IDENTIFICATION OF ENDOGENOUS FUNCTION AND SUBSTRATE-DEPENDENT INTERACTIONS OF ORGANIC CATION TRANSPORTER 1

BY

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Submitted to the graduate degree program in Pharmacology, Toxicology and Therapeutics and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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ABSTRACT

The human organic cation transporter 1 (hOCT1) is a polyspecific transporter, primarily expressed in the liver, which is known to interact with a large number of structurally dissimilar compounds. Several clinically-relevant drugs, as well as some endogenous compounds and other xenobiotics have been shown to be transported by or inhibit hOCT1. Due to its hepatic expression and general ADME function, hOCT1 has been implicated in adverse drug events (ADEs), including drug-drug interactions. As such, multiple regulatory agencies recommend including hOCT1, in pre-clinical transporter interaction studies. Limited structural information is available for hOCT1, and recently, endogenous functions and substrate-dependent effects have been identified for close relatives of hOCT1. Taken together, these suggest a need for further scrutiny of hOCT1 structure-activity relationships for development of critical drug-transporter interaction studies.

The hypothesis was developed that both endogenous and xenobiotic compounds modulate the functional activity of hOCT1 in a substrate-dependent manner through interaction with specific, but perhaps distinct ligand-binding domains within the transporter. The hypothesis was tested via the following specific aims: 1) investigate the effect of xenobiotics on endogenous substrate transport by hOCT1, 2) identify and characterize substrate-dependent interactions with hOCT1, and 3) examine the role of the extracellular loop domain of hOCT1 in substrate affinity and translocation.

In the first specific aim, dopamine and serotonin were identified as substrates for hOCT1. Serotonin proved to be a moderate-affinity substrate, while hOCT1 was able to

transport it at high capacity. Several clinically-relevant drugs inhibited hOCT1-mediated serotonin transport, and these results were capitulated in primary human hepatocytes. Combined data from this inhibition screen and those previously published by other groups suggested the possibility of substrate-dependent effects.

In specific aim two, substrate-dependent effects were screened for in a relatively new assay method, competitive counterflow (CCF). The CCF assay allowed for identification of novel substrates for hOCT1, including negatively-charged bromosulfophthalein (BSP). CCF results also identified numerous substrate-dependent effects which were explored further using computational (homology) modeling and ligand docking. Docking experiments identified three distinct binding sites within the hOCT1 homology model which explain several of the overserved substrate-dependent interactions, and supports previous claims that hOCT1 has a large substrate binding region versus a singular binding site and may be the reason for hOCT1's polyspecificity.

In the final specific aim, an attempt was made to generate human and rat OCT1 chimeric proteins. The goal of this study was to examine the role of the extracellular loop (ECL) domain in the observed differences in substrate affinity between species. Issues during the cloning process prevented the completion of this aim. However, had the chimeras successfully been generated, important information relating hOCT1 structure to its function could have been collected.

This dissertation demonstrates that hOCT1 possesses important endogenous function and exhibits substrate-dependent effects, while also revealing important structural information relating to its function. Ultimately, this knowledge will be useful in

improving pre-clinical trials for new drugs in the hope of identifying and preventing dangerous adverse drug events.

DEDICATION

The work herein is dedicated, with love, to Jace Otto Boxberger.

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LIST OF ABBREVIATIONS

ABC – ATP-binding cassette

ADE – adverse drug event

ADME – absorption, distribution, metabolism, and excretion

AhR – aryl hydrocarbon receptor

AMPK – adenosine monophosphate-activated protein kinase

APDA – N-(4,4-azo-n-pentyl)-21-deoxyajmalinium

APQ – N-(4,4-azo-n-pentyl)-quinuclidine

ASP^+ – 4-4-dimethylaminostyryl-N-methylpyridinium

ATP – adenosine triphosphate

AUC – area under the curve

B_1 – $beta_1$ adrenergic receptor

β_2 – $beta_2$ adrenergic receptor

BCA – bicinchoninic acid assay

BCRP – breast cancer resistance protein

BHM – build homology models

Bmim – 1-butyl-3-methylimidazolium chloride

BmPy – N-butyl-N-methylpyrrolidinium

BPH – benign prostatic hyperplasia

BSEP – bile salt efflux pump

BSP – bromosulfophthalein

CaM – calmodulin

CaMKII – calmodulin-dependent kinase II

CAR – constitutive androstane receptor

CC – cholangiocarcinoma

CCF – competitive counterflow

CDPCP – cis-diammine(pyridine)chloroplatinum(II)

CHO – Chinese hamster ovary

CL – cytoplasmic loop

CLL – chronic lymphocytic leukemia

C_{max} – maximal plasma concentration

CML – chronic myeloid leukemia

DAT – dopamine transporter

DDI – drug-drug interaction

DHEAS – dehydroepiandrosterone sulfate

DMSO – dimethylsulfoxide

DOPE – discrete optimized protein energy

DTP – Developmental Therapeutics Program

E3S – estrone-3-sulfate

E17βG – estradiol-17β-glucuronide

ECL – extracellular loop

EGCG – epigallocatechin gallate

EMA – European Medicines Agency

ENT – equilibrative nucleoside transporter

EtOH – ethanol

EV – empty vector

FDA – Food and Drug Administration

FRET – Förster resonance energy transfer/fluorescence resonance energy transfer

FXR – farnesoid X receptor

GlpT – glycerol 3-phosphate transporter

H_2 – histamine H_2 receptor

HAART – highly active antiretroviral therapy

HCC – hepatocarcinoma

HEK – human embryonic kidney

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV – human immunodeficiency virus

HNF1 – hepatocyte nuclear factor 1

HNF4α – hepatocyte nuclear factor 4α

HTS – high-throughput screening

IC_{50} – half maximal inhibitory concentration

IND – investigational new drug

IPTG – isopropyl β-D-1-thiogalacto-pyranoside

LacY – lactose permease

LB – Luria Bertani

kD – kilodalton

K_m – Michaelis-Menten constant

LacY – lactose permease

MATE – multidrug and toxin extrusion protein

MDMA – 3,4-methylenedioxymethamphetamine

MDR1 – multidrug resistance protein 1; P-glycoprotein

MeOH – methanol

MFS – major facilitator superfamily

mIBG – [^{123}I]-meta-iodobenzylguanidine

MPP^+ – 1-methyl-4-phenylpyridinium

MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA – messenger RNA

MRP – multidrug resistance-associated protein

NASH – nonalcoholic steatohepatitis

NBD-MTMA – N,N,N-trimethyl-2-[methyl(7-nitrobenzo[c][1,2,5]oxadiazol-4-yl)amino] ethanaminium

NCI – National Cancer Institute

NET – norepinephrine transporter

NMN – N-1-methylnicotinamide

NMR – nuclear magnetic resonance

NRTI – nucleoside reverse transcriptase inhibitor

NTCP – sodium/taurocholate co-transporting polypeptide

OAT – organic anion transporter

OATP – organic anion transporting polypeptide

OCT – organic cation transporter

OCTN – organic cation/carnitine transporter

ORF – open reading frame

PDB – Protein Data Bank

P-gp – (see MDR1)

PEPT- peptide transporter

PGE2 – prostaglandin E_2

PiPT – *Piriformospora indica* phosphate transporter

PKA – protein kinase A

PKC – protein kinase C

PKG – protein kinase G

PMAT – plasma membrane monoamine transporter

PXR – pregnane X receptor

RACE – rapid amplification of cDNA ends

rOCT1 – rat organic cation transporter 1

RT-PCR – reverse transcription polymerase chain reaction

SAR – structure-activity relationship

SD – standard deviation

SEM – standard error of the mean

SERT – serotonin reuptake transporter

SHP – small heterodimer partner

SLC – solute carrier

SLCO – solute carrier family of the OATPs

SRC-1 – steroid receptor coactivator 1

TBA – tetrabutylammonium

TBE – tris/borate/EDTA

TBuMA – tributylmethylammonium

TCDD – dioxin; 2,3,7,8-tetrachlorodibenzo-p-dioxin

TEA – tetraethylammonium

THA – tetrahexylammonium

TKI – tyrosine kinase inhibitor

TM/TMD – transmembrane domain

TEMA – triethylmethylammonium

TMA – tetramethylammonium

TPeA – tetrapentylammonium

TPrA – tetrapropylammonium

V_{max} – maximal rate of transport

LIST OF APPENDICES

Appendix I: Citations of published papers

Appendix II: List of license agreements for copyrighted materials

CHAPTER 1 : INTRODUCTION

1.1 INTRODUCTION TO PHARMACOKINETICS

The field of pharmacology can be loosely divided into two categories: pharmacodynamics, described as the effect(s) a drug exerts on the body, and pharmacokinetics, or the effect the body has on a drug. Pharmacokinetics describes how a drug is taken in, disbursed throughout the body, and expelled from the body. These processes are commonly referred to using the acronym ADME, which stands for absorption, distribution, metabolism, and excretion (Doogue and Polasek, 2013). These four factors collectively determine the disposition of a given drug in the body.

The first step, absorption, is the means by which a drug gains access to the body. The most common route of drug administration is oral, and therefore absorption into the bloodstream via the intestine is a critical first step. The next step, distribution of the drug into various tissues of the body, is achieved through systemic circulation which allows for the delivery of drug to the target tissue (where it will exert the desired effects), as well as into detoxifying organs, most notably the liver and kidneys, for metabolism and excretion. It is estimated that nearly 60% of prescribed drugs must undergo hepatic metabolism (Cascorbi, 2006) to convert the drug into a more hydrophillic entity before it can be excreted. The final component of drug disposition described by ADME is excretion, or the process by which a drug or metabolite leaves the body. The most common routes of excretion are through the kidneys, or the intestines by way of the liver. Collectively, the processes in ADME are crucial to drug efficacy and detoxification.

Generally, a required process for all of these steps is the passing of drug molecules across cell membranes. There are three methods by which molecules can

pass through cell membranes: passive diffusion, facilitated diffusion, and active transport. Passive diffusion is the movement of molecules through a cell membrane from high concentration to low concentration without the aid of carriers or energy expenditure; whereas facilitated diffusion involves the passage of molecules across a membrane along a concentration gradient by utilizing a carrier protein, or transporter. Active transport moves molecules across cell membranes, via a transport protein, against a concentration gradient, and therefore energy expenditure, usually in the form of ATP hydrolysis, is required.

Several factors determine a molecule's ability to passively diffuse across the plasma membrane, including size and lipophilicity. Due to the selectively permeable nature of cell membranes, many drugs are incapable of entering cells via passive diffusion, and thus must enter cells via facilitated diffusion or active transport. As a result, there is an ever-increasing appreciation for the role of transporters in ADME.

1.2 TRANSPORTERS INVOLVED IN ADME

Transporters expressed at the cell membrane have been described as "gatekeepers" that govern selective cellular permeability (Kaback, et al., 2001, Sprowl, et al., 2016). They regulate uptake and efflux of essential cellular components, including amino acids, sugars, nucleosides, and inorganic ions (Kaback, et al., 2001, Wu, et al., 2011). Transporters are also largely responsible for the movement of pharmaceutical agents across cell membranes, and thus are key determinants of therapeutic response

to drugs (Borst and Elferink, 2002, Hillgren, et al., 2013, International Transporter, et al., 2010, Nies, et al., 2011b).

Transporters are ubiquitously expressed throughout the body. However, drug transporters are generally concentrated in tissues with barrier functions, namely the liver, kidneys, intestine, and brain, the same tissues highly involved in ADME (You and Morris, 2007). A summary of transporters expressed in these tissues that have been identified as important in drug disposition can be seen in Figure 1.1 (Hillgren, et al., 2013, International Transporter, et al., 2010, You and Morris, 2007). These transporters are divided into two large superfamilies of transporters: ATP-binding cassette (*ABC*) and solute carrier (*SLC*) transporters.

ABC transporters generally function as effluxers, pumping substrates out of the cell against their concentration gradients via active transport using energy derived from ATP hydrolysis. Important drug transporters in the *ABC* superfamily include the multidrug resistance protein (MDR1; also known as P-glycoprotein, or P-gp), multidrug resistance-associated proteins (MRPs), and breast cancer resistance protein (BCRP) (Hillgren, et al., 2013, You and Morris, 2007).

SLC transporters operate as facilitated diffusion carriers and are generally influx transporters, though some function as exchangers or effluxers (Koepsell, 2015, You and Morris, 2007). *SLC* transporters which have been identified as important in drug disposition include organic cation transporters (OCTs), organic cation/carnitine transporters (OCTNs), multidrug and toxin extrusion proteins (MATEs), organic anion transporters (OATs), organic anion transporting polypeptides (OATPs),

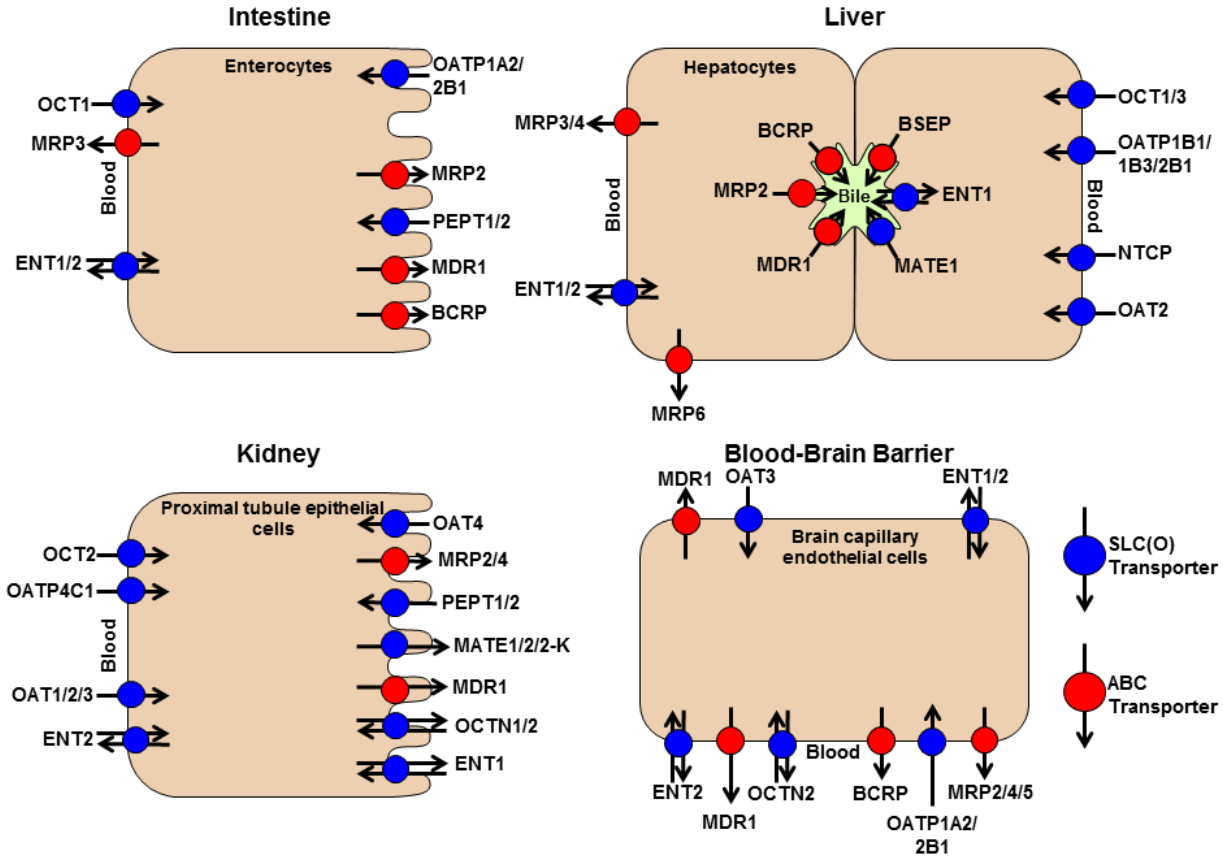

Figure 1.1. Important drug transporters expressed in intestine, liver, kidney, and at the blood-brain barrier. Relevant *ABC* (red) and *SLC/SLCO* (blue) transporters expressed in enterocytes, hepatocytes, kidney proximal tubule cells, and brain capillary endothelial cells. Schematic adapted from (Hillgren, et al., 2013, You and Morris, 2007, Zamek-Gliszczynski, et al., 2012).

sodium/taurocholate co-transporting polypeptide (NTCP), equilibrative nucleoside transporters (ENTs), and peptide transporters (PEPTs) (Hillgren, et al., 2013, You and Morris, 2007).

The work presented in this dissertation focuses on organic cation transporters, and specifically hOCT1 (*SLC22A1*).

1.3 INTRODUCTION TO ORGANIC CATION TRANSPORTER 1

Organic cation transporter 1 (OCT1) is a multispecific transporter belonging to the *SLC22* superfamily (identified by gene name *SLC22A1*). Homologs exist in humans, several species of rodents, insects, and bacteria. OCT1 is expressed in several tissues and is involved in the transport of numerous clinically-relevant drugs and endogenous compounds.

1.3.1 Cloning and Functional Characterization of OCT1

The first OCT1 homolog was isolated from rat kidney using functional expression cloning (rOCT1; Grundemann, et al., 1994). They reported the identification of a novel 556 amino acid transmembrane protein which demonstrated saturable uptake of model cation tetraethylammonium (TEA) in *Xenopus laevis* oocytes. Uptake of TEA was independent of sodium and potassium concentration, as well as alterations to intra- and extracellular pH, but appeared to be dependent on membrane potential. Transport was also inhibited by several structurally dissimilar hydrophobic and hydrophilic cations. Further characterization of rOCT1 identified *N*-1-methylnicotinamide (NMN), 1-methyl-4-

phenylpyridinium (MPP^+), and choline as additional substrates (Busch, et al., 1996b). It was also determined that rOCT1 could translocate electrical charge across the membrane and transport function could be measured using electrophysiology techniques, which lead to the classification of rOCT1 as an electrogenic transporter (Busch, et al., 1996b).

Shortly after isolation of rOCT1, two groups independently cloned human OCT1 (hOCT1) using homology screening of human kidney and liver cDNA libraries (Gorboulev, et al., 1997) and human liver mRNA subjected to RT-PCR and RACE (Zhang, et al., 1997b). The resultant cDNAs encoded proteins of 553 and 554 amino acids, respectively, and shared a 78% homology with rOCT1. Like rOCT1, hOCT1 demonstrated saturable transport of MPP^+, NMN, and TEA, which was inhibited by both small and large organic cations, though some inhibitor-specificity was observed (Gorboulev, et al., 1997, Zhang, et al., 1997b). Consistent with rOCT1, hOCT1 transport rates did not show a significant difference when pH was varied, but did appear to be dependent on membrane potential (Zhang, et al., 1997b). It was reasoned that human and rat OCT1 functioned by similar mechanisms, though with important species-dependent differences in kinetics of interactions (Zhang, et al., 1997b, Zhang, et al., 1998).

1.3.2 hOCT1 Expression and Tissue Distribution

When first cloned, it was determined that hOCT1 was predominantly expressed in the liver (Gorboulev, et al., 1997, Zhang, et al., 1997b). Immunohistochemistry techniques demonstrated that hOCT1 is localized to the basolateral, or sinusoidal

membranes of hepatocytes (Nies, et al., 2008, Nies, et al., 2009). Subsequent studies demonstrated expression of hOCT1 expression at the apical membrane of kidney proximal and distal tubules (Nies, et al., 2008, Tzvetkov, et al., 2009), on the luminal side of brain microvascular endothelial cells (Lin, et al., 2010), at the apical membrane of airway epithelia (Lips, et al., 2005), and at the luminal and lateral membranes of the small intestine (Muller, et al., 2005). hOCT1 mRNA is found in several other tissues, including heart, pancreas, spleen, stomach, skeletal muscle, large intestine, adipose, adrenal glands, thyroid, salivary glands, bone marrow, thymus, ovaries, uterus, placenta, mammary glands, prostate, and testis (Hilgendorf, et al., 2007, Jung, et al., 2008, Moreno-Navarrete, et al., 2011, Nies, et al., 2009, Nishimura and Naito, 2005, Zhang, et al., 1997b).

Aberrant expression of hOCT1 is observed in various disease states. Leukocytes and lymphocytes from chronic myeloid leukemia (CML) and chronic lymphocytic leukemia (CLL) patients exhibited significantly higher levels of hOCT1 expression than control cells, which expressed very little (Gupta, et al., 2012, Thomas, et al., 2004). Additionally, hOCT1 expression was elevated and correlated with disease stage in lymphocytes isolated from HIV-infected patients, while expression in cells from healthy controls was negligible (Jung, et al., 2008). In certain colorectal cancer patients, hOCT1 mRNA expression was found to be significantly increased compared to normal samples (Zhang, et al., 2006). Interestingly, hepatic expression of hOCT1 is decreased in advanced hepatocarcinoma (HCC) and cholangiocarcinoma (CC) patients (Heise, et al., 2012, Herraez, et al., 2013, Schaeffeler, et al., 2011), as well as those suffering from cholestasis (Nies, et al., 2009). The changes observed in hOCT1 expression in disease

states are likely due to altered expression or function of transcription factors known to regulate hOCT1 (see section 1.3.3). A summary of hOCT1 expression can be found in Table 1.1.

Several groups have characterized the expression profiles of hOCT1 in cultured cell lines. hOCT1 expression is observed in: SK-MG-1 and Ski-1 cells derived from human glioma; human hepatoma cell lines Hep3b, Huh7, HepG2 and HepaRG; Caki-1 cells derived from human kidney carcinoma; human colon adenocarcinoma cell lines Caco-2, LS180, DLD, SW620, and HT29; human colorectal carcinoma cell lines HCT116 and RKO; JAR, a human placenta choriocarcinoma cell line; hCMEC/D3, an immortalized human brain endothelial cell line; TOV2223G cells, an ovarian cancer cell line; and human leukemia, lymphoma, or lymphoblastoid cell lines MT2, MT4, Molt4/CCR5, Hut78, A3F7, CEM13, KYO1, LAMA84, KCL22, K562, Karpas-422, Raji, SUDHL-4, JURKAT, and L-428 (Andreev, et al., 2016, Dickens, et al., 2012, Gupta, et al., 2012, Hayer-Zillgen, et al., 2002, Heise, et al., 2012, Hilgendorf, et al., 2007, Le Vee, et al., 2006, Minuesa, et al., 2008, Muller, et al., 2005, Thomas, et al., 2004, Zhang, et al., 2000, Zhang, et al., 2006). Expression profiles for hOCT1 in cultured cell lines appear to be similar to those of the human tissues they’re derived from, whether under normal or disease conditions, and therefore are useful models for exploring drug transport under normal physiological conditions or in disease states.

1.3.3 Molecular Regulation of hOCT1

hOCT1 regulation is two-fold: regulation of expression, and regulation of function. Reports on regulation of both expression and function are briefly summarized below.

Table 1.1. Human OCT1 mRNA and protein expression in various tissues.

	Tissue	mRNA	Protein	Reference
Normal Expression	Liver	+	+	Dickens, et al., 2012, Gorboulev, et al., 1997, Hilgendorf, et al., 2007, Jung, et al., 2008, Nies, et al., 2008, Nies, et al., 2009, Nishimura and Naito, 2005, Tzvetkov, et al., 2009, Zhang, et al., 1997b, Zhang, et al., 2000
	Heart	+		Jung, et al., 2008, Nishimura and Naito, 2005, Zhang, et al., 1997b
	Skeletal muscle	+		Gorboulev, et al., 1997, Jung, et al., 2008, Nishimura and Naito, 2005, Zhang, et al., 1997b
	Kidney	+	+	Dickens, et al., 2012, Gorboulev, et al., 1997, Hilgendorf, et al., 2007, Nies, et al., 2008, Nies, et al., 2009, Nishimura and Naito, 2005, Pietig, et al., 2001, Tzvetkov, et al., 2009, Zhang, et al., 1997b
	Lung	+	+	Jung, et al., 2008, Lips, et al., 2005, More, et al., 2010, Nies, et al., 2009, Nishimura and Naito, 2005
	Small intestine	+	+	Gorboulev, et al., 1997, Hilgendorf, et al., 2007, Jung, et al., 2008, Muller, et al., 2005, Nishimura and Naito, 2005, Tzvetkov, et al., 2009, Zhang, et al., 2000
	Large intestine	+		Gorboulev, et al., 1997, Hilgendorf, et al., 2007, Jung, et al., 2008, Nishimura and Naito, 2005
	Stomach	+		Gorboulev, et al., 1997, Nishimura and Naito, 2005
	Prostate	+		Jung, et al., 2008, Nishimura and Naito, 2005
	Ovary	+		Jung, et al., 2008
	Uterus	+		Nishimura and Naito, 2005

	Placenta	+		Gorboulev, et al., 1997, Nishimura and Naito, 2005
	Mammary gland	+		Jung, et al., 2008
	Adrenal gland	+		Nies, et al., 2009, Nishimura and Naito, 2005
	Salivary gland	+		Nishimura and Naito, 2005
	Thyroid	+		Nishimura and Naito, 2005
	Pancreas	+		Jung, et al., 2008, Nies, et al., 2009, Nishimura and Naito, 2005
	Thymus	+		Nishimura and Naito, 2005
	Spleen	+		Gorboulev, et al., 1997, Jung, et al., 2008, Nies, et al., 2009, Nishimura and Naito, 2005
	Testis	+		Jung, et al., 2008, Nies, et al., 2009, Nishimura and Naito, 2005
	Brain	+	+	Dickens, et al., 2012, Gorboulev, et al., 1997, Lin, et al., 2010, More, et al., 2010
	Adipose	+		Moreno-Navarrete, et al., 2011
	Bone marrow	+		Nishimura and Naito, 2005
Aberrant Expression	CML (leukocytes)	+		Thomas, et al., 2004
	CLL (lymphocytes)	+		Gupta, et al., 2012
	HIV (lymphocytes)	+	+	Jung, et al., 2008, Minuesa, et al., 2008

1.3.3.1 Regulation of hOCT1 Expression

The robust expression of hOCT1 in the liver suggests the existence of a hepatocyte-specific expression regulation system. Saborowski, et al., (2006) identified putative hepatocyte nuclear factor 4α (HNF4α) response elements within the hOCT1 gene promoter. Indeed, they were able to demonstrate that HNF4α regulates hOCT1 gene expression. Additionally, they determined that bile acids can suppress hOCT1 gene activation via the nuclear receptor FXR which induces the transcriptional repressor SHP, effectively repressing HNF4α transactivation of the hOCT1 promoter (Saborowski, et al., 2006). This finding may explain the decreased levels of hepatic hOCT1 expression observed in cholestasis patients (Nies, et al., 2009).

High levels of hepatic expression in hOCT1 may also be attributed to hepatocyte nuclear factor 1 (HNF1). Bioinformatics analyses identified strong HNF1 binding motifs within the evolutionary conserved region in intron 1 of the hOCT1 gene (O'Brien, et al., 2013). Follow-up studies confirmed HNF1 binding to the identified sequence in intron 1, and demonstrated a robust upregulation of hOCT1 expression when overexpressed in hepatocyte-derived cell lines (O'Brien, et al., 2013). Furthermore, high HNF1 expression levels significantly correlated with high hOCT1 expression in human liver samples, suggesting that HNF1 and HNF4α may work in parallel to regulate hepatic expression of hOCT1.

In addition to regulation by hepatocyte nuclear factors, hOCT1 expression appears to be controlled by several xenobiotic-sensing nuclear receptors. Jigorel, et al., (2016) examined the effect of xenobiotic activation of various nuclear receptors, known

to induce drug-metabolizing enzymes, on expression of several hepatic drug transporters in isolated human hepatocytes. When hepatocytes were treated with TCDD, an activator of the aryl hydrocarbon receptor (AhR), phenobarbital, activator of the constitutive androstane receptor (CAR), and rifampicin, activator of the pregnane X receptor (PXR), hOCT1 mRNA expression decreased. Their findings suggest that, when activated, AhR, CAR, and PXR all downregulate hOCT1 expression, either individually or in concert. Further studies substantiated the role of PXR in downregulation of hOCT1 expression. Hyrsova, et al., (2016) verified a decrease in hOCT1 expression at the mRNA and protein levels in primary human hepatocytes, and demonstrated diminished hOCT1-mediated transport, confirming a reduction in functional hOCT1 protein levels following activation of PXR. They determined that the mechanism of PXR-mediated suppression of hOCT1 involves the transcriptional coactivator SRC-1. Their results suggest that SRC-1 is a required coactivator for HNF4α, and PXR competes for, or “squelches” SRC-1, thereby inhibiting HNF4α-mediated hOCT1 expression (Hyrsova, et al., 2016).

The findings by Jigorel, et al., (2006) and Hyrsova, et al., (2016) that PXR downregulates hOCT1 expression are contrary to conclusions drawn by other groups. Cho, et al., (2011) reported an observed increase in the glucose-lowering effect of metformin, a hOCT1 substrate, when given to patients after treatment with PXR activator, rifampicin. They followed up this observation by determining hOCT1 mRNA levels in peripheral blood cells with and without rifampicin treatment. Their results demonstrated an increase in hOCT1 mRNA in peripheral blood cells following rifampicin treatment. The authors surmised that PXR can also induce hOCT1 expression in the

liver, thus explaining the observed changes in glucose-lowering effect of metformin, though this hypothesis was not tested. Furthermore, freshly isolated human blood mononuclear cells from CML patients exhibit increased hOCT1 expression following treatment with rifampicin (Austin, et al., 2015). Combined, these data indicate that PXR upregulates hOCT1 expression, though it's important to note that these observations were made from studies using blood cells, not hepatocytes, which may explain the discrepancies with other reports in regards to the role of PXR in regulation of hOCT1 expression, and suggests the possibility of tissue-specific regulation.

1.3.3.2 hOCT1 Functional Regulation

Post-translational modifications, particularly phosphorylation, appear to be significant in modulating hOCT1 function. Multiple groups have reported that hOCT1 has predicted phosphorylation sites for protein kinases A, C, and G (highlighted in Figure 1.2; Ciarimboli and Schlatter, 2005, Gorboulev, et al., 1997). *In vitro* studies in transfected Chinese hamster ovary (CHO) and human embryonic kidney (HEK) cells demonstrated that hOCT1 transport is inhibited when protein kinase A (PKA) is activated (Ciarimboli, et al., 2004). Interestingly, it was found that PKC activation stimulates uptake by rOCT1 (Mehrens, et al., 2000), but appears to exert no effect on hOCT1 (Ciarimboli, et al., 2004). To date, there have been no reports regarding modulation of hOCT1 by PKG.

It appears that phosphorylation by additional kinases may be important in hOCT1 functional regulation. For example, published data suggest that Src family tyrosine kinases can regulate OCT function. Sprowl, et al. (2016) demonstrated that tyrosine

kinase inhibitors (TKIs) that deactivate Src family tyrosine kinases noncompetitively and reversibly inhibit OCT2 function. They also determined that tyrosine phosphorylation is critical for OCT2 function, and proposed this same mechanism of regulation for all organic cation transporters. Indeed, this may be the case for hOCT1, as Ciarimboli, et al. (2004) revealed that the Src-like tyrosine kinase $p56^{lck}$ appears to regulate hOCT1 activity.

Endogenous calcium signaling pathways also appear to functionally regulate hOCT1. It was reported that hOCT1 activity is endogenously stimulated by calmodulin (CaM) and calmodulin-dependent kinase II (CaMKII), evidenced by an observed decrease in hOCT1 activity when both enzymes were pharmacologically inhibited by CaM inhibitor calmidazolium (Ciarimboli, et al., 2004).

1.3.4 hOCT1 Structure

hOCT1 is a large, 554 amino acid, transmembrane glycoprotein, with a predicted molecular weight of approximately 61 kD, based on its amino acid sequence. Hydropathy analysis suggests that the protein contains twelve α-helical transmembrane (TM) domains, a common trait among all members of the *SLC22* family, with cytoplasmic amino- and carboxy-termini (Gorboulev, et al., 1997, Zhang, et al., 1997b). The predicted membrane topology also includes a large extracellular loop (ECL), comprised of 110 amino acids located between the first and second TM domains, as well as a smaller, 66 amino acid cytoplasmic loop (CL) between the sixth and seventh TM domains. The ECL contains three putative N-glycosylation sites, at asparagine

residues 71, 96, and 112 (indicated by black stars in Figure 1.2), two of which are conserved within the OCT family (Gorboulev, et al., 1997, Zhang, et al., 1997b).

Data suggests that the ECL is essential for expression of functional transporters. When the six ECL cysteine residues of rOCT1 were individually mutated to serine, no discernable transport of TEA was observed (Sturm, et al., 2007). The authors of this study speculate that the sulfhydryl groups in these six conserved cysteines form disulfide bonds that are critical for stabilization of tertiary structure of the ECL. Combining results from their own studies with information published regarding the effect of glycosylation on proper folding of the ECL in rabbit OCT2 (Pelis, et al., 2006), Sturm, et al. conclude that proper ECL tertiary structure is essential for OCT insertion into the membrane. This was later confirmed for rOCT1 (Keller, et al., 2011) and hOCT1 (Arimany-Nardi, et al., 2016).

