulted in complete loss of TEA

transport activity was identified [23]; however, the allele frequency of this variant was not reported.

In this study, we comprehensively examined the coding region of OCTN1 by direct sequencing to identify all OCTN1 protein sequence variants in an ethnically diverse sample of healthy volunteers. We then assessed the functional significance of these variants by investigating their transport function, expression, and subcellular localization. We discovered several missense and one nonsense mutation with significant functional alterations from the reference OCTN1. In addition to the previously described L503F polymorphism, these rarer variants may contribute to interindividual variability in the disposition of organic cations and provide new information on OCTN1 structure-function relationships.

Materials and Methods

Chemicals

[^{14}C]-Betaine (57 mCi/mmol) and [^{14}C]-tetraethylammonium (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Flp-In-293 Cells, Lipofectamine 2000 and pcDNA5/FRT expression vector were purchased from Invitrogen (Carlsbad, CA, USA). BCA Protein Assay Kit was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Unlabeled betaine, L-ergothioneine and tetraethylammonium bromide were purchased from Sigma (St. Louis, MO, USA). Cell culture supplies were purchased from the Cell Culture Facility (UCSF, San Francisco, CA, USA). All other chemicals were of reagent grade and commercially available.

Identification of SLC22A4 Variants

Genomic DNA samples were collected from unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. *SLC22A4* variants were identified by direct sequencing of genomic DNA as previously described [24] from an ethnically diverse population of 270 individuals: 80 African-Americans, 80 European-Americans, 60 Asian-Americans (50 Chinese-Americans and 10 Japanese-Americans), and 50 Mexican-Americans. The reference cDNA sequence of *SLC22A4* was obtained from GenBank (http://www.ncbi.nlm.nih.gov, accession number NM_003059). Primers were designed manually to span the exons and 50–200 bp of flanking intronic sequence per exon. The primer sequences can be found at http://www.pharmgkb.org. Variant positions are relative to the ATG start site and are based on the reference cDNA sequence of *SLC22A4.*

Construction of OCTN1 Variants

Human OCTN1 cDNA (GenBank accession number NM_003059) was subcloned into the mammalian expression vector pcDNA5/FRT to obtain OCTN1-reference, which corresponds to the highest-frequency amino acid sequence in all ethnic groups. Variant cDNA clones were constructed by site-directed mutagenesis of the reference clone using Pfu Turbo DNA polymerase (Invitrogen, Carlsbad, CA, USA). The reference sequence clone was used as the template for mutagenesis, except for the reverse-mutagenesis studies (see Figure 3.2b) in which the corresponding variant cDNA was mutagenized to restore the reference sequence. Sequences of variant cDNA clones were confirmed by

direct sequencing, and the full cDNA of each variant was sequenced to verify that only the intended mutation was introduced.

Transport Studies

HEK-293 cells were routinely cultured in Dulbecco's modified Eagle's medium (DME H-21) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum. For transport studies, cells were seeded onto 24-well poly-D-lysine coated plates (BD Discovery Labware, Bedford, MA, USA) at a density of 1.5 X 10^5 cells per well in antibiotic-free media and grown for 24 h. Cells were then transfected with 1 μg OCTN1-reference or variant DNA and 3 μg Lipofectamine 2000 per well following the manufacturer's protocol. Cells were incubated for 48 h and assayed for activity by measurement of cellular uptake of radiolabeled probe substrates.

For the common variants of OCTN1, detailed functional studies were performed using stably transfected Flp-In-293 cells generated according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). The Flp-In system generates stable cell lines by Flp recombinase-mediated integration, creating single-copy, site-specific stable integrants. This allows comparison among allelic variants of a gene while avoiding concerns about gene dose-dependent or integration site-dependent differences in expression level. Briefly, cells were plated at a density of 6 X 10^5 cells per well in six-well plates and incubated overnight in antibiotic-free media. At 24 hours, cells were transfected with 0.4 μg OCTN1-reference or variant cDNA, 3.6 μg pOG44 DNA, and 20 μg Lipofectamine 2000 per well. Two days after transfection, cells were split 1:4 into six-well plates and selected for stable transfectants by addition of hygromycin B (75 μg/mL) to the standard

growth media. Media were replaced every two to three days. After two weeks under selective media, multiple colonies of stable cells were obtained for each variant. Since the Flp-In system generates single, site-specific integration of the transfected cDNA, these colonies were pooled and expanded to obtain the OCTN1 reference and variant cell lines used for functional studies.

Uptake studies were performed by first washing the cells with warm Hank's Buffered Salt Solution (HBSS) and incubating at 37ºC for 15 min, after which the wash buffer was removed and replaced with HBSS containing either [^{14}C]-betaine (10 μM) or [^{14}C]-tetraethylammonium (10 μM). Cells were returned to 37ºC for 10 min. Uptake was terminated by rapidly removing the extracellular media and washing three times with ice-cold HBSS. Cells were lysed in 1 mL 0.1 N NaOH/0.1% SDS and 800 μL of the lysate was added to 3 mL Ecolite scintillation fluid (ICN Biomedicals, Costa Mesa, CA, USA). Intracellular radioactivity was determined by scintillation counting and normalized to per-well protein content as measured using the BCA protein assay. Results were expressed as the percent of activity of the OCTN1-reference wells.

Kinetics studies were performed as described above, with varying concentrations of unlabeled substrate (betaine or TEA) added to the uptake buffer. Rates of uptake (V), expressed as pmol/min/mg protein, were fit to the equation:

$$V = V_{max}*[S]/(K_m + [S]) + K_o*[S]$$

where K_o represents the first-order rate constant for non-OCTN1-mediated uptake, [S] is the substrate concentration, and V_{max} and K_m are the Michaelis-Menten kinetic parameters.

Inhibition studies were performed in a similar manner, with varying concentrations of L-ergothioneine added to an uptake buffer containing a single concentration (10 μM) of either [^{14}C]-betaine or [^{14}C]-TEA. Rates of uptake were expressed as percent of uptake in the absence of inhibitor for each variant, and fit to the equation:

$$V = V_0 * [I]/(IC_{50} + [I])$$

where V_0 is the uptake rate in the absence of inhibitor (fixed at 100%), [I] is the inhibitor concentration, and IC_{50} is the concentration of inhibitor that produces 50% inhibition. IC_{50} values were converted to K_i values by the Cheng-Prusoff identity [25],

$$K_i = (IC_{50})/(1 + [S]/ K_m)$$

where [S] is the concentration of the probe substrate, and K_m is the Michaelis-Menten constant for the probe substrate.

Statistical differences between variant and reference OCTN1 activity were assessed by Student's t-test. $p<0.05$ was used as the threshold for significance.

GFP Fusion Constructs and Confocal Microscopy

To visualize subcellular localization of OCTN1 variants, variant cDNA constructs were subcloned in frame with green fluorescent protein (GFP) at the carboxy-terminus of OCTN1 the pcDNA5/FRT expression vector. The GFP fusion constructs thus obtained were used to generate stable cell lines using the Flp-In system as described above. For localization studies, cells were plated on poly-D-lysine coated glass coverslips (BD Biosciences, San Jose, CA, USA) in 24-well plates at a density of 1.5 X 10^5 cells per well. At 24 hours after seeding, cells were washed three times in HBSS, followed by incubation with 5 μg/mL AlexaFluor 594 wheat germ agglutinin and 1 μM Hoechst 33342 in HBSS for 10 minutes. Cells were then washed three times with blank HBSS to remove the stain, after which cells were fixed with 4% paraformaldahyde in HBSS for 15 minutes. Coverslips were then removed from the 24-well plate and mounted in Vectashield antifade solution (Vector Technologies, Inc., Burlingame, CA, USA) on glass microscope slides. Cells were visualized by confocal microscopy using a Zeiss LSM 510 laser scanning microscope.

Determination of mRNA Expression

Stable cell lines expressing GFP-tagged OCTN1 variants were used to assay for mRNA or protein expression differences among variants. Total RNA was extracted from 5 X 10^6 cells per line using the RNeasy Mini Kit (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed using Superscript III Reverse Transcriptase (Invitrogen) according to the manufacturer's protocols. Resulting cDNA was used as template for quantitative PCR using Taqman primers and probes specific to OCTN1 or GAPDH (Applied Biosystems,

Foster City, CA, USA) using 100 ng cDNA per reaction in triplicate wells (20 μL/reaction). OCTN1 expression was normalized to GAPDH and expressed as relative expression *vs*. the control (mock-transfected) cell line.

Results

Discovery of Novel OCTN1 Variants

We discovered a total of 22 nucleotide substitutions in the survey region of the *SLC22A4* gene, which included the coding region and 50-200 base pairs of flanking intronic sequence per exon. Only eight of these variants had been identified previously. The nucleotide positions and allele frequencies of the variants are shown in Tables 3.1 and 3.2. Of the 22 variants identified, 11 occurred in the coding region, and 11 were found in the noncoding regions of the sequence interrogated in our screen. Variants in noncoding regions included a polymorphism in the 5'-UTR, nine variants in the intronic sequence, and one variant in the 3' UTR of SLC22A4. The intronic variant IVS6(+5) G>A, which occurs five bp downstream of the last nucleotide in exon six, was polymorphic in all ethnic groups, but occurred at a particularly high allele frequency (~28%) in the Asian American subset of our sample. This variant disrupts a consensus splice donor sequence, and may influence *SLC22A4* mRNA splicing.

Of the 11 coding region variants, five were synonymous, *i.e.*, they did not alter the amino acid sequence of the OCTN1 protein. The six non-synonymous variants included five amino acid substitutions, as well as a nonsense mutation (c.844C>T) that results in a premature stop codon at amino acid position 282 (R282X), and is predicted to result in a protein product that is truncated approximately in half relative to the reference OCTN1.

Table 3.1. Synonymous and noncoding variants in *SLC22A4*

dbSNP ID#	CDS Position	Nucleotide Substitution	Functional Effect	Allele Frequency AA (n=160)*	EA (n=160)	AS (n=120)	ME (n=100)
Synonymous Variants:							
rs11568502	339	C->T	Cys113Cys	0	0.006	0	0
rs11568511	465	C->T	Leu155Leu	0	0.006	0	0
rs11568498	606	C->T	Ile202Ile	0.000	0	0.008	0
rs272879	1182	G->C	Thr394Thr	0.369	0.369	0.717	0.250
rs12777	1413	C->G	Gly471Gly	0.025	0.056	0	0.040
Noncoding Variants:							
rs11568501	5' UTR (-10)	A->T	unknown	0.013	0	0	0
rs11568499	IVS2 (-7)	T->C	unknown	0	0.006	0	0
rs270600	IVS4 (+37)	C->T	unknown	0.0	0	0.042	0
rs273909	IVS5 (-96)	A->G	unknown	0.006	0.069	0.017	0.020
rs11568507	IVS5 (-93)	G->T	unknown	0.069	0.013	0	0
rs2304081	IVS6 (+5)	G->A	Splice Donor	0.050	0.056	0.283	0.010
rs11568504	IVS7 (+19)	T->C	unknown	0.013	0	0	0
rs11568506	IVS7 (-51)	G->A	unknown	0	0.013	0	0.020
rs11568505	IVS7 (-23)	T->A	unknown	0.006	0	0	0
rs272878	IVS8 (+76)	C->T	unknown	0.219	0.287	0.392	0.23
rs11568508	3' UTR (+3)	A->G	unknown	0.006	0	0	0

ID numbers in bold indicate variants that were not identified prior to this study.CDS (nucleotide) position is given relative to the "A" in the ATG start codon for OCTN1. AA, African American; EA, European American; AS, Asian American; ME, Mexican American. *Number of chromosomes screened.

Table 3.2. Protein-altering variants of *SLC22A4*

dbSNP ID#	Amino Acid Substitution	CDS Position	Nucleotide Substitution	Grantham Score	EC/EU	Allele Frequency AA (n=160)*	EA (n=160)	AS (n=120)	ME (n=100)
Rare Variants:									
rs11568509	Val159Met	475	G->A	21	EC	0.006	0	0	0
rs11568510	Asp165Gly	494	A->G	94	EC	0	0	0.008	0
rs11568500	Met205Ile	615	G->A	10	EC	0.006	0	0	0
rs11568503	Arg282STOP	844	C->T	N/A	EC	0.006	0	0	0
Common Variants:									
rs272893	Thr306Ile	917	C->T	89	EC	0.269	0.338	0.642	0.240
rs1050152	Leu503Phe	1507	C->T	22	EU	0.088	0.412	0.000	0.230

ID numbers in bold indicate variants that were not identified prior to this study. CDS (nucleotide) position is given relative to the "A" in the ATG start codon for OCTN1. Grantham score is a measure of chemical distance between alternative amino acid residues. EC, evolutionarily conserved; EU, evolutionarily unconserved; AA, African American; EA, European American; AS, Asian American; ME, Mexican American. *Number of chromosomes screened.

Four of the non-synonymous variants were singletons, *i.e.*, they were found on only one chromosome in our sample. Two were highly polymorphic: c.917C>T (T306I), which was found in all ethnic groups at varying allele frequency in the different groups; and c.1507C>T (L503F), with an allele frequency of 42% in the European American sample, and lower allele frequencies in other ethnic groups (Table 3.2).

The locations of the nonsynonymous variants in the predicted secondary structure of OCTN1 are shown in Figure 3.1. Three of the variants (c.475G>A, V159M; c.615G>A, M205I; and c.1507C>T, L503F) occur in the transmembrane domains (TMDs) of OCTN1. The c.494A>G (D165G) variant occurs at the interface between the second TMD and the intracellular loop. The common c.917C>T variant leads to the T306I substitution in the large extracellular loop between TMDs 5 and 6, and disrupts a predicted N-glycosylation site on the extracellular surface of the protein.

Functional Effects of OCTN1 Nonsynonymous Variants

The nonsynonymous variants of OCTN1 were constructed by site-directed mutagenesis of the reference (wild-type) cDNA and assayed for functional effects by transient transfection in HEK-293 cells, using the typical cation tetraethylammonium (TEA) and the zwitterion betaine (trimethylglycine) as probe substrates (Figure 3.2a). Among the rare variants, a high degree of functional polymorphism was observed, with several loss-of-function alleles and one hypomorph. The nucleotide substitution D165G and the nonsense mutation R282X showed a complete loss of transport activity toward both substrates: cells transfected with D165G or R282X showed uptake similar to control

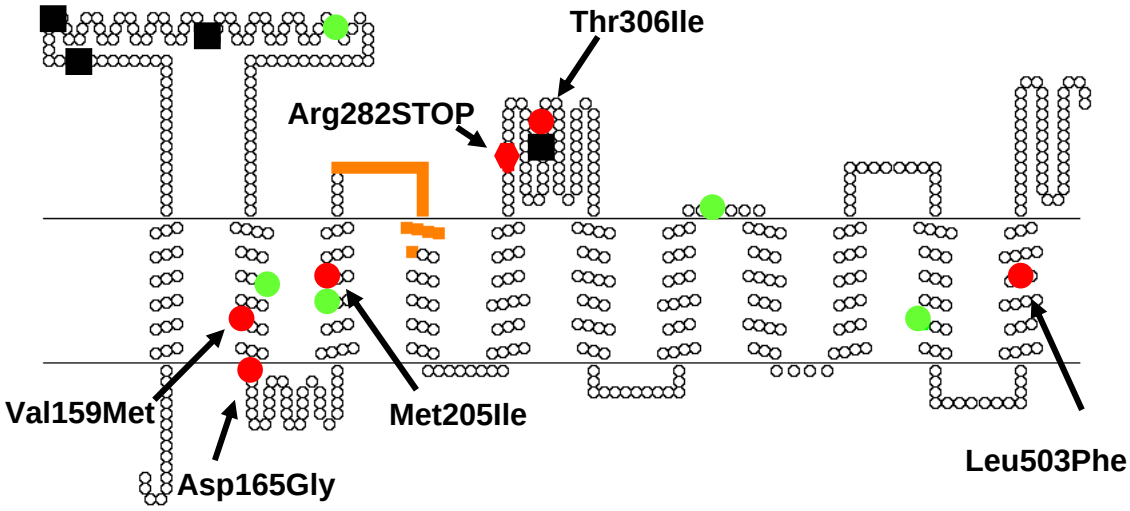

Figure 3.1. Location of nonsynonymous variants in the predicted secondary structure of OCTN1. Amino acid substitutions are shown as red circles. The nonsense mutation R282X is shown as a red hexagon. Protein-altering substitutions are indicated by arrows. Synonymous variant sites are shown in green. Predicted N-glycosylation sites are shown as black squares, and an ATP-binding motif is shown in orange. The transmembrane topology diagram was rendered using TOPO2 (SJ Johns (UCSF, San Francisco) and RC 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).

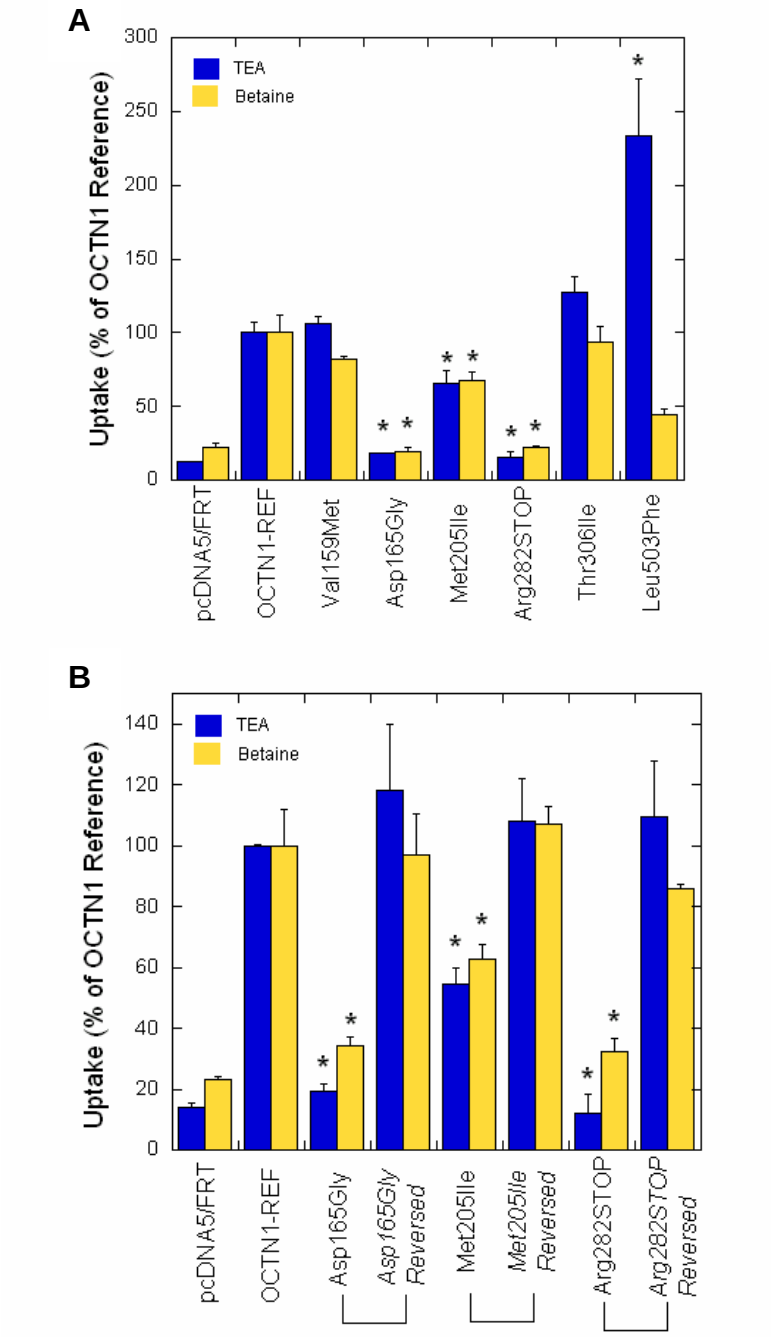

Figure 3.2. Functional screen of protein-altering variants of OCTN1. Variants of OCTN1 were expressed by transient transfection in HEK-293 cells and assayed for activity by measurement of uptake of radiolabeled probe substrates (10 µM [^{14}C]-TEA or [^{14}C]-betaine) at 10 min. (A) Functional activity of all nonsynonymous variants was assessed. (B) Variant cDNAs for those variants found to have altered function were reversed to the reference sequence by site-directed mutagenesis and assayed for restoration of wild-type activity. Mock-transfected cells (pcDNA5/FRT) serve as the negative control. Results are expressed as percent of the reference sequence clone (OCTN1-REF) after correcting for per-well protein content. Values represent mean +/- S.D. from triplicate wells in a representative experiment. *$p<0.05$ *vs.* OCTN1-Reference.

(mock-transfected) cells for both TEA and betaine. One of the variants, M205I, was hypomorphic, showing approximately a 50% reduction in specific transport activity for both substrates when compared with the reference OCTN1 (*i.e.*, after subtracting background uptake). The V159M variant showed no difference in activity compared with the reference OCTN1.

When the reduced-function variant cDNA clones were reverted to the reference sequence by site-directed mutagenesis, wild-type activity was restored, confirming that the functional deficiencies observed in the screen were due to the intended mutations (Figure 3.2b).

The common polymorphism T306I demonstrated activity similar to the reference OCTN1 for both substrates (Figure 3.2a). In contrast, the common L503F variant showed a marked substrate selectivity difference from the reference OCTN1, with a 2-fold increase in TEA transport, despite wild-type activity toward betaine. Substrate selectivity differences between the reference OCTN1 and the L503F variant had been recognized previously [20]. As shown in Figure 3.3, when kinetics of TEA transport were compared between the reference and L503F variant, L503F showed a significantly reduced K_m (310 +/- 13 μM vs. 1800 +/- 96 μM for the reference OCTN1) along with a slightly increased V_{max} (663 +/- 9 pmol/min/mg protein vs. 497 +/- 11 pmol/min/mg protein for the reference OCTN1). Betaine transport by L503F exhibited kinetics similar to the reference OCTN1. The T306I variant did not show any appreciable difference in the

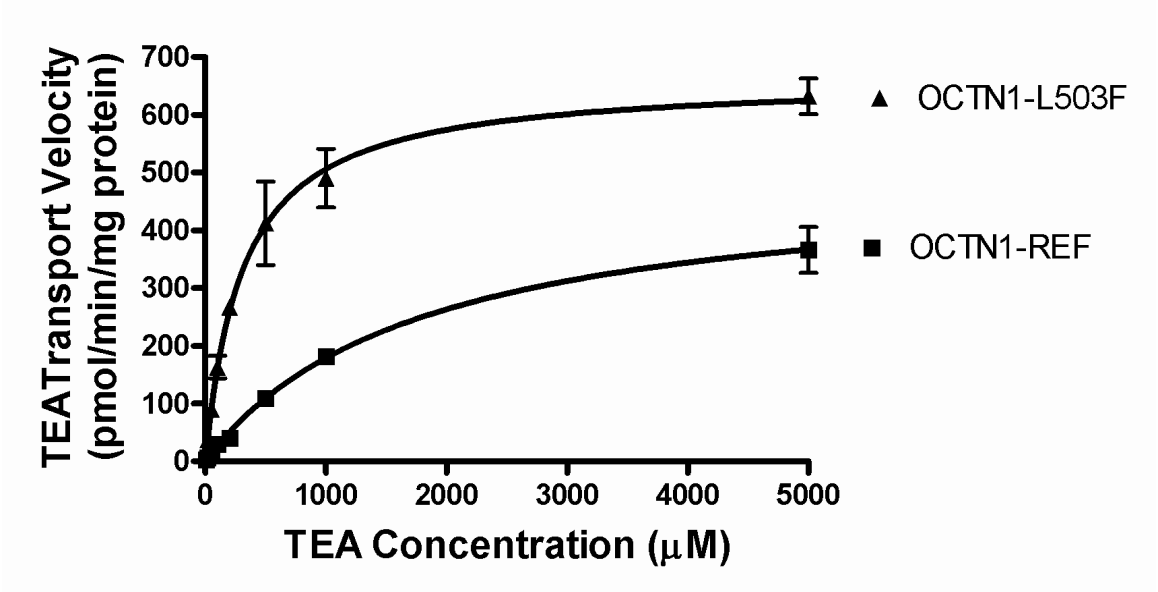

Figure 3.3. Kinetics of TEA transport by reference OCTN1 and L503F variant. HEK-293 cells stably expressing either OCTN1-Reference (squares) or OCTN1-L503F (triangles) were incubated in the presence of 10 μM [^{14}C]-TEA and varying concentrations of unlabeled compound for 10 minutes. Data were fit to the Michaelis-Menten equation after subtracting background uptake. Results are expressed as pmol/min/mg protein. OCTN1-REF: K_m = 1800 +/- 96 μM, V_{max} = 497 +/- 11 pmol/min/mg protein; OCTN1-L503F: K_m = 310 +/- 13 μM, V_{max} = 663 +/- 8.8 pmol/min/mg protein. Data points represent the mean +/- S.D. from triplicate wells in a representative experiment.

transport kinetics of either substrate compared with the reference OCTN1 (data not shown).

Since the antioxidant L-ergothioneine is thought to be the endogenous substrate of OCTN1, we compared the common nonsynonymous variants of OCTN1 in terms of their interaction with this compound. As seen in Figure 3.4, the inhibition kinetics of L-ergothioneine showed no differences among the common OCTN1 variants. The K_i for inhibition of betaine transport was nearly identical among the reference, T306I and L503F variants. The estimate for L-ergothioneine K_i in this study was approximately 9 μM, which is similar to the K_m for L-ergothioneine transport in previous reports [10,26].

Expression and Localization of Nonsynonymous OCTN1 Variants

To further characterize the functional consequences of natural variation in the OCTN1 amino acid sequence, and to determine the mechanism for altered transport function of these variants, we stably transfected HEK-293 cells with OCTN1 variant cDNA clones tagged at the C-terminus with green fluorescent protein (GFP). When visualized by confocal microscopy, the subcellular localization of the OCTN1-GFP fusion protein was similar among all of the OCTN1 amino acid substitutions, with the OCTN1-GFP-derived signal restricted to the plasma membrane (Figure 3.5). The loss-of-function variant D165G and the hypomorph M205I showed somewhat reduced fluorescence at the plasma membrane, suggesting a slight reduction in protein expression level, but did not appear to alter trafficking or localization of OCTN1. Analysis of protein expression by Western blot showed a similar trend, with equivalent levels of OCTN1-GFP expression among all the variants, but slightly lower expression for D165G and M205I (data not shown). The

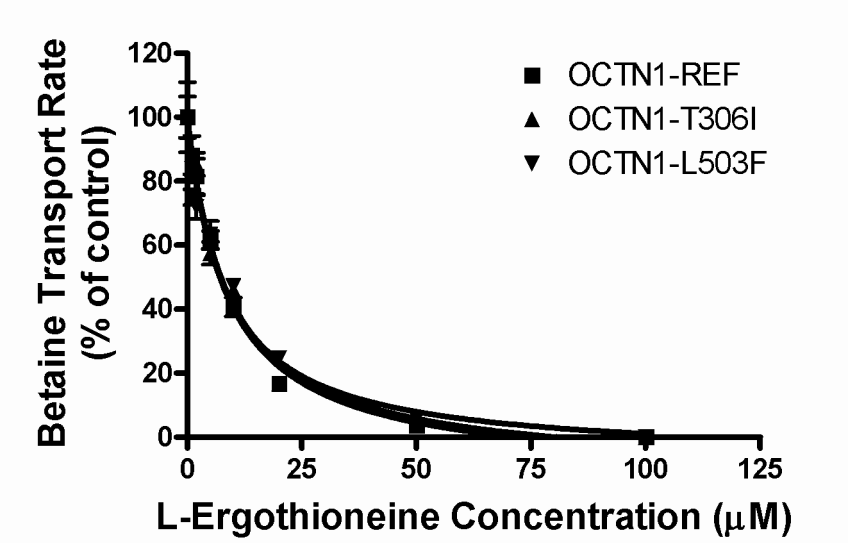

Figure 3.4. Ergothioneine inhibition kinetics of common OCTN1 polymorphisms. HEK-293 cells stably expressing the common variants of OCTN1 (OCTN1-Reference, OCTN1-T306I, and OCTN-L503F) were incubated with 10 μM [^{14}C]-betaine in the presence or absence of various concentrations of unlabeled L-ergothioneine for 10 minutes. Results are expressed as a percent of uptake in the absence of L-ergothioneine for each variant. Data points represent the mean +/- S.D. from triplicate wells in a representative experiment.

