SUBSTRATE DEPENDENT ALTERATIONS OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3 (OATP1B3)

By

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ABSTRACT

Organic anion-transporting polypeptides (OATPs) are multispecific transporters that mediate the uptake of numerous drugs and xenobiotics into cells. Alterations in the function of the liver-specific OATP1B1 and OATP1B3 have been shown to affect the disposition of drugs throughout the body. It has been proposed that new drug candidates should be screened for possible OATP inhibition using a prototypical substrate such as estradiol-17β-glucuronide. However, there is evidence that OATPs may have multiple binding sites, and therefore screening with a single compound may be ineffective. Therefore, I tested the hypothesis that OATP1B3 has multiple overlapping but distinct binding sites, which are affected in substrate-dependent ways. This hypothesis was tested via two specific aims: 1) to identify and characterize substrate-dependent effects of plant compounds on OATP1B3-mediated transport, and 2) to identify regions of OATP1B3 involved in the binding and/or translocation of individual model substrates.

In the first specific aim, interacting compounds were identified by screening a library of plant compounds for inhibition or stimulation of OATP-mediated uptake of two model substrates. Completion of this specific aim identified two structurally similar compounds that produce substrate-dependent effects on OATP1B3-mediated transport. These compounds stimulate transport of estrone-3-sulfate by increasing substrate affinity. However, the compounds either inhibit or have no effect on the uptake of estradiol-17β-glucuronide. These results demonstrate that estrone-3-sulfate and estradiol-17β-glucuronide have distinct binding sites on OATP1B3.

In specific aim two, thirty-three amino acids in the first transmembrane domain and extracellular loop of OATP1B3 were individually mutated to cysteines, and I determined the effect of these mutations on the transport of estradiol-17β-glucuronide and estrone-3-sulfate. Five of the cysteine-substituted OATP1B3 mutants produced different effects on transporter function depending upon the substrate tested. These results suggest that this region of OATP1B3 is involved in the recognition and translocation of individual model substrates.

This dissertation demonstrates that OATP1B3 has distinct binding sites for estradiol-17β-glucuronide and estrone-3-sulfate. Furthermore, it shows that transport of these two model substrates is affected in different ways by the same compounds. This knowledge can be used to improve screening of drug candidates to prevent adverse drug-drug interactions prior to the occurrence of adverse events.

Acknowledgements

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List of Abbreviations

ABC	ATP-binding cassette
ATP	adenosine triphosphate
BCRP	breast cancer resistance protein (ABCG2)
BUOH	butanol
CCK8	cholecystokinin-octapeptide
CHO	Chinese hamster ovary
CoMFA	comparative molecular field analysis
DCM	dichloromethane
DHEAS	dehydroepiandosterone sulfate
DMSO	dimethyl sulfoxide
E3S	estrone-3-sulfate
E17b	estradiol-17β-glucuronide
E17β	estradiol-17β-glucuronide
EC_{50}	half maximal effective concentration
EC	epicatechin
ECG	epicatechin gallate
ECL	extracellular loop
EDTA	ethylenediaminetetraacetic acid
EGC	epigallocatechin
EGCG	epigallocatechin gallate
EtOAc	ethyl acetate

HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-peperazineethanesulfonic acid
HEX	hexane
HIV	human immunodeficiency virus
HPLC	high pressure liquid chromatography
HRMS	high resolution mass spectroscopy
HTS	high throughput screening
IC_{50}	half maximal inhibitory concentration
IR	infrared
K_m	Michaelis-Menten constant
MDR1	multidrug resistance protein (P-glycoprotein)
MeOH	methanol
mRNA	messenger RNA
MRP	multidrug resistance associated protein
MTSES	sodium (2-sulfonatoethyl)methanethiosulfonate
NMR	nuclear magnetic resonance
OATP/Oatp	organic anion transporting polypeptide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
P-gp	P-glycoprotein (MDR1)
SD	standard deviation
SDS	sodium dodecyl sulfate
SEM	standard error of the mean

Si-Gel CC	silica gel column chromatography
SLC	solute carrier
SLCO	solute carrier family of the OATPs
TCL	thin layer chromatography
TM	transmembrane domain
UV	ultraviolet
V_{max}	maximal rate of transport

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Chapter 1

Background and Significance

1.1: Drug Absorption and Distribution

The field of pharmacology, which is the study of drugs, can be broadly divided into two disciplines: pharmacokinetics and pharmacodynamics. These disciplines have been described as the study of what drugs do to the body (pharmacodynamics), and what the body does to drugs (pharmacokinetics). The pharmacokinetics of a drug describes its disposition throughout the body and are determined by four factors commonly described by the acronym ADME: absorption, distribution, metabolism and excretion.

The first step, absorption, involves entry of the drug into the bloodstream. This step is not always necessary: a drug may exert its effect where it is applied (e.g. the surface of the skin) or absorption may be bypassed by injecting the drug directly into the bloodstream. However, to exert their effect, most drugs must enter the bloodstream by passing through one of the barriers that protect the body from the outside world. The most common means by which a drug is absorbed is by passing through the intestinal epithelium after oral administration, although drugs may also be absorbed through the skin or lungs, or may be injected into muscle, peritoneal fluid, or under the skin. The distribution of drug involves its delivery into tissues from the bloodstream. The site of action for a drug is known as the target tissue, but this is rarely the only tissue exposed. Most drugs are converted into other compounds after entering the body through enzyme-catalyzed metabolism. The metabolites generally have different chemical characteristics

than the parent drug and may be active (having the desired effect) or, more commonly, be easier for the body to excrete. Excretion, the final component of drug disposition, is the elimination of drugs from the body. The most common routes of excretion are through the kidney (into urine) or the liver (into bile eliminated through feces).

With the exception of metabolism, all steps of drug disposition involve passage of the drug or its metabolites through cell membranes. Whereas some small, hydrophobic, and uncharged drugs may passively diffuse through cell membranes, many drugs and other xenobiotics require transporter proteins to mediate their translocation through the cell membrane. Therefore, transporters are essential to the pharmacokinetics of many drugs.

1.2: Transporters Involved in Drug Absorption, Distribution and Elimination

There are two major superfamilies of multispecific drug transporters: solute carrier (SLC) transporters and ATP-binding cassette (ABC) transporters. Many of these transporters are capable of bi-directional transport. In practice, however, they act in opposition, with SLC transporters mediating uptake of substrates into cells whereas ABC transporters pump substrates out of cells. SLC transporters involved in drug influx include the organic cation transporters (OCTs), organic anion transporters (OATs), organic cation/carnitine transporters (OCTNs), organic anion transporting polypeptides (OATPs), petptide transporters (PEPTs), monocarboxylate transporters (MCTs, SMCTs), nucleoside transporters (CNTs, ENTs) and bile acid transporters (NTCP, ASBT) (You and Morris, 2007). Drug efflux is mediated by ABC

transporters, such as the multidrug resistance protein (MDR, also known as P-glycoprotein), multidrug resistance-associated proteins (MRPs), breast cancer resistance proteins (BCRP), and the bile acid transporter BSEP (You and Morris, 2007). This dissertation focuses on four transporters that are involved in drug absorption and first-pass clearance from the blood stream: OATP1A2, OATP1B1, OATP1B3, and OATP2B1.

1.3: The Organic Anion Transporting Polypetide (OATP) Superfamily of Drug Uptake Transporters

Organic anion transporting polypeptides (OATPs in humans, Oatps in other species) are multispecific transporters expressed on epithelia throughout the body that mediate the cellular uptake of a broad range of substrates.

1.3.1: Nomenclature

Originally classified as superfamily *SLC21A*, the genes encoding the OATP proteins were reclassified as the *SLCO* (solute carrier family of the OATPs) superfamily in 2004, to create a standard nomenclature based on phylogenetic relationships (Hagenbuch and Meier, 2004). The eleven members of the OATP superfamily in humans are classified into six families based on a 40% amino acid identity, and further divided into 10 subfamilies based on a 60% amino acid identity. The nomenclature consists of OATP followed by the family number, subfamily letter, and a number based on the historical order of identification. For example, OATP1A2 is a human

transporter in family 1, subfamily A, which was the second OATP1A family member discovered. The gene symbols are named with the same system, substituting *SLCO* for OATP.

A significant amount of gene duplication and divergence has occurred in this superfamily. OATP1A2 has 5 rodent orthologs: Oatp1a1, Oatp1a3 (in rats only), Oatp1a4, Oatp1a5 and Oatp1a6, whereas OATP1B1 and OATP1B3 have a single rodent ortholog, Oatp1b2. The other OATPs and their rodent orthologs are OATP1C1 (Oatp1c1), OATP2A1 (Oatp2a1), OATP2B1 (Oatp2b1), OATP3A1 (Oatp3a1), OATP4A1 (Oatp4a1), OATP4C1, OATP5A1, and OATP6A1 (Oatp6b1, Oatp6c1, and Oatp6d1). The phylogenetic tree for human OATPs and their rodent orthologs are shown in Figure 1-1. The best characterized OATPs belong to families 1 and 2, and include OATP1A2, OATP1B1, OATP1B3, and OATP2B1.

1.3.2: Tissue Distribution

1.3.2a: Normal Expression

OATP1A2, the first human OATP identified, is expressed widely throughout the body. Northern blots show that the highest mRNA expression is in the brain, liver, lung, kidney and testes (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004). Protein has been localized to the brush border membrane of enterocytes (Glaeser et al., 2007), to cholangiocytes in the liver (Lee et al., 2005), at the apical membrane of distal nephrons in the kidney (Lee et al., 2005), and at the luminal membrane of endothelial cells of brain capillaries (Bronger et al., 2005). OATP1B1 and OATP1B3 are both thought to be exclusively expressed at the basolateral membrane of

Figure 1-1:

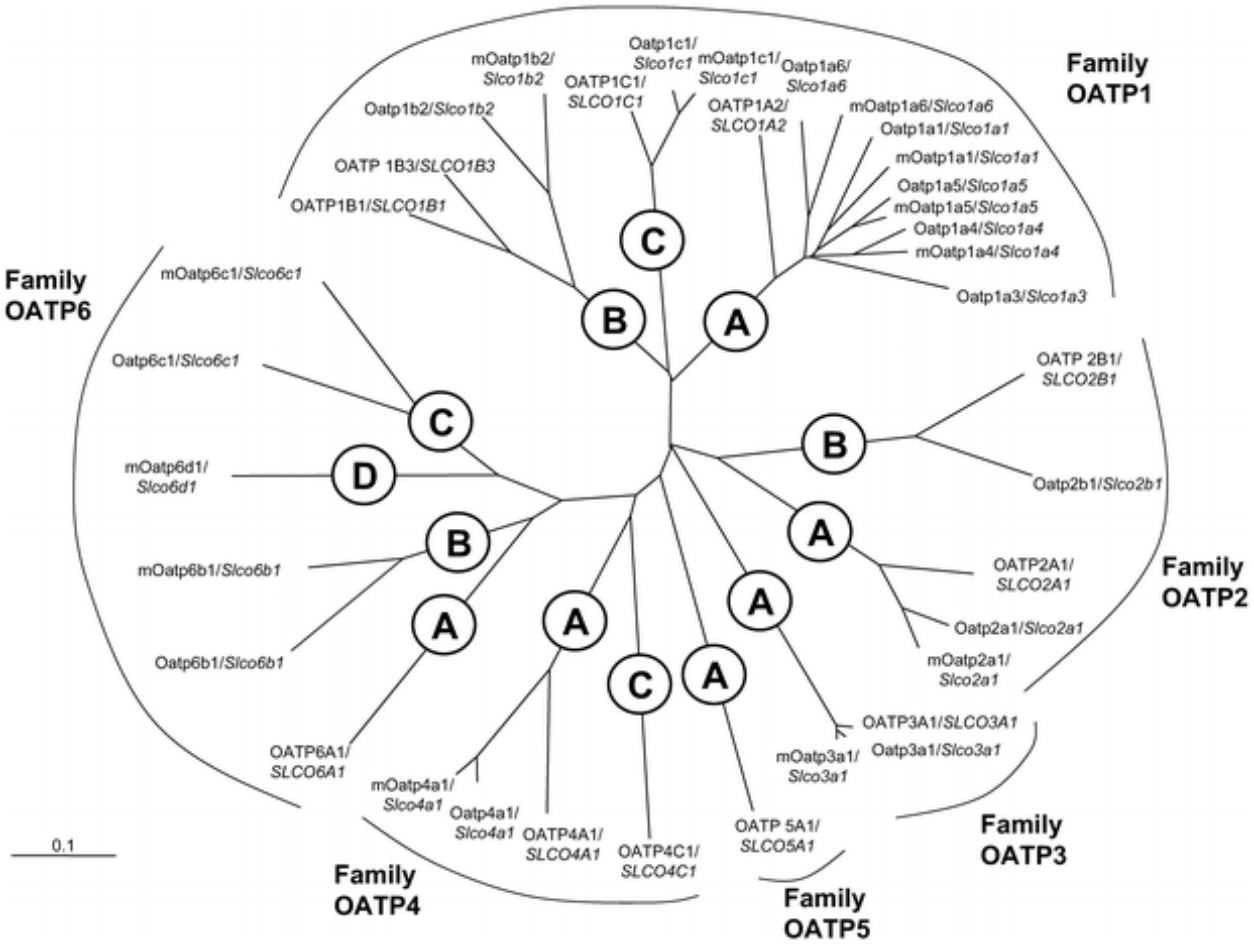
Family OATP1
Family OATP2
Family OATP3
Family OATP4
Family OATP5
Family OATP6
mOatp1b2/ Slco1b2
Oatp1c1/ Slco1c1
mOatp1c1/ Slco1c1
Oatp1a6/ Slco1a6
OATP1C1/ SLCO1C1
OATP1A2/ SLCO1A2
mOatp1a6/Slco1a6
Oatp1b2/Slco1b2
Oatp1a1/Slco1a1
OATP 1B3/SLCO1B3
mOatp1a1/Slco1a1
OATP1B1/SLCO1B1
Oatp1a5/Slco1a5
mOatp1a5/Slco1a5
Oatp1a4/Slco1a4
mOatp1a4/Slco1a4
Oatp1a3/Slco1a3
mOatp6c1/Slco6c1
Oatp6c1/Slco6c1
OATP 2B1/ SLCO2B1
mOatp6d1/ Slco6d1
Oatp2b1/Slco2b1
mOatp6b1/Slco6b1
OATP2A1/ SLCO2A1
Oatp2a1/Slco2a1
Oatp6b1/Slco6b1
mOatp2a1/ Slco2a1
OATP3A1/SLCO3A1
Oatp3a1/Slco3a1
mOatp3a1/ Slco3a1
OATP6A1/ SLCO6A1
mOatp4a1/ Slco4a1
Oatp4a1/ Slco4a1
OATP4A1/ SLCO4A1
OATP4C1/ SLCO4C1
OATP 5A1/ SLCO5A1
0.1

Figure 1-1:

Phylogenetic tree of human, rat and mouse OATPs/Oatps. Oatps/OATPs with amino acid sequence identities ≥ 40% among each other belong to the same OATP family (e.g. OATP1, OATP2, OATP3, OATP4, OATP5, OATP6). Proteins with amino acid sequence identities ≥ 60% are grouped into subfamilies and denoted with a capital letter after the family number (e.g. OATP1A, OATP1B, OATP1C, OATP2A, OATP2B, etc.). Individual proteins (genes) are continuously numbered according to the chronology of their identification. The "Oatp" (rodents)/"OATP" (human) symbols denote proteins, whereas the "Slco"/"SLCO" symbols indicate the respective genes. Mouse Oatps are indicated by "m". Only the families OATP1 to OATP6 are indicated, since only these six families contain human OATPs. Reproduced with kind permission from Springer Science+Business Media: (Hagenbuch and Meier, 2004).

hepatocytes under normal physiological conditions (Abe et al., 1999; Konig et al., 2000a; Abe et al., 2001) al., 2001); (Hsiang et al., 1999; Konig et al., 2000b; Kullak-Ublick et al., 2001; Cui et al., 2003). OATP1B1 has higher mRNA levels in total liver than does OATP1B3 (Michalski et al., 2002; Briz et al., 2006); however, it is expressed in hepatocytes throughout the lobule, while OATP1B3 is primarily expressed around the central vein (Konig et al., 2000b). OATP1C1 protein is expressed at the basolateral membrane of choroid plexus epithelial cells (Roberts et al., 2008) and in the Leydig cells of the testes (Pizzagalli et al., 2002).

OATP2A1 is ubiquitously expressed throughout the body, with mRNA identified in brain, colon, heart, kidney, liver, lung, ovary, pancreas, placenta, prostate, skeletal muscle, spleen, and small intestine (Schuster, 2002; Nomura et al., 2004; Nomura et al., 2005). OATP2B1 is also widely expressed throughout the body (Tamai et al., 2000; Kullak-Ublick et al., 2001), with protein localized to the basolateral membrane of hepatocytes (Kullak-Ublick et al., 2001), the apical membrane of enterocytes (Kobayashi et al., 2003), the basolateral membrane of syncytiotrophoblasts in the placenta (St-Pierre et al., 2002), and the luminal membrane of endothelial cells of the blood-brain barrier (Bronger et al., 2005), as well as in epidermal keratinocytes (Schiffer et al., 2003), in vascular endothelial cells in the heart (Grube et al., 2006b), and in the myoepithelium surrounding ductal epithelial cells in human mammary glands (Pizzagalli et al., 2003).

The less well studied OATP3A1 and OATP4A1 are also expressed at the mRNA level in a wide variety of tissues (Tamai et al., 2000; Fujiwara et al., 2001; Adachi et al., 2003; Huber et al., 2007). OATP4C1 mRNA has only been detected in kidney (Mikkaichi et al., 2004), whereas

OATP5A1 expression has not yet been determined. OATP6A1 mRNA has highest levels in the testes, although it is also expressed at low levels in spleen, brain, fetal brain, and placenta (Suzuki et al., 2003; Lee et al., 2004).

1.3.2b: Dysregulated Expression

Normal OATP expression patterns are altered in a wide variety of cancers. OATP1A2 is expressed at decreased levels in colon polyps and cancer tissue when compared to healthy colon tissue (Ballestero et al., 2006). Conversely, OATP1A2 expression is significantly higher in malignant breast tissue than in adjacent non-malignant tissue (Meyer zu Schwabedissen et al., 2008). Expression levels of both liver-specific proteins OATP1B1 and OATP1B3 are decreased in hepatocellular carcinomas (Cui et al., 2003; Zollner et al., 2005; Tsuboyama et al., 2010) and adenomas (Vander Borght et al., 2005). OATP1B3, which is normally restricted to hepatocytes, is also expressed in many different tumors and cancer cell lines, including gastric, colon, and pancreatic cancers (Abe et al., 2001; Lee et al., 2008), lung cancer (Monks et al., 2007), breast cancer (Muto et al., 2007), and prostate cancer (Hamada et al., 2008). Table 1-1 shows the dysregulated expression patterns of the normally liver-specific OATP1B1 and OATP1B3.

1.3.2c: Physiological Consequences of Expression

The expression pattern of OATPs illustrates their potential involvement in drug absorption and distribution. Both OATP1A2 and OATP2B1 are expressed on the apical membrane of enterocytes, where they may mediate the absorption of drugs and other xenobiotics

from the intestinal lumen. OATP1B1, OATP1B3, and OATP2B1 are all expressed on the basolateral membrane of hepatocytes, and are involved in the first-pass clearance of many drugs from the bloodstream. Figure 1-2 illustrates the role these OATP family members may play in drug absorption and first-pass clearance from the blood-stream. OATP1A2 is also expressed in liver, but is localized to cholangiocytes, where it may be involved in reabsorbing xenobiotics that would otherwise be excreted through the bile. Similarly, OATP1A2 is expressed at the apical membrane of distal nephrons in the kidney, where it could be involved in the secretion of xenobiotics into the urine or, conversely, could mediate the reabsorption of xenobiotics from the urine. The expression of OATP1A2 and OATP2B1 in the endothelial cells of brain capillaries also suggests that these proteins could play a role in either maintaining or bypassing the blood-brain barrier. Finally, OATP2B1 is expressed in epithelial cells in placenta and mammary glands, and could be involved in bypassing or maintaining the fetal-maternal blood barrier and blood-milk barrier.

The abnormal expression of OATPs in many cancer types may also have physiological consequences. OATPs transport numerous hormones and their conjugates, which could provide a survival benefit to hormone-dependent cancers. Additionally, as will be described in the following section, OATPs are capable of transporting a variety of anti-cancer drugs. Theoretically, this ability could be taken advantage of to better target chemotherapeutic drugs to cancer tissues. Of particular interest is the expression of the normally hepatocyte-specific OATP1B3 in numerous cancer tissues. By improving OATP1B3-mediated transport of anticancer drugs, those drugs could be better directed into the cancer tissues as well as into the slowly dividing hepatocytes, where their toxicity would be much lower.

Table 1-1: Expression of OATP1B1 and OATP1B3 in cancer tissues. Modified from Obaidat et al. (2012).

OATP	**Cancer tissue expression**	**Reference**
OATP1B1	*Reduced* in Hepatocellular carcinoma	(Cui et al., 2003; Vander Borght et al., 2005; Zollner et al., 2005; Libra et al., 2006; Monks et al., 2007; Tsuboyama et al., 2010)
OATP1B3	*Reduced* in Hepatocellular carcinoma	(Vavricka et al., 2004)
	Expressed in:	
	Colorectal adenocarcinoma tissues	(Lee et al., 2008)
	Non-small cell lung tumors	(Monks et al., 2007)
	Prostate cancer tissue	(Hamada et al., 2008; Wright et al., 2011)
	Invasive ductal carcinoma breast cells	(Muto et al., 2007)
	Cell lines of stomach, colon, pancreatic and gall bladder cancers	(Abe et al., 1999)

Figure 1-2:

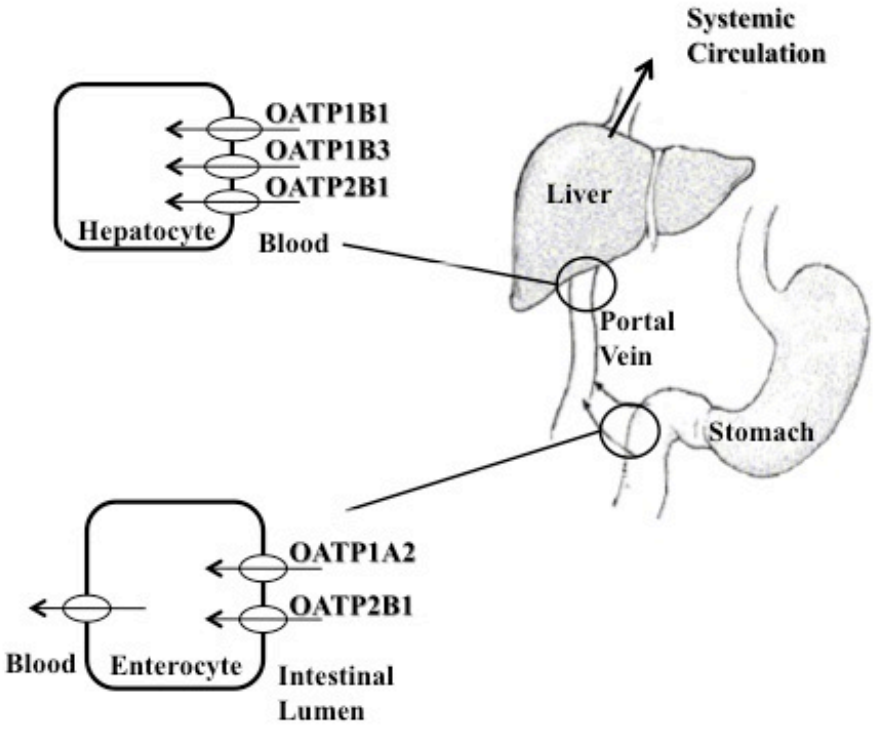
Systemic
Circulation
OATP1B1
OATP1B3
OATP2B1
Hepatocyte
Blood
Liver
Portal
Vein
Stomach
OATP1A2
OATP2B1
Blood
Enterocyte
Intestinal
Lumen

Figure 1-2:

OATPs involved in xenobiotic absorption and first-pass clearance. Xenobiotics may be absorbed into enterocytes from the intestinal lumen through OATP1A2- or OATP2B1-mediated transport. Absorbed xenobiotics enter the portal blood from the enterocytes through uncharacterized transporters. Portal blood then flows through the liver, where xenobiotics may be taken up into hepatocytes through OATP1B1-, OATP1B3- or OATP2B1-mediated transport.

1.3.3: Substrate Specificity

OATPs mediate the uptake of a wide range of structurally diverse, amphipathic compounds, both endogenous and exogenous. Among the endogenous OATP substrates are bile acids, conjugated steroids, thyroid hormones, and linear and cyclic peptides. Xenobiotic substrates include mushroom toxins, the cholesterol-lowering statins, sartans, numerous antibiotics, and some anticancer drugs. Selected substrates of OATP1A2, OATP1B1, OATP1B3, and OATP2B1 are listed in Tables 1-2 through 1-5.

OATPs have a partially overlapping yet distinct substrate specificity. For example, all four proteins described above transport the model substrates estrone-3-sulfate and bromosulfophthalein; fluvastatin is transported by OATPs 1B1, 1B3, and 2B1; and methotrexate is transported by OATPs 1A2, 1B1, and 1B3, however cholecystokinin-octapeptide (CCK8) is selectively transported by OATP1B3. The diverse range of substrates for OATPs suggests the presence of multiple binding sites. Indeed, OATP1B1-mediated transport of estrone-3 sulfate has biphasic saturation kinetics, suggesting the presence of both a high-affinity, low-capacity binding site and a low-affinity, high-capacity binding site (Tamai et al., 2001; Noe et al., 2007; Gui and Hagenbuch, 2009). Similarly, it has been shown that OATP4C1 has distinct binding sites for estrone-3-sulfate and digoxin (Yamaguchi et al., 2010), and that rat Oatp1a4 has at least two distinct binding sites, with estradiol-17β-glucuronide being handled differently than digoxin or taurocholate (Sugiyama et al., 2002). In addition, compounds may have inhibitory, stimulatory, or no effect on OATP-mediated transport, depending on the model substrate used. Clotrimazole stimulates transport of estradiol-17β-glucuronide by OATP1B3, whereas it inhibits transport of

Table 1-2: Selected Substrates of OATP1A2. Modified from Roth et al. (Submitted).