The ECL is also implicated in OCT oligomerization. Keller, et al. (2008) synthesized His- and FLAG-tagged rOCT1 by a cell-free expression system. After affinity purification over a nickel column, which binds His-tag, FLAG-tagged rOCT1 constructs could be detected by Western blot, suggesting rOCT1 oligomerizes. When the cysteine residues within the ECL were mutated or the amino acid sequence of the ECL was replaced with that of the ECL from rOAT1, oligomerization was significantly reduced (Keller, et al., 2011), suggesting that the ECL is pivotal for OCT oligomerization.

To date, a crystal structure has not been determined for any *SLC22* family member. This is likely due to the high degree of hydrophobicity in the large number of

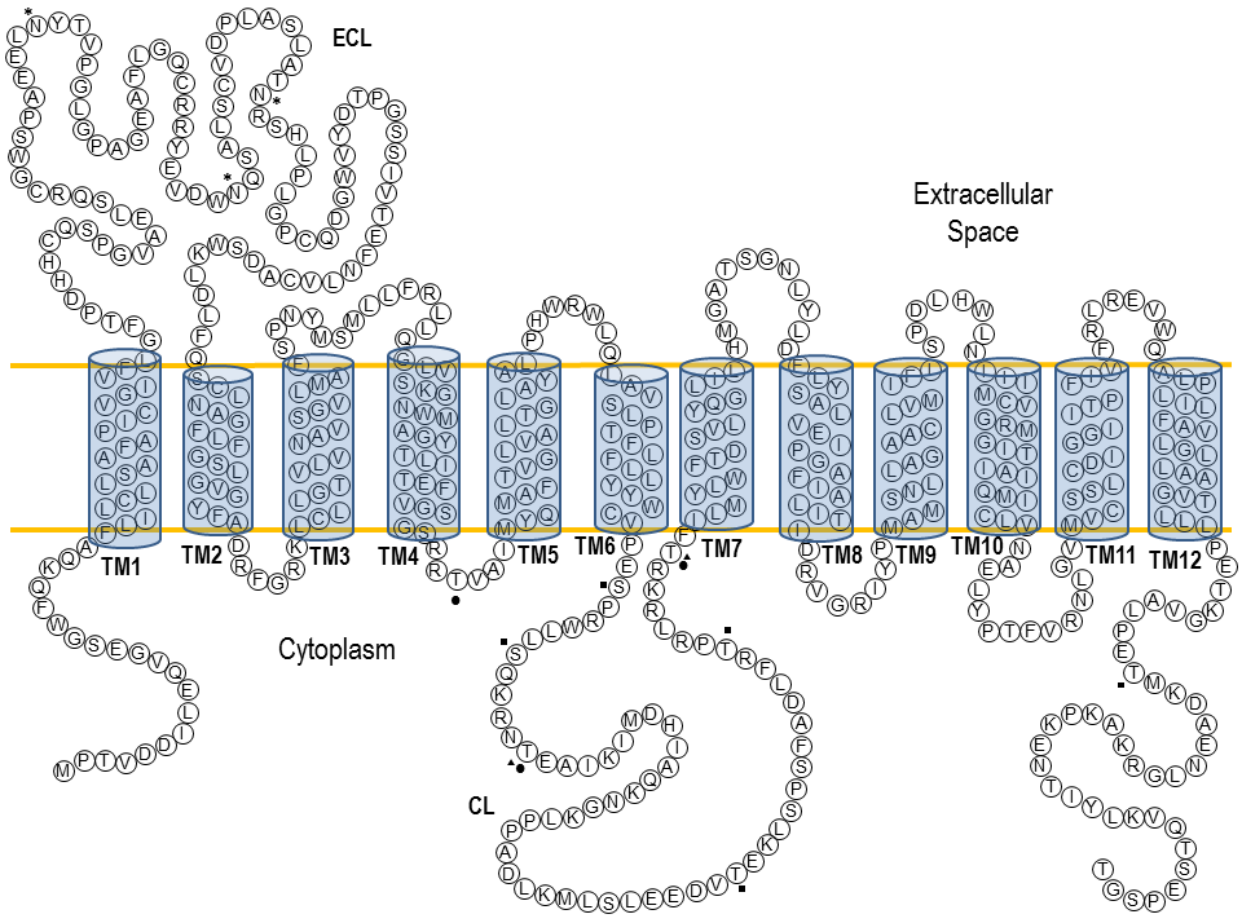

Figure 1.2. Amino acid sequence and predicted membrane topology of hOCT1. The predicted structure of human OCT1 includes twelve putative transmembrane domains (blue cylinders), a large extracellular loop (ECL), a smaller cytoplasmic loop (CL), and intracellular amino- and carboxy-termini. Potential N-glycosylation sites are designated by an asterisk (*****). Predicted phosphorylation sites are notated by a dot (•) for PKA, a square (■) for PKC, and a triangle (▲) for PKG (Ciarimboli and Schlatter, 2005). Amino acid arrangement in this figure adapted from sequence, hydropathy, and topology analysis (Gorboulev, et al., 1997, Koepsell, et al., 2003, Zhang, et al., 1997b).

TM domains. The lack of crystal structures can make structural analysis of transporters difficult. Computational modeling is a tool that can be useful in overcoming this obstacle. Indeed, homology modeling is a useful tool for identifying potential substrate binding sites and relating functional data to transporter structure. A rOCT1 homology model, which modeled the TM domains of rOCT1 based on the crystal structure of bacterial major facilitator superfamily member lactose LacY (Abramson, et al., 2003), was published in 2005 (Popp, et al., 2005). Using this homology model, the authors related rOCT1 mutagenesis data to structural components and elucidated a putative binding pocket. Comparative modeling of hOCT1 substrate binding regions to the crystal structure of fungal transporter PiPT (Pedersen, et al., 2013) is also useful in the prediction of competitive and noncompetitive ligands (Chen, et al., 2017), though it is important to note that subsequent *in vitro* and *in vivo* studies are necessary to validate findings derived from computational models.

Very few structural analyses have been performed using hOCT1. While the majority of structural information available for OCT1 homologs is derived from the above reports utilizing rOCT1 constructs, it is likely that these data are relatable to hOCT1, with some important caveats, due to primarily differences in substrate specificity and affinity, because of the high similarity in secondary structure and conserved regions (Gorboulev, et al., 1997, Zhang, et al., 1997b).

1.3.5 Mechanisms of Transport for hOCT1

SLC family transporters are facilitated diffusion systems which do not require the energy of ATP hydrolysis. However, the driving forces for members of this family are

vastly varied. hOCT1, like other OCTs, is an electrogenic transporter, meaning electric current (measured by electrophysiology techniques) can be detected during substrate transport (Dresser, et al., 2000). This is likely due to the charged nature of substrates. The driving force of transport appears to be a combination of the substrate concentration gradient and membrane potential, as hOCT1 functions independently of sodium, chloride, and pH (Koepsell, 2004, Zhang, et al., 1997b).

Another common property of OCT transport is the ability to translocate substrates across the cell membrane bi-directionally; thus, OCTs effectively function as both influx and efflux transporters (Koepsell, et al., 2003). Indeed, several groups have confirmed that OCT1 (human and rat) can transport substrates in both directions, sometimes seemingly exchanging an intracellular substrate for a different substrate outside the cell (Arimany-Nardi, et al., 2014, Lips, et al., 2005, Zhang, et al., 1998).

OCTs transport a wide array of structurally dissimilar cationic and uncharged organic molecules (see a summary of hOCT1 substrates in Tables 1.2-1.4). The ability to translocate such diverse chemical structures is a property common to many of the *SLC22* family members. The mechanism behind this is not well understood and is the subject of a great deal of research. Several mutagenesis studies have identified certain amino acids within putative OCT binding sites that alter the transport of one substrate, but not others (Gorboulev, et al., 1999, Gorboulev, et al., 2005, Popp, et al., 2005, Zhang, et al., 2005). Additional reports describe substrate-dependent ligand interactions for OCTs and other *SLC22* family transporters (see section 1.5 for further discussion; Belzer, et al., 2013, Harper and Wright, 2013, Martínez-Guerrero and Wright, 2013). Based on these observations, several groups hypothesize that OCTs have multiple

substrate and inhibitor binding pockets or one large binding region, wherein specific binding sites may overlap (Chen, et al., 2017, Ciarimboli, et al., 2004, Ciarimboli and Schlatter, 2005, Koepsell, et al., 2003). This may explain how hOCT1 facilitates the binding and translocation of such structurally diverse substrates with varying degrees of affinity.

It is also important to consider the consequences of microenvironment on transporter function. In view of the fact that substrate affinity can be different for multiple substrates depending on the overexpression system employed (Nies, et al., 2011b), it is quite possible that the lipid composition and protein content of the plasma membrane may influence transporter function (Umehara, et al., 2007).

1.3.6 hOCT1 Substrate and Inhibitor Specificity

As discussed above, hOCT1 interacts with a wide array of structurally diverse molecules. These include numerous drugs from various drug classes and other xenobiotics, as well as endogenous compounds. A summary of hOCT1 substrates and inhibitors can be found in Tables 1.2-1.5.

Multiple groups have analyzed the physicochemical properties of hOCT1 ligands (Ahlin, et al., 2008, Bednarczyk, et al., 2003, Chen, et al., 2017, Liu, et al., 2016, Moaddel, et al., 2007). In general, the molecular weight of hOCT1 ligands is less than 500 Daltons. Ligands tend to be fairly hydrophobic and, as the transporter name would suggest, carry a net positive charge, though neutral and negatively charged substrates

Table 1.2. Select drug substrates of hOCT1.

Substrate	Drug Classification	Reference
Disopyramide	Antiarrhythmic	Hendrickx, et al., 2013
Procainamide	Antiarrhythmic	Hendrickx, et al., 2013
Quinidine	Antiarrhythmic	van Montfoort, et al., 2001
Norfloxacin	Antibiotic	Ciarimboli, et al., 2013
Ofloxacin	Antibiotic	Ciarimboli, et al., 2013
Trimethoprim	Antibiotic	Hendrickx, et al., 2013, Jung, et al., 2008
Atropine	Anticholinergic	Hendrickx, et al., 2013
Clidinium	Anticholinergic	Hendrickx, et al., 2013
Glycopyrrolate	Anticholinergic	Hendrickx, et al., 2013
Ipratropium	Anticholinergic/ bronchodilator	Hendrickx, et al., 2013
Mepenzolate	Anticholinergic	Hendrickx, et al., 2013
Oxyphenonium	Anticholinergic	Hendrickx, et al., 2013
Tiotropium	Anticholinergic/ bronchodilator	Hendrickx, et al., 2013
Lamotrigine	Anticonvulsant	Dickens, et al., 2012
Metformin	Antidiabetic	Ahlin, et al., 2011, Kimura, et al., 2005
Phenformin	Antidiabetic	Hendrickx, et al., 2013
Metoclopramide	Antiemetic/prokinetic	Hendrickx, et al., 2013
Tropisetron	Antiemetic	Tzvetkov, et al., 2012
Atenolol	Antihypertensive	Ciarimboli, et al., 2013, Hendrickx, et al., 2013
Pindolol	Antihypertensive	Ciarimboli, et al., 2013
Bamet-R2	Antineoplastic (experimental)	Briz, et al., 2002
Bamet-UD2	Antineoplastic (experimental)	Briz, et al., 2002
Bendamustine	Antineoplastic	Arimany-Nardi, et al., 2015
Carboplatin	Antineoplastic	Zhang, et al., 2006
CDPCP	Antineoplastic	Li, et al., 2011
Cisplatin	Antineoplastic	Yonezawa, et al., 2006, Zhang, et al., 2006
Daunorubicin	Antineoplastic	Andreev, et al., 2016
Imatinib[a]	Antineoplastic/ tyrosine kinase inhibitor	Burger, et al., 2013, Hu, et al., 2008, Nies, et al., 2014, Thomas, et al., 2004, Wang, et al., 2008

Irinotecan	Antineoplastic	Gupta, et al., 2012
Oxaliplatin	Antineoplastic	Li, et al., 2011, Minematsu and Giacomini, 2011, Yonezawa, et al., 2006, Zhang, et al., 2006
Paclitaxel	Antineoplastic	Gupta, et al., 2012
Picoplatin	Antineoplastic	More, et al., 2010
Sorafenib	Antineoplastic/ tyrosine kinase inhibitor	Herraez, et al., 2013
YM155	Antineoplastic (experimental)	Minematsu, et al., 2010
Zebularine	Antineoplastic	Arimany-Nardi, et al., 2014
Furamidine	Antiparasitic	Ming, et al., 2009
Pentamidine	Antiparasitic	Jung, et al., 2008, Ming, et al., 2009
Amisulpride	Antipsychotic	Dos Santos Pereira, et al., 2014
Sulpiride	Antipsychotic	Dos Santos Pereira, et al., 2014
Perphenazine	Antipsychotic	Hendrickx, et al., 2013
Acyclovir	Antiviral	Takeda, et al., 2002
Efavirenz	Antiviral	Moss, et al., 2015
Ganciclovir	Antiviral	Takeda, et al., 2002
Lamivudine	Antiviral	Jung, et al., 2008, Minuesa, et al., 2009
Zalcitabine	Antiviral	Jung, et al., 2008
Albuterol (Salbutamol)	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013, Salomon, et al., 2015
Fenoterol	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013
Formoterol	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013
Procaterol	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013
Terbutaline	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013
Xamoterol	β_2-agonist/ bronchodilator	Hendrickx, et al., 2013
Aminoguanidine	Diabetic nephropathy therapeutic (experimental)	Kimura, et al., 2009
Amiloride	Diuretic	Hendrickx, et al., 2013
Triamterene	Diuretic	Hendrickx, et al., 2013
Cimetidine	H_2-antagonist	Hendrickx, et al., 2013
Famotidine	H_2-antagonist	Bourdet, et al., 2005

Nizatidine	H_2-antagonist	Hendrickx, et al., 2013
Ranitidine	H_2-antagonist	Bourdet, et al., 2005, Hendrickx, et al., 2013
Varenicline	Nicotinic receptor partial agonist/ smoking cessation aid	Hendrickx, et al., 2013
Morphine	Opioid	Tzvetkov, et al., 2013
O-desmethyltramadol	Opioid metabolite	Tzvetkov, et al., 2011
mIBG	Radiopharmaceutical	Bayer, et al., 2009
Sumatriptan	Serotonin receptor agonist	Hendrickx, et al., 2013
Eltrombopag	Thrombopoietin receptor agonist	Takeuchi, et al., 2011

[a] Status of imatinib as a substrate is controversial. See references.

Table 1.3. Other xenobiotic and model substrates of hOCT1.

Substrate	Classification	Reference
Quercetin	Flavanoid	Glaeser, et al., 2014
DAPI	Fluorescent dye	Yasujima, et al., 2011
Rhodamine 123	Fluorescent dye	Jouan, et al., 2014
Ethidium	Fluorescent DNA intercalating agent	Lee, et al., 2009
Paraquat	Herbicide	Chen, et al., 2007
APDA	Model cation	van Montfoort, et al., 2001
APQ	Model cation	van Montfoort, et al., 2001
ASP^+	Model cation/ fluorescent dye	Ahlin, et al., 2008
Azidoprocainamide	Model cation	van Montfoort, et al., 2001
MPP^+	Model cation	Gorboulev, et al., 1997, Grundemann, et al., 2003, Umehara, et al., 2007, Zhang, et al., 1997b
N-methylquinidine	Model cation	van Montfoort, et al., 2001
N-methylquinine	Model cation	van Montfoort, et al., 2001
TBA	Model cation	Dresser, et al., 2000
TBuMA	Model cation	van Montfoort, et al., 2001
TEA	Model cation	Bednarczyk, et al., 2003, Gorboulev, et al., 1997, Umehara, et al., 2007, Zhang, et al., 1998
TMA	Model cation	Dresser, et al., 2000
TPrA	Model cation	Dresser, et al., 2000
Aflatoxin B1	Mycotoxin	Tachampa, et al., 2008
MPTP	Neurotoxin (prodrug of MPP^+)	Lin, et al., 2010
Berberine	Plant alkaloid	Nies, et al., 2008
Berberrubine	Plant alkaloid	Li, et al., 2016
Coptisine	Plant alkaloid	Li, et al., 2016
Epiberberine	Plant alkaloid	Li, et al., 2016
Jatrorhizine	Plant alkaloid	Li, et al., 2016
Monocrotaline	Plant alkaloid	Tu, et al., 2013
Nitidine	Plant alkaloid	Li, et al., 2014b
Retrorsine	Plant alkaloid	Tu, et al., 2014

Table 1.4. Endogenous substrates of hOCT1.

Substrate	Classification	Reference
Prostaglandin E_2[a]	Eicosanoid	Harlfinger, et al., 2005, Kimura, et al., 2002
Prostaglandin F_2[a]	Eicosanoid	Harlfinger, et al., 2005, Kimura, et al., 2002
Agmatine	Metabolite	Grundemann, et al., 2003, Winter, et al., 2011
N-methylnicotinamide	Metabolite	Gorboulev, et al., 1997
Putrescine	Metabolite	Winter, et al., 2011
Spermidine	Metabolite	Sala-Rabanal, et al., 2013
Acetylcholine	Neurotransmitter	Lips, et al., 2005
Dopamine	Neurotransmitter	Bayer, et al., 2009, Boxberger, et al., 2014
Norepinephrine	Neurotransmitter	Bayer, et al., 2009, Boxberger, et al., 2014
Serotonin	Neurotransmitter	Boxberger, et al., 2014, Kerb, et al., 2002
Thiamine	Vitamin	Chen, et al., 2014

[a] Status of prostaglandins as substrates is controversial. See references.

Table 1.5. Select inhibitors of hOCT1.

Substrate	Classification	Reference
Alfuzosin	α_1-antagonist (BPH therapeutic)	Chen, et al., 2017
Acetaminophen	Analgesic	Khamdang, et al., 2002
Phenacetin	Analgesic	Khamdang, et al., 2002
Salicylamide	Analgesic	Yasujima, et al., 2011
Flecainide	Antiarrhythmic	Umehara, et al., 2008
Lidocaine	Antiarrhythmic/anesthetic	Yasujima, et al., 2011
Propafenone	Antiarrhythmic	Ahlin, et al., 2008
Chloramphenicol	Antibiotic	Yasujima, et al., 2011
Ciprofloxacin	Antibiotic	Mulgaonkar, et al., 2013
Ethambutol	Antibiotic	Pan, et al., 2013
Fleroxacin	Antibiotic	Mulgaonkar, et al., 2013
Gatifloxacin	Antibiotic	Mulgaonkar, et al., 2013
Levofloxacin	Antibiotic	Mulgaonkar, et al., 2013
Lomefloxacin	Antibiotic	Mulgaonkar, et al., 2013
Moxifloxacin	Antibiotic	Mulgaonkar, et al., 2013
Perfloxacin	Antibiotic	Mulgaonkar, et al., 2013
Prulifloxacin	Antibiotic	Mulgaonkar, et al., 2013
Rufloxacin	Antibiotic	Mulgaonkar, et al., 2013
Sparfloxacin	Antibiotic	Mulgaonkar, et al., 2013
Butylscopalomine	Anticholinergic	Muller, et al., 2005
Camylofine	Anticholinergic	Chen, et al., 2017
Orphenadrine	Anticholinergic	Ahlin, et al., 2008
Oxybutynin	Anticholinergic	Ahlin, et al., 2008
Pancuronium	Anticholinergic	Zhang, et al., 1997b
Propantheline	Anticholinergic	Yasujima, et al., 2011
Tubocurarine	Anticholinergic	Minematsu, et al., 2010
Rocuronium	Anticholinergic	van Montfoort, et al., 2001
Vecuronium	Anticholinergic	Zhang, et al., 1997b
Trihexylphenidyl	Anticholinergic (Parkinson's therapeutic)	Ahlin, et al., 2008
Clopidogrel	Anticoagulant	Li, et al., 2014a
Carbamazepine	Anticonvulsant	Yasujima, et al., 2011
Phenobarbital	Anticonvulsant	Yasujima, et al., 2011
Phenytoin	Anticonvulsant	Yasujima, et al., 2011
Primidone	Anticonvulsant	Yasujima, et al., 2011
Amitriptyline	Antidepressant	Ahlin, et al., 2008

Bupropion	Antidepressant	Haenisch, et al., 2012
Citalopram	Antidepressant	Ahlin, et al., 2008
Clomipramine	Antidepressant	Ahlin, et al., 2008
Desipramine	Antidepressant	Ahlin, et al., 2008, Zhang, et al., 1998
Doxepin	Antidepressant	Haenisch, et al., 2012
Fluoxetine	Antidepressant	Haenisch, et al., 2012
Fluvoxamine	Antidepressant	Haenisch, et al., 2012
Imipramine	Antidepressant	Ahlin, et al., 2008
Maprotiline	Antidepressant	Haenisch, et al., 2012
Mianserin	Antidepressant	Haenisch, et al., 2012
Mirtazapine	Antidepressant	Haenisch, et al., 2012
Nefazodone	Antidepressant	Haenisch, et al., 2012
Nisoxetine	Antidepressant (experimental)	Haenisch, et al., 2012
Nomifensine	Antidepressant	Haenisch, et al., 2012
Paroxetine	Antidepressant	Haenisch, et al., 2012
Reboxetine	Antidepressant	Haenisch, et al., 2012
Sertraline	Antidepressant	Haenisch, et al., 2012
Tianeptine	Antidepressant	Haenisch, et al., 2012
Trimipramine	Antidepressant	Ahlin, et al., 2008
Venlafaxine	Antidepressant	Haenisch, et al., 2012
Glyburide	Antidiabetic	Yasujima, et al., 2011
Repaglinide	Antidiabetic	Ahlin, et al., 2008, Bachmakov, et al., 2008
Rosiglitazone	Antidiabetic	Bachmakov, et al., 2008
Sitagliptin	Antidiabetic	Choi, et al., 2010
Ondansetron	Antiemetic	Ahlin, et al., 2008, Tzvetkov, et al., 2012
Clotrimazole	Antifungal	Ahlin, et al., 2008
Fluconazole	Antifungal	Yasujima, et al., 2011
Ketoconazole	Antifungal	Ahlin, et al., 2008
Clemastine	Antihistamine/ anticholinergic	Ahlin, et al., 2008
Diphenhydramine	Antihistamine	Lee, et al, 2009, Muller, et al., 2005
Loratadine	Antihistamine	Ahlin, et al., 2008
Promethazine	Antihistamine (antiemetic)	Ahlin, et al., 2008
Terfenadine	Antihistamine	Ahlin, et al., 2008
Acebutolol	Antihypertensive	Zhang, et al., 1998

Bucindolol	Antihypertensive (experimental)	Ahlin, et al., 2008
Carvedilol	Antihypertensive	Chen, et al., 2017
Clonidine	Antihypertensive	Ahlin, et al., 2008, Bednarczyk, et al., 2003, Zhang, et al., 1997b
Diltiazem	Antihypertensive/ calcium channel blocker	Ahlin, et al., 2008, Umehara, et al., 2008
Guanabenz	Antihypertensive	Chen, et al., 2017
Methoxyverapamil	Antihypertensive/ calcium channel blocker	Ahlin, et al., 2008
Metoprolol	Antihypertensive	Umehara, et al., 2008
Nitroprusside	Antihypertensive	Chen, et al., 2017
Oxprenolol	Antihypertensive	Ahlin, et al., 2008
Phenoxybenzamine	Antihypertensive	Ahlin, et al., 2008, Hayer-Zillgen, et al., 2002
Prazosin	Antihypertensive	Ahlin, et al., 2008, Hayer-Zillgen, et al., 2002
Propranolol	Antihypertensive	Ahlin, et al., 2008
Terazosin	Antihypertensive	Ahlin, et al., 2008
Verapamil	Antihypertensive/ calcium channel blocker	Ahlin, et al., 2008, Zhang, et al., 1997b
Cochicine	Anti-inflammatory	Yasujima, et al., 2011
Sulfasalazine	Anti-inflammatory	Yasujima, et al., 2011
Pyrimethamine	Antimalarial	Chen, et al., 2017
Quinine	Antimalarial	Ahlin, et al., 2008, Zhang, et al., 1997b
Afatinib	Antineoplastic	Johnston, et al., 2014
Amsacrine	Antineoplastic	Ahlin, et al., 2008
Bosutinib	Antineoplastic	Johnston, et al., 2014
Cediranib	Antineoplastic	Johnston, et al., 2014
Cepharanthine	Antineoplastic	Moss, et al., 2015
Dasatinib	Antineoplastic	Minematsu and Giacomini, 2011
Erlotinib	Antineoplastic	Minematsu and Giacomini, 2011
Etoposide	Antineoplastic	Gupta, et al., 2012
Foretinib	Antineoplastic	Johnston, et al., 2014
Gefitinib	Antineoplastic	Minematsu and Giacomini, 2011
Ifosfamide	Antineoplastic	Gupta, et al., 2012
Lapatinib	Antineoplastic	Minematsu and Giacomini, 2011
Mitaxantrone	Antineoplastic	Gupta, et al., 2012
Neratinib	Antineoplastic	Johnston, et al., 2014

Nilotinib	Antineoplastic	Minematsu and Giacomini, 2011
Pelitinib	Antineoplastic	Johnston, et al., 2014
Sorafenib	Antineoplastic	Minematsu and Giacomini, 2011
Sunitinib	Antineoplastic	Minematsu and Giacomini, 2011
Tamoxifen	Antineoplastic	Ahlin, et al., 2008
Vandetanib	Antineoplastic	Johnston, et al., 2014
Vinblastine	Antineoplastic	Moaddel, et al., 2005b
Bithionol	Antiparasitic	Chen, et al., 2017
Closantel	Antiparasitic	Chen, et al., 2017
Dichlorophen	Antiparasitic	Chen, et al., 2017
Chlorpromazine	Antipsychotic	Ahlin, et al., 2008, Bednarczyk, et al., 2003
Chlorprothixene	Antipsychotic	Ahlin, et al., 2008
Clozapine	Antipsychotic	Haenisch, et al., 2012
Flupentixol	Antipsychotic	Ahlin, et al., 2008
Fluphenazine	Antipsychotic	Ahlin, et al., 2008
Haloperidol	Antipsychotic	Ahlin, et al., 2008
Levomepromazine	Antipsychotic	Haenisch, et al., 2012
Olanzapine	Antipsychotic	Haenisch, et al., 2012
Perazine	Antipsychotic	Haenisch, et al., 2012
Prochlorperazine	Antipsychotic	Ahlin, et al., 2008
Promazine	Antipsychotic	Ahlin, et al., 2008
Quetiapine	Antipsychotic	Dickens, et al., 2012, Haenisch, et al., 2012
Remoxipride	Antipsychotic	Haenisch, et al., 2012
Risperidone	Antipsychotic	Haenisch, et al., 2012
Sertindole	Antipsychotic	Haenisch, et al., 2012
Spiperone	Antipsychotic	Haenisch, et al., 2012
Thioridazine	Antipsychotic	Haenisch, et al., 2012
Zotepine	Antipsychotic	Haenisch, et al., 2012
Zuclopenthixol	Antipsychotic	Haenisch, et al., 2012
Carbapentane	Antitussive	Chen, et al., 2017
Cloperastine	Antitussive	Chen, et al., 2017
Dextromethorphan	Antitussive	Chen, et al., 2017
Abacavir	Antiviral	Minuesa, et al., 2009
Amprenavir	Antiviral	Moss, et al., 2015
Azidothymidine	Antiviral	Minuesa, et al., 2009
Darunavir	Antiviral	Moss, et al., 2015
Emtricitabine	Antiviral	Minuesa, et al., 2009

Etravirine	Antiviral	Moss, et al., 2015
Indinavir	Antiviral	Jung, et al., 2008, Zhang, et al., 2000
Lopinavir	Antiviral	Moss, et al., 2015
Nelfinavir	Antiviral	Jung, et al., 2008, Zhang, et al., 2000
Nevirapine	Antiviral	Moss, et al., 2015
Rilpivierine	Antiviral	Moss, et al., 2015
Ritonavir	Antiviral	Jung, et al., 2008, Zhang, et al., 2000
Saquinavir	Antiviral	Jung, et al., 2008, Zhang, et al., 2000
Stavudine	Antiviral	Moss, et al., 2015
Tenofovir	Antiviral	Minuesa, et al., 2009
Denopamine	β_1-agonist	Ahlin, et al., 2008
Dobutamine	β_1-agonist	Chen, et al., 2017
Etilefrine	β_1-agonist	Muller, et al., 2005
Salmeterol	β_2-agonist/ bronchodilator	Salomon, et al., 2015
Isoproterenol	β-agonist (non-selective)	Moaddel, et al., 2007
Midazolam	Benzodiazepine	Zhang, et al., 1998
Taurocholate	Bile acid	Zhang, et al., 1997b
Histamine	Biogenic amine	Bednarczyk, et al., 2003
Ouabain	Cardiac glycoside	Yasujima, et al., 2011
Tacrine	Cholinesterase inhibitor	Chen, et al., 2017
Hemicholinium-3	Choline transporter inhibitor	Yasujima, et al., 2011
Spironolactone	Diuretic	Ahlin, et al., 2008
Apomorphine	Dopaminergic (Parkinson's therapeutic)	Ahlin, et al., 2008
Bromosulfophthalein	Dye	Yasujima, et al., 2011
Crystal violet	Dye	Bednarczyk, et al., 2003
EGCG	Flavonoid	Knop, et al., 2015
Kaempferol	Flavonoid	Yasujima, et al., 2011
Naringenin	Flavonoid	Yasujima, et al., 2011
Phlorizin	Flavonoid	Yasujima, et al., 2011
Rutin	Flavonoid	Yasujima, et al., 2011
Azathioprine	Immunosuppressant	Yasujima, et al., 2011
Creatinine	Metabolite	Zhang, et al., 1998
Guanidine	Metabolite	Zhang, et al., 1997b
Guanidinosuccinic acid	Metabolite	Kimura, et al., 2009
Guanidinovaleric acid	Metabolite	Kimura, et al., 2009

Methylguanidine	Metabolite	Kimura, et al., 2009
Tyramine	Metabolite	Bednarczyk, et al., 2003
Cyclohexylamine	Model cation	Bednarczyk, et al., 2003
Decynium-22	Model cation	Hayer-Zillgen, et al., 2002, Zhang, et al., 1997b
THA	Model cation	Zhang, et al., 1999
TPeA	Model cation	Bednarczyk, et al., 2003, Zhang, et al., 1998
Citreoviridin	Mycotoxin	Tachampa, et al., 2008
Gliotoxin	Mycotoxin	Tachampa, et al., 2008
Rubratoxin B	Mycotoxin	Tachampa, et al., 2008
Zearalenone	Mycotoxin	Tachampa, et al., 2008
α-Zearalenol	Mycotoxin metabolite	Tachampa, et al., 2008
Amantadine	NMDA receptor antagonist/ dopaminergic (Parkinson's therapeutic)	Amphoux, et al., 2006, Bednarczyk, et al., 2003, Zhang, et al., 1998
Dizocilpine	NMDA receptor antagonist	Amphoux, et al., 2006
Ethopropazine	NMDA receptor antagonist	Chen, et al., 2017
Ketamine	NMDA receptor antagonist (dissociative anesthetic)	Amphoux, et al., 2006
Memantine	NMDA receptor antagonist (Alzheimer's therapeutic)	Ahlin, et al., 2008
Phencyclidine	NMDA receptor antagonist (dissociative anesthetic)	Amphoux, et al., 2006
Acetylsalicylic acid	NSAID	Khamdang, et al., 2002
Diclofenac	NSAID	Khamdang, et al., 2002
Ibuprofen	NSAID	Khamdang, et al., 2002
Indomethacin	NSAID	Khamdang, et al., 2002
Ketoprofen	NSAID	Khamdang, et al., 2002
Mefenamic acid	NSAID	Khamdang, et al., 2002
Naproxen	NSAID	Khamdang, et al., 2002
Piroxicam	NSAID	Khamdang, et al., 2002
Sulindac	NSAID	Khamdang, et al., 2002
Inosine	Nucleoside	Zhang, et al., 1997b
Thymidine	Nucleoside	Zhang, et al., 1997b
Uridine	Nucleoside	Yasujima, et al., 2011
Nitrobenzylthioinosine	Nucleoside analogue	Yasujima, et al., 2011
Fentanyl	Opioid	Ahlin, et al., 2008
Tramadol	Opioid	Ahlin, et al., 2008, Tzvetkov, et al., 2011

Loperamide	Opioid/antidiarrheal	Ahlin, et al., 2008
Corydaline	Plant alkaloid	Li, et al., 2016
Palmatine	Plant alkaloid	Li, et al., 2016
Tetrahydropalmitine	Plant alkaloid	Tu, et al., 2014
Gugglesterone	Phytosteroid	Ahlin, et al., 2008
Lansoprazole	Proton pump inhibitor	Nies, et al., 2011a
Omeprazole	Proton pump inhibitor	Nies, et al., 2011a
Pantoprazole	Proton pump inhibitor	Nies, et al., 2011a
Rabeprazole	Proton pump inhibitor	Nies, et al., 2011a
Tenatoprazole	Proton pump inhibitor	Nies, et al., 2011a
6-Fluorodopamine	Radiopharmaceutical	Bayer, et al., 2009
Androstenedione	Steroid	Ahlin, et al., 2008
β-estradiol	Steroid	Ahlin, et al., 2008, Hayer-Zillgen, et al., 2002
Beclometasone	Steroid	Lips, et al., 2005
Budesonide	Steroid	Lips, et al., 2005
Corticosterone	Steroid	Hayer-Zillgen, et al., 2002, Zhang, et al., 1998
Cortisone	Steroid	Yasujima, et al., 2011
Cyproterone	Steroid/ androgen antagonist	Ahlin, et al., 2008
Estrone-3-sulfate	Steroid	Yasujima, et al., 2011
Hydrocortisone	Steroid	Yasujima, et al., 2011
Nandrolone	Steroid	Ahlin, et al., 2008
Prednisolone	Steroid	Yasujima, et al., 2011
Progesterone	Steroid	Ahlin, et al., 2008, Hayer-Zillgen, et al., 2002
2-methoxyestradiol	Steroid metabolite/ experimental antineoplastic	Ahlin, et al., 2008
Cocaine	Stimulant	Amphoux, et al., 2006
Dextroamphetamine	Stimulant	Amphoux, et al., 2006
MDMA	Stimulant/hallucinogen	Amphoux, et al., 2006
Nicotine	Stimulant	Bednarczyk, et al., 2003, Zhang, et al., 1998
Pseudoephedrine	Stimulant	Moaddel, et al., 2005a
Papaverine	Vasodilator/antispasmodic	Ahlin, et al., 2008
Choline	Vitamin	Zhang, et al., 1997b
Pyridoxine	Vitamin	Yasujima, et al., 2011

and inhibitors have been identified. Structures containing bulkier hydrophobic regions tend to be better ligands for hOCT1 than planar molecules. Additionally, hOCT1 ligands generally have fewer hydrogen-bond donors and are less polar than non-interacting structures.