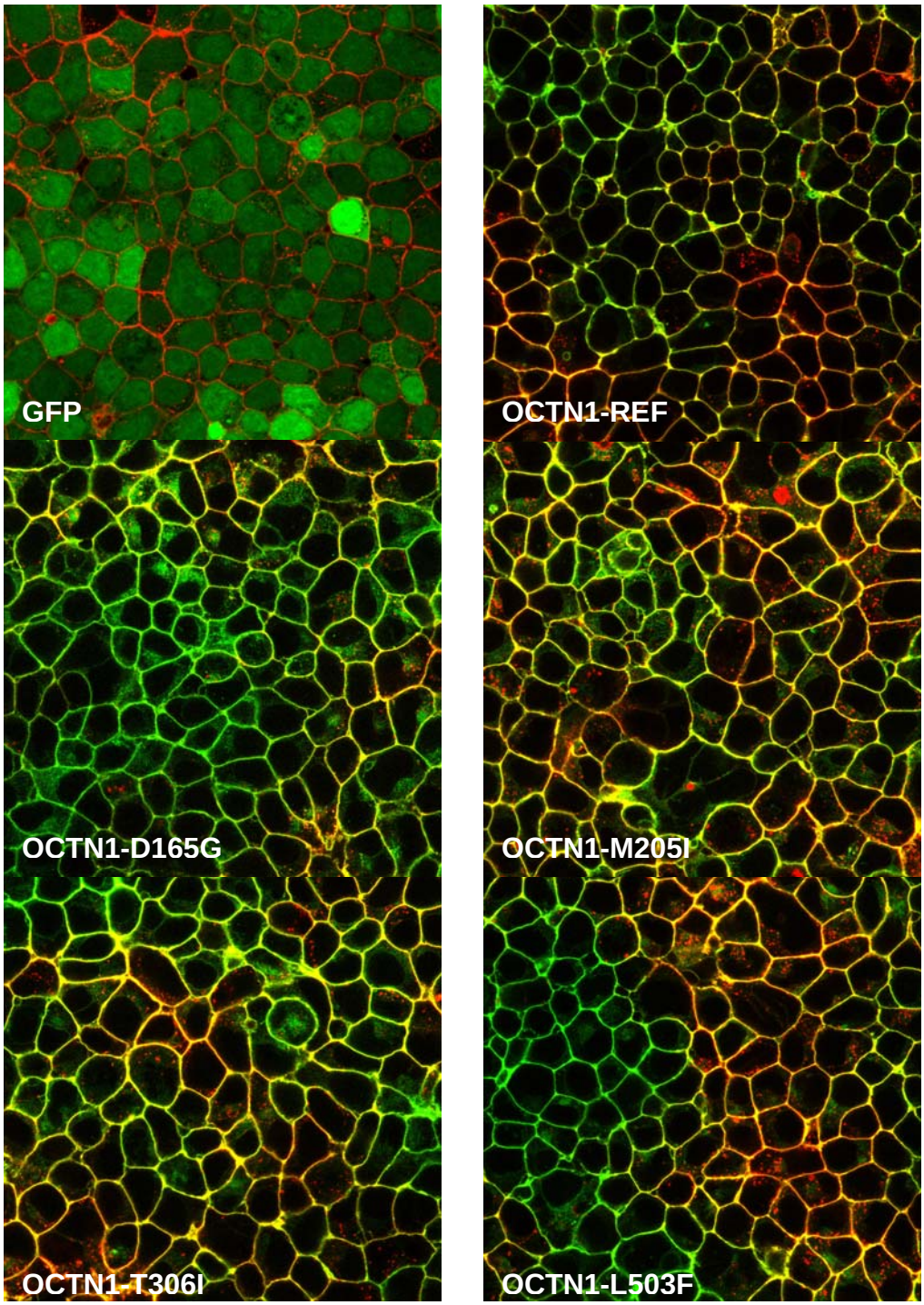

Figure 3.5. Subcellular localization of OCTN1 variants. OCTN1 variant GFP-fusion constructs were stably expressed in HEK-293 cells and visualized by confocal microscopy. The plasma membrane was stained using AlexaFluor 594-labeled wheat germ agglutinin, shown in red. OCTN1-GFP protein is shown in green. Colocalization of OCTN1-GFP-derived signal and that from the plasma membrane stain is shown in yellow.

mRNA expression level of these variants was assessed by quantitative real-time PCR (Figure 3.6). OCTN1 mRNA expression in the stably-transfected cells was much higher than that in mock-transfected cells, with a ~60,000-fold higher expression for the OCTN1-transfected cells *vs*. control. There was little difference among the variants with respect to OCTN1 mRNA expression, with the reduced-function variants D165G and M205I showing slightly lower mRNA expression *vs*. the reference OCTN1.

Discussion

Variation in genes responsible for the absorption, distribution, metabolism and elimination of drugs and toxic xenobiotics has the potential to explain a large amount of population variability in the clinical pharmacokinetics of many drugs. Particularly in the case of multispecific drug transporters and metabolizing enzymes, where a single protein may be an important determinant of the disposition of a wide array of xenobiotics, knowledge of the allelic spectrum of a "pharmacokinetic" gene may ultimately aid prediction of *in vivo* drug disposition of all, or most, substrates of that protein. The clinical relevance of functional polymorphism in genes encoding drug metabolizing enzymes (*e.g.*, CYP2D6) is now widely appreciated, and a similar appreciation for the role of drug transporters (*e.g.*, P-glycoprotein and other organic cation transporters) in drug response is now emerging [19,27-29].

Here, we report on a systematic screen of coding region variation in the multispecific organic cation transporter, OCTN1. Due to the large sample size and ethnic diversity of our sample, we were able both to identify rare, ethnic-specific alleles and to reliably

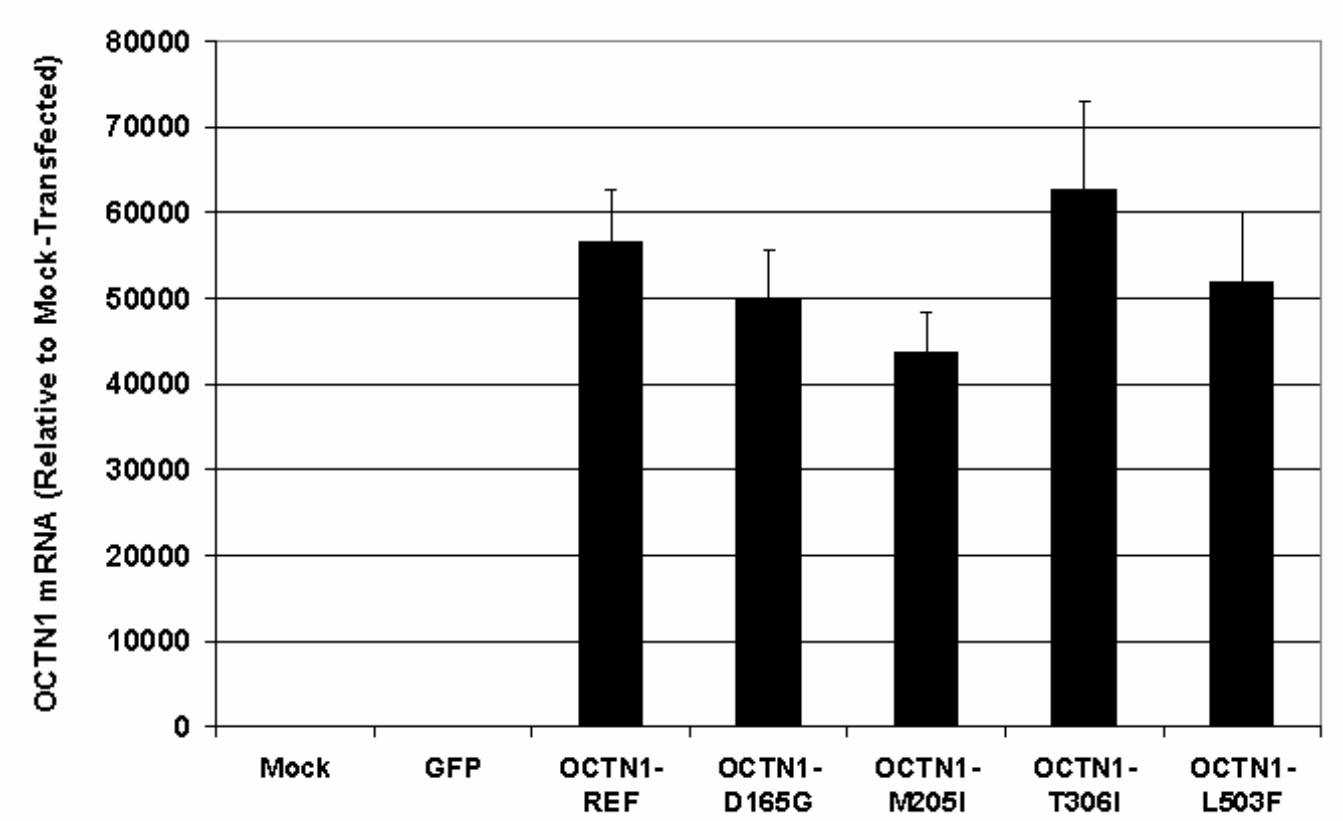

Figure 3.6. OCTN1 mRNA expression in variant cell lines. Total RNA was extracted from HEK-293 cells stably expressing OCTN1-GFP variant proteins, reversed transcribed, and used as template for quantitative PCR using Taqman primers and probes specific to OCTN1. OCTN1 mRNA expression was normalized to GAPDH expression for each cell line, and the results expressed as fold-change *vs*. the control (mock-transfected) cell line. Data are shown as mean +/- S.D. from a single experiment.

estimate ethnic-specific allele frequencies for the most common polymorphisms. We identified 22 nucleotide substitutions in the *SLC22A4* gene, 14 of which had not been reported in public databases prior to this screen. We discovered six protein sequence-altering variants, of which only the two most common (T306I and L503F) had been identified previously.

Using heterologous expression of amino acid-altering variants of OCTN1, we discovered several rare variants with significantly reduced function (M205I) or complete loss of transport function (D165G and R282X) (Figure 3.2). The nonsense mutation, R282X, results in a premature stop codon at amino acid position 282, leading to a truncated protein (*cf.* the 554-amino acid wild-type protein). Studies of mRNA expression and subcellular localization revealed that the amino acid substitutions D165G and M205I did not appreciably affect expression or subcellular localization of these variants, suggesting that the impairment of transport function seen with these variants occurs at the level of the mature protein. Asp-165 is predicted to lie at the interface between the second transmembrane domain and the intracellular membrane surface, while Met-205 is located in the third transmembrane domain. Thus, these sites occur in regions of the OCTN1 protein that are potentially important for either substrate recognition or global protein function.

Among the more common protein sequence variants, we found that the cosmopolitan (pan-ethnic) variant T306I did not result in any appreciable alteration in transport function. Interestingly, although Ile-306 is the minor allele at this position in all

ethnicities studied apart from the Asian population, nearly all mammalian OCTN1 orthologs contain Ile at this position, suggesting that Ile-306 is the ancestral allele. The substitution of Thr at this position introduces a potential N-glycosylation site in the third extracellular loop. Previous studies have suggested that variants at evolutionarily conserved amino acid postions may have functional effects *in vivo* that are not detectable (or are difficult to detect) based on cell-based assays [30]. Thus, it remains possible that the Thr306Ile variant, by disrupting a potential regulatory site in the protein, may have effects on the function of OCTN1 *in vivo* that were not discovered using our assay.

The L503F variant has previously been shown to affect the substrate selectivity of OCTN1, and specifically to increase preference for tetraalkylammonium compounds. We reproduce these results here, showing a decreased K_m and increased V_{max} for TEA transport by the L503F variant compared to the reference OCTN1. Although the breadth of OCTN1 substrate specificity is relatively unexplored, it is likely that other OCTN1 substrates, such as cationic drugs, will also be found to differ in their interactions with L503F *vs*. the reference OCTN1. Since the L503F variant is particularly common, especially among individuals of European descent, this variant may help to explain population variability in the pharmacokinetics of OCTN1 substrate drugs, and thus may be helpful in predicting drug response.

L-ergothioneine, which is believed to be the endogenous substrate of OCTN1, is an antioxidant whose precise physiological role is poorly understood. It has been suggested that a major role of ergothioneine is as a free radical scavenger in erythrocytes, in which

ergothioneine is found in particularly high concentrations (100 μM – 1 mM), and in which OCTN1 expression is also particularly high [10,31,32]. Ergothioneine has also been implicated in inflammatory processes due to its presence in CD14+ monocytes, and has been shown to influence cell proliferation [26]. A previous study using stably-transfected HEK293 cells showed that the L503F variant of OCTN1 was associated with increased affinity (3-fold lower K_m) and reduced capacity (2-fold lower V_{max}) for ergothioneine, resulting in a ~50% higher intrinsic transport clearance (V_{max}/K_m) compared with the reference OCTN1 [26]. In this study, we determined the kinetics of OCTN1 inhibition by ergothioneine among the common OCTN1 variants (Figure 3.4), and found that the interaction affinity (K_i) of ergothioneine for OCTN1 was similar among T306I, L503F, and the reference protein. This was true whether betaine or TEA was used as the probe substrate (data not shown). The reasons for the discrepancy between our results and those of the previous authors are unclear, although differences in expression systems (Flp-In *vs*. standard methods for generation of stable cell lines) may provide some explanation. More likely, because we measured inhibition potency rather than transport of ergothioneine, the affinity measured in our study may reflect a noncompetitive interaction with transport of the probe substrate by OCTN1. Given that the K_m for ergothioneine transport is different between the L503F and reference proteins, but the K_i for inhibition of betaine or TEA transport is almost identical between the two alleles, we suspect that the active site for ergothioneine transport is different from that for betaine and/or TEA. Although the common L503F variant may have altered function with respect to ergothioneine transport, perhaps more interesting are the rare loss-of-function alleles of OCTN1 identified here (D165G, R282X) and elsewhere (G462E) [23].

Since these variants result in a complete loss of OCTN1 activity, it may be fruitful to study the significance of OCTN1 (and of ergothioneine) *in vivo* by examination of individuals and families carrying these alleles.

In summary, we identified several protein sequence polymorphisms in OCTN1 with functional significance in biochemical assays, including several rare loss-of-function mutations and a common variant that alters substrate selectivity. Studies are underway to determine the influence of these polymorphisms on clinical drug disposition.

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CHAPTER 4

FUNCTIONAL GENETIC DIVERSITY IN THE HIGH-AFFINITY CARNITINE TRANSPORTER OCTN2 (*SLC22A5*)[2]

Introduction

Primary systemic carnitine deficiency (SCD) is an early-onset disorder marked by cardiac and skeletal myopathy, hypoketotic hypoglycemia, hyperammonemia, encephalopathy, and in some cases acute liver failure [1,2]. The symptoms are reversible on administration of high doses of oral L-carnitine [3]. SCD has been shown to result from mutations in the high-affinity plasma membrane carnitine transporter, OCTN2 (*SLC22A5*) [4-6] (reviewed in [1,2]). The primary defect in SCD appears to be a severe reduction in active reabsorption of carnitine in the kidney [7], highlighting the importance of OCTN2 in renal salvage of carnitine, and thus maintenance of total body stores of carnitine, an essential cofactor in mitochondrial fatty acid oxidation [8]. Mutations identified in patients with SCD include both missense and nonsense mutations as well as insertions/deletions [1,2]. With few exceptions, unique OCTN2 mutations have been found in each of the unrelated cases of SCD for which a causative mutation was identified, *i.e.*, they are private mutations. Thus, the allele frequencies of these loss-of-function mutations are not known, but are thought to be very rare. Estimates of the overall carrier frequency have ranged from 1:100 in Akita, Japan[9], to as low as 1:150-1:480 in an Australian population[10].

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OCTN2 (*SLC22A5*) is a member of the SLC22 family of plasma membrane solute carrier proteins, which includes multispecific transporters of organic cations, anions, and zwitterions. OCTN2 is unique in that it is a bifunctional transporter, facilitating the transport of both organic cations (such as the xenobiotic tetraethylammonium, or TEA) in a pH-dependent and Na^+-independent manner, and of zwitterions (such as the prototypical substrate, L-carnitine) in a Na^+-dependent manner [11,12]. OCTN2 is expressed ubiquitously, with high expression in kidney and lower expression in heart, skeletal muscle and other tissues [13,14].

A mouse model of systemic carnitine deficiency exists (the *jvs* mouse), and exhibits a phenotype very similar to human SCD, including cardiomyopathy and fatty infiltration of visceral organs [15]. The *jvs* mouse, which has a mutation in the mouse ortholog of OCTN2, exhibits decreased renal secretory clearance of TEA and increased renal secretory clearance of carnitine, consistent with the dual function of OCTN2 [12], and suggesting that humans with genetic defects in OCTN2 may exhibit abnormalities in the disposition of carnitine and in the pharmacokinetics of organic cations (such as cardiovascular drugs[16]) that are OCTN2 substrates.

Given that complete loss-of-function mutations in OCTN2 cause severe abnormalities in carnitine disposition and fatty acid oxidation (*i.e.*, SCD), and that this syndrome is extremely rare, it is unlikely that null alleles of this gene exist at high frequency in the general population. However, it is possible that more moderate defects in OCTN2 activity may be caused by genetic polymorphisms in the *SLC22A5* gene, and may explain

variability in the disposition of carnitine and other OCTN2 substrates. In both humans [9] and mice [17,18], heterozygosity for *SLC22A5* mutations has been shown to produce a moderate carnitine deficiency phenotype, demonstrating that even partial loss of OCTN2 function may be detrimental.

Peltekova *et al*. recently identified a single-nucleotide polymorphism (SNP) in the promoter region of *SLC22A5*, -207G>C, that in combination with an amino acid substitution in the paralog OCTN1 (OCTN1-Leu503Phe) formed a two-point haplotype that was enriched in patients with Crohn's disease in a case-control study of individuals of European descent [19]. This SNP was found to disrupt a heat shock element in the *SLC22A5* promoter, and biochemical assays showed that the -207C allele had reduced responsiveness to heat shock and arachidonic acid treatment. While a causal role for this polymorphism in the pathogenesis of Crohn's disease remains controversial[20,21], based on the biochemical evidence it appears likely that this SNP may modify the functional expression of OCTN2, and thus may help to explain interindividual variability in carnitine homeostasis as well as drug response.

Coding region variants, specifically nonsynonymous variants (*i.e.*, amino acid substitutions), may result in changes in protein function. To systematically investigate functional genetic variation in OCTN2, we resequenced the *SLC22A5* coding region in a large (n=270) ethnically diverse sample of healthy volunteers and identified 20 nucleotide substitutions, including 15 previously unreported variants, in this region. We then examined the functional effects in cellular assays of amino acid sequence variants of

OCTN2 identified in this screen. Since OCTN2 is a bifunctional transporter, we assessed the activity of OCTN2 variants with respect to both the endogenous substrate, L-carnitine, and the xenobiotic TEA. We further characterized the subcellular localization of the most common protein sequence variants of OCTN2. Additionally, we established immortalized lymphoblasts from subjects homozygous for either allele of the promoter region polymorphism -207G>C, to determine the effects of this polymorphism on L-carnitine transport and OCTN2 mRNA expression *ex vivo*.

Methods

Chemicals

[^{3}H]-L-carnitine (80 Ci/mmol) and [^{14}C]-tetraethylammonium (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO, USA). Lipofectamine 2000 and pcDNA3 were purchased from Invitrogen (Carlsbad, CA, USA). BCA Protein Assay Kit was purchased from Pierce Biotechnology Inc. (Rockford, IL, USA). Unlabeled L-carnitine and tetraethylammonium bromide were purchased from Sigma (St. Louis, MO, USA). Cell culture supplies were purchased from the Cell Culture Facility (UCSF, San Francisco, CA, USA). All other chemicals were of reagent grade and commercially available.

Identification of SLC22A5 Variants

Genomic DNA samples were collected from unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. *SLC22A5* variants were identified by direct sequencing of genomic DNA as previously described [22] from an ethnically diverse population of 270 individuals: 80 African-Americans, 80 European-Americans, 60 Asian-Americans (50 Chinese-Americans and 10 Japanese-Americans), and 50 Mexican-Americans. The reference cDNA sequence of *SLC22A5* was obtained from GenBank (http://www.ncbi.nlm.nih.gov, accession number NM_003060). Primers were designed manually to span the exons and 50–200 bp of flanking intronic sequence per exon. The primer sequences can be found at http://www.pharmgkb.org. Variant positions are relative to the ATG start site and are based on the reference cDNA sequence of *SLC22A5*.

Genetic Analysis of SLC22A5

The neutral parameter (θ), nucleotide diversity (π), and Tajima's *D* statistic were calculated as described by Tajima [23]. Each parameter was determined for various sites within the coding region of *SLC22A5* (*e.g.*, synonymous and nonsynonymous sites, and sites within transmembrane domains and loops) for the entire population and for each ethnic group. Synonymous and nonsynonymous sites were defined as described by Hartl and Clark [24]. Evolutionarily conserved amino acid residues were defined as residues identical among human, mouse and rat orthologs of OCTN2. Chemical distance, or the degree of difference between alternative amino acid residues, was taken from the amino acid substitution matrix of Grantham[25].

Construction of OCTN2 Variants

Human OCTN2 cDNA (GenBank accession number NM_003060) was subcloned into the mammalian expression vector pcDNA3 to obtain OCTN2-reference, which corresponds to the highest-frequency amino acid sequence in all ethnic groups. Variant cDNA clones were constructed by site-directed mutagenesis of the reference clone using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). Variants that showed functional differences from OCTN2-reference were reverted to the reference sequence by site-directed mutagenesis and assayed further, to confirm that the functional effect was due to the intended mutation. In all cases, OCTN2-reference in pcDNA3 was used as the template, except for OCTN2-Phe17Leu-reversed, OCTN2-Tyr449Asp-reversed, and OCTN2-Val481Phe-reversed, for which OCTN2-Phe17Leu, OCTN2-Tyr449Asp, and OCTN2-Val481Phe were used as templates, respectively. Sequences of variant cDNA clones were confirmed by direct sequencing, and the full cDNA was sequenced to verify that only the intended mutation was introduced.

Cellular Assays of OCTN2 Variants

HEK-293 cells were maintained in Dulbecco's modified Eagle's medium (DME H-21) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum. Cells were seeded onto 24-well poly-D-lysine coated plates (BD Discovery Labware, Bedford, MA, USA) at a density of 1.5 X 10^5 cells per well and grown for 24 h. Cells were then transfected with 1 μg OCTN2-reference or variant DNA and 3 μg Lipofectamine 2000 per well following the manufacturer's protocol. Cells were incubated for 48 h and assayed for activity by measurement of cellular uptake of radiolabeled probe substrates. For studies of transport kinetics, OCTN2-reference and

the polymorphic variants (Phe17Leu, Leu144Phe, and Pro549Ser) were subcloned into the expression vector pcDNA5/FRT and used to generate stable cell lines using the Flp-In System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. This system, which generates single-copy, site-specific integration of target constructs, allows for comparisons among allelic variants in stable cell lines by eliminating concerns regarding gene-dose-dependent or integration site-dependent difference among cell lines. Briefly, Flp-In-293 cells were plated at a density of 6 X 10^5 cells per well in 6-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 0.4 μg OCTN2 (reference or variant) cDNA, 3.6 μg pOG44 DNA, and 20 μg Lipofectamine 2000. Two days after transfection, cells were trypsinized and split 1:4 into new 6-well plates and selected for stable transfectants by addition of hygromycin B (75 μg/mL) to the growth media. After 10-14 days under selection, colonies were pooled and expanded in 25 cm^2 flasks and used for transport experiments by seeding in 24-well poly-D-lysine coated plates as described above.

Uptake studies were performed by first washing the cells with warm Hank's Buffered Salt Solution (HBSS) and incubating at 37ºC for 15 min, after which the wash buffer was removed and replaced with HBSS containing either [^{3}H]-L-carnitine (10 nM [^{3}H]-L-carnitine, 1 μM unlabeled L-carnitine) or [^{14}C]-tetraethylammonium (10 μM radiolabeled). Cells were returned to 37ºC for 10 min, which was determined to be within the linear range of uptake *vs*. time. Uptake was terminated by rapidly removing the extracellular media and washing three times with ice-cold HBSS. Cells were lysed in

1 mL 0.1 N NaOH/0.1% SDS and 800 μL of the lysate was added to 3 mL Ecolite scintillation fluid (ICN Biomedicals, Costa Mesa, CA, USA). Intracellular radioactivity was determined by scintillation counting and normalized to per-well protein content as measured using the BCA protein assay. Results were expressed as the percent of activity of the OCTN2-reference wells. Kinetics studies were performed as described above, with varying concentrations of unlabeled substrate (L-carnitine or TEA) added to the uptake buffer. Rates of uptake (V), expressed as pmol/min/mg protein, were fit to the equation:

$V = V_{max}*[S]/(K_m + [S])$

or

$V = V_{max}*[S]/(K_m + [S]) + K_o*[S]$

where K_o represents the first-order rate constant for non-OCTN2-mediated uptake, [S] is the substrate concentration, and V_{max} and K_m are the Michaelis-Menten kinetic parameters.

Statistical differences between variant and reference OCTN2 activity were assessed by paired t-tests using mean values from replicate experiments. $p<0.05$ was used as the threshold for significance.

Subcellular Localization Studies

Polymorphic OCTN2 variant cDNA clones were used to make GFP fusion constructs in order to determine their subcellular localization. The terminal codon in OCTN2 was mutated to introduce a *Bst*BI recognition site, and the GFP coding sequence was ligated

to the 3'-end of the OCTN2 cDNA in the expression vector pcDNA5/FRT. The resulting clones were used to generate stable cell lines as described above. For localization studies, cells were seeded at 1.5 X 10^5 cells per well on 12 mm poly-D-lysine coated glass coverslips (BD Discovery Labware, Bedford, MA, USA) in 24 well plates. Cells were stained using the Image-IT™ Live labeling kit (Molecular Probes, Eugene, OR, USA) and fixed in 4% paraformaldehyde according to the manufacturer's protocol. Coverslips were mounted in Vectashield antifade solution (Vector Laboratories, Inc., Burlingame, CA, USA) on glass microscope slides and visualized by confocal microscopy using a Zeiss 510 laser scanning microscope.