Substrates	References
Acebutolol	(Kato et al., 2009)
Atenolol	(Kato et al., 2009)
Atrasentan	(Katz et al., 2006)
Bamet-R2	(Briz et al., 2002)
Bamet-UD2	(Briz et al., 2002)
Bilirubin	(Briz et al., 2003)
BQ-123	(Kullak-Ublick et al., 2001)
Bromosulfophthalein	(Kullak-Ublick et al., 1995)
Celiprolol	(Kato et al., 2009)
Chlorambucil-taurocholate	(Kullak-Ublick et al., 1997)
Cholate	(Kullak-Ublick et al., 1995; Meier et al., 1997)
Ciprofloxacin	(Maeda et al., 2007)
CRC220	(Meier et al., 1997)
Darunavir	(Hartkoorn et al., 2010)
Dehydroepiandrosterone-3-sulfate	(Kullak-Ublick et al., 1998)
Deltorphin II	(Gao et al., 2000)
[D-penicillamine^{2,5}]enkephalin	(Gao et al., 2000)
Enoxacin	(Maeda et al., 2007)

Epicatechin gallate	(Roth et al., 2011b)
Epigallocatechin gallate	(Roth et al., 2011b)
Erythromycin	(Franke et al., 2008)
Estradiol-17β-glucuronide	(Meier et al., 1997; Kullak-Ublick et al., 2001; Briz et al., 2003)
Estrone-3-sulfate	(Lee et al., 2005)
Fexofenadine	(Cvetkovic et al., 1999)
Gatifloxacin	(Maeda et al., 2007)
Gd-B20790	(Pascolo et al., 1999)
Glycocholate	(Kullak-Ublick et al., 1995; Meier et al., 1997; Kullak-Ublick et al., 2001)
Hydroxyurea	(Walker et al., 2011)
Imatinib	(Hu et al., 2008)
Labetalol	(Kato et al., 2009)
Levofloxacin	(Maeda et al., 2007)
Lomefloxacin	(Maeda et al., 2007)
Lopinavir	(Hartkoorn et al., 2010)
Methotrexate	(Badagnani et al., 2006)
Microcystin	(Fischer et al., 2005)
Nadolol	(Kato et al., 2009)
Norfloxacin	(Maeda et al., 2007)
Ouabain	(Bossuyt et al., 1996)
Pitavastatin	(Fujino et al., 2005)

Prostaglandin E_2	(Kullak-Ublick et al., 2001)
Reverse triiodothyronine (rT3)	(Fujiwara et al., 2001)
Rocuronium	(van Montfoort et al., 1999)
Rosuvastatin	(Ho et al., 2006)
Saquinavir	(Su et al., 2004)
Sotalol	(Kato et al., 2009)
Talinolol	(Shirasaka et al., 2010)
Taurocholate	(Kullak-Ublick et al., 1995)
Taurochenodeoxycholate	(Kullak-Ublick et al., 1995)
Tauroursodeoxycholate	(Kullak-Ublick et al., 1995)
Thyroxine (T4)	(Fujiwara et al., 2001)
Tebipenem pivoxil	(Kato et al., 2010)
TR-14035	(Tsuda-Tsukimoto et al., 2006)
Triiodothyronine (T3)	(Fujiwara et al., 2001)
Unoprostone metabolite	(Gao et al., 2005)

Bamet-R2: *cis*-diammine-chloro-cholylglycinate-platinum(II); Bamet-UD2: *cis*-diammine-bisursodeoxycholate-platinum(II); BQ-123: cyclic pentapeptide endothelin receptor antagonist; CRC220: peptidomimetic thrombin inhibitor; TR-14035: a4b1/a4b7 integrin dual antagonist

Table 1-3: Selected Substrates of OATP1B1. Modified from Roth et al. (submitted).

Substrates	References
Arsenic (arsenite, arsenate)	(Lu et al., 2006)
Atorvastatin	(Lau et al., 2007)
Atrasentan	(Katz et al., 2006)
Bamet-R2	(Briz et al., 2002)
Bamet-UD2	(Briz et al., 2002)
Benzylpenicillin	(Tamai et al., 2000)
BDE47	(Pacyniak et al., 2010)
BDE99	(Pacyniak et al., 2010)
BDE153	(Pacyniak et al., 2010)
Bilirubin	(Briz et al., 2003)
Bisglucuronosyl bilirubin	(Cui et al., 2001)
Bosentan	(Treiber et al., 2007)
BQ-123	(Kullak-Ublick et al., 2001)
Bromosulfophthalein	(Cui et al., 2001; Kullak-Ublick et al., 2001)
Caspofungin	(Sandhu et al., 2005)
Cefazolin	(Nakakariya et al., 2008)
Cefditoren	(Nakakariya et al., 2008)
Cefoperazone	(Nakakariya et al., 2008)

Cerivastatin	(Shitara et al., 2003)
Cholate	(Cui et al., 2001)
[D-Ala2, D-Leu5]enkephalin	(Nozawa et al., 2003)
Darunavir	(Hartkoorn et al., 2010)
Dehydroepiandrosterone-3-sulfate (DHEAS)	(Abe et al., 1999; Hsiang et al., 1999; Abe et al., 2001; Cui et al., 2001; Kullak-Ublick et al., 2001)
Demethylphalloin	(Meier-Abt et al., 2004)
[D-penicillamine^{2,5}]enkephalin (DPDPE)	(Abe et al., 2001)
Eltrombopag	(Takeuchi et al., 2011)
Enalapril	(Liu et al., 2006)
Estradiol-17β-glucuronide	(Abe et al., 1999; Konig et al., 2000b; Tamai et al., 2000; Cui et al., 2001; Kullak-Ublick et al., 2001; Nakai et al., 2001; Tamai et al., 2001; Hirano et al., 2004)
Estrone-3-sulfate	(Cui et al., 2001; Hirano et al., 2004) (Tamai et al., 2001; Noe et al., 2007)
Ezetimibe glucuronide	(Oswald et al., 2008)
Fluvastatin	(Kopplow et al., 2005; Noe et al., 2007)
Gimatecan	(Oostendorp et al., 2009)
Glycocholate	(Kullak-Ublick et al., 2001)

Glycoursodeoxycholate	(Maeda et al., 2006b)
Hydroxyurea	(Walker et al., 2011)
Leukotriene C4	(Abe et al., 1999)
Leukotriene E4	(Abe et al., 1999)
Lopinavir	(Hartkoorn et al., 2010)
Mesalazine	(Konig, 2011)
Methotrexate	(Abe et al., 2001)
Microcystein	(Fischer et al., 2005)
Monoglyucuronosyl bilirubin	(Cui et al., 2001)
Mycophenolic acid-7-O-glucuronide	(Picard et al., 2010)
Nafcillin	(Nakakariya et al., 2008)
Olmesartan	(Nakagomi-Hagihara et al., 2006; Yamada et al., 2007)
Phalloidin	(Fehrenbach et al., 2003; Meier-Abt et al., 2004)
Pitavastatin	(Hirano et al., 2004; Fujino et al., 2005)
Pravastatin	(Hsiang et al., 1999; Nakai et al., 2001; Sasaki et al., 2002)
Prostaglandin E_2	(Abe et al., 1999; Tamai et al., 2000; Kullak-Ublick et al., 2001)
Rifampicin	(Vavricka et al., 2002; Tirona et al., 2003)

Rosuvastatin	(Ho et al., 2006)
Saquinavir	(Hartkoorn et al., 2010)
Simvastatin acid	(Pasanen et al., 2006)
SN-38	(Nozawa et al., 2005a)
Taurocholate	(Abe et al., 1999; Hsiang et al., 1999; Abe et al., 2001; Cui et al., 2001; Kullak-Ublick et al., 2001)
Tauroursodeoxycholate	(Maeda et al., 2006b)
Temocapril	(Maeda et al., 2006a)
Thromboxane B2	(Abe et al., 1999)
Thyroxine (T4)	(Abe et al., 1999)
Torasemide	(Vormfelde et al., 2008; Werner et al., 2008)
Triiodothyronine (T3)	(Abe et al., 1999)
Troglitazone sulfate	(Nozawa et al., 2004b)
Valsartan	(Yamashiro et al., 2006)

Bamet-R2: *cis*-diammine-chloro-cholylglycinate-platinum(II); Bamet-UD2: *cis*-diammine-bisursodeoxycholate-platinum(II); BDE47: 2,2',4,4'-Tetrabromodiphenyl ether; BDE99: 2,2',4,4',5-pentabromodiphenyl ether; BDE153: 2,2',4,4',5,5'-hexabromodiphenyl ether; BQ-123: cyclic pentapeptide endothelin receptor antagonist; SN-38: 7-ethyl-10-hydroxycamptothecin (active metabolite of irinotecan)

Table 1-4: Selected Substrates of OATP1B3. Modified from Roth et al. (submitted).

Substrates	References
Amanitin	(Letschert et al., 2006)
Atrasentan	(Katz et al., 2006)
Benzylpenicillin (Penicillin G)	(Letschert et al., 2006)
BDE47	(Pacyniak et al., 2010)
BDE99	(Pacyniak et al., 2010)
BDE153	(Pacyniak et al., 2010)
Bilirubin	(Briz et al., 2003)
Bosentan	(Treiber et al., 2007)
BQ-123	(Kullak-Ublick et al., 2001)
Bromosulfophthalein	(Kullak-Ublick et al., 2001)
Cefadroxil	(Nakakariya et al., 2008)
Cefazolin	(Nakakariya et al., 2008)
Cefditoren	(Nakakariya et al., 2008)
Cefmetazole	(Nakakariya et al., 2008)
Cefoperazone	(Nakakariya et al., 2008)
Cephalexin	(Nakakariya et al., 2008)
CDCA-NBD	(Yamaguchi et al., 2006)
Cholate	(Briz et al., 2006)
Cholecystokinin octapeptide (CCK-8)	(Ismair et al., 2001; Hirano et al.,

	2004)
Cholyl-glycylamido-fluorescein (CGamF)	(Annaert et al., 2010)
Dehydroepiandrosterone-3-sulfate (DHEAS)	(Konig et al., 2000a; Cui et al., 2001; Kullak-Ublick et al., 2001)
Deltorphin II	(Kullak-Ublick et al., 2001)
Demethylphalloin	(Meier-Abt et al., 2004)
Diclofenac	(Kindla et al., 2011)
Digoxin	(Kullak-Ublick et al., 2001)
Docetaxel	(Smith et al., 2005a)
[D-penicillamine^{2,5}]enkephalin (DPDPE)	(Kullak-Ublick et al., 2001)
Enalapril	(Liu et al., 2006)
Epicatechin gallate	(Roth et al., 2011b)
Epigallocatechin gallate	(Roth et al., 2011b)
Erythromycin	(Franke et al., 2008)
Estradiol-17β-glucuronide	(Konig et al., 2000a; Cui et al., 2001; Hirano et al., 2004)
Estrone-3-sulfate	(Kullak-Ublick et al., 2001; Nozawa et al., 2004b; Nozawa et al., 2005b)
Fexofenadine	(Shimizu et al., 2005)
Fluvastatin	(Kopplow et al., 2005)
Glutathione	(Briz et al., 2006)
Glycocholate	(Kullak-Ublick et al., 2001; Briz et al., 2006)

Glycoursodeoxycholate	(Maeda et al., 2006b)
Hydroxyurea	(Walker et al., 2011)
Imatinib	(Hu et al., 2008)
Leukotriene C4	(Konig et al., 2000a; Kullak-Ublick et al., 2001)
Mesalazine	(Konig, 2011)
Methotrexate	(Abe et al., 2001)
Microcystin	(Fischer et al., 2005; Komatsu et al., 2007)
Monoglyucuronosyl bilirubin	(Cui et al., 2001)
Mycophenolic acid-7-O-glucuronide	(Picard et al., 2010)
Nafcillin	(Nakakariya et al., 2008)
Olmesartan	(Nakagomi-Hagihara et al., 2006; Yamada et al., 2007)
Ouabain	(Kullak-Ublick et al., 2001)
Paclitaxel	(Smith et al., 2005a)
Phalloidin	(Meier-Abt et al., 2004)
Pitavastatin	(Hirano et al., 2004; Fujino et al., 2005)
Rifampicin	(Vavricka et al., 2002; Tirona et al., 2003)
Ro 48-5033	(Treiber et al., 2007)
Rosuvastatin	(Ho et al., 2007)

S-8921G	(Sakamoto et al., 2008)
Saquinavir	(Hartkoorn et al., 2010)
Taurocholate	(Abe et al., 2001; Kullak-Ublick et al., 2001; Letschert et al., 2004; Briz et al., 2006)
Taurochenodeoxycholate	(Briz et al., 2006)
Taurodeoxycholate	(Briz et al., 2006)
Tauroursodeoxycholate	(Maeda et al., 2006b)
Telmisartan	(Ishiguro et al., 2006)
Thyroxine (T4)	(Kullak-Ublick et al., 2001)
TR-14035	(Tsuda-Tsukimoto et al., 2006)
Triiodothyronine (T3)	(Abe et al., 2001; Kullak-Ublick et al., 2001)
Valsartan	(Yamashiro et al., 2006)

BDE47: 2,2',4,4'-Tetrabromodiphenyl ether; BDE99: 2,2',4,4',5-pentabromodiphenyl ether; BDE153: 2,2',4,4',5,5'-hexabromodiphenyl ether; BQ-123: cyclic pentapeptide endothelin receptor antagonist; CDCA-NBD: chenodeoxycholyl-(Ne-NBD)-lysine; Ro 48-5033: Bosentan metabolite; S-8921G: methyl 1-(3,4-dimethoxyphenyl)-(3-ethylvaleryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate glucuronide (inhibitor of the ilial apical sodium-dependent bile acid transporter); TR-14035: a4b1/a4b7 integrin dual antagonist

Table 1-5: Selected Substrates of OATP2B1 Modified from Roth et al. (submitted).

Substrates	References
Aliskiren	(Vaidyanathan et al., 2008)
Atorvastatin	(Grube et al., 2006b)
Benzylpenicillin	(Tamai et al., 2000)
BDE47	(Pacyniak et al., 2010)
BDE99	(Pacyniak et al., 2010)
BDE153	(Pacyniak et al., 2010)
Bosentan	(Treiber et al., 2007)
Bromosulfophthalein	(Kullak-Ublick et al., 2001)
Dehydroepiandrosterone-3-sulfate (DHEAS)	(Pizzagalli et al., 2003)
Eltrombopag	(Takeuchi et al., 2011)
Estrone-3-sulfate	(Tamai et al., 2001; Pizzagalli et al., 2003; Nozawa et al., 2004a; Grube et al., 2006a; Hirano et al., 2006)
Ezetimibe glucuronide	(Oswald et al., 2008)
Fexofenadine	(Nozawa et al., 2004a)
Fluvastatin	(Kopplow et al., 2005; Noe et al., 2007)
Glibenclamide	(Satoh et al., 2005)
Latanoprost acid	(Kraft et al., 2010)

Mesalazine	(Konig, 2011)
Montelukast	(Mougey et al., 2009)
Pravastatin	(Nozawa et al., 2004a)
Pitavastatin	(Hirano et al., 2006)
Pregnenolone sulfate	(Grube et al., 2006a)
Prostaglandin E_2	(Tamai et al., 2000)
Rosuvastatin	(Ho et al., 2006)
Talinolol	(Shirasaka et al., 2010)
Taurocholate	(Kobayashi et al., 2003)
Tebipenem pivoxil	(Kato et al., 2010)
Thyroxine (T4)	(Leuthold et al., 2009)
Unoprostone metabolite	(Gao et al., 2005)

BDE47: 2,2’,4,4’-Tetrabromodiphenyl ether; BDE99: 2,2’,4,4’,5-pentabromodiphenyl ether; BDE153: 2,2’,4,4’,5,5’-hexabromodiphenyl ether

Fluo-3, and has little effect on the uptake of estrone-3-sulfate by the same transporter (Gui et al., 2008). Gemfibrozil inhibits OATP1B1-mediated uptake of pravastatin, fluvastatin, simvastatin and taurocholate, but not estrone-3-sulfate or troglitazone sulfate (Noe et al., 2007). Rosiglitazone inhibits OATP1B1- and OATP1B3-mediated transport of bromosulfophthalein, while stimulating uptake of pravastatin (Bachmakov et al., 2008).

Substrates of the OATPs generally have molecular weights greater than 350 Daltons, and as the name suggests are usually anionic, although some OATPs can also transport neutral or cationic compounds (Bossuyt et al., 1996). Attempts to define the structural or chemical requirements for transport by OATPs have included the development of a pharmacophore model based on published apparent affinity (K_m values) of substrates (Chang et al., 2005), and a CoMFA model based on 25 competitive inhibitors for estradiol-17β-glucuronide (Gui et al., 2009). The first model suggests that substrates generally contain two hydrogen bond acceptors, one hydrogen bond donor, and two hydrophobic regions (Chang et al., 2005). The substrate binding site for estradiol-17β-glucuronide may consist of a large hydrophobic region with basic residues at both ends (Gui et al., 2009). However, the predicted presence of multiple binding sites or translocation pathways complicates the *in silica* identification of binding sites.

1.3.4: Mechanism of Transport

There are three main classes of mediated transport: passive, primary active, and secondary active. Passive transport is driven by the electrochemical gradient of the substrate being transported, whereas primary active transport uses the hydrolysis of ATP to directly drive

the transport of substrates. Secondary active transport is driven by an electrochemical gradient generated by a primary active transporter. It is generally accepted that OATPs operate through secondary active transport, but the driving force has not yet been identified. Several studies have suggested that OATPs work as electroneutral exchangers, and bicarbonate, glutathione, and glutathione conjugates have all been proposed as intracellular compounds that may be exchanged for extracellular substrates (Satlin et al., 1997; Li et al., 1998; Franco and Cidlowski, 2006; Leuthold et al., 2009). However, there is some evidence that the driving force may vary either by transporter or by substrate: for example, intracellular glutathione trans-stimulates uptake of multiple substrates by Oatp1a1 (Li et al., 1998), but not by OATP1B1 or OATP1B3 (Mahagita et al., 2007).

There is considerable evidence that OATP2B1 has both a broader substrate specificity and increased transport ability at acidic pH (Kobayashi et al., 2003; Nozawa et al., 2004a; Sai et al., 2006; Varma, 2011). The increased transport can be caused by either increased affinity for substrate, or increased turnover rate of the transporter (Nozawa et al., 2004a; Leuthold et al., 2009). A recent study suggested that this increased substrate affinity was due to the protonation of a conserved histidine residue on the extracellular portion of transmembrane domain 3 (TM3) (Leuthold et al., 2009). However, this effect seems to be substrate dependent, as numerous substrates of OATP2B1 do not show increased transport at reduced pH. OATP2B1 is widely expressed throughout the body, including at the apical membrane of enterocytes. The phenomenom of increased transport under acidic conditions could result in compounds being transported by OATP2B1 into the body, but not being distributed into other OATP2B1-expressing tissues. For other OATPs, the role of pH is more controversial. A recent study

demonstrated that the transport of taurocholate, estradiol-17β-glucuronide, and estrone-3-sulfate by OATP1B1 and OATP1B3 is influenced by both pH and membrane potential (Martinez-Becerra et al., 2011), although a previous report showed no pH effect on estrone-3-sulfate transport by OATP1B1 or OATP1B3 (Mahagita et al., 2007).

1.3.5: Structure of OATPs

OATPs are large, membrane-spanning glycoproteins. Several structural features are shared by all OATPs. Hydropathy analyses indicate that all OATPs contain 10 or 12 transmembrane domains, with cytosolic termini. Recently, the 12-transmembrane domain model was confirmed for Oatp1a1 (Wang et al., 2008). The OATP "superfamily signature" is a consensus sequence found on the c-terminal end of extracellular loop 3, and has been used to identify additional family members (Hagenbuch and Meier, 2003). The large extracellular loop (ECL5) between transmembrane domains IX and X is conserved in all OATPs/Oatps, and contains 10 cysteines that are fully conserved in all human OATPs. In OATP2B1, removing this extracellular loop or mutating any of the cysteines into alanine resulted in a protein with limited to no surface expression, indicating that disulfide bonds formed by these cysteines may be essential for correct protein folding (Hanggi et al., 2006). Both the second and fifth extracellular loops (EL2 and EL5) contain multiple potential N-glycosylation sites, some of which have been confirmed for individual proteins. Most OATPs also contain a PDZ consensus sequence (Wang et al., 2005a) that may be involved in localization to the cell membrane (Kato et al., 2004); (Choi et al., 2011). In addition, several of the intracellular loops contain potential

phosphorylation sites, which may provide a mechanism for regulation of cell surface expression (Choi et al., 2011; Kock et al., 2010).

The large number of transmembrane domains in most mammalian multispecific transporters make crystallization extremely difficult. As a result, very few crystal structures exist within the major facilitator superfamily. Due to the continuing difficulties associated with crystallization of multi-pass transmembrane proteins, other experimental methods are required to identify structural components of OATPs that are involved in substrate binding and translocation. Three homology models of the putative structure of OATPs have been generated (Meier-Abt et al., 2005; Gui and Hagenbuch, 2008; Glaeser et al., 2010). Each model was created with comparative modeling, based on the crystal structures of transmembrane transporters in the major facilitator superfamily: glycerol-3-phosphate, lactose permease, and EmrD, all from *E. coli*.

Based on one of these homology models, it was proposed that OATPs transport substrates through a central positively charged pore via a rocker-switch mechanism (Meier-Abt et al., 2005). Several positively charged amino acids that are predicted to line the central pore have been mutated to other amino acids to test this hypothesis. Amino acids R57, K361 and R580 in OATP1B1 (Weaver and Hagenbuch, 2010) and K41, R580 and K361 in OATP1B3 (Glaeser et al., 2010; Mandery et al., 2011) were found to be important for full transport activity, suggesting that positive charges within the pore are involved in the transport of anionic substrates. Indeed, in the case of R57 in OATP1B1 and all three amino acids in OATP1B3, replacing the amino acid with another positively charged amino acid restored function of the protein (Glaeser et al., 2010;

Weaver and Hagenbuch, 2010; Mandery et al., 2011). For OATP1B1, the mutations also produced variant effects on transport, depending on the substrate being tested (Weaver and Hagenbuch, 2010). This suggests that estrone-3-sulfate, estradiol-17β-glucuronide, and bromosulfophthalein have at least partially distinct binding sites.

Closely related OATPs have distinct substrate specificities, and transport common substrates with different kinetic characteristics. Chimeras between OATPs can therefore be used to identifiy regions of each protein that are involved in substrate recognition or translocation. Several such studies have been conducted using chimeras between OATP1B1 and OATP1B3, which share 80% sequence identity. Three neutral amino acids in the tenth transmembrane domain (TM10) of OATP1B3 (Y537, S545, and T550) are important for transport of CCK-8, which is a substrate of OATP1B3 but not of OATP1B1 (Gui and Hagenbuch, 2008). OATP1B1 has biphasic saturation kinetics for estrone-3-sulfate transport, with both high-affinity, low-capacity (K_m = 0.1 – 0.6 μM) and low-affinity, high-capacity (K_m = 7 – 45 μM) binding sites (Tamai et al., 2001; Noe et al., 2007; Gui and Hagenbuch, 2009). TM10 in OATP1B1, specifically amino acids L545, F546, L550 and S554, were identified as essential for the high affinity portion of estrone-3-sulfate transport (Gui and Hagenbuch, 2009). Similar studies showed that transmembrane domains 8 and 9 in OATP1B1 are also critical for estrone-3-sulfate transport, and that TM8 but not TM9 contributes to estradiol-17β-glucuronide transport (Miyagawa et al., 2009), again suggesting that different binding sites are involved in the transport of these two substrates.

Comparative models provide a useful basis for developing hypotheses about the three-

dimensional structure of a protein, and aid in designing experiments to test proposed mechanisms of transport. However, as such models are based on distantly related transporters with low sequence similarity and different functions, they are of limited use in practical applications such as *in silico* predictions of substrate specificity or drug-drug interactions. Before the homology models can be useful for these applications, they must be improved and key characteristics confirmed through experimental procedures.

1.3.6: Polymorphisms

Numerous polymorphisms exist in OATPs, and many have been described in the most highly studied OATP family members. OATP1A2 polymorphisms were first described in 2005, when Lee et al. identifed 6 nonsynonymous polymorphisms (Lee et al., 2005). Four variants had reduced transport activity, two of which showed substrate-dependent effects. Shortly thereafter, seven additional nonsynonymous polymorphisms were identified, 4 of which had altered transport activity (Badagnani et al., 2006). Two polymorphisms in the promoter region of OATP1A2 have been associated with altered clearance of orally-administered imatinib, suggesting that OATP1A2 is involved in imatinib absorption and/or distribution (Yamakawa et al., 2011). Polymorphisms in OATP2B1 have been less frequently studied, although they have been associated with the pharmacokinetics of fexofenadine (Akamine et al., 2010) and montelukast (Mougey et al., 2009).