Perhaps unsurprisingly, ligand structure influences the mechanism of interaction with hOCT1. After analyzing hundreds of ligand interactions with hOCT1, both *in silico* and *in vitro*, it was determined that noncompetitive inhibitors were significantly larger and more hydrophobic than competitive ligands (Chen, et al., 2017), suggesting that these two groups bind to different sites on the transporter. This corroborates the hypothesis discussed in the previous section of multiple binding sites or a large binding "pocket" within the transporter. The identification of multiple, high- and low-affinity binding sites for various ligands (Gorbunov, et al., 2008, Minuesa, et al., 2009), both substrates and inhibitors, further supports this hypothesis.

1.3.7 Clinical Significance and Polymorphisms of hOCT1

In 2010, the International Transporter Consortium, a group of transporter experts from across academia, government, and industry, published a white paper discussing the clinical importance of several drug transporters (International Transporter, et al., 2010). According to this publication, hOCT1 is included in a group of transporters deemed key in drug disposition and response. Several publications have identified polymorphisms (summarized in Table 1.6) which affect hOCT1 function, and emphasized the clinical implications of hOCT1 transport of several drugs, especially the antidiabetic drug metformin.

Table 1.6. Select hOCT1 variants and their functional consequences (increased function [↑], decreased function [↓], or no change [-]).

Amino Acid Position	Mutation	Functional Consequence	Reference
14	Ser→Phe	↑/↓	Shu, et al., 2003, Shu, et al., 2007
61	Arg→Cys	↓ (due to expression)	Kerb,et al., 2002, Shu, et al., 2003, Shu, et al., 2007
88	Cys→Arg	↓[a]	Kerb, et al., 2002
97	Glu→Lys	↓	Chen, et al., 2010
117	Pro→Leu	↓	Chen, et al., 2010
189	Ser→Leu	−/↓	Shu, et al., 2003, Shu, et al., 2007
206	Arg→Cys	↓ (due to expression)	Chen, et al., 2010
220	Gly→Val	↓	Shu, et al., 2003, Shu, et al., 2007
283	Pro→Leu	↓	Sakata, et al., 2004, Takeuchi, et al., 2003
287	Arg→Gly	↓	Sakata, et al., 2004, Takeuchi, et al., 2003
341	Pro→Leu	↓	Shu, et al., 2003, Takeuchi, et al., 2003
401	Gly→Ser	↓	Kerb, et al., 2002, Sakata, et al., 2004, Shu, et al., 2003, Shu, et al., 2007
408	Met→Val	−	Shu, et al., 2007
420	Deletion	−/↓	Kerb, et al., 2002, Shu, et al., 2003, Shu, et al., 2007
465	Gly→Arg	↓ (due to expression)	Shu, et al., 2003, Shu, et al., 2007

[a] Surface expression of C88R was not examined, but the observed decrease in function is likely due to decreased expression of the transporter due to the location of the mutation in the ECL. ECL cysteines have been determined to be critical for proper insertion of hOCT1 into the membrane (Sturm, et al., 2007, Keller, et al., 2011, Arimany-Nardi, et al., 2016).

In 2002, Kerb, et al., (2002) identified 25 single nucleotide polymorphisms (SNPs) in hOCT1, five of which resulted in amino acid changes: R61C, C88R, F160L, G401S, and M420del. The R61C, C88R, and G401S mutants demonstrated significantly decreased transport capacity. Furthermore, C88R and G401S exhibited significant changes in substrate specificity. A later study identified these variants and others in an ethnically diverse population sample (Leabman, et al., 2003), and subsequent characterization of the new variants identified two amino acid substitutions which yielded decreased hOCT1 function (G220V and P341L), and one variant, S14P, which exhibited increased transport of MPP^+ (Shu, et al., 2003). Interestingly, mutants which demonstrated no functional change or increased transport of MPP^+, including S14F, S189L, and M420del, showed significantly decreased uptake of metformin (Shu, et al., 2007). Furthermore, several reduced function variants led to decreased activation of AMPK, a putative target for metformin, after metformin treatment (Chen, et al., 2010, Shu, et al., 2007).

In addition to biochemical data demonstrating hOCT1 variant roles in metformin action, several hOCT1 polymorphisms appear to be clinically relevant. Shu, et al., (2008) demonstrated that metformin pharmacokinetics were altered in individuals carrying the reduced function hOCT1 alleles R61C, G401S, M420del, and G465R. These alleles resulted in higher metformin AUC, higher C_{max}, and lower oral volume of distribution, all of which are consistent with these variants' significantly decreased metformin transport. Patients expressing these variants also demonstrated a reduced glucose-lowering effect following treatment with metformin (Shu, et al., 2008), suggesting that hOCT1 polymorphisms which demonstrate decreased transport function

affect metformin efficacy. Furthermore, hOCT1 polymorphisms are associated with adverse drug events in metformin users. The M408V (Tarasova, et al., 2012), R61C, and M420del (Dujic, et al., 2016) SNPs are all associated with unfavorable gastrointestinal side effects of oral metformin therapy.

An intronic hOCT1 polymorphism is also implicated in metformin action. The intronic SNP rs622342 A>C is seen in several ethnically diverse populations, and is associated with a decrease in metformin efficacy, as determined by a decrease in HbA1c lowering (Becker, et al., 2009, 2010, Du Plessis, et al., 2015, Ohishi, et al., 2014, Umamaheswaran, et al., 2015). This polymorphism is also associated with increased dosage and lower response rates to the Parkinson's drug levodopa (Becker, et al., 2011). Becker, et al. also demonstrated a 1.5-fold increase in mortality ratio after starting levodopa in Parkinson's patients with one or two copies of the minor allele. While these studies demonstrated a putative role for this intronic SNP in the efficacy of metformin and levodopa, the mechanism(s) are yet unknown.

Though hOCT1 is, perhaps, most clinically implicated in metformin disposition and efficacy, it is also recognized in the clinical outcomes of certain chemotherapies. hOCT1 expression and genetic polymorphisms are associated with imatinib response and resistance in chronic myeloid leukemia patients (Bazeos, et al., 2010, Crossman, et al., 2005, Giannoudis, et al., 2013, Kim, et al., 2009, Marin, et al., 2010, Wang, et al., 2008, White, et al., 2007, White, et al., 2010), though this association is somewhat controversial (Hu, et al., 2008, Nies, et al., 2014, Zhang, et al., 2009). Furthermore, hOCT1 genetic variants demonstrated decreased sorafenib uptake in hepatocellular

carcinoma and cholangiocarcinoma, due to both altered expression and function, leading to chemoresistance (Herraez, et al., 2013).

The clinical relevance of hOCT1 has been established for various other drugs as well. Several groups have shown that the pharmacokinetics of the opioid analgesics morphine and tramadol are dependent on hOCT1 genotype (Tzvetkov, et al., 2011, Tzvetkov, et al., 2013, Venkatasubramanian, et al., 2014). In addition, plasma drug concentrations and efficacy of the antiemetic drugs ondansetron and tropisetron are reduced by loss-of-function hOCT1 polymorphisms (Tzvetkov, et al., 2012). With the wide array of clinically-relevant drugs that have been established as hOCT1 substrates, it is likely that further studies will identify other medications whose pharmacokinetics and/or pharmacological response are varied due to hOCT1 polymorphisms. Altered drug pharmacokinetics can lead to decreased drug efficacy and potentially increased toxicity, and therefore hOCT1 variants are an important consideration in the preclinical and clinical realm during drug development.

1.4 TRANSPORTER-MEDIATED ADVERSE DRUG INTERACTIONS

Polymorphisms aside, adverse drug interactions, like drug-drug interactions (DDIs), are another key factor in altered pharmacokinetics and drug response. DDIs occur when the ADME properties of a drug are altered because of co-administration of another drug. With the prominent practice of polypharmacy, or the simultaneous use of multiple drugs by one patient for one or more conditions, in the US today, DDIs are becoming increasingly common, especially in the geriatric population. It is estimated

that nearly 40% of the population age 65 and older take five or more medications concurrently (Maher, et al., 2014). Furthermore, an estimated 10% of emergency department visits are attributed to adverse drug events (ADEs), including DDIs (Maher, et al., 2014). The effects of DDIs include lowered drug efficacy, as a result of decreased absorption, and perhaps more importantly, increased drug-induced toxicities resulting from decreased metabolism or excretion. For these reasons, DDIs are studied extensively, particularly in preclinical studies for investigational new drugs (INDs) seeking FDA approval.

There is a growing appreciation for the role of drug transporters in DDIs. Many clinically relevant DDIs have been described for drug transporters in both the *ABC* and *SLC* transporter families, including P-gp, BCRP, OCTs, OATs, and OATPs (International Transporter, et al., 2010). As such, the International Transporter Consortium has published a series of papers outlining recommendations for regulatory agencies regarding transporter-mediated DDIs (Hillgren, et al., 2013, International Transporter, et al., 2010).

1.4.1 hOCT1-Mediated Drug-Drug Interactions

While the US Food and Drug Administration (FDA) does not currently include hOCT1 in their guidelines for preclinical studies (U.S. Food and Drug Administration, 2017), the European Medicines Agency (EMA) recommends *in vitro* inhibition studies against hOCT1 for investigational drugs in its Guideline on the Investigation of Drug Interactions (European Medicines Agency, 2012). To date, multiple hOCT1 mediated-DDIs have been identified.

Perhaps the most relevant DDIs mediated by hOCT1 involve metformin. Administration of the calcium channel blocker verapamil, a potent inhibitor of hOCT1, reduces patient response to metformin, presumably through reducing hepatic drug levels (Cho, et al., 2014). Various *in vitro* interactions with metformin have been identified as well. Metformin uptake has been shown to be strongly inhibited by several tyrosine kinase inhibitors (Minematsu and Giacomini, 2011), the antiarrhythmic drug quinidine (Umehara, et al., 2008), and other antidiabetic drugs commonly taken in combination with metformin, repaglinide, rosiglitazone (Bachmakov, et al., 2008), and sitagliptin (Choi, et al., 2010). With metformin being co-administered with other antidiabetic agents or drugs used for treating comorbidities, efficacy can be decreased, as described above in the verapamil study. In addition, certain toxicities associated with metformin action are possible.

Antiretroviral drugs employed in HAART therapy for HIV patients comprise another set of drugs which can lead to hOCT1-mediated DDIs. Studies *in vitro* have identified several antiretrovirals, including abacavir, azidothymidine, indinavir, nelfinavir, ritonavir, saquinavir, and zalcitabine, as hOCT1 inhibitors (Jung, et al., 2008, Minuesa, et al., 2009, Zhang, et al., 2000). Furthermore, the NRTI lamivudine was identified as a hOCT1 substrate, and all of the antiretrovirals tested potently inhibited lamivudine transport by hOCT1 (Jung, et al., 2008, Minuesa, et al., 2009). Lamivudine is frequently included in HAART regimens in combination with several other antiretrovirals, including those listed above. Therefore, combination therapy may lead to a decrease in lamivudine efficacy and potentially adverse side effects.

The prevalence of transporter-mediated DDIs is a strong impetus for identification of new DDIs through low-cost and high-throughput drug screens. Several groups, therefore, have designed *in silico* and *in vitro* high-throughput screening (HTS) methods to speed the process of identifying putative DDIs mediated by hOCT1 (Ahlin, et al., 2008, Chen, et al., 2017). *In vitro* studies involved high-throughput inhibition testing in homologous expression systems employing a probe substrate. *In silico* studies included modeling physicochemical properties and structure-activity relationships of known inhibitors (Ahlin, et al., 2008), and generation of a hOCT1 homology model with subsequent docking of inhibitors and substrates (Chen, et al., 2017). These screens identified hundreds of new interactions, and even delineated competitive versus noncompetitive inhibitors (Chen, et al., 2017). However, care must be taken in the conclusions drawn from these studies as both the modeling and HTS methods have limitations, as discussed in their respective reports. Further characterization of these putative DDIs is necessary.

1.5 SUBSTRATE-DEPENDENT EFFECTS OF DRUG TRANSPORTERS

The push to elucidate novel DDIs has presented a new challenge for the transporter field. As discussed above, many investigators have turned to high-throughput screening to speed the process of DDI identification. While HTS can be a valuable tool in identifying new interactions, there are disadvantages associated with these assays. High-throughput screens typically utilize only one probe substrate at a single concentration. However, there is growing evidence that transporter interactions

are substrate-dependent, meaning, the effects of ligand interactions (i.e. inhibition, stimulation) are conditional, subject to the substrate employed. In the realm of DDI identification, substrate-dependent modulation may lead to false-negatives, or missed interactions, which may have important implications in drug development.

Substrate-dependent interactions exhibited by drug transporters were first described for efflux pumps of the *ABC* transporter family, including P-gp, BCRP, and some MRPs (Bodo, et al., 2003, Giri, et al., 2009, Honda, et al., 2004). Shortly after, several OATPs, members of the *SLC* transporter superfamily, were implicated in substrate-dependent interactions as well. Noé, et al., (2007) demonstrated that OATP1B1-mediated transport of several statins and taurocholate was strongly inhibited by gemfibrozil, while uptake of troglitazone sulfate was not affected. Interestingly, the high-affinity component of estrone-3-sulfate (E3S) transport was inhibitable by gemfibrozil, but the low-affinity site was not. Similar substrate-dependent modulation has been observed for the other liver-specific OATP, 1B3. The antifungal drug, clotrimazole, strongly inhibited OATP1B3-mediated uptake of Fluo-3, had no effect on E3S transport, and interestingly, stimulated uptake of E17βG (Gui, et al., 2008). Conversely, the green tea catechin, EGCG, inhibited Fluo-3 uptake, exerted no effect on E17βG, but markedly stimulated E3S transport, while its close relative, ECG, inhibited all three substrates to varying degrees (Roth, et al., 2011). These differential effects were mirrored by multiple quercetin derivatives when tested against substrates E3S, E17βG, and DHEAS (Zhang, et al., 2013).

Substrate-dependent effects have been described for other *SLC* drug transporters, including MATEs, which are arguably more closely related to hOCT1.

Martínez-Guerrero and Wright (2013) compared the inhibitor potency of three different cationic liquids against three separate MATE substrates. For MATE1, IC_{50} values for Bmim and BmPy were significantly higher when TEMA and NBD-MTMA were employed as substrates versus those when MPP^+ was used. This inhibitor profile was shared with MATE2-K as well, though NBD-MTMA was not tested as a substrate (Martínez-Guerrero and Wright, 2013). Similarly for MATE1 and MATE2-K, inhibitor IC_{50} values were comparable when screened against metformin, thiamine, and MPP^+, but up to ten-fold higher with rhodamine 123 (Lechner, et al., 2016).

Members of the organic cation transporter subfamily, most commonly hOCT2, have exhibited substrate-dependent modulation. Several structurally diverse cationic drugs were markedly more potent inhibitors of hOCT2-mediated metformin transport than of MPP^+ transport (Zolk, et al., 2009). In addition, hOCT2 exhibited widely varied IC_{50} values for multiple drugs when tested against model substrates MPP^+ and NBD-MTMA (Belzer, et al., 2013). Furthermore, Hacker, et al., (2015) compared the inhibitory effect of numerous hOCT2 ligands against probe substrates MPP^+, ASP^+, and metformin. While they identified several paninhibitors, or ligands which inhibited all three substrates, they also discerned selective inhibitors which affected transport of only one or two substrates. While these data further validate the existence of substrate-dependent interactions, they also demonstrate that not every ligand exhibits substrate-dependent effects.

To date, no direct studies of substrate-dependent effects have been published for hOCT1. However, myriad reports in the literature have established comparatively different inhibitor interactions depending on the probe substrate employed, as discussed

by Nies, et al., (2011b). Due to deviations in transport assays developed by different groups, results can't be directly compared between researchers; thus, there is a need for identification and characterization of putative substrate-dependent effects for hOCT1.

1.6 SPECIFIC AIMS OF THIS DISSERTATION

Adverse drug interactions are an increasingly common consequence of the practice of polypharmacy in treating comorbidities. There is growing appreciation for the role of drug transporters in drug-drug interactions (DDIs). Drug transporters are expressed in organs key in drug disposition and are known to interact with a wide variety of structurally dissimilar compounds (International Transporter, et al., 2010). Due to these inherent characteristics, DDIs frequently occur at the transporter level, and as such, regulatory guidelines include recommendations for screening investigational new drugs for transporter-mediated DDIs (European Medicines Agency, 2012, U.S. Food and Drug Administration, 2017).

The human organic cation transporter 1 (hOCT1) mediates the uptake of several cationic and uncharged drugs, including the antidiabetic agent metformin. Indeed, hOCT1 is critical in metformin pharmacokinetics and efficacy. Furthermore, several hOCT1-mediated DDIs are implicated in a loss of metformin action and increase in certain metformin-associated adverse drug reactions.

There is currently limited structural information available for hOCT1, therefore, little is known about the mechanism of ligand interaction and substrate translocation.

Without a comprehensive understanding of these mechanisms, it will remain challenging to accurately predict hOCT1-mediated DDIs. Prediction of DDIs is a critical first step in their prevention. Therefore, the *long term goal* of the studies presented in this dissertation is to improve methods for accurate prediction of DDIs. The *objective* of this dissertation was to address the lack of understanding, on multiple levels, of the mechanisms through which adverse drug interactions occur via hOCT1.

Several studies have demonstrated that hOCT1 is capable of transporting endogenous substrates, though little has been done to characterize the effects of endogenous substrate-drug interactions (Kerb, et al., 2002, Kimura, et al., 2002, Lips, et al., 2005). In addition, recent studies have suggested that the mechanisms of ligand interactions of close relatives of hOCT1 are substrate-dependent (Belzer, et al., 2013, Martínez-Guerrero and Wright, 2013), further complicating DDI prediction. The *central hypothesis* of this dissertation is that both endogenous and xenobiotic compounds modulate the activity of hOCT1 in a substrate-dependent manner through interaction with specific ligand-binding domains within the transporter. Therefore, a multifaceted approach was necessary to comprehend the mechanisms of ligand interactions with hOCT1. To elucidate structural components key in ligand interactions and determine the functional consequences, the following specific aims were employed to test this hypothesis:

1.6.1 Specific Aim 1: Investigate the effect of xenobiotics on endogenous substrate transport by hOCT1

Much, if not all of the focus of studies regarding adverse drug interactions of hOCT1 are dedicated to DDIs, but little attention has been paid to endogenous compound-drug interactions. Monoamine neurotransmitters are substrates for rOCT1, but have not been confirmed for hOCT1. In addition, little work has been done to characterize inhibitory effects of drugs on monoamine transport. The *working hypothesis* was that hOCT1 mediates the uptake of monoamine neurotransmitters in the liver. Furthermore, uptake of these monoamines, which rely on entry into hepatocytes for metabolism, can be inhibited by commonly-prescribed drugs. The uptake of monoamine neurotransmitters dopamine, norepinephrine, and serotonin by hOCT1 was measured. Inhibition of serotonin transport by various drugs was tested and characterized in a homologous overexpression system and in primary human hepatocytes.

1.6.2 Specific Aim 2: Identify and characterize substrate-dependent interactions with hOCT1

The *working hypothesis* of this aim was that the mechanisms of ligand interactions with hOCT1 were dependent upon the probe substrate employed. Multiple combinations of hOCT1 drug substrates and inhibitors were screened using *in vitro* transporter assays to identify substrate-dependent interactions. The mechanisms of these interactions were explored further using *in silico* docking and structure-activity relationship modeling.

1.6.3 Specific Aim 3: Examine the role of the extracellular loop domain of hOCT1 in substrate affinity and translocation

The extracellular loop (ECL) domain of OCT1 homologs is critical to substrate recognition and binding, and initiation of translocation. While there exists an overlap in substrate specificities between rat and human OCT1, significant differences in substrate affinities exist between orthologs. The *working hypothesis* was that the ECL domain is important in determining substrate affinity and is the driving force behind the observed species differences. To examine this, attempts were made to generate chimeric transporters containing the transmembrane domains of hOCT1 but the ECL of rOCT1, and vice versa. The goal of this study was to characterize the kinetics of transport for several substrates and compare parameters between generated chimeras and wild-type transporters to elucidate binding regions within the transporters.

CHAPTER 2 : COMMON DRUGS INHIBIT HUMAN OCT1-MEDIATED NEUROTRANSMITTER UPTAKE

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2.1 INTRODUCTION

It is widely accepted that the liver plays an essential role in removing drugs, toxins and other xenobiotics from circulation in the human body. The liver is also involved in the clearance of several endogenous compounds, including circulating monoamine neurotransmitters (Chu et al., 1999, Eisenhofer et al., 2004). As early as 1967, it was demonstrated that the liver is capable of removing more than 70% of the serotonin in portal blood by filtration and metabolism (Thomas and Vane, 1967, Tyce, 1990). Several endogenous compounds, particularly the monoamines, are positively charged at physiologic pH, and therefore require transport proteins to facilitate crossing the plasma membrane into hepatocytes. However, the high-affinity dopamine (DAT), norepinephrine (NET), and serotonin (SERT) transporters are not expressed in the liver (Eisenhofer, 2001, Ramamoorthy et al., 1993), leaving open to question the transporter(s) responsible for monoamine clearance in the human liver.

Organic cation transporters (OCTs), a subset of the *SLC22* superfamily of transporters, are polyspecific transporters that mediate the uptake of a wide variety of positively- and neutrally- charged compounds (Koepsell, 2013). Broad substrate specificity combined with tissue localization, primarily in detoxifying organs, has recently coupled OCTs to the elimination of several drugs and toxins as well as endogenous compounds (Koepsell et al., 2007, Nies et al., 2011). Expressed primarily in the liver, hOCT1 is involved in the hepatic elimination of numerous small molecules, and has been linked to the transport of biogenic amines. Previously, the rat organic cation transporter, rOCT1, was shown to transport catecholamines (Breidert et al., 1998, Busch et al., 1996b, Jonker and Schinkel, 2004). Additionally, human OCT1 has been

associated with neurotransmitter transport, however there exists some controversy in the literature as to substrate specificity and transport efficiency (Amphoux et al., 2006, Kerb et al., 2002, Lips et al., 2005).

Moreover, the interference of drugs with endogenous neurotransmitter clearance, particularly at the transporter level, has not been investigated. While it is becoming increasingly necessary to identify transporter-mediated drug-drug interactions in the modern age of polypharmacy, little is currently known about the effects that therapeutics have on transport and elimination of endogenous substrates. To elucidate interactions of common medications and other xenobiotics with endogenous substrates of human OCT1, transport and inhibition of the biogenic amines dopamine, norepinephrine, and serotonin were characterized in both Human Embryonic Kidney 293 (HEK293) cells and primary human hepatocytes.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Radiolabeled [^{3}H]-1-methyl-4-phenylpyridinium iodide (MPP^+, 85.0 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Radiolabeled [^{3}H]-dihydroxyphenylethylamine (dopamine, 46.0 Ci/mmol), [^{3}H]-norepinephrine hydrochloride (14.9 Ci/mmol), and [^{3}H]-hydroxytryptamine creatinine sulfate (serotonin, 28.3 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). NaCl was purchased from Amresco (Solon, OH). HEPES sodium salt and KCl were purchased from Fisher Scientific (Fair Lawn, NJ). Imatinib (Gleevec) was purchased from Toronto Research

Chemicals (North York, ON, Canada). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

2.2.2 Cell Culture

HEK293 cells were grown at 37ºC under humidified 5% CO_2 in Dulbeccos's Modified Eagle Medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were seeded on 24-well plates coated with 0.1 mg/mL poly-D-lysine (Invitrogen) at a density of 175,000 cells/well. Twenty-four hours after plating, cells were transiently transfected with pcDNA5/FRT ("empty vector", Invitrogen) or pcDNA5/FRT-hOCT1 ("OCT1") using the FuGENE HD transfection reagent (Promega, Madison, WI). Transfection mixtures contained 0.25 µg plasmid cDNA, 0.75 µL FuGENE HD, and Opti-MEM I + GlutaMAX–I (Invitrogen) to a final volume of 25 µL per well. Transfected cells were incubated at 37ºC as above for 24 hours before use. Freshly isolated human hepatocytes seeded on collagen-coated 24-well plates at a density of 350,000 cells/well were obtained from the KUMC Department of Pharmacology, Toxicology and Therapeutics Cell Isolation Core lab. Hepatocytes were isolated from livers of male and female patients, with ages ranging from 30 to 57.

2.2.3 Transport Assays

HEK293 uptake assays were performed 24 hours post-transfection at 37ºC. Media were aspirated, and cells washed three times with warm (37ºC) uptake buffer (116 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose, and 20 mM HEPES sodium salt, pH 7.4). After washing, cells were incubated with 200 µL

uptake buffer containing radiolabeled substrate and sufficient unlabeled substrate to reach the specified substrate concentration, as well as putative inhibitors (drugs) where indicated, for the specified amount of time. Transport for all substrates was measured within the initial linear time range (at 30 seconds for MPP^+, and at five minutes for all other substrates). Uptake was terminated by washing four times with ice-cold uptake buffer. To quantify uptake, cells were lysed with 300 µL/well 1% TX-100 in PBS, of which 200 µL were transferred to 24-well scintillation plates (Perkin Elmer, Waltham, MA) and mixed with 750 µL Optiphase Supermix scintillation cocktail (Perkin Elmer). Radioactivity was measured using a MicroBeta Trilux liquid scintillation counter (Perkin Elmer). The remaining cell lysate was used for protein determination for normalization by BCA Protein Assay with bovine serum albumin standards (Pierce). Transporter-specific uptake (net uptake) was determined by subtracting uptake into empty vector cells from the uptake into hOCT1-expressing cells. Each data point represents the average of three independent experiments, in which each condition was performed in triplicate. Transport assays were performed with hepatocytes between 20 and 24 hours after plating according to the method described above for HEK293 cells, using uptake buffer modified from Jigorel et al., (2005) (136 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM D-glucose, 10 mM HEPES, pH 7.4). Each data point represents the average of three independent experiments, in which each condition was performed in duplicate.

2.2.4 Western Blotting

Cultured cells and hepatocytes were lysed in hypotonic homogenization solution (1mM NaCl, 5mM Tris-HCl, pH 7.5) with protease inhibitor cocktail (Roche, Indianapolis,

IN) using a tissue homogenizer on ice. Lysates were subjected to centrifugation at 900 *g* for 10 minutes, after which supernatant was collected and subjected to further centrifugation at 10,000 *g* for 20 min. The resulting pellets which contained protein-enriched plasma membrane were resuspended in hypotonic homogenization solution including protease inhibitor, and protein concentration was determined by BCA protein assay (Pierce, Rockford, IL). Proteins (0.5 µg for HEK lysates, 50 µg for hepatocyte lysates) were resolved by SDS-PAGE on 4-15% Mini-PROTEAN® TGX™ polyacrylamide gels (Bio-Rad, Hercules, CA). Subsequently, proteins were transferred to nitrocellulose membrane. Immunoblotting was performed using standard procedures, with anti-OCT1 primary antibody (Novus Biologicals, Littleton, CO) at a concentration of 1:2000, and HRP-conjugated secondary antibody at 1:10,000. Proteins were detected with ECL substrate (Pierce).

2.2.5 Kinetic Analysis

For kinetic analysis of serotonin transport, net uptake values from each individual experiment (n=3) were averaged, and the mean was analyzed by nonlinear regression and fit to the Michaelis-Menten equation to obtain maximal transport velocity (V_{max}) and affinity constant (K_m) using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA).

2.2.6 IC_{50} Determination

For determination of IC_{50} values, net uptake from each individual experiment (n=3) was converted to percent of control. These values were averaged, and the mean values were plotted in GraphPad Prism 6 and subjected to nonlinear regression.

2.2.7 Statistical Analysis

Statistical significance was calculated using two-tailed unpaired t-tests. A *P* value <0.05 was considered significant.

2.3 RESULTS

2.3.1 Functional Characterization of Human OCT1 in Transiently-transfected HEK293 Cells

To establish that our model of transient expression of hOCT1 in HEK293 cells was functional, transport of the model cation [^{3}H]-1-methyl-4-phenylpyridinium (MPP^+) was characterized. Initial time dependencies at low (0.5 µM) and high (100 µM) concentrations demonstrated uptake of MPP^+ to be linear through one minute (Figure 2.1) and kinetics experiments yielded a K_m of 35 ± 7 µM and a V_{max} of 500 ± 36 pmol/mg protein/min (Figure 2.2). This compares well with previously published values (32 µM, Grundemann et al., 2003; 25 µM, Umehara et al., 2007) and suggested that our model of hOCT1 transport was functional and suitable for further experiments.

2.3.2 hOCT1-mediated Neurotransmitter Transport

In order to study the effect of drugs on hOCT1-mediated uptake of endogenous substrates, it was first necessary to identify suitable endogenous substrates. To establish monoamine neurotransmitters as substrates of hOCT1, transport of 100 µM (0.8 µCi/mL) [^{3}H]-dopamine, [^{3}H]-norepinephrine, and [^{3}H]-serotonin was measured in pcDNA5/FRT- (empty vector) and hOCT1-transfected HEK293 cells at 37ºC for five

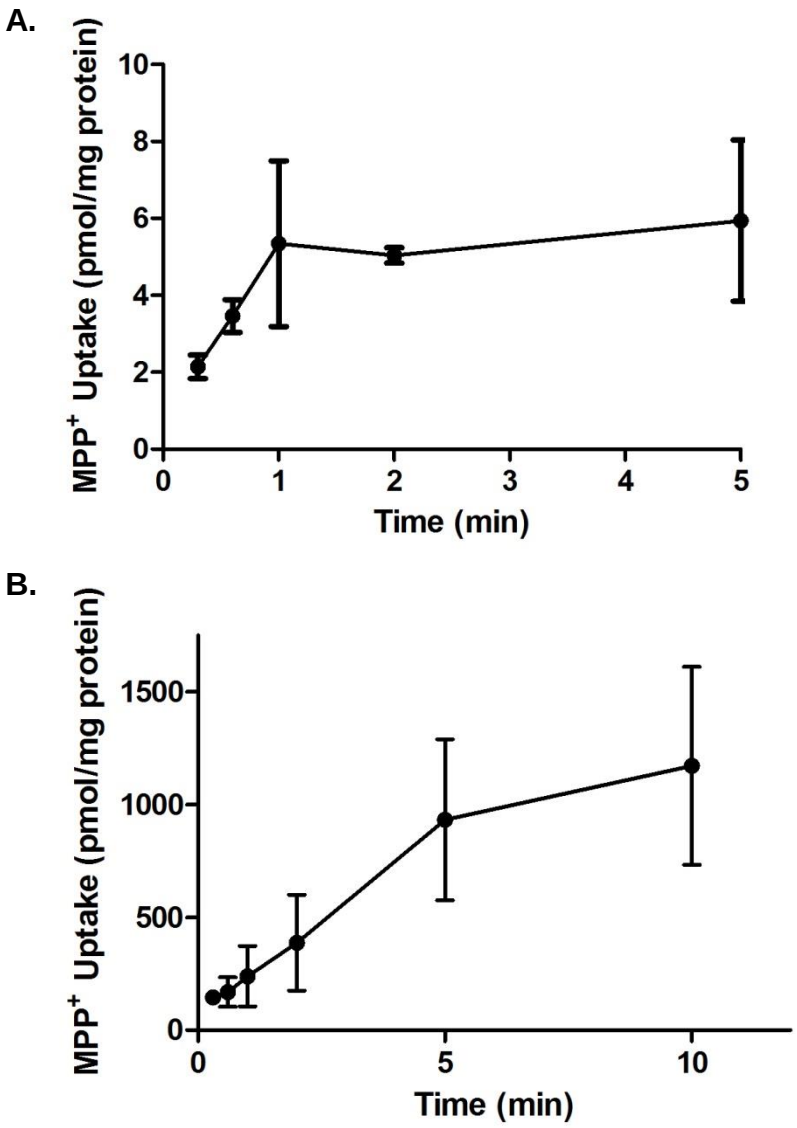

Figure 2.1. Time-dependent uptake of MPP^+ by hOCT1. HEK293-OCT1 and empty vector cells were incubated with (A) 0.5 µM, or (B) 100 µM radiolabeled MPP^+ for given amount of time to determine the linear range of uptake. Each time point done in triplicate; mean ± SD is shown.