Phenotyping of SLC22A5 -207G>C Promoter Polymorphism in Immortalized Lymphoblasts

Subjects in the SOPHIE cohort were screened by direct sequencing of the promoter region of *SLC22A5* to identify individuals with the -207G/G or -207C/C genotypes. Because this polymorphism had only been described in individuals of European ancestry, this screen was restricted to the European American subset of SOPHIE. Homozygotes were recruited into a clinical study designed to assess differences between subject groups in carnitine renal clearance and in carnitine transport activity and OCTN2 mRNA expression in lymphoblastoid cell lines (LCLs). To establish these cell lines, 10 mL whole blood was drawn from each subject (-207G/G, n=7; -207C/C, n=8), and the buffy coat isolated by separation of blood using Ficoll Paque Plus (Amersham Biosciences, Pittsburgh, PA, USA). White blood cells were washed twice in phosphate-buffered saline, and transformed with Epstein-Barr virus (EBV) in the presence of 1 μg/mL

cyclosporine in growth media (RPMI-1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin). Cells were incubated in 25 cm^2 flasks at 37°C in 5% CO_2 for 7-10 days before feeding. Once colonies were established, cells were expanded by feeding every 2-3 days, then transferred to 75 cm^2 flasks. L-carnitine transport studies were performed using the method described by Tein *et al.* [26]. Briefly, 6 X 10^6 cells were incubated in the presence of 5 μM or 5 mM L-carnitine (10 nM [^{3}H]-L-carnitine plus unlabeled L-carnitine to achieve the final desired concentration) for three hours in triplicate 1.5 mL Eppendorf tubes. Cells were then washed three times in ice-cold PBS and lysed in 1 mL 0.1N NaOH/0.1% SDS, and 800 μL of cell lysate was used for scintillation counting. Active transport was determined by subtracting non-specific uptake (at 5 mM) from total uptake (at 5 μM) after correcting for total protein as determined by the BCA protein assay. Transport studies were performed at least twice for each cell line, and average values from replicate experiments were used in the final analysis. To measure OCTN2 mRNA expression, 5 X 10^6 cells from each line were pelleted and total RNA extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and reverse-transcribed using Superscript III Reverse Transcription Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturers' protocols. The resulting cDNA was used as template for real-time quantitative PCR using TaqMan primers and probes specific for human OCTN2 and GAPDH (Applied Biosystems, Foster City, CA, USA). OCTN2 expression was normalized to GAPDH mRNA and expressed relative to the lowest-expressing cell line. To determine the effect of heat shock on OCTN2 expression, cells were incubated in water baths in room air at either 42°C or 37°C for two hr and returned to a 37°C, 5% CO_2 incubator. Cells were pelleted for RNA extraction at various

time points (0, 1, 2, or 6 hr) after termination of heat shock. Differences between treatments (for heat shock studies) were tested for significance using a paired t-test. Differences between genotype groups were assessed using an unpaired t-test.

Results

Variant Discovery and Population Genetics of OCTN2 Coding Region Variants

We identified 20 nucleotide substitution variants in the survey region, including 10 in coding and 10 in noncoding regions of the OCTN2 gene. Five of these had previously been reported on the NCBI single nucleotide polymorphism database (dbSNP): two high-frequency synonymous SNPs (c.285C>T and c.807G>A), one amino acid substitution (L144F), one intronic SNP (IVS4+13C>T) and one SNP in the 3'-untranslated region (UTR). Additionally, one rare variant (Tyr449Asp) had been identified previously in a patient with a suspected carnitine transport defect [27].

Of the variants we identified in noncoding regions, nine were intronic and one was found in the 3'-UTR, 47 bp downstream of the stop codon. Among coding region variants, two were synonymous (*i.e.*, did not alter the amino acid sequence) and eight were non-synonymous. Although a total of eight nonsynonymous variants were found, only seven nonsynonymous sites were identified, as one of the sites was triallelic (c.1441G>T, c.1441G>A). The variant identification data have been deposited in the public databases PharmGKB (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Table 4.1 summarizes the population genetics statistics for the OCTN2 coding region. Of note is the fact that while only two synonymous variants were found *vs*. eight nonsynonymous variants, the nucleotide diversity at synonymous sites (π_S) was much higher than that at nonsynonymous sites (π_{NS}). The ratio of π_{NS}/π_S is frequently used as a measure of the extent of selective pressure on a gene, with low π_{NS}/π_S corresponding to a high degree of negative selection (*i.e*., low tolerance for nonsynonymous substitution *vs*. synonymous substitution); the ratio of 0.04 for OCTN2 is among the lowest of all transporter genes surveyed by our group [22,28], suggesting a very low evolutionary tolerance for alterations in OCTN2 protein structure. This is consistent with the observation that a potentially lethal early-onset disorder (SCD) results from null alleles of this gene.

Figure 4.1 shows the predicted secondary structure of OCTN2 and the location of the non-synonymous coding region variants. Only one of the eight non-synonymous variants is predicted to occur in the transmembrane domain (TMD), five in the intracellular loop and two in the extracellular loop regions of the protein. Variants occurring in the loop regions do not disrupt any predicted N-glycosylation or phosphorylation motifs. All but one (Met530Val) of the nonsynonymous variants occur in evolutionarily conserved amino acid residues, defined as residues identical among human, mouse, and rat orthologs of OCTN2.

Notably, one OCTN2 variant, Tyr449Asp, had been identified previously in the heterozygous state in a patient who died of sudden cardiac arrest at 3 months of age

Table 4.1. Population genetics statistics for variation in *SLC22A5*

Population	Region/Type	Base Pairs Surveyed	# of Chromosomes	Total bp Sequenced	# of Variable Sites	θ (X 10^4)	π (X 10^4)	*D*
Total	All	2996	552	1653792	18	8.72 ± 2.62	6.47 ± 4.07	-0.64
	Coding	1674		924048	9	7.80 ± 2.98	6.58 ± 4.77	-0.26
	Noncoding	1322		729744	9	9.88 ± 3.77	6.34 ± 5.00	-0.58
	Synonymous	407		224664	2	7.14 ± 5.22	23.93 ± 18.01	1.38
	Nonsynonymous	1267		699384	7	8.02 ± 3.38	1.01 ± 1.70	-1.21
AA	All	2996	160	479360	12	7.09 ± 2.60	8.01 ± 4.84	0.30
	Synonymous	407		65120	2	8.71 ± 6.46	23.49 ± 17.87	1.2
	Nonsynonymous	1267		202720	4	5.59 ± 3.07	2.73 ± 2.99	-0.58
EA	All	2996	160	479360	8	4.73 ± 1.98	5.48 ± 3.60	0.29
	Synonymous	407		65120	2	8.71 ± 6.46	22.7 ± 17.4	1.14
	Nonsynonymous	1267		202720	1	1.40 ± 1.43	0.10 ± 0.51	-0.40
AS	All	2996	120	359520	11	6.85 ± 2.63	5.24 ± 3.48	-0.54
	Synonymous	407		48840	2	9.17 ± 6.85	19.8 ± 16.0	0.87
	Nonsynonymous	1267		152040	2	2.94 ± 2.20	0.39 ± 1.04	-0.65
ME	All	2996	100	299600	9	5.80 ± 2.41	4.32 ± 3.02	-0.53
	Synonymous	407		40700	2	9.50 ± 7.11	18.6 ± 15.3	0.74
	Nonsynonymous	1267		126700	2	3.05 ± 2.28	0.32 ± 0.93	-0.69

The neutral parameter (θ), nucleotide diversity (π) and Tajima's *D* statistic were calculated as described by Tajima [23]. π for synonymous and nonsynonymous sites was calculated as described by Hartl and Clark [24]. AA, African American; EA, European American; AS, Asian American; ME, Mexican American.

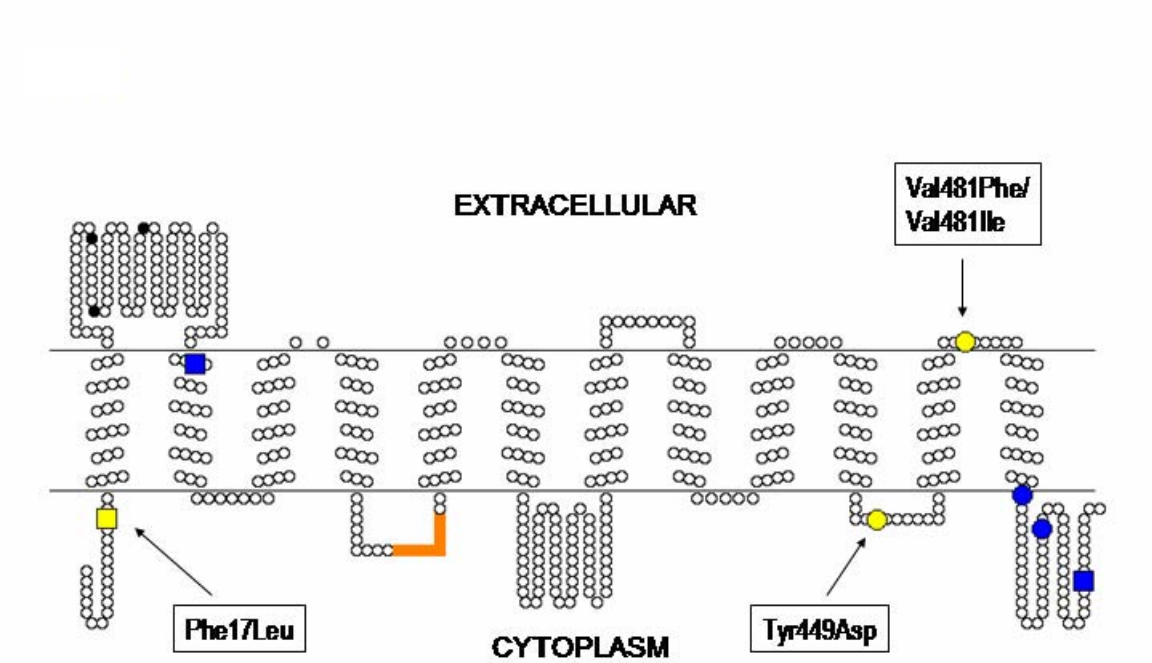

Figure 4.1. Predicted secondary structure of OCTN2 showing the position of the eight nonsynonymous coding region variants. The transmembrane topology diagram was rendered using TOPO2 (SJ Johns (UCSF, San Francisco) and RC Speth (Washington State University, Pullman), transmembrane protein display software available at the UCSF Sequence Analysis Consulting Group website, http://www.sacs.ucsf.edu/TOPO/topo.html). Polymorphic variant sites (allele frequency >1%) are shown as squares; rare variants are shown as circles. Variants with significant functional differences from the reference OCTN2 are shown in yellow, all other variants are shown in blue, putative N-glycosylation sites are shown in black, and an ATP-binding motif is boxed in orange. The three functionally significant nonsynonymous variants are indicated by arrows.

[27,29]. This patient was found on autopsy to have moderately reduced carnitine transport activity (to 57% of control) and reduced very long-chain acyl-CoA dehydrogenase (VLCAD) activity (to 46% of control) in cultured fibroblasts. The patient was also heterozygous for a point mutation in the VLCAD gene, and death is presumed to have resulted from synergism between defects in multiple steps in the fatty acid oxidation pathway (see Vockley *et al.* [29]). This variant occurs at an evolutionarily conserved residue in an intracellular loop that has been shown to influence sodium activation of OCTN2 [27].

Of the eight nonsynonymous variants identified, five were singletons, that is, were found on only one chromosome in our sample. Three were polymorphic (defined as ≥1% allele frequency in at least one ethnic group): Phe17Leu, found at an allele frequency of 1.7% in the Asian American sample; Leu144Phe, found at a 7.5% frequency in the African American sample; and Pro549Ser, found at a frequency of 10.0% in the African American sample. The characteristics of the coding region variants and their population-specific allele frequencies are shown in Table 4.2.

Activity of OCTN2 Protein Sequence Variants in Cellular Assays

We probed OCTN2 activity using 2 compounds: the endogenous substrate L-carnitine, and the synthetic molecule TEA. When expressed in mammalian cells, the majority of nonsynonymous variants of OCTN2 retained function, having activity approximately equal to OCTN2-reference (Figure 4.2a). None of the variants tested showed complete loss of function; however, one rare variant (Val481Phe) and one polymorphism

Table 4.2. Coding region variants of OCTN2.

dbSNP ID#	CDS Position[a]	Nucleotide Change	Amino Acid Position	Amino Acid Change	Grantham Score[b]	EC/EU[c]	Allele Frequency[d] AA (n=160)[e]	EA (n=160)	AS (n=120)	ME (n=100)	Total (n=540)
rs11568520	51	C->G	17	Phe->Leu	22	EC	0	0	1.7	0	0.4
rs2631365	285	T->C	95	Syn	--	EC	38.7	35.6	70.8	25.0	42.8
rs10040427	430	C->T	144	Leu->Phe	22	EC	7.5	0	0	1.0	2.4
rs274558	807	A->G	269	Syn	--	EC	38.7	35.6	70.8	25.0	42.8
rs11568514	1345	T->G	449	Tyr->Asp	160	EC	0.6	0	0	0	0.2
rs11568513	1441	G->T	481	Val->Phe	50	EC	0	0.6	0	0	0.2
rs11568513	1441	G->A	481	Val->Ile	29	EC	0.6	0	0	0	0.2
rs11568521	1522	T->C	508	Phe->Leu	22	EC	0	0	0	1.0	0.2
rs11568524	1588	A->G	530	Met->Val	21	EU	0	0	0.8	0	0.2
rs11568525	1645	C->T	549	Pro->Ser	74	EC	10.0	0	0	0	2.9

[a]CDS position is given in reference to the ATG start site (where position +1 is the "A" in ATG). [b]Grantham score is shown as a measure of chemical distance between alternative residues, with high Grantham score corresponding to large chemical changes [25]. [c]Evolutionarily conserved (EC) residues are defined as residues identical among human, mouse and rat orthologs of OCTN2; all other residues were classified as evolutionarily unconserved (EU). [d]Population-specific allele frequencies are given: AA=African American; EA=European American; AS=Asian American; ME=Mexican American. [e]Number of chromosomes screened per ethnicity. Variant information has been deposited in PharmGKB (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

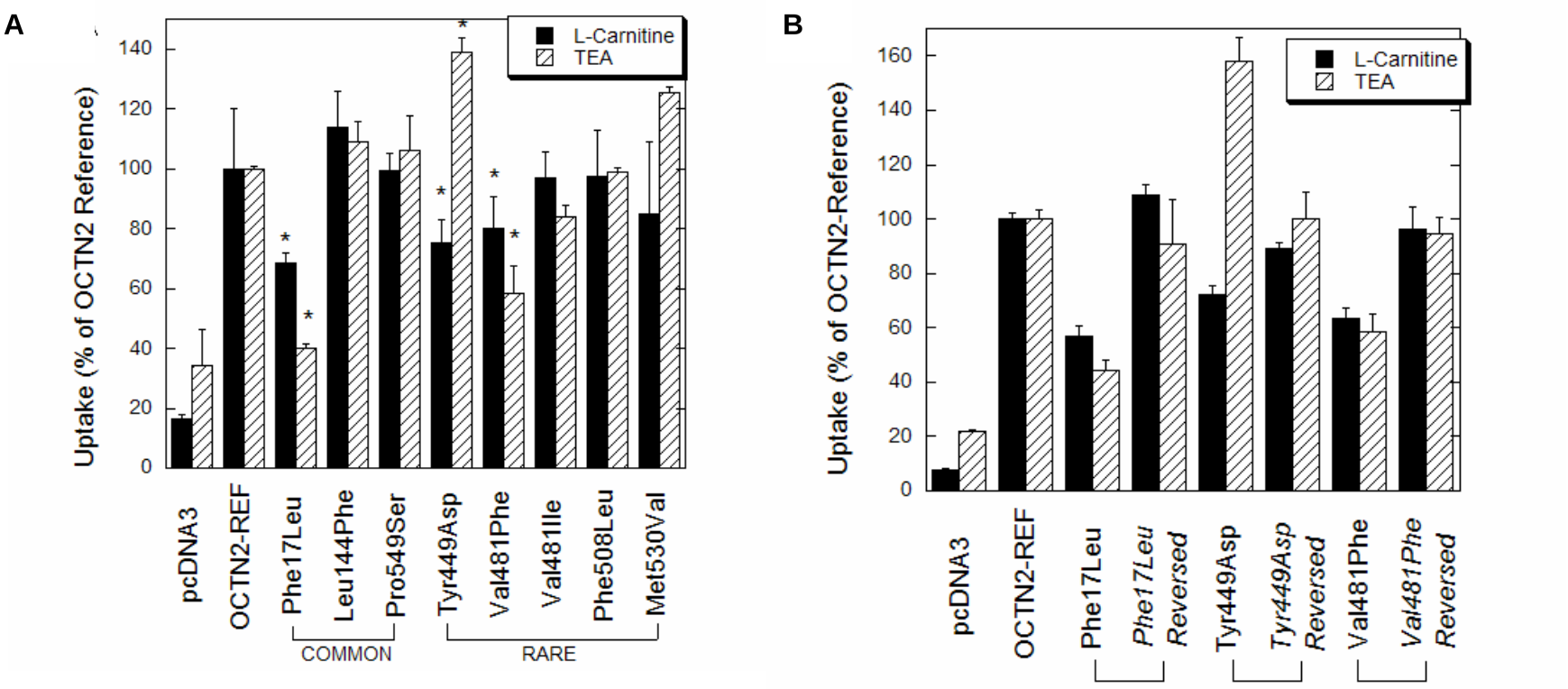

Figure 4.2. Functional screen of protein-altering variants of OCTN2. Variants of OCTN2 were expressed by transient transfection in HEK-293 cells and assayed for activity by measurement of uptake of radiolabeled probe substrates (1 µM [^{3}H]-L-carnitine or 10 µM [^{14}C]-TEA) at 10 min. (A) Functional activity of all nonsynonymous variants was assessed. (B) Variant cDNAs for those variants found to have altered function were reversed to the reference sequence by site-directed mutagenesis and assayed for restoration of wild-type activity. Mock-transfected cells (pcDNA3) serve as the negative control. Results are expressed as percent of the reference sequence clone (OCTN2-REF) after correcting for per-well protein content. Values represent mean +/- S.D. from triplicate wells in a representative experiment. *$p<0.05$ *vs*. OCTN2-Reference.

(Phe17Leu) showed reduced function. Val481Phe exhibited reduced activity to approximately 71% of OCTN2-reference activity with respect to L-carnitine transport (after subtracting background, *i.e.*, uptake in mock-transfected cells), and 53% activity with respect to transport of TEA when averaged over triplicate experiments ($p<0.05$). Interestingly, this variant occurred at a triallelic site: two rare substitutions (Val481Phe and Val481Ile) were found at the same amino acid position, but only Val481Phe exhibited a functional difference from the reference protein. The more common Phe17Leu variant showed a greater decrease in function, reduced to 51% of OCTN2-reference activity toward L-carnitine ($p<0.05$) and to only 20% activity toward TEA ($p<0.01$).

The Tyr449Asp variant, previously identified in a patient heterozygous for a carnitine transport defect, showed increased preference for TEA *vs.* L-carnitine: 150% of OCTN2-reference activity toward TEA ($p<0.0001$), compared to a reduction in L-carnitine transport to 62% of the reference clone ($p<0.05$). The kinetic constant (K_m) of Tyr449Asp toward L-carnitine was not different from the reference OCTN2; however, sodium activation kinetic studies showed reduced sodium stimulation of L-carnitine transport by Tyr449Asp compared with the reference OCTN2 (data not shown), consistent with previous findings [27].

To confirm that the differences in functional activity exhibited by these variants was due to the intended mutation, and not due to unidentified mutations introduced through site-directed mutagenesis in generating the variant cDNAs, we reversed these variant cDNAs

back to the reference sequence and assayed the resulting cDNA clones for activity. In all cases, reversing the mutation back to the reference sequence restored wild-type function (Figure 4.2b), demonstrating that the functional differences observed were specifically caused by the intended nonsynonymous variants.

Detailed Phenotyping of OCTN2 Protein Sequence Polymorphisms

Among the most common OCTN2 variants, Leu144Phe and Pro549Ser, no obvious functional differences were identified in the initial screen. However, the Phe17Leu polymorphism showed significant reduction in activity toward both substrates. In order to determine whether more subtle quantitative differences may exist among the Leu144Phe and Pro549Ser variants, and to further characterize the alterations in activity of the Phe17Leu variant, concentration dependence of L-carnitine and TEA transport was examined. Kinetics of both L-carnitine and TEA transport were nearly identical between OCTN2-reference and both Leu144Phe and Pro549Ser (Figure 4.3). For the Phe17Leu variant, the decrease in L-carnitine transport was explained by a reduction in V_{max} to approximately 50% of OCTN2-reference (446 +/- 18 pmol/min/mg protein *vs*. 861 +/- 18 pmol/min/mg protein for OCTN2-reference) with no effect on K_m (12.5 +/- 0.5 μM vs. 13.5 +/- 0.8 μM for OCTN2 reference). Phe17Leu was also shown to lower V_{max} of TEA transport by OCTN2 (470 +/- 74 pmol/min/mg protein vs. 720 +/- 47 pmol/min/mg protein for OCTN2-reference), as well as increase K_m (1.09 +/- 0.28 mM vs. 0.53 +/- 0.07 mM for OCTN2-reference).

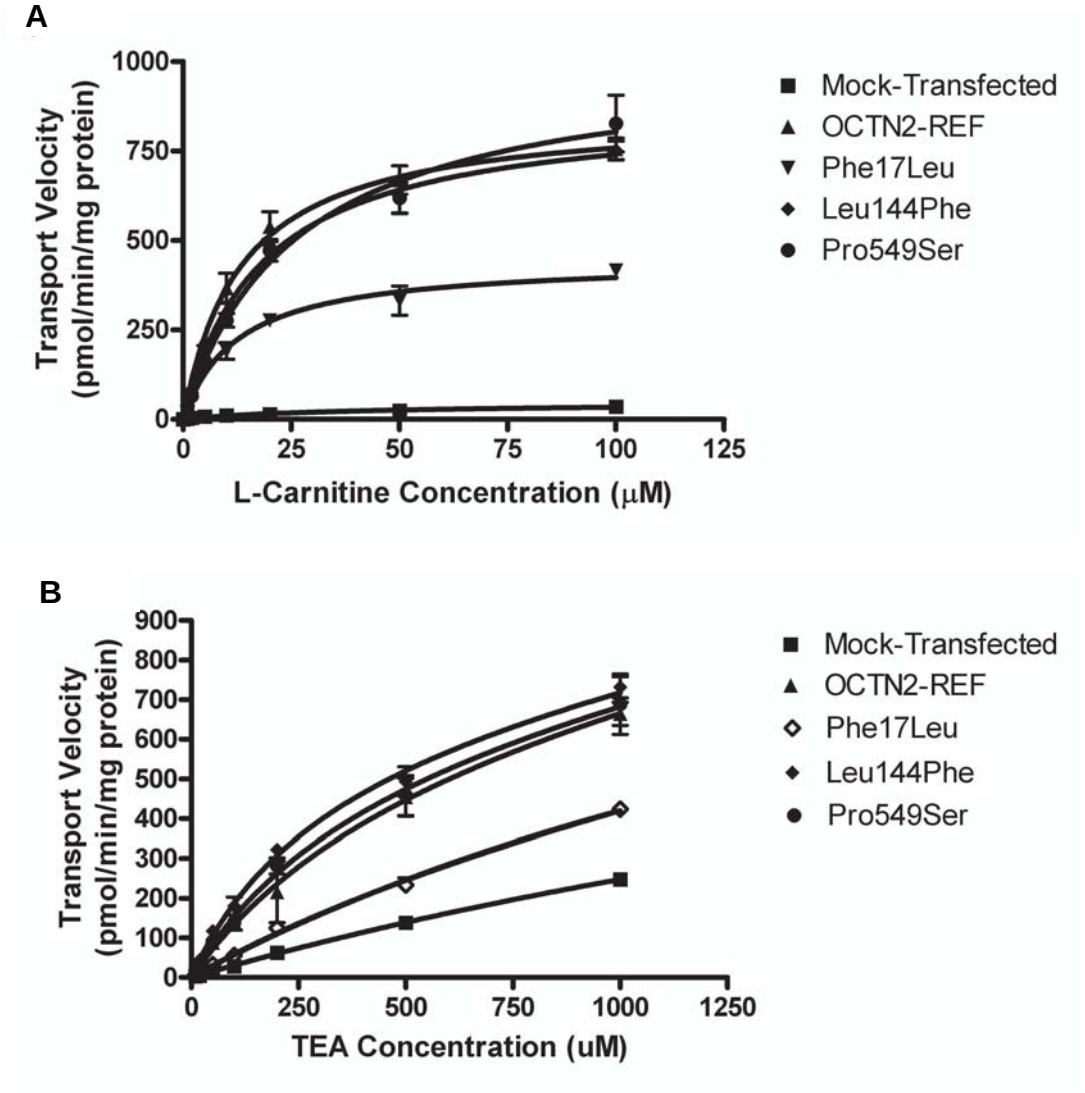

Figure 4.3. Concentration-dependence of L-carnitine and TEA transport by common OCTN2 protein sequence variants. OCTN2-reference, Phe17Leu, Leu144Phe and Pro549Ser were expressed in stably transfected Flp-In-293 cells. Kinetics of L-carnitine (A) and TEA (B) transport were measured by incubation with radiolabeled probe compounds (10 nM [^{3}H]-L-carnitine or 10 μM [^{14}C]-TEA) and varying concentrations of unlabeled substrate for 10 min. Data for TEA transport were fit to a modified Michaelis-Menten equation that included a first-order non-saturable component. The non-saturable uptake rate constant was 0.195. Mock-transfected cells served as a negative control. Results are expressed as pmol/min/10^{6} cells. Values represent mean +/- S.D. from triplicate wells.

In order to further address the mechanism for reduced activity of the Phe17Leu polymorphism, GFP fusion proteins were constructed and expressed by stable transfection in Flp-In-293 cells. It was determined by confocal microscopy that, while OCTN2-reference (as well as Leu144Phe and Pro549Ser) showed strict localization to the plasma membrane, Phe17Leu showed a more diffuse pattern of localization, with some plasma membrane staining as well as marked cytosolic retention of the variant protein (Figure 4.4).