Polymorphisms in OATP1B1 have clearly demonstrated the importance of this transporter in the disposition of several drugs. A genome-wide scan of patients with simvastatin-

induced myopathy identified a strong association with the noncoding single-nucleotide polymorphism rs4363657 in OATP1B1 (Link et al., 2008). Altered pharmacokinetics of pravastatin and pitavastatin are associated with OATP1B1*1b and OATP1B1*15 polymorphisms, both of which contain the amino acid substitution N130D (Nishizato et al., 2003; Mwinyi et al., 2004; Niemi et al., 2004; Chung et al., 2005; Wen and Xiong, 2010). Neonates with either of these polymorphisms are at a higher risk for developing severe hyperbilirubinemia (Huang et al., 2004; Buyukkale et al., 2011), and the OATP1B1*15 polymorphism is also associated with higher serum bilirubin levels in adults (Ieiri et al., 2004). OATP1B1*5 and OATP1B1*15, which both contain the V174A allele, are associated with reduced efficacy of multiple cholesterol-lowering statins (Tachibana-Iimori et al., 2004), with increased systemic exposure of the antidiabetic nateglinide (Zhang et al., 2006), and with higher system levels of the HIV protease inhibitor lopinavir (Hartkoorn et al., 2010). This allele has not been associated with altered pharmacokinetics of rosiglitazone and pioglitazone (Kalliokoski et al., 2008), torasemide (Werner et al., 2008), mycophenolic acid (Miura et al., 2007) or telmisartan (Miura et al., 2009), indicating either that the amino acid substitution does not alter transport of those drugs, or that OATP1B1-mediated transport into hepatocytes does not play a significant role in their distribution.

Seven nonsynonymous polymorphisms have been identified in the coding region of OATP1B3. Two of these variants, S112A and M233I, were shown to have genotypic frequencies of greater than 70% among individuals of European descent, but did not have altered transport of six model substrates when compared to the reference sequence (Letschert et al., 2004). A separate study performed on an ethnically diverse population revealed lower genotypic

frequency of each allele in the African American population than in other ethnic groups and, in contrast to the earlier study, found that CCK8 transport activity was significantly reduced in the M233I variant compared to the reference sequence (Schwarz et al., 2011). Similar reductions were seen in the transport of rosuvastatin. The M233I allele is associated with altered pharmacokinetic parameters of the immunosuppressant mycophenolic acid (Miura et al., 2007; Picard et al., 2010). Although no associations have been identified between OATP1B3 geneotype and pharmacokinetics of telmisartan (Miura et al., 2009), or paclitaxel (Smith et al., 2007), the rs11045585 genotype (IVS12-5676A>G) is associated with higher systemic concentrations of docetaxel (Chew et al., 2011) and is also associated with docetaxel-induced neutropenia (Kiyotani et al., 2008).

1.4: Interactions with Drug Transporters: Drugs, Foods, and Small Molecules

1.4.1: Adverse drug-drug interactions

Adverse drug-drug interactions pose a significant health threat to older Americans, who frequently take a large number of medications concurrently. Pharmacokinetic drug-drug interactions can take place at any step during drug disposition: absorption, distribution, metabolism or excretion. Traditionally, it has been thought that most drug-drug interactions are caused by changes in the activity or expression of drug-metabolizing enzymes. However, a rapidly growing field of research is demonstrating that these interactions may also be caused by

changes in the other three steps of drug disposition, all of which may involve the participation of transporters.

The multidrug resistance transporter protein (MDR1, P-glycoprotein, P-gp) has been implicated in numerous drug-drug interactions, including those between digoxin and quinidine (Fromm et al., 1999), fexofenadine and ketoconazole (Cvetkovic et al., 1999), and paclitaxel and valspodar (ten Tije et al., 2003). Uptake transporters may also be involved in drug-drug interactions, and may explain the interactions between probenecid and cephalosporin antibiotics (Brown, 1993), cerivastatin and cyclosporine (Shitara et al., 2003), rosuvastatin and cyclosporine (Simonson et al., 2004), or fexofenadine and grapefruit juice (Bailey et al., 2007; Glaeser et al., 2007). The latter three interactions are thought to occur at organic anion transporting polypeptides. Cerivastatin was withdrawn from the US market due to many deaths from severe rhabdomyolysis, which is caused by toxic concentrations of statins in muscles. Many of these deaths occurred in patients who were concomitantly taking gemfibrozil (Charatan, 2001), which inhibits OATP1B1 transport function, and it has been proposed that inhibition of OATP1B1-mediated uptake of cerivastatin into liver contributed to its toxicity (Shitara et al., 2004).

To reduce the possibility of such adverse drug-drug interactions, the International Transporter Consortium recently suggested that new molecular entities should be tested for possible OATP inhibition using a prototypical substrate such as estradiol-17β-glucuronide (Giacomini et al., 2010). However, as was discussed in sections 1.3.3 and 1.3.5, there is considerable evidence that OATPs have multiple substrate binding sites. Therefore, screening for inhibition of a single substrate may be insufficient to identify potential drug-drug interactions

1.4.2: Adverse drug-food interactions

Just as drugs can interfere with the disposition of other drugs, so too can food components and nutrient supplements. Interactions occurring at drug-metabolizing enzymes, such as those caused by grapefruit juice or St. John's wort, are well known. Recent studies have also indicated that these compounds can affect drug uptake and efflux transporters. Such interactions can affect the bioavailability of drugs such as fexofenadine and celiprolol, known OATP substrates (Greenblatt, 2009). In vitro studies have shown that components of fruit juices, teas, and dietary supplements can alter transport mediated by MDR1, MRP2, OATP1A2, OATP1B1 and OATP2B1 (Honda et al., 2004; Wang et al., 2005b; Fuchikami et al., 2006; Bailey et al., 2007). Although many in vivo studies have shown altered pharmacokinetics of drugs when co-administered with these dietary components, it is very difficult to determine whether they are caused by altered uptake, efflux, or metabolism. It seems likely that the overall effect is a result of all three interactions, and that the ultimate determinant of the pharmacokinetic alteration depends on the concentrations of both food and drug, the expression levels of proteins, and the identity of the rate-determining step.

1.4.3: Beneficial drug interactions: targeted treatment

Both drug-drug interactions and drug-food interactions are generally unintended, and the consequences are adverse to health. However, the principle of altered drug pharamcokinetics through concurrent administration of another drug or food can just as easily be beneficial as long as the administration is intentional. During World War II, penicillin was frequently administered

along with the uricosuric agent probenecid, which drastically reduces the urinary excretion of penicillin and thus reduces the required effective dose. By administering the MDR1-inhibiting cyclosporine along with paclitaxel or docetaxel, the absorption of taxanes is improved, presumably due to reduced efflux from enterocytes (Meerum Terwogt et al., 1998; Malingre et al., 2001). Similarly, co-administrating the efflux inhibitor GF120918 with topotecan increased the systemic bioavailability of topotecan two-fold (Kruijtzer et al., 2002). Just as inhibiting efflux from enterocytes can increase systemic bioavailability, stimulating or inhibiting uptake into enterocytes can alter absorption of drugs from the intestine. Selective inhibition of the liver-specific OATP1B1 or OATP1B3 could also be used to inhibit the first-pass clearance of drugs, thereby increasing their systemic bioavailability. Conversely, selective stimulation of OATP1B1 or OATP1B3 uptake could be used to increase drug delivery to hepatocytes, or to stimulate OATP1B3-mediated anticancer drug uptake into OATP1B3-expressing cancer cells.

1.5: Specific Aims of this Dissertation

Adverse drug-drug interactions are a common result of comorbidity and polypharmacy, and pose a significant health threat to older Americans. Such interactions occurring at drug-metabolizing enzymes are well studied and characterized; however, recent research has shown that these interactions may also be caused by alterations in transporter activity. Organic anion transporting polypeptides (OATPs) are transporters that mediate the uptake of a broad range of drugs and other xenobiotics into cells. Among the drugs transported by OATPs are numerous statins and antidiabetic agents, the pharmacokinetics of which depend upon OATP-mediated uptake into hepatocytes. Drug-drug interactions at OATPs can alter the pharmacokinetic profiles

of these or other substrates, leading to decreased efficacy or increased toxicity. Due to the limited structural information available for OATPs, little is known about the mechanism of substrate binding and translocation. Subsequently, little is known about the mechanism of drug-drug interactions that occur at OATPs. Without a detailed understanding of these mechanisms, it will remain impossible to predict potentially hazardous OATP-mediated drug-drug interactions.

The studies presented in this dissertation were designed to address the lack of understanding of the mechanism through which drug-drug interactions occur. In order to decrease the frequency of OATP-mediated drug-drug interactions by improving pre-clinical screening methods, the objective of this dissertation is to characterize the mechanism of drug-drug interactions at OATP1B1 and OATP1B3. By testing the central hypothesis that OATP1B1 and OATP1B3 have overlapping but distinct binding sites, which are affected in substrate-dependent ways, I have discovered novel information about the mechanism of drug-drug interactions occurring at OATPs. A more detailed understanding of this mechanism will allow more accurate prediction and prevention of such occurrences.

Recent studies suggest that these liver-specific OATPs contain multiple substrate binding sites. Experiments in our laboratory demonstrate that mutations in OATPs have varied effects on the transport of different substrates. Furthermore, our laboratory has demonstrated that clotrimazole can stimulate, inhibit, or have no effect on OATP1B3 function, depending upon the substrate transported. Based on these observations, I generated the ***central hypothesis*** of this dissertation: **OATP1B1 and OATP1B3 have overlapping but distinct binding sites, which**

are affected in substrate-dependent ways. This hypothesis was tested via the following specific aims:

1.5.1: Specific Aim 1: Identify and characterize substrate-dependent effects of plant compounds on OATP-mediated transport.

Our working hypothesis was that plants are a rich source of compounds that have substrate-dependent interactions with OATPs. Interacting compounds were identified by screening a library of plant compounds for inhibition or stimulation of OATP-mediated uptake of two model substrates. Substrate-dependent interactions were further studied with additional model substrates and characterized by determining potency (IC_{50} or EC_{50}). Completion of this specific aim identified two plants that contained OATP-modifying compounds, and provides a basis for identifying stimulators of OATP1B3-mediated transport.

1.5.1a: Interaction of green tea catechins with organic anion transporting polypeptides. To determine whether green tea catechins were a potential cause of drug-food interactions occurring at OATP transporters, uptake of the model substrate estrone-3-sulfate by cells expressing OATP1A2, OATP1B1, OATP1B3, or OATP2B1 was quantified in the absence and presence of the four most abundant flavonols found in green tea. In addition, uptake of green tea catechins by the four OATPs was quantified and characterized.

1.5.1b: Isolation of modulators of organic anion transporting polypeptides 1B1 and 1B3 from *Rollinia emarginata* Schlecht (Annonaceae). In this study, a bioassay guided isolation

approach was used to identify specific modulators of OATP1B1 and OATP1B3 from the organic extract of *Rollinia emarginata* Schlecht (Annonaceae). Fractions of the plant extract were screened for effects on OATP1B-mediated transport of the model substrates estradiol-17β-glucuronide and estrone-3-sulfate.

1.5.2. Identify regions of OATP1B3 involved in the binding and/or translocation of individual model substrates.

Our working hypothesis was that the mechanism of substrate-specific drug-drug interactions is dependent on the presence of multiple binding sites. As the first step toward characterization of this mechanism, cysteine-substituted OATP1B3 mutants were produced and tested to identify regions that are involved in substrate recognition and translocation.

Chapter 2

Experimental Materials and Methods

2.1: Materials

[^{3}H]Estrone-3-sulfate and [^{3}H]estradiol-17β-glucuronide were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). [^{3}H]Epigallocatechin gallate (10 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis. MO). Unlabeled estrone-3-sulfate, estradiol-17β-glucuronide, (−)-epicatechin, (−)-epigallocatechin, (−)-epicatechin gallate, and (−)-epigallocatechin gallate and rifampicin were purchased from Sigma-Aldrich (St. Louis, MO). Fluo-3, pentapotassium salt was purchased from Invitrogen (Carlsbad, CA). Green tea (*Camellia sinensis*) biomass was provided by the Royal Estates Tea Company, a Division of Thomas J. Lipton, Co. (Englewood Cliffs, NJ). Above-ground plant material of *Rollina emarginata* was collected and identified in February 1999 in Argentina by R. Fortunato & A. Cabral (INTA) collection # ARP 613. LAT: 25°14'0"5 South LON:57°57'0"0 West. RN 86, 2Km NE of Patino, Department Primavera, Province Formosa.

Human embryonic kidney (HEK-293) cells, Eagle's minimum essential medium, and Dulbecco's modified Eagle's medium (high glucose) were from American Type Culture Collection (Manassas, VA). Dulbecco's modified Eagle's medium (low glucose) was purchased from Caisson Laboratories (North Logan, UT). Fetal bovine serum was obtained from HyClone (Logan, UT). Sulfo-NHS-SS-biotin, NeutrAvidin agarose beads and BCA protein assay kits were from Thermo Fisher Scientific (Rockford, IL). Maleimide-PEG_2-biotin was purchased from

Invitrogen (Carlsbad, CA), and sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) was purchased from Toronto Research Chemicals (North York, Ontario). All other materials were purchased from Sigma-Aldrich or Invitrogen.

2.2: Plant Compound Extraction and Isolation.

Plant compound extraction and isolation was performed by Gemma O'Donnell, Ph.D. (green tea extraction) or by Juan J. Araya (*Rollinia emarginata* extraction and isolation) under the supervision of Barbara N. Timmermann, Ph.D., in the Department of Medicinal Chemistry at the University of Kansas, Lawrence, Kansas, according to the following protocols.

Green Tea Extraction. A sample of green tea biomass was extracted exhaustively with 10 ml of H_2O (70°C, 10 min). The extract was concentrated *in vacuo* and dried overnight at 30°C in a vacuum oven. ***Rollinia emarginata*** **Extraction and Isolation.** The extraction and isolation protocol is outlined in the separation diagram shown in Figure 4-1. Dried and ground plant material (562 g) was extracted with methanol (MeOH) and dichloromethane (CH_2Cl_2) mixture (1:1, v/v) three times for 24 h. periods at room temperature. Organic solvents were removed *in vacuo* at 35°C; the residue was suspended in MeOH:H_2O (9:1, v/v) and partitioned with hexanes (HEX fraction). After removal of MeOH, the aqueous layer was extracted successively with CH_2Cl_2 (DCM fraction) and butanol (BUOH fraction). The HEX fraction was then subjected to silica gel column chromatography (Si-Gel CC) (32-64 μm, 36x460 mm) and eluted with a gradient of hexane-ethyl acetate (EtOAc) (20:1 to 0:100, v/v) to afford 20 subfractions (A to T), which were combined according to TLC analysis. Subfraction HEX-G (310 mg) was submitted

to Si-Gel CC (12-26 μm, 36×230 mm) using a gradient of hexane and acetone (15:1 to 5:1, v/v) to obtain three subfractions (G1-G3). Subfraction HEX-G1 (205 mg) was purified using Si-Gel CC (CH_2Cl_2:EtOAc, 20:1, v/v) to afford Compound 3 (120 mg). Subfraction HEX-G2 was purified with Si-Gel CC (12-16 μm, 20×460 mm) using hexane, CH_2Cl_2 and methyl *tert*-butyl ether (20:15:1, v/v/v) as mobile phase to yield Compound 4 (10.4 mg). Also, subfraction HEX-N (284 mg) was separated using Si-gel CC (32-64μm, 36×230 mm) and CH_2Cl_2-EtOAc (10:1, v/v) as a solvent system to yield a mixture of Compounds 1 and 2 (103 mg), which was resolved by means of semi preparative HPLC (reverse phase C-18, 10×250 mm, 5μm, solvent A: acetonitrile, solvent B :water, gradient: 80%A to 100%A in 45 min). Fraction BUOH (19.7 g) was subject to MCI-Gel CHP20P CC (65×350 mm) and eluted with various mixtures of water and MeOH (100:0, 25:75, 50:50,75:25, 0:100; v/v) to afford four fractions (A-D). Subfraction BUOH-B (2.2g) was submitted to Sephadex LH-20 with MeOH as a mobile phase and a total of 180 fractions (7.5 mL each) were collected and combined into nine fractions (1-9) after TLC analysis. Pigments present in fraction BUOH-B7 (530 mg) were removed with a small Si-gel plug using CH_2Cl_2:MeOH:H_2O (4:1:0.1, v/v/v) as eluent to obtain a mixture of Compounds 5 and 6 (450 mg). A portion of this mixture (40 mg) was purified using semi-preparative HPLC (reverse phase C-18, 10×250 mm, 5μm, solvent A: acetonitrile, solvent B: water, isocratic 18%A) to obtain Compounds 5 (24 mg) and 6 (6.2 mg).

2.3: Plant Compound Identification

Plant compound identification was performed by Juan J. Araya under the supervision of Barbara N. Timmermann, Ph.D., in the Department of Medicinal Chemistry, at the University of

Kansas, Lawrence, Kansas. The structures of compounds isolated from *Rollinia emarginata* were established by one and two dimension NMR experiments and compared with those in the literature (Seebacher et al., 2003; Zhang et al., 2005; Muzitano et al., 2006; Faini et al., 2007). IR, UV, and HRMS were also in agreement with the proposed structures. NMR experiments were performed in a Bruker AVIII 500 instrument with a dual C/H cryoprobe. Standard ^{1}H-NMR, ^{13}C-NMR, COSY, HSQC and HMBC experiments were recorded of each of the pure compounds. An Agilent 1200 system with a 6300 Series Ion Trap detector was used for LCMS experiments. An Agilent RP-C18 (15x4.1mm, 5μm) column was used and different gradients of acetonitrile and water were applied as the mobile phase depending on polarity of samples. HRMS was obtained with a LCT Premier instrument (Waters Corp., Milford, MA). The purity of each compound was determined to be over 95% by HPLC analysis.

2.4: Site-directed Mutagenesis

The backbone for all OATP1B3 mutagenesis experiments is the previously described His-tagged OATP1B3 in the pcDNA5/FRT expression vector (Gui and Hagenbuch, 2008). Site-directed mutagenesis was performed with primers custom synthesized by Invitrogen, using the Quikchange II Site-Directed Mutagenesis Kit from Agilent Technologies (Santa Clara, CA), as per the manufacturer's instructions. Cysteine-substituted constructs were fully sequenced on both strands. Mutant Q54C could not be propagated in XL1-Blue Supercompetent cells (Agilent Technologies) or in One Shot® TOP10 Chemically Competent E. coli (Invitrogen), and is not included in the analysis.

2.5: Cell Culture

CHO cells stably transfected with human OATP1B1, OATP1B3, and OATP2B1 were generated in our laboratory previously and were cultured as described previously (Gui et al., 2008; Pacyniak et al., 2010). HeLa cells were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. HEK-293 cells were grown in Dulbecco's modified Eagle's medium containing 4.5 g/l D-glucose, 2 mM L-glutamine, 25 mM HEPES, and 110 mg/l sodium pyruvate, supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cells were maintained in a humidified environment at 37°C and 5% CO_2.

2.6: OATP Expression

CHO cells stably expressing OATP1B1 or OATP1B3 and wild-type CHO cells were seeded on 24- or 96-well plates and grown for 48-72 hours. Once cells had reached visual confluency, gene expression was non-specifically induced in the absence of geneticin by exchanging medium for that containing 5 mM sodium butyrate (Palermo et al., 1991). Experiments were performed 24 h after induction. CHO cells stably expressing OATP2B1 or the empty vector were seeded on 24-well plates 48 h before uptake experiments; OATP2B1 expression in this cell line did not require sodium butyrate induction. OATP1A2 was transiently expressed in HeLa or HEK-293 cells. HeLa cells were seeded on 12-well plates and transfected using the vaccinia virus T7 system, essentially as described previously (Lee et al., 2005). Between 16 and 20 h before uptake experiments, cells were infected with vaccinia virus in

serum-free Opti-MEM medium and incubated for 1 h at 37°C. After washing, cells were transfected with pcDNA5/FRT containing the open reading frame of a His-tagged OATP1A2 or with the empty vector using Lipofectamine 2000, as per the manufacturer's instructions. HEK-293 cells were seeded on 24-well plates pretreated with poly-D-lysine and were transfected with pExpress-1 (Express Genomics, Inc., Frederick, MD) containing OATP1A2 or with the empty vector approximately 48 h before uptake experiments. OATP1B3 mutant constructs were also transiently expressed in HEK-293 cells. Cells were seeded in 12- or 24-well tissue culture plates pre-treated with poly-D-lysine. At approximately 90% visual confluence, cells were transfected with wild-type or mutant OATP1B3 constructs or the empty plasmid using FuGENE-HD transfection reagent from Roche (Basel, Switzerland), following the manufacturer's instructions. All experiments were performed forty-eight hours after transfection.

2.7: Transport Assays

Uptake experiments were performed essentially as described previously for CHO (Gui et al., 2008) and HEK-293 cells (Weaver and Hagenbuch, 2010). CHO uptake buffer contained 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM HEPES; pH was adjusted to 7.4 using Trizma base. HeLa uptake buffer contained 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM Hepes and was adjusted to pH 7.5 with Trizma base. For experiments involving OATP1A2, HEK-293 uptake buffer contained 142 mM NaCl, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose, and 12.5 mM HEPES, and was adjusted to pH 7.4 with Trizma base. For experiments with OATP1B3 transiently expressed in HEK-293 cells, CHO uptake buffer was used.

Cells were washed three times with prewarmed (37°C) uptake buffer, then cells were incubated at 37°C with uptake buffer containing substrate for a given timepoint. Uptake was terminated by quickly removing the uptake solution and washing four times with ice-cold uptake buffer. To quantify uptake of radiolabeled substrates, cells were lysed with 1% Triton X-100 in phosphate-buffered saline, and the radioactivity was quantified with liquid scintillation counting. To measure uptake of Fluo-3, cells were lysed with 1% Triton X-100 in phosphate-buffered saline containing 1 mM $CaCl_2$, and fluorescence was quantified on a Synergy HT microplate reader (BioTek Instruments, Winooski, VT) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Unlabeled catechins were detected using a Quattro Premier high-performance liquid chromatography tandem mass spectrometer (Waters, Milford, MA) in electrospray negative ion mode using a C18 column (50 × 2.1 mm, 5 μm; Phenomenex, Torrance, CA) at 40°C. The mobile phase consisted of 60:40 acetonitrile and 1% acetic acid, and was eluted isocratically with a flow rate of 0.3 ml/min. Cells were lysed in mobile phase containing 1 μM ethyl gallate, and lysate was centrifuged at 20,000 xg for 20 min to remove protein before injection. The transitions monitored were 441.15 > 169.1 for ECG, 457.05 > 169.1 for EGCG, and 197.17 > 124.2 for ethyl gallate (internal standard). QuanLynx software (Waters) was used to quantify mass spectrometry data. Protein concentrations were determined with BCA assay kits (Thermo Fisher Scientific, Waltham, MA), and uptake was corrected for protein. Net OATP-mediated uptake was defined as the uptake by OATP-expressing cells minus the uptake by the appropriate control cell line (wild-type CHO cells for OATP1B1 and OATP1B3, CHO cells stably expressing empty vector for OATP2B1, and HeLa or HEK-293 cells transiently expressing the empty vector for transiently expressed OATP1A2 and OATP1B3).

2.8: Cell Surface Biotinylation and Immunoblot Analysis

Cell surface expression was quantified by incubating cells with 1 mg/ml sulfo-NHS-SS-biotin in PBS for 1 h. at 4°C. After quenching the reaction with 100 mM glycine in PBS, cells were lysed for 10 min. on ice, in buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100 and protease inhibitors (Roche). Lysate was centrifuged at 10,000 xg for 2 min. Supernatant was added to neutravadin agarose beads, and incubated for 1 h. at room temperature. Beads were washed three times for 5 min. with lysis buffer, then biotinylated proteins were eluted by incubating in 2x Laemmli buffer containing 5% 2-mercaptoethanol for 30 min. Protein was run on a 7.5% SDS-PAGE gel and transferred to nitrocellulose membrane. Western blots were probed with a monoclonal tetra-His antibody from Qiagen (Valencia, CA), following the manufacturer's recommendations. The plasma membrane marker Na^+/K^+ ATPase was detected as a control for biotinylation.

2.9: Methanethiosulfonate and Maleimide Accessibility Reactions

HEK293 cells transiently expressing OATP1B3 were washed three times with prewarmed (37°C) uptake buffer, then incubated with uptake buffer containing 10 mM MTSES for ten min. at 37°C. Cells were then washed two times with prewarmed uptake buffer before performing uptake experiments as described above. Uptake was normalized for protein concentration, and uptake by cells transfected with the empty vector was subtracted from each uptake value.

To determine accessibility of inserted cysteines to the aqueous extracellular environment, cells transiently expressing wild-type or mutant OATP1B3 were incubated with the membrane impermeable, thiol-specific reagent maleimide-PEG_2-biotin (0.5 mg/ml in PBS, pH 7.0) for one h. at 4°C. After thorough washing, cells were lysed and affinity purified with neutravidin-agarose beads as described above. Biotinylated proteins were eluted by boiling in 2x Laemmli sample buffer containing 5% 2-Mercaptoethanol for five min. Both eluted proteins and total cleared lysate (as a control for transfection) were run on 7.5% SDS-PAGE gels and transferred to nitrocellulose membranes. Western blots were probed with a monoclonal tetra-His antibody from Qiagen (Valencia, CA), following the manufacturer's recommendations.

2.10: Calculations and Statistics

All calculations were performed using Prism 5 (GraphPad Software Inc., San Diego, CA). IC_{50} values and kinetic parameters were determined within the initial linear period of uptake after correction for protein and subtraction of uptake by the control cell line. Statistical analyses were performed with two-way analysis of variance followed by the Bonferroni post-test, or with two-tailed paired t-tests.