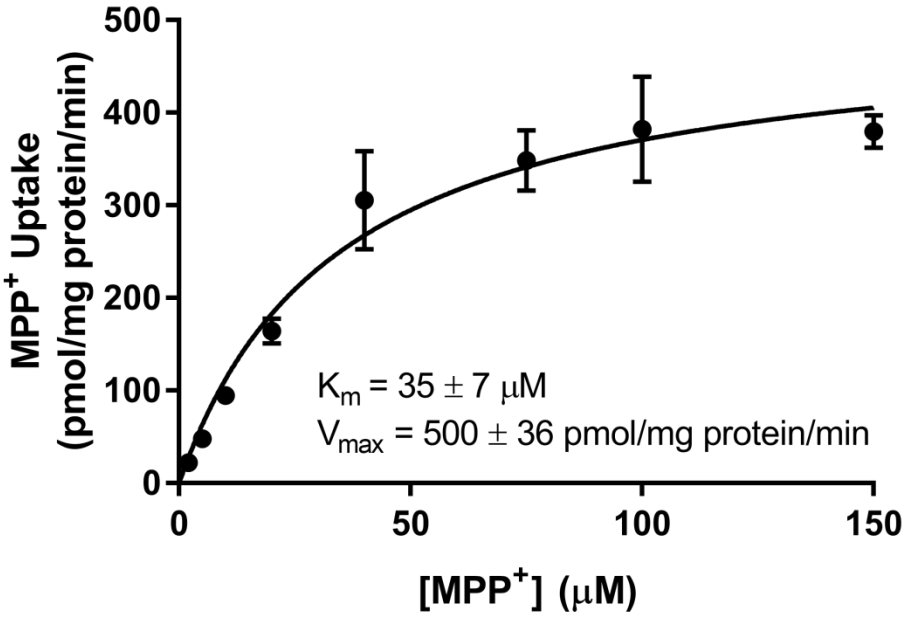

Figure 2.2. Kinetics of hOCT1-mediated MPP^+ uptake. HEK293-OCT1 and empty vector cells were incubated with increasing concentrations of radiolabeled MPP^+ for 30 seconds. The mean value (net uptake) ± SD for two independent experiments is shown. K_m and V_{max} values were determined to be 35 ± 7 μM and 500 ± 36 pmol/mg protein/min, respectively.

minutes. Uptake of these monoamines was significantly higher in hOCT1-expressing cells than in empty vector cells for all three neurotransmitters (Figure 2.3A). hOCT1-mediated transport (net uptake) was obtained by subtracting the uptake into empty vector cells from that of hOCT1-expressing cells (Figure 2.3B). At a time point of five minutes, serotonin appeared to be the most efficiently transported substrate. Dopamine uptake was approximately 50% that of serotonin, while norepinephrine uptake was approximately 20% compared with serotonin. The observed norepinephrine uptake was minimal, and therefore unlikely to be physiologically relevant. Dopamine uptake by hOCT1, though significant, is again unlikely to be germane to the liver, due to low circulating levels of this neurotransmitter. Serotonin, however, is found at high concentrations in the gut and consequently portal blood levels are significantly higher than arterial blood, reportedly as much as three-fold, (Gershon and Tack, 2007, Toh 1954), making it the best candidate of the three neurotransmitters to be transported by hOCT1 *in vivo*. Because of this, and the data suggesting that serotonin was the superior neurotransmitter substrate for hOCT1, serotonin was selected as the model endogenous substrate for further study.

2.3.3 Kinetic Characterization of Serotonin Uptake by hOCT1

To further characterize serotonin transport, uptake kinetics were performed. Serotonin influx was assessed in empty vector- and hOCT1-expressing cells after incubation with increasing concentrations of [^{3}H]-serotonin, from 50 µM to 2 mM (0.8-2.0 µCi/mL), for five minutes at 37ºC. Net uptake was fit to the Michaelis-Menten equation

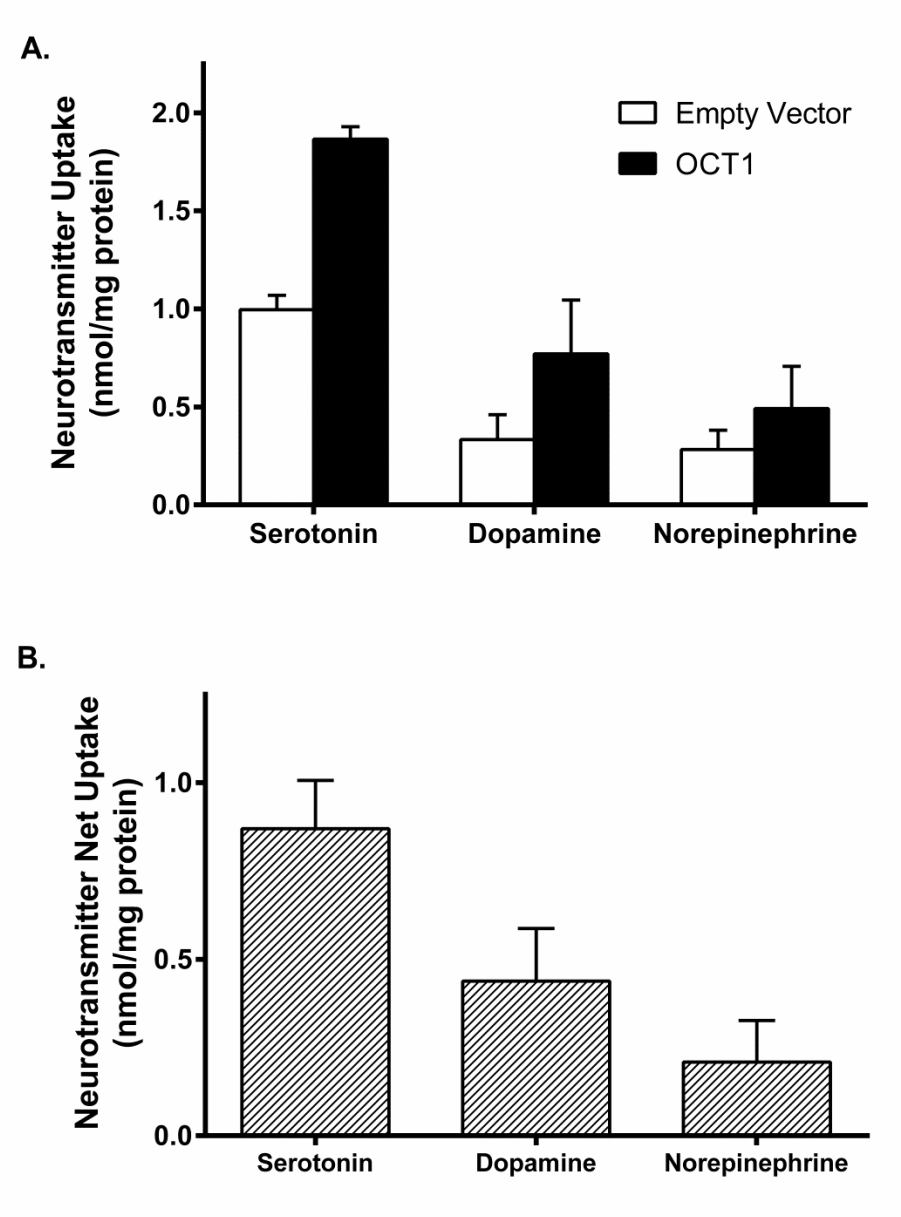

Figure 2.3. Transport of serotonin, dopamine, and norepinephrine by hOCT1. (A) HEK293 cells transiently transfected with empty vector and hOCT1 plasmid were incubated with 100 µM radiolabeled serotonin, dopamine, or norepinephrine (0.8 µCi/mL) at 37ºC for five minutes. (B) hOCT-mediated uptake (net uptake) was determined by subtracting uptake into empty vector cells from that of hOCT1-expressing cells. Mean + SD of three independent experiments is shown.

to yield a K_m of 197 ± 42 µM and V_{max} of 561 ± 36 pmol/mg protein/min (Figure 2.4). These data suggest that serotonin is transported by hOCT1 with affinity and capacity comparable to other hOCT1 substrates.

2.3.4 Inhibition of Serotonin Transport

Because hOCT1 has moderate affinity for serotonin, it is feasible to hypothesize that hOCT1-mediated uptake of serotonin could be inhibited by drugs or other xenobiotics. To evaluate this, the following eight compounds were selected: acyclovir, cimetidine, diphenhydramine, fluoxetine, imatinib, metformin, tyramine, and verapamil. These potential inhibitors were selected based on previous reports that showed interactions with hOCT1 or serotonin-transporting proteins (Dresser et al., 2001, Nies et al., 2011, Sitte et al., 1998). Uptake of 200 µM (1.2 µCi/mL) serotonin was measured for five minutes at 37ºC in the absence and presence of 10 µM and 100 µM of each compound (Figure 2.5). While there appeared to be a trend of inhibition for almost all of the drugs screened, serotonin uptake was significantly (P<0.05) inhibited by diphenhydramine, fluoxetine, imatinib, and verapamil at both concentrations (Figure 2.5). Inhibition by these four drugs was characterized further by determining IC_{50} values for serotonin transport (Figure 2.6). Transfected HEK293 cells were incubated with 100 µM serotonin and increasing concentrations of each drug for five minutes at 37ºC. Net uptake was converted to percent of control and was analyzed by nonlinear regression to obtain IC_{50} values. IC_{50} values for diphenhydramine, fluoxetine, imatinib and verapamil were determined to be 4.1 ± 1.4 µM, 6.2 ± 1.2 µM, 10.2 ± 1.2 µM, and 1.5 ± 1.4 µM

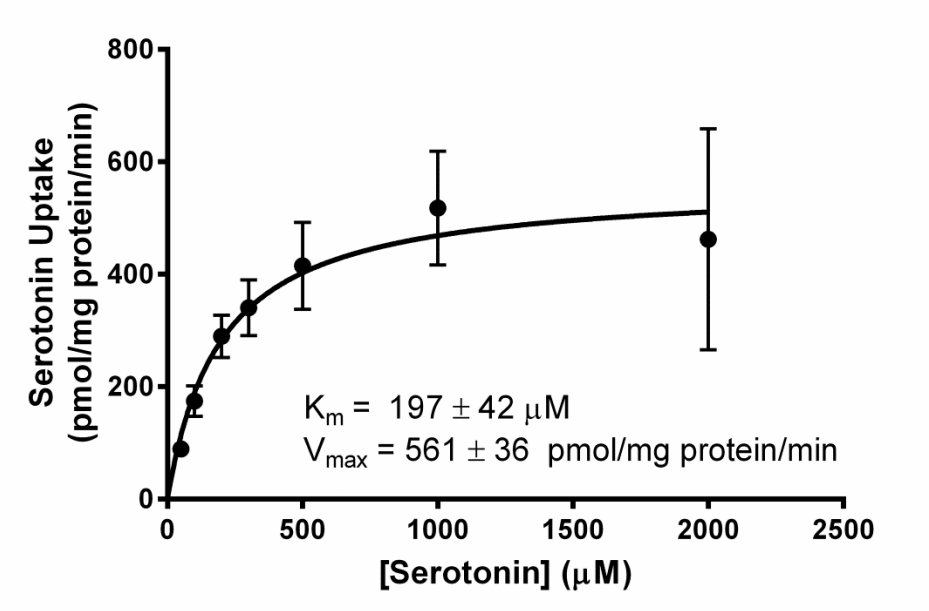

Figure 2.4. Kinetics of hOCT1-mediated serotonin transport. Empty vector- and hOCT1-transfected HEK293 cells were incubated with increasing concentrations of radiolabeled serotonin, ranging from 50 µM (0.8 µCi/mL) to 2 mM (2.0 µCi/mL), for five minutes at 37ºC. Net uptake was fit to the Michaelis-Menten equation to obtain the affinity constant, K_m = 197 ± 42 µM, and maximum transport velocity, V_{max} = 561 ± 36 pmol/mg protein*min. Values ± SEM are the result of three independent experiments.

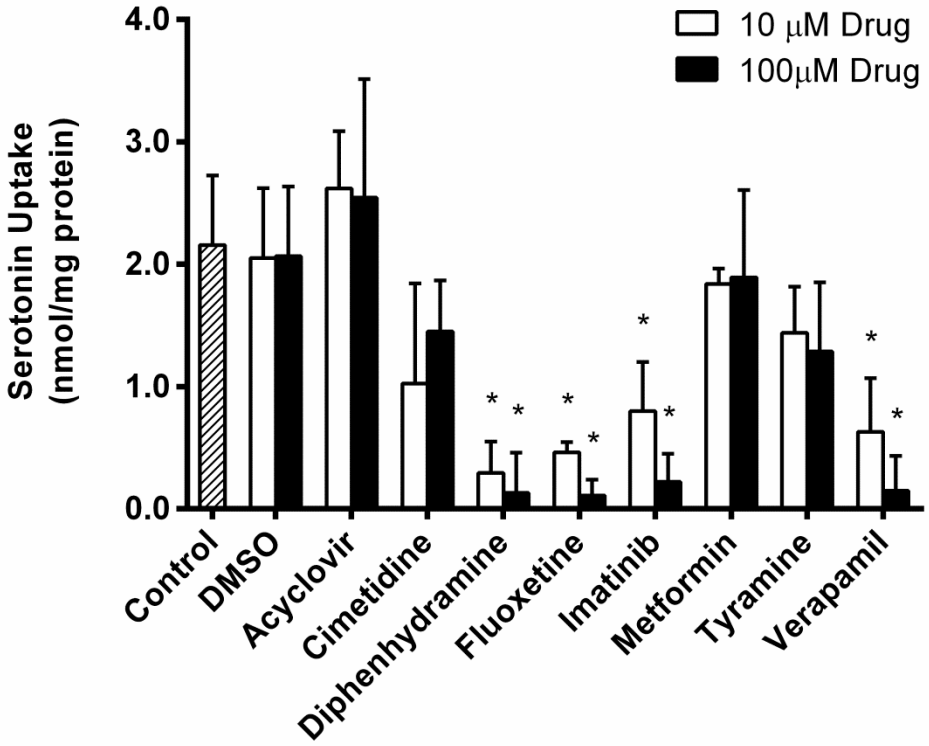

Figure 2.5. Inhibition of serotonin transport by common drugs. Transport of 200 μM radiolabeled serotonin (1.2 μCi/mL) was measured in HEK293 cells transfected with empty vector or hOCT1 plasmid cDNA in the presence of 10 μM or 100 μM drug for five minutes at 37ºC. DMSO controls were included at concentrations equivalent to those of imatinib preparations (≤ 1%). Mean ± SD of three independent experiments (net uptake) is shown. P <0.05 is indicated by (*).

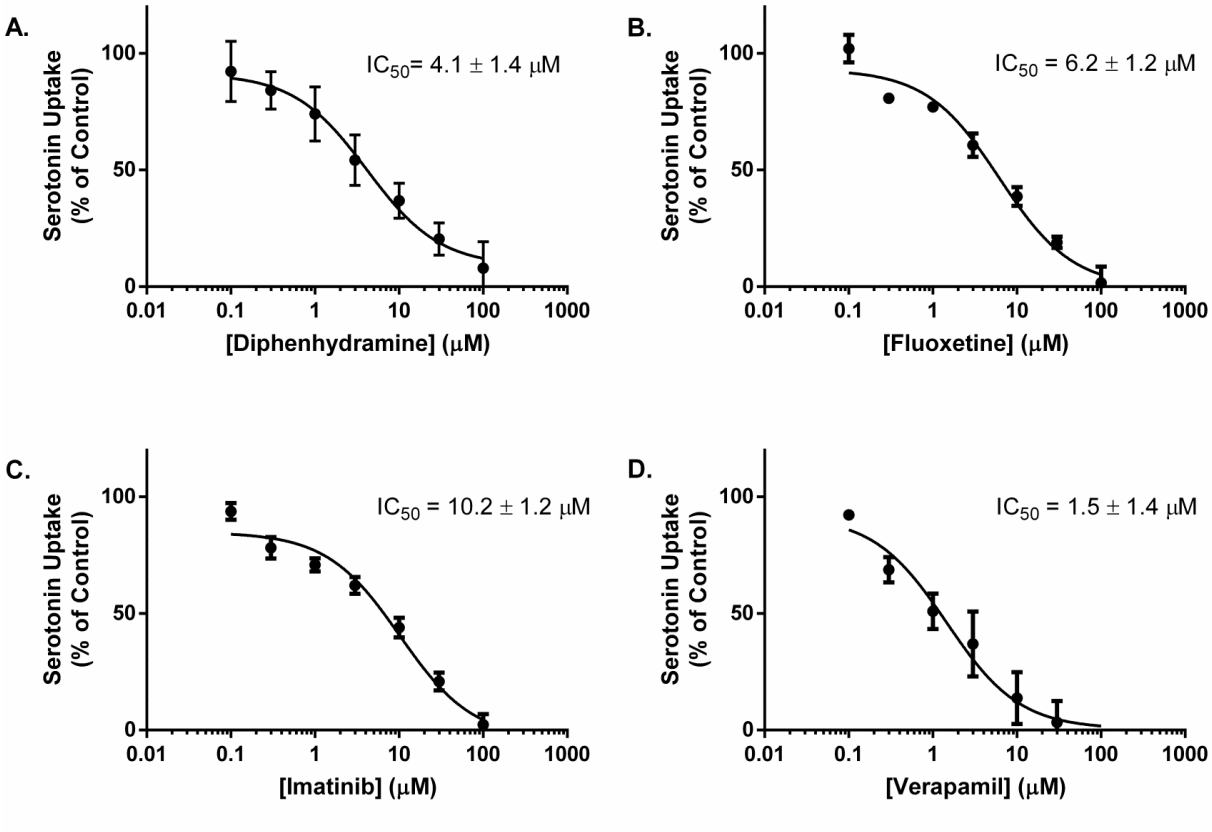

Figure 2.6. Concentration-dependent inhibition of serotonin uptake by hOCT1. In HEK293 cells transfected with empty vector or hOCT1, uptake of 100 µM radiolabeled serotonin (0.4 µCi/mL) was measured in the presence of increasing concentrations of (A) diphenhydramine, (B) fluoxetine, (C) imatinib, or (D) verapamil. Uptake values are expressed as percent of control and the mean ± SD of three independent experiments were subjected to nonlinear regression analysis for IC_{50} determination.

respectively. With IC_{50} values in the low micromolar range, all four drugs appear to be fairly potent inhibitors of hOCT1-mediated serotonin transport.

2.3.5 Serotonin transport and inhibition in primary human hepatocytes

To determine whether the observed transport and inhibition of serotonin is likely to be physiologically relevant, uptake and inhibition studies were conducted with freshly isolated and plated primary human hepatocytes. First, serotonin uptake into hepatocytes was assessed by incubating cells with 1.2 µCi/mL (42 nM) [^{3}H]-serotonin at 37ºC for five minutes (Figure 2.7A). Significant serotonin uptake was observed, even at this very low substrate concentration. To establish that the observed uptake of serotonin was carrier-mediated, hepatocytes were incubated with [^{3}H]-serotonin in the presence of 1 mM unlabeled serotonin intended to inhibit any carrier-mediated transport of radiolabeled serotonin. Addition of 1 mM unlabeled serotonin decreased uptake of [^{3}H]-serotonin by more than 50% (Figure 2.7A), suggesting that the majority of serotonin uptake in hepatocytes is carrier-mediated. To confirm expression of hOCT1 in the hepatocytes used to measure serotonin transport, western blotting was performed using a commercially-available anti-OCT1 antibody. HEK-EV and HEK-OCT1 lysates were used as controls (Figure 2.7B). For each batch of hepatocytes, hOCT1 expression levels were high, and total hOCT1 expression was consistent between batches. Next, the ability of the four drugs to inhibit serotonin uptake into hepatocytes was investigated. Hepatocytes were incubated with [^{3}H]-serotonin as described before in the presence of 10 µM and 100 µM diphenhydramine, fluoxetine, imatinib, or verapamil (Figure 2.7C). Each drug significantly (P<0.05) inhibited serotonin uptake into hepatocytes at both concentrations.

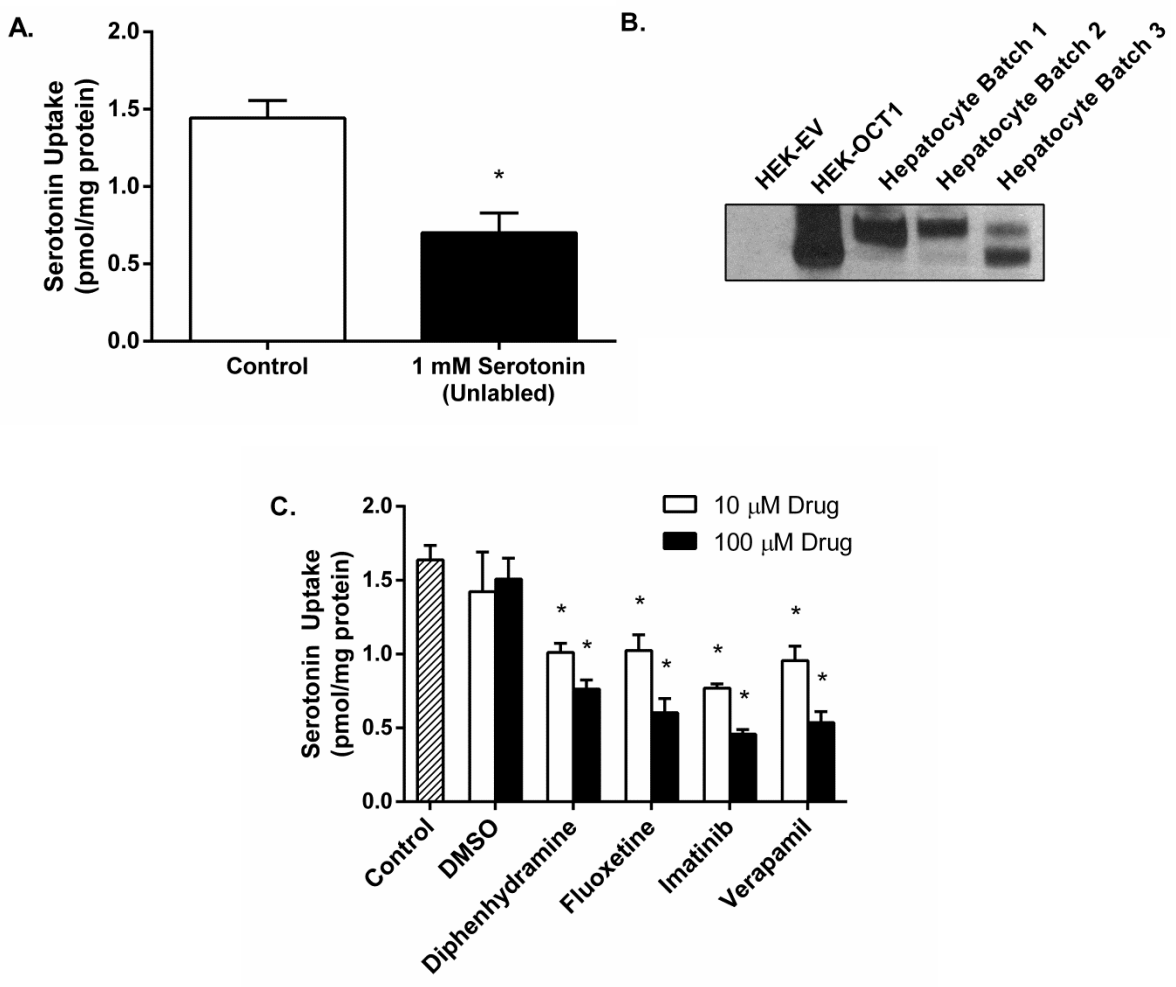

Figure 2.7. Serotonin transport in primary human hepatocytes. (A) Between 20 and 24 hours after plating, freshly isolated primary human hepatocytes were incubated for five minutes with 1.2 µCi/mL (radiolabeled only, 42 nM) serotonin. Carrier-mediated transport was inhibited by the presence of 1 mM unlabeled serotonin (right). (B) hOCT1 expression was confirmed in hepatocytes used to measure serotonin uptake. 50 µg of protein isolated from hepatocytes, was resolved by SDS-PAGE and blotted with anti-OCT1 antibody; 0.5 µg of protein isolated from HEK293 cells transfected with empty vector (HEK-EV) and hOCT1 (HEK-OCT1) was included as control. (C) Drug-mediated inhibition of serotonin transport was conducted as in (A) in the presence of 10 µM and 100 µM diphenhydramine, fluoxetine, imatinib, or verapamil. DMSO controls were included at sufficient concentrations to match those in imatinib preparations. Net uptake is represented as the mean + SD of three independent experiments with (*) indicating P <0.05.

2.4 DISCUSSION

This study demonstrates that serotonin is a substrate of human OCT1 and, more importantly, that hOCT1-mediated serotonin transport can be inhibited by several commonly-prescribed drugs. Taken together, these findings suggest that hepatic clearance of endogenous substrates, including biogenic amines, can be affected by small molecule therapeutics at the transporter level. Our results illustrate that diphenhydramine, fluoxetine, imatinib, and verapamil inhibit serotonin uptake in hOCT1-expressing HEK293 cells and in primary human hepatocytes.

The liver has been established as a key organ in the elimination of endogenous compounds, including monoamine neurotransmitters, from the body. As previously mentioned, it is unlikely that hOCT1 plays a major role in the uptake of dopamine or norepinephrine in the liver, due to both low circulating concentrations and relatively low transport, as documented in Figure 2.3. However, the same cannot be said for serotonin. Approximately 95% of the body's serotonin is synthesized and stored in the gut, where it is released to initiate peristalsis and activate secretory reflexes (Gershon and Tack, 2007). While the serotonin transporter (SERT) is expressed in the gut and functions in the reabsorption of released serotonin, a significant portion of serotonin reaches portal circulation; in fact, serotonin concentrations in portal blood can be as much as three-fold higher than in arterial blood (Toh, 1954). Additionally, the liver is responsible for the removal of up to 70% of the serotonin from portal blood (Thomas and Vane, 1967, Tyce, 1990). Given that SERT is not expressed in the liver (Ramamoorthy et al., 1993), we hypothesized that hOCT1 may be one transporter involved in serotonin uptake in the liver. Previous studies have investigated serotonin

transport by hOCT1, though with conflicting results. Kerb et al., (2002) demonstrated serotonin transport by human OCT1 as a test probe for comparison of wild-type and polymorphic transporters. Conversely, Amphoux, et al., (2006) reported that human OCT1 showed very little specific transport of serotonin, among other neurotransmitters. However, Amphoux, et al,. also failed to show OCT3-mediated transport of several monoamines known to be OCT3 substrates, and those that were transported yielded K_m values much higher than established elsewhere (Duan and Wang, 2010), suggesting a potential flaw in the employed expression system or other methods. In any case, we have demonstrated hOCT1-mediated serotonin transport in both HEK293 cells and in hepatocytes, confirming that serotonin is indeed a substrate of hOCT1, and our results indicate that hOCT1 is an important element in the elimination of serotonin from portal blood.

While this study suggests that hOCT1 is a key component in hepatic elimination of serotonin, we cannot completely rule out minor contributions of other cation transporters that have also been reported to transport biogenic amines. Organic cation transporter 3 (OCT3) and plasma membrane monoamine transporter (PMAT) are both high-capacity neurotransmitter transporters and together comprise the "uptake$_2$" mechanism for monoamine clearance in the brain (Wu et al., 1998, Zhou et al., 2007). Studies have shown that, in addition to hOCT1, both OCT3 and PMAT are expressed in the liver, though at very low levels. PMAT mRNA levels were nearly undetectable in the liver (Engel et al., 2004), suggesting its function pertains primarily to the brain, and hepatic OCT3 mRNA levels were shown to be between 6-30% that of hOCT1 (Chen et al., 2010, Nies et al., 2009). Additionally, the affinities of PMAT and OCT3 for serotonin

are relatively low (Duan and Wang, 2010) compared to that established in this study for hOCT1 (Figure 2.4). Combined, the low expression levels and relative transport affinities of OCT3 and PMAT suggest that they are likely minor components of serotonin uptake in hepatocytes, further solidifying the role of hOCT1 in serotonin elimination in the liver.

Because hepatic clearance of serotonin may rely heavily on hOCT1 transport, it is important to understand the effects that drug interaction with hOCT1 has on serotonin uptake in the liver. The inhibition screen performed in this study (Figure 2.5) indicates that several drugs are capable of inhibiting serotonin transport by hOCT1. Diphenhydramine, fluoxetine, imatinib, and verapamil all significantly inhibited serotonin uptake in both HEK293 cells and human hepatocytes (Figures 2.5-2.7). Furthermore, even though uptake was not significantly inhibited, a trend of inhibition was observed for cimetidine and metformin, as well as the notorious monoamine neurotransmitter transporter inhibitor, tyramine. Additionally, IC_{50} values determined for diphenhydramine, fluoxetine, imatinib, and verapamil were all in the low micromolar range, 4.1 ± 1.4 µM, 6.2 ± 1.2 µM, 10.2 ± 1.2 µM, and 1.5 ± 1.4 µM respectively (Figure 2.6). Given that substrate-dependent inhibition has been reported for OCT2 (Belzer et al., 2013) and MATE1 (Martínez-Guerrero and Wright, 2013), comparison of the IC_{50} values obtained in the present study with previously reported values obtained using other substrates might give some insight into how different substrates are handled by hOCT1. Previously, diphenhydramine and fluoxetine inhibited MPP^+ uptake with IC_{50}'s of 3.4 µM and 2.8 µM, respectively (Müller et al., 2005, Haenisch et al., 2012), and verapamil inhibited TEA^+ transport with an IC_{50} of 2.9 µM (Zhang et al., 1998). These

values are comparable to the IC_{50} values we obtained for hOCT1-mediated uptake of serotonin, which suggests that serotonin and the two model substrates MPP^+ and TEA are transported in a very similar way by hOCT1. In contrast, previous reports demonstrated that imatinib inhibited hOCT1-mediated metformin uptake with an IC_{50} value of 1.5 µM (Minematsu and Giacomini, 2011), while in our study imatinib inhibited serotonin uptake with an IC_{50} value of 10.2 µM. This confirms the substrate-dependent inhibition seen with other organic cation transporters (Belzer et al., 2013, Martínez-Guerrero and Wright, 2013) and suggests that these transporters have complex binding pockets, with different interaction sites for different substrates. Regardless, these novel results are strong evidence that xenobiotics may inhibit serotonin uptake in the liver, potentially hindering proper hepatic clearance of serotonin *in vivo*, and it is plausible that these same effects would be seen with other endogenous substrates as well.

Undoubtedly, drug-mediated inhibition of serotonin transport would be dependent upon drug concentrations achieved *in vivo*. Peak plasma drug concentrations have been shown to reach 66 ng/mL (0.3 µM) for diphenhydramine, 302 ng/mL (1 µM) for fluoxetine, 3380 ng/mL (6.8 µM) for imatinib, and 400 ng/mL (0.9 µM) for verapamil (Blyden et al. 1986, Peng et al., 2004, U.S. NLM Daily Med a-b). These plasma levels are not necessarily high enough to elicit significant hOCT1 inhibition. However, in this case, because hOCT1 is localized to the liver, portal blood drug concentrations are likely more relevant. Though drug concentrations have not been measured in portal blood, it is likely that portal drug concentrations are significantly higher than those measured in plasma, given that all of the drugs tested are dosed orally. This suggests that these drugs may indeed affect serotonin transport in the liver.