Ex Vivo Phenotyping of -207G>C Promoter Polymorphism

To determine the effect of the -207G>C polymorphism in the promoter of the *SLC22A5* gene, lymphoblastoid cell lines (LCLs) were derived from subjects homozygous for either -207G/G or -207C/C and assayed for L-carnitine uptake and OCTN2 mRNA expression. As shown in Figure 4.5a, cell lines from -207C/C subjects had significantly lower total and specific (*i.e.*, saturable) transport of L-carnitine compared with -207G/G subjects ($p<0.05$). When OCTN2 mRNA levels were compared between groups, -207C/C subjects showed a trend toward reduced mRNA expression in LCLs. This difference was not significant ($p=0.20$), though the lack of statistical significance may be explained by a single -207C/C cell line that showed the highest OCTN2 mRNA expression among all cell lines. When this cell line was excluded from the analysis, -207C/C subjects also showed reduced OCTN2 mRNA expression compared with -207G/G subjects ($p<0.05$). There was, however, a significant correlation between L-carnitine transport activity and OCTN2 mRNA level when all cell lines were included (Figure 4.5b, $p<0.05$). No effect of heat shock on OCTN2 mRNA expression was

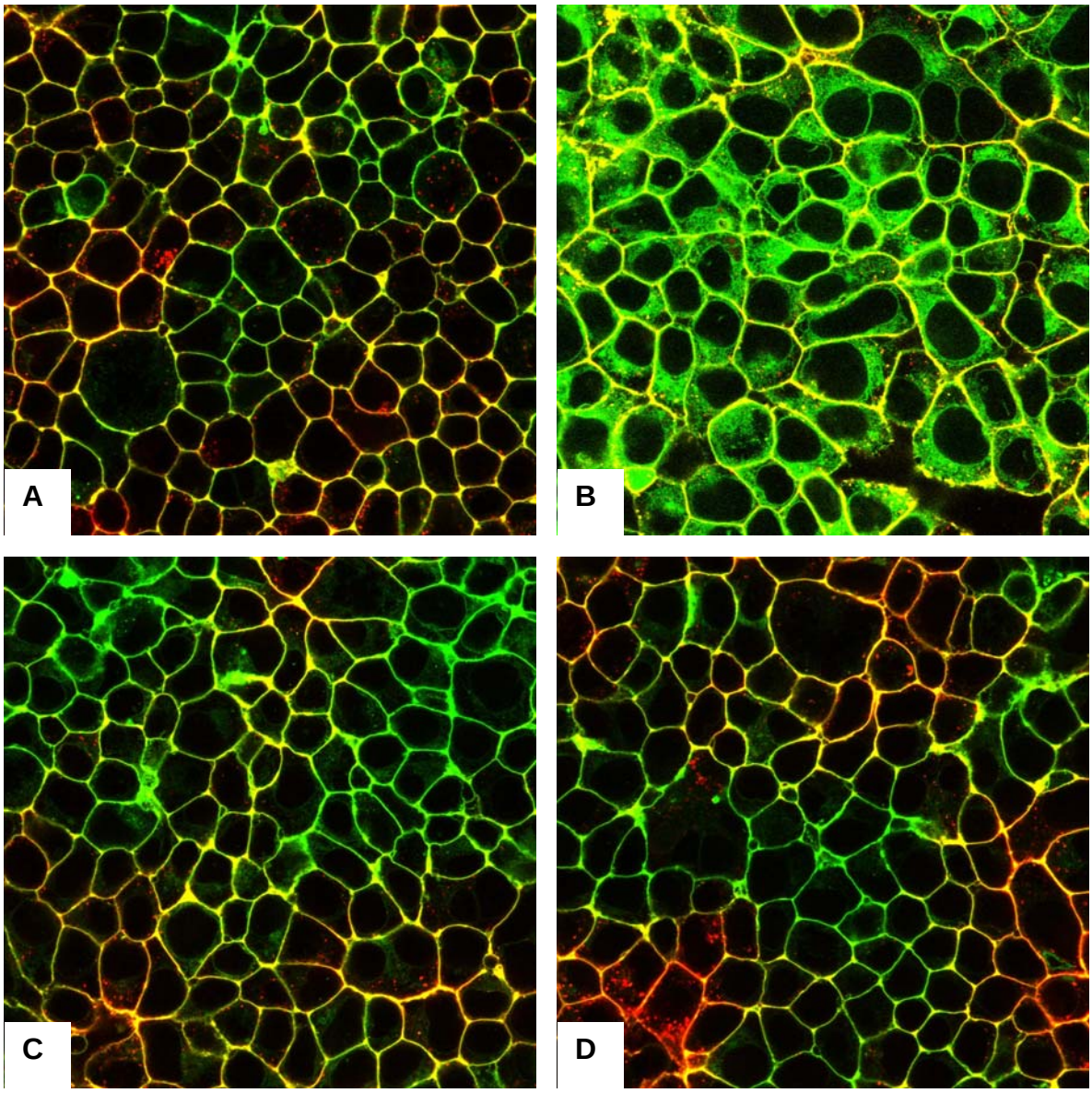

Figure 4.4. Subcellular Localization of Polymorphic OCTN2 Variants. GFP fusion contructs were generated for the OCTN2 variants with population-specific allele frequencies >1% and used to generate stable cell lines using Flp-In-293 cells. The plasma membrane was stained using AlexaFluor594 WGA and cells were visualized by confocal microscopy. A, OCTN2-reference; B, Phe17Leu; C, Leu144Phe; D, Pro549Ser.

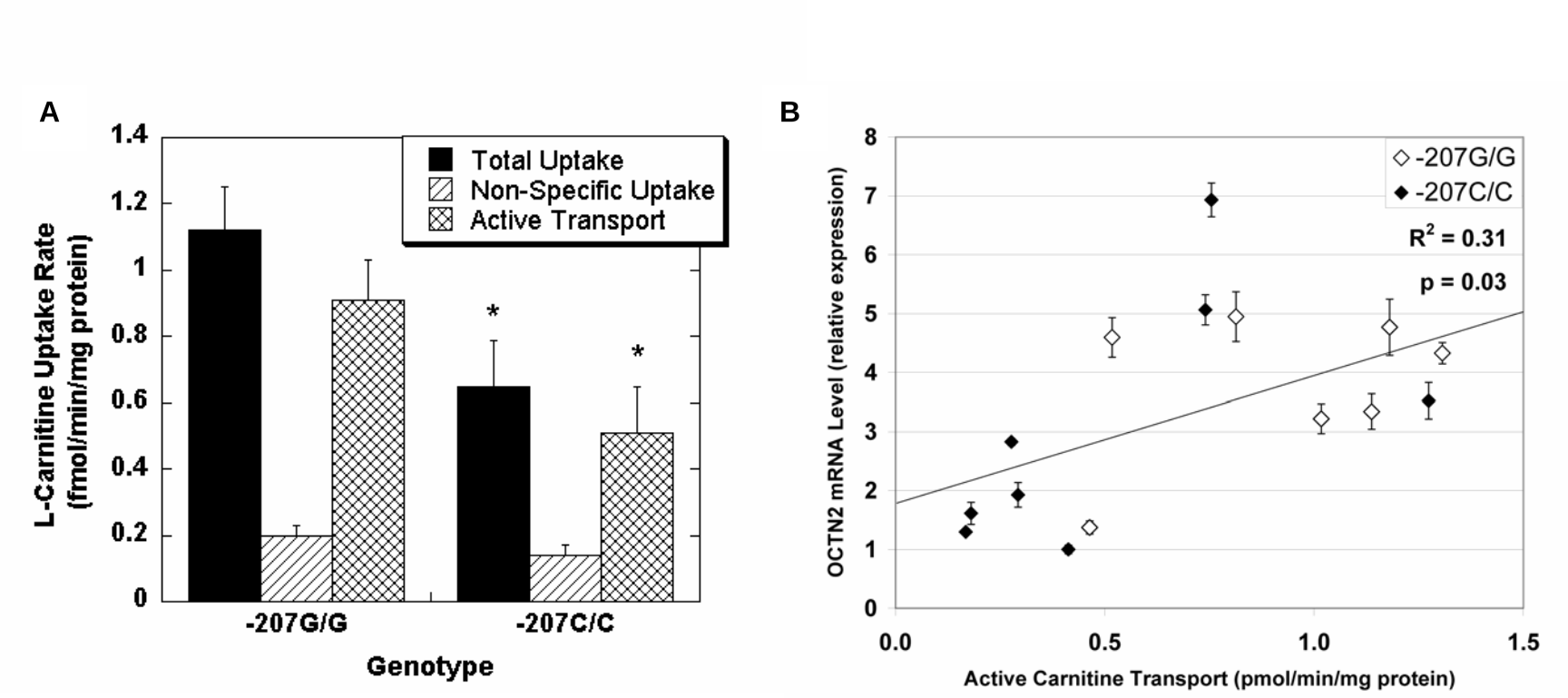

Figure 4.5. Effect of OCTN2 -207G>C Promoter Polymorphism on L-Carnitine Transport and OCTN2 mRNA Expression in Lymphoblastoid Cell Lines. LCLs were derived from healthy human subjects homozygous for -207G/G or -207C/C and assayed for L-carnitine transport activity and OCTN2 mRNA expression. A, Subjects homozygous for -207C/C showed significantly reduced total and specific L-carnitine transport (*$p<0.05$ vs. -207G/G). Transport experiments were performed twice for each cell line, and the average values over two experiments were used in the final analysis. Data shown here represent the mean and standard deviation for all cell lines in each genotype group. B, Correlation between OCTN2 mRNA level and transport activity in LCLs. -207G/G, open symbols; -207C/C, closed symbols.

observed in either genotype group at any of the time points tested (0 hr, 1 hr, 2 hr, or 6 hr after heat shock, data not shown).

Discussion

Although rare null mutations in OCTN2 have been identified in patients with primary systemic carnitine deficiency, this study represents a systematic survey of the coding region of OCTN2 for functionally significant genetic polymorphisms in an ethnically diverse sample. Others have previously surveyed the entire *SLC22A5* gene for novel polymorphisms; however, the samples used for SNP identification were restricted to 48 individuals from a single ethnic group (Japanese) and did not identify any amino acid sequence variants [30]. Another group used an "extreme phenotype" approach to estimate the carrier frequency of loss-of-function mutations in OCTN2; however, this study was restricted to individuals in the Akita prefecture of Japan [9]. We did not identify any of the variants reported by Koizumi *et al*. [9]in our sample. Based on the results of a newborn screening program to detect SCD and other fatty acid oxidation disorders in New South Wales, Wilcken *et al.* anticipate a carrier frequency of 1:240 (<0.5%) for defective alleles of *SLC22A5* in their study population [10].

We did not identify any complete loss-of-function mutations in our cohort of healthy volunteers, which suggests that the carrier frequency for loss-of-function alleles of OCTN2 is lower in our study populations than can be reliably estimated in a sample of this size (*i.e*., less than 1% in each ethnic group included in our sample); however,

several variants exhibited significant alterations in function compared with the reference sequence protein. We found two variants with significantly decreased function in heterologous expression systems: Phe17Leu and Val481Phe (Figure 4.2). Individuals who carry these variants may be at risk for toxicity related to insufficient renal tubular reabsorption of carnitine, or to reduced clearance of substrate drugs. Recent studies have shown that heterozygous *jvs* mice, for example, have significantly reduced free carnitine and increased triglycerides in the liver [18], and are at increased risk for age-associated cardiomyopathy [17]. Humans who are heterozygous for loss-of-function alleles of *SLC22A5* have also been shown to be at increased risk for late-onset cardiac hypertrophy [9]. Thus, even partial loss of OCTN2 function may produce clinical symptoms of carnitine insufficiency. In addition, there is growing evidence for the concept of synergistic heterozygosity, in which heterozygosity for a reduced-function allele of one gene may produce a severe phenotype in the context of heterozygosity for a mutation in another gene in the same pathway [29]. This phenomenon has already been described for mutations in *SLC22A5*, and for other genes involved in fatty acid metabolism [29,31]. Thus, individuals carrying the reduced-function *SLC22A5* alleles identified here may be at risk for defects in fatty acid oxidation as a result of these mutations *per se*, or may be at increased risk of fatty acid oxidation defects resulting from synergism of *SLC22A5* mutations with mutations in other genes involved in mitochondrial fatty acid oxidation.

Of particular interest in this regard is the Phe17Leu polymorphism, which was found at an allele frequency of 1.7% in the Asian American subset of our sample, and showed the most marked reduction in transport activity. The impairment in L-carnitine transport

activity by Phe17Leu was found to be due to a reduction in V_{max}, which suggests a reduction in functional expression of this variant (as contrasted with a reduced affinity for L-carnitine). Indeed, subcellular localization studies confirmed that the expression of Phe17Leu differed from OCTN2-reference, with a lower fraction of the total protein localizing to the plasma membrane, and diffuse intracellular retention of the variant protein. Thus, Phe17Leu disrupts, but does not completely abolish, the normal trafficking of OCTN2 to the plasma membrane. These results suggest that Phe17 may be important for proper folding of the OCTN2 protein, or for optimal interaction with proteins involved in membrane trafficking. Defects in plasma membrane sorting have recently been shown for disease-causing mutations of *SLC22A5* [32]. With regard to TEA transport, Phe17Leu showed an even greater deficiency, to only 20% of OCTN2-reference activity at non-saturating concentrations. This was explained by a reduced V_{max} for TEA transport (presumably also a result of reduced surface expression) as well as a ~2-fold increase in K_m. Thus, in addition to its effects on protein localization, this variant appears to also affect interactions of the mature transporter with particular substrates. Since this allele is particularly common in individuals of Asian ancestry, Phe17Leu may contribute significantly to population variation in carnitine and organic cation transport, and may be an important modifier of fatty acid oxidation disorders, in this population.

We also identified one variant, Tyr449Asp, that had been previously identified in a patient suspected to be heterozygous for a carnitine transport defect, and offered as an example of synergistic heterozygosity [29]. Although this mutation had previously been identified in only a single patient, the fact that the same variant was found in an unrelated

individual in our cohort of healthy subjects suggests that this variant may be more common than other risk alleles for systemic carnitine deficiency. We show that Tyr449Asp influenced the substrate selectivity of OCTN2; that is, Tyr449Asp showed increased preference for TEA and reduced activity toward L-carnitine (Figure 4.2). A reduction in function with respect to L-carnitine transport is most easily explained by the fact that Tyr449 appears to be involved in sodium stimulation of L-carnitine transport [27]. The degree of impairment in carnitine transport activity was less severe in our study compared to the previous report (previously, carnitine transport activity by Tyr449Asp was found to be reduced to 18% of reference OCTN2, *vs*. 62% in the current study [27]). Although the reduction in L-carnitine transport activity was not as great as that for Phe17Leu, it is possible that even a mild reduction in OCTN2-mediated L-carnitine transport in the kidney may have physiological consequences. As tubular reabsorption of carnitine is normally a very efficient process (>95% of filtered carnitine is reabsorbed in a single pass), even a small reduction in reabsorption could lead to significant increases in renal clearance (and thus reduced total body and plasma carnitine levels). While OCTN2-mediated L-carnitine is Na^+-dependent, transport of the synthetic organic cation TEA by OCTN2 occurs via a pH-dependent, Na^+-independent mechanism. Our finding that the uptake of TEA is stimulated by Tyr449Asp suggests that Tyr449 is also important for TEA transport by OCTN2.

For variants that showed alterations in function, the degree of change in function was greater for the synthetic substrate (TEA) compared to the physiologic substrate

(L-carnitine). This was true for both of the reduced-function SNPs (Phe17Leu and Val481Phe) and the selectivity variant, Tyr449Asp (Figure 2). These findings are consistent with previous studies demonstrating that the OCTN2 protein has distinct recognition sites for TEA *vs.* L-carnitine [27,33-36]. This also suggests that for genes with dual functions (*i.e.*, a physiological and a pharmacological role), mutations that disrupt the essential physiological function may be less tolerated than those that affect less essential functions, such as interactions with drugs or other xenobiotics.

Recently, Peltekova *et al*. demonstrated that a SNP in the promoter region of *SLC22A5* (-207G>C) causes reduced promoter activity as measured by response to heat-shock or treatment with arachidonic acid in reporter and gel-shift assays [19]. This SNP occurs at particularly high allele frequency (approximately 50%) in individuals of European descent. In this study, we found that this SNP is associated with basal L-carnitine transport *ex vivo* in lymphoblastoid cell lines, in the absence of heat shock (Figure 4.5). We also found that OCTN2 mRNA expression in LCLs derived from -207C/C homozygotes tended to be lower than that in -207G/G cell lines, consistent with the supposition that the effect of this SNP on transport activity is related to transcriptional activity at the OCTN2 promoter. In contrast to the previous study, we found that heat shock had no effect on mRNA expression of OCTN2 or of a positive control, MDR1, in LCLs of either genotype. The reasons for this discrepancy are not clear, though one possibility is that the functional elements required for heat shock-inducible expression of OCTN2 (and of MDR1) are constitutively active in our cell system, but not in that used by Peltekova *et al.* [19](HeLa cells and GM10665 fibroblasts). Alternatively, it is

possible that the elements required for heat-shock pathways are not active in this cell system, but the -207G>C polymorphism affects basal expression of OCTN2 in the absence of heat shock. Others have investigated the effect of the -207G>C polymorphism in human cardiac muscle, and found that OCTN2 expression in this tissue was not affected by genotype [16]. Thus, we conclude that the -207G>C polymorphism in the *SLC22A5* promoter may have variable effects on OCTN2 expression depending on the cell system, tissue type, and experimental conditions. This SNP is in linkage disequilibrium with SNPs in *SLC22A4*, a paralog of *SLC22A5* that may also be involved in carnitine disposition and has overlapping substrate specificity with OCTN2 [19,37]. Thus, multipoint haplotypes comprised of polymorphisms in both *SLC22A4* and *SLC22A5* may have additive or synergistic effects, as has been suggested for the OCTN1-Leu503Phe/ *SLC22A5* (-207)G>C haplotype. Individuals who harbor these haplotypes together with reduced function variants identified in this study may be at risk for pathological phenotypes associated with carnitine insufficiency. Recent advances in prospective newborn screening may contribute additional information on common reduced-function alleles of OCTN2, as has been shown for other genes [38].

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CHAPTER 5

EFFECTS OF GENETIC VARIATION IN THE NOVEL ORGANIC CATION TRANSPORTER, OCTN1, ON THE RENAL CLEARANCE OF GABAPENTIN

Introduction

Gabapentin is a broad-spectrum anticonvulsant that is commonly used as adjunctive therapy in a variety of seizure disorders and types [1]. There is increasing evidence for the efficacy of gabapentin as monotherapy in refractory partial epilepsy. Additionally, the list of alternative uses of gabapentin is large and growing, and includes many neurological and psychological disorders, such as diabetic neuropathy, migraine, and bipolar disorder [2-5]. The precise mechanisms of gabapentin action are unclear, but its anticonvulsant activity is believed to be due to a combination of factors, including increasing synthesis and synaptic release of GABA, a major inhibitory neurotransmitter, and decreasing synthesis of the excitatory neurotransmitter glutamate [1].

Gabapentin, which is mostly unbound in plasma ($f_u > 97\%$) is eliminated almost exclusively by the kidney as unchanged parent drug [6]. Although gabapentin is cleared primarily by glomerular filtration, several lines of evidence suggest that the drug is also eliminated by active secretion. In studies of the pharmacokinetics of gabapentin in both children [7] and adults [8], mean gabapentin renal clearance (CL_R) exceeded creatinine clearance (CL_{CR}). Coadministration of cimetidine with gabapentin caused a small but significant reduction in gabapentin renal clearance [9]. Additionally, interindividual variation in gabapentin renal clearance has been shown to be higher than that for

creatinine clearance [7]. Boyd *et al*. observed that the correlation between gabapentin CL_R and CL_{CR} was poor (R^2=0.234), though statistically significant [8]; thus, although gabapentin is primarily cleared by filtration, creatinine clearance explains a surprisingly small fraction of gabapentin CL_R. Collectively, these studies suggest that secretory transporters may play a role in the clearance of gabapentin by the kidney.

Intestinal absorption of gabapentin appears to involve a saturable transport mechanism, as evidenced by its dose-dependent bioavailability [1,9-12]. Interindividual variability in gabapentin absorption is large even at a constant dose, with some subjects having very high (>75%), whereas others having very low (5%) bioavailability [12]. Intersubject variation in the bioavailability was much higher than intrasubject variation after repeated doses, which is consistent with a high heritability component to gabapentin oral absorption [12-14]. These results suggest that gabapentin transporter(s) in the intestine are the limiting factors in gabapentin bioavailability, and that genetic variation in the transporter(s) may explain a large fraction of variability in gabapentin absorption.

Gabapentin has been shown to be transported by the L-type amino acid transporter LAT1, which is expressed in abundance at the basolateral membranes of intestinal and renal epithelial cells, and is widely believed to be responsible for the intestinal absorption of gabapentin [1,15]. However, because gabapentin is relatively hydrophilic (a zwitterion at physiological pH), its permeation across the apical membrane of both intestinal and renal epithelia may require a complementary transport protein. Here, we show that OCTN1

(*SLC22A4*), a transporter of ergothioneine and a variety of organic cations, also mediates gabapentin transport. We also show that a common genetic variant of OCTN1 (L503F), which has previously been shown to alter the substrate selectivity of this protein [16,17], is deficient in gabapentin transport activity. In clinical studies, we then investigate the significance of OCTN1 to the oral absorption and renal secretion of gabapentin by studying the pharmacokinetics of gabapentin in subjects homozygous for either the reference (503L) or variant (503F) allele of OCTN1.

Methods

Cellular Assays

HEK-293 cells were routinely cultured in Dulbecco's modified Eagle's medium (DME H-21) supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% fetal bovine serum. Studies of gabapentin transport by OCTN1 were performed using stably transfected Flp-In-293 cells generated according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

For transport studies, cells were seeded onto 24-well poly-D-lysine coated plates (BD Discovery Labware, Bedford, MA, USA) at a density of 1.5 X 10^5 cells per well in normal growth media and grown for 24 h. Uptake studies were performed by first washing the cells with warm Na^+-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 incubating at 37ºC for 15 min, after which the wash buffer was removed and replaced with buffer containing 25 nM [^{3}H]-gabapentin and 5 μM unlabeled gabapentin, in the presence or absence of 1 mM L-

phenylalanine, which served to inhibit the background (LAT1-mediated) uptake. Uptake was allowed to proceed at 37ºC for 10 min, which was within the linear range of uptake *vs*. time (data not shown). Uptake was terminated by removing the extracellular media and washing three times with ice-cold buffer. Cells were lysed in 1 mL 0.1 N NaOH/0.1% SDS and 800 µL of the lysate was added to 3 mL Ecolite scintillation fluid (ICN Biomedicals, Costa Mesa, CA, USA). Intracellular radioactivity was determined by scintillation counting and normalized to per-well protein content as measured using the BCA protein assay. Results were expressed as the initial rate of uptake (pmol/min/mg protein). For studies of Na^+-dependence of OCTN1-mediated gabapentin transport, cells were treated as above, in the presence or absence of Na^+. To obtain Na^+-free conditions, Na^+ was replaced iso-osmotically with *N*-methyl-D-gluconate (NMDG). For L-ergothioneine *trans*-stimulation studies, cells were preloaded with L-ergothioneine by incubation with 100 µM L-ergothioneine in Na^+-containing buffer for 30 minutes prior to initiating the uptake study. Cells were washed three times with warm Na^+-containing buffer before addition of the radiolabeled gabapentin uptake solution. Uptake of gabapentin after L-ergothioneine preload was then measured as described above.

Pharmacokinetic Study Cohort

Genomic DNA samples were collected from unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project, as described previously [18]. Subjects in the SOPHIE cohort were screened by direct sequencing of exon nine of *SLC22A4* to identify individuals homozygous for either c.1507C/C (encoding 503L/503L) or c.1507T/T (encoding 503F/503F). The reference cDNA sequence of *SLC22A4* was obtained from

GenBank (http://www.ncbi.nlm.nih.gov, accession number NM_003059). Primers were designed manually to span exon nine, and included 80 bp of 5'-flanking intronic sequence and 140 bp of 3'-intronic sequence. Primer sequences were: 5'-CCAACTTCACAAAATGATGCTC-3' (sense) and 5'-CCCAGCCAACAATATGCTTTAT-3' (antisense). Because the 503F polymorphism has been described primarily in individuals of European ancestry [16,19], this screen was restricted to the European American subset of SOPHIE. Subjects homozygous for 503L or 503F were recruited into a clinical study designed to assess the effect of *SLC22A4* genotype on the pharmacokinetics of gabapentin.

Subjects in the study group were between the ages of 18 and 40 years old, and were selected as healthy by medical history questionnaire and screening blood work (CBC, Comprehensive Metabolic panel). Subjects were taking no regular medications and had normal renal function. Subjects were excluded from participation if they were: pregnant; had a new history indicating they are no longer healthy; taking a medication that could confound study results; or did not consent to participate in the study. Individuals with anemia (hemoglobin < 12 g/dL), an elevation in liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase) to higher than double the respective normal value, or elevated creatinine concentrations (males ≥ 1.5 mg/dL, females ≥ 1.4 mg/dL), were excluded.

Study Design

A total of twenty subjects homozygous for either genotype participated in a 36-hour

study of gabapentin pharmacokinetics. Informed consent was obtained from each subject. Subjects reported to the SFGH GCRC in a fasting state, and voided all urine at the start of the study. A single indwelling catheter was placed in an arm vein for serial blood collections. A blood sample (20 ml) was collected at baseline to measure serum creatinine and to serve as a blank for determination of gabapentin in plasma. A single dose of gabapentin (400 mg) was administered with 240 mL water, and subjects resumed a normal meal schedule starting two hours after drug administration. Blood samples (6 mL each) were collected at 0.25, 0.75, 1, 1.5, 2, 3, 4, 6, 8, 12, 18, 24, 30, and 36 hours after dosing. Urine was collected over the course of 36 hours in the following time periods: 0-2 hr, 2-4 hr, 4-8 hr, 8-12 hr, 12-24 hr, and 24-36 hr.

Gabapentin was measured in plasma and urine using an LC/MS/MS method developed by the UCSF Drug Studies Unit. The method reproducibly detected gabapentin concentrations as low as 75 ng/ml. Data from both plasma and urine were simultaneously fit to a one-compartment closed model defined by the following differential equations:

(1) $df(1) = -f(1)*K_a \mid f(1) = F_{po}*\text{Dose at } t = 0$

(2) $df(2) = f(1)*K_a/V - f(2)*CL_R \mid f(2) = 0 \text{ at } t = 0$

(3) $df(3) = f(2)*CL_R \mid f(3) = 0 \text{ at } t = 0$

Where K_a is the first-order absorption rate constant, F_{po} is the oral bioavailability, V is the volume of distribution, and CL_R is the renal clearance of gabapentin. These four primary parameters were estimated by nonlinear regression of the above set using WinNonlin (Pharsight Inc., Research Triangle Park, NC, USA). The dose (400 mg) was a constant. Equation (1) represents the change in amount of drug in the gut compartment *vs*. time.

Equation (2) describes the change in plasma concentration *vs*. time. Equation (3) describes the rate of gabapentin excretion in urine *vs*. time. Since gabapentin is eliminated exclusively by the kidney as unchanged parent compound, total clearance was assumed to be equal to the renal clearance, and the total bioavailable dose of gabapentin is theoretically equal to the total amount collected in urine divided by the dose (A_e/Dose). This parameter was estimated both by nonlinear regression (as above) and by computing the total amount collected in urine and dividing by the dose administered. The two methods for estimating F_{po} yielded almost identical results. Urine samples were analyzed for creatinine, and creatinine clearance (CL_{CR}) was estimated by dividing the 24-hr excretion rate by the plasma creatinine concentration at baseline.

Pharmacokinetic parameters were summarized for each genotype group by the mean, standard deviation, median, and range. Differences in pharmacokinetic parameters between the two genotype groups were evaluated with a *t*-test.

Nineteen of the 20 subjects completed the study. Subjects who completed the study included 10 subjects homozygous for the reference (503L) allele of OCTN1 and nine subjects homozygous for the L503F variant. A twelfth subject in the reference (c1507C/C) genotype group did not adhere to the urine collection protocol, and data from this subject were omitted from the analysis. Because a major aim in designing the study was to investigate the role of OCTN1 in gabapentin bioavailability, a sample size of 10 subjects in each genotype group was desired in order to achieve 80% power to detect a difference in F_{po} of 0.25 between the two groups, assuming a standard deviation of 0.2 (coefficient of variation in bioavailability is *ca*. 25%) as seen in healthy volunteers.