Chapter 3

Interactions of Green Tea Catechins with Organic Anion-Transporting Polypeptides

3.1: Abstract

Organic anion-transporting polypeptides (OATPs) are multispecific transporters that mediate the uptake of numerous drugs and xenobiotics into cells. Here, we examined the effect of green tea (*Camellia sinensis*) catechins on the function of the four OATPs expressed in human enterocytes and hepatocytes. Uptake of the model substrate estrone-3-sulfate by cells expressing OATP1A2, OATP1B1, OATP1B3, or OATP2B1 was measured in the absence and presence of the four most abundant flavonols found in green tea. Uptake by OATP1A2, OATP1B1, and OATP2B1 was inhibited by epicatechin gallate (ECG) and epigallocatechin gallate (EGCG) in a concentration-dependent way. In contrast, OATP1B3-mediated uptake of estrone-3-sulfate was strongly stimulated by EGCG at low substrate concentrations. The effect of EGCG on OATP1B3 was also studied with additional substrates: uptake of estradiol-17β-glucuronide was unchanged, whereas uptake of Fluo-3 was noncompetitively inhibited. Both ECG and EGCG were found to be substrates of OATP1A2 (K_m values of 10.4 and 18.8 μM, respectively) and OATP1B3 (34.1 and 13.2 μM, respectively) but not of OATP1B1 or OATP2B1. These results indicate that two of

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the major flavonols found in green tea have a substantial effect on the function of OATPs expressed in enterocytes and hepatocytes and can potentially alter the pharmacokinetics of drugs and other OATP substrates. In addition, the diverse effects of EGCG on the transport of other OATP1B3 substrates suggest that different transport/binding sites are involved.

3.2: Introduction

Adverse drug-drug interactions are a common result of comorbidity and polypharmacy and pose a significant health threat. Furthermore, dietary supplements are increasingly popular, and some of their ingredients have the potential for additional drug interactions. These adverse drug interactions may be caused by alterations in efflux (Durr et al., 2000) and uptake transporters (Fattinger et al., 2000), such as the organic anion-transporting polypeptides (OATPs). OATPs are multispecific transporters that mediate the cellular uptake of a wide range of amphipathic compounds, including numerous drugs (Hagenbuch and Gui, 2008). Four well characterized OATPs, OATP1A2, OATP1B1, OATP1B3 and OATP2B1, are expressed in the small intestine and the liver, where the likelihood of drug-drug or drug-food interactions is the greatest.

Both OATP1A2 and OATP2B1 are expressed at the apical membrane of enterocytes (Kobayashi et al., 2003; Glaeser et al., 2007) where they can contribute to the absorption of drugs such as statins, sartans, fexofenadine, talinolol, and methotrexate (Shimizu et al., 2005; Badagnani et al., 2006; Ho et al., 2006; Kitamura et al., 2008; Shirasaka et al., 2010). In the liver, OATP1B1, OATP1B3, and OATP2B1 are expressed at the basolateral membrane of hepatocytes

(Abe et al., 1999; Konig et al., 2000b; Kullak-Ublick et al., 2001). Here, these proteins are involved in the removal of drugs from the bloodstream into hepatocytes. With their broader substrate specificity, OATP1B1 and OATP1B3 are thought to play a more important role in hepatocellular drug uptake than OATP2B1 (Smith et al., 2005b; Hagenbuch and Gui, 2008; Kindla et al., 2009).

The importance of OATPs to drug disposition has been highlighted by pharmacokinetic studies that correlated changes in the bioavailability of drugs with polymorphisms of OATPs (Kalliokoski and Niemi, 2009). Thus, inhibition or stimulation of OATP function by food or dietary supplements can alter the pharmacokinetics of OATP substrates and potentially lead to adverse effects. Recent studies have indicated that flavonoids found in fruit juices, in green tea, and in many dietary supplements can alter the function of OATP1A2, OATP1B1, and OATP2B1 (Wang et al., 2005b; Fuchikami et al., 2006; Bailey et al., 2007). Such interactions can affect the bioavailability of drugs such as fexofenadine and celiprolol, known OATP substrates (Greenblatt, 2009).

Green tea is a commonly consumed beverage and has received much attention for its reputed health benefits. Several epidemiological studies have shown a reduced risk of gastrointestinal cancers among those who regularly consume green tea (Liu et al., 2008). Compared with other tea preparations, green tea is characterized by very high concentrations of catechins, which make up 30 to 40% of its dry weight. The catechins include epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG), and epigallocatechin gallate (EGCG). EGCG, the most predominant catechin in green tea, has been highly studied for its in vitro

effects. Because of the many apparent health benefits of green tea and EGCG, green tea beverages and extract supplements are widely used, creating an increased risk of adverse interactions. EGC and EGCG have been shown to inhibit OATP1B1-mediated uptake of dehydroepiandrosterone sulfate (Wang et al., 2005b), whereas all four catechins inhibited estrone-3-sulfate uptake mediated by OATP2B1 (Fuchikami et al., 2006). However, the effect of catechins on the function of OATP1A2 and OATP1B3 has not been reported, and it is not known whether any of these four catechins are transported by any of the OATPs. Therefore, in the present study we asked the question whether all four major catechins inhibit OATP-mediated uptake and whether they are transported by OATPs.

3.3: Results

3.3.1 Characterization of OATP1A2 and OATP2B1 Expression Systems.

OATP2B1 was expressed at high levels on the plasma membrane of the stably transfected OATP2B1-expressing CHO cells as confirmed using an anti-His antibody (data not shown). To minimally characterize OATP2B1 and the transiently expressed OATP1A2 at a functional level, we used the model substrate estrone-3-sulfate. Uptake of 100 μM estrone-3-sulfate was linear for at least 30 s for both OATP1A2 and OATP2B1 (data not shown); therefore, subsequent experiments were performed at 30 s for OATP1A2 and 20 s for OATP2B1. In both systems, transport of estrone-3-sulfate was saturable, with apparent K_m and V_{max} values of 16.1 ± 0.2 μM and 640 ± 150 pmol/mg · min for OATP1A2 and 14.8 ± 4.0 μM and 2.54 ± 0.57 nmol/mg · min for OATP2B1, respectively.

3.3.2: Effect of Green Tea Extract and Catechins on OATP-Mediated Uptake of Estrone-3-Sulfate.

To determine the effects of green tea components on OATP function, we measured OATP-mediated uptake of 0.1 μM estrone-3-sulfate in the presence of 0.03 μg/ml green tea extract or 100 μM green tea catechin under initial linear rate conditions. EC and EGC did not significantly affect estrone-3-sulfate uptake by any of the four cell lines (Figure 3-1, A–D). ECG and EGCG inhibited uptake of estrone-3-sulfate by OATP1A2, OATP1B1, and OATP2B1. Of interest, OATP1B3-mediated uptake of estrone-3-sulfate was unaffected by ECG but strongly stimulated by EGCG (Figure 3-1C). To further characterize the effect of the gallated catechins on OATP-mediated uptake of estrone-3-sulfate, we determined uptake of 0.1 μM estrone-3-sulfate in the presence of increasing concentrations of ECG or EGCG. As shown in Figure 2, both ECG and EGCG exhibited a concentration-dependent inhibition of estrone-3-sulfate uptake mediated by OATP1A2, OATP1B1, and OATP2B1 (Figure 3-2, A, B, and D). Uptake by OATP1A2 and OATP2B1 was more strongly inhibited by ECG (IC_{50} values of 10.2 and 35.9 μM, respectively) than by EGCG (54.8 and 101 μM, respectively), whereas uptake by OATP1B1 was more strongly inhibited by EGCG than by ECG (IC_{50} values of 7.8 and 58.6 μM, respectively). EGCG stimulated estrone-3-sulfate uptake by OATP1B3 5-fold at concentrations of 30 to 300 μM. The stimulatory effect remained at 1 mM EGCG; however, it was greatly reduced (Figure 3-2C).

Figure 3-1:

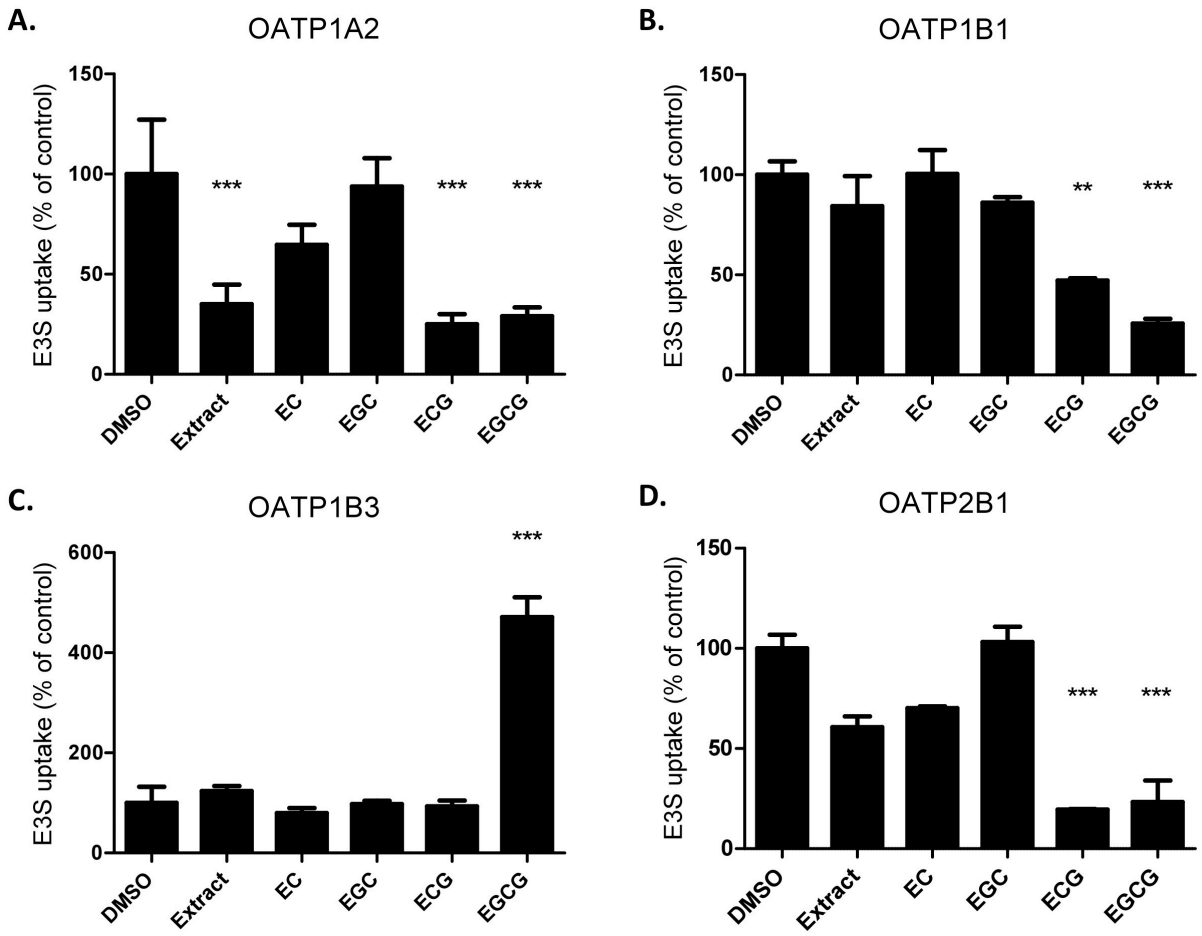
A.
OATP1A2
E3S uptake (% of control)
150
100
50
0
***
***
***
DMSO
Extract
EC
EGC
ECG
EGCG
B.
OATP1B1
E3S uptake (% of control)
150
100
50
0
**
***
DMSO
Extract
EC
EGC
ECG
EGCG
C.
OATP1B3
E3S uptake (% of control)
600
400
200
0
***
DMSO
Extract
EC
EGC
ECG
EGCG
D.
OATP2B1
E3S uptake (% of control)
150
100
50
0
***
***
DMSO
Extract
EC
EGC
ECG
EGCG

Figure 3-1:

Effect of green tea extract and catechins on OATP-mediated estrone-3-sulfate uptake. Cells were coincubated with 0.1 μM [^{3}H]estrone-3-sulfate (E3S) and 0.03 μg/ml green tea extract, 100 μM EC, EGC, ECG, EGCG, or the vehicle control (1% DMSO) at 37°C for 20 s [OATP1B1 (B), OATP1B3 (C), and OATP2B1 (D)] or 30 s [OATP1A2 (A)]. After correction for protein, uptake into empty vector (OATP1A2 and OATP2B1) or wild-type control cells (OATP1B1 and OATP1B3) was subtracted to determine OATP-mediated uptake. Values are expressed as a percentage of vehicle control; each value is the mean ± S.E.M. of three independent experiments. Asterisks represent statistically significant differences from the DMSO control (**, $p < 0.005$; ***, $p < 0.001$).

Figure 3-2:

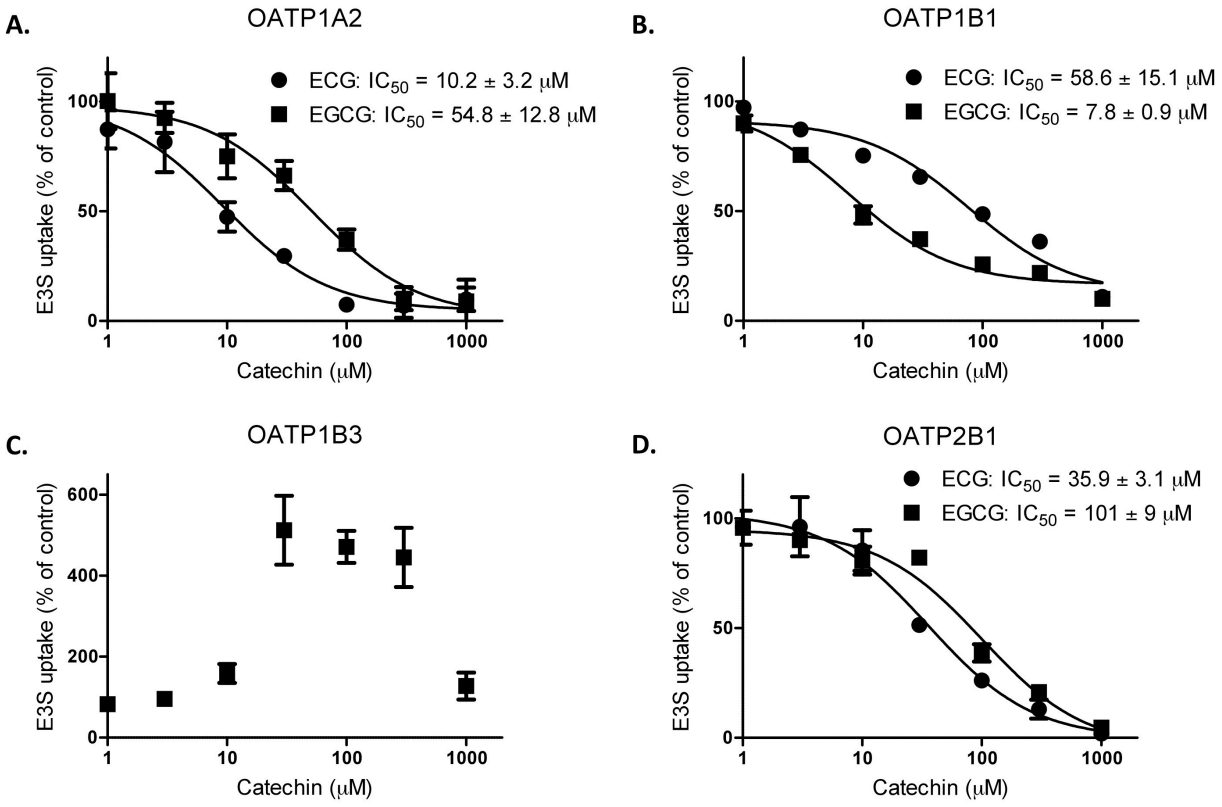
A.
OATP1A2
ECG: IC50 = 10.2 ± 3.2 μM
EGCG: IC50 = 54.8 ± 12.8 μM
E3S uptake (% of control)
Catechin (μM)
B.
OATP1B1
ECG: IC50 = 58.6 ± 15.1 μM
EGCG: IC50 = 7.8 ± 0.9 μM
E3S uptake (% of control)
Catechin (μM)
C.
OATP1B3
E3S uptake (% of control)
Catechin (μM)
D.
OATP2B1
ECG: IC50 = 35.9 ± 3.1 μM
EGCG: IC50 = 101 ± 9 μM
E3S uptake (% of control)
Catechin (μM)

Figure 3-2:

Concentration-dependent effects of green tea catechins on OATP-mediated estrone-3-sulfate uptake. Cells were coincubated with 0.1 μM [^{3}H]estrone-3-sulfate (E3S) and increasing concentrations of ECG or EGCG at 37°C as described in the legend to Figure 3-1. A, OATP1A2. B, OATP1B1. C, OATP1B3. D, OATP2B1. Values are expressed as a percentage of vehicle control; each value represents the mean ± S.E.M. of three independent experiments.

3.3.3: Substrate-Dependent Effect of ECG and EGCG on OATP1B3-Mediated Uptake.

On the basis of previous evidence for substrate-dependent stimulation of OATP1B3 (Gui et al., 2008), we measured the effects of increasing concentrations of ECG and EGCG on the uptake of two additional model substrates of OATP1B3, estradiol-17β-glucuronide (0.1 μM) and Fluo-3 (1 μM), as well as estrone-3-sulfate (0.1 μM). As can be seen in Figure 3-3A, ECG inhibited uptake of estradiol-17β-glucuronide with an IC_{50} value of 130 μM, slightly inhibited estrone-3-sulfate uptake at concentrations higher than 100 μM, and strongly inhibited uptake of Fluo-3 (IC_{50} = 6.9 μM). EGCG had no effect on the uptake of estradiol-17β-glucuronide; however, it strongly inhibited uptake of Fluo-3 with an IC_{50} value of 8.4 μM, whereas it stimulated estrone-3-sulfate uptake with an EC_{50} value of 10.5 μM (Figure 3-3B).

To investigate the mechanism of this substrate dependence, we determined the effect that ECG and EGCG had on the kinetic parameters of each affected OATP1B3 substrate (Figure 3-4). Both ECG and EGCG noncompetitively inhibited OATP1B3-mediated uptake of Fluo-3, reducing the V_{max} from 9.8 ± 0.6 to 4.3 ± 0.8 and 5.6 ± 0.6 pmol/mg · min, respectively, whereas they had no effect on the K_m (2.5 ± 0.8, 2.1 ± 1.8, and 2.7 ± 1.4 μM) (Figure 3-4, A and B). ECG also demonstrated noncompetitive inhibition of estradiol-17β-glucuronide uptake, decreasing the V_{max} from 240 ± 40 to 110 ± 40 pmol/mg · min, while not affecting K_m (19 ± 3 to 17 ± 5 μM) (Figure 4C). Surprisingly, EGCG also significantly decreased the maximal rate of estrone-3-sulfate transport, reducing the V_{max} from 2.1 ± 0.1 to 0.36 ± 0.03 nmol/mg · min (Fig. 3-4D). However, the K_m was also strongly decreased, from 95 ± 9 to 12 ± 5 μM. This 5- to 10-fold

Figure 3-3:

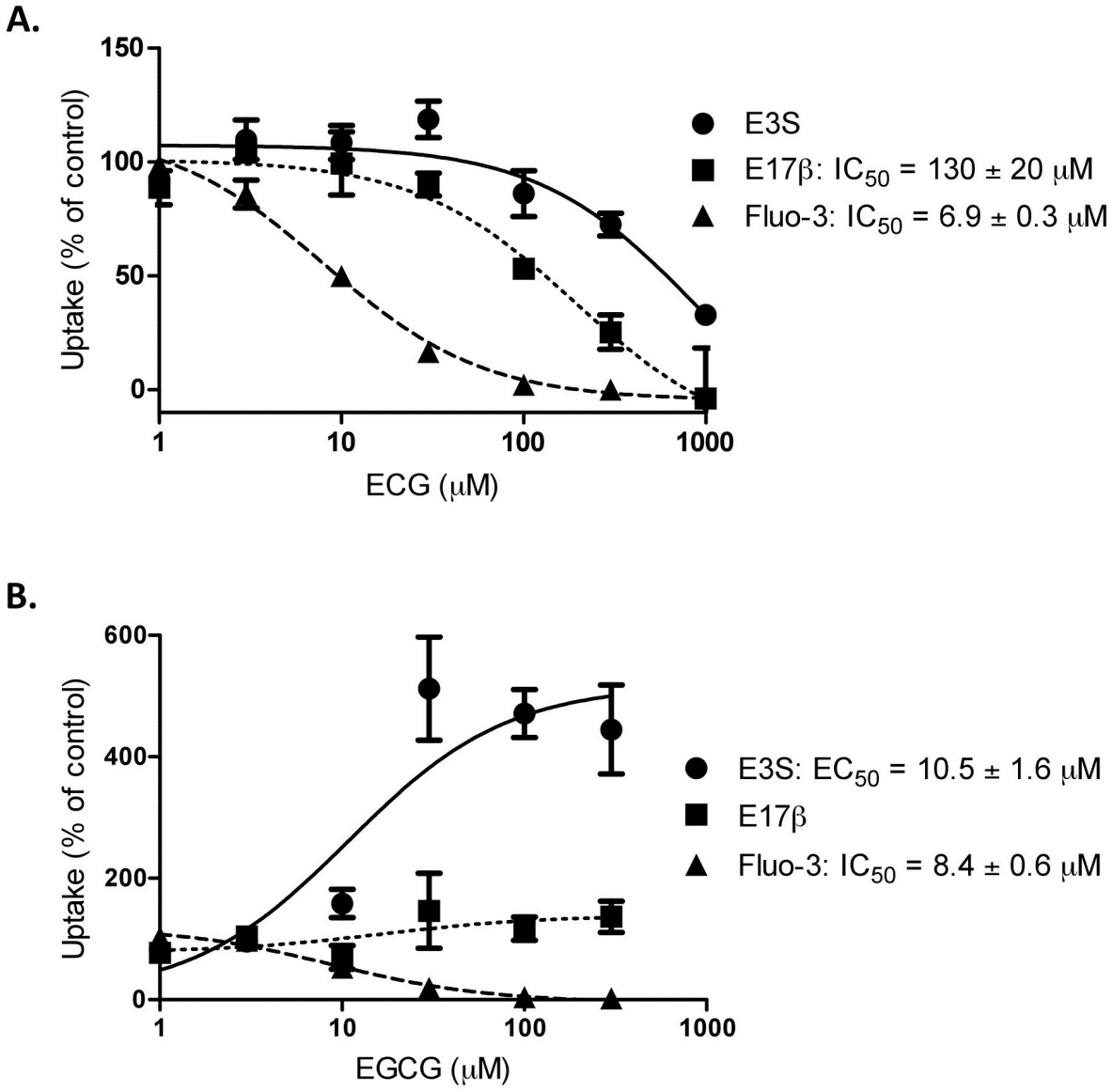
A.
150
100
50
0
Uptake (% of control)
1
10
100
1000
ECG (μM)
E3S
E17β: IC50 = 130 ± 20 μM
Fluo-3: IC50 = 6.9 ± 0.3 μM
B.
600
400
200
0
Uptake (% of control)
1
10
100
1000
EGCG (μM)
E3S: EC50 = 10.5 ± 1.6 μM
E17β
Fluo-3: IC50 = 8.4 ± 0.6 μM

Figure 3-3:

Substrate-dependent effects of ECG and EGCG on OATP1B3-mediated transport. OATP1B3-expressing and wild-type CHO cells were coincubated with 0.1 μM [^{3}H]estrone-3-sulfate (E3S, ●), 0.1 μM [^{3}H]estradiol-17β-glucuronide (E17β, ■), or 1 μM Fluo-3 (▲) and increasing concentrations of ECG (A) or EGCG (B) at 37°C as described in the legend to Figure 3-1. Values are expressed as a percentage of vehicle control; each value represents the mean ± S.E.M. of three independent experiments.

Figure 3-4:

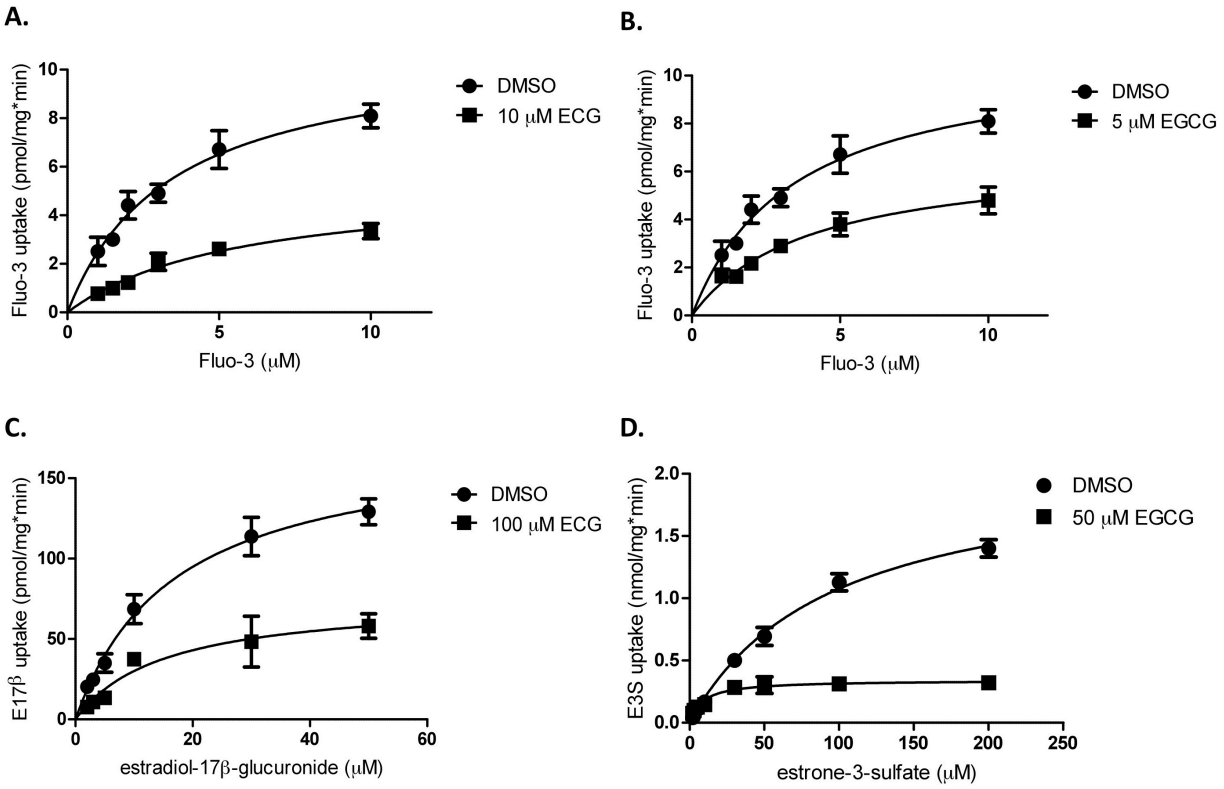
A.
Fluo-3 uptake (pmol/mg*min)
Fluo-3 (μM)
DMSO
10 μM ECG
B.
Fluo-3 uptake (pmol/mg*min)
Fluo-3 (μM)
DMSO
5 μM EGCG
C.
E17β uptake (pmol/mg*min)
estradiol-17β-glucuronide (μM)
DMSO
100 μM ECG
D.
E3S uptake (nmol/mg*min)
estrone-3-sulfate (μM)
DMSO
50 μM EGCG

Figure 3-4:

Effect of ECG and EGCG on the kinetics of OATP1B3-mediated transport. OATP1B3-expressing and wild-type CHO cells were coincubated with the stated concentrations of ECG or EGCG (■) or the vehicle control (1% DMSO, ●) and increasing concentrations of Fluo-3 (A and B), estradiol-17β-glucuronide (E17β) (C), or estrone-3-sulfate (E3S) (D). A–C are representative graphs from at least three independent experiments. Each value shown in D represents the mean ± S.E.M. of at least three independent experiments.

increase in affinity results in the stimulation of estrone-3-sulfate transport observed at the low (0.1 μM) concentrations used in the initial inhibition experiments.