Inhibition of serotonin uptake in the liver may bear several implications, including a potential increase in circulating serotonin levels as well as locally increased extracellular serotonin concentrations in the liver. Increased serotonin levels in the circulation has the potential to lead to certain specific toxicities, including alterations in the blood coagulation cascade. When activated, platelets degranulate, releasing a variety of factors, including serotonin, to initiate coagulation (Troxler et al., 2007). Recently, SSRIs have been shown to exert an anticoagulative effect, due to decreases in serotonin levels in the blood (Bottlender et al., 1998). Conversely, drug-mediated inhibition of serotonin uptake in the liver could increase blood serotonin levels, potentially resulting in hypercoagulopathy. In addition, increases in circulating serotonin has the potential to cause acute changes in blood pressure due to its vasoactive properties (Page and McCubbin, 1953, Rapport, 1949). Furthermore, serotonin has been implicated in changes in renal blood flow (Blackshear et al., 1986), which might suggest a role for increased circulating serotonin levels in kidney dysfunction. Additionally, the importance of serotonin in both liver injury and regeneration has recently been established. Work completed by Pierre-Alain Clavien and others exposed a critical function of serotonin in liver regeneration (Lesurtel et al., 2006, Nocito et al., 2007b). Conversely, serotonin has also been implicated in mitochondrial dysfunction and hepatocellular injury in nonalcoholic steatohepatitis (NASH) (Nocito et al., 2007a). Inhibition of serotonin transport by hOCT1 could potentiate the pathogenesis of NASH. In contrast, increased serotonin levels in resection patients could be beneficial for liver regeneration. In any case, drug-mediated inhibition of serotonin uptake by hOCT1 may well have important physiological consequences.

In conclusion, we have established that serotonin is a viable substrate for human OCT1, and more importantly, that commonly-prescribed drugs inhibit its uptake. Diphenhydramine, fluoxetine, imatinib, and verapamil significantly inhibited serotonin transport in both HEK293 cells and in primary human hepatocytes. Moreover, these compounds appear to be fairly potent inhibitors of serotonin uptake, as IC_{50} values were determined to be in the low micromolar range for all four drugs. The implications of serotonin uptake inhibition in the liver may be several, and the results of this study bring new insights to the potential for drugs and other xenobiotics to interfere with endogenous substrate transport and elimination.

CHAPTER 3 : SUBSTRATE-DEPENDENT LIGAND INTERACTIONS WITH HUMAN ORGANIC CATION TRANSPORTER 1

3.1 INTRODUCTION

The human organic cation transporter 1 (hOCT1) is a liver-specific drug transporter that mediates the uptake of numerous drugs and endogenous compounds into the liver for metabolism and/or excretion. Its location and function makes it a prime candidate for drug-drug interactions (Bachmakov, et al., 2008, Cho, et al., 2014, Choi, et al., 2010, Minematsu and Giacomini, 2011). As such, regulatory agencies include hOCT1 inhibition studies in their recommended preclinical testing of new drug candidates (European Medicines Agency, 2012).

Recently, substrate-dependent interactions have been described for several drug transporters, including close relatives of hOCT1. For example, when incubated with OATP1B3, the antifungal drug clotrimazole strongly inhibits uptake of substrate Fluo-3, stimulates uptake of estradiol-β17-glucuronide, but exhibits no effect on estrone-3-sulfate uptake (Gui, et al., 2008). Similar substrate-dependent effects were described for green tea catechins interacting with OATP1B3 (Roth, et al., 2011). Several studies identified substrate-dependent modulation for hOCT2, a member of the same subfamily of transporters and which shares 70% sequence identity with hOCT1 (Gorboulev, et al., 1997). Zolk, et al., (2009) demonstrated that several structurally diverse compounds were markedly more potent inhibitors of hOCT2-mediated uptake of metformin than MPP^+, as determined by IC_{50} values. In addition, widely-varied IC_{50} values were observed for hOCT2 inhibitors when screened against MPP^+ versus novel substrate NBD-MTMA (Belzer, et al., 2013). Furthermore, Hacker, et al., (2015) compared the inhibitory effect of a number of hOCT2 ligands on transport of MPP^+, ASP^+, and

metformin, and observed that several of the tested compounds only inhibited one or two substrates.

Recent publications also indicate the possibility of substrate-dependent effects for hOCT1. A previous publication from our lab demonstrated that acyclovir, cimetidine, and metformin, all reported substrates or inhibitors of hOCT1, did not inhibit serotonin uptake (Boxberger, et al., 2014). Also, in our hands, imatinib elicited a 10-fold higher IC_{50} with serotonin employed as probe substrate versus that published using metformin as probe substrate (Minematsu and Giacomini, 2011). Additionally, IC_{50} values determined for MPP^+, TEA, and cimetidine were much higher when ethidium was used as probe substrate than previously reported values utilizing other probe substrates (Lee, et al., 2009). Furthermore, quercetin and lamivudine did not inhibit hOCT1-mediated transport of MPP^+ (Mandery, et al., 2012) and TEA (Moss, et al., 2015), respectively, despite being substrates of hOCT1 (Glaeser, et al., 2014, Jung, et al., 2008, Minuesa, et al., 2009). While it is difficult to directly compare IC_{50} values between laboratories due to inter-assay variability, all of these studies taken together with reports that describe multiple binding sites and/or translocation pathways within hOCT1 (Chen, et al., 2017, Ciarimboli, et al., 2004, Ciarimboli and Schlatter, 2005, Koepsell, et al., 2003) strongly suggest the potential for substrate-dependent modulation of hOCT1.

If substrate-dependent interactions occur with hOCT1, guidelines recommending inhibition studies utilizing one or two model probe substrates (European Medicines Agency, 2012) may not be sufficient to identify potentially dangerous drug-drug interactions. Therefore, concrete identification of substrate-dependent effects of hOCT1 is crucial. In the present study, we characterized substrate-dependent interactions of

hOCT1 with competitive counterflow (CCF) experiments, utilizing multiple hOCT1 probe substrates and numerous known ligands, and explored the observed interactions through homology modeling and docking studies.

3.2 MATERIALS AND METHODS

3.2.1 Materials

[^{3}H]-1-methyl-4-phenylpyridinium iodide (MPP^{+}, 85.0 Ci/mmol), and [^{14}C]-metformin (0.1 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]-hydroxytryptamine creatinine sulfate (serotonin, 28.3 Ci/mmol), and [^{14}C]-tetraethylammonium bromide (3.5 mCi/mmol) were purchased from Perkin Elmer (Boston, MA). [^{3}H]-bromosulfophthalein (BSP, 11.5 Ci/mmol) was purchased from International Isotopes Clearing House, Inc. (Shawnee Mission, KS). Sodium chloride, potassium chloride, and ethidium bromide were obtained from Fisher Scientific (Waltham, MA). Dolutegravir was purchased from Advanced ChemBlocks (Burlingame, CA). Prostaglandin E_2 and imatinib were purchased through Toronto Research Chemicals (Toronto, ON, Canada). Erlotinib, gefitinib, and oxaliplatin were obtained from the NCI/DTP Open Chemical Repository (http://dtp.cancer.gov) as part of the approved oncology drug set II library. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

3.2.2 Generation and Culture of Stable Cell Lines

Flp-In^{TM}-293 cells (Invitrogen, Carlsbad, CA), derived from Human Embryonic Kidney (HEK) cells, were cultured in Dulbecco's Modified Eagle's medium (ATCC, Manassas, MA) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich), and 100 µg/mL Zeocin (Invitrogen) at 37°C under humidified 5% CO_2. Per manufacturer's protocol, to generate HEK–EV and –hOCT1 cells, Flp-In^{TM}-293 cells were co-transfected with pcDNA5/FRT plasmid (empty vector, EV) or pcDNA5/FRT-hOCT1 (hOCT1) plasmid, respectively, and pOG44 using FuGENE HD® transfection reagent (Promega, Madison, WI). After transfection, cells were grown in medium, using conditions described above, with hygromycin (100 µg/mL, Invitrogen) replacing Zeocin to select for stable transfectants. Colonies were isolated and propagated through clonal expansion. Clones were assayed for hOCT1 transport activity. HEK-hOCT1 clones, which demonstrated high transport of [^{3}H]-MPP$^+$, and HEK-EV clones, which did not show significant uptake of [^{3}H]-MPP$^+$ were selected, expanded, frozen, and stored per manufacturer protocol for future use. Cells were seeded on 48-well plates coated with 0.1 mg/mL poly-D-lysine (Invitrogen) at a density of 160,000 cells/well, and incubated at 37°C for approximately 48 hours until confluency was reached. For BSP transport assays, cells were seeded on 24-well plates coated with 0.1 mg/mL poly-D-lysine (Invitrogen) at a density of 250,000 cells/well, and incubated as described above.

3.2.3 Transport Assays

Transport assays were performed approximately 48 hours after cells were plated. All assays (excluding BSP transport assays) were performed at room temperature, using room-temperature uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM NaH_2PO_4, 1.2

mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM D-glucose, and 12.5 mM HEPES, adjusted to pH 7.4 with Tris base) to prewash cells, and ice-cold uptake buffer to stop transport. BSP transport assays were performed at 37°C, using pre-warmed uptake buffer to prewash the cells. Buffer components (with the addition of bovine serum albumin, during washes only, to limit non-specific binding of BSP) and stop conditions were as described above.

Time-dependent uptake was measured by incubating HEK-EV and -hOCT1 cells with 150 µL of uptake buffer containing radiolabeled [^{3}H]-MPP^+ (0.4 µCi/mL), [^{3}H]-serotonin (0.4 µCi/mL), [^{14}C]-metformin (0.2 µCi/mL), or [^{14}C]-TEA (0.2 µCi/mL) for 30 seconds to 180 minutes. Net uptake was calculated by subtracting uptake into HEK-EV cells from that of HEK-hOCT1 cells.

Competitive counterflow (CCF) experiments involved pre-loading HEK-hOCT1 cells with radiolabeled probe substrates (as described above) for 90 minutes. Following preload incubation, 1.5 µL of concentrated efflux solution containing water (control) or unlabeled test compound was added to the wells and plates were vortexed briefly on low to initiate efflux. Compounds used to induce CCF were ideally employed at concentrations 10-fold higher than published K_m values (see Table 3.1 for concentrations of test compounds employed). For time-dependent efflux, cells were incubated with efflux solutions for 15 seconds to 10 minutes. For CCF assays, cells were incubated with efflux solutions for 30 seconds (MPP^+) or 5 minutes (serotonin, TEA).

Once the assays were complete, all cells were lysed with 150 µL/well for 48-well plates or 300 µL/well for 24-well plates of 1% Triton X-100 in PBS. Then, 100 or 200 µL

Table 3.1. Published K_m/IC_{50} values and concentrations of test compounds employed in CCF assays.

Drug List	K_m or [K_i/IC_{50}] (μM)	References	Desired Concentration (mM)	Actual Concentration (mM)[a]
MPP^+	15, 32, 35	Boxberger, et al., 2014, Grundemann, et al., 2003, Zhang, et al., 1997b	0.1	0.1
Serotonin	197	Boxberger, et al., 2014	1	1
Metformin	1470	Kimura, et al., 2005	10	10
TEA	168, 229	Bednarczyk, et al., 2003, Zhang, et al., 1998	1	1
BSP	---[b]		1	0.59
Glucose	---[b]		1	1
Acyclovir	151	Takeda, et al., 2002	1	1
Dolutegravir	---[b]		1	1
Ritonavir	[5]	Zhang, et al., 2000	0.1	0.1
Lamivudine	249, 1250	Jung, et al., 2008, Minuesa, et al., 2009	1	1
Cimetidine	[95], [166]	Ciarimboli, et al., 2004, Zhang, et al., 1998	10	10
Ranitidine	[22], 70	Bednarczyk, et al., 2003, Bourdet, et al., 2005	1	1
Famotidine	[28]	Bourdet, et al., 2005	1	1
Diphenhydramine	[4]	Boxberger, et al., 2014, Muller, et al., 2005	0.1	0.1
Imatinib	[0.1], [1.5], [10]	Boxberger, et al., 2014, Minematsu and Giacomini, 2011, Nies, et al., 2014	0.1	0.1
Gefitinib	[1]	Minematsu and Giacomini, 2011	0.1	0.1
Erlotinib	[0.35]	Minematsu and Giacomini, 2011	0.1	0.1
Oxaliplatin	[4][c]	Zhang, et al., 2006	0.1	0.1
Verapamil	[1.5], [1.8], [3]	Boxberger, et al., 2014, Dickens, et al., 2012, Zhang, et al., 1998	0.1	0.1

Midazolam	[3.7]	Zhang, et al., 1998	0.1	0.1
Fluoxetine	[3], [6]	Boxberger, et al., 2014, Haenisch, et al., 2012	0.1	0.1
Lamotrigine	62	Dickens, et al., 2012	1	1
Acebutolol HCl	[96]	Zhang, et al., 1998	1	1
Salbutamol	[277]	Salomon, et al., 2015	1	1
Thiamine	[434], 780	Bednarczyk, et al., 2003, Chen, et al., 2014	10	10
Prostaglandin E_2	[0.7]	Kimura, et al., 2002	0.1	0.1
EGCG	[140][d]	Knop, et al., 2015	1	0.93
Quercitin	2.2	Glaeser, et al., 2014	0.1	0.1
Berberine	15	Nies, et al., 2008	0.1	0.09
Ethidium	0.8	Lee, et al., 2009	0.1	0.1

[a] In some instances, actual concentration varied from desired concentration due to solubility issues when preparing stock solutions.

[b] Affinity has not been determined for hOCT1.

[c] IC_{50} determined by cytotoxicity.

[d] Value calculated from data presented in Knop, et al., 2015.

of each lysate was transferred to a 96- or 24-well scintillation plate (Perkin Elmer, Waltham, MA), and mixed with 100 or 300 µL Optiphase HiSafe 3 scintillation cocktail (Perkin Elmer). Radioactivity was determined by liquid scintillation counting using a MicroBeta Trilux liquid scintillation counter (Perkin Elmer). Where protein concentration was determined, protein was measured in duplicate for each condition on 96-well plates using the Pierce BCA assay (Thermo Fisher Scientific, Waltham, MA). Each data point represents the average of three independent experiments, wherein each condition was completed in triplicate.

3.2.4 Determination of Kinetic Parameters

To determine kinetic parameters, net uptake values, obtained by subtracting uptake into HEK-EV cells from that of HEK-hOCT1 cells, from each of three independent experiments were averaged and plotted using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). Subsequently, the data were analyzed by nonlinear regression and, for kinetics analysis, fit to the Michaelis-Menten equation.

3.2.5 Generation of a hOCT1 Homology Model

A homology model of human hOCT1 (GenPept accession #: O15245) was prepared using the BIOVIA Discovery Studio's version 4.5 (http://accelrys.com/) BHM (Build Homology Models) protocol, which supports a software plugin for UCSF Modeller automodel, ver. 9.14 (https://salilab.org/modeller/contact.html). Chain A of the crystal structure of the bacterial glycerol-3-phosphate transporter from *E.Coli*, GlpT (PDB ID # 1PW4_A; Huang, et al., 2003), was used as an input template. The GlpT transporter was chosen due to the fact that it belongs to the MFS class of proteins, shares

approximately 18% sequence identity (~50% sequence similarity) to hOCT1, and functionally parallels hOCT1 in regards to its propensity for substrate promiscuity. In order to facilitate efficient modeling of the hOCT1 transporter, the large extracellular loop region between helical TMDs 1 and 2, and the intercellular loop region between helical TMDs 6 and 7, were both removed from the structure prior to model construction. Parameters in the Parameters Explorer box of the BHM menu in Discovery Studio were set to the following: Cut Overhangs was set to True in order to cut the terminal residues of the input model sequence that did not align properly with the input templates, Number of Models was set to 5 in order to define the number of models to create from an initial structure, and the Optimization Level was set to Low in order to specify the amount of molecular dynamics to perform with simulated annealing.

In order to build refinement models on the detected loop regions, i.e., the model sequence segments of at least 5 residues length which are not specifically aligned with the templates, the LOOPER function was used to systematically search loop conformations and rank them using CHARMm, with Refine Loops set to True. The BHM protocol uses the DOPE (Discrete Optimized Protein Energy; Shen and Sali, 2006) method to refine loops, which results in a more energetically accurate portrait of the loop structure. Refine Loops, Number of Models was set to 5 to specify the number of models to be created by loop optimization, and the Refine Loops Optimization Level was set to Low. Refine Loops with the DOPE method was set to High Resolution. After running the BHM protocol, the Best Model Structure Superimposed to Templates was chosen from the generated output models for the final three-dimensional model structure of the human hOCT1 transporter.

3.2.6 Docking of Ligands into the hOCT1 Homology Model

In order to identify the potential binding modes and protein interaction sites of various ligands with hOCT1, an *in silico* molecular docking strategy was employed using the UCSF DOCK 6.7 software suite, under academic license (University of California, San Francisco: http://dock.compbio.ucsf.edu/DOCK_6/index.htm; Allen, et al., 2015, Fan, et al., 2009, Huang, et al., 2006). The 3D conformer for each ligand was downloaded directly from the PubChem website (https://pubchem.ncbi.nlm.nih.gov/), parameterized for partial charges, and used for all docking studies described below. Extraneous information not required for the docking simulation, including the headers, connect records, and waters, were deleted from the PDB text file. Additionally, all hydrogens were removed from the receptor using the SELECT function from the UCSF Chimera 1.11.2 software suite (Pettersen, et al., 2004).

The hOCT1 receptor was then prepared for docking by using the UCSF Chimera 1.11.2 DOCK PREP function. Once preparation of the receptor had been completed, it was saved in the MOL2 format. Sphere generation was accomplished by inputting a dot molecular surface (DMS) parameterization of the receptor surface into the SPHGEN module of the DOCK software suite. The DMS parameterization is accomplished by rolling a ball the size of a water molecule over the Van der Waal's surface of the receptor. The surface normal vector at each surface point is computed and used to calculate the size of each sphere generated. After initial sphere generation, the total number of sphere clusters was pruned to only the clusters representing the active site of the protein using the DOCK SHOWSPHERE module. This resulted in the largest sphere cluster being contained within the active site. Next, a molecular docking grid was

generated by enclosing the spheres in a rectangular box and using the GRID utility to obtain files representing both the electrostatic and Van der Waals forces according to the following equation:

$$E = \sum_{i=1}^{lig} \times \sum_{j=1}^{rec} \times \left(\frac{A_{ij}}{r_{ij}^{a}} - \frac{B_{ij}}{r_{ij}^{b}} + 332 \frac{q_i q_j}{D r_{ij}} \right)$$

where each term is a double sum over ligand atoms *i* and receptor atoms *j*, A_{ij} and B_{ij} are the attraction and repulsion parameters, respectively, *r* is the distance between the van der Waals radii of particles *i* and *j* in the case of repulsion *a* or attraction *b*, *q* is the electrostatic surface potential of *i* or *j*, and *D* is the well depth of the interaction between the ligand and receptor atoms, as defined by Kuntz, et al., (1982). The GRID utility then generated files representing the contact score and the energy score which are utilized in the docking routine. The final size of the docking grid box was 16.7 Å x 25.9 Å x 16.7 Å. The docking parameters were as follows: maximum number of orientations was set to between 50 and 1000, Van der Waals energy component from between 20 to 25, and maximum number of iterations to 500 (except for berberine, which was set to 1,000). The VIEWDOCK utility of UCSF Chimera 1.11.2 was used for visualization of the docking poses and measuring distances and angles between atoms of interest.

3.3 RESULTS

3.3.1 Functional Validation of Generated hOCT1 Stable Transfectants

HEK293 cells were used to generate stable cells lines containing pcDNA5/FRT empty vector (control) and pcDNA5/FRT-hOCT1 vectors using the Flp-In^{TM} system. Clones stably-expressing hOCT1 were assayed for transport of model cation MPP^+ and probe substrate serotonin (Figure 3.1). Significant uptake of both substrates was observed in the selected hOCT1-expressing cells at multiple time points, compared to control cells, demonstrating adequate functionality of the stably-expressed transporters.

3.3.2 Substrate-dependent Interactions with hOCT1 as Determined by Competitive Counterflow

To characterize the previously observed putative substrate-dependent interactions of hOCT1, the competitive counterflow (CCF) assay, developed by Harper and Wright (2013), was employed. CCF capitalizes on the observation that OCTs transport substrates in both directions, and therefore can function as exchangers by transporting one substrate into the cell and subsequently effluxing another from the cytoplasm. In this study, CCF was employed to examine substrate-dependent effects by utilizing four different radiolabeled probe substrates: MPP^+, serotonin, metformin, and TEA.

Before CCF could be performed, the time point where substrate transport reached steady-state equilibrium needed to be determined. For this, time-dependent uptake assays were performed for all four substrates (Figure 3.2A-D). MPP^+, metformin, and TEA uptake reached steady-state by 60 minutes, while serotonin uptake plateaued around 90 minutes. Therefore, pre-load incubations prior to CCF were done for 90 minutes. To determine the time point with the best efflux signal, time-dependent efflux

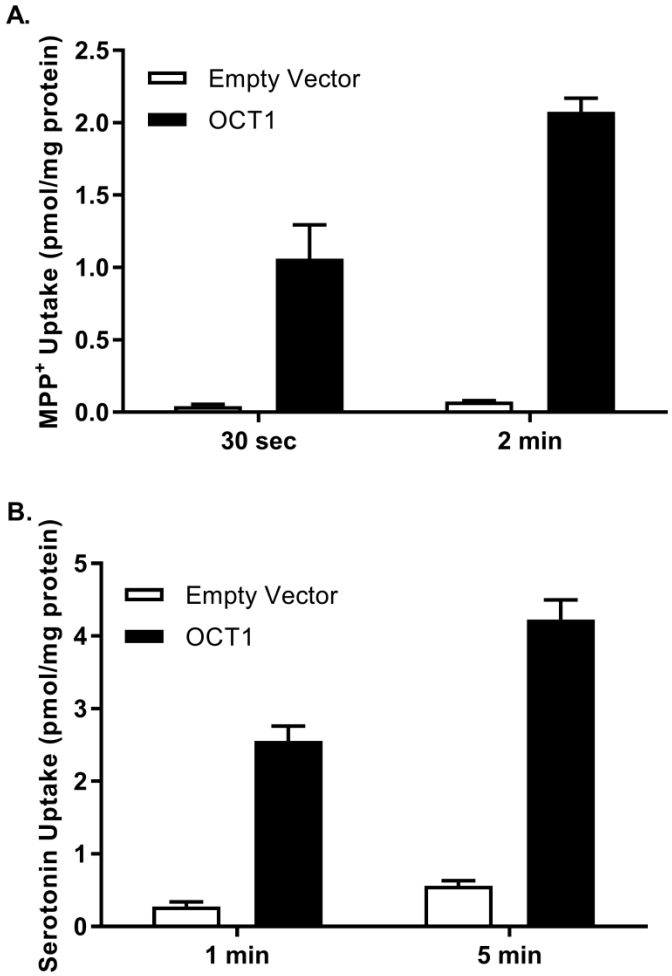

Figure 3.1. Uptake of MPP^+ and Serotonin by HEK-EV and -hOCT1 stable transfectants. To functionally validate the generated HEK-EV (empty vector) and -hOCT1 stable cell lines, cells from each pool were incubated with radiolabeled (A) MPP^+ (0.9 µCi/mL, 11 nM) and (B) serotonin (1.2 µCi/mL, 42 nM) at 37°C for the indicated amount of time. Each condition was completed in triplicate and the mean ± SD is shown.

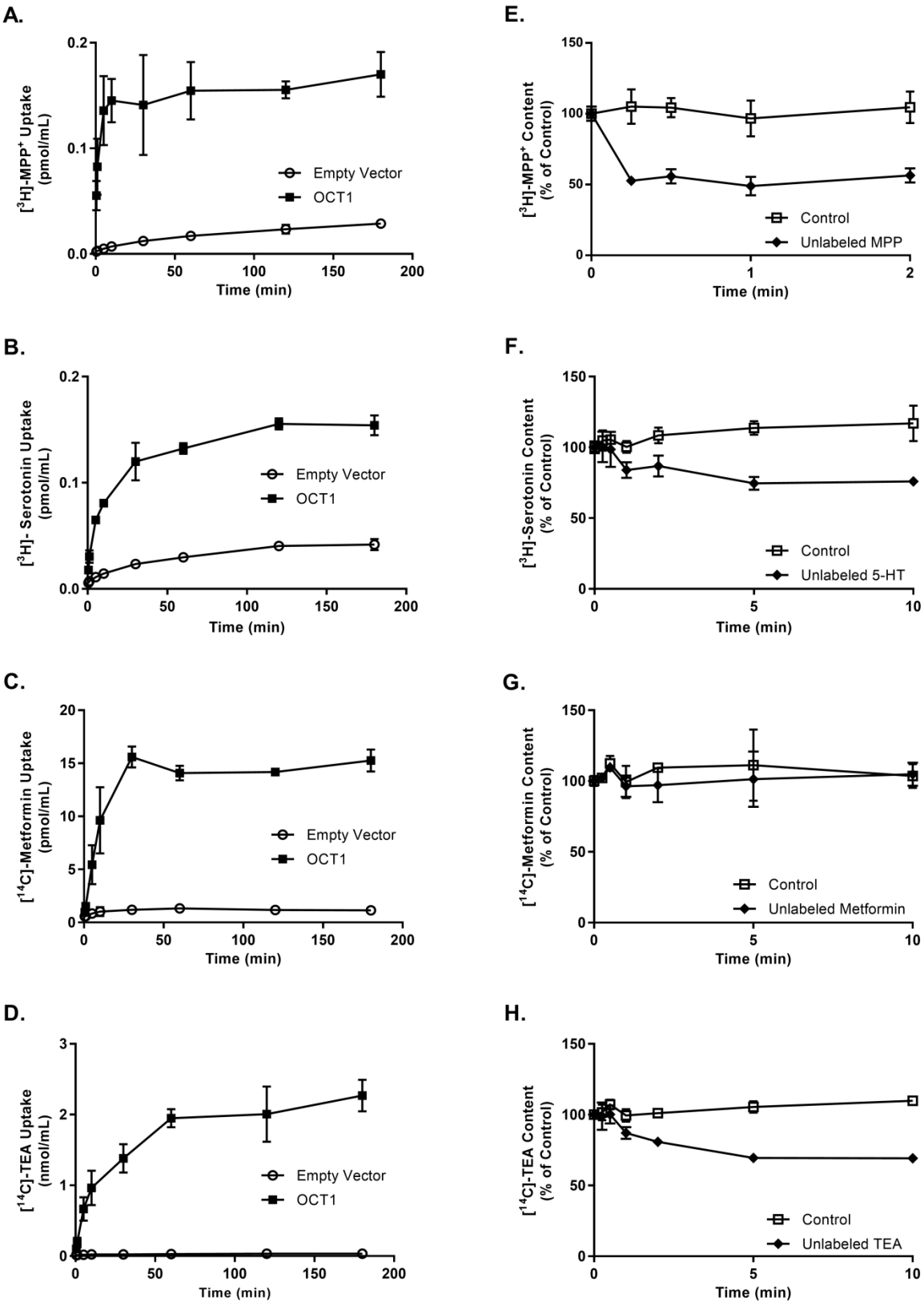
A.
[3H]-MPP+ Uptake (pmol/mL)
Time (min)
Empty Vector
OCT1
B.
[3H]- Serotonin Uptake (pmol/mL)
Time (min)
Empty Vector
OCT1
C.
[14C]-Metformin Uptake (pmol/mL)
Time (min)
Empty Vector
OCT1
D.
[14C]-TEA Uptake (nmol/mL)
Time (min)
Empty Vector
OCT1
E.
[3H]-MPP+ Content (% of Control)
Time (min)
Control
Unlabeled MPP
F.
[3H]-Serotonin Content (% of Control)
Time (min)
Control
Unlabeled 5-HT
G.
[14C]-Metformin Content (% of Control)
Time (min)
Control
Unlabeled Metformin
H.
[14C]-TEA Content (% of Control)
Time (min)
Control
Unlabeled TEA

Figure 3.2. Time-dependent uptake and efflux of MPP^+, serotonin, metformin, and TEA by hOCT1. To establish the time when hOCT1 transport reaches steady-state, HEK-EV and -hOCT1 cells were incubated with radiolabeled (A) MPP^+ (0.4 µCi/mL, 4.7 nM), (B) serotonin (0.4 µCi/mL, 14 µM), (C) metformin (0.2 µCi/mL, 2 µM), or (D) TEA (0.2 µCi/mL, 54 µM) at room temperature for 15 seconds to 180 minutes. Once steady-state was determined, HEK-hOCT1 cells were pre-loaded with (E) MPP^+ (0.4 µCi/mL, 4.7 nM), (F) serotonin (0.4 µCi/mL, 14 µM), (G) metformin (0.2 µCi/mL, 2 µM), or (H) TEA (0.2 µCi/mL, 54 µM), for 90 minutes to reach equilibrium, at which point CCF (efflux) was initiated by adding unlabeled MPP^+ (100 µM), serotonin (1 mM), metformin (10 mM), or TEA (1 mM) to each well and incubated for 15 seconds to 10 minutes (2 minutes for MPP^+). Each condition was done in triplicate and the mean ± SD of three independent experiments is shown.

was performed with all four substrates, using unlabeled MPP^+, serotonin, metformin, and TEA as the efflux solution for their respective pre-load substrates. As can be seen in Figure 3.2E-H, maximum efflux was reached by 30 seconds for MPP^+, and 5 minutes for serotonin and TEA. Interestingly, unlabeled metformin did not induce efflux of radiolabeled metformin at any time point measured (Figure 3.2G). This may suggest a metformin "sink" within the cell. In any case, because of this, metformin was excluded as a probe substrate for further CCF studies, and 30 seconds (MPP^+) and 5 minutes (serotonin and TEA) were used as CCF efflux time points.

For CCF, several known hOCT1 substrates and inhibitors were employed as test compounds, as well as some compounds which haven't been tested for interactions with hOCT1. MPP^+, serotonin, metformin, and TEA were used as positive controls for efflux, while glucose and bromosulfophthalein (BSP) were selected as negative controls, as glucose does not interact with hOCT1 and as a negatively-charged molecule, BSP is not expected to interact. A "cut-off" value for efflux was set at 80% substrate content to distinguish substrates from non-substrates.

Because the maximum efflux varied for each of the pre-load substrates, as seen in Figures 3.2E-G and 3.3A, a correction factor for CCF efflux values was necessary to compare the efficiency of efflux elucidated by the test compounds for each probe substrate. The data shown in Figure 3.3B were corrected for the observed differences in maximum efflux between probe substrates by setting the observed value of substrate content when TEA and thiamine were employed as test compounds as the maximum efflux for each respective probe substrate. This factor effectively changed the previously

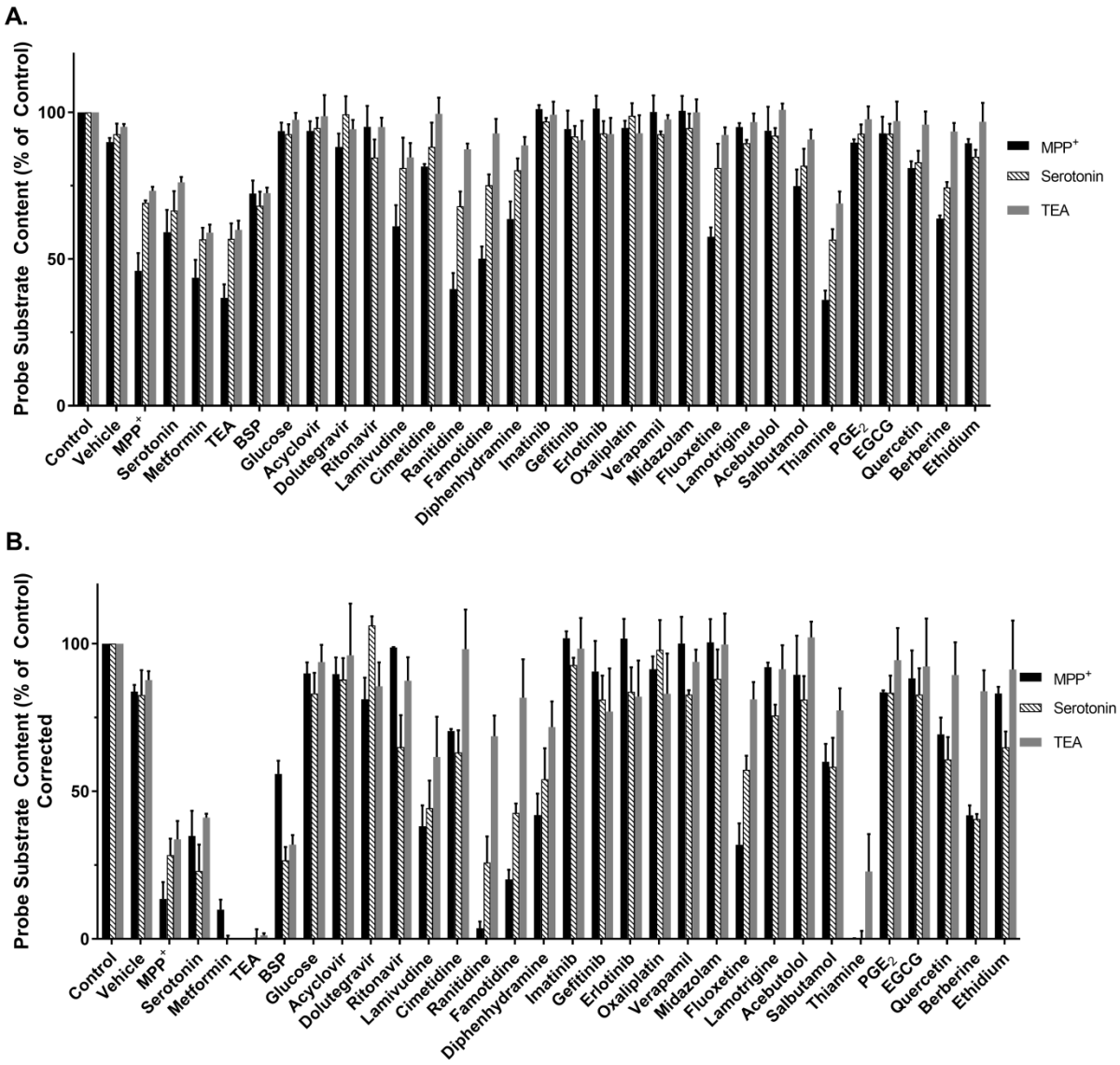

Figure 3.3. Substrate-dependent ligand interactions as determined by competitive counterflow. HEK-hOCT1 cells were pre-loaded with radiolabeled MPP^+ (0.4 µCi/mL, 4.7 nM; black bars), serotonin (0.4 µCi/mL, 14 nM; hatched bars), or TEA (0.05 µCi/mL, 14 µM; grey bars) for 90 minutes to reach equilibrium. CCF was initiated by adding unlabeled test compounds at concentrations approximately equal 10-times the published K_m values for hOCT1 (see Table 3.1 for concentrations used) to the respective wells. CCF was measured for 30s when MPP^+ was employed as the pre-load substrate, and 5 minutes when serotonin and TEA were used. Raw data are shown in panel (A).The data shown in (B) corrects for the observed differences in maximum efflux between pre-load substrates. Each condition was done in triplicate and the mean ± SD of three independent experiments is shown.

assigned “cut-off” value from 80% (uncorrected data, Figure 3.3A) to 65% (corrected data, Figure 3.3B).