Results

OCTN1-mediated Transport of Gabapentin and Effects of OCTN1 Genetic Variants

Because of the structural similarity between gabapentin and L-carnitine (a natural substrate of OCTN transporters), and because the expression of OCTN1 in the intestine and kidney suggests it may play a role in gabapentin pharmacokinetics, we assessed the ability of OCTN1 to facilitate uptake of gabapentin in a heterologous expression system. As shown in Figure 5.1, uptake of gabapentin in cells expressing the reference OCTN1 was enhanced approximately four-fold compared to that in mock-transfected cells (mean +/- S.D.: 18.3 +/- 2.1 pmol/min/mg protein in OCTN1-503L-transfected cells *vs*. 4.6 +/- 0.6 pmol/min/mg protein in mock-transfected cells). In contrast, uptake of gabapentin in cells transfected with the common L503F variant of OCTN1 was enhanced only two-fold (10.2 +/- 0.4 pmol/min/mg protein) *vs*. control cells. Since gabapentin is a zwitterion with structural similarity to L-carnitine, and L-carnitine and other zwitterions (*e.g.*, L-ergothioneine) are transported by the OCTNs in a Na^+-dependent manner [16,20], we investigated whether gabapentin transport by OCTN1 was dependent on the sodium gradient. Iso-osmotic replacement of Na^+ with *N*-methyl-D-gluconate (NMDG) in the transport buffer had no negative effect on OCTN1-mediated gabapentin transport (Figure 5.1); rather, the uptake of gabapentin appeared to be slightly enhanced in the absence of Na^+. *Trans*-stimulation experiments using L-ergothioneine, the natural substrate of OCTN1, were also performed (Figure 5.2). Regardless of whether the buffer used for gabapentin uptake contained Na^+, uptake of gabapentin was enhanced when the cells

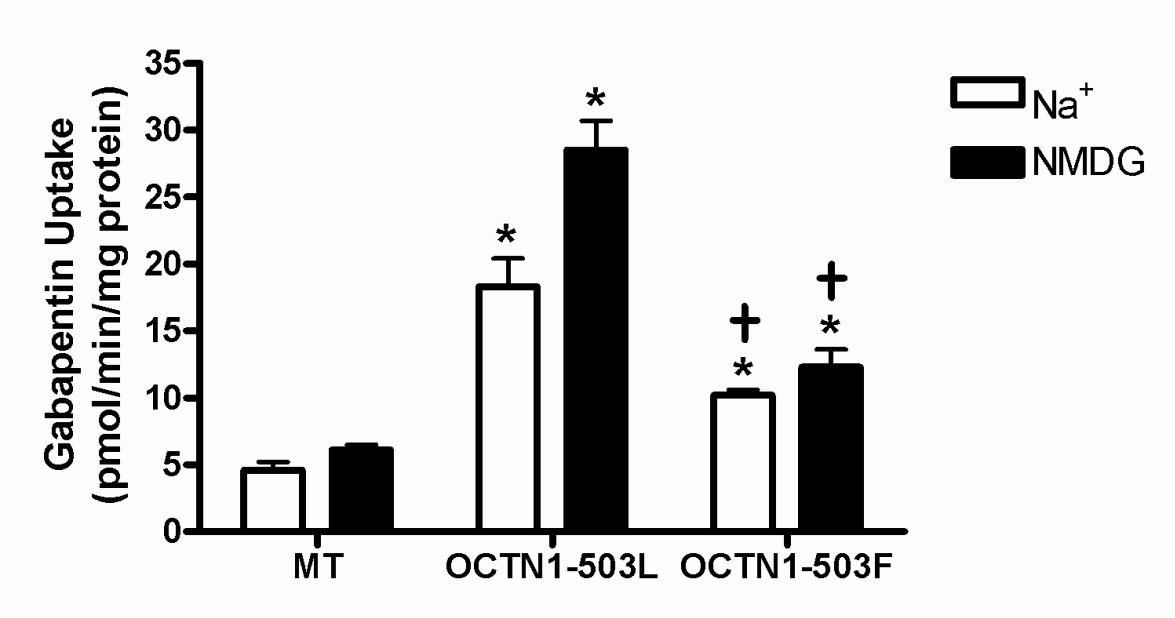

Figure 5.1. Na^+-independent gabapentin transport by OCTN1 and L503F variant. HEK-293 cells stably expressing OCTN1-reference or OCTN1-L503F were incubated in Na^+-containing (Na^+) or Na^+-depleted (NMDG) buffer in the presence of 10 μM [^{3}H]-gabapentin and 1 mM L-phenylalanine (a LAT1 inhibitor) for 10 minutes. Mock-transfected cells (MT) served as a negative control. Data shown represent mean +/- S.D. from triplicate wells in a representative experiment. *$p<0.01$ vs. control (mock-transfected) cells. $^{\dagger}p<0.01$ vs. OCTN1-REF.

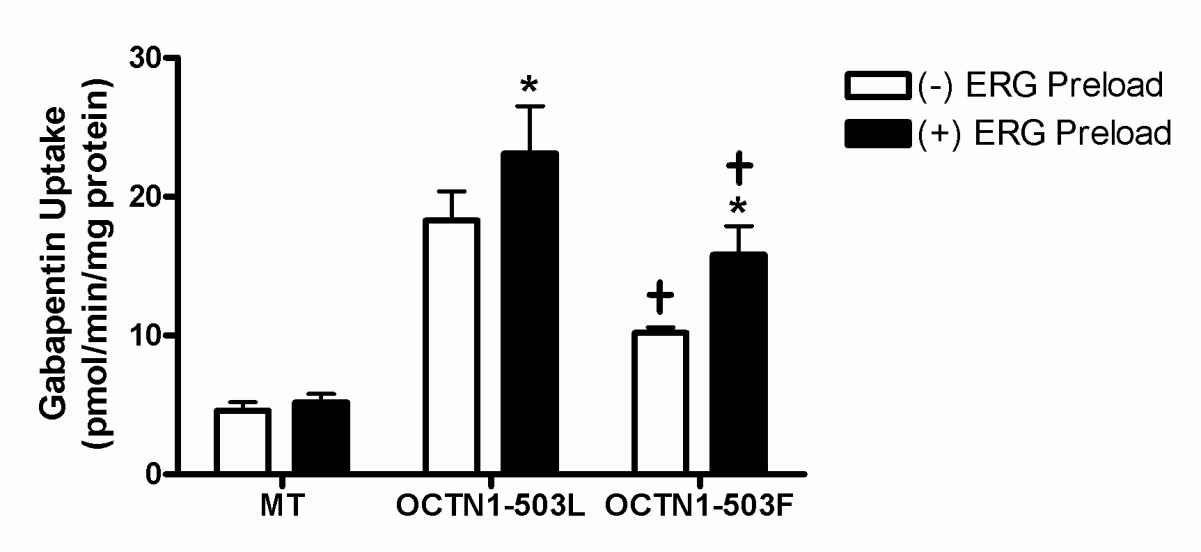

Figure 5.2. L-Ergothioneine trans-stimulates OCTN1-mediated gabapentin transport. HEK-293 cells stably expressing OCTN1-reference or OCTN1-L503F were preloaded with L-ergothioneine by incubation with 100 μM L-ergothioneine for 30 minutes, washed thrice with blank Na^+-containing buffer, and incubated for 10 minutes in the presence of 10 μM [^{3}H]-gabapentin and 1 mM L-phenylalanine. Mock-transfected cells (MT) served as a negative control. Data shown represent mean +/- S.D. from triplicate wells in a representative experiment. *$p<0.05$ vs. non-preloaded (ERG(-)) cells. $^{\dagger}p<0.01$ vs. OCTN1-REF.

were preloaded with unlabeled L-ergothioneine. The data show that gabapentin is transported by OCTN1 in a sodium-independent manner, and that this transport activity is reduced for the L503F variant compared with the reference OCTN1.

Effect of OCTN1 Genetic Variants on Clinical Pharmacokinetics of Gabapentin

The pharmacokinetics of gabapentin were evaluated in 11 subjects homozygous for the common allele (503L) of OCTN1 (group *1 subjects), and 9 gender-matched subjects homozygous for the common variant L503F (group *2). Data from a single *1 subject who did not comply with the urine collection protocol were omitted from the analysis. Demographic characteristics of the study group are shown in Table 5.1. The two genotype groups were similar in terms of gender, age, height and weight.

Mean gabapentin plasma concentration *vs.* time curves are shown in Figure 5.3. Gabapentin concentrations were significantly lower in the *1 group (503L/503L) than the *2 group (503F/503F) at 3, 6, 8, 18, and 24 hours after gabapentin administration. The total area under the plasma concentration vs. time curve (AUC) was significantly lower in the *1 group (mean +/- S.D.: 23.1 +/- 3.75 mg*hr/L) than in the *2 group (28.6 +/- 4.98 mg*hr/L, $p = 0.014$). The mean peak plasma concentration (C_{max}) was lower in *1 subjects vs. *2 subjects, though this difference was not significant.

Pharmacokinetic parameters for the two genotype groups are summarized in Table 5.2. Because gabapentin is eliminated exclusively by the kidney as unchanged parent compound, bioavailability of gabapentin was estimated using the cumulative amount

Table 5.1. Demographics of study participants by genotype.

	Genotype		
	c.1507C/C (503L/503L, n=10)	c.1507T/T (503F/503F, n=9)	p-value
Gender (% female)	50%	56%	1.000
Height (cm)	174.2 +/- 3.6	174.3 +/- 4.1	0.975
Weight (kg)	77.8 +/- 4.1	77.6 +/- 7.5	0.982
Age (yr)	31.5 +/- 1.4	29.7 +/- 1.9	0.455

Height, weight and age are given as the mean +/- standard error for each genotype group. Differences between groups with respect to gender were tested using Fisher's exact test. Differences between genotype groups with respect to other variables were assessed by t-test.

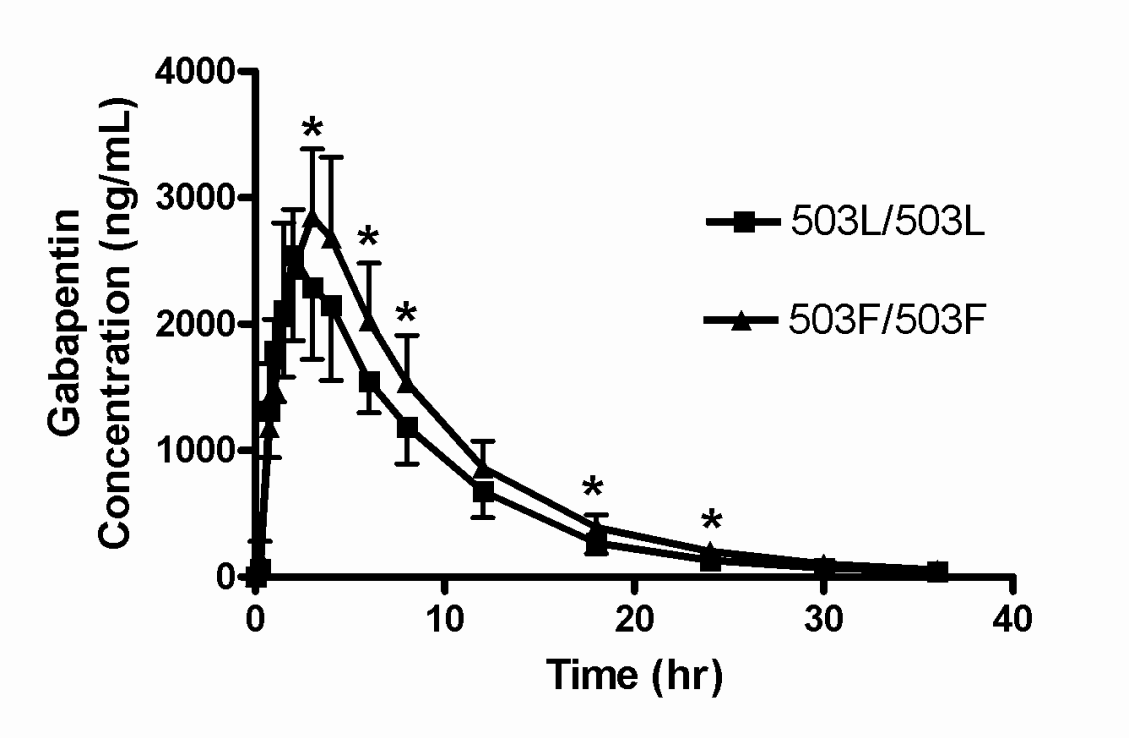

Figure 5.3. Gabapentin pharmacokinetics in OCTN1-503L and OCTN1-503F homozygotes. A single 400 mg dose of gabapentin was administered orally to subjects homozygous for the reference (squares, n=10) or variant (triangles, n=9) allele of OCTN1. Data are shown as mean +/- standard deviation for each time point. Significant differences in plasma concentration between genotype groups (as assessed by unpaired two-tailed t-test) are indicated by asterisks.

Table 5.2. Summary of gabapentin pharmacokinetic parameters by genotype

	Total Sample	By Genotype		
		c.1507C/C	c.1507T/T	p-value
		(503L/503L, n=10)	(503F/503F, n=9)	
F_{po} (%)	44.4 +/- 11.7	45.1 +/- 11.3	43.7 +/- 12.7	0.801
C_{max} (mg/mL)	2.83 +/- 0.52	2.69 +/- 0.52	2.98 +/- 0.51	0.233
T_{max} (hr)	2.81 +/- 0.91	2.75 +/- 0.98	2.89 +/- 0.89	0.751
K_a (hr^{-1})	0.548 +/- 0.286	0.647 +/- 0.338	0.438 +/- 0.172	0.107
K_e (hr^{-1})	0.220 +/- 0.071	0.219 +/- 0.070	0.222 +/- 0.076	0.936
V_d (L)	38.2 +/- 16.2	42.8 +/- 16.5	33.1 +/- 15.3	0.202
AUC (mg*hr/L)	25.7 +/- 5.10	23.1 +/- 3.75	28.6 +/- 4.98	0.014
CL_R (mL/min)	126 +/- 35	141.4 +/- 26.2	109.9 +/- 36.8	0.045
CL_{CR} (mL/min)	105 +/- 20	104.8 +/- 19.0	106.1 +/- 22.4	0.891
ATS (mL/min)	21.0 +/- 28.5	36.6 +/- 25.0	3.83 +/- 21.9	0.008

Parameter estimates are given as mean +/- standard deviation. F_{po}: oral bioavailability; C_{max}: peak plasma concentration; T_{max}: time to peak plasma concentration; K_a: absorption rate constant; K_e: first-order elimination rate constant; CL_R: renal clearance; CL_{CR}: 24-hour measured creatinine clearance; ATS: active tubular secretion (CL_R - CL_{CR}). Differences between genotype groups were assessed by two-tailed unpaired *t*-test.

excreted in urine at the end of the study. Gabapentin bioavailability in the total sample was 0.44 +/- 0.117 (mean +/- S.D.). However, the bioavailability of gabapentin was not different between subject groups. There was a trend toward reduced absorption rate constant (K_a) in the *2 subjects vs. *1 subjects (0.438 +/- 0.172 hr^{-1} *vs.* 0.647 +/- 0.338 hr^{-1}), but this difference was not significant (p = 0.11). Additionally, there was a trend toward reduced volume of distribution (V_d) in the *2 subjects (33.1 +/- 15.3 L, *vs.* 42.8 +/- 16.5 L in *1 subjects), but this was also not significant (p = 0.20).

A significant difference between genotype groups was found with respect to renal clearance of gabapentin: *1 subjects showed a higher gabapentin CL_R (141 +/- 26 mL/min) compared to the *2 subjects (110 +/- 37 mL/min, p = 0.045). This difference was not explained by the creatinine clearance, which was nearly identical between genotype groups (105 +/- 19 mL/min for *1 subjects vs. 106 +/- 22 mL/min for *2 subjects, p = 0.891).

The renal clearance of gabapentin was 126 +/- 35 mL/min in the total sample, which was higher than creatinine clearance (105 +/- 20 mL/min). When the genotype groups were compared in terms of net secretion of gabapentin (gabapentin CL_R – CL_{CR}), an interesting finding emerged. In *1 subjects, renal clearance of gabapentin significantly exceeded the creatinine clearance, whereas in *2 subjects, there was, on average, almost no active secretion of gabapentin. Net secretion in *1 subjects was 36.6 +/- 25 mL/min, vs. 3.8 +/- 22 mL/min in the *2 group (p=0.008). The effect of genotype on gabapentin net

secretion is shown graphically in Figure 5.4. The large increase in active secretion of gabapentin in the *1 genotype group vs. the *2 subjects was still observed, and to a similar degree, when estimates of glomerular filtration rate were used (Cockcroft-Gault, MDRD) rather than the measured creatinine clearance [21,22].

Using linear regression, we then tested the following variables as predictors of gabapentin renal clearance: age, height, weight, gender, CL_{CR}, and OCTN1 genotype. Of these, only CL_{CR} (p=0.01) and OCTN1 genotype (p=0.045) were significant predictors of gabapentin CL_R, with CL_{CR} alone explaining only slightly more of the total variance in gabapentin CL_R than OCTN1 genotype ($R^2 = 0.33$ for the regression of gabapentin CL_R on CL_{CR}, *vs.* $R^2 = 0.22$ for regression of gabapentin CL_R on OCTN1 genotype). When both CL_{CR} and OCTN1 genotype were included in a multiple linear regression model, 56% of the variance in gabapentin CL_R was explained (*i.e.* $R^2 = 0.56$ for the model including both CL_{CR} and OCTN1 genotype terms), and the p-value for the model fit ($p < 0.001$) and both predictors (CL_{CR}, $p < 0.003$; OCTN1 genotype, $p < 0.01$) improved. There were no significant interaction effects between these two predictor variables. OCTN1 genotype was the only significant predictor of gabapentin net secretion ($R^2 = 0.35$, $p < 0.008$).

Discussion

Although the importance of drug transporters to clinical pharmacokinetics has been well described, there exist very few examples of pharmacogenetic studies showing an influence of genetic variation in drug transporter genes on pharmacokinetics of substrate drugs [23]. Most studies have focused on the role of transporters in hepatic uptake and

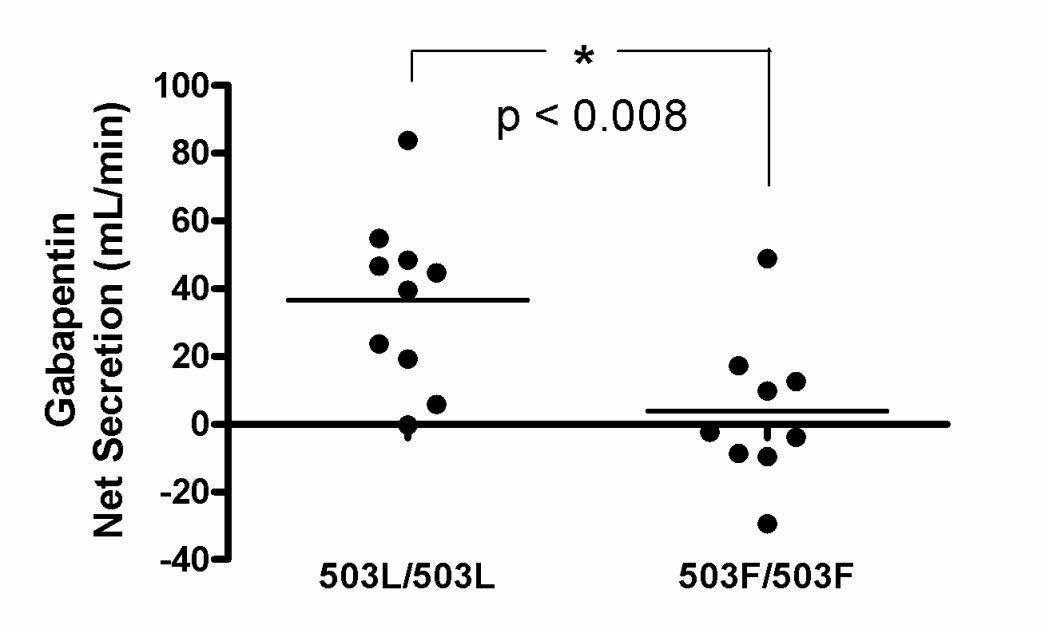

Figure 5.4. Effect of OCTN1 genotype on net tubular secretion of gabapentin. Glomerular filtration rate was estimated by 24-hour creatinine clearance (CL_{CR}) measurements, and net secretion of gabapentin was calculated by substracting CL_{CR} from the total gabapentin renal clearance. Subjects were homozygous for either the reference (503L/503L) allele of OCTN1 or the variant (503F/503F) allele. Net secretion of gabapentin was significantly different between genotype groups (p=0.008) by unpaired two-tailed t-test.

biliary excretion, or intestinal drug efflux with few studies focusing on other aspects of drug disposition such as renal clearance.

In this study, we describe the interaction of gabapentin with OCTN1. Although gabapentin has been shown previously to be a substrate of the LAT1 amino acid transporter [15], to our knowledge no other transporters have been shown to interact with gabapentin. LAT1 is expressed at the basolateral membrane of intestine and kidney, and is likely responsible for basolateral transport of this drug. Since the expression of OCTN1 is restricted to the apical membrane [24], it is possible that OCTN1 contributes to the apical transport of gabapentin in the intestine and kidney. We show that transport of gabapentin by OCTN1 is independent of the sodium gradient, unlike some naturally occurring OCTN1 substrates (*e.g.*, L-carnitine, L-ergothioneine), and that OCTN1-mediated transport of gabapentin is trans-stimulated by L-ergothioneine, the preferred substrate of OCTN1 [20]. These findings suggest that renal transport of gabapentin by OCTN1 may occur in the secretory direction (from cell to lumen), and may be driven by exchange for reabsorbed L-ergothioneine *in vivo*. An analogous situation has been observed for the closely related bifunctional transporter OCTN2, which facilitates the active reabsorption of some substrates (*e.g.*, L-carnitine) via Na^+-symport at the apical membrane [25], but acts as a secretory transporter for others (*e.g.*, tetraethylammonium) that are transported in a Na^+-independent manner [26].

We found that a genetic variant of OCTN1, L503F, exhibits significantly reduced transport of gabapentin in cellular assays (Figures 5.1 and 5.2). Because the L503F

variant is relatively common (with a minor allele frequency of approximately 42% in individuals of European descent), this finding allowed us to investigate the role of OCTN1 in drug disposition *in vivo*, using gabapentin as a probe drug to assess differences between individuals homozygous for either the reference or variant alleles of OCTN1 with respect to intestinal drug absorption and renal drug secretion. Our study suggests that OCTN1 does not contribute substantially to the oral absorption rate or bioavailability of gabapentin (see Table 5.2), which is in agreement with previous studies implicating LAT1-mediated transport as the limiting step in the oral absorption of gabapentin [10,15].

In contrast, our study suggests that OCTN1 is responsible for the active secretory component of the renal clearance of gabapentin. We found a striking difference between genotype groups in the net secretion of gabapentin (Figure 5.4), with almost all of the *1 (503L/503L) subjects showing positive net secretion of gabapentin (95% CI: 18.7 to 54.5 mL/min), and the *2 (503F/503F) subjects exhibiting values close to zero (95% CI: -13.0 to 20.7 mL/min). Of the subject characteristics that were measured in this study, only CL_{CR} and OCTN1 genotype were significant predictors of gabapentin renal clearance. Creatinine clearance was the best predictor of gabapentin CL_R; however, similar to Boyd *et al*. [8], we found that CL_{CR} explained a surprisingly small fraction (33%) of the variance in gabapentin CL_R given that gabapentin clearance occurs primarily through filtration. OCTN1 genotype was responsible for a significant fraction (22%) of the variance in gabapentin CL_R, and was the only significant predictor of net secretion of gabapentin. When both CL_{CR} and OCTN1 genotype were included in a multiple

regression model, >56% of the total variance in gabapentin CL_R was explained by these two predictors, which suggests that CL_{CR} and OCTN1 genotype contribute complementary (*i.e.*, non-overlapping) information on gabapentin CL_R, with CL_{CR} acting as a predictor of gabapentin filtration clearance, and OCTN1 genotype predicting active secretory clearance of gabapentin. These findings, coupled with the fact that the L503F variant of OCTN1 has reduced activity toward gabapentin in cellular assays, support the conclusion that OCTN1 is responsible for the renal secretion of gabapentin.

Gabapentin is a widely prescribed drug for a variety of neurological and psychological disorders. The popularity of the use of gabapentin may be explained, in part, by its relatively benign toxicity profile and wide therapeutic range, particularly in comparison to other anticonvulsant drugs for which the side effects and clinical manageability are a major problem in pharmacotherapy. As such, the clinical utility of predictors of gabapentin pharmacokinetics is likely to be limited. However, the current study provides a proof of principle regarding the relationship between genetic variation in renal drug transporters and drug pharmacokinetics that may by applied to other drugs or other renal transporters. Our findings represent a first step in understanding the potential effects of genetic variants in renal drug transporters on drug clearance generally.

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CHAPTER 6

INFLUENCE OF GENETIC VARIANTS OF THE NOVEL ORGANIC CATION TRANSPORTERS, OCTN1 AND OCTN2, ON CARNITINE HOMEOSTASIS AND PLASMA LIPIDS

Introduction

The novel organic cation transporter 2 (OCTN2) is a member of the solute carrier family of transporters that facilitate the flux of small molecules across cell membranes. As its name suggests, OCTN2 was discovered as a multispecific transporter of organic cations [1]; however, it was soon shown to be more important as a high-affinity transporter of L-carnitine [2]. L-carnitine is important physiologically as an essential cofactor in mitochondrial beta-oxidation of fatty acids [3]. Rare inherited deficiencies in the *SLC22A5* gene cause systemic carnitine deficiency (SCD; OMIM#212140) or primary carnitine uptake deficiency (CUD), a disease in which loss of OCTN2-mediated carnitine transport leads to loss of renal tubular reabsorption of filtered carnitine and resultant deficits in systemic carnitine levels, as well as reduced uptake of carnitine into tissues [4,5]. This, in turn, leads to impaired utilization of fatty acids for energy, and lipid deposition in energetic tissues such as heart and skeletal muscle. The clinical presentation of SCD is variable, but includes early-onset skeletal myopathy, cardiomyopathy, encephalopathy, hepatic steatosis and, in some cases, acute liver failure [4,5]. Given the severe phenotype caused by rare null mutations in *SLC22A5*, it is reasonable to inquire whether more common variants of this gene may cause a less severe, but clinically important phenotype. In fact, evidence from both humans and mice

heterozygous for loss-of-function alleles of *SLC22A5* suggests that even a partial defect in OCTN2 activity is sufficient to produce phenotypic carnitine insufficiency, including hepatic steatosis and benign cardiac hypertrophy [6-8]. Thus, it is possible that common reduced-function alleles of the OCTN genes may produce physiological abnormalities related to reduced carnitine transport.

Information on the relationship between OCTN transporters and plasma lipids is provided by the del^{11}/del^{11} mouse (in which the *Octn* locus has been deleted), which exhibits carnitine deficiency that is associated with a severe increase in triglycerides in plasma (~10-fold), heart (~4-fold) and liver (~14-fold) [9]. In addition, it has previously been noted that supplemental carnitine reduces plasma triglyceride concentrations in hypertriglyceridemic hemodialysis patients [10], in patients with type IV hyperlipoproteinemia [11], and in HIV-infected patients receiving highly active antiretroviral therapy (HAART) [12]. Hypertriglyceridemia is known to be an independent risk factor for coronary heart disease [13-15]. Although the genetic basis for certain rare familial hyperlipidemias has been studied extensively, information on the genetic determinants of susceptibility to hyperlipidemias is currently lacking [16-18]. The high-affinity carnitine transporter, OCTN2, represents an interesting candidate gene for hypertriglyceridemia.

We and others have identified both common polymorphisms and rare variants in the *SLC22A5* gene that are predicted to have functional significance [19,20]. We have also identified several SNPs in *SLC22A4*, a close homolog of *SLC22A5* that transports carnitine with lower affinity. As a result of their proximity to each other, a specific locus haplotype of OCTN1 and OCTN2 (*SLC22A4-1507T, SLC22A5-(-207C)*) is found in

tandem on chromosome 5. The *SLC22A4-1507T* results in a non-synonymous amino acid change in OCTN1 (L503F) and the *SLC22A5-(-207C)* is a variant that disrupts a heat shock element in the OCTN2 promoter region. Studies by Peltekova *et al.* showed that OCTN1-L503F exhibits reduced transport of carnitine and other substrates of these transporters [20]. In fact, the K_i of carnitine for OCTN1-L503F was one thousand fold higher than that for the reference OCTN1. Peltekova *et al.* have shown that the promoter region variant *SLC22A5-(-207C)* results in reduced binding to nuclear extracts, and reduced inducibility by heat shock in reporter assays [20]. We previously showed that this promoter variant was associated with reduced basal L-carnitine transport activity and reduced OCTN2 mRNA expression in immortalized human lymphoblasts [19].