3.3.4: OATP-Mediated Uptake of ECG and EGCG.

Given that inhibitors of transport are sometimes also substrates, we tested whether either ECG or EGCG was transported by these four OATPs. We measured accumulation of 100 μM ECG or EGCG in the OATP-expressing or control cells after a 10-min incubation. As summarized in Figure 3-5, both OATP1A2 and OATP1B3 transported ECG and EGCG. Although ECG and EGCG were clear inhibitors of OATP1B1 and OATP2B1, we did not detect significant uptake by either OATP. Uptake of both catechins by OATP1A2 and OATP1B3 increased with time and was linear for at least 2 to 5 min (data not shown). Uptake was saturable, with apparent K_m values between 10 and 34 μM (Figure 3-6). OATP1A2 transports EGCG with a maximal rate of transport (V_{max}) almost twice that of ECG (100 and 60 pmol/mg*min, respectively), whereas OATP1B3 has a V_{max} approximately 6 times higher for ECG than for EGCG (2.2 and 0.340 nmol/mg*min, respectively).

Figure 3-5:

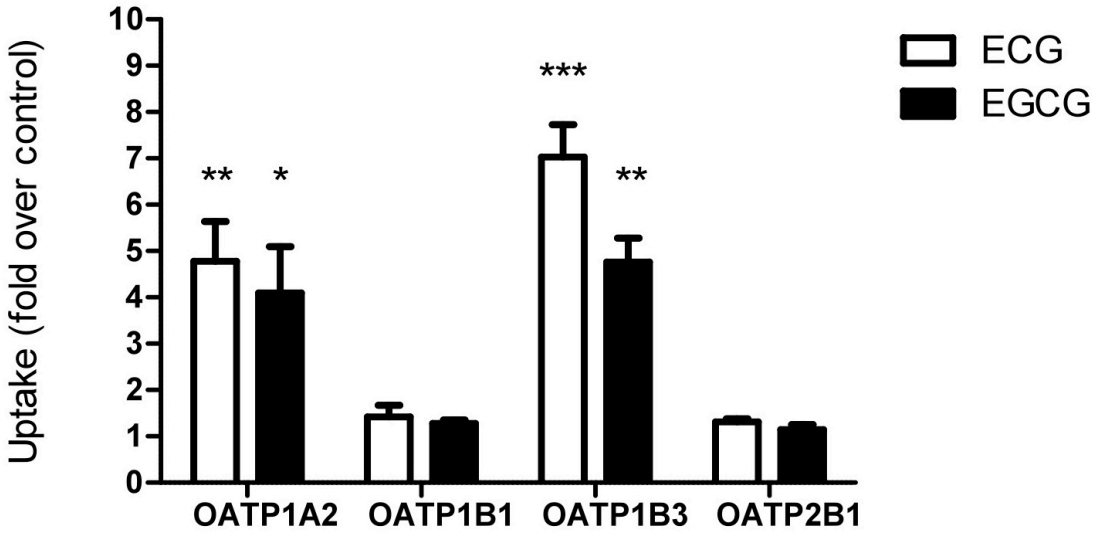
Uptake (fold over control)
10
9
8
7
6
5
4
3
2
1
0
**
*
***
**
ECG
EGCG
OATP1A2
OATP1B1
OATP1B3
OATP2B1

Figure 3-5:

Uptake of green tea catechins by OATPs. Cells were incubated with 100 μM ECG or EGCG at 37°C for 10 min. Uptake by each OATP-expressing cell line was divided by the uptake by its appropriate control cell and is expressed as fold uptake over control. Each value represents the mean ± S.D. of at least two experiments performed in triplicate. Asterisks represent statistically significant uptake compared with control cell lines (*, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.001$).

Figure 3-6:

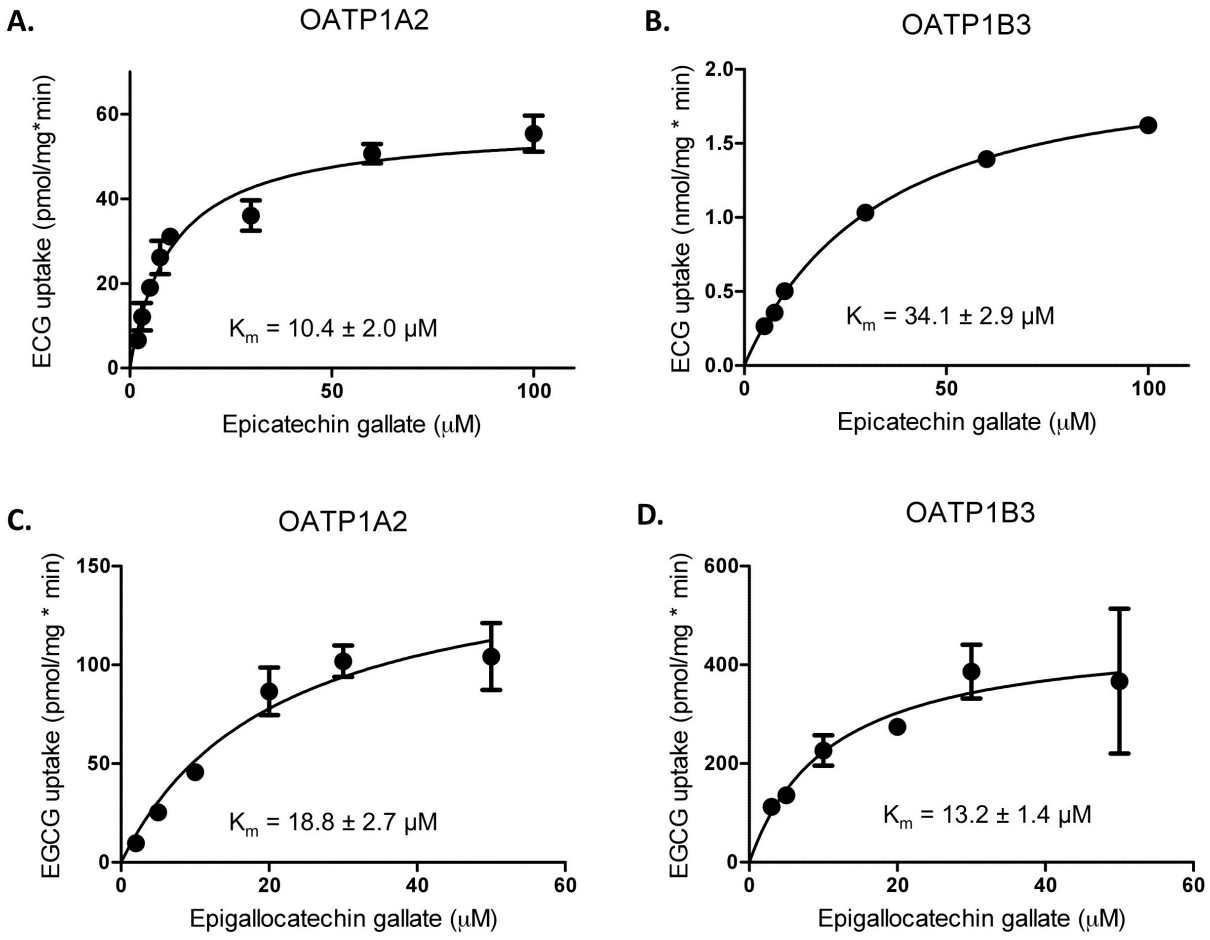
A.
OATP1A2
ECG uptake (pmol/mg*min)
K_m = 10.4 ± 2.0 μM
Epicatechin gallate (μM)
B.
OATP1B3
ECG uptake (nmol/mg * min)
K_m = 34.1 ± 2.9 μM
Epicatechin gallate (μM)
C.
OATP1A2
EGCG uptake (pmol/mg * min)
K_m = 18.8 ± 2.7 μM
Epigallocatechin gallate (μM)
D.
OATP1B3
EGCG uptake (pmol/mg * min)
K_m = 13.2 ± 1.4 μM
Epigallocatechin gallate (μM)

Figure 3-6:

Kinetics of epicatechin gallate and epigallocatechin gallate uptake mediated by OATP1A2 or OATP1B3. Uptake of increasing concentrations of ECG (A and B) or [^{3}H]EGCG (C and D) was measured at 37°C under the initial linear rate conditions. After subtraction of the values obtained with control cells, net OATP1A2-mediated (A and C) or OATP1B3-mediated (B and D) uptake was fitted to the Michaelis-Menten equation to determine K_m and V_{max} values. A and B, plot of mean data points from at least three independent experiments. C and D, representative graphs with mean ± S.E.M. of three independently determined K_m values.

3.4: Discussion

The present study addressed the question of whether the four major green tea catechins affect the activity of all OATPs expressed in the small intestine and liver. In addition, we investigated whether the catechins that do alter OATP activity are transported by OATPs. Our results demonstrate that although EC and EGC have minimal effect on OATPs, ECG and EGCG significantly alter the function of all four OATPs investigated. We found that the effects of ECG and EGCG on OATP1B3-mediated transport were substrate-dependent and could cause noncompetitive inhibition or stimulation of activity. In addition, we showed that both ECG and EGCG are substrates of OATP1A2 and OATP1B3 but are not transported by OATP1B1 or OATP2B1, despite the strong inhibition of estrone-3-sulfate transport by these two proteins.

ECG and EGCG significantly inhibited the uptake of estrone-3-sulfate by all four OATPs at a concentration of 100 μM (Figure 3-2). The U.S. Department of Agriculture Database for the Flavonoid Content of Selected Foods reported average concentrations in brewed green tea to be 19.73 mg/100 ml (450 μM) ECG and 77.81 mg/100 ml (430 μM) EGCG, with the maximal concentrations of each catechin in the low millimolar range (U.S. Department of Agriculture, www.nal.usda.gov/fnic/foodcomp/Data/Flav/Flav02-1.pdf). These compounds inhibited estrone-3-sulfate uptake by OATP1A2 and OATP2B1, expressed at the lumen of enterocytes, with IC_{50} values ranging from 10 to 100 μM (Figure 3-2). Assuming a gastric fluid volume of 100 to 500 ml, drinking a cup or two of tea on an empty stomach would result in intestinal concentrations of ECG and EGCG within the range that alters OATP transport. The physiological relevance of altered OATP1B1 and OATP1B3 transport is more ambiguous, because the bioavailability of

catechins is low. A single-dose study in healthy volunteers showed that consumption of 1600 mg of EGCG resulted in mean peak plasma concentrations (C_{max}) of 7.4 μM, with values ranging from 5.8 to 11.3 μM (Ullmann et al., 2003). The IC_{50} and EC_{50} values of EGCG on OATP1B3-mediated uptake of Fluo-3 and estrone-3-sulfate (8.4 and 10.5 μM, respectively) and on OATP1B1-mediated transport of estrone-3-sulfate (IC_{50} = 7.8 μM), are well within this range, indicating the physiological relevance of these interactions for those who take high-dose supplements. The same authors found that daily consumption of 800 mg of EGCG resulted in an average C_{max} of 5.3 μM after 10 days (Ullmann et al., 2004). In addition, the bioavailability of EGCG was shown to increase with increasing doses, indicating that a saturable presystemic elimination process is involved in the low systemic bioavailability (Chow et al., 2001). If this presystemic elimination occurs via the liver, the OATP1B1- and OATP1B3-expressing hepatocytes may be exposed to these EGCG concentrations at more moderate doses as well.

We identified both ECG and EGCG as novel substrates for OATP1A2 and OATP1B3 (Figs. 3-5 and 3-6). It is important to note that although these two catechins were inhibitors of OATP1B1 and OATP2B1 (Figs. 3-1 and 3-2), we did not see any uptake by either of these OATPs (Figure 3-5). This result corroborates the finding that many inhibitors of transporters are not substrates of those transporters. It has been shown that ECG and EGCG are taken up into Caco-2 cells and that uptake of ECG was saturable and stimulated by low pH (Vaidyanathan and Walle, 2003). The authors suggested that this transport was mediated by the monocarboxylate transporter MCT1 (Vaidyanathan and Walle, 2003). However, direct transport of ECG or EGCG by MCT1 to our knowledge has not been reported, and so far no uptake transporter has been identified for green tea catechins. Given that OATP1A2 is expressed in enterocytes, our results

suggest that OATP1A2 could be involved in the absorption of ECG and EGCG from the gut. Furthermore, given that OATP1A2 can transport numerous drugs including fexofenadine (Cvetkovic et al., 1999), several antibiotics such as levofloxacin (Maeda et al., 2007), methotrexate (Badagnani et al., 2006), statins (Fujino et al., 2005; Ho et al., 2006), and talinolol (Shirasaka et al., 2010), there is the potential for food-drug interactions such as the ones described for fruit juices (Dresser et al., 2002; Lilja et al., 2004; Greenblatt, 2009) in patients that complement their prescription drugs with over-the-counter green tea supplements. A similar danger may exist for OATP1B3. It is not known whether the low systemic bioavailability of ECG and EGCG is due to efflux from enterocytes or to a high first-pass effect. However, efficient uptake via OATP1B3 into hepatocytes could contribute to the low bioavailability of these compounds.

This study clearly demonstrates that OATP-mediated transport may be affected in different ways by the same compound, depending on the substrate being transported. We found that ECG inhibited OATP1B3-mediated uptake of estrone-3-sulfate, estradiol-17β-glucuronide, and Fluo-3, but to very different extents (Figure 3-3A). Inhibition of estrone-3-sulfate transport was too weak to further characterize; however, estradiol-17β-glucuronide and Fluo-3 were both inhibited in a noncompetitive manner. EGCG, which differs from ECG by a single hydroxyl group, stimulated OATP1B3 activity with respect to estrone-3-sulfate transport, inhibited transport of Fluo-3, and had no effect on the transport of estradiol-17β-glucuronide (Figure 3-3B). Uptake of Fluo-3 was noncompetitively inhibited, as was uptake of estrone-3-sulfate at high substrate concentrations. The stimulation of estrone-3-sulfate at low substrate concentrations was found to be caused by increased substrate affinity. In addition, although 100 μM ECG did not

inhibit OATP1B3-mediated estrone-3-sulfate uptake and although EGCG did not affect OATP1B3-mediated estradiol-17β-glucuronide uptake, both catechins are substrates of OATP1B3 (Figs. 3-1, 3-5, and 3-6). Together, these results suggest the presence of multiple substrate binding sites or translocation pathways on OATP1B3.

These results emphasize that, at least in the case of OATP1B1- and OATP1B3-mediated transport, it is crucial to test more than one substrate when screening for potential inhibitors. The International Transporter Consortium recently suggested that possible OATP inhibition by new molecular entities should be tested using a prototypical substrate such as estradiol-17β-glucuronide (Giacomini et al., 2010). However, we showed that OATP1B3-mediated uptake of estradiol-17β-glucuronide was not affected by EGCG, whereas uptake of Fluo-3 was inhibited and uptake of estrone-3-sulfate was stimulated (Figure 3-3). Likewise, although a previous study found that EGC inhibited OATP1B1-mediated uptake of dehydroepiandrosterone sulfate (Wang et al., 2005b), we did not see significant inhibition of OATP1B1-mediated uptake of estrone-3-sulfate in the presence of EGC. Thus, we propose that for OATP1B1 and OATP1B3, the effect of potential inhibitors should always be tested by using several substrates instead of a single prototypical substrate. Similar substrate-dependent effects have previously been observed for rat Oatp1a4 (Sugiyama et al., 2002), for human OATP1B1 (Noe et al., 2007), and for human OATP1B3 (Gui et al., 2008). In our previous study (Gui et al., 2008), the non-OATP substrate clotrimazole stimulated OATP1B3-mediated estradiol-17β-glucuronide uptake, did not affect uptake of estrone-3-sulfate, and inhibited uptake of Fluo-3. Together, these data clearly demonstrate that stimulation as well as inhibition are substrate-dependent and indicate the presence of multiple substrate binding sites.

A previous study showed inhibition of OATP2B1-mediated uptake of estrone-3-sulfate by all four catechins (Fuchikami et al., 2006). However, we found that only ECG and EGCG significantly inhibit OATP2B1-mediated estrone-3-sulfate transport. These differences could be explained by the 10-fold higher substrate concentration used in the current study and highlights the difficulty in predicting in vivo effects based on in vitro data. However, in both studies the effects of EC and EGC on OATP2B1-mediated transport were much weaker than the effects of ECG and EGCG, suggesting that ECG and EGCG are the green tea catechins most likely to alter OATP-mediated drug uptake.

In conclusion, we have demonstrated that the green tea compounds ECG and EGCG are substrates for OATP1A2 and OATP1B3 suggesting that these two transporters could be involved in the disposition of these two catechins. We also demonstrated that compounds such as ECG and EGCG can affect OATPs in a substrate-dependent manner. This finding highlights the importance of using multiple and clinically relevant substrates when screening for potential drug-drug interactions with this family of transporters. Because of increasing use of green tea catechins, particularly EGCG, in dietary supplements and because ECG and EGCG can significantly alter the function of OATPs involved in drug disposition, the results of this study suggest that there is a significant possibility of adverse drug-catechin interactions.

Chapter 4

Isolation of modulators of the liver specific Organic Anion Transporting Polypeptides (OATPs) 1B1 and 1B3 from *Rollinia emarginata* Schlecht (Annonaceae)

4.1 Abstract

Organic anion transporting polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) are liver-specific transporters that mediate the uptake of a broad range of drugs into hepatocytes, including statins, antibiotics and many anticancer drugs. Compounds which alter transport by one or both of these OATPs could potentially be used to target drugs to hepatocytes or to improve bioavailability of drugs that are cleared by the liver. In this study, we applied a bioassay guided isolation approach to identify such compounds from the organic extract of *Rollinia emarginata* Schlecht (Annonaceae). Fractions of the plant extract were screened for effects on OATP1B1- and OATP1B3-mediated transport of the model substrates estradiol-17β-glucuronide and estrone-3-sulfate. We isolated three compounds, ursolic acid, oleanolic acid, and 8-*trans*-*p*-coumaroyloxy-α-terpineol, which inhibited estradiol-17β-glucuronide uptake by OATP1B1 but not OATP1B3. In addition, a rare compound, quercetin 3-*O*-α-L-arabinopyranosyl(1→2) α-L-

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rhamnopyranoside, was identified that had distinct effects on each OATP. OATP1B1 was strongly inhibited, as was OATP1B3-mediated transport of estradiol-17β-glucuronide. However, OATP1B3-mediated uptake of estrone-3-sulfate was stimulated 4- to 5-fold. Kinetic analysis of this stimulation revealed that the apparent affinity for estrone-3-sulfate was increased (decreased K_m) while the maximal rate of transport (V_{max}) was significantly reduced. These results demonstrate a mechanism through which the hepatic uptake of drug OATP substrates could be stimulated.

4.2 Introduction

Organic anion transporting polypeptides (OATPs) are multispecific transporters that mediate the uptake of a broad range of drugs and other xenobiotics into cells. Two members of this superfamily, OATP1B1 and OATP1B3, are selectively expressed on the basolateral membrane of hepatocytes under normal physiological conditions. Among the substrates of OATP1B1 and OATP1B3 are numerous drugs used to treat hypertension, hypercholesterolemia, and diabetes (Hagenbuch and Gui, 2008).

Due to the localization of OATP1B1 and OATP1B3 at the basolateral membrane of hepatocytes, these transporters can have a significant impact upon the first-pass effect of orally administered drugs. Alterations in the function of these OATPs have been shown to affect the disposition of drugs throughout the body. Polymorphisms in OATP1B1 have been associated with altered pharmacokinetics of several of the HMG-CoA reductase inhibitors (statins). Patients with OATP1B1*1b have lower plasma levels of pravastatin and pitavastatin than those with the

*1a variant, while the *15 variant is associated with higher plasma concentrations (Nishizato et al., 2003; Chung et al., 2005; Maeda et al., 2006a). OATPs have also been implicated as a cause of several known drug-drug interactions, including those between cerivastatin and gemfibrozil (Shitara et al., 2003), rosuvastatin and cyclosporine (Simonson et al., 2004), and fexofenadine and grapefruit juice (Bailey et al., 2007). This suggests that interactions at OATP1B1 or OATP1B3 may alter the disposition of drugs throughout the body.

Co-administration of a drug with a small molecule that alters transporter function can be used therapeutically to improve drug bioavailability or distribution. The bioavailability of orally administered paclitaxel and docetaxel is improved by co-administration with Cyclosporin A, which inhibits P-gp-mediated efflux from enterocytes (Meerum Terwogt et al., 1998; Malingre et al., 2001). Similarly, co-administration of the BCRP and P-gp inhibitor GF120918 with topotecan increased systemic bioavailability two-fold when dosed orally (Kruijtzer et al., 2002). Just as inhibiting efflux from enterocytes can increase systemic bioavailability, so could inhibiting the first-pass effect of uptake into the liver. Selective inhibition of OATP1B1 or OATP1B3 could therefore be used to increase the systemic bioavailability of OATP drug substrates. Conversely, selective stimulation of OATP1B1 or OATP1B3 uptake could be used to increase drug delivery to hepatocytes.

Among the many OATP substrates are several anticancer drugs, such as docetaxel and paclitaxel (OATP1B3) (Smith et al., 2005a), methotrexate (OATP1B1 and OATP1B3) (Abe et al., 2001), and the active metabolite of irinotecan, SN-38 (OATP1B1) (Nozawa et al., 2005a). Thus, stimulation of OATP1B1 or OATP1B3 transport activity could also be a method of

improving cancer treatments. Recently, OATP1B1 and OATP1B3 have also been identified in certain cancer tissues, including cancers of the breast, colon, lung, pancreas, prostate, and stomach (Abe et al., 2001; Monks et al., 2007; Muto et al., 2007; Hamada et al., 2008). Most of these studies have only identified mRNA expression, and neither membrane localization nor function has been shown. However, if OATPs are expressed and functional on the surface of these cancer cells, stimulation of OATP function could be a promising technique for increasing uptake of cytotoxic drugs into cancer cells.

So far, specific modulators of OATPs are limited. One method for identifying additional specific modulators is using high throughput screening (HTS). A recent HTS method for detecting OATP1B3 modulators identified several compounds that preferentially modulated OATP1B1 or OATP1B3, however this assay is not sufficiently stringent for OATP1B1 (Gui et al., 2010). Another way to identify modulators is through bioassay-guided fractionation. This is a technique frequently used to identify active compounds from plants. Plants are a potentially rich source of OATP modulators - herbal extracts used in dietary supplements have been found to affect transport by OATP1B1, OATP1B3 (Roth et al., 2011) and by OATP2B1 (Fuchikami et al., 2006), and interactions between OATPs and fruit juices are well-documented (Dresser et al., 2002; Bailey et al., 2007; Glaeser et al., 2007; Greenblatt, 2009).

In this study, we applied a bioassay guided isolation approach to identify OATP modulators from *Rollinia emarginata* Schlecht (Annonaceae), a plant that grows in several regions of South America. This plant extract was a positive hit during an initial screening of several South American plant extracts for their effect on OATP-mediated transport. The stem

barks have been used in combination with *Ilex paraguayensis* St Hilaire (Aquifoliaceae) (common name: hierba mate), to treat migraine and as a relaxant. In addition, antiprotozoal and antifeedant properties have been reported (Fevrier et al., 1999; Colom et al., 2007). Fractions of plant extract were screened for effects on OATP1B1- and OATP1B3-mediated uptake of the two model substrates estradiol-17β-glucuronide and estrone-3-sulfate.

4.3: Results

4.3.1: Identification of Compounds with Modulating Effects on OATP1B1 and OATP1B3.