As expected, MPP^+, serotonin, metformin, and TEA induced efflux of all three probe substrates, as did thiamine (Figure 3.3). Interestingly and unexpectedly, BSP also induced efflux of all three substrates, indicating that BSP may be a substrate of hOCT1. Lamivudine, ranitidine, famotidine, diphenhydramine, fluoxetine, and berberine all induce efflux of radiolabeled MPP^+ and serotonin, but not TEA (Figure 3.3). The remaining test compounds did not induce efflux of any of the probe substrates.

3.3.3 Characterization of hOCT1-mediated Bromosulfophthalein Uptake

To confirm the results of the CCF assay, which suggested that BSP is a substrate of hOCT1, direct uptake studies using [^{3}H]-BSP were performed. Time-dependent uptake studies using 0.1 µM and 50 µM BSP demonstrated an increase of uptake into HEK-hOCT1 cells over HEK-EV cells by five minutes (Figure 3.4A-B), indicating that BSP is indeed a substrate for hOCT1. Kinetics analysis was then performed to further characterize BSP transport by hOCT1 (Figure 3.4C). Michaelis-Menten analysis yielded a K_m of 13.6 ± 2.6 µM and V_{max} of 55.1 ± 4.1 pmol/mg protein/min for BSP.

3.3.4 Computational Modeling of Ligand Interactions

To further investigate the observed interactions in the CCF assay, we constructed a hOCT1 homology model and performed *in silico* docking of several of the employed test compounds. The hOCT1 homology model generated by BIOVIA Discovery Studio’s UCSF Modeller automodel plug-in produced 9 helical TMD regions with an inward facing conformation (Figure 3.5), similar to the original GlpT structure. Previous

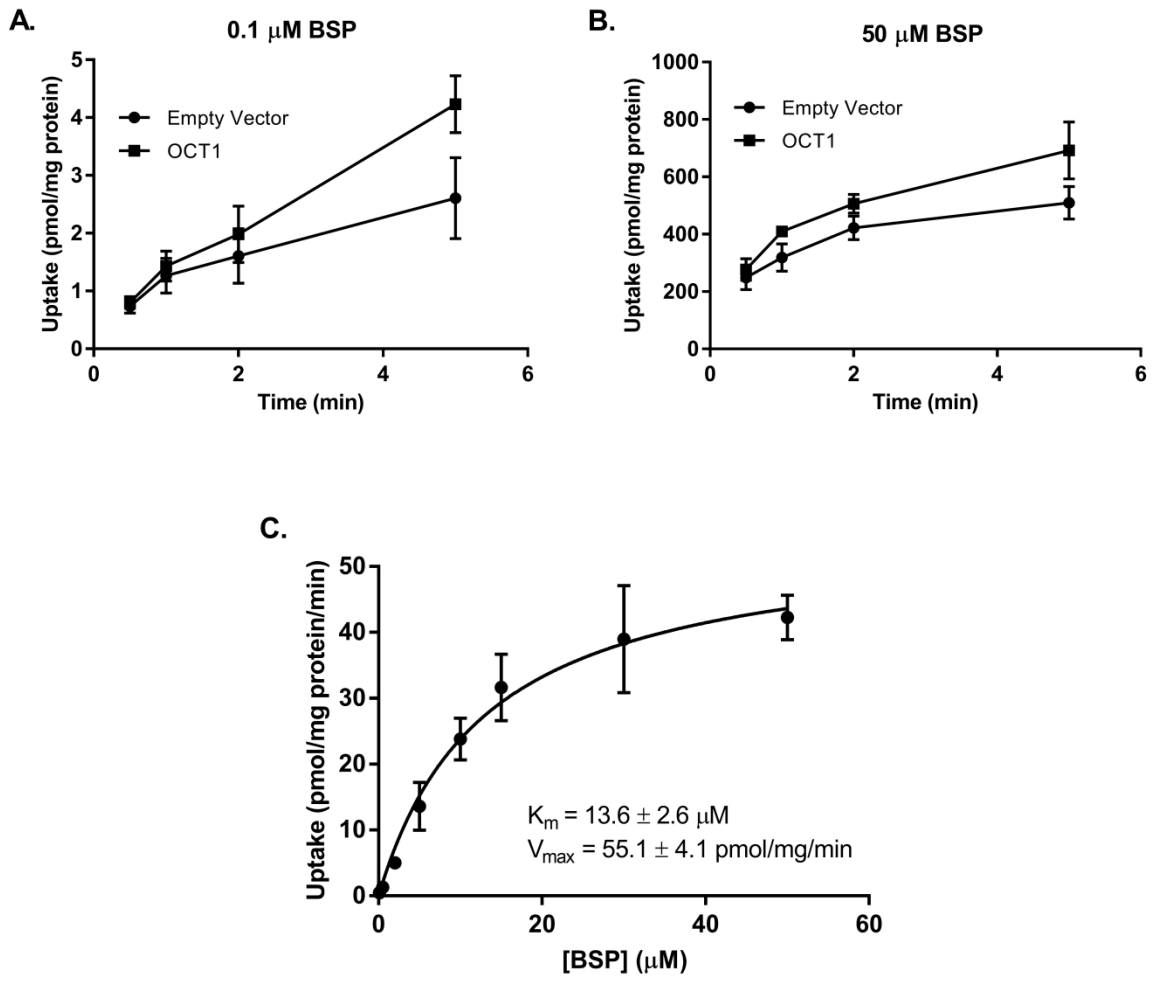

Figure 3.4. Bromosulfophthalein is a substrate of hOCT1. HEK-EV and -hOCT1 cells were incubated in the presence of (A) 0.1 μM (0.3 μCi/mL) or (B) 50 μM (0.9 μCi/mL) radiolabeled BSP for increasing amounts of time to demonstrate time-dependent transport. To evaluate the kinetic parameters of BSP transport, (C) HEK-EV and -hOCT1 cells were incubated with increasing concentrations of radiolabeled BSP, ranging from 0.1 μM (0.3 μCi/mL) to 50 μM (0.9 μCi/mL), for 2 minutes at 37ºC. Net uptake, obtained by subtracting uptake in empty vector cells from that of hOCT1-expressing cells, was fit to the Michaelis-Menten equation to obtain a K_m of 13.6 ± 2.6 μM and V_{max} of 55.1 ± 4.1 pmol/mg protein/min. Each condition was completed in triplicate and the plotted values ± SD are the result of three independent experiments.

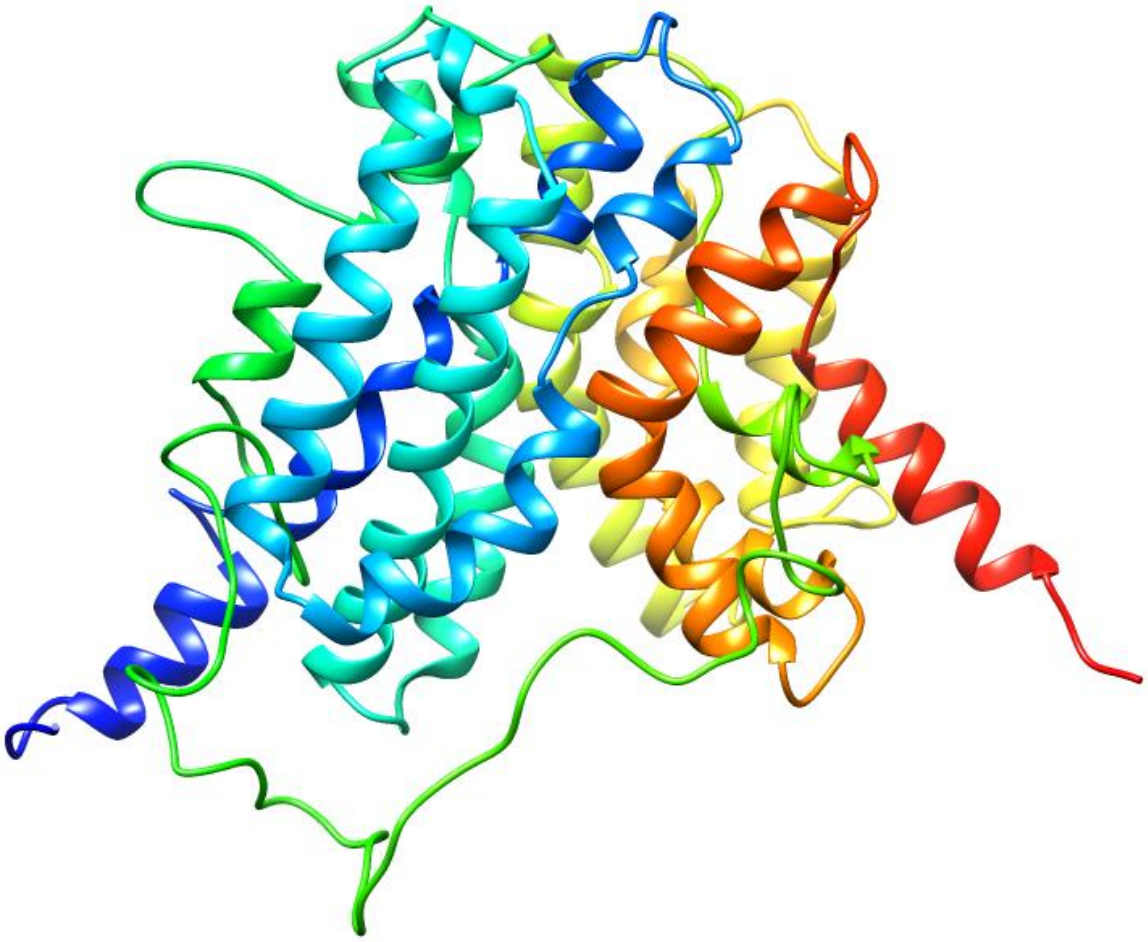

Figure 3.5. Homology model of hOCT1. A homology model for hOCT1 was constructed using BIOVIA Discovery Studio's UCSF Modeller. Chain A of the GlpT (*E.coli*) crystal structure served as the input template from which this model was built. The model excludes the extracellular loop between TMD 1 and 2, and the intracellular loop between TMD 6 and 7. The model is depicted here colored by helix from N-terminus (blue) to C-terminus (red).

homology models have been constructed using both the *E. coli* lactose permease, LacY (Popp, et al., 2005), and more recently, the high affinity phosphate transporter from *Piriformospora indica*, PiPT (Chen, et al., 2017). Our rationale in choosing the GlpT protein was that it, and PiPT, are more functionally similar to hOCT1 in that they both transport ions that are charged at physiological pH (cations in the case of hOCT1, and anions in the cases of GlpT and PiPT), in contrast to LacY, which transports uncharged lactose and closely-related analogues. The structural model of hOCT1 was well resolved and overall closely resembled the tertiary structure of GlpT. In particular, residues W218, Y222, and T226 are all located in TMD4, with their respective side chains pointed toward the interior of the translocation channel. These residues were previously implicated in substrate translocation via site-directed mutagenesis experiments (Gorboulev, et al., 2005). Similarly, I443, I447, and Q475, which are also implicated in substrate translocation (Gorboulev, et al., 1999), are located in TM helix 10, again near the putative active site cavity. These results increased our confidence in the accuracy of the homology model for use as a docking template.

In order to understand the structural implications of multiple ligand modulation of hOCT1 transport activity, we used our constructed hOCT1 homology model as a receptor template to dock several of the more interesting drugs that demonstrated significant differences in the CCF assay. The majority of compounds tested in the CCF assay (MPP^+, serotonin, TEA, metformin, BSP, acyclovir, ritonavir, lamivudine, cimetidine, famotidine, imatinib, fluoxetine, lamotrigine, thiamine, prostaglandin E_2, quercetin, berberine, and ethidium) were docked into the homology model using UCSF DOCK 6.7. Figure 3.6A illustrates all of the ligand structures docked into the homology

Figure 3.6A

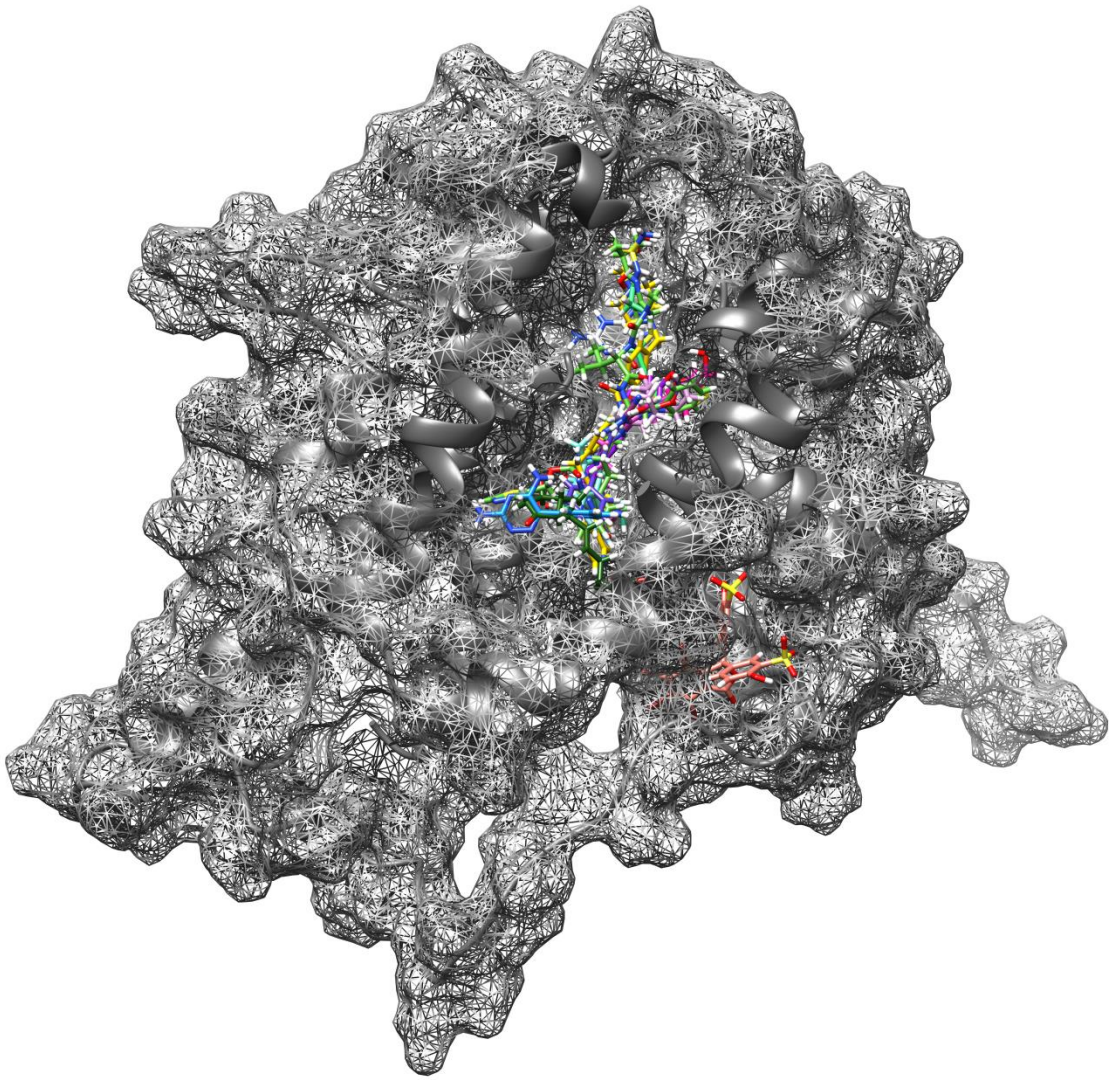

Figure 3.6B

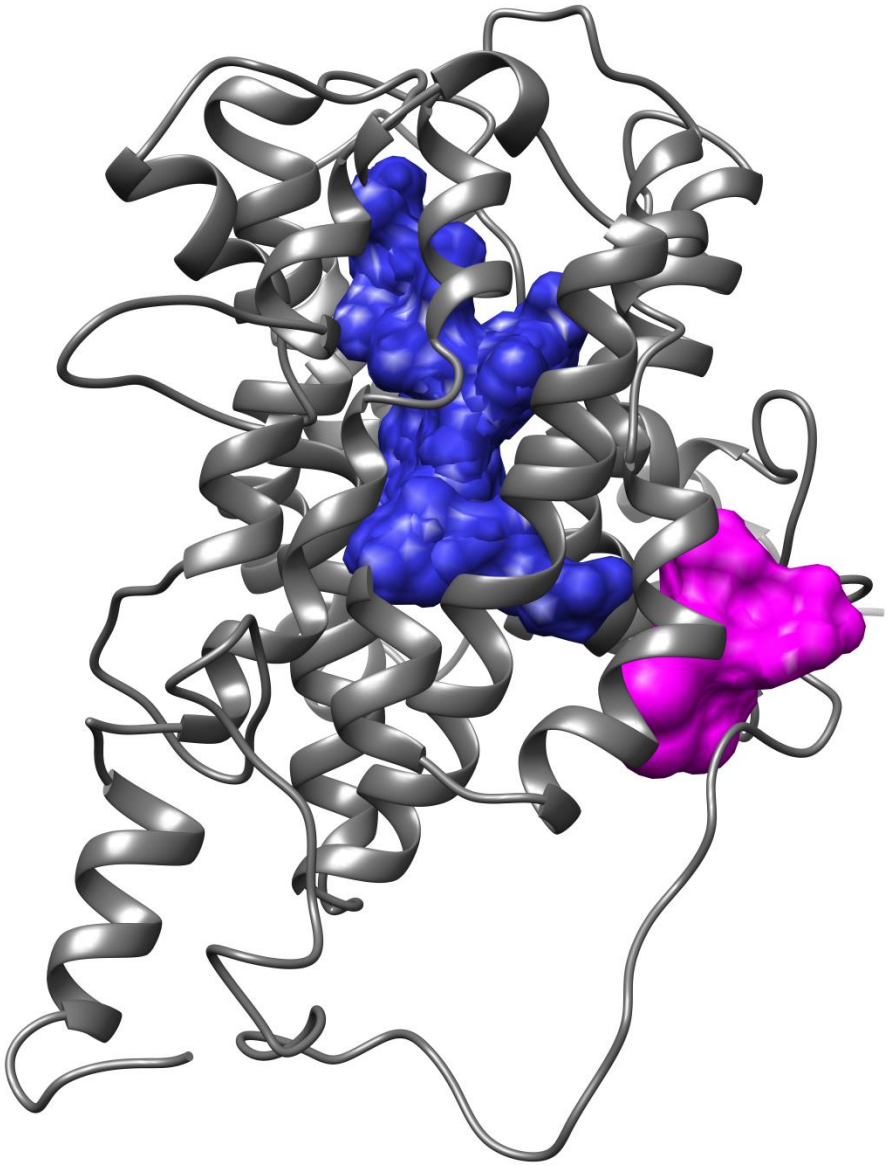

Figure 3.6. Ligand structures docked into hOCT1 homology model. The chemical structures of ligands yielding interesting results in the CCF assay were docked into a hOCT1 homology model using UCSF DOCK. These ligands included: MPP^+, serotonin, metformin, TEA, BSP, acyclovir, ritonavir, lamivudine, cimetidine, famotidine, imatinib, fluoxetine, lamotrigine, thiamine, PGE_2, quercetin, berberine, and ethidium. (A) The chemical structures are shown in multiple colors within the pore of the transporter, which is shown as a grey mesh of the van der Waals surface of the transporter. (B) The van der Waal's surfaces of the docked ligands are illustrated magenta (BSP) and blue (remaining ligands docked), and the backbone structure of protein shown in grey. The equivalent of one helix was removed from these graphics to better visualize the ligand structures. With the exception of BSP, all 19 ligands included in the docking study docked within a central pore of the transporter.

model, exhibited as a mesh of the protein surface. All ligands, with the exception of BSP, docked within the putative translocation pore, in close proximity to residues that are crucial for transporter-ligand interactions (e.g., W218, Y222, T226, I443, I447, and Q475), further enhancing our confidence in the structural validity of the hOCT1 homology model. This can be viewed more clearly in Figure 3.6B, which reveals putative binding pockets within the transporter (grey ribbon) shown as the van der Waals surfaces of all the ligand structures docked.

Interestingly, most docked ligands, with the exception of BSP, appeared to segregate into three categories: 1) those that docked in a binding site overlapping with MPP^+, 2) those that docked in a binding site overlapping with TEA, and 3) those that overlapped with the binding site of acyclovir. BSP was an outlier from all the other ligands in that it bound to the hOCT1 receptor outside the confines of the translocation channel (Figure 3.6), likely due to its inherent negative charge, and, therefore, was not considered further. These observations led us to identify three distinct, but not mutually exclusive, ligand binding sites within the substrate translocation channel: the "MPP^+ binding pocket", the "TEA binding pocket", and the "acyclovir binding pocket". These separate binding pockets are illustrated in Figure 3.7, as both the chemical structures of ligands docked within the ribbon structure (Figure 3.7A,C), and the van der Waal's surface of residues lining the binding regions (Figure 3.7B,D). These pockets were defined by residues Y240, Q241, F244, E386, I446, S470, and C473 ("MPP^+ pocket"), A33, C36, V359, L360, C473, D474, G477, and I478 ("TEA pocket"), and Q362, G363, N374, L377, D378, Y381, and A383 ("acyclovir pocket"), all located within 3Å of the ligand. Principal component analysis was performed comparing the ligands docked

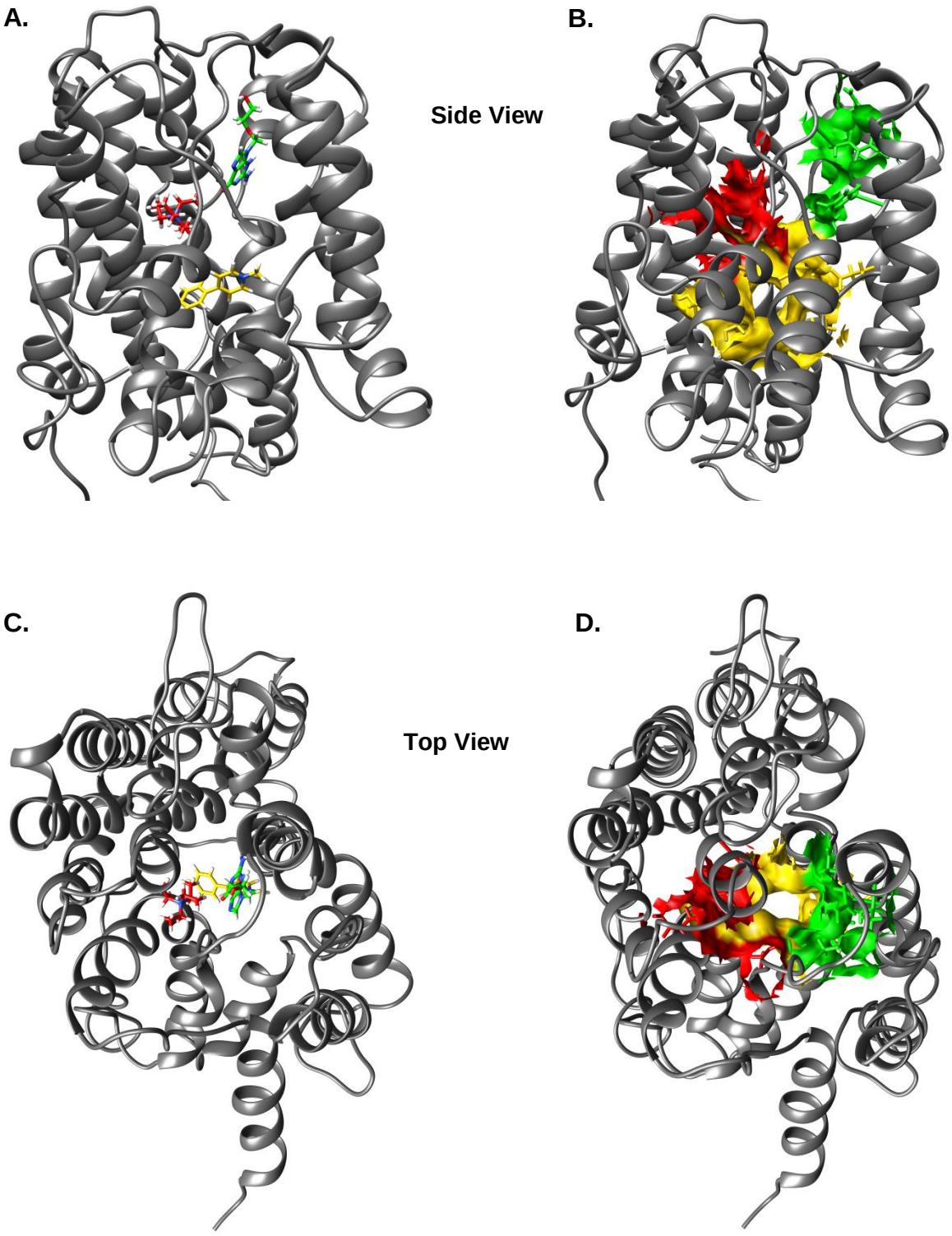

Figure 3.7. Ligands docked in disparate pockets within hOCT1 homology model. In general, all ligands docked within one of three separate "binding pockets". These pockets are demonstrated here by (A,C) the chemical structures and (B,D) the van der Waal's surface of amino acid residues lining the binding pockets of MPP^+ (yellow), TEA (red), and acyclovir (green). A portion of one helix was removed from (A) to better visualize the MPP^+ chemical structure.

within each pocket and various ligand-specific properties which identified trends for each binding site. These aspects are addressed further in the Discussion.

3.4 DISCUSSION

Substrate-dependent interactions have been confirmed for several drug transporters, including close relatives of hOCT1 (Belzer, et al., 2013, Martínez-Guerrero and Wright, 2013, Noe, et al., 2007, Roth, et al., 2011), and as discussed in the introduction, a number of studies suggest substrate-dependent effects for hOCT1. This study identified substrate-dependent interactions for several hOCT1 ligands and employed computer modeling simulations to predict the structural basis underlying potential mechanisms.

The CCF assay employed in this study was originally developed to identify novel substrates (Harper and Wright, 2013). As discussed previously, for the purpose of our study, a “cut-off” value of 80% efflux for the uncorrected data (Figure 3.3A) or 65% for corrected values (Figure 3.3B) was used to distinguish substrates from non-substrates. Based on the CCF results, MPP^+, serotonin, metformin, TEA, BSP, lamivudine, ranitidine, famotidine, diphenhydramine, fluoxetine, thiamine, and berberine were all identified as substrates, given that they induced efflux of at least one radiolabeled probe substrate. MPP^+, serotonin, metformin, TEA, lamivudine, ranitidine, famotidine, thiamine, and berberine were all previously reported as substrates (Bourdet, et al., 2005, Boxberger, et al., 2014, Chen, et al., 2014, Gorboulev, et al., 1997, Jung, et al., 2008, Kimura, et al., 2005, Nies, et al., 2008). This study confirms their status as such. However, the CCF assay also identified the negatively-charged BSP, as well as

diphenhydramine and fluoxetine as novel substrates of hOCT1. Diphenhydramine and fluoxetine have been reported to interact with hOCT1 in the past (Boxberger, et al., 2014, Haenisch, et al., 2012, Muller, et al., 2005). However we are the first to demonstrate that BSP, a molecule which possesses two negative charges at physiological pH, interacts with and is transported by hOCT1. The importance of this novel substrate will be discussed further below.

Acyclovir, ritonavir, cimetidine, imatinib, gefitinib, erlotinib, oxaliplatin, verapamil, midazolam, lamotrigine, acebutolol, salbutamol, prostaglandin E_2, EGCG, quercetin, and ethidium did not elicit significant efflux (Figure 3.3). Interestingly, acyclovir, imatinib, oxaliplatin, lamotrigine, salbutamol, prostaglandin E_2, quercetin, and ethidium have all been reported as substrates (Dickens, et al., 2012, Glaeser, et al., 2014, Kimura, et al., 2002, Lee, et al., 2009, Salomon, et al., 2015, Takeda, et al., 2002, Thomas, et al., 2004, Yonezawa, et al., 2006). The fact that they did not induce efflux any of the radiolabeled probe substrates employed in the assay suggests that their interaction with hOCT1 is substrate-dependent.

Substrate-dependent interactions were observed with those test compounds which were determined to be substrates, as well. Several substrates induced efflux of radiolabeled MPP^+ and serotonin, but not TEA (Figure 3.3), indicating an interaction at the MPP^+ binding site but not the TEA pocket. And, even within this group of compounds, there appears to be variation in the magnitude of efflux between probe substrates. However, these differences cannot necessarily be attributed to differences in substrate affinities. Some of these interactions are corroborated by previous reports. For example, berberine, which induced significant efflux of radiolabeled MPP^+ and

serotonin, but not TEA, was a more potent inhibitor of MPP^+ than of TEA in a study by Nies, et al., (2008) characterizing berberine transport by hOCT1.

In this study, the CCF assay proved useful in identifying substrate-dependent modulation, though limitations exist. Due to the nature of the assay, wherein an extracellular substrate is exchanged with a cytoplasmic substrate, substrate-dependent effects cannot be observed for test compounds which are exclusively inhibitors, as they would not induce efflux of probe substrates. To determine substrate-dependent interactions with hOCT1 inhibitors, inhibition studies must be completed analyzing inhibitors versus multiple substrates. However, in this study, test compounds which had previously only been described as inhibitors (diphenhydramine and fluoxetine) and compounds which had never been reported and were not expected to interact with hOCT1 (BSP) were identified as novel substrates (Figure 3.3).

The observation that BSP induced efflux of any of the probe substrates, let alone all three (Figure 3.3), was particularly unexpected. BSP was selected as a negative control for hOCT1 CCF assays because it possesses a negative charge (-2). To date, hOCT1 was thought to transport only positively-charged or neutral compounds (Koepsell, 2015, Nies, et al., 2011b), but not negatively-charged compounds. Therefore, it was believed that the negative charge associated with BSP would prevent its interaction with hOCT1. However, as is evident in Figure 3.3, BSP induced efflux of each of the probe substrates, which suggests that it is a hOCT1 substrate. To confirm that BSP is a substrate, direct uptake was measured. HEK-hOCT1 cells demonstrated significant uptake of BSP compared to HEK-EV cells, and time-dependent uptake was linear over two minutes (Figure 3.4A-B). To further characterize BSP uptake, kinetics

analysis was completed. The K_m and V_{max} were determined to be 13.6 ± 2.6 µM and 55.1 ± 4.1 pmol/mg protein/min, respectively (Figure 3.4C), indicating that hOCT1 is a high affinity, but low capacity transporter of BSP. This appears to be the first negatively-charged substrate characterized for hOCT1, and the implications of this are noteworthy; perhaps the most significant: a negatively-charged substrate for hOCT1 may call into question its classification as a cation transporter and the mechanism of transport established shortly after its cloning (Gorboulev, et al., 1997, Zhang, et al., 1997b). More work must be done to elucidate the mechanism of transport of a negatively-charged substrate for hOCT1.