Therefore, we hypothesized that individuals who are homozygous for the haplotype *SLC22A4-1507T, SLC22A5-(-207C)* will have a "double hit", that is, a reduced expression level of OCTN2 and a reduced activity of OCTN1 thereby conferring an overall reduced carnitine transport activity. We anticipated that reduced carnitine transport activity would be expressed as a reduction in active reabsorption of carnitine in the kidney, leading to increased renal clearance and reduced plasma carnitine levels. We also hypothesized that reduced carnitine levels would be associated with increased plasma triglyceride levels as seen in the del^{11}/del^{11} mouse model [9].

To address this, we used a genotype-to-phenotype strategy to inquire whether individuals homozygous for these common haplotypes of the OCTN genes differed with respect to carnitine renal clearance, carnitine plasma concentrations, or plasma lipid profiles.

Materials and Methods

Study Cohort

Genomic DNA samples were collected from unrelated healthy individuals in the San Francisco Bay Area as part of the Studies of Pharmacogenetics in Ethnically Diverse Populations (SOPHIE) project. Variants in the *SLC22A4* and *SLC22A5* genes were identified by direct sequencing of genomic DNA as previously described [21] from an ethnically diverse population of 270 individuals: 80 African-Americans, 80 European-Americans, 60 Asian-Americans (50 Chinese-Americans and 10 Japanese-Americans), and 50 Mexican-Americans. The reference cDNA sequences of *SLC22A4* and *SLC22A5* were obtained from GenBank (http://www.ncbi.nlm.nih.gov, accession numbers NM_003059 and NM_003060). Primers were designed manually to span the exons and 50–200 bp of flanking intronic sequence per exon. The primer sequences can be found at http://www.pharmgkb.org.

Subjects in the SOPHIE cohort were screened by direct sequencing of exon nine in *SLC22A4* and of the promoter region of *SLC22A5* to identify individuals homozygous for either of the two major haplotypes: (*SLC22A4-1507C, SLC22A5-(-207G)*), which we called the *1 haplotype, or (*SLC22A4-1507T, SLC22A5-(-207C)*), which we called the *2 haplotype. Because this haplotype had only been described in individuals of European ancestry, this screen was restricted to the European American subset of SOPHIE. Homozygotes were recruited into a clinical study designed to assess differences between

subject groups in carnitine renal clearance and in carnitine transport activity and OCTN2 mRNA expression in lymphoblastoid cell lines (LCLs).

Subjects in the SOPHIE cohort were between the ages of 18 and 40 years old, and were selected as healthy by medical history questionnaire and screening blood work (CBC, Comprehensive Metabolic panel). Subjects were taking no regular medications and had normal renal function. Subjects were excluded from participation if they were: pregnant; had a new history indicating they are no longer healthy; taking a medication that could confound study results; or did not consent to participate in the study. Individuals with anemia (hemoglobin < 12 g/dL), an elevation in liver enzymes (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyltransferase) to higher than double the respective normal value, or elevated creatinine concentrations (males ≥ 1.5 mg/dL, females ≥ 1.4 mg/dL), were excluded.

Study Design

Sixteen subjects, eight in each haplotype group, were enrolled into a genotype-to-phenotype study that attempted to discover an effect of OCTN haplotype on carnitine transport activity and plasma triglycerides. The primary measure was renal clearance of carnitine, assessed following a 2-hour urine collection period. Informed consent was obtained from each subject. Subjects reported to the SFGH GCRC in a fasting state, and voided all urine at the start of the study. A blood sample (20 mL) was drawn at t=0 for determination of serum creatinine, carnitine, and acylcarnitine esters, and for transformation of lymphoblasts for *ex vivo* studies of carnitine transport (Chapter 4).

Urine was collected over 2 hours after the initial void, and urine pH was measured. A second blood sample (20 mL) was collected at 2 hours after the initial urine void for measurement of carnitine and acylcarnitines. Carnitine was measured in plasma and urine using an LC/MS/MS method developed for clinical use by the Stanford University Biochemical Genetics Laboratory. The method reliably detected carnitine concentrations as low as 300 nM in plasma and urine.

Renal clearance of carnitine was estimated by dividing the urinary excretion rate by the average plasma concentration ($CL_R = A_{e,0\text{-}2hr}/(2\ hr * (C_{p,\ t=0\ hr} + C_{p,\ t=2\ hr})/2)$. This value was normalized to the glomerular filtration rate (GFR) estimated by a two-hour creatinine clearance measurement.

Plasma lipids were measured by CLIA-certified assays (Quest Diagnostics), including total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, very-low-density lipoprotein (VLDL) cholesterol, and triglycerides.

Statistical analyses

Differences in pharmacokinetic parameters, cholesterol and triglyceride levels, and carnitine transport activity between the two haplotype groups were evaluated by *t*-test or nonparametric tests where appropriate. Relationships between study measures were assessed by multivariate linear regression using JMP (SAS Institute, Inc., Cary, NC, USA), correcting for effects of age, gender, and weight where appropriate. Since extreme deficiencies in carnitine transport produce obvious disease, we did not expect to

find a complete loss of carnitine transport activity in our healthy test subjects; however, more modest decreases were hypothesized. Renal elimination of carnitine occurs by filtration, followed by extensive active tubular reabsorption, so that >98% of filtered carnitine is reabsorbed into the systemic circulation. Therefore, a modest decrease in active reabsorptive transport of carnitine (e.g., to 93% efficiency) produces a significant increase in renal clearance (a 2-fold increase, in this case). A sample size of n=8 per group was chosen to achieve 80% power to detect a 2-fold increase in renal clearance of carnitine assuming a within-group coefficient of variation of 50% at a significance level α=0.05.

Results

Effect of OCTN Haplotype on Carnitine Disposition

We enrolled a total of 16 healthy adult volunteers into a 2-hour study of carnitine renal clearance, including seven subjects homozygous for the *1 haplotype (*SLC22A4-1507C, SLC22A5-(-207G)*) and nine homozygous for the *2 haplotype (*SLC22A4-1507T, SLC22A5-(-207C)*). The subjects were recruited so as to achieve gender balance between haplotype groups, but subjects were not matched. The demographic and clinical characteristics of the subjects are shown in Table 6.1. The two haplotype groups did not differ with respect to gender, age, height, or weight.

The primary study measures are summarized in Table 6.2. There were no significant differences between haplotype groups in terms of carnitine renal clearance, fractional excretion (*i.e.*, carnitine CL_R as a percent of creatinine clearance), or plasma carnitine concentration. We determined both the total (carnitine plus acylcarnitine esters) and free

Table 6.1. Demographic characteristics of study sample by haplotype.

	Total Sample	By Haplotype		p-value
		*1/*1 (n = 7)	*2/*2 (n = 9)	
Gender (Male/Female)	7/9	4/3	3/6	0.614
Age (yr)	31.9 +/- 5.1	30.1 +/- 5.4	33.3 +/- 4.6	0.223
Height (cm)	176 +/- 11	181 +/- 12	172 +/- 8.8	0.184
Weight (kg)	74.4 +/- 13.1	79.4 +/- 15.1	70.2 +/- 10.8	0.270
BMI	23.7 +/- 2.5	24.0 +/- 3.4	23.5 +/- 1.9	0.749

Healthy subjects aged 18-40 years and of European descent were recruited for the study based on haplotype at the OCTN locus. Subjects were homozygous for either the common *1 haplotype (*SLC22A4-1507C, SLC22A5-(-207G)*) or the *2 haplotype (*SLC22A4-1507T, SLC22A5-(-207C)*). Values are shown as mean +/- standard deviation. Baseline differences between subject groups were assessed using a two-tailed unpaired t-test. Gender differences were assessed by Fisher's exact test.

Table 6.2. Summary of carnitine disposition by haplotype.

	Total Sample	By Haplotype		p-value
		*1/*1 (n = 7)	*2/*2 (n = 9)	
Plasma Carnitine (μM)				
Free	31.2 +/- 5.3	32.5 +/- 5.9	30.2 +/- 4.9	0.410
Esters	8.35 +/- 2.45	7.90 +/- 1.51	8.71 +/- 3.04	0.532
Total	39.6 +/- 5.7	40.4 +/- 6.2	38.9 +/- 5.6	0.622
Ester/Free Ratio	0.274 +/- 0.093	0.249 +/- 0.057	0.295 +/- 0.11	0.342
Carnitine CL_R (mL/min)				
Free	2.77 +/- 2.25	3.20 +/- 2.97	2.44 +/- 1.61	0.527
Total	5.77 +/- 3.24	5.78 +/- 3.58	5.76 +/- 3.17	0.993
Fractional Excretion (% of GFR)				
Free	1.92 +/- 1.29	2.06 +/- 1.60	1.82 +/- 1.08	0.724
Total	4.16 +/- 2.05	3.93 +/- 2.11	4.34 +/- 2.12	0.707
Creatinine Clearance (mL/min)	138 +/- 34	145 +/- 40	132 +/- 30	0.478

After a 12-hour fast, blood was drawn from subjects homozygous for either the *1 haplotype (*SLC22A4-1507C, SLC22A5-(-207G)*, n = 7) or *2 haplotype (*SLC22A4-1507T, SLC22A5-(-207C)*, n = 9) at the OCTN locus. Urine was collected over the following two hours, and a final blood draw was collected at two hours after initiating the study. Plasma and urine samples were analyzed for free (non-esterified) carnitine and total (free plus esterified) carnitine as well as creatinine. The mean analyte concentration (from the baseline and 2-hour blood draws) for each subject was used in the analysis. Values are shown as mean +/- standard deviation. Differences between haplotype groups were assessed using a two-tailed unpaired t-test.

(non-esterified) carnitine in both plasma and urine. OCTN haplotype did not have any significant effect on renal clearance of total or free carnitine. Likewise, subjects did not differ between haplotype groups with respect to plasma free carnitine or acylcarnitines, or with respect to the ratio of free/esterified carnitine.

We performed multiple linear regression to determine whether carnitine renal clearance could be predicted by measured variables other than OCTN haplotype, and to determine whether correcting for these effects would allow detection of a phenotypic effect of OCTN haplotype. We discovered that gender was significantly associated with carnitine plasma concentrations in our sample (Figure 6.1), with male subjects having significantly higher plasma carnitine levels than female subjects (mean +/- S.D.: 34.8 +/- 4.6 μM for males *vs*. 28.5 +/- 4.2 μM for females, p=0.01). This difference was not explained by decreased carnitine clearance in male subjects, who had, on average, higher renal clearance of carnitine (3.57 +/- 2.89 mL/min *vs*. 2.15 +/- 1.49 mL/min for females), though this difference was not significant (p=0.28). Multiple regression of carnitine disposition parameters (carnitine plasma levels, carnitine CL_R, and fractional excretion) on gender and OCTN haplotype was performed, but did not reveal any significant effect of OCTN haplotype on carnitine CL_R. Notably, carnitine plasma levels were not significantly influenced by carnitine renal clearance in either univariate or multiple regression analyses (*i.e.*, after correcting for gender effects).

Effect of OCTN Haplotype on Plasma Lipids

Next, we compared the OCTN haplotype groups in terms of plasma lipid levels (Table 6.3). Similar to the results for carnitine disposition, we found that the haplotype groups

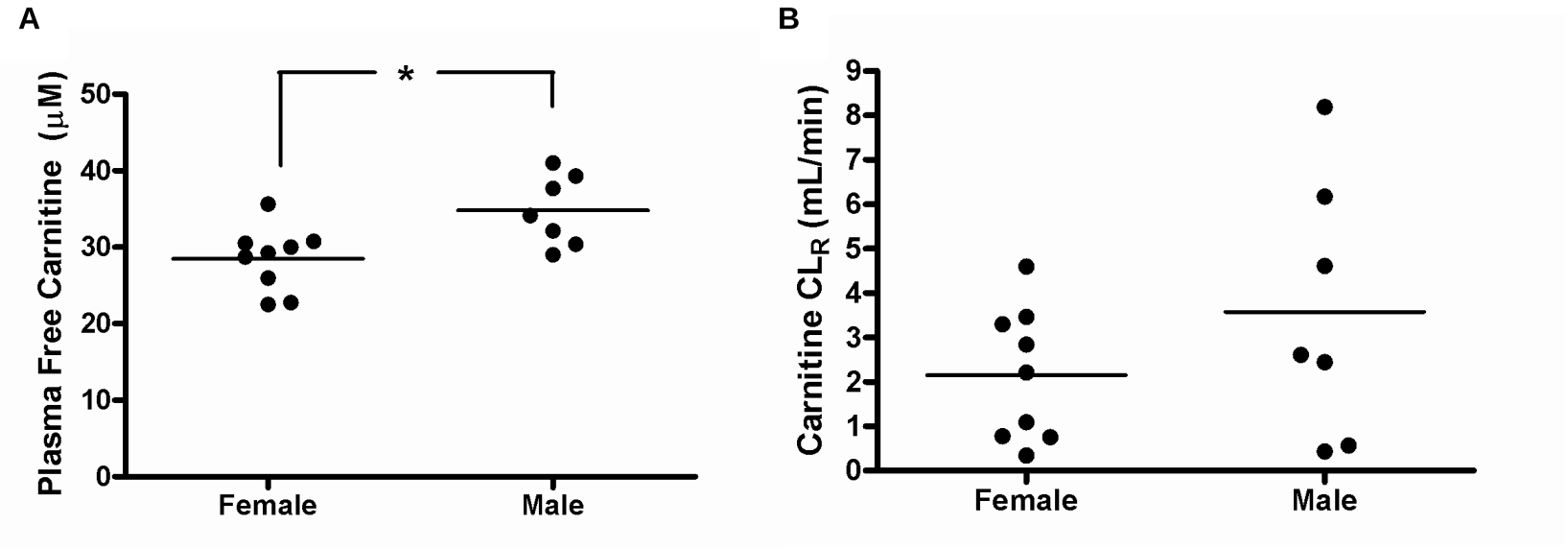

Figure 6.1. Gender differences in carnitine status.
Blood samples were drawn from subjects homozygous for either the *1 or *2 haplotype (n = 7 or 9 per group) at 0 hours and 2 hours after voiding urine. (A) Data points indicate the average total carnitine concentration from the two samples for each subject, grouped by gender. Plasma carnitine levels were significantly different between males and females (p=0.01). (B) Data points indicate the renal clearance of total carnitine for each subject. Subjects did not differ significantly by gender in terms of carnitine renal clearance (p=0.28)

Table 6.3. Summary of plasma lipid profile by haplotype.

	Total Sample	By Haplotype		p-value
		*1/*1 (n = 7)	*2/*2 (n = 9)	
Total Cholesterol	173 +/- 32	174 +/- 38	173 +/- 28	0.965
Triglycerides	63.9 +/- 18.2	58.7 +/- 20.8	67.9 +/- 16.0	0.334
VLDL-C	12.9 +/- 3.60	12.0 +/- 4.08	13.7 +/- 3.24	0.377
LDL-C	96.5 +/- 26.8	99.7 +/- 28.7	94.0 +/- 26.7	0.687
HDL-C	64.0 +/- 14.1	62.1 +/- 12.7	65.4 +/- 15.7	0.658
Total/HDL-C Ratio	2.81 +/- 0.64	2.84 +/- 0.47	2.78 +/- 0.77	0.847

VLDL-C: very low-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
All measurements are in mg/dL (with the exception of the total cholesterol/HDL-C ratio, which is dimensionless) and are given as mean +/- standard deviation. Differences between haplotype groups were assessed using a two-tailed unpaired t-test.

were similar in their plasma lipid profiles, with no significant differences between groups in any of the lipid fractions measured (total cholesterol, triglycerides, LDL-C, VLDL-C, or HDL-C). We then assessed the relationship between carnitine levels and the various lipid measures (Figure 6.2). We found that plasma carnitine was positively associated with triglycerides and VLDL-C, and negatively associated with HDL-C, though these trends were not statistically significant. A significant effect of plasma carnitine on the total cholesterol/HDL-C ratio was observed. Given that gender was a significant predictor of plasma carnitine levels, and that gender may also influence plasma lipid profile, we then tested whether the correlation of carnitine and lipid measures was independent of gender by multiple linear regression. We found that several lipid measures (HDL-C and total cholesterol/HDL-C ratio) were significantly different between males and females ($p<0.01$). When gender was included as a covariate, plasma carnitine levels were no longer significantly associated with total cholesterol/HDL-C ratio, and the predictive value of carnitine for all lipid fractions became weaker. Gender remained a significant predictor of HDL-C and TC/HDL-C ratio despite inclusion of plasma carnitine levels in the model.

Discussion

The current study exemplifies an unusual approach to human genetics, in which subjects are prospectively recruited based on known genotype, at a variant position(s) at which there are known functional effects in cellular assays. In this study, we recruited subjects homozygous for either of two common alleles at the OCTN locus that had been shown previously to differ with respect to carnitine transport activity or transcriptional activity.

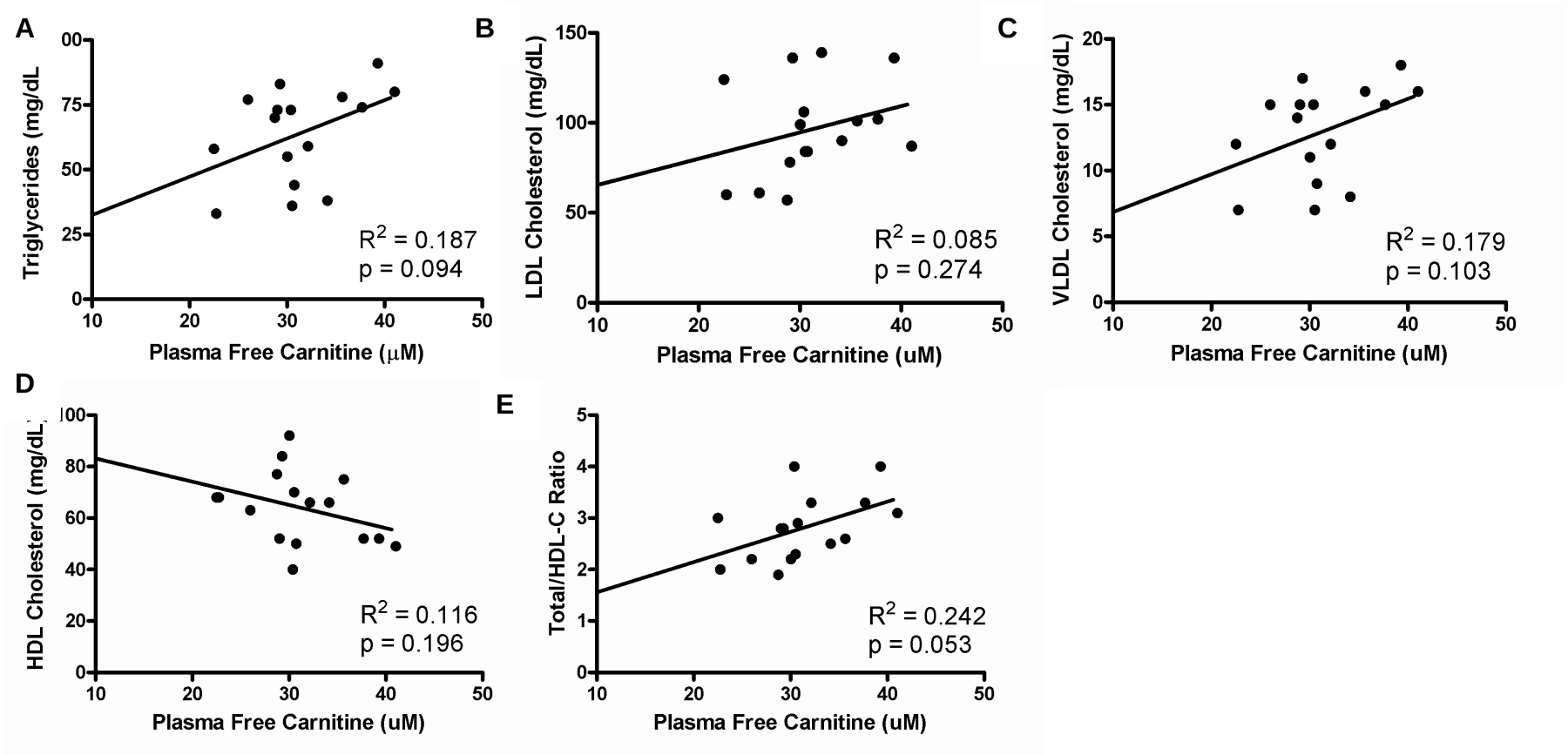

Figure 6.2. Relationship between plasma carnitine and lipid levels. A plasma lipid profile was determined from baseline (0 hr) blood samples for each subject and plotted vs. average plasma carnitine concentration. Plots show the relationship between average total carnitine and (A) triglycerides, (B) low-density lipoprotein cholesterol (LDL-C), (C) very-low-density lipoprotein cholesterol (VLDL), (D) high-density lipoprotein cholesterol (HDL-C), and (E) total cholesterol/HDL ratio. Significance of the relationship between carnitine levels and lipid measures was assessed by the correlation coefficient (R^2) and by linear regression/ANOVA.

The subjects were then characterized phenotypically by measurement of carnitine plasma levels and carnitine renal clearance. Since the primary defect in systemic carnitine deficiency, which results from genetic defects in OCTN2, is renal loss of carnitine (*i.e.*, reduced active reabsorption), these primary study measures are thought to be the most proximal measure of OCTN activity *in vivo*. Despite the evidence from *in vitro* [20] and *ex vivo* [19] studies, however, we found that OCTN haplotype, at least for the common haplotypes as defined here, did not predict carnitine plasma concentrations or carnitine renal clearance *in vivo*. Of course, one explanation for this finding is that there is truly no effect of the OCTN*2 haplotype *vs*. OCTN*1 on carnitine disposition *in vivo*. Alternatively, there may be some heterogeneity within each haplotype group that offsets or masks the effects of the *SLC22A4-1507C/T and SLC22A5-(-207)G/C* SNPs. For example, OCTN genetic variants with strong functional consequences *in vivo*, but not located or discovered in the region of the genes surveyed, may be carried by subjects in both haplotype groups, and offset the effects of the variants that define our haplotypes. Another consideration is the magnitude of the effect of the *1 vs. *2 haplotype *in vivo*, and the power of our study to detect that effect. We anticipated that a within-group coefficient of variation (CV) of 50% was a liberal estimate for variance in the primary study measure, carnitine renal clearance. However, the results of the study showed a much larger variance: within-group CV for carnitine CL_R was over 60% for both haplotype groups, and for fractional excretion, CV was greater than 50% in both groups. The expected between-group difference in mean values for the phenotypic measures (*i.e.*, the effect size of the haplotype on carnitine CL_R), which was chosen arbitrarily as a reasonable difference for clinical relevance, may also have been an overestimate. Thus, it

remains possible that our hypothesis regarding the effect of OCTN haplotype on carnitine disposition is correct, but that the effect is too small to be measured in our study.

Interestingly, the only study measure that was significantly associated with carnitine status was gender, with males having significantly higher plasma carnitine than females. This difference was not explained by reduced carnitine renal clearance in male subjects, who, paradoxically, had a higher average carnitine clearance than females, though that difference was not significant. These findings require two complementary explanations. First, since higher plasma carnitine levels in males were not explained by lower renal clearance values, the difference must be explained by a greater carnitine intake in male subjects. While there is some endogenous synthesis of carnitine in humans, the majority (>75%) of total body carnitine is supplied by diet [22]. Our data suggests that dietary intake of carnitine may be greater in males, at least in the population represented by our sample. There is a lack of consensus on gender differences in carnitine levels in the existing literature, with some reports of higher carnitine levels in males [23-26], but others showing no gender differences [27-29]. Second, since higher carnitine plasma concentrations are associated with higher carnitine renal clearance (and not the reverse), it is possible that a negative feedback mechanism for carnitine reabsorption may be at work. That is, at higher plasma carnitine concentrations, the efficiency of reabsorption is decreased, leading to greater renal losses. This effect has been demonstrated previously: in a study of carnitine renal clearance in strict vegetarians before and after carnitine supplementation, higher carnitine plasma concentrations after supplementation were associated with greater renal losses, a phenomenon the authors described as “renal

adaptation" to higher carnitine levels [30]. This may be due either to saturation of the reabsorption process, or to transcriptional or protein-level regulation of reabsorptive transporter (*i.e.*, OCTN) expression.

When we evaluated the relationship between carnitine and various plasma lipid fractions, we found trends toward a positive correlation of carnitine with atherogenic species such as VLDL-C and triglycerides, and negative correlation with the cardioprotective HDL-C. These relationships are in the opposite direction from what was originally hypothesized based on the pharmacology of carnitine and from animal models of carnitine deficiency. Notably, multiple regression analysis suggested that the covariation of carnitine with plasma lipids was explained by gender differences in both parameters. That is, gender independently predicted both carnitine plasma levels and plasma HDL-C, as well as VLDL-C and triglycerides, though these latter two were not statistically significant; on the other hand, carnitine was not an independent predictor of HDL-C when gender was included in the model. This finding, coupled with our supposition that the observed gender differences in carnitine levels were due to differences in dietary intake of carnitine, suggests that dietary intake of cholesterol-rich foods may explain the carnitine-lipid correlations in our study. This is consistent with the fact that foods rich in carnitine (e.g., meat, fish, and dairy products) are also major sources of dietary fat. However, intrinsic differences between males and females with respect to lipid storage and metabolism, independent of carnitine intake, cannot be excluded. Rather, gender-dependent differences in lipid metabolism have been previously described, and may have important clinical implications [31]; it is unknown whether gender differences in

carnitine levels are causally related to these differences in lipid metabolism.

Despite our knowledge of the importance of cholesterol and triglycerides in the development of heart disease, relatively little is known about the genetic determinants of hypertriglyceridemia. We hypothesized that common genetic variation in the OCTN genes may explain population variation in triglyceride levels, and therefore may influence risk for heart disease. Knowledge of the mechanisms underlying heart disease and lipid disorders is crucial to improving the prevention and treatment of these disorders. However, the results of this study provided no positive evidence of an effect of OCTN*1 or OCTN*2 haplotype on either carnitine disposition (the more proximal measure of OCTN activity) or plasma lipid profiles. Data from animal models and humans with SCD clearly demonstrate the significance of carnitine in lipid metabolism, and in certain patient populations carnitine has been shown to reduce atherogenic lipid species, particularly triglycerides. For these reasons and those described above, we believe the negative results of the current study should be interpreted narrowly, and that the broader hypothesis, that genetic variation in OCTN genes influences carnitine disposition and lipid metabolism, may still be valid. Future studies of the influence of OCTN variants on these phenotypes using larger samples and a retrospective, association-based approach to this question may prove to be more fruitful.

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CHAPTER 7

FUNCTIONAL GENOMICS OF MEMBRANE TRANSPORTERS IN HUMAN POPULATIONS[3]

Introduction

Since the completion of the human genome project, considerable progress has been made in characterizing the nature and degree of human DNA sequence variation [1-5]. Numerous single nucleotide polymorphisms in all regions of the human genome have been identified, including coding and non-coding regions of genes and large intergenic regions. However, there remains a lack of information connecting genetic polymorphisms to human phenotypic variation. As a consequence, current attempts to predict the functional significance of genetic variants from sequence data alone (from interspecies sequence comparisons, or degree of chemical change for amino acid substitutions, as examples) [6-15] are lacking in support from empirical data. Furthermore, we have little information on the fraction of natural variants with altered or potentially deleterious function that are harbored in healthy populations. In order to understand variation in normal human physiology and responses to the environment (including, for example, drug response phenotypes), it is important to understand the range of phenotypic variation that is associated with genetic variation in human populations.