To identify components of *Rollinia emarginata* that affect OATP1B1 and OATP1B3 function, the organic components were extracted with MeOH:CH_2Cl_2, and fractionated with various solvents (Figure 4-1). Fractions were solubilized in DMSO, and functional assays were performed in triplicate on 96-well plates. Two model substrates, estradiol-17β-glucuronide (OATP1B1: K_m = 5.4 μM; OATP1B3: K_m = 15.8 μM) and estrone-3-sulfate (OATP1B1 high affinity component: K_m = 0.22 μM; OATP1B3: K_m = 58 μM) (Gui et al., 2008; Gui et al., 2009) were used to identify compounds that have substrate-dependent effects on transport. Active fractions were identified by co-incubating wild-type or OATP-expressing cells with uptake buffer containing 0.03 μg/ml *Rollinia emarginata* extracts and 0.1 μM estradiol-17β-glucuronide or 1 μM estrone-3-sulfate for 5 minutes at 37˚C. Results from selected fractions are shown in Figure 4-2. The whole plant extract inhibited uptake of both substrates by both transporters; detannification increased the inhibitory effect, possibly due to increased availability of formerly tannin-bound compounds (data not shown). The hexane (HEX) and butanol (BUOH) fractions

Figure 4-1a:

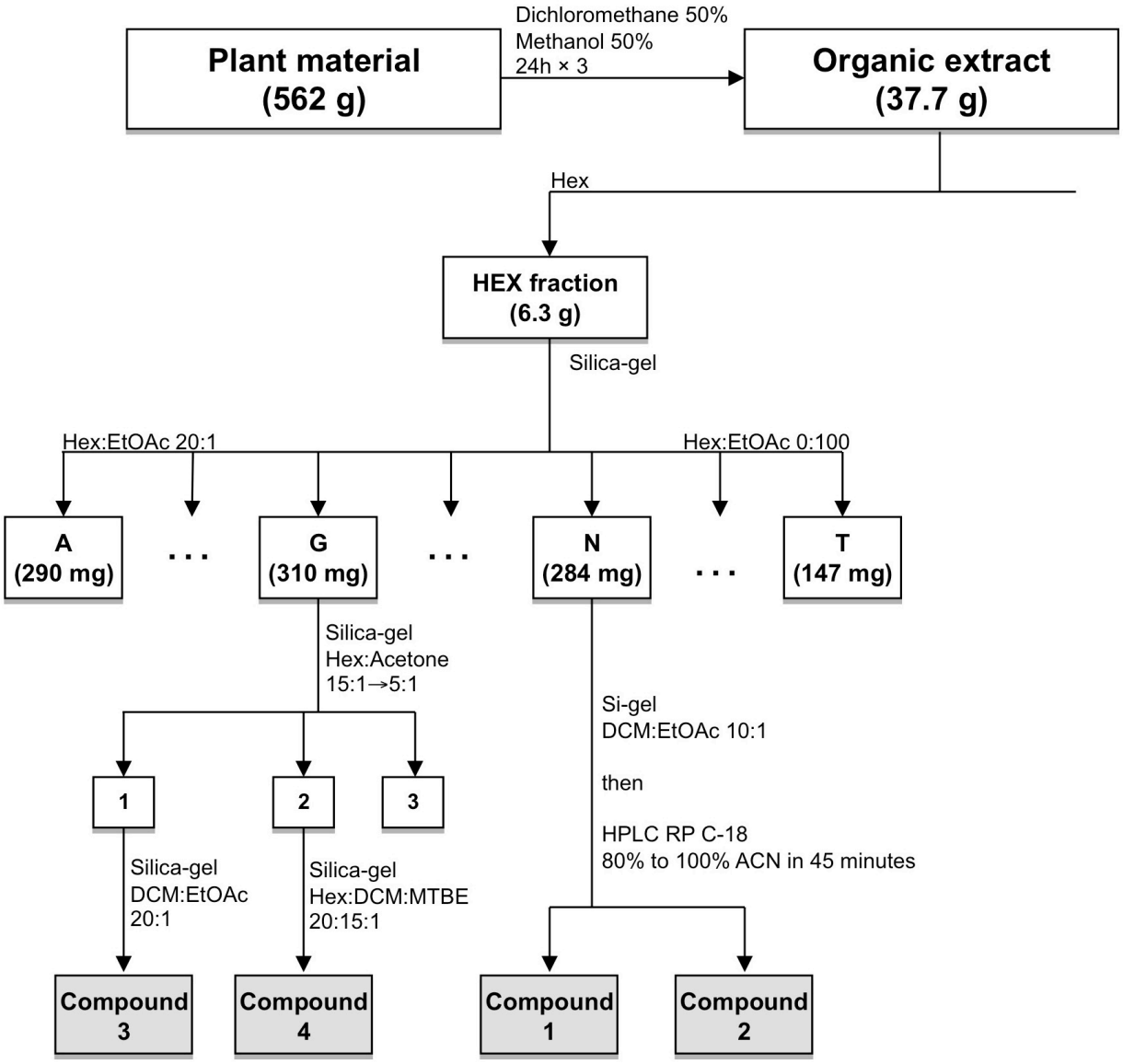

Plant material
(562 g)
Dichloromethane 50%
Methanol 50%
24h × 3
Organic extract
(37.7 g)
Hex
HEX fraction
(6.3 g)
Silica-gel
Hex:EtOAc 20:1
Hex:EtOAc 0:100
A
(290 mg)
G
(310 mg)
N
(284 mg)
T
(147 mg)
Silica-gel
Hex:Acetone
15:1→5:1
Si-gel
DCM:EtOAc 10:1
then
HPLC RP C-18
80% to 100% ACN in 45 minutes
1
2
3
Silica-gel
DCM:EtOAc
20:1
Silica-gel
Hex:DCM:MTBE
20:15:1
Compound
3
Compound
4
Compound
1
Compound
2

Figure 4-1b:

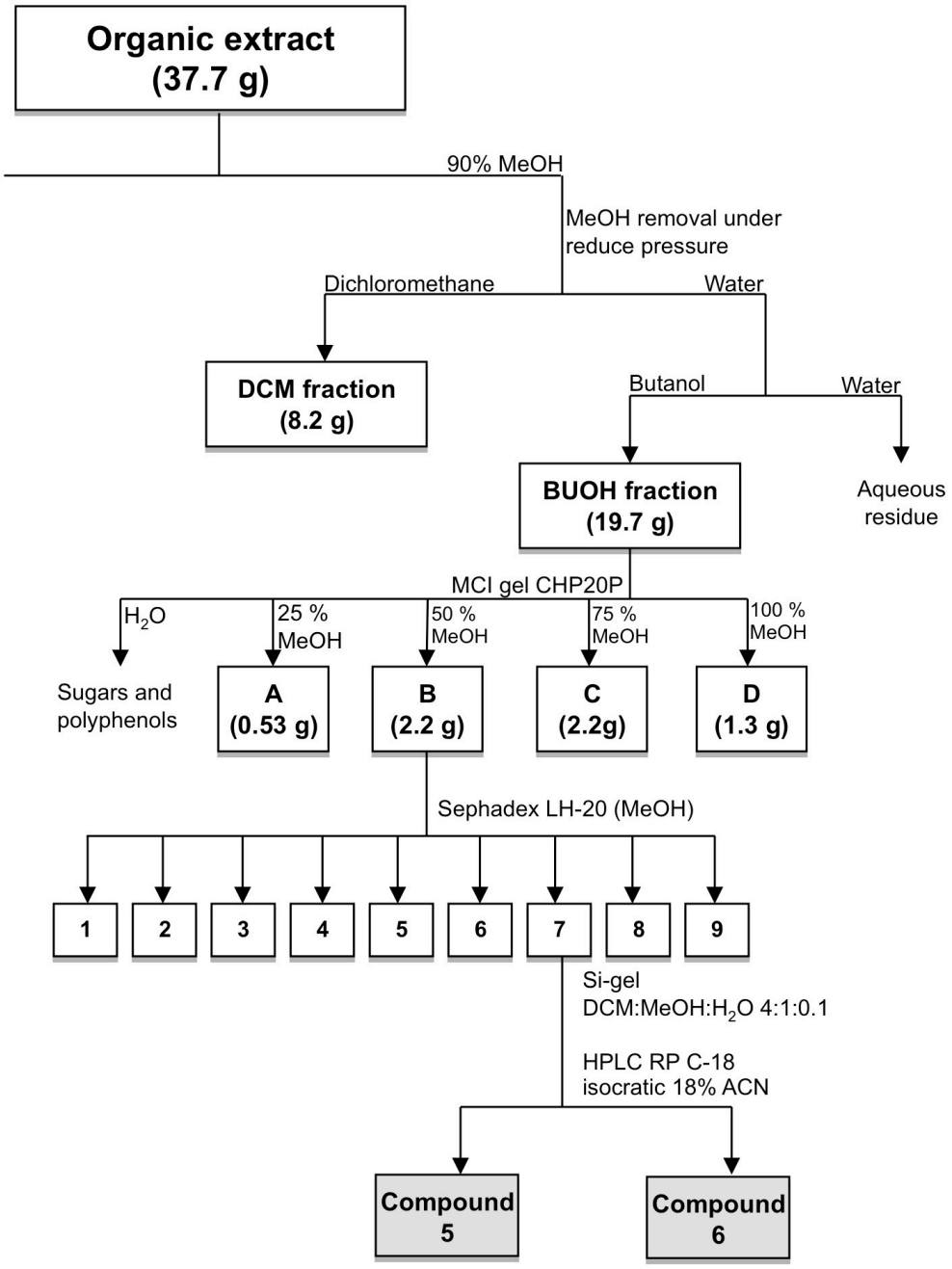

Organic extract
(37.7 g)
90% MeOH
MeOH removal under
reduce pressure
Dichloromethane
Water
DCM fraction
(8.2 g)
Butanol
Water
BUOH fraction
(19.7 g)
Aqueous
residue
MCI gel CHP20P
H2O
25 %
MeOH
50 %
MeOH
75 %
MeOH
100 %
MeOH
Sugars and
polyphenols
A
(0.53 g)
B
(2.2 g)
C
(2.2g)
D
(1.3 g)
Sephadex LH-20 (MeOH)
1
2
3
4
5
6
7
8
9
Si-gel
DCM:MeOH:H2O 4:1:0.1
HPLC RP C-18
isocratic 18% ACN
Compound
5
Compound
6

Figure 4-1: Separation diagram of *Rollinia emarginata* organic extract.

both showed preferential inhibition of OATP1B1-mediated transport of estradiol-17β-glucuronide (Figure 4-2A), and were further fractionated. An active subfraction of the hexane fraction (data not shown) contained four compounds, which were identified as (1) ursolic acid, (2) oleanolic acid, (3) β-sitosterol, and (4) 8-*trans*-*p*-coumaroyloxy-α-terpineol (Compound 4). Ursolic acid, oleanolic acid, and Compound 4 inhibited OATP1B1 transport of estradiol-17β-glucuronide by more than 50% while having a minimal effect on OATP1B1 transport of estrone-3-sulfate (Figure 4-2). The butanol fraction contained a subfraction (Frac B) that strongly stimulated uptake of estrone-3-sulfate by OATP1B3, while inhibiting uptake of estradiol-17β-glucuronide by both OATPs. Frac B was found to mainly consist of two structurally similar compounds: (5) rutin and (6) quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (Compound 6; Figure 4-3). Compound 6 stimulated uptake of estrone-3-sulfate by OATP1B3, while inhibiting uptake of estrone-3-sulfate by OATP1B1 (Figure 4-2B) and uptake of estradiol-17β-glucuronide by both transporters (Figure 4-2A). To further examine the effects of these compounds on OATP-mediated transport, we purified ursolic acid, oleanolic acid, Compound 4 and Compound 6 to greater than 95% by HPLC analysis, and used these purified compounds for all additional experiments.

4.3.2: Ursolic Acid, Oleanolic Acid, and 8-*trans*-*p*-coumaroyloxy-α-terpineol Selectively Inhibit OATP1B1-Mediated Transport of Estradiol-17β-glucuronide.

To examine the selectivity of inhibition of the three compounds isolated from the hexane fraction, OATP1B1- and OATP1B3-mediated transport of 0.1 μM estradiol-17β-glucuronide or estrone-3-sulfate was measured for 20 seconds at 37˚C in the presence of 100 μM ursolic acid,

Figure 4-2a:

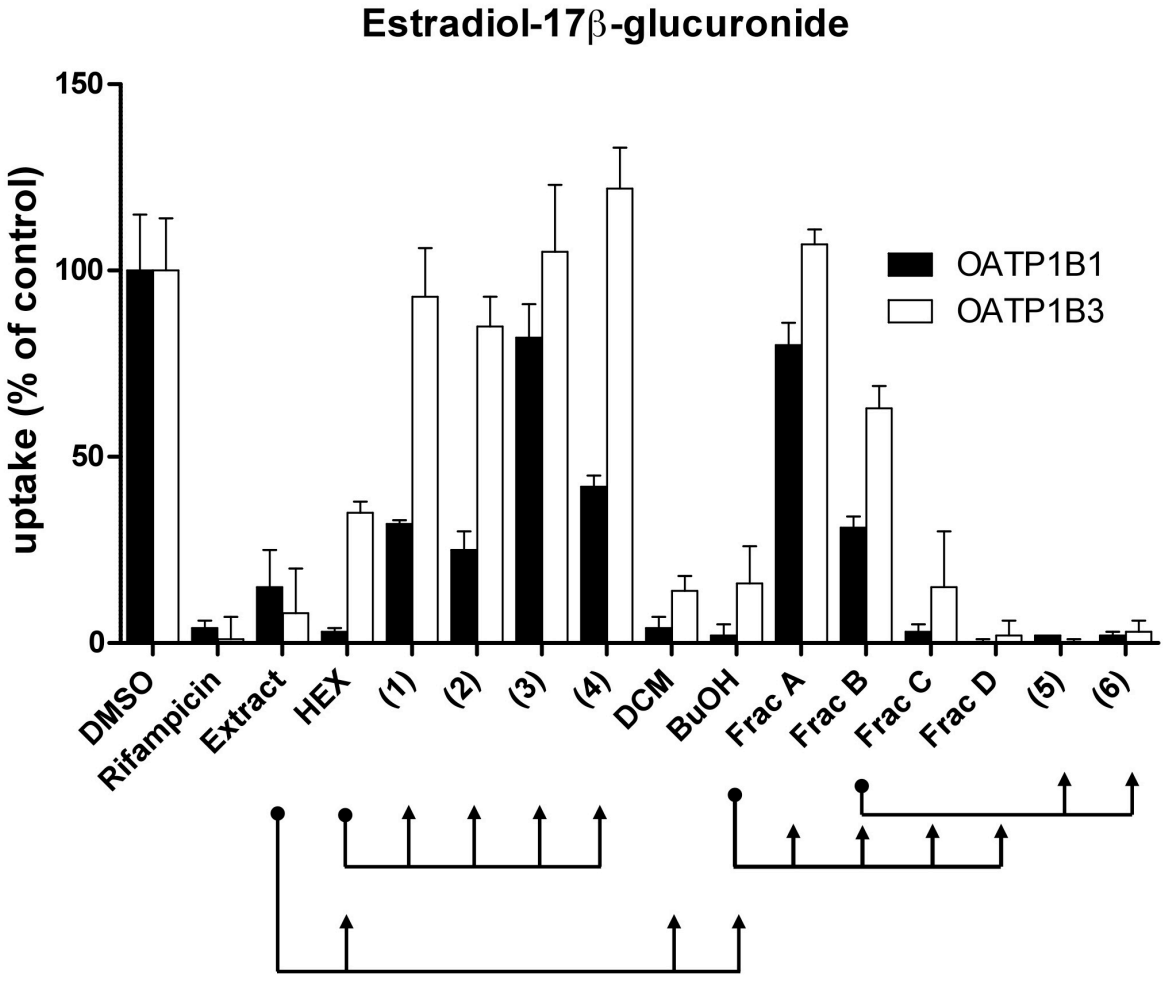

Estradiol-17β-glucuronide
uptake (% of control)
150
100
50
0
OATP1B1
OATP1B3
DMSO
Rifampicin
Extract
HEX
(1)
(2)
(3)
(4)
DCM
BuOH
Frac A
Frac B
Frac C
Frac D
(5)
(6)

Figure 4-2b:

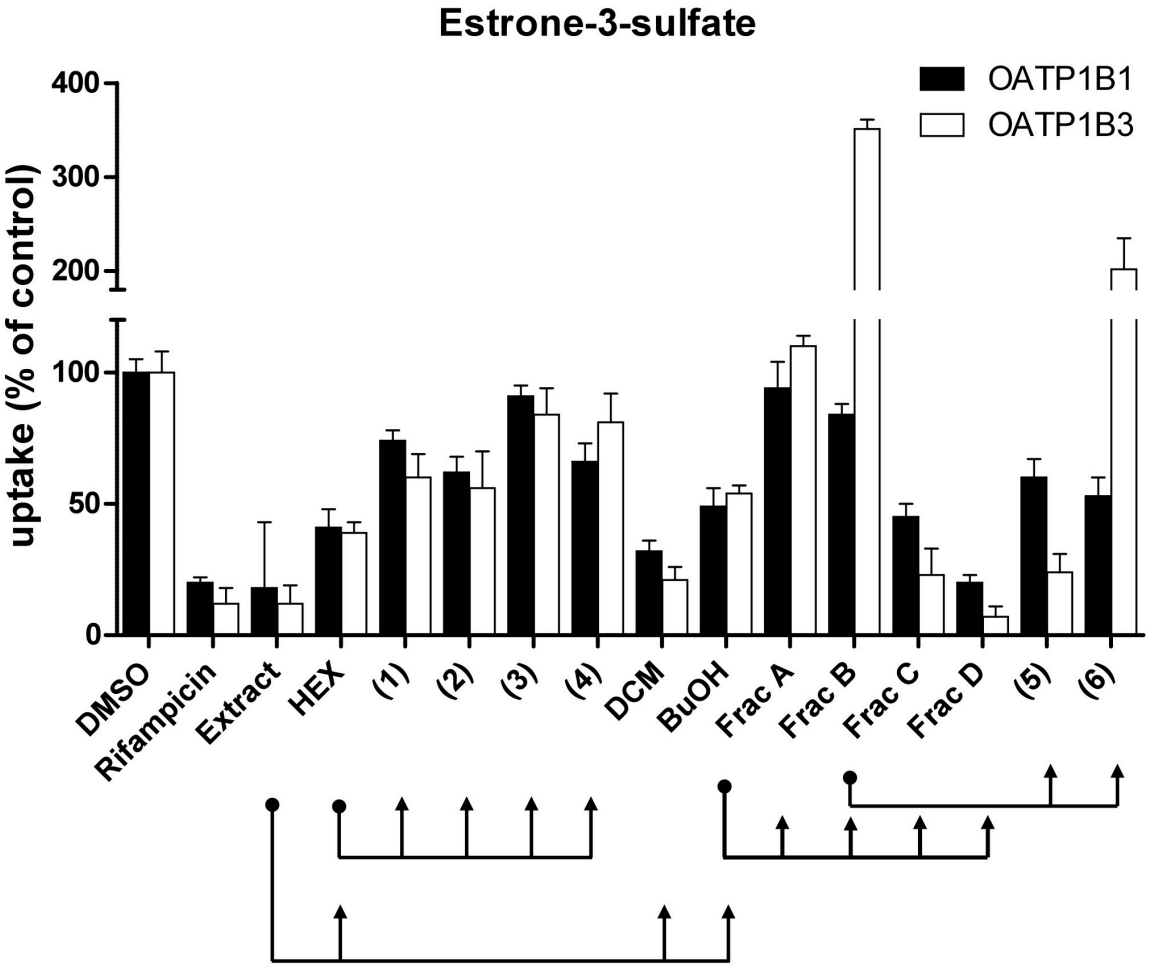

Estrone-3-sulfate
OATP1B1
OATP1B3
uptake (% of control)
400
300
200
100
50
0
DMSO
Rifampicin
Extract
HEX
(1)
(2)
(3)
(4)
DCM
BuOH
Frac A
Frac B
Frac C
Frac D
(5)
(6)

Figure 4-2:

Effect of *Rollina emarginata* extract and fractions on OATP1B1- and OATP1B3–mediated uptake. Cells were coincubated for 5 minutes with 0.1 μM ^{3}H-estradiol-17β-glucuronide (a) or 1 μM ^{3}H-estrone-3-sulfate (b) and 0.03 μg/ml of plant extract or fraction or 100 μM pure compound (1-6). After subtracting the values obtained in wild type cells from those obtained by OATP1B1- or OATP1B3-expressing cells, net transporter-mediated uptake was expressed as a percentage of the vehicle control (1% DMSO). Each value is the mean ± SD from one experiment done in triplicate. Separation steps from initial extract to pure compounds are shown with arrows.

Figure 4-3:

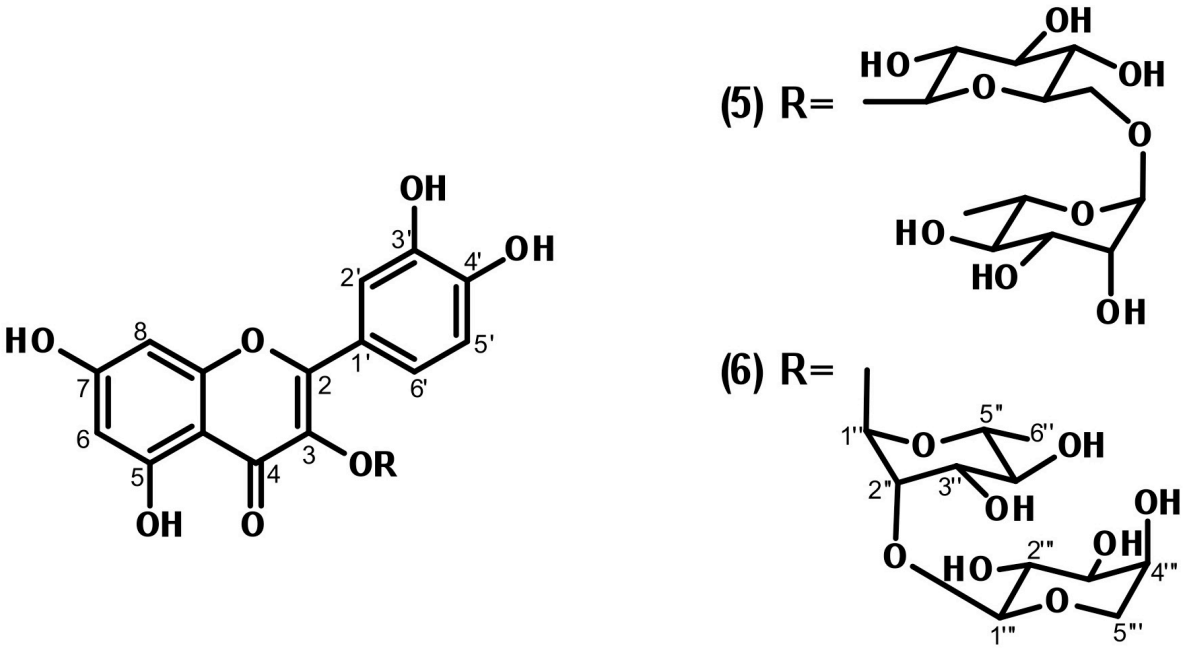


Figure 4-3:

Chemical structures of OATP modulators isolated from *Rollina emarginata* butanol fraction. Compounds were identified as: (5) rutin and (6) quercetin 3-*O*-α-L-arabinopyranosyl(1→2) α-L-rhamnopyranoside (Compound 6).

oleanolic acid, 8-*trans-p*-coumaroyloxy-α-terpineol (Compound 4), or 1% DMSO (vehicle control). All three compounds significantly inhibited uptake of estradiol-17β-glucuronide by OATP1B1 ($p < 0.001$), while having no effect on uptake by OATP1B3 (Figure 4-4A). Compound 4 had a similar effect on uptake of estrone-3-sulfate, inhibiting OATP1B1- but not OATP1B3-mediated transport (Figure 4-4B). However, uptake of estrone-3-sulfate by both transporters was inhibited to an equal extent by ursolic acid and oleanolic acid (Figure 4-4B). Inhibition of estradiol-17β-glucuronide transport by OATP1B1 was further studied with a concentration dependency. Ursolic acid and oleanolic acid inhibited uptake of estradiol-17β-glucuronide with IC_{50} values of 15.3 μM (Figure 4-5A) and 4.2 μM (Figure 4-5B). Compound 4 was the weakest inhibitor; the full plateau of inhibition could not be determined due to limited solubility (Figure 4-5C).

4.3.3: Quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside has Concentration-dependent Effects on OATP1B1- and OAPT1B3-mediated Uptake.