Interestingly, several test compounds previously reported as hOCT1 substrates, including imatinib, lamotrigine, and prostaglandin E_2, did not induce efflux of any probe substrate in the CCF assay (Figure 3.3). Controversy exists regarding the status of imatinib and prostaglandin E_2 as OCT substrates (Harlfinger, et al., 2005, Hu, et al., 2008, Kimura, et al., 2002, Nies, et al., 2014, Thomas, et al., 2004, White, et al., 2006). Our data may add further strength to the argument that imatinib and prostaglandins are not substrates for hOCT1. In our model, lamotrigine docked closest to the "MPP^+ binding site," however, it did not appear to overlap with any of the docked probe substrates (Figure 3.6). Therefore, it is possible that the lack of overlap causes a change in conformation within the translocation pathway that prevents exchange of probe substrates for CCF.

The observed interactions in the CCF assay could be attributed to differential binding regions for specific ligands. It has been proposed that hOCT1 contains multiple binding sites within the transporter (Chen, et al., 2017, Ciarimboli, et al., 2004,

Ciarimboli and Schlatter, 2005). As indicated above, our homology model appears to align closely with previously published models. Amino acids reported to interact with ligands in previous reports, including C36, F244, S358, I446, C473, D474, also outlined the binding regions identified in our docking studies. However, the model produced by Chen, et al., (2017) appeared to contain two distinct binding sites, while our model suggests a third binding site. This discrepancy may be due, in part, to the fact that Chen, et al., (2017) pre-determined the two putative binding sites and subsequently docked ligands into both sites, selecting the site which yielded the most favorable score as the binding site for that ligand. However, our docking studies allowed for docking ligands anywhere within the transporter, and therefore allowed for the distinction of three binding pockets.

As discussed previously, MPP^+, TEA, and acyclovir docked within the translocation pore, but in disparate locations (Figure 3.7). Accordingly, we termed these pockets the "MPP^+ binding pocket," the "TEA binding pocket," and the "acyclovir binding pocket." A majority of the test compounds docked seemed to dock within one of these pockets, with some overlap. Many of the docked substrates docked in or around the MPP^+ binding pocket, a handful docked within the TEA and acyclovir pockets, and two ligands, imatinib and ritonavir, spanned the entire translocation pore. Principal component analysis indicated that the MPP^+ pocket allows for greater flexibility in ligand binding, as there were no specific trends in docked-ligand properties, other than a tendency to be smaller in size and ionizable, trends that held true for both endogenous ligands and drugs examined in this study. Conversely, ligands docked in the acyclovir pocket tended to be larger, more hydrophobic and flexible (based on the number of

rotatable bonds) molecules. No trends emerged when relating ligand properties to the TEA pocket; however, interestingly, all of the ligands which docked in or near the TEA, berberine, cimetidine, and lamivudine, did not induce any efflux of radiolabeled TEA in the CCF assay, even though they are substrates of hOCT1 (Hendrickx, et al., 2013, Minuesa, et al., 2009, Nies, et al., 2008). This may suggest an occlusion of this site, either by TEA or the test compounds, which prevents substrate exchange. Ligands that spanned both the TEA and MPP^+ binding sites (imatinib and ritonavir) tended to be larger ligands dominated by H-bond donors, with a high clogP and a large number of rotatable bonds. Notably, a similar GRID docking score was associated with ligands that occupied both the MPP^+ and TEA pockets. The fact that these molecules effectively spanned the entire translocation pore may explain why they function more as inhibitors of hOCT1 rather than substrates, as recent reports suggest is the case for imatinib (Hu, et al., 2008, Nies, et al., 2014). BSP was an outlier in all cases, largely due to the nature and location of its unique binding pocket, lying outside of the translocation channel.

While the number of test compounds employed in this study is small and doesn't allow for development of concrete conclusions, it could be considered a proof of principle study, and as such, certain trends were uncovered which can be followed-up on in larger future studies. Despite the small sample size, clear substrate-dependent interactions were observed in our CCF assay, confirming substrate-dependent effects for hOCT1. The observed substrate-dependent effects strengthen the argument for investigating multiple probe substrates in preclinical transport studies to improve identification of potentially dangerous drug-drug interactions.

CHAPTER 4 : GENERATION OF HUMAN/RAT CHIMERIC OCT1 PROTEINS TO EXAMINE THE ROLE OF THE EXTRACELLULAR LOOP DOMAIN IN SUBSTRATE AFFINITY AND SPECIFICITY

4.1 INTRODUCTION

Organic cation transporters (OCTs) are a subset of the *SLC22* superfamily of transporters which mediate the uptake of a wide variety of structurally dissimilar charged and uncharged compounds, and as such play a pivotal role in absorption, disposition, and excretion of endogenous and exogenous molecules (Koepsell, 2013). OCT1, a member of this family, was first identified in rat kidney, and shortly thereafter in human liver (Gorboulev, et al., 1997, Grundemann, et al., 1994, Zhang, et al., 1997b). Since their cloning, many substrates have been identified for both rat and human OCT1, and there is a wide overlap in transported substrates (Dresser, et al., 2001, Jonker and Schinkel, 2004, Koepsell, et al., 2003, Koepsell, et al., 2007). However, despite the similarities in amino acid sequence between the two transporters, significant differences in substrate affinity have been observed (Table 4.1).

Because of the diverse chemical structures of substrates transported by OCTs, the mechanism of substrate recognition, binding, and translocation has been of particular interest to the field. Putatively, OCTs contain twelve alpha-helical transmembrane (TM) domains, with a large extracellular loop domain situated between TM 1-2 and an intracellular loop located between TM 6-7 (Meyer-Wentrup, et al., 1998). Recent publications described a potential role for the extracellular loop domain of OCT1 in substrate affinity and translocation. Keller et al., (2011) demonstrated a decrease in affinity and transport efficiency of multiple substrates when mutations were introduced within the extracellular loop of rat OCT1. Furthermore, Kerb, et al., (2002) functionally characterized multiple human OCT1 mutations which found in the general population.

Table 4.1. Affinities of select substrates of rat and human OCT1.

Substrate	Affinity (K_m [μM])		References
	Human OCT1	Rat OCT1	
MPP^+	35	9.6	Boxberger, et al., 2014, Busch et al., 1996b
TEA	168, 229	95	Bednarczyk, et al., 2003, Nies, et al., 2011b, Otsuka, et al., 2005, Grundemann, et al., 1994
Serotonin	197	37.6	Boxberger, et al., 2014, Busch, et al., 1996a
Dopamine	(>Serotonin)[a]	19.4	Boxberger, et al., 2014

[a] Transport of dopamine by human OCT1 was demonstrated by Boxberger, et al., (2014), however transport levels were too low for accurate determination of affinity.

Their study identified two mutations, R61C and C88R, located in the extracellular loop domain of human OCT1, that negatively impact the transport of model cation MPP^+. Taken together, these studies suggest that the extracellular loop region of OCT1 may indeed play a role in substrate binding and initiating translocation, and this, therefore, may be the driving force behind the observed differences in substrate affinity between species.

Structural characterization of OCTs has thus far proven somewhat difficult. Because of the largely hydrophobic nature of the transmembrane protein, OCTs have yet to be successfully crystalized, making determination of binding regions nearly impossible. In lieu of utilizing crystal structures for identifying key binding regions in drug transporters, homology modeling and mutagenesis techniques have been employed. One such method of exploring key binding regions within a transporter is the generation of chimeric transporters. In 2009, Gui and Hagenbuch developed mutagenic chimeras of organic anion transporting polypeptides (OATP) 1B1 and 1B3, members of the *SLCO* family, to identify TM 10 as a critical region for the function of OATP1B1. Furthermore, Gorboulev, et al., (2005) generated rat OCT1/OCT2 chimeras, and were able to demonstrate that exchanging three key amino acids in TM 10 raised the affinity of rOCT1 for corticosterone to that of rOCT2. By the same token, human and rat OCT1 loop chimeras could be employed to examine the role of the extracellular loop domain in substrate recognition, binding, and translocation, and determine if the loop domain is, at least in part, responsible for the observed differences in substrate affinity between rat and human OCT1. In this study, we aimed to generate rat and human OCT1

extracellular loop chimeras, as depicted in Figure 4.1, to functionally characterize the role of the loop region in substrate affinity and translocation.

4.2 MATERIALS AND METHODS

4.2.1 Materials

The rat OCT1 ORF, cloned into pCMV6-Entry expression vector, was purchased from Origene (Rockville, MD). The TOPO TA cloning kit, Taq polymerase, T4 DNA ligase, and all chemically competent bacteria were purchased from Invitrogen (Carlsbad, CA). Loading buffers, restriction enzymes, and Phusion-HF polymerase were purchased from New England Biolabs (Ipswich, MA). DNA ladders, dNTPs, and alkaline phosphatase were purchased from Fermentas (Waltham, MA). QuikChange Site-Directed Mutagenesis kit and *Pfu* Turbo polymerase were purchased from Agilent Technologies (Santa Clara, CA). LB agar powder and ethidium bromide were purchased from Fisher Scientific (Hampton, NH). LB broth powder, tris-acetate-EDTA (TAE), and tris-borate-EDTA (TBE) buffers were purchased from Sigma-Aldrich (St. Louis, MO). All antibiotics were purchased from Amresco, LLC (Cleveland, OH). Agarose was purchased from Bio-Rad (Hercules, CA). Gel purification kits and PCR clean-up kits were purchased from Qiagen (Valencia, CA). Isopropyl β-D-1- thiogalacto-pyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). X-gal was purchased from Bioline Reagents, Ltd. (Taunton, MA).

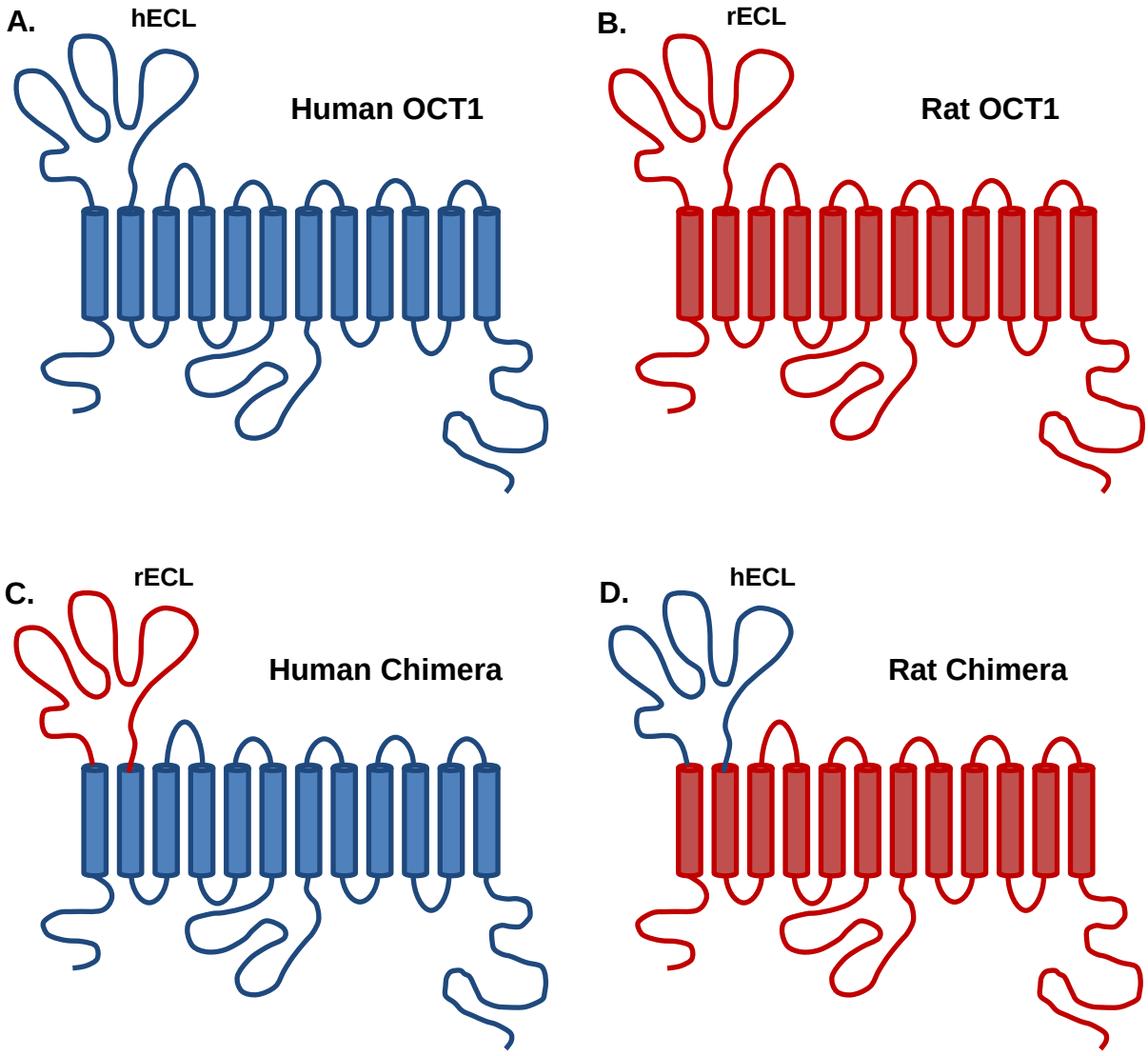

Figure 4.1. Schematic representation of human and rat OCT1 chimeric proteins. In order to examine the role of the extracellular loop (ECL) domain in substrate recognition, binding, and transport, chimeric human (C) and rat (D) OCT1 proteins were designed from human (A) and rat (B) wild-type OCT1 proteins by exchanging the ECL regions. For the purpose of these studies, the extracellular loop domains are defined as amino acids 47-153 for rat OCT1, and 46-152 for human OCT1, as described by Grundemann, et al., (1994) and Gourbulev, et al., (1997), respectively.

4.2.2 Primer Design

Primer sequences were designed based on the *SLC22A1* coding sequences for human (NCBI reference sequence: NM_003057.2) and rat (NCBI reference sequence: NM_012697.1) using the BioEdit sequence alignment tool, and the Agilent Genomics BioCalculators T_m calculator. All primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Primer sequences are listed in Table 4.2.

4.3 RESULTS

4.3.1 Generation of Human and Rat OCT1 Chimeric Proteins

In order to determine if disparities observed in substrate affinities between human and rat OCT1 are indeed due to the extracellular loop domains, human/rat OCT1 chimeric proteins needed to first be developed. Figure 4.2 depicts a schematic of the proposed generation of both human and rat OCT1 chimeric transporters. The method proposed for mutation of wild-type human and rat OCT1 was overlap extension PCR, adapted from Protocol 6 in Sambrook and Russell's *Molecular Cloning*, volume 2 (2001). Looking forward, however, it would be necessary to accurately quantitate and compare expression levels of both chimeras and wild-type transporters. Thus it was necessary to introduce an affinity tag to each construct before generating the chimeric proteins. Addition of an affinity tag, such as a polyhistidine-tag, would allow for quantification of the expressed transporters, via Western blot detection, using a single primary antibody that detects the affinity tag. This removes the issue of comparing

Table 4.2. Oligonucleotide primer sequences for addition of C-terminal His-tag to wild-type human and rat OCT1.

	Oligonucleotide Name[a]	Sequence	Method
1	OCT1 SDM C-term His-tag Fwd	5'- CCC TCG GGC ACC CAC CAC CAC CAC CAC CAC CAC TGA CTC GAG TCT AGA -3'	QuikChange
2	OCT1 SDM C-term His-tag Rev	5'- TCT AGA CTC GAG TCA GTG GTG GTG GTG GTG GTG GGT GCC CGA GGG -3'	QuikChange
3	F-hOCT1 Cterm 6xHis (HindIII)	5'- TTA AAG CTT ATG GTG GAT GAC ATT CTG GAG CAG GTT GGG GAG -3'	Insert via restriction site/TOPO cloning
4	R-hOCT1 Cterm 6xHis (BamHI)	5'- TAT GGA TCC TCA GTG GTG GTG GTG GTG GTG GGT GCC CGA GGG TTC TG -3'	Insert via restriction site/TOPO cloning
5	F-rOCT1 Cterm 6xHis (HindIII)	5'- TTA AAG CTT ATG CCC ACC GTG GAT GAT GTC CTG GAG CAA GTT GGA GAG -3'	Insert via restriction site/TOPO cloning
6	R-rOCT1 Cterm 6xHis (BamHI)	5'- TAT GGA TCC TCA GTG GTG GTG GTG GTG GTG GGT ACT TGA GGA CTT GCC TG -3'	Insert via restriction site/TOPO cloning
7	F-hOCT1 Chis HindIII 6bp tail	5'- ATA TTA AAG CTT ATG CCC ACC GTG GAT GAC ATT CTG GAG CAG GTT GGG GAG -3'	Insert via restriction site (redesigned primers)
8	R-hOCT1 Chis BamHI 6bp tail	5'- TTA TAT GGA TCC TCAA GTG GTG GTG GTG GTG GTG GGT GCC CGA GGG TTC TG -3'	Insert via restriction site (redesigned primers)
9	F-rOCT1 Chis HindIII 6bp tail	5'- ATA TTA AAG CTT ATG CCC ACC GTG GAT GAT GTC CTG GAG CAA GTT GGA GAG -3'	Insert via restriction site (redesigned primers)
10	R-rOCT1 Chis BamHI 6bp tail	5'- TTA TAT GGA TCC TCA GTG GTG GTG GTG GTG GTG GGT ACT TGA GGA CTT GCC TG -3'	Insert via restriction site (redesigned primers)

[a] For an explanation of primer names, see text.

Figure 4.2A

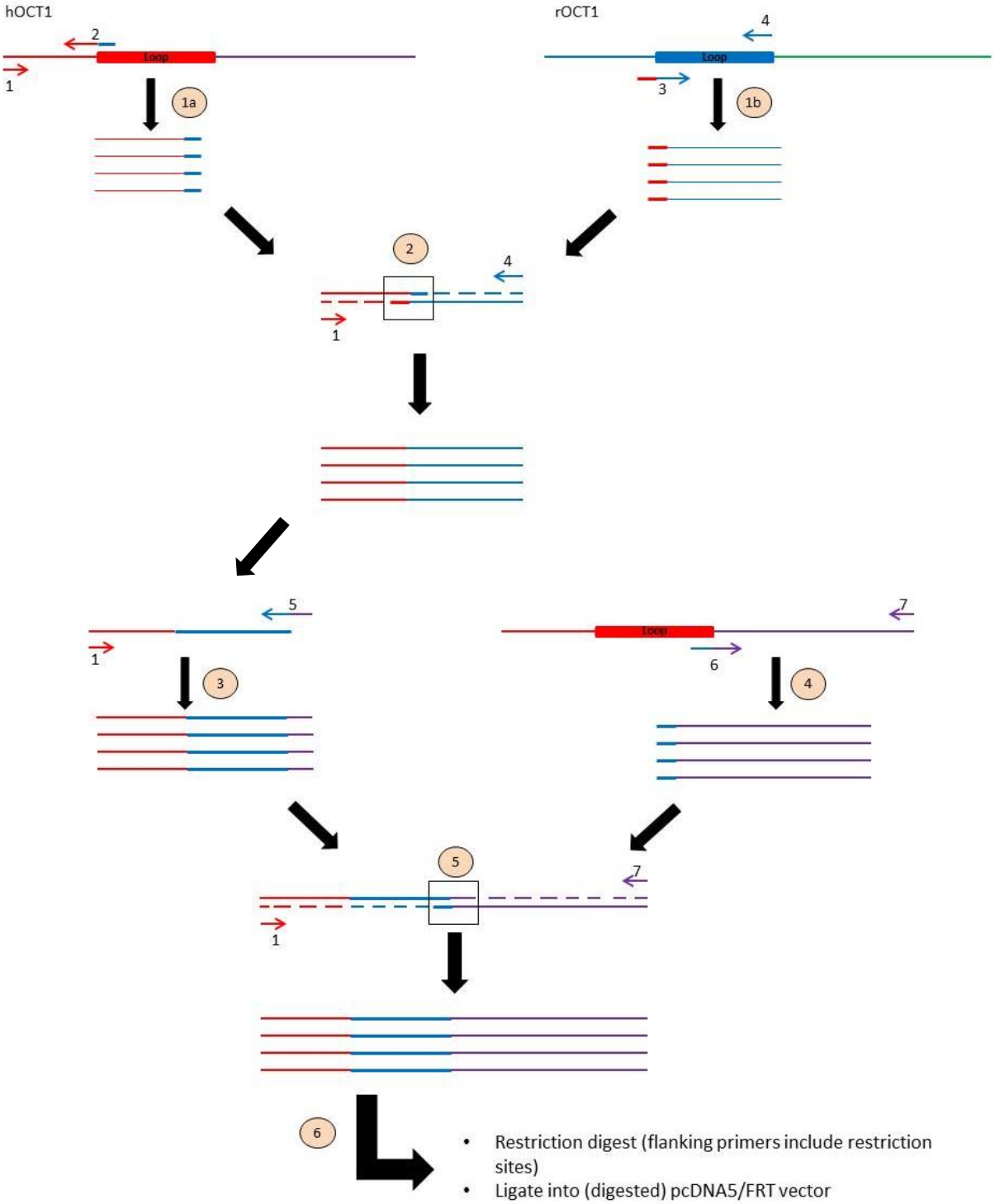
hOCT1
2
Loop
1
1a
rOCT1
4
Loop
3
1b
2
4
1
5
1
3
7
Loop
6
4
5
7
1
6
• Restriction digest (flanking primers include restriction sites)
• Ligate into (digested) pcDNA5/FRT vector

Figure 4.2B

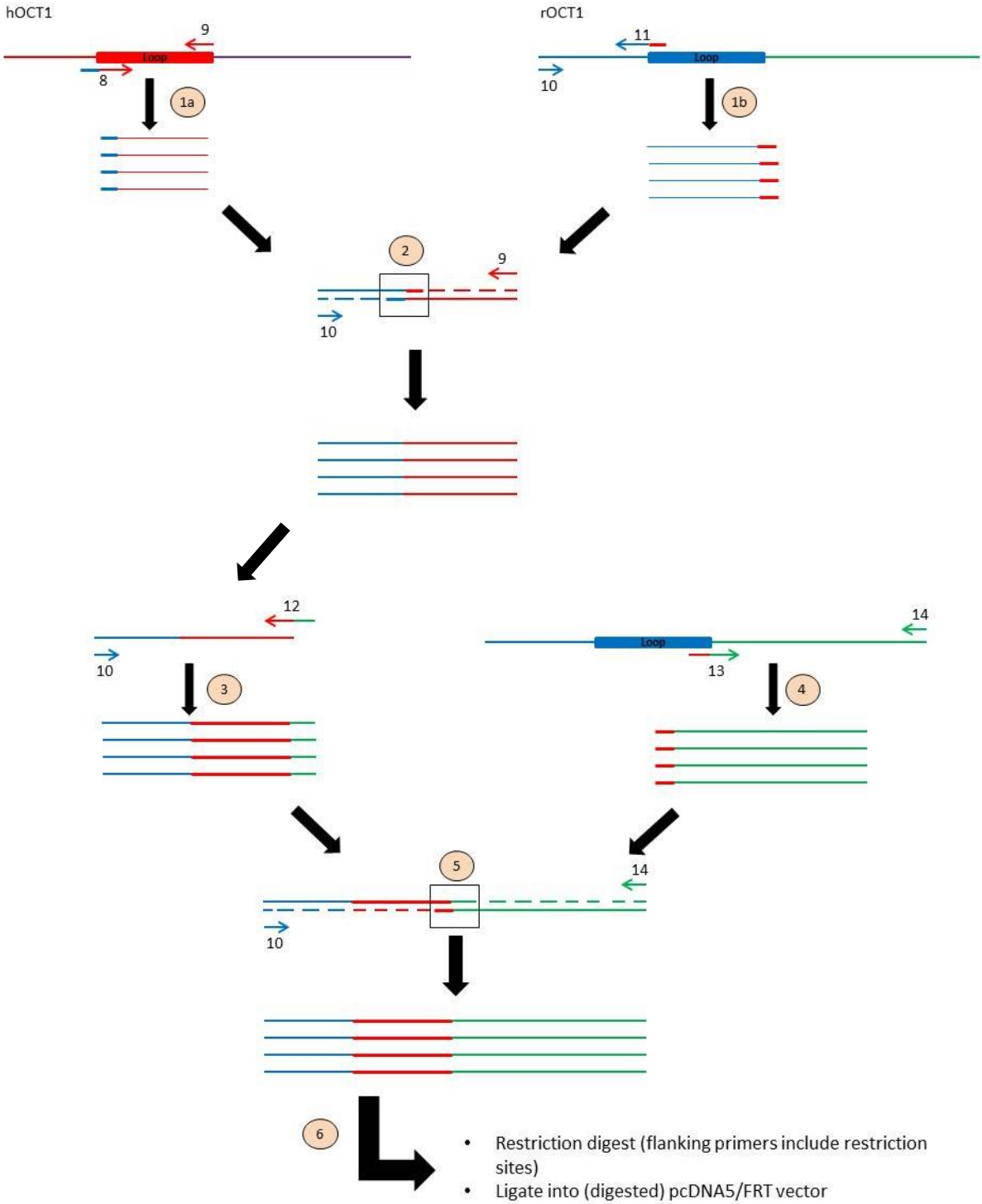
hOCT1
9
Loop
8
1a
rOCT1
11
Loop
10
1b
2
9
10
12
10
3
Loop
14
13
4
5
14
10
6
• Restriction digest (flanking primers include restriction sites)
• Ligate into (digested) pcDNA5/FRT vector

Figure 4.2. Generation of human and rat OCT1 chimeric proteins. A schematic of the planned generation of human (A) and rat (B) extracellular loop chimeras. The scheme depicts mutations of the extracellular loop domains, defined as amino acids 47-153 for rat OCT1 (Grundemann, et al., 1994), and 46-152 for human OCT1 (Gourbulev, et al., 1997), of each transporter by the overlap extension PCR method. Primers 1 (A) and 10 (B) match the beginning sequence of the first transmembrane (TM) domain of their respective transporters. Primers 2 and 11 consist of a sequence matching the end of TM1 of their respective proteins, followed by a sequence which overlaps with the loop region of the opposite protein. Performing PCR with these primers (step 1a) yields a DNA sequence corresponding TM1 of one transporter and an overlapping region of the loop domain of the other transporter. Primers 3 and 8 consist of sequences matching the beginning of the loop domain of one transporter, preceded by a sequence which overlaps with the end of TM1 of the opposite protein. Primers 4 and 9 contain sequences that match the end of the loop region for their respective transporters. PCR products resulting from these primers (step 1b) consist of a small portion of the end of TM1 from one transporter, and the complete loop domain of the other. Combining these two PCR products, and melting, reannealing, and extending them (step 2) yields DNA sequences containing the complete TM1 of one transporter followed by the complete loop domain of the other transporter. Similar processes are performed to combine the previous DNA product with TMs 2-12 (steps 3-5). Following generation of the mutant sequences, restriction digest (primers 1,7,10, and 14 contain added 5' restriction sites) and ligation into pcDNA5/FRT vector are performed (step 6).

binding affinities for multiple primary antibodies. Based on success of cloning with other transporters (Gui and Hagenbuch, 2009), a polyhistidine-tag (6xHis-tag) was selected.

4.3.2 Insertion of His-tag into Human and Rat OCT1 DNA Sequences

Several methods were employed to introduce a His-tag into wild-type human and rat OCT1. The first method involved the QuikChange II Site-Directed Mutagenesis kit (AgilentTechnologies, Santa Clara, CA). Primers were designed to overlap with the end sequence of each wild-type protein, with an insertion of six repeating histidine codons between the end of the protein coding sequence and the stop codon (primers 1-2, Table 4.2). The codon 'CAC' was selected for histidine based on codon bias in mammalian expression systems (Lavner and Kotlar, 2005). PCR reactions with the QuikChange II kit did not yield a product of appropriate size (Figure 4.3). It is likely that the complementarity of overlapping primers led to primer-dimer formation, meaning the primers annealed to each other and not the DNA template as desired, and thus no product is visible within the size range observed. Following multiple attempts, this method was abandoned.

Addition of the His-tag by inclusion in PCR primers, followed by insertion into the expression vector via restriction sites was attempted next. Primers were designed for C-terminal His-tag insertion into both human and rat OCT1 (primers 3-4 and 5-6, Table 4.2). Primers included 5' HindIII and 3' BamHI restriction sites for subsequent subcloning into the pcDNA5/FRT expression vector. PCR product for both constructs (human/rat OCT1-Cterm-His) was successfully produced via this method (Figure 4.4), however subsequent attempts to digest with restriction enzymes, ligate into the

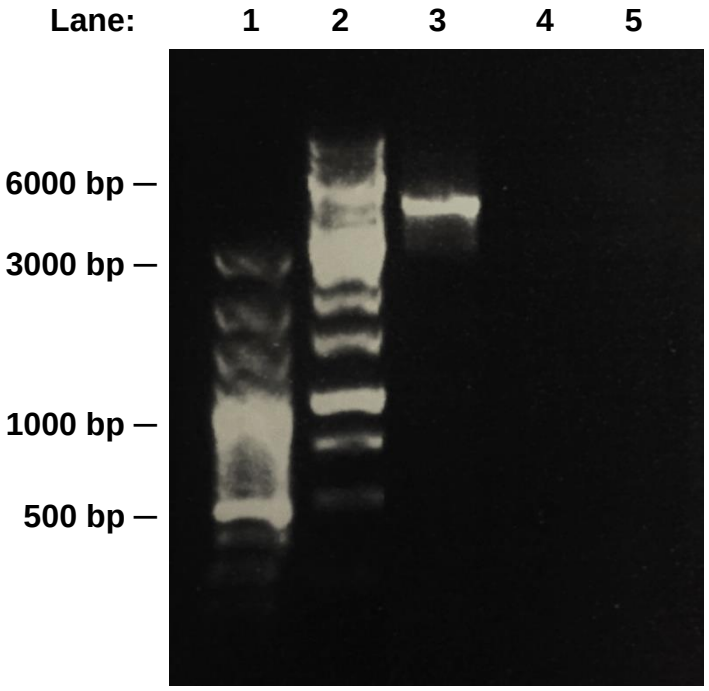

Figure 4.3. PCR products from QuikChange II Site-Directed Mutagenesis kit method. Products from PCR using primers and pWhitescript (control) template DNA from the QuikChange II kit, and primers 1-2 and human OCT1 WT DNA coding sequence template were separated by electrophoresis on a 0.8% agarose in TBE gel. Lane 1: 100 bp ladder. Lane 2: 1 kb ladder. Lane 3: pWhitescript control PCR products (expected amplicon size: 4.5 kb). Lane 4: hOCT1-C-term-His insertion PCR products (5 ng template DNA; expected amplicon size: 6.8 kb). Lane 5: hOCT1-C-term-His insertion PCR products (20 ng template DNA; expected amplicon size: 6.8 kb).

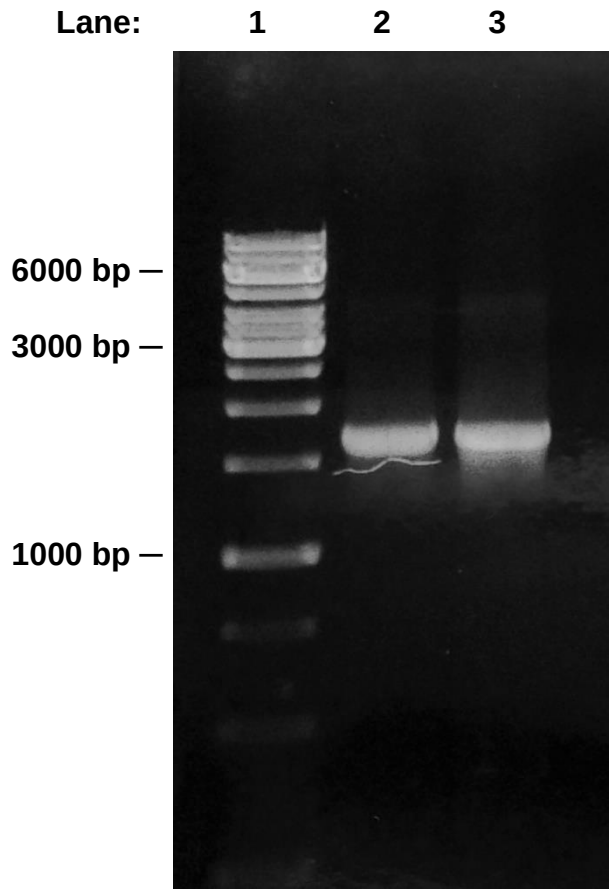

Figure 4.4. PCR amplicons for human and rat OCT1-Cterm-His. PCR products generated from primers 3-4 and 5-6, flanking the coding sequence of human/rat WT OCT1, which included a C-terminal His-tag and HindIII and BamHI restriction sites. Products were separated by electrophoresis on a 1% agarose in TBE gel. Lane 1: 1 kb ladder. Lane 2: rOCT1-Cterm-His amplicon (expected amplicon size: 1.7 kb). Lane 3: hOCT1-Cterm-His (expected amplicon size: 4.5 kb).

expression vector, and transform chemically competent *E. coli* failed to yield any bacterial colonies containing human or rat pcDNA5/FRT-OCT1-Cterm-His. The only bacterial colonies observed following ligation and transformation attempts contained only pcDNA5/FRT empty vector (Figure 4.5), and frequently, no colonies formed. To be sure that enough base pairs were present around the restriction sites for enzymes to bind and digestion to occur, primers were redesigned to include an additional six base pairs outside the restriction sites (primers 7-10, Table 4.2). PCR amplicons were successfully generated with these primers, but subsequent subcloning steps again yielded no complete constructs. Several alterations were made to the protocols provided by manufacturers (summarized in Table 4.3), but all attempts to obtain His-tagged OCT1 constructs via this method were unsuccessful. Possible reasons for this are outlined in the discussion section of this chapter (section 4.4).