[3] This chapter has been published previously: Urban T.J., R. Sebro, E.H. Hurowitz, M.K. Leabman, I. Badagnani, L.L. Lagpacan, N. Risch, and K.M. Giacomini. "Functional genomics of membrane transporters in human populations." *Genome Res* 16(2): 223-230, 2006.

Analysis of causal mutations in Mendelian diseases has demonstrated that the majority of these diseases are caused by rare non-synonymous variants, specifically amino acid substitutions [16]. Splice site mutations and insertions or deletions, although rarer occurrences, account for most other causative mutations in Mendelian diseases. The preponderance of non-synonymous variants in disease association may also be true for more common disease [16]. Several recent studies have revealed that among disease-associated polymorphisms with the strongest evidence for true association, almost all are amino acid substitutions [16,17]. Attempts have been made to estimate the fraction of non-synonymous variants in the human genome that is functionally deleterious, with estimates ranging from 10% to upwards of 50% of all non-synonymous mutations [6,8,18].

One of the primary goals of current large-scale sequencing projects is to identify SNPs that may be used in candidate gene association studies. However, a major obstacle in designing association studies is choosing appropriate SNPs to genotype. One strategy is to choose SNPs that are expected *a priori* to affect protein function and are therefore more likely to be associated with an altered phenotype. A variety of algorithms and bioinformatics tools have been developed in recent years to predict the functional consequences of protein-altering variants [7,10,15,19]. These algorithms attempt to predict the effect of an amino acid substitution on protein function based on the nature of the chemical change, the structural location of the substitution, and/or the evolutionary conservation of the residue, rather than from direct measurement of the function of the variant protein. As amino acid substitutions are particularly amenable to study in

biochemical assays, systematic investigation of the functional consequences of these variants in cellular assays represents a first step toward cataloging human phenotypic variation.

Solute Carrier (SLC) transporters maintain cellular and total body homeostasis by importing nutrients and exporting cellular waste products and toxic compounds. These transporters also play a critical role in drug response, serving as drug targets and facilitating drug absorption, metabolism, and elimination. The SLC superfamily is comprised of transporters from a wide range of functional classes, including neurotransmitter, nutrient, heavy metal, and xenobiotic transporters. Genetic defects in SLC transporters have been associated with a variety of Mendelian diseases, including metabolic disorders such as glucose-galactose malabsorption [20] and neurologic disorders such as peripheral neuropathy with agenesis of the corpus callosum (ACCPN) [21], demonstrating the diverse physiological functions of these proteins.

In this study, we characterized the function of protein-altering variants of eleven SLC transporters belonging to three different families: *SLC22, SLC28*, and *SLC29*. These transporters are present in a variety of epithelial tissues and have diverse biological roles. Although several of these transporters have specific functions that are important for normal human physiology, they are all capable of transporting xenobiotic small molecules (i.e., drugs), and were selected for screening as candidate genes to explain variability in drug response. In addition to identifying and functionally characterizing all naturally-occurring protein-altering variants of these transporter genes, we attempted to

identify characteristics of protein-altering variants that are predictive of alterations in protein function, both in biochemical assays and *in vivo*.

Methods

Variant Identification

The coding regions (all exons and 50-100 bp of flanking intronic region per exon) of eleven membrane transporter genes (*SLC22A1* (OCT1), U77086; *SLC22A2* (OCT2), X98333; *SLC22A4* (OCTN1), NM_003059; *SLC22A5* (OCTN2), NM_003060; *SLC22A6* (OAT1), AF097490; *SLC22A8* (OAT3), NM_004254; *SLC28A1* (CNT1), U62968; *SLC28A2* (CNT2), U84392; *SLC28A3* (CNT3), AF305210; *SLC29A1* (ENT1), U81375; *SLC29A2* (ENT2), AF029358) were screened for polymorphism by denaturing HPLC or by direct sequencing of a large number of DNA samples collected from ethnically diverse populations. Set I genes (*SLC22A1, SLC22A2, SLC28A1, SLC28A2*, *SLC29A1*, and *SLC29A2*) were screened using ethnically identified DNA samples (100 African-Americans and 100 European-Americans) from the Coriell Institute; Set II genes (*SLC22A4*, *SLC22A5, SLC22A6, SLC22A8,* and *SLC28A3*) were screened using a cohort of individuals (80 African-Americans, 80 European-Americans, 60 Asian-Americans, 50 Mexican-Americans, and 6 Pacific Islanders) from the San Francisco Bay Area enrolled in the SOPHIE project (Studies of Pharmacogenetics in Ethnically Diverse Populations). Nucleotide diversity (π), which is the average proportion of nucleotide differences between all possible pairs of sequences in the sample, was used to estimate nucleotide diversity at synonymous sites (π_S) and amino acid-altering or non-synonymous sites (π_{NS}) [22,23]. All variants identified and their ethnic-specific allele frequencies have been

deposited in the public databases PharmGKB (http://www.pharmgkb.org) and dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/).

Functional Characterization

Uptake studies for OCT1, OCT2, OAT1, CNT1, CNT2, CNT3 and ENT2 were performed using *X. laevis* oocytes as described previously [24-29]. Studies of ENT1 were performed in *S. cerevisiae* using cytotoxicity and cell growth assays as described previously [30]. Studies of OAT3, OCTN1 and OCTN2 were performed by transient transfection of HEK-293 cells using the Lipofectamine 2000 reagent as per the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Reference and variant cDNA clones in the expression vector pcDNA5/FRT (OAT3 and OCTN1) or pcDNA3 (OCTN2) were used to transfect HEK-293 cells at 90% confluence in 24-well poly-D-lysine coated plates (BD Discovery Labware, Bedford, MA, USA) using 1 μg DNA and 3 μg Lipofectamine 2000 per well. Cells were assayed for activity at 48 hours post-transfection by measurement of initial rate uptake of radiolabeled probe substrates: 0.1 μM ^{3}H-estrone-3-sulfate (OAT3), 10 μM ^{14}C-tetraethylammonium (OCTN1), or 1 μM ^{3}H-L-carnitine (OCTN2). For each transporter, variant cDNAs were constructed by site-directed mutagenesis of the reference sequence clone, defined as the most common amino acid sequence in our sample. Initial rate of uptake of radiolabeled probe compound was measured for each variant, and the results expressed as a percent of the uptake of the reference sequence clone after subtracting background uptake. Experiments were performed several times and the average values from multiple experiments were used in the analysis.

Data Analysis

Prediction of function in cellular assays:

The rate of uptake of the radiolabeled probe compound for each variant as a percentage of the reference sequence clone was used as the measure of biochemical function of the variant. The distribution of this variable was multimodal, with some variants having biochemical function less than 55% of the control and other variants having function of greater than 60% of the control in cellular assays. We used the value of 60% of the control uptake value, which marked the lower quartile of uptake values, as a cut-off point to demarcate reduced or loss-of-function variants from "normal" function variants.

Each amino acid substitution was then evaluated for characteristics that might be expected to aid in prediction of functional activity: evolutionary conservation, degree of chemical change, and location in the protein (transmembrane domain *vs*. intracellular or extracellular loop regions). The degree of chemical change for each amino acid substitution was scored using the substitution matrix of Grantham [31]. For evolutionary conservation, two methods were used to score the variants. In the first method, the human amino acid sequence of each of our eleven transporter genes was aligned with three to six known vertebrate orthologs (chimp, dog, mouse, rat, pig, cow, chicken, and/or frog). Residues that were identical across 80% or more of the reference sequences of all comparator species were classified as evolutionarily conserved (EC); all other residues were classified as evolutionarily unconserved (EU). The second method utilized the prediction algorithm SIFT (for Sort Intolerant From Tolerant amino acid

substitutions)[7]. In contrast to our alignments with only a few orthologs, the SIFT algorithm generates alignments with a large number of homologous sequences and assigns scores to each residue, ranging from zero to one. Scores close to zero indicate evolutionary conservation and intolerance to substitution while scores close to one indicate tolerance to substitution. SIFT scores less than 0.05 are predicted by the algorithm to be intolerant or deleterious amino acid substitutions, whereas scores greater than 0.05 are considered tolerant. SIFT analysis was performed by allowing the algorithm to search for homologous sequences (i.e., without inputting known homologs) and using the default settings (SWISS-PROT 45 and TREMBL 28 databases, median conservation score 3.00, remove sequences >90% identical to query sequence). To determine the structural location of each variant, the secondary structure of the reference sequence of each protein was estimated by hydropathy analysis (Pepplot, GCG sequence analysis suite). Each variant was scored as occurring in either the transmembrane domain (TMD) or loop region of the protein. The variables scored were tested as predictors of functional activity in biochemical assays using the χ^2 test for association.

Prediction of *in vivo* function:

Alleles that are deleterious in nature suffer strong selection pressure and are therefore more likely to be found at low frequency. The allele frequency of each variant was used as an estimator for the effect on human fitness of that allele. The probability that a SNP had a minor allele frequency greater than some frequency, x, was modeled. Plots of these probability curves were generated, stratifying over dichotomous variables (function/no function in cellular assays, evolutionary conservation, SIFT) to determine whether these

variables were predictive of allele frequency distribution, and thus might be correlated with gene function *in vivo*. The curves generated were analogous to Kaplan-Meier survival curves with allele frequency replacing time. These curves were compared using the Log-Rank Test.

Results

We systematically analyzed the function of all protein-altering variants of eleven membrane transporters in the Solute Carrier families *SLC22*, *SLC28* and *SLC29* in heterologous expression systems. Coding region variants were identified by screening many DNA samples (n=247 to 276) from ethnically diverse human populations. The transporters are dispersed throughout the human genome on five chromosomes, although some pairs (OCT1-OCT2, OAT1-OAT3, OCTN1-OCTN2) are found in tandem at a single locus and presumably arose by gene duplication (Table 7.1). The amino acid diversity (π_{NS}) of the nine transporters ranges from 0.11×10^{-4} to 8.7×10^{-4}. Previous large-scale sequencing studies have found that the average amino acid diversity in the human genome is approximately 2.0×10^{-4} [4,5]. Therefore, this subset of genes includes a representative sampling of genetic diversity within the human genome. We expressed all protein-altering variants in heterologous systems and determined activity by measuring the uptake of radiolabeled probe substrates (Figure 7.1). We include in the analysis new information on functional variation in four membrane transporter genes and pooled analysis of variants of seven membrane transporters for which functional data have been reported previously [24-30]. In total, functional analysis of 88 protein-altering

Table 7.1. Summary of population genetics statistics of eleven membrane transporters in the SLC family.

Gene	HGNC Name	Chromosome	PAV[a] (#)	RFV[b] (#)	π_{coding} (x 10^4)[c]	π_S (x 10^4)	π_{NS} (x 10^4)	π_{NS}/π_S	Orthologs[d]
OCT1	*SLC22A1*	6q26	15	5	6.58 ± 4.78	11.20 ± 11.00	5.11 ± 4.40	0.46	7
OCT2	*SLC22A2*	6q26	9	1	6.99 ± 4.99	22.54 ± 17.50	2.23 ± 2.64	0.10	7
OCTN1	*SLC22A4*	5q31.1	6	3	8.13 ± 5.58	13.98 ± 12.65	6.25 ± 5.04	0.45	5
OCTN2	*SLC22A5*	5q31.1	8	1	6.58 ± 4.77	23.93 ± 18.01	1.01 ± 1.70	0.04	6
OAT1	*SLC22A6*	11q13.1-q13.2	6	1	1.25 ± 1.70	3.87 ± 5.88	0.37 ± 1.02	0.10	7
OAT3	*SLC22A8*	11q11	10	5	4.25 ± 3.57	15.20 ± 13.42	0.74 ± 1.46	0.05	6
CNT1	*SLC28A1*	15q25-26	12	2	11.96 ± 7.24	22.55 ± 16.51	8.56 ± 5.98	0.38	5
CNT2	*SLC28A2*	15q15	5	0	7.64 ± 5.09	7.61 ± 8.22	7.64 ± 5.47	1.00	6
CNT3	*SLC28A3*	9q22.2	10	1	5.54 ± 3.97	18.13 ± 14.19	1.81 ± 2.13	0.10	5
ENT1	*SLC29A1*	6p21.1-p21.2	2	0	0.38 ± 0.97	0.60 ± 2.47	0.30 ± 1.00	0.50	6
ENT2	*SLC29A2*	11q13	5	3	0.54 ± 1.18	1.81 ± 4.27	0.11 ± 0.61	0.06	4

[a]PAV: protein-altering variants. [b]RFV: reduced-function variants. [c]Values of π are listed as mean ± standard deviation. π_{coding} is the nucleotide diversity in the entire coding region of each gene, π_S is the nucleotide diversity at synonymous sites and π_{NS} is the nucleotide diversity at non-synonymous sites. [d]Number of orthologs used in the alignments for manual scoring of evolutionarily conserved residues

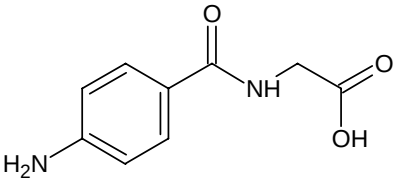
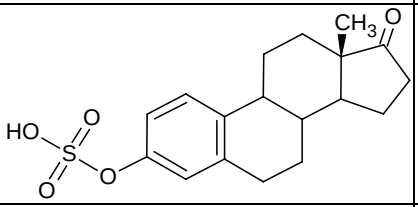
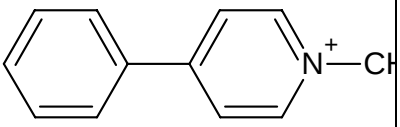
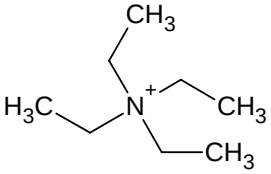
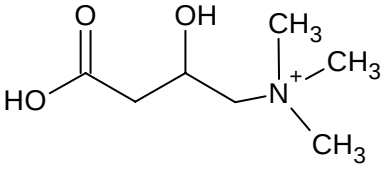
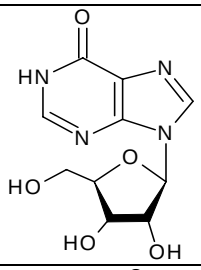
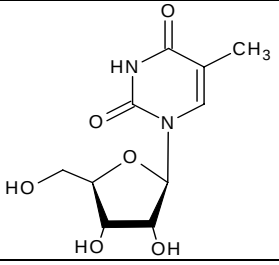
TRANSPORTER	MODEL SUBSTRATE	STRUCTURE	OTHER SUBSTRATES
OAT1	p-Aminohippuric acid (PAH)		NA
OAT3	Estrone Sulfate		Cimetidine
OCT1, OCT2	1-Methyl-4-phenylpyridinium (MPP+)		Metformin, Phenformin, Procainamide, Quinidine, Tetrabutyl-ammonium
OCTN1	Tetraethylammonium (TEA)		Betaine
OCTN2	Carnitine		Tetraethyl-ammonium
CNT2, ENT1, ENT2	Inosine		Uridine, Guanosine, Ribavirin, 5-Fluorouracil, Fludarabine, Gemcitabine, Cytarabine
CNT1, CNT3	Thymidine		Adenosine, Inosine, Gemcitabine, Cladribine, Fludarabine

Figure 7.1. Model substrates of eleven SLC transporters.

variants is presented, including 80 amino acid substitutions, two insertions, four deletions, and two nonsense mutations.

The distribution of uptake values for all variants analyzed is shown in Figure 7.2. The uptake values show a multimodal distribution, with breaks in the distribution at 25-40%, 55-60%, and 150-175% of the activity of the control. Twenty-two (25%) of the 88 variants tested exhibited decreased transport function (defined as uptake <60% of control) (Table 7.2). Of the 88 protein-altering variants tested, 50 (57%) of the variants were polymorphic (defined as allele frequency ≥1% in at least one ethnic population), and 7 (14%) of those 50 polymorphisms had decreased transport function. Interestingly, three variants appeared to be hyperfunctional, that is, had uptake values greater than 150% of the control. These three variants shared the properties (discussed below) of the other variants with greater than 60% activity. Therefore, we used a bimodal retained-function *vs*. reduced-function model (as opposed to a trimodal normal-function *vs*. altered-function model) to analyze the data.

Evolutionary Conservation vs. Biochemical Function

To learn about the characteristics of variants that decrease function and thus aid in the development of prediction tools, we evaluated the amino acid substitutions in our data set using several measures (based on degree of chemical change, evolutionary conservation, and/or location in the protein) and examined correlations between the nature of the amino acid substitution and protein function. For these analyses, only amino acid substitutions were considered due to problems inherent in quantifying the chemical change or

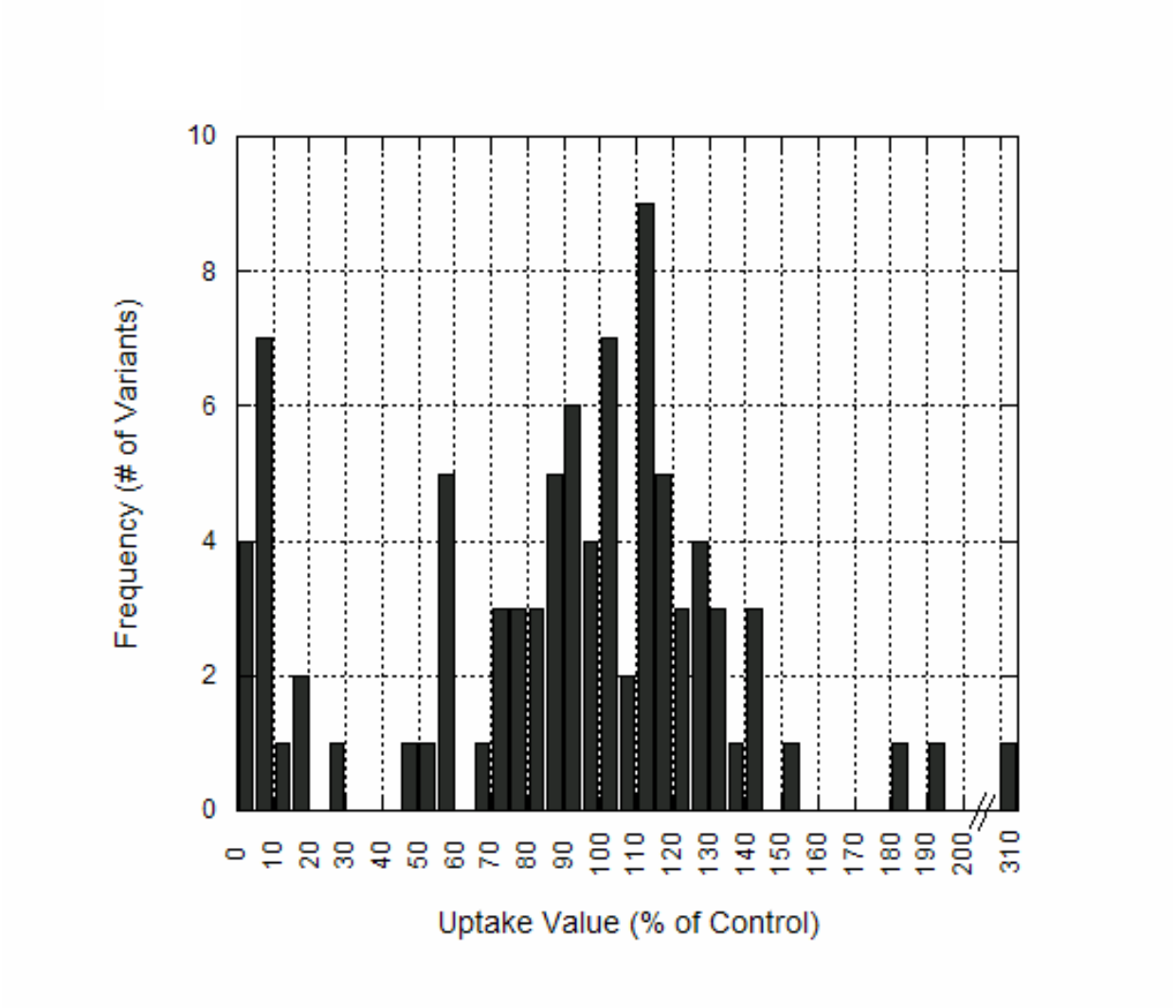


Figure 7.2. Distribution of uptake values for protein-altering variants in eleven SLC transporters. Initial rate of uptake of radiolabeled probe substrate was measured and the results expressed as a percent of the activity of the reference sequence clone after subtracting background uptake. Uptake values used to construct the histogram reflect the mean of several experiments.

Table 7.2. Characteristics of variants that exhibit reduced function in biochemical assays.

Variant	Total Freq.[a]	AA Freq.[b]	EA Freq.	AS Freq.	ME Freq.	Grantham	EC/EU[c]	Loop/TMD[d]
OCT1-R61C	0.036	0.000	0.072	0.000	0.056	85	EC	L
OCT1-G220V	0.002	0.005	0.000	0.000	0.000	109	EC	T
OCT1-P341L	0.041	0.082	0.000	0.117	0.000	98	EU	L
OCT1-G401S	0.009	0.007	0.011	0.000	0.000	56	EC	L
OCT1-G465R	0.020	0.000	0.040	0.000	0.000	125	EC	L
OCT2-F45Ins	0.002	0.000	0.005	0.000	0.000	n/a	n/a	T
OCTN1-D165G	0.002	0.000	0.000	0.008	0.000	94	EC	L
OCTN1-M205I	0.002	0.006	0.000	0.000	0.000	10	EC	T
OCTN1-R282X	0.002	0.006	0.000	0.000	0.000	n/a	n/a	L
OCTN2-F17L	0.004	0.000	0.000	0.017	0.000	22	EC	L
OAT1-R454Q	0.002	0.006	0.000	0.000	0.000	43	EC	L
OAT3-R149S	0.004	0.000	0.006	0.008	0.000	177	EC	L
OAT3-Q239X	0.002	0.000	0.000	0.008	0.000	n/a	n/a	T
OAT3-I260R	0.002	0.000	0.000	0.008	0.000	97	EC	L
OAT3-R277W	0.002	0.007	0.000	0.000	0.000	101	EU	L
OAT3-I305F	0.009	0.000	0.000	0.035	0.011	21	EC	L
CNT1-V385Del	0.015	0.030	0.000	0.000	0.000	n/a	n/a	L
CNT1-S546P	0.003	0.005	0.000	0.000	0.000	74	EC	T
CNT3-G367R	0.002	0.000	0.000	0.008	0.000	125	EU	T
ENT2-D5Y	0.003	0.005	0.000	0.000	0.000	160	EU	L
ENT2-S184Del	0.003	0.000	0.005	0.000	0.000	n/a	n/a	L
ENT2-S282Del	0.003	0.005	0.000	0.000	0.000	n/a	n/a	L

[a]For OCT1, OCT2, CNT1, and ENT2 variants, total population includes 100 AA and 100 EA samples. For OCTN1, OCTN2, OAT1, OAT3, and CNT3, total population includes 80 AA, 80 EA, 60 AS and 50 ME samples.
[b]AA=African American; EA=European American; AS=Asian American; ME=Mexican American.
[c]EC=evolutionarily conserved; EU=evolutionarily unconserved.
[d]Variant residue is located in predicted transmembrane domain (T) or loop region (L) of protein.

evolutionary conservativeness of insertions, deletions, and nonsense mutations. Of the five frameshift and nonsense mutations in the dataset, all showed virtually no activity.

The amino acid substitution matrix of Grantham [31] is commonly used to measure the degree of chemical similarity or difference between alternative residues. The variants that retained function had lower Grantham values than the variants that decreased function (65 ± 48 versus 87 ± 48, respectively), though this difference narrowly missed significance (p=0.052 by one-tailed t-test).

The amino acid residues found in the transmembrane regions of proteins are highly conserved throughout evolution, owing to unique physical constraints on membrane-spanning helices [5]. Transmembrane regions had a lower fraction of reduced-function variants than loop regions (18% vs. 30%). However, the difference was not significant (χ^2=1.60, p=0.66).

We have used two methods to evaluate evolutionary conservation of the variant sites in our dataset. In the first method, based on multiple sequence alignment with known vertebrate orthologs, each amino acid substitution was scored as either evolutionarily conserved (EC) or evolutionarily unconserved (EU). We observed that 12 of the 35 (34%) EC variants resulted in decreased function compared to only 4 of the 45 (9%) EU variants (χ^2=7.93, p=0.047). Twelve of the 16 variants that resulted in loss of function were EC, giving a sensitivity of 75%. However, 23 of 64 variants that retained function also occurred at EC residues, giving a specificity of only 64%. In the second method, we

used the prediction algorithm SIFT, which scores each variant as either "tolerant" or "intolerant" to the indicated substitution. Out of the 80 single amino acid substitution variants, SIFT correctly predicted as "intolerant" 75% of the variants that decreased function and predicted as "tolerant" 75% of the variants that retained function. Overall, SIFT performed better, mispredicting only 20 variants (25%) compared to a misprediction of 27 variants (34%) by our EC/EU method.

Relationship Between Biochemical Function, Evolutionary Conservation, and Negative Selection

We plotted the fraction of variants with decreased function versus allele frequency and compared that distribution to the distribution of the variants that retained function (Figure 7.3a). Our results demonstrated that there was a significantly lower allele frequency distribution of variants with decreased function compared to those that retained function in cellular assays (Log-Rank test, $p=9.3 \times 10^{-3}$). These data are consistent with decreased function variants being selected against. It is notable that all four ethnic populations had variants with decreased function. In fact, 17 of the 22 variants with decreased function (77%) were present in only a single ethnic or racial population sample. Fourteen of the 17 were singletons (only found on a single chromosome in our sample) and by definition, present in only one population. However, three were non-singletons: OCT1-G465R (MAF=0.04 in European Americans), OCTN2-F17L (MAF=0.02 in Asian Americans), and CNT1-V385del (MAF=0.03 in African Americans). The existence of functional variants common in some ethnicities and absent in others highlights the importance of considering race and ethnicity in human genetics, as different populations may carry a

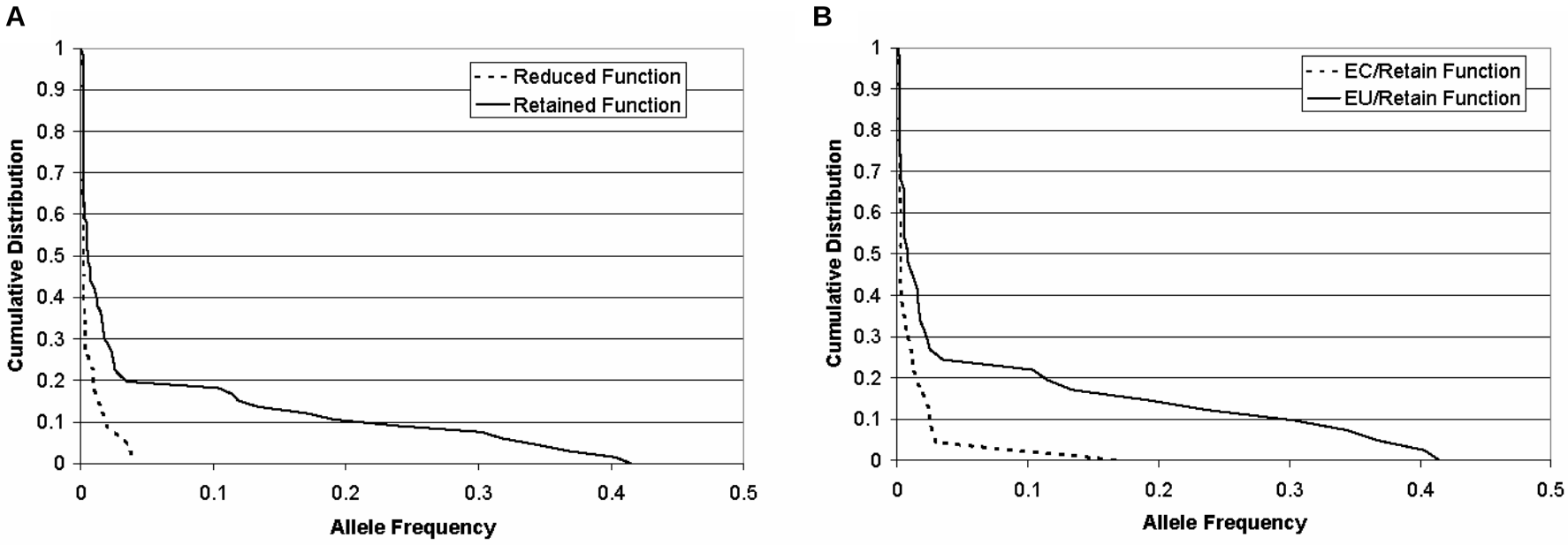


Figure 7.3. Retention or loss of function in cellular assays predicts function *in vivo*. Variants were classified as having reduced function if they exhibited uptake values <60% of control. (A) Allele frequency distributions between variants that retained function vs. those that exhibited loss of function in cellular assays. The resulting curves were significantly different (Log-Rank test, $p=9.3 \times 10^{-3}$), with a skew toward lower population allele frequencies for reduced-function variants. (B) Allele frequency distributions between variants at evolutionarily conserved (EC) positions that retained function vs. variants at evolutionarily unconserved (EU) positions that retained function. EC variants showed a significant shift toward lower allele frequencies (Log-Rank test, p=0.02), even for variants that retained function in cellular assays.

different set of deleterious polymorphisms, especially when the causative mutations are of low frequency (<10%) [32].