To further investigate the substrate-dependent effects of quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (Compound 6), uptake of 0.1 μM estradiol-17β-glucuronide or estrone-3-sulfate by OATP1B1 and OATP1B3 was measured for 20 seconds at 37˚C in the presence of increasing concentrations of Compound 6. Uptake of estradiol-17β-glucuronide was inhibited by Compound 6 to a similar extent for both transporters (Figure 4-6A, 6B). OATP1B1-mediated uptake of estrone-3-sulfate was inhibited to a lesser extent (IC_{50} = 130 μM, Figure 4-6C). The stimulation of OATP1B3-mediated uptake of estrone-3-sulfate was also concentration dependent, with an EC_{50} of 6.8 μM (Figure 4-6D). At concentrations higher than

Figure 4-4:

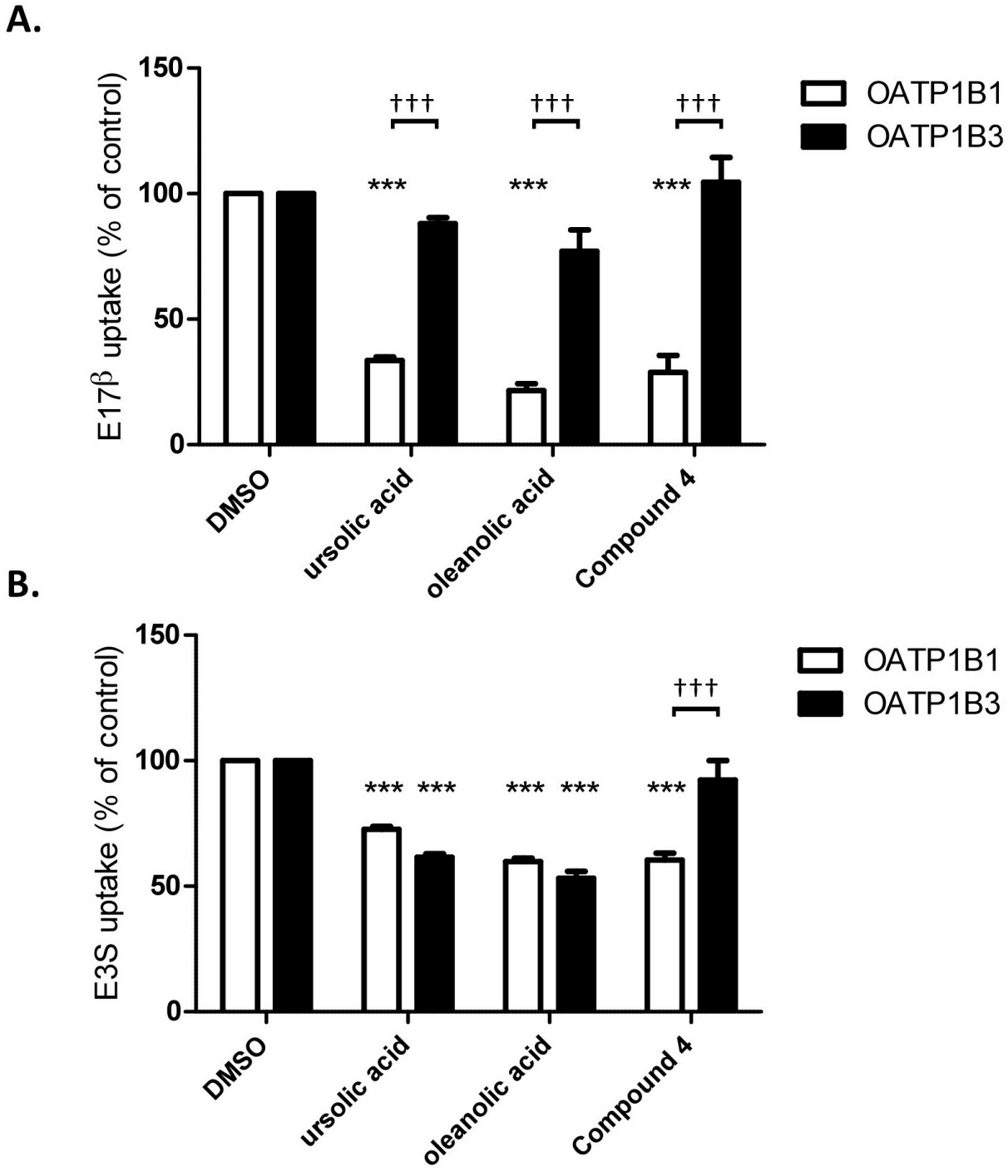

A.
E17β uptake (% of control)
150
100
50
0
†††
†††
†††
***
***
***
OATP1B1
OATP1B3
DMSO
ursolic acid
oleanolic acid
Compound 4
B.
E3S uptake (% of control)
150
100
50
0
†††
*** ***
*** ***
***
OATP1B1
OATP1B3
DMSO
ursolic acid
oleanolic acid
Compound 4

Figure 4-4:

Effect of ursolic acid, oleanolic acid, and 8-*trans-p*-coumaroyloxy-α-terpineol on OATP-mediated uptake. Cells were coincubated with 0.1 μM ^{3}H-estradiol-17β-glucuronide (E17β; **A**) or ^{3}H-estrone-3-sulfate (E3S; **B**) and 100 μM ursolic acid, oleanolic acid, or 8-*trans-p*-coumaroyloxy-α-terpineol (Compound 4) for 20 seconds. After subtracting the values obtained in wild type cells from those obtained by OATP1B1- or OATP1B3-expressing cells, net transporter-mediated uptake was expressed as a percentage of the vehicle control (1% DMSO). Each value is the mean ± SEM of three independent experiments, each performed in triplicate. Asterisks (***) represent statistically significant differences ($P < 0.001$) from the vehicle control, daggers (†††) represent statistically significant differences ($P < 0.001$) between OATP1B1 and OATP1B3.

Figure 4-5:

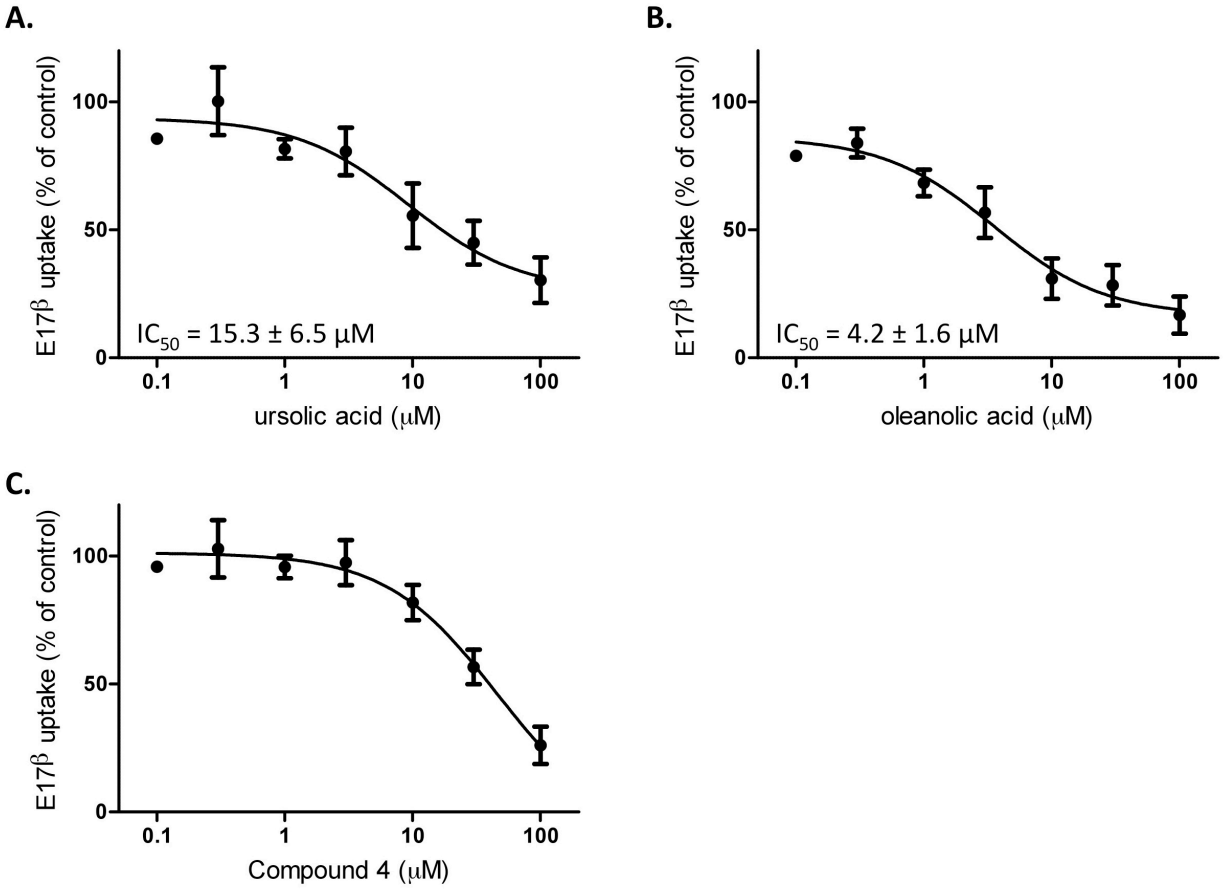

A.
E17β uptake (% of control)
100
50
0
IC50 = 15.3 ± 6.5 µM
0.1
1
10
100
ursolic acid (µM)
B.
E17β uptake (% of control)
100
50
0
IC50 = 4.2 ± 1.6 µM
0.1
1
10
100
oleanolic acid (µM)
C.
E17β uptake (% of control)
100
50
0
0.1
1
10
100
Compound 4 (µM)

Figure 4-5:

Concentration-dependent effect of ursolic acid, oleanolic acid, and Compound 4 on OATP1B1-mediated uptake of estradiol-17β-glucuronide. Cells were coincubated with 0.1 μM ^{3}H-estradiol-17β-glucuronide (E17β) and increasing conentrations of ursolic acid (**A**), oleanolic acid (**B**), or Compound 4 (**C**) for 20 seconds. After subtracting the values obtained in wild type cells from those obtained by OATP1B1-expressing cells, net transporter-mediated uptake was expressed as a percentage of the vehicle control (1% DMSO). Each value shown is the mean ± SEM of three independent experiments, each performed in triplicate.

100 μM, the effect of Compound 6 on estrone-3-sulfate decreased, although it remained stimulatory to the highest tested concentration of 1 mM Compound 6 (data not shown).

4.3.4: Quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside Decreases OATP1B3's Maximal Transport Rate for Estradiol-17β-glucuronide and Estrone-3-sulfate, and Increases its Affinity for Estrone-3-sulfate.

To characterize the mechanism by which these four compounds interact with OATP1B1 and OATP1B3, we examined their effects on the kinetic parameters of OATP1B-mediated transport. Kinetic analysis of estradiol-17β-glucuronide and estrone-3-sulfate uptake was performed in the presence of each interacting compound or the vehicle control, and results are shown in Table 4-1. The affinity of estradiol-17β-glucuronide for OATP1B1 was slightly decreased by each of the four substrates tested. The maximal rate of transport (V_{max}) was not changed by ursolic acid or Compound 4, but was somewhat decreased by both oleanolic acid and Compound 6. However, none of these changes reached statistical significance. In initial experiments, we determined that ursolic acid, oleanolic acid, and Compound 4 did not alter the low-affinity, high-capacity component of OATP1B1-mediated transport of estrone-3-sulfate (data not shown); therefore only the high-affinity component was studied. As for estradiol-17β-glucuronide transport, all four compounds caused small but non-significant decreases in substrate affinity, although none altered the maximal rate of transport.

Figure 4-6:

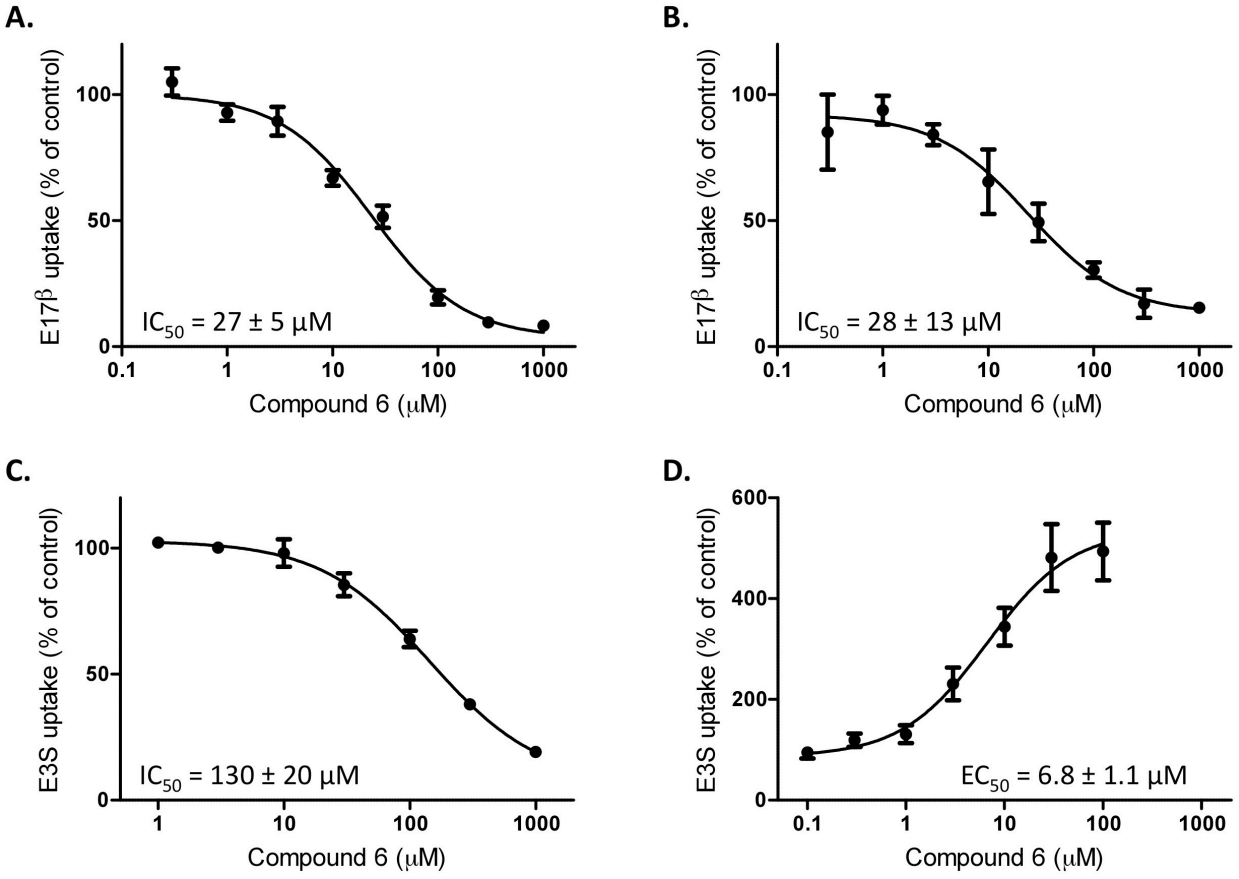

A.
E17β uptake (% of control)
100
50
0
IC50 = 27 ± 5 µM
0.1
1
10
100
1000
Compound 6 (µM)
B.
E17β uptake (% of control)
100
50
0
IC50 = 28 ± 13 µM
0.1
1
10
100
1000
Compound 6 (µM)
C.
E3S uptake (% of control)
100
50
0
IC50 = 130 ± 20 µM
1
10
100
1000
Compound 6 (µM)
D.
E3S uptake (% of control)
600
400
200
0
EC50 = 6.8 ± 1.1 µM
0.1
1
10
100
1000
Compound 6 (µM)

Figure 4-6:

Concentration-dependent effect of Compound 6 on OATP-mediated uptake of estradiol-17β-glucuronide and estrone-3-sulfate. Cells were coincubated with 0.1 μM ^{3}H-estradiol-17β-glucuronide (E17β; **A, B**) or ^{3}H-estrone-3-sulfate (E3S; **C,D**) and increasing concentrations of Compound 6 under for 20 seconds. After subtracting the values obtained in wild type cells from those obtained by OATP1B1- (**A, C**) or OATP1B3-expressing cells (**B, D**), net transporter-mediated uptake was expressed as a percentage of the vehicle control (1% DMSO). Each value shown is the mean ± SEM of three independent experiments, each performed in triplicate.

At the substrate concentrations necessary to determine the kinetics of estrone-3-sulfate uptake by OATP1B3, ursolic acid and oleanolic acid had no effect on transport. Compound 6, however, significantly altered the kinetic parameters of OATP1B3-mediated transport of both model substrates, as illustrated in Figure 4-7. OATP1B3-mediated estradiol-17β-glucuronide uptake was inhibited in a non-competitive manner (Figure 4-7A). Inclusion of 25 μM (squares) or 75 μM (triangles) Compound 6 in the uptake media decreased the maximal rate of transport (V_{max}) from 280 ± 45 to 188 ± 40 (not statistically significant) and 83 ± 9 pmol/mg*min ($p < 0.05$), respectively. Compound 6 had no effect on the apparent affinity (K_m) for estradiol-17β-glucuronide (16 ± 9, 17 ± 5, and 18 ± 9 μM, respectively). Uptake of estrone-3-sulfate by OATP1B3 was measured in the presence of 50 μM Compound 6 or 1% DMSO (Figure 4-7B). As was the case with estradiol-17β-glucuronide, the V_{max} was decreased, from 2.12 ± 0.34 to 1.07 ± 0.05 nmol/mg*min ($p < 0.05$). However, the K_m was also decreased nearly 10-fold, from 93 ± 38 μM to 15 ± 3 μM ($p < 0.005$). This explains the stimulation of transport seen at low estrone-3-sulfate concentrations despite the decrease in V_{max}.

Table 4.1a:

OATP1B1			
Substrate	Inhibitor	K_m (μM)	V_{max} (pmol/mg protein/min)
Estradiol-17β-glucuronide	None	7 ± 1	175 ± 11
	Ursolic acid	20 ± 11	217 ± 60
	Oleanolic acid	13 ± 3	117 ± 10
	Compound 4	36 ± 19	258 ± 86
	Compound 6	15 ± 6	92 ± 18
Estrone-3-sulfate[1]	None	0.5 ± 0.1	96 ± 10
	Ursolic acid	0.8 ± 0.1	105 ± 8
	Oleanolic acid	0.7 ± 0.1	85 ± 5
	Compound 4	1.3 ± 0.3	102 ± 10
	Compound 6	0.8 ± 0.2	93 ± 8

Table 4.1b:

OATP1B3			
Substrate	Inhibitor	K_m (μM)	V_{max} (pmol/mg protein/min)
Estradiol-17β-glucuronide	None	16 ± 9	280 ± 45
	Ursolic acid	n/a	n/a
	Oleanolic acid	n/a	n/a
	Compound 4	n/a	n/a
	Compound 6 (25 μM)	17 ± 5	188 ± 40
	Compound 6 (75 μM)	18 ± 9	83 ± 9*
Estrone-3-sulfate[1]	None	93 ± 38	2120 ± 340
	Ursolic acid	---	---
	Oleanolic acid	---	---
	Compound 4	n/a	n/a
	Compound 6 (50 μM)	15 ± 3**	1070 ± 50

Table 4.1:

Kinetics of OATP-mediated transport in the absence and presence of modulators. All inhibitors were used at 100 μM unless indicated. [1] high affinity component; * = $p < 0.05$; ** = $p < 0.005$; n/a = no significant inhibition of 0.1 μM substrate by 100 μM inhibitor; --- = no apparent inhibition at the concentrations required to determine kinetics

Figure 4-7:

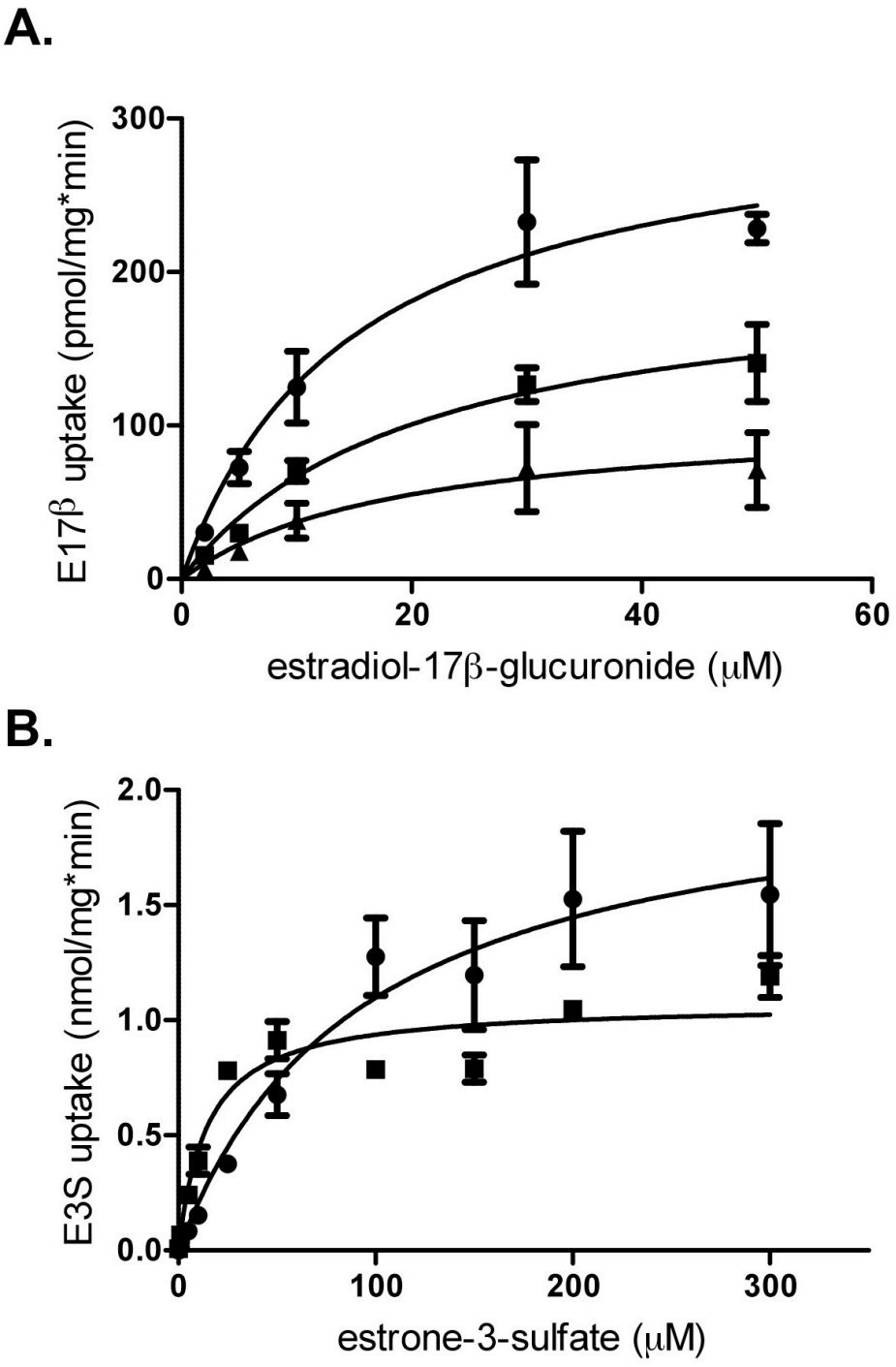

A.
E17β uptake (pmol/mg*min)
300
200
100
0
0
20
40
60
estradiol-17β-glucuronide (μM)
B.
E3S uptake (nmol/mg*min)
2.0
1.5
1.0
0.5
0.0
0
100
200
300
estrone-3-sulfate (μM)

Figure 4-7:

Effect of Compound 6 on OATP1B3-mediated transport. A. Cells were incubated with increasing concentrations of estradiol-17β-glucuronide (E17β) in the presence of 25 μM (squares) or 75 μM (triangles) quercetin 3-*O*-α-L-arabinopyranosyl (1→2)α-L-rhamnopyranoside (Compound 6), or the vehicle control (0.5% DMSO, circles) for 20 seconds. **B.** Cells were incubated with increasing concentrations of estrone-3-sulfate (E3S) in the presence of 50 μM Compound 6 (squares) or the vehicle control (circles) under initial linear rate conditions. After subtracting the values obtained in wild type cells, net transporter-mediated uptake was fitted to the Michaelis-Menten equation to determine K_m and V_{max} values. Each value shown is the mean ± SEM of at least three independent experiments, each performed in triplicate.

4.4: Discussion

Many naturally occurring plant compounds have been identified that interact with OATPs (Dresser et al., 2002; Fuchikami et al., 2006; Bailey et al., 2007; Glaeser et al., 2007; Greenblatt, 2009), making plants a potentially rich source of OATP modulators. In the present study, we used a bioassay-guided approach to isolate and identify four compounds from the organic extract of *Rollinia emarginata* Schlecht (Annonaceae) that selectively modulate OATP function. This approach enabled us to quickly focus in on fractions of plant extract containing compounds that had substrate-specific or transporter-specific effects on OATP function. As is illustrated in Figure 4-1, only 3 to 4 fractionation steps were required to isolate pure compounds with the desired activity from the plant extract. A downfall of this approach is the likelihood of missing OATP modulating compounds, whose effects may be masked in the early stages of fractionation due to the large number of compounds in each fraction. However, it does allow for the early elimination of many general OATP modulators, increasing the efficiency of this approach when compared to that of high-throughput screening of large compound libraries.

We demonstrated that ursolic acid, oleanolic acid, and Compound 4 selectively inhibit estradiol-17β-glucuronide transport by OATP1B1 compared to OATP1B3 (Figure 4-4A). Several other compounds have previously been identified as selective inhibitors of OATP1B1. Uptake of estradiol-17β-glucuronide by OATP1B1 was inhibited to a greater extent than was uptake by OATP1B3 by both indocyanine green (Cui et al., 2001) and the proposed cardiotonic agent YM758 (Umehara et al., 2008). Taurocholate uptake by OATP1B1 was inhibited by the glycocholic acid derivatives BAPA-8, BAPA-6, and BAPA-3 more strongly than was uptake by

OATP1B3 (Vicens et al., 2007). However, we found that the selective inhibition of OATP1B1 by ursolic acid and oleanolic acid was dependent upon the substrate being transported. While Compound 4 selectively inhibited OATP1B1 transport of both estradiol-17β-glucuronide and estrone-3-sulfate but had no significant effect on transport by OATP1B3, ursolic acid and oleanolic acid inhibited the uptake of estrone-3-sulfate by both transporters (Figure 4-4B). Furthermore, in a previous study using fluorescein-methotrexate (FMTX) we identified ursolic acid as a preferential OATP1B3 inhibitor (Gui et al., 2010). Ursolic acid inhibited OATP1B3-mediated FMTX uptake with an IC_{50} of 2.3 μM while the IC_{50} value for OATP1B1 was 12.5 μM.

Substrate–dependent modulation of OATP1B3 was also produced by Compound 6. Transport of estradiol-17β-glucuronide by both OATP1B1 and OATP1B3 was inhibited to the same extent (Figures 4-6A, 4-6B). However, uptake of estrone-3-sulfate by OATP1B1 was inhibited, while uptake by OATP1B3 was stimulated (Figures 4-6C, 4-6D). Further investigation of this effect on OATP1B3 revealed that the maximal rate of transport (V_{max}) was reduced for both substrates (Figure 4-7). The apparent substrate affinity (K_m) for estradiol-17β-glucuronide was unchanged, causing inhibition of transport at all concentrations studied. In contrast, the affinity for estrone-3-sulfate was increased 10-fold, leading to stimulation of transport at low substrate concentrations, and inhibition of transport at high substrate concentrations.