The third subcloning method selected was the TOPO® TA Cloning® kit (Invitrogen, Carlsbad,CA). TOPO® cloning, touted as one of the fastest, easiest methods of subcloning, involves insertion of a PCR product containing 3' adenine (A) overhangs into the pCR2.1-TOPO® vector with the aid of covalently attached topoisomerases. Primers 3-4 and 5-6 were used to generate fresh OCT1-Cterm-His PCR amplicons using proofreading polymerase *Pfu* Turbo (Agilent Technologies, Santa Clara, CA), which were then incubated with Taq polymerase to add 3'-A overhangs, as prescribed by the manufacturer's protocol. These PCR products were subsequently incubated with the TOPO® vector for ligation, and transformed into chemically competent *E.coli*, per manufacturer's directions, for later digestion with restriction

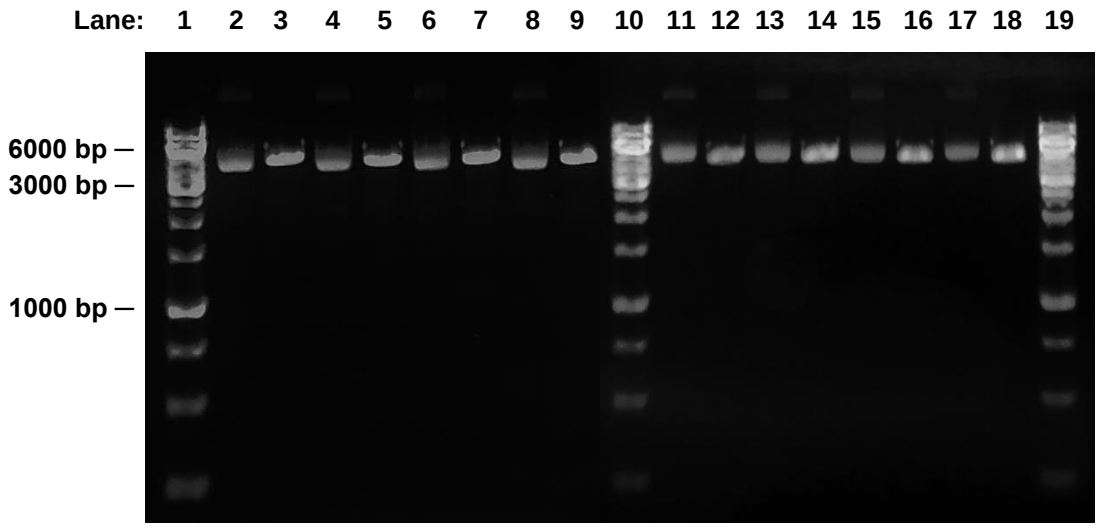

Figure 4.5. DNA isolated from bacterial colonies following ligation of pcDNA5/FRT and human and rat OCT1-Cterm-His. Chemically competent TOP10 E. coli were transformed with DNA products generated by ligating digested human and rat OCT1-Cterm-His amplicons and digested pcDNA5/FRT vector. Transformants were grown overnight at 37°C on LB agar plates containing ampicillin. Colonies were selected, and grown overnight at 37°C in LB broth with ampicillin. DNA was isolated from overnight cultures using the Qiagen mini-prep kit. A portion of DNA isolated from each colony was digested using HindIII and BamHI, then whole and digested DNA were separated by electrophoresis on a 1% agarose in TBE gel. Expected bands: 6.7 kb (undigested); 5 kb and 1.7 kb (digested). Lane 1: 1 kb ladder. Lane 2: Colony1 (rat). Lane 3: Colony 1-digested. Lane 4: Colony 2 (rat). Lane 5: Colony 2-digested. Lane 6: Colony 3 (rat). Lane 7: Colony 3-digested. Lane 8: Colony 4 (rat). Lane 9: Colony 4-digested. Lane 10: 1 kb ladder. Lane 11: Colony 9 (human). Lane 12: Colony 9-digested. Lane 13: Colony 10 (human). Lane 14: Colony 10-digested. Lane 15: Colony 11 (human). Lane 16: Colony 11-digested. Lane 17: Colony 12 (human). Lane 18: Colony 12-digested. Lane 19: 1 kb ladder.

Table 4.3. Troubleshooting of subcloning methods for generating human and rat pcDNA5/FRT-OCT1-Cterm-His.

Alteration to Method	Result
Used purified DNA products from both PCR clean-up kit and gel purification kit, as well as unpurified DNA products in ligation reactions	No colonies
Purchased new, and changed brands of restriction enzymes (New England Biosciences, Fermentas Fast Digest)	No colonies
Redesigned primers to include extra base pairs around restriction sites to enable proper digestion	PCR successful, colonies contained only pcDNA5/FRT
Dephosphorylated digested vector before ligation	No colonies
Tried several vector:insert ratios for ligation (1:1, 1:3, 1:5, 1:9, 1:10	1:5 yielded colonies containing only pcDNA5/FRT; other ratios yielded no colonies
Varied ligation reaction time (1 hour-overnight)	No colonies
Varied ligation reaction temperature (4-25ºC)	No colonies
Purchased new T4 DNA ligase	No colonies
Changed cell type transformed (TOP10, TOP10F', DH5α)	No colonies
Transformed cells with diluted and undiluted ligation reaction mixtures	No colonies

enzymes and ligation into pcDNA5/FRT vector. Each attempt at TOPO® cloning human OCT1-Cterm-His yielded no bacterial colonies following transformation. One attempt at TOPO® cloning rat OCT1-Cterm-His yielded several colonies (white and blue; Figure 4.6A). Eight white colonies were selected for overnight cultures. DNA isolated from these colonies was digested with EcoRI. EcoRI was selected due to the presence of EcoRI restriction sites flanking the gene insertion site on the pCR2.1 vector, thus eliminating the need for double digestion at this step. Digestion of isolated DNA with EcoRI yielded two DNA segments, one near 1.2 kb and one around 5.5 kb, when separated by gel electrophoresis (Figure 4.6B). The insert of interest, rat OCT1-Cterm-His, is approximately 1.7 kb, and pCR2.1 empty vector is 3.9 kb. Because the sizes of the generated fragments did not match what was expected, this result was somewhat confusing. Utilization of the NEBcutter tool (New England Biolabs, Ipswich, MA) identified an EcoRI cut site within the rat OCT1 coding sequence at base pair 1159. Further analysis demonstrated that the two fragments were likely derived from the original rat OCT1 template, pCMV6-Entry-ratOCT1-WT. There is an EcoRI restriction site near the beginning of the gene insertion location on the pCMV6-Entry vector. When combined with the EcoRI restriction site within the rat OCT1 coding sequence, digestion with EcoRI would yield the two observed fragments. pCMV6-Entry also contains a kanamycin resistance gene, the antibiotic used for selection with the TOPO® cloning kit. Therefore, it was determined that these colonies contained only rat OCT1 template vector (pCMV6-Entry-ratOCT1), and not pCR2.1-ratOCT1-Cterm-His, and therefore TOPO® cloning failed for both human and rat OCT1-Cterm-His. Subsequently, subcloning attempts were arrested, and alternative studies were pursued.

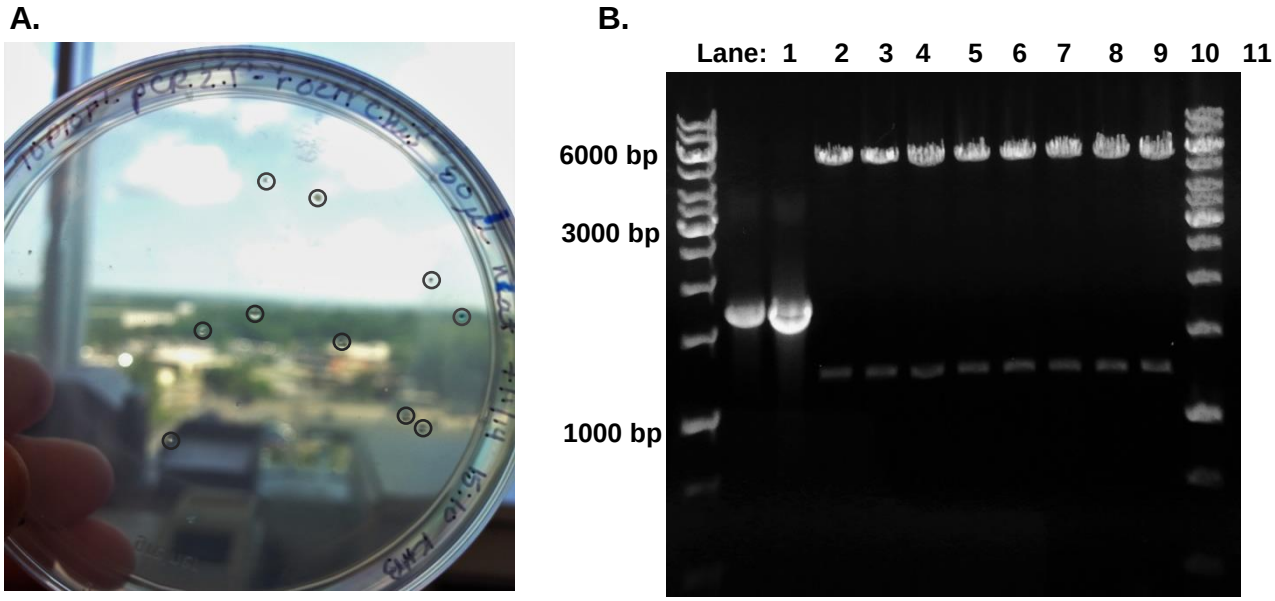

Figure 4.6. TOPO® cloning of rat OCT1-Cterm-His. (A) Following TOPO® cloning or rat OCT1-Cterm-His, chemically competent TOP10F' E. coli were transformed with 2 µL of the cloning reaction, spread on an LB agar plate containing kanamycin for selection, and IPTG and X-gal for blue-white screening, and incubated overnight at 37°C. Colonies observed following overnight incubation are circled. (B) Eight colonies were selected from the LB agar plate and grown in overnight cultures. DNA was isolated from the overnight cultures by mini-prep and digested with EcoRI, then separated by electrophoresis on a 1% agaose in TBE gel. Lane 1: 1 kb ladder. Lane 2: TOPO® cloning reaction. Lane 3: rat OCT1-Cterm-His amplicon. Lane 4: Colony 1. Lane 5: Colony 2. Lane 6: Colony 3. Lane 7: Colony 4. Lane 8: Colony 5. Lane 9: Colony 6. Lane 10: Colony 7. Lane 11: Colony 8. Lane 12: 1 kb ladder.

4.4 DISCUSSION

The goal of this study was to investigate the role of the extracellular loop domain of OCT1 on substrate affinity by developing and utilizing human/rat chimeric transporters. Generation of OCT1 extracellular loop chimeras would allow for the direct comparison of substrate affinities associated with whole, wild-type transporters and those associated with specific ECL domains, without altering the entire transporter, and therefore yield important information regarding the ECL's role in substrate binding. However, in order to be sure that measured transport values are a result of the exchange of the ECL, and not due to protein expression, and therefore can be directly compared, it was necessary to have a means of accurately quantifying and comparing the expression levels of both the wild-type and chimeric transporters. Hence, the need for addition of the His-tag to human and rat OCT1.

There are myriad reasons why subcloning attempts of human and rat pcDNA5/FRT-OCT1-Cterm-His were unsuccessful. The first and most obvious potential issue surrounds ligation of the gene(s) into the pcDNA5/FRT vector. Although multiple alterations were made to manufacturer's protocols to troubleshoot ligation (summarized in Table 4.3), further adjustments could have been made. Due to the size of the gene insert, it's possible that the optimum ratio of insert-to-vector was not achieved, and therefore ligation could not occur. To remedy this, more insert-to-vector ratios could be tried. Additionally, ligation incubation time and temperature are critical in achieving ligation. Further varying the length and temperature of ligation reactions, or changing the concentration or type of ligase, may lead to successful ligation, and production of the desired constructs. Furthermore, it is possible that too little or too much DNA (from

ligation reactions) was used in transformation, so varying the amount of DNA added to *E. coli* during transformation procedures may yield colonies. In any case, the modifications that can be made to subcloning protocols are infinite, and the cost-benefit of continuing the troubleshooting process must be weighed.

Further causes for the failed subcloning attempts involve properties inherent to the gene and bacterial host, rather than subcloning methods. It is possible that the mutations introduced into wild-type human and rat OCT1 made the transporters toxic, in some way, to *E. coli*, and therefore inhibited growth of colonies when transformed. Toxicity of heterologous proteins, particularly membrane proteins, has been described numerous times (Brosius, 1984, Doherty, et al., 1993, Dong, et al., 1995, Dumon-Seignovert, et al., 2004, Miroux and Walker, 1996, Montigny, et al., 2004). Although in this case protein expression was not being induced, it is common for protein expression to occur due to the "leaky" *lac* promoter, which can drive transcription even when not stimulated (Nielsen, et al., 2007). Several methods have been proposed to limit toxicity resulting from basal expression via the *lac* promoter. Utilization of a bacterial strain which over expresses the lac repressor, *lac*I or *lac*I^Q, inhibits transcription from the T7 promoter until induced, and therefore limits basal expression of the heterologous protein (Rosano and Ceccarelli, 2014). Additionally, "leaky" expression can be suppressed by co-transforming a plasmid which expresses T7 lysozyme, which binds and destabilizes T7 RNA polymerase (Moffatt and Studier, 1987, Stano and Patel, 2004), thus repressing transcription and eliminating the toxic effects of the heterologous protein.

Likewise, it is also plausible that the lack of colony growth following transformation is due to toxic effects of a protein(s) other than the heterologous

transporters. The mutations introduced into wild-type human and rat OCT1 may have introduced or uncovered a cryptic promoter, a normally silent promoter that when activated can lead to transcription and translation of aberrant peptides (Hennig and Fischer, 2013, Islam, et al., 2011). Previous reports describe just such an event during cloning of other genes (Jakab, et al., 1997, Li, et al., 2011, Saida, et al., 2006), and interestingly, a cryptic bacterial promoter was identified within the coding sequence of the mouse *Mdr*1 (P-gp) gene (Pluchino, et al., 2015). Suppression of cryptic promoters can be achieved by introduction of Rho-independent transcription terminators (T1 and T2) into the vector (Brosius, 1984, Brown and Campbell, 1993, Saida, et al., 2006), eliminating the production of the toxic peptides.

Affinity-tagged OCT1 (rat and human) constructs have been described in the literature, however most contain a FLAG- (DYKDDDK) tag (Egenberger, et al., 2012, Keller, et al., 2008, Popp, et al., 2005). Only one group, in two separate publications, has described the inclusion of a His-tag in an OCT1 expression construct. Keller and colleagues (2008, 2011) inserted full-length, wild-type rat OCT1 into the pET21a vector, which contains a His-tag sequence a few amino acids downstream of the gene insertion site. Our attempts at cloning OCT1-Cterm-His placed the His-tag immediately following the OCT1 coding sequences. The inclusion of several amino acids between the end of the OCT1 coding sequence and His-tag may be enough of a difference to overcome any possible toxicity to transformed bacteria, and allow for colony formation and plasmid isolation.

Had we been able to successfully subclone the His-tag and generate the proposed OCT1 chimeras, we would have hoped to find that the ECL is indeed involved

in substrate affinity. If this were the case, we would have expected to see a switch in substrate affinities that correlated with the exchange of human and rat OCT1 ECLs similar to that observed by Gorboulev et al., (2005) when they exchanged amino acids in rat OCT1 for those of rat OCT2, as described in the introduction of this chapter. However, because the ECLs of human and rat OCT1 share approximately 80% homology, it is possible that significant differences would not be observed between wild-type and chimeric transporters. In this case, it may be still be possible to study the role of the ECL domain's role in substrate binding and affinity by generating a "loopless" mutant. To achieve a "loopless" mutant, we would simply replace the ECL on human and/or rat OCT1 with the linker region of a close bacterial homolog, lactose permease, or LacY (Abramson, et al., 2003). Interestingly, there is literature precedent for a "loopless" mutant of OCT1. Shortly after the initial cloning and characterization of rat OCT1, Zhang et al., (1997a) isolated an mRNA splice variant of OCT1 from rat kidney, dubbed rOCT1A, which lacked the first two TMs and extracellular loop. When expressed in *Xenopus laevis* oocytes, rOCT1A demonstrated saturable and inhibitable transport of model cation TEA, though with altered kinetics. The K_m for rOCT1A transport of TEA was determined to be 42 µM, whereas the established K_m for wild-type rat OCT1 TEA transport is 95 µM (Grundemann, et al., 1994). Therefore, while the loop is not essential for transport, it appears to be involved in substrate binding and affinity. Further characterization of a "loopless" mutant may provide critical insight into the structure-function relationship of OCT1.

While generation of OCT1 chimeric proteins proved too difficult at this time, there is still a multitude of information that could be derived from studies of such transporters.

As demonstrated by others, important data relating to substrate binding, affinity, and translocation, as well as structural insights may be obtained from studies with chimeric transporters. And because OCT1 has a solidly established role in the ADME and efficacy of several clinically important drugs, it would be worthwhile for these to be attempted again in the future.

CHAPTER 5 : DISCUSSION AND FUTURE DIRECTIONS

5.1 SIGNIFICANCE

Drug-drug interactions (DDIs) resulting from the concurrent use of multiple medications are increasing, particularly in the geriatric population. One report estimates that nearly 10% of emergency department visits are the result of adverse drug events (ADEs), which include DDIs (Maher, et al., 2014). DDIs can occur through several processes in the body, including drug absorption, distribution, metabolism, and excretion, as discussed in Chapter 1. Most frequently, DDIs occur during metabolism, as a result of unfavorable interactions with cytochrome P450 enzymes. However, there is a growing appreciation for the role of drug transporters in DDIs, as DDIs often occur at the transporter level. As such, government-issued guidelines now include recommendations for screening INDs for transporter-mediated DDIs (European Medicines Agency, 2012, U.S. Food and Drug Administration, 2017). These guidelines recommend including OATPs, OATs, and OCTs, among others, in preclinical studies.

hOCT1, expressed primarily in the liver, interacts with more than 100 clinically-prescribed drugs (Nies et al., 2011), and thus serves as an important target for transporter-mediated DDIs. While there is much information available regarding hOCT1 interactions with xenobiotics, little information is available regarding endogenous roles for the transporter. Endogenous function is equally important to consider when identifying ADEs. Furthermore, several reports demonstrate substrate-dependent effects for close relatives of hOCT1 (Belzer, et al., 2013, Martínez-Guerrero and Wright 2013, Roth, et al., 2011, Zhang, et al., 2013), and suggest the same for hOCT1. However, substrate-dependent effects have yet to be confirmed for hOCT1, and limited

structural information is available, making it challenging to define ligand binding domains and predict unfavorable drug interactions.

Without a comprehensive understanding of the structure and mechanisms of ligand interaction with hOCT1, identification and prevention of DDIs involving this transporter remains difficult. Therefore, the studies performed in this dissertation were designed to address the lack of understanding, on multiple levels, of the mechanisms through which adverse drug interactions occur via hOCT1. I formed the central hypothesis that both endogenous and xenobiotic compounds modulate the functional activity of hOCT1 in a substrate-dependent manner through interaction with specific ligand-binding domains within the transporter. This hypothesis was tested via three specific aims: 1) investigation of the effect of xenobiotics on endogenous substrate transport by hOCT1, 2) identification and characterization of substrate-dependent interactions with hOCT1, and 3) examination of the role of the extracellular loop domain of hOCT1 in substrate affinity and translocation. The findings resulting from these aims are discussed further in the following sections.

5.2 SPECIFIC AIM 1

For the first specific aim, a role for hOCT1 in transport of monoamine neurotransmitters was established. Uptake of radiolabeled serotonin, dopamine, and norepinephrine by hOCT1 was determined in a heterologous expression system. Next, inhibition of hOCT1-mediated uptake of serotonin by several commonly-prescribed

drugs was examined. And finally, uptake and inhibition studies were completed for serotonin in primary human hepatocytes.

While it is widely accepted that hOCT1 is involved with the clearance of many xenobiotics, but an endogenous function of hOCT1 has not been definitively determined, though some reports suggest the possibility, particularly for monoamine neurotransmitters (Amphoux et al., 2006, Breidert et al., 1998, Busch et al., 1996, Jonker and Schinkel, 2004, Kerb et al., 2002, Lips et al., 2005). To confirm a role for hOCT1 in neurotransmitter transport, direct transport of radiolabeled serotonin, dopamine, and norepinephrine was observed. Of the three, serotonin was by far the superior substrate of hOCT1, with only modest transport of dopamine, and very little uptake of norepinephrine measured. This was particularly significant because of the high levels of serotonin contained within the small intestine, and furthermore in the portal blood supply, and the fact that the liver is known to be involved in the clearance of up to 70% of circulating serotonin (Thomas and Vane, 1967, Toh, 1954), making hOCT1 a potential target for drug-induced inhibition of serotonin clearance. Kinetic characterization of serotonin transport determined that hOCT1 is likely a low-to-mid affinity but high capacity transporter of serotonin, yielding solid evidence of its role in serotonin clearance.

Increased serotonin uptake into hepatocytes has been implicated in the pathogenesis and progression of non-alcoholic steatohepatitis (NASH) (Nocito, et al., 2007a). Furthermore, OCT1 expression is increased at the mRNA level in a mouse model of NASH (Clarke, et al., 2015). If the same is true in human NASH, hOCT1-mediated serotonin transport may be implicated in NASH progression. Studies in human

hepatocytes or humanized mouse models may aid in fleshing out a role for hOCT1 in NASH progression.

Because hOCT1 is known to interact with many drugs, it is possible that the newly discovered endogenous function could be disrupted. To evaluate the potential for drug-endogenous substrate interactions, a small drug screen was performed with radiolabeled serotonin as the probe substrate. The screen identified multiple prescription and over-the-counter drugs as inhibitors of hOCT1-mediated serotonin uptake. This was confirmed in primary human hepatocytes, an arguably more relevant model. Inhibition of serotonin uptake into the liver effectively increases circulating serotonin levels, which bears several implications, many of which were discussed in Chapter 2. Further implications of increased circulating serotonin levels involve serotonin signaling in both the heart and the bone. Cardiac valve abnormalities, including fibrodysplasia and calcification, have been described in patients with carcinoid syndrome, due to increased serotonin signaling as a result of excessive serotonin secretion from carcinoid tumors in the gut (Levy, 2006). Yadav et al., (2008) described misregulation of bone remodeling, leading to osteoporosis, as a result of increased serotonin synthesis in the gut consequently increasing serotonin signaling in osteoblasts. Drug-mediated inhibition of OCT1 serotonin transport has the potential to increase serotonin levels in the blood, which may lead to pathological signaling in several tissues, and thus deserves further investigation but was outside the scope of this dissertation.

Studies in this aim also identified putative substrate-dependent interactions, which were explored further in specific aim 2.

5.3 SPECIFIC AIM 2

Results from experiments completed for aim 1 suggested substrate-dependent effects for hOCT; several drugs known to be substrates or inhibitors of hOCT1 did not inhibit hOCT1-mediated uptake of serotonin. Therefore, for specific aim 2, we explored substrate-dependent effects experimentally and *in silico* by employing a novel competitive-counterflow (CCF) assay and docking ligands into a homology model for hOCT1.

The CCF assay identified several ligands which induced efflux of one or two probe substrates, but not all three. Several of these interactions were explained by data extracted from the docking studies, which suggested multiple binding pockets within the hOCT1 translocation pore. The identification of multiple binding sites in our study corroborates hypotheses from several groups that hOCT1 and other OCTs possess a large substrate binding region versus a specific binding site, as is common in enzymes (Chen, et al., 2017, Ciarimboli, et al., 2004, Ciarimboli and Schlatter, 2005, Koepsell, et al., 2003). The existence of multiple binding pockets may complicate the binding kinetics of multiple ligands at the same time. One can imagine that the binding of a ligand in one site may cause conformational changes within the transporter that occlude another ligand from binding in a different site. Indeed, this may explain the lack of efflux induced by acyclovir, lamotrigine, salbutamol, and ethidium, which have all been reported as substrates, as discussed in Chapter 3. Further mechanistic studies need to be performed to fully understand the binding kinetics of multiple substrates.

Another notable finding in the CCF assay was the identification of negatively-charged BSP as a substrate for hOCT1. As discussed in Chapter 3, hOCT1 is considered a transporter of positively-charged and neutral compounds (Koepsell, 2015, Nies, et al., 2011b). The identification of a negatively-charged compound not only calls into question the nomenclature of the transporter (organic *cation* transporter), but also potentially the mechanism of transport. hOCT1 is described as an electrogenic transporter which facilitates the movement of positively charged molecules across cell membranes along an electrochemical gradient (Gorboulev, et al., 1997, Koepsell, 2004, Zhang, et al., 1997b). While transporting BSP from an extracellular region of high concentration to an intracellular location of low concentration would follow the chemical gradient, transporting negatively-charged BSP from extracellular space to the cytoplasm would mean transporting against the electric gradient (membrane potential) which generally requires a source of energy. Organic anion transporting polypeptides (OATPs) transport numerous negatively-charged substrates, as the name suggests, and thus facilitate the transport of compounds against the electric gradient. It has been hypothesized that this is made possible by the fact that OATPs can transport in both directions, and therefore exchange extracellular substrates with a negatively-charged molecule within the cell, effectively functioning as exchangers, though a counter ion has not yet been identified (Roth, et al., 2012). OCTs are also capable of transporting in both directions (indeed, this serves as the foundation of the CCF assay; Arimany-Nardi, et al., 2014, Lips, et al., 2005, Zhang, et al., 1998), and thus may rely on a similar mechanism to transport negatively-charged substrates like BSP. Certainly, more studies are needed to examine this possibility.

In our studies, docking ligands into a homology model proved useful, to an extent, in explaining substrate-dependent interactions observed in the CCF assay. In industry, the employment of docking studies could expedite pre-clinical inhibition studies and save money long-term. Docking studies could be employed prior to transporter assays to identify potential interactions which can be examined further in the lab, and potentially eliminate the need to test a large group of compounds which would not demonstrate any significant interactions. However, this is not likely to occur in the very near future, as several improvements to modeling large hydrophobic proteins are necessary, particularly for flexible, non-static models which require large computing capacity. Additionally, improving model accuracy would ideally require a crystal structure for hOCT1. Improvement in modeling techniques, and ultimately solving a crystal structure for hOCT1 could improve the process of identifying potential transporter interactions which could be highly beneficial for drug companies entering pre-clinical drug trials.

5.4 SPECIFIC AIM 3

Substrate-dependent modulation from within the translocation pore was explored in aim 2. However, due to unfavorable energetics and intrinsic disorder, the extracellular loop (ECL) domain of hOCT1 couldn't be modeled, a limitation of our homology model. Information from several reports suggests that the ECL is important in ligand recognition and binding (Keller et al., 2011, Kerb, et al., 2002). Therefore, it was necessary to

examine the role of the ECL in these interactions, and specific aim 3 attempted to address this.

The goal of specific aim 3 was to generate chimeric transporters containing specific regions from both human and rat OCT1 to explore the differences in substrate affinities between species. However, this goal proved too lofty to obtain, at least within the scope of this dissertation, despite the same having been completed for relatives of OCT1 (Gorboulev, 2005, Gui and Hagenbuch, 2009). Putatively, this was due to a toxic protein product resulting from insertion of the His-tag at the C-terminus of the transporters. Perhaps future attempts at generating tagged constructs could be successful by including a FLAG-tag instead of a His-tag. If the histidine codon or series of repeating codons employed in our primers to introduce the tag was ultimately what lead to a failure of bacterial colonies to grow during subcloning, utilizing a FLAG-tag instead of the histidine should allow for colony growth. Conversely, changing the codon employed for histidine may yield similar results. For our ultimate purpose of expression in a mammalian cell line, the codon CAC was utilized for histidine, as this is this optimum codon for mammalian protein expression. The codon CAT could be used in place of CAC to encourage colony growth.

Had we been successful in producing the desired chimeras, uptake studies comparing the chimeras with wild-type transporters from both species would have been conducted. It was hoped that these studies would yield observable differences in substrate affinity that could ultimately be traced back to specific amino acid regions, thus highlighting key binding regions. These studies would also determine whether the ECL is critical for substrate binding and initiating translocation, as has been previously

suggested. Additionally, chimeric OCT1 may prove useful in further explaining substrate-dependent interactions observed in other aims of this dissertation.

5.5 FUTURE DIRECTIONS

The studies performed in aim 1 determined that hOCT1 serves an important endogenous function, and transport of xenobiotics can disrupt this function. Likely, OCTs didn't evolve to serve as xenobiotic transporters (Nigam, 2015), so determining endogenous functions of hOCT1 is critical to preventing adverse drug events relating to inhibition of endogenous function. While we have identified one function in serotonin clearance, the polyspecificity of hOCT1 might suggest that it serves multiple physiological functions. Chen et al., (2016) describe hOCT1 as a high capacity thiamine transporter and suggest a role for the transporter in hepatic steatosis, though one could argue the authors over-emphasize the implications of hOCT1-mediated thiamine transport. In any case, a full understanding of the endogenous roles played by hOCT1 is critical to predicting and preventing drug-mediated interruption of endogenous function at the transporter level, and as such, further studies are necessary.

As discussed above, the studies performed for aim 2 identified multiple substrate-dependent interactions for hOCT1, as well as potential mechanistic explanations for the observations. However, not all of the observed interactions could be explained by docking ligands into our model. In our study, ligands were docked into a rigid model, meaning that the computer fit the ligands to available space within the transporter. Ideally, further mechanistic studies would allow for development of a flexible

model which could incorporate transporter movement constraints obtained from observations of conformational changes during transport. Techniques such as NMR and FRET may be useful in elucidating the structural changes during transport to produce such a model. However, laboratory techniques to solubilize the transporter and perform such studies must be optimized before such studies could be conducted. Combining a flexible transporter model with simultaneous and/or chronological docking of multiple ligands may aid in further explanation of several of the interactions observed in Chapter 3.

The list of compounds tested in the CCF assays served sufficiently as an introductory study in substrate-dependent interactions for hOCT1. However, further expansion of the group of test compounds could allow for more information to be extracted from the combination of *in* vitro and *in silico* methods. If enough compounds were screened in a similar fashion to those in this dissertation, one could compile the information and develop a pharmacophore model for each binding site identified. A similar study was performed by Ahlin, et al., (2008), but for a single substrate, and thus a single binding site. Because we have defined multiple binding sites for hOCT1, new studies are necessary to identify comprehensive pharmacophore(s) for all binding sites.

Specific amino acids within hOCT1 have been identified, via mutagenesis experiments, as important in binding certain substrates (Gorboulev, et al., 1999, Gorboulev, et al., 2005, Popp, et al., 2005). Furthermore, mutants observed in different populations demonstrate altered transport of one substrate but not another (Kerb, et al., 2002), as discussed in section 1.3.5. Therefore, it is possible that hOCT1 variants may exhibit altered substrate-dependent effects. Mutagenesis experiments, replacing amino

acids previously identified as important to substrate binding or those that line the binding sites identified in aim 2, combined with uptake and inhibition studies could elucidate mutants which exhibit altered substrate-dependent effects.

Ultimately, crystallization of hOCT1 by itself and, ideally, co-crystalized with various ligands would yield important structural information with regards to binding sites and protein conformation. A hOCT1 crystal structure could guide a multitude of structural and SAR studies toward a more comprehensive understanding of substrate binding and ligand interactions.

In conclusion, the studies in this dissertation have identified important functional qualities for hOCT1, and attempted to elucidate the underlying structural mechanisms involved. Our studies have demonstrated that the current guidelines for preclinical determination of potential DDIs should be reassessed. However, much work still needs to be completed to obtain a comprehensive understanding of the mechanisms of ligand interaction and structure-function relationships for hOCT1, to further aid the goal of predicting and preventing adverse drug interactions.

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