We then plotted the allele frequency distributions of the EC variants that retained function and the EU variants that retained function (Figure 7.3b). Interestingly, the EC variants that retained function had an allele frequency distribution that was skewed toward lower frequencies and was significantly different from that of the EU variants that retained function (Log-Rank, p=0.02). The data suggest that variants that appear to retain function in biochemical assays, but alter evolutionarily conserved residues, may affect some function important in organism fitness that is not measured in these assays. For example, this function may be an entirely different (i.e., non-transport) function mediated by the same gene, or may simply be the transport activity with respect to substrates that were not studied. Figure 7.4 shows one variant of OAT3, OAT3-I305F, that retained activity toward one substrate, the peptic ulcer drug, cimetidine, but had reduced activity toward the model substrate estrone sulfate, an endogenous steroid hormone.

Since the uptake of multiple substrates had been measured for variants of 9 out of the 11 transporters in our dataset, we calculated the fraction of variants that showed substrate-specific changes in uptake activity. Out of the 58 variants for which multiple substrates had been assayed, eight (14%) showed substrate-specific differences (Table 7.3). The distribution of allele frequencies for those eight substrate-specificity variants was comparable to that of the entire dataset, and contained both rare (<1%) EC variants and common (>10%) EU variants. Notably, however, the allele frequency distribution of

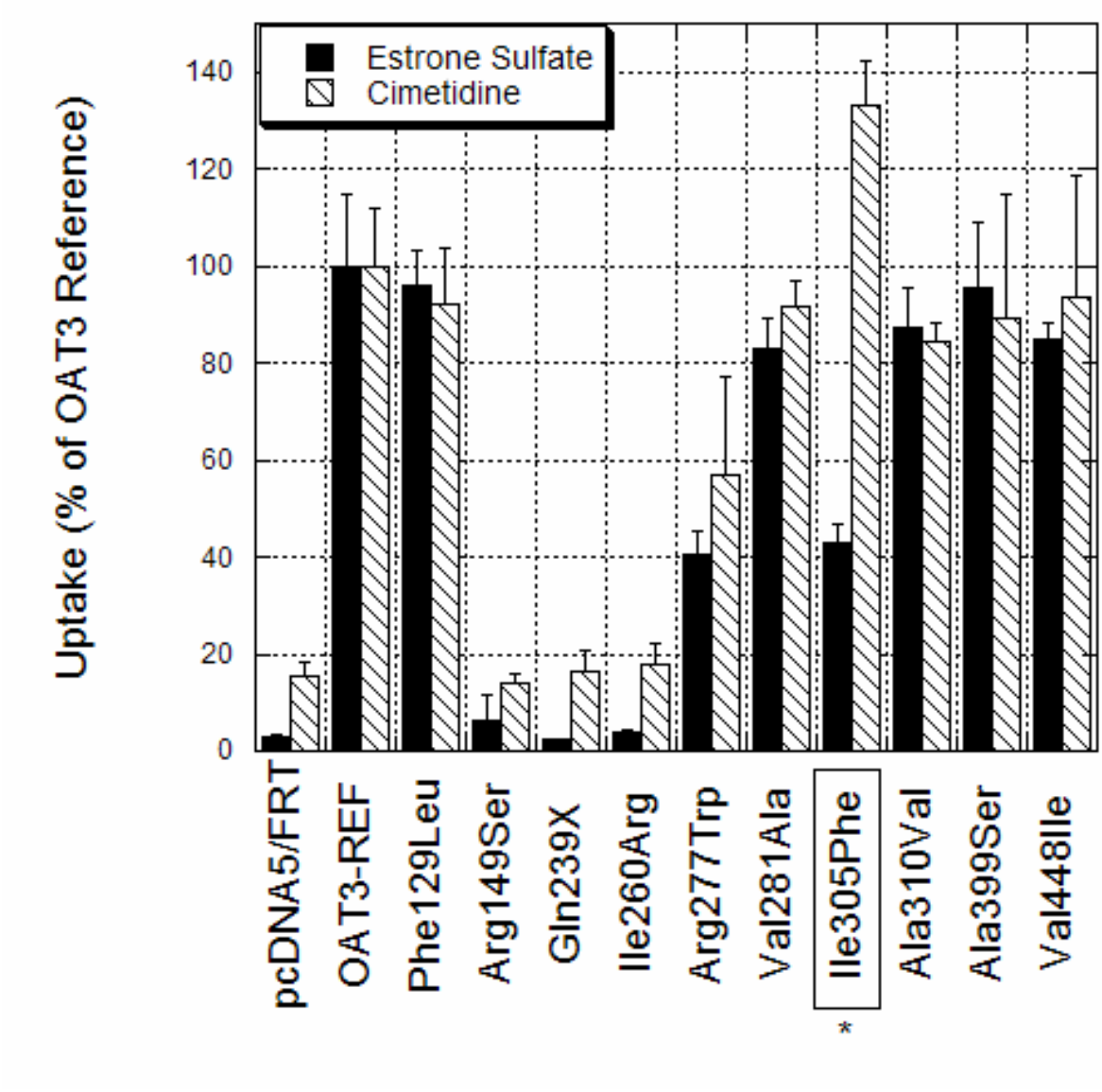


Figure 7.4. Functional characterization of protein-altering variants of OAT3 (*SLC22A8*). Uptake of estrone sulfate and cimetidine in HEK-293 cells expressing reference OAT3 and OAT3 protein-altering variants. Uptake values are expressed as a percentage of reference OAT3. Each value represents mean ± SD from triplicate wells in a representative experiment. *Indicates the selectivity variant Ile305Phe.

Table 7.3. Number of variants in nine SLC transporters that change substrate specificity.

Gene	Substrates tested (#)	Variants tested (#)	Number of Variants with Altered Substrate Specificity (%)
OCT2	6	4	3
	7	5	0
OCTN1	2	6	1
OCTN2	2	8	1
OAT3	2	10	1
CNT1	2	4	1
CNT2	2	3	0
	3	1	1
CNT3	2	7	0
	5	3	0
ENT1	2	1	0
	7	1	0
ENT2	4	5	0
	Total	58	8 (14%)

these specificity variants was significantly different from that of the reduced function variants, with the specificity variants having higher allele frequencies than variants that exhibited reduced activity toward the prototypical substrate (Log-Rank, p=0.01).

Discussion

Our study suggests that healthy human populations harbor a significant number of severely reduced function polymorphisms and rare variants. In a set of 88 protein-altering variants from eleven membrane transporter genes, we found that 14% of the polymorphic (allele frequency ≥1% in at least one ethnic population) variants had decreased transport function (see Figure 7.2). We then examined the variants to identify any characteristics that could be used to predict a reduction in function. First, we found that mutations that alter more than a single amino acid (*e.g.*, frameshift and nonsense mutations) all showed virtually complete loss of function. For the amino acid substitutions, we examined the magnitude of the chemical change, and found that there was a trend toward larger chemical changes in variants with decreased function compared to those that retained function. These data are consistent with Miller and Kumar who demonstrated that amino acid substitutions associated with disease had higher Grantham values (larger chemical changes) than amino acid substitutions across species [33].

We then assessed the ability of evolutionary conservation to predict effects on protein function. Previous studies have demonstrated that evolutionarily conserved (EC) residues are under stronger purifying selection than evolutionarily unconserved (EU) residues, suggesting that variation at EC residues is more likely to affect protein function

than variation at EU residues. For example, Miller and Kumar demonstrated that non-synonymous variants associated with disease occur at EC sites more frequently than expected by chance [33]. We found that a simple measure of evolutionary conservation using a small number of known orthologs does reasonably well at predicting decreased function variants, but less well at predicting variants that retain function. The prediction algorithm SIFT, which uses a much larger set of homologous sequences, provides a similar sensitivity for prediction of functional significance, but had higher specificity compared to the EC/EU method. These results suggest that evolutionary conservation across larger distances may more accurately predict effects on protein function, or, in other words, that knowledge of the *degree* of conservation allows for more specific prediction of functionally significant amino acid substitutions [34]. New efforts at sequencing the genomes of additional species should therefore facilitate the improvement of predictive algorithms [35].

We found that our measure of protein function in cellular assays correlated significantly with allele frequency, a measure of effect on human fitness. That is, variants with grossly impaired function in biochemical assays were present at lower allele frequencies than variants that retained function, consistent with the idea that biochemical assays should be performed as a confirmatory measure for variants found to be associated with a disease phenotype. However, we found that alleles that altered evolutionarily conserved amino acid residues, but retained apparently normal function in biochemical assays, were also under negative or purifying selection. This finding suggests that even direct biochemical assay of variant protein function is not perfectly predictive of function *in vivo*, and that

evolutionary conservation contains residual information independent of loss/retention of function in biochemical assays. An implication of this is that a negative finding in a biochemical assay of a disease-associated polymorphism is not necessarily evidence against a role of that variant in the phenotype of interest. This may be particularly important to the genetics of complex disease, in which the contribution of any individual risk-conferring polymorphism is expected to be very small, and thus may not be detectable in cellular assays.

Negative selection may act on variants that appear to "retain" function in cellular assays when those variants specifically alter occult functions of the protein that aren't measured in the assay or when small changes in protein function have large physiological consequences. For the membrane transport proteins in our study, possible occult functions include the transport of physiologically-relevant substrates other than the model substrate. We examined this possibility by calculating the fraction of variants for which the transport of more than one substrate had been measured that showed substrate-specific changes in transport. Although relatively few variants (14%) showed substrate-specific changes in function, we likely underestimated the true fraction of these variants since not all of the physiologically relevant substrates are known for each transporter and not all known substrates were tested. Variants with substrate-specific effects on function are probably not unique to membrane transporters, but common to all proteins that have multiple catalytic activities, multiple substrates, or multiple binding partners. This has important implications for pharmacogenetic association studies since some of the protein variants that associate with variation in response to one drug may not associate with

variation in the other drugs that interact with the same protein. Future biochemical assays of variant protein function should be interpreted with respect to how well the pertinent functions of the studied protein are known and how many of those functions are measured by the assay. Likewise, our best measure of evolutionary conservation (SIFT) failed to predict 25% of the reduced-function variants. Since measures of evolutionary conservation ignore species-specific physiology and are extremely sensitive to the availability of homologous sequence, they cannot substitute for direct measurement of protein function to predict and understand phenotypic diversity.

Early successes in pharmacogenetics (for example, the identification of the genetic determinants of polymorphism in debrisoquine metabolism [36] or succinylcholine sensitivity [37]), gave hope that the genetic determinants of drug-response phenotypes would be relatively easy to detect and verify. The underlying assumption of this optimism was that the genes involved in such non-essential functions as the elimination of xenobiotics would be under relatively little selective pressure, such that loss-of-function or severely reduced-function alleles would be expected to be found at high frequencies in healthy individuals. This assumption, that variation in "drug-response genes" is selectively neutral, may be incorrect. Experience has shown us that many genes that were discovered as drug metabolizing enzymes or (as in this case) drug transport proteins are under significant negative selection. Whether this relates to possible homeostatic/physiological roles of these genes, or to an unrecognized importance of their protective effects in evolutionary fitness, is unknown. It is apparent, however, that high-frequency null alleles in drug-response genes have not often been observed. However,

our identification of protein variants with substrate-specific changes in function suggests that there may exist variants in drug-response genes that are normal-function with respect to endogenous or common environmental molecules but reduced function with respect to recently developed, clinically-relevant drugs. These variants would be free of negative selection and could therefore reach high frequencies in healthy individuals. Indeed, of the small set of substrate selectivity variants identified in the current study, nearly half occurred at allele frequencies >10%. Thus, in addition to the reduced function variants identified here, these selectivity variants represent plausible candidates for association with drug response phenotypes.

As we and others have seen, the relative frequency of deleterious mutations (the allelic spectrum) varies from gene to gene, and it may be that for many genes, association with a particular phenotype cannot be explained by one or a small number of high-frequency variants, even when the effect of that gene is significant and the phenotype is relatively common. A well-studied example is the association between the *MC4R* gene and obesity, in which no single variant occurs at a sufficiently high frequency to establish a significant association, yet the sum of deleterious variants of the *MC4R* gene has been shown consistently to be higher in obese individuals than in non-obese controls [38-40]. A more recent example is the association of rare amino acid substitutions in several candidate genes (*ABCA1*, *APOA1*, and *LCAT*) with variation in plasma HDL cholesterol levels, in which various deleterious amino acid substitutions in these genes were found to be more common in individuals with low levels of HDL than in those with high HDL [41]. For pharmacogenetics, in cases similar to this, it may be possible to study the role

of a candidate gene prospectively, by taking a "genotype-to-phenotype" approach. That is, identification of (rare) variants of severely reduced function, followed by phenotyping the (rare) individuals carrying these variants by drug administration. The knowledge that a particular variant alters function in cellular assays will greatly strengthen our confidence in positive associations found using such a strategy.

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CHAPTER 8

CONCLUSIONS

The overall goal of this dissertation was to elucidate the physiological and pharmacological roles of the novel organic cation transporters, OCTN1 and OCTN2. We used a sequence-based, or "genotype-to-phenotype" approach, to investigate the influence of genetic variation in these genes on drug and nutrient disposition.

Summary of Findings

In Chapter 2, we identified genetic variants in the coding region of the *SLC22A4* and *SLC22A5* genes encoding OCTN1 and OCTN2 in DNA samples from a large, ethnically-diverse healthy population, and estimated population-specific haplotype structures and allele frequencies. We identified several variants, including common variants in the coding region and potential regulatory regions (splice donor site and 3'-untranslated region) of these genes that were at least partially correlated with variants that had been associated with inflammatory diseases (specifically Crohn's disease) [1]. We pointed out that the pathological consequences of OCTN deficiency, as determined by studies in rodent models and in humans carrying deleterious OCTN2 mutations, argue against their involvement in inflammatory bowel disease. We noted that the *SLC22A4* and *SLC22A5* genes reside in a cytokine-rich gene cluster that includes several genes with stronger *a priori* evidence for involvement in the pathogenesis of Crohn's. In light of this, we argued for further study before concluding that the *SLC22A4*-1507C/T and *SLC22A5*-(-207)C/G polymorphisms are the causal variants at the IBD5 susceptibility locus.

The protein sequence-altering variants identified in Chapter 2 were then phenotyped in biochemical assays (Chapters 3 and 4), revealing a number of variants with abnormal activity or subcellular localization in heterologous expression systems.

In Chapter 3, the characterization of six protein-altering variants of OCTN1, including five amino acid substitutions and one nonsense mutation, was described. Two rare variants (D165G and R282X) were found to result in complete loss of transport function, and one (M205I) was found to cause a reduction in transport of TEA and betaine to approximately 50% of that of the reference OCTN1. The subcellular localization of the D165G and M205I variants was similar to that of the reference sequence protein, suggesting that the impairment in transport activity seen with these variants occurs at the level of the mature protein. We also found two common variants of OCTN1, T306I and L503F. The T306I polymorphism disrupts a potential N-glycosylation site; however, this variant had no effect on transport activity or membrane expression in cellular assays. In contrast, the L503F polymorphism was shown to influence the substrate selectivity of OCTN1, causing increased affinity and transport capacity for the organic cation TEA, despite having activity similar to the reference allele toward betaine. Because this variant exhibited significantly altered biochemical function, and was particularly common (with a minor allele frequency ~42% in the European American population), we focused on this variant in our clinical genotype-to-phenotype study (Chapter 5).

In Chapter 4, we characterized the transport function and subcellular localization of eight amino acid substitutions in the *SLC22A5* gene. We found that a rare variant, Y449D,

resulted in altered substrate selectivity, with decreased activity toward the endogenous substrate, L-carnitine, but increased activity toward the prototypical cation TEA. The Y449D substitution caused a defect in sodium activation of L-carnitine transport, as had been described previously [2]. We discovered a rare variant, V481F, that caused a decrease in transport activity toward both L-carnitine and TEA. Interestingly, this variant occurred at a triallelic site, *i.e.*, we discovered two amino acid substitutions at this position (V481I and V481F), but only the V481F substitution resulted in altered function, suggesting that the substitution of phenylalanine at this position may result in steric hindrance of substrate binding or of protein folding during the transport cycle. The two most common protein sequence-altering polymorphisms, L144F and P549S, did not differ from the reference OCTN2 in any of our assays, including kinetics of L-carnitine transport and subcellular localization studies. In contrast, the polymorphism F17L, which occurred at an allele frequency of 2% in the Asian American subset of our sample, showed decreased transport activity toward both L-carnitine and TEA. The reduction in substrate transport exhibited by the F17L variant was shown to be caused by a defect in trafficking to the plasma membrane. Thus, this variant may help to explain variability in the disposition of carnitine and other OCTN2 substrates in Asian populations. We also found that a common polymorphism in the promoter of OCTN2, (-207)G>C, was associated with decreased carnitine transport activity and reduced OCTN2 mRNA levels in immortalized lymphoblasts. This variant occurs at a particularly high frequency (~50%) in individuals of European ancestry, and is in linkage disequilibrium with the common OCTN1-L503F variant described in chapters 3 and 5, which had been shown to cause reduced carnitine transport by OCTN1. These two SNPs form a two-point locus

haplotype that was expected to cause a reduction in carnitine transport activity (specifically, a deficiency in active tubular reabsorption of carnitine), a hypothesis that was tested in Chapter 7.

Although the variants that resulted in complete loss of function were extremely rare, the *SLC22A4*-L503F and *SLC22A5*-(-207)G>C variants were highly polymorphic, with particularly high allele frequencies in individuals of European ancestry. This allowed us to employ a genotype-to-phenotype strategy to study the effects of functional genetic variation in the OCTNs *in vivo*.

In Chapter 5, we investigated the role of OCTN1 in the pharmacokinetics of gabapentin. We discovered that OCTN1 facilitated the transport of gabapentin in cellular assays, and that the OCTN1-L503F variant was deficient in gabapentin transport. In clinical studies, we showed that the active secretion of gabapentin in the kidney was strongly associated with OCTN1 genotype: individuals homozygous for the 503L (reference) allele uniformly showed active secretion of gabapentin, whereas in subjects homozygous for the 503F allele, renal clearance was essentially equal to the glomerular filtration rate. Because the effect of OCTN1 genotype on total clearance of gabapentin was relatively small and gabapentin has a fairly wide therapeutic range, this finding may not have great clinical relevance. However, these studies provide a proof of concept regarding the importance of OCTN1 to renal drug clearance, and may serve as a guide to future pharmacogenetics studies of renal drug transporters.

In Chapter 6, we studied the effect of the common two-point haplotype *SLC22A4*-L503F/*SLC22A5*-(-207)G>C on carnitine disposition and plasma lipid profile. We failed to detect an effect of OCTN haplotype on any of the parameters measured, including plasma carnitine concentrations and carnitine renal clearance. We found a significant difference between males and females in terms of plasma carnitine levels, with males having higher carnitine concentrations in plasma *vs*. females. This was not explained by reduced carnitine renal clearance in males *vs*. females; rather, carnitine renal clearance was higher in males than in females. This suggested that the efficiency of carnitine reabsorption is related to plasma concentrations, with a tendency toward saturation or down-regulation of carnitine reabsorption in the presence of high plasma carnitine levels. Carnitine plasma concentrations were positively correlated with atherogenic lipid species in plasma, which also tended to be higher in males than in females. Taken together, these results may be explained by higher dietary intake of carnitine-rich and lipid-rich foods in male subjects than in female subjects in our sample population, although intrinsic differences between males and females in the absorption or clearance of both carnitine and lipids cannot be ruled out. Although we hypothesized, based on the phenotypes of OCTN2-deficient humans and animal models, that genetic variation at the OCTN locus may influence both carnitine homeostasis and lipid metabolism, it appears that within the normal range of carnitine plasma concentrations and in healthy individuals, diet is a controlling factor in these parameters.

Most of the work presented in this dissertation focused on the OCTN transporters, and some of the findings with respect to OCTN functional variation fit a general trend in functional genetic variation among solute carrier transporters. In Chapter 7, we performed a meta-analysis of the properties of 88 functionally characterized protein-altering variants in the *SLC22A*, *SLC28A* and *SLC29A* families. We found a high percentage (25%) of variants with complete loss of function or significantly reduced function in cellular assays. These reduced-function variants tended to be very rare, with only one reduced-function variant having an allele frequency greater than 3%. This suggests that complete null alleles of membrane transporters, including drug transporters, are unlikely to be found at frequencies high enough to be useful for genetic association studies. This is in contrast to the well-described common loss-of-function variants of drug metabolizing enzymes such as CYP2D6 or NAT1 [3]. However, rare loss-of-function variants of drug transporters may still be relevant to rare phenotypes such as idiosyncratic drug toxicities. In constrast, we found that substrate selectivity variants, ***i.e.***, variants with altered activity toward at least one, but not all substrates tested in cellular assays, had an allele frequency distribution that was similar to neutral (normal-function) variants. This suggests that common polymorphisms in drug transporters may explain a considerable amount of population variation in drug disposition and drug response, but that these effects are likely to be substrate-dependent. The data presented in Chapters 3 and 5 provide a detailed example of this: the OCTN1-L503F variant, which had increased activity toward TEA and normal activity toward the amino acid betaine, was found to have reduced activity toward the anticonvulsant gabapentin in cellular assays, and this allele was associated with an absence of active secretion of gabapentin *in vivo*.

Similar examples of this sort of substrate-specific effect are likely to be found for variants in other membrane transporters and perhaps other classes of genes, such as drug metabolizing enzymes or drug receptors.

From Genotype to Phenotype

The sequence-based, or genotype-to-phenotype, studies described in this dissertation represent an unconventional approach to human genetics. Traditionally, attempts to identify genetic determinants of human disease or other phenotypes (including drug response) have been carried out using forward-genetic approaches such as linkage mapping (in pedigrees or founder populations) or genetic association studies (in unrelated individuals).

Genotype-to-phenotype studies can be thought of as a reverse-genetics approach, akin to functional deletion of genes in animal models, such as "knockout" mouse models. Using the information gathered by sequencing of known functional (coding or regulatory) regions of the human genome, and empirically testing variant DNA sequences for functional significance in biochemical or cellular assays, it is possible to identify humans that are likely to exhibit reduced activity of a particular gene *in vivo*. Individuals carrying these putative functional polymorphisms may then be prospectively studied to test hypotheses regarding the *in vivo* function of these genes, and importantly, to make predictions regarding the likelihood of a particular outcome (such as a disease, or a drug toxicity) in individuals carrying these alleles. An elegant feature of this approach is that, at least initially, it restricts the analysis to a test of a single hypothesis (or, where possible,

a small number of hypotheses) regarding a single predictor variable – genotype at the lone variant position used to identify potential subjects. This essentially makes the study design similar to a comparison between two treatment groups, and avoids the concerns raised in genetic association studies with regard to false positive associations arising from multiple testing of independent SNP markers. An important practical advantage to a genotype-to-phenotype approach is that it allows the investigator to manipulate the genotype distribution of the sample to achieve the greatest statistical power at the lowest cost. For pharmacogenetics studies, this is of great importance, as the collection of phenotype data frequently requires expensive and time-consuming studies of drug pharmacokinetics in healthy individuals. This is in contrast to studies of complex disease genetics, in which DNA samples may be collected from individuals who are phenotyped as part of routine clinical care. In the latter case, the marginal cost of ascertainment of study subjects consists solely of acquiring consent for the study, collection of DNA, and genotyping. For pharmacogenetics studies, such as those described in Chapters 5 and 6, the time and expense of phenotyping far outweighs the cost of DNA collection and genotyping. In cases such as these, it is ideal to recruit subjects that are homozygous for either of two functionally different alleles, as heterozygotes would be expected to have an intermediate phenotype. For common polymorphisms, heterozygotes would be expected to make up the majority of a randomly-collected sample, while contributing little to the phenotypic variation in the sample.

Thus, genotype-to-phenotype studies represent a complementary approach to human genetics that is likely to be useful in addition to, but not in place of, traditional

approaches to human genetics. It would seem that a genotype-to-phenotype approach would be most useful in cases in which the function of the gene being studied is fairly well understood, but still requires investigation in human subjects. The reason for this is that the phenotype must be chosen extremely carefully, and the investigator must have very strong prior evidence that the gene under study influences that phenotype, in order to justify the cost of recruiting individuals specifically based on genotype at a particular position in the genome.

Conclusions and Future Studies

The results described in this dissertation have significantly extended our knowledge of the nature and extent of functional genetic variation in the *SLC22A4* and *SLC22A5* genes, and have provided the first evidence of the importance of OCTN1 to renal drug elimination. These studies provide a model for investigation of the influence of genetic variation on drug disposition or drug response that may be extended to other genes (*e.g.*, other drug transport proteins, or drug metabolizing enzymes or receptors), or to other substrates of the OCTNs (*e.g.*, ergothioneine, cephalosporin or quinolone antibiotics) [4-6]. These approaches may be complemented by genetic association studies, by testing the functionally significant polymorphisms described here for association with drug response or drug toxicity, or with phenotypes related to carnitine or ergothioneine disposition. Additionally, genotype-to-phenotype studies of other genes known or suspected to regulate OCTN transcription (*e.g.*, PPARα, cartregulin) or activity (*e.g.*, PDK1, PDK2), or to function in concert with the OCTNs (*e.g.*, OCT1, OCT2, MATE1,

MATE2) may provide further evidence of the role of these transporters in drug response, and improve prediction of drug response using pathway analysis [7-14].

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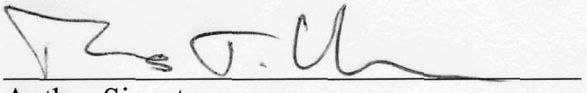

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