There are many mechanisms by which transporter function may be inhibited or stimulated. Changes in the expression of transporter protein on the cell surface, whether through transcriptional regulation or post-translational modifications, would affect uptake of all

substrates in the same manner, and thus cannot explain the substrate-specific changes in transporter activity produced by ursolic acid, oleanolic acid, and Compound 6. Compound 4 inhibited transport of both substrates by OATP1B1 but not by OATP1B3, which could be explained by rapid internalization of OATP1B1. However, the full inhibitory effect described here took place during 20 seconds of exposure, whereas phosphorylation-induced internalization of OATP2B1 and rat Oatp1a1 proteins appears to take at least 10 to 30 minutes (Kock et al., 2010; Choi et al., 2011). However, post-translational modifications such as phosphorylation may also directly alter activity of a transporter, as is seen with the glucose transporter (Berridge and Tan, 1995), and theoretically could do so in a substrate-dependent way. Alternatively, if the isolated compounds are substrates of OATP1B1 or OATP1B3, they could exert their effects through competitive inhibition. There is considerable evidence that OATPs have multiple binding sites, therefore a compound may inhibit uptake of a substrate that shares its binding site while having no effect on a substrate that has a distinct binding site. This competitive inhibition would be expected to decrease affinity for the substrate that shares a binding site, while not affecting the V_{max}. Unfortunately, as relatively weak inhibitors, ursolic acid, oleanolic acid and Compound 4 did not have statistically significant effects on the kinetic parameters of OATP1B1- or OATP1B3-mediated uptake, suggesting that if they are substrates, they are very low-affinity. A final explanation for these results is that the compounds may bind to a portion of OATP1B1 or OATP1B3, sterically hindering either the binding or the translocation of substrates. In the case of R002, this binding could also cause a conformational change in OATP1B3 that increases the affinity of the transporter for estrone-3-sulfate while not affecting the estradiol-17β-glucuronide binding site.

Interestingly, although Compound 6 stimulated transport of estrone-3-sulfate, the structurally similar rutin (Compound 5) did not show this effect. However, rutin was previously shown to stimulate uptake of 0.5 μM DHEAS by OATP1B1 (Wang et al., 2005b). This demonstrates that stimulation of uptake is not a phenomenon specific to OATP1B3, but may be shared by all OATPs. This is further supported by a previous study on OATP2B1 where different steroids stimulated the uptake of either estrone-3-sulfate or DHEAS by OATP2B1 (Grube et al., 2006a). Substrate-dependent effects of OATP modulators as shown in this study have also been seen previously. We found that EGCG inhibits OATP1B3-mediated uptake of Fluo-3, has no effect on uptake of estradiol-17β-glucuronide, and stimulates uptake of estrone-3-sulfate (Roth et al., 2011b). We also discovered that clotrimazole stimulates OATP1B3-mediated transport of estradiol-17β-glucuronide, inhibits transport of Fluo-3, and has little effect on uptake of estrone-3-sulfate (Gui et al., 2008). Gemfibrozil inhibits OATP1B1 uptake of pravastatin, fluvastatin, simvastatin and taurocholate, but not of estrone-3-sulfate or troglitazone sulfate (Noe et al., 2007). Low concentrations of rosiglitazone (10 μM) inhibit OATP1B1 and OATP1B3 transport of bromosulfophthalein, but stimulate uptake of pravastatin by both transporters (Bachmakov et al., 2008).

Inhibition and stimulation of OATPs can clearly be dependent on the substrate being transported. Although there are compounds that can nonspecifically inhibit transport of all OATP substrates, as rifampicin seems to do, there are no compounds which selectively inhibit transport of all substrates by a single OATP. Using a general OATP inhibitor to reduce the hepatic first-pass effect of a drug is likely to cause a wide range of effects, due to inhibition of transport of other OATP substrates by OATPs expressed throughout the body. Therefore, to be used

therapeutically, it is essential to identify compounds that selectively inhibit either OATP1B1 or OATP1B3 while having no effect on transport mediated by other OATPs. Ideally, the inhibition would also be less potent for endogenous substrates or other drugs like statins than for the drug with the disadvantageous first-pass effect. Similarly, to increase uptake of drugs by hepatocytes or by OATP-expressing cancer cells, stimulation of OATP transport should be restricted to the OATP being targeted, while having little effect on uptake of other substrates by that transporter. It is therefore essential to identify selective OATP modulators.

In conclusion, we have identified four compounds that modulate OATP function. Ursolic acid, oleanolic acid, and 8-*trans*-*p*-coumaroyloxy-α-terpineol, inhibit estradiol-17β-glucuronide uptake by OATP1B1 but not by OATP1B3, while ursolic acid and oleanolic acid inhibit estrone-3-sulfate uptake by both transporters. Quercetin 3-*O*-α-L-arabinopyranosyl(1→2) α-L-rhamnopyranoside (Compound 6) inhibits transport by OATP1B1, but has substrate-dependent effects on OATP1B3, non-competitively inhibiting uptake of both substrates at high substrate concentrations, but stimulating estrone-3-sulfate uptake at low substate concentrations by increasing affinity. The results of this study show that plant materials are a good starting point for the isolation of OATP modulating compounds, and that a bioassay-guided approach can be used to efficiently identify selective OATP modulators.

Chapter 5

Cysteine Scanning Mutagenesis on Transmembrane Domain 1 of the Liver-Specific Organic Anion Transporting Polypeptide 1B3 (OATP1B3)

5.1: Abstract

The liver-specific organic anion transporting polypeptide 1B3 (OATP1B3) is a multispecific transporter that mediates the uptake of a broad range of drugs and other xenobiotics into hepatocytes. To identify regions in OATP1B3 important for substrate binding and/or translocation, we performed cysteine scanning mutagenesis. Thirty-three amino acids in transmembrane domain 1 (TM1) and extracellular loop 1 (ECL1) were individually mutated to cysteines, and mutant proteins were transiently expressed in HEK293 cells. To determine the effect of each mutation on protein function, we measured uptake of estrone-3-sulfate and estradiol-17β-glucuronide. Transport was significantly reduced in several mutants after correcting for surface expression, indicating that this stretch of amino acids may be important for protein function. Functional cysteine mutants were then incubated with the cysteine-modifying reagent sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) prior to measuring uptake. MTSES pre-incubation strongly inhibited the function of those mutants that could be labeled with biotinylated maleimide. These data suggest that TM1 and ECL1 in OATP1B3 play a significant role in substrate binding and/or translocation.

5.2: Introduction

The efficient absorption and distribution of many xenobiotics depends on carrier-mediated transport through cell membranes. Organic anion transporting polypeptides (OATPs) are multispecific transporters that are expressed in epithelia throughout the body and are largely responsible for the Na^+-independent uptake of large amphipathic organic anions into cells. There are eleven human members of the OATP superfamily, classified into six families based on a 40% amino acid sequence identity (Hagenbuch and Meier, 2004). Two of these proteins, OATP1B1 and OATP1B3, are normally exclusively expressed at the basolateral membrane of hepatocytes (Abe et al., 1999; Konig et al., 2000a; Konig et al., 2000b; Abe et al., 2001), where they can have a significant effect on the first-pass clearance of orally administered drugs. Substrates of OATP1B1 and OATP1B3 include both endogenous compounds, such as bile acids, bilirubin, steroid conjugates, and thyroid hormones, and numerous xenobiotics, including statins, sartans, β-lactam antibiotics, anticancer drugs, fexofenadine and HIV protease inhibitors (Hagenbuch and Gui, 2008).

OATP1B1 and OATP1B3 share 80% amino acid identity with each other and have a single rodent ortholog, Oatp1b2. Because these three proteins have overlapping but distinct substrate specificities, potential drug-drug interactions can be missed in pre-market safety testing. OATPs have indeed been implicated in multiple adverse drug-drug interactions (DDIs), such as those between cerivastatin and gemfibrozil (Shitara et al., 2003), rosuvastatin and cyclosporine (Simonson et al., 2004), and fexofenadine and grapefruit juice (Bailey et al., 2007; Glaeser et al., 2007). In addition, OATP1B3 is expressed in a wide variety of cancer cell lines

and tumors, including cancers of the breast (Muto et al., 2007), lung (Monks et al., 2007), and prostate (Hamada et al., 2008), as well as gastric, colon and pancreatic cancers (Abe et al., 2001; Lee et al., 2008). Among the many OATP1B3 substrates are several anti-cancer drugs, including methotrexate (Abe et al., 2001), the taxanes paclitaxel and docetaxel (Smith et al., 2005a; Letschert et al., 2006), imatinib (Hu et al., 2008), and SN-38, the active metabolite of irinotecan (Yamaguchi et al., 2008). This raises the possibility that cytotoxic OATP1B3 substrates may be targeted to tumor tissues.

OATP1B3 is a 691 amino acid glycoprotein with an apparent molecular mass of 120 kDa, which is reduced to about 60 kDa after deglycosylation. Like all OATPs, OATP1B3 is predicted to contain 12 transmembrane domains with both termini located in the cytosol (Wang et al., 2008). However, due to the lack of a crystal structure, little is known about either the three-dimensional structure of OATPs or the mechanism by which they bind and transport substrates. Multiple groups have created homology models for OATP1B3 based on the crystal structures of the glycerol-3-phospate transporter and lactose permease (Meier-Abt et al., 2005; Glaeser et al., 2010; Frandoloso et al., 2011) or the glycerol-3-phosphate transporter and multidrug transporter EmrD (Gui and Hagenbuch, 2008). These models suggest that the transmembrane domains of OATP1B3 form a central positively charged pore. Numerous amino acids within this pore have been identified as important for transport, including the positively charged K41 and R580 (Glaeser et al., 2010), and K361 (Mandery et al., 2011). Similar studies with OATP1B1 confirmed that the positively charged K361 and R580 are involved in transport by both OATP1B family members, as well as identifying a positive charge at R57 as being essential for OATP1B1 transport (Weaver and Hagenbuch, 2010). In addition, chimera studies

between OATP1B1 and OATP1B3 identified several neutral amino acids in transmembrane domain 10 that are important for transport of the OATP1B3-specific substrate cholecystokinin octapeptide (CCK-8), possibly due to their involvement in forming hydrogen bonds with the substrate (Gui and Hagenbuch, 2008). The same study indicated that replacing TM1 of OATP1B3 with the corresponding transmembrane domain from OATP1B1 resulted in a greater than 50% loss of function for CCK-8 transport. This, combined with the findings that K41 in OATP1B3 and R57 in OATP1B1 are important for transporter function, suggests that transmembrane domain 1 of the OATP1B family forms part of the positively charged central pore, and provides critical interactions with the substrates. To further study the role of this region in the binding and translocation of substrates, we performed cysteine scanning mutagenesis studies on the first transmembrane domain and extracellular loop of OATP1B3. Our results indicate that TM1 does form part of the substrate translocation pathway, and that both TM1 and ECL1 contain numerous amino acids that are important for substrate transport.

5.3: Results

5.3.1 Cysteine Scanning Mutagenesis of OATP1B3.

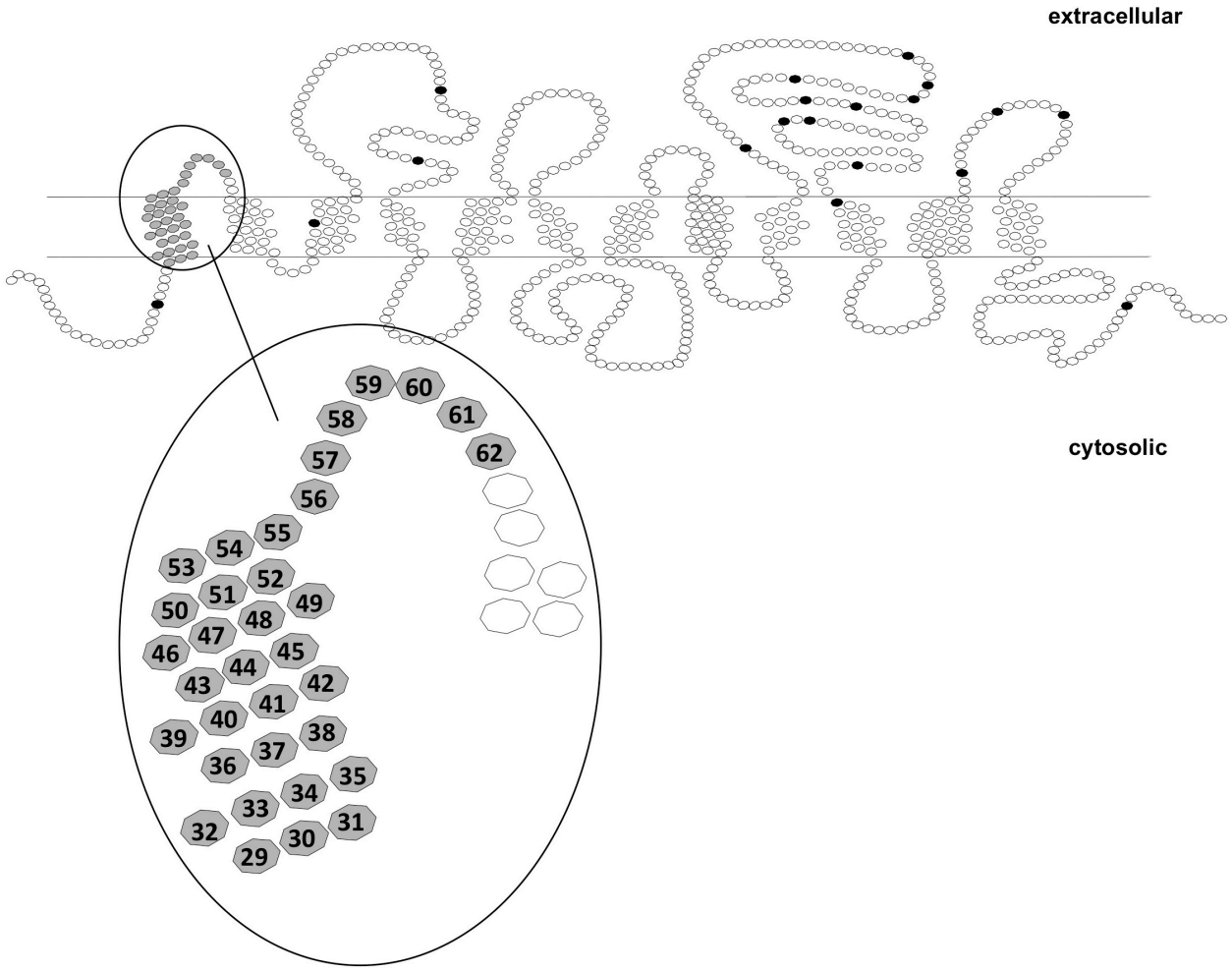
OATP1B3 is predicted to have twelve transmembrane domains, with both termini being located in the cytoplasm. Previous studies have indicated that amino acids in the first transmembrane domain (TM1) and extracellular loop (ECL1) are important for transport function of OATP1B3. To determine the role that amino acids in TM1 and ECL1 of OATP1B3 play in substrate recognition and translocation, we performed cysteine scanning mutagenesis.

Thirty-three amino acids predicted to be located in the first transmembrane domain (TM1) and extracellular loop (ECL1) were individually mutated into cysteines (M29 – S62, Figure 5-1). Mutant Q54C could not be propagated and is not included in the analysis. Vectors containing wild-type or mutant OATP1B3 were transiently expressed in HEK293 cells. To measure the level of surface expression of each mutant, we biotinylated and purified surface proteins and performed a western blot, detecting OATP1B3 expression using an anti-His antibody. Western blots were also probed with an antibody against the alpha subunit of Na^+/K^+ ATPase, as a control for biotinylation. OATP1B3 and all mutant proteins were expressed on the cell surface to approximately the same extent (Figure 5-2).

5.3.2: Function of Cysteine-Substituted Mutants.

Due to observed differences in substrate specificity, we screened all cysteine-substituted mutants for transport activity by measuring uptake of two different radiolabeled model substrates: estrone-3-sulfate and estradiol-17β-glucuronide. Uptake of each substrate was measured after incubating at 37˚C for 5 minutes, and is expressed for each cysteine mutant as a percentage of uptake by wild-type OATP1B3. Most mutant transporters retained at least partial transport activity, however six mutants (K41C, K49C, I50C, T53C, I55C and E56C) had very little or no transport activity, despite being expressed on the cell surface (Figure 5-3). In general, mutations predicted to fall within the cytoplasmic half of the transmembrane domain (M29C-S37C) had little effect on function, while mutations in the predicted extracellular loop (T53C-D60C) all caused significant reductions in substrate transported (>50%). Within the area of the

Figure 5-1:


extracellular
cytosolic
59
60
61
58
62
57
56
55
54
53
52
51
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50
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44
43
42
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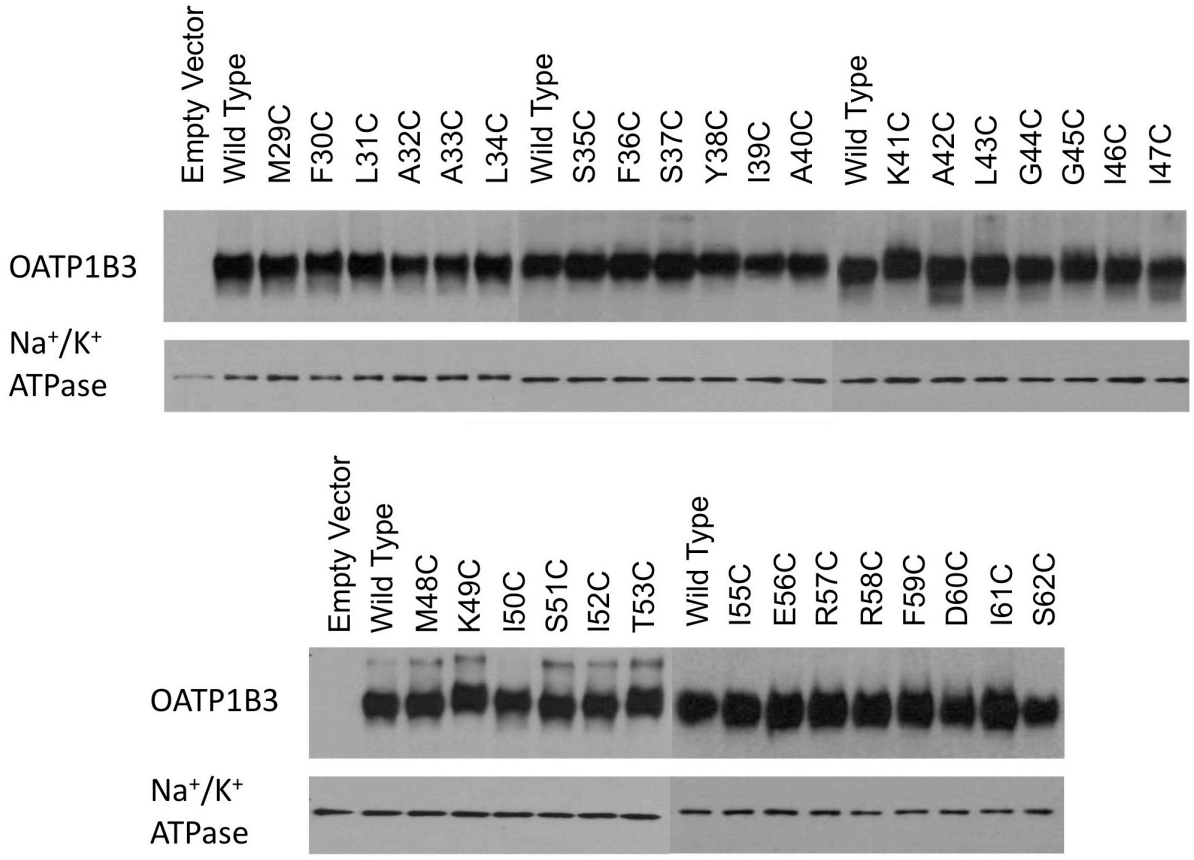
32
30
29

Figure 5-1:

Predicted transmembrane location of the cysteine-substituted amino acids. Membrane topology as predicted from three-dimensional homology model of OATP1B3 (Gui and Hagenbuch, 2008). Black filled circles represent naturally occurring cysteine residues; amino acids represented by gray filled circles were individually mutated to cysteine (inset).

Figure 5-2: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F30C
L31C
A32C
A33C
L34C
Wild Type
S35C
F36C
S37C
Y38C
I39C
A40C
Wild Type
K41C
A42C
L43C
G44C
G45C
I46C
I47C
OATP1B3
Na+/K+
ATPase
Empty Vector
Wild Type
M48C
K49C
I50C
S51C
I52C
T53C
Wild Type
I55C
E56C
R57C
R58C
F59C
D60C
I61C
S62C
OATP1B3
Na+/K+
ATPase

Figure 5-2:

Surface expression of cysteine-substituted mutants. HEK293 cells transiently expressing wildt-ype or cysteine mutated OATP1B3 were incubated with Sulfo-NHS-SS-Biotin, and surface proteins were purified with Neutravidin. Western blots were incubated with monoclonal anti-his or anti-Na^+/K^+ ATPase antibodies. Shown are representative blots of at least three independent experiments.

Figure 5-3:

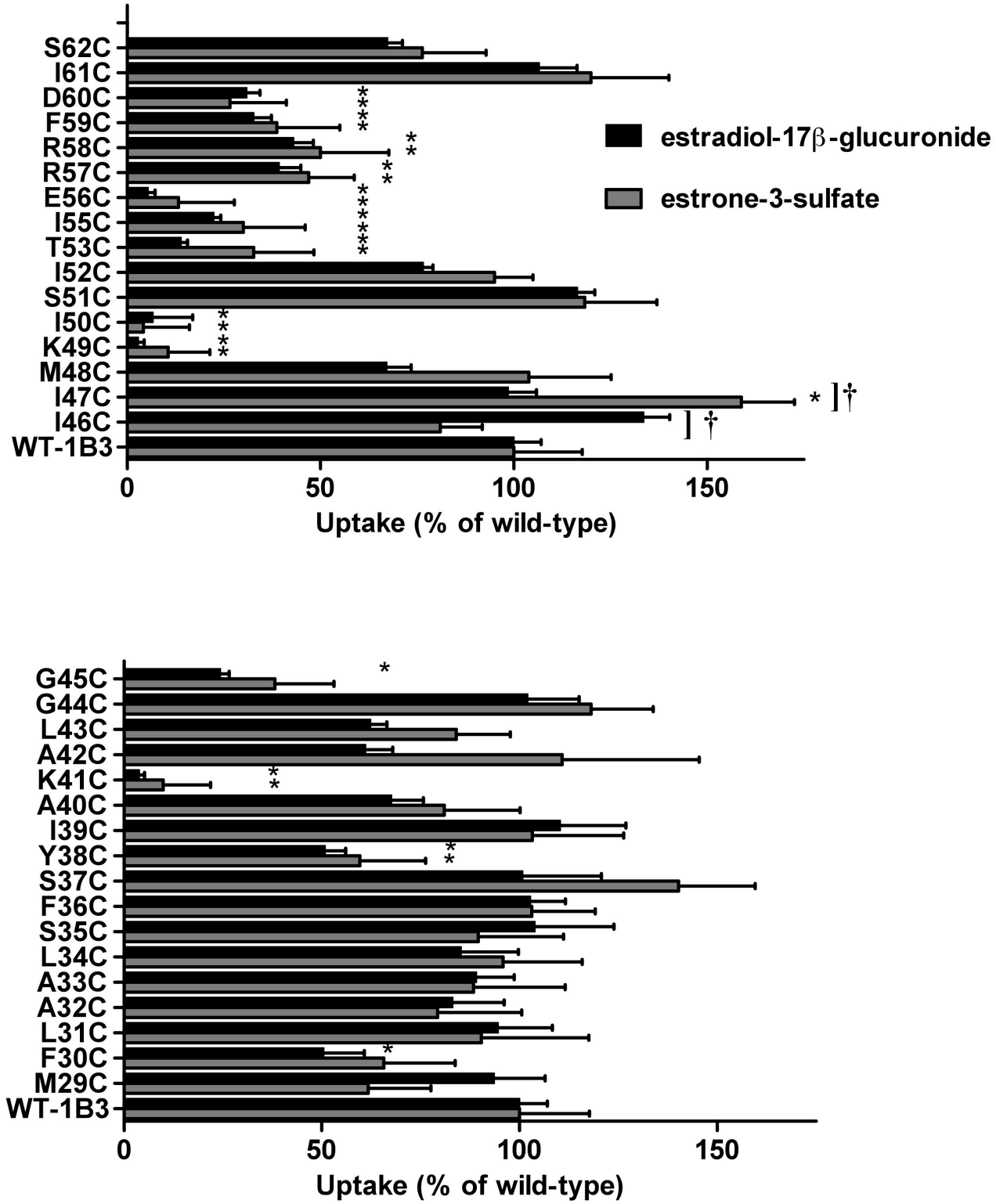

S62C
I61C
D60C
F59C
R58C
R57C
E56C
I55C
T53C
I52C
S51C
I50C
K49C
M48C
I47C
I46C
WT-1B3
estradiol-17β-glucuronide
estrone-3-sulfate
0
50
100
150
Uptake (% of wild-type)
G45C
G44C
L43C
A42C
K41C
A40C
I39C
Y38C
S37C
F36C
S35C
L34C
A33C
A32C
L31C
F30C
M29C
WT-1B3
0
50
100
150
Uptake (% of wild-type)

Figure 5-3:

Function of cysteine-substituted mutants. Cells were incubated with 0.1 μM ^{3}H-estrone-3-sulfate (E3S) or ^{3}H-estradiol-17β-glucuronide (E17β) for 5 minutes at 37˚C. After subtracting the values obtained in cells expressing the empty vector, uptake was expressed as a percentage of wild-type. Each value is the mean ± SEM of three independent experiments. Asterisks represent significant differences from the wild-type control ($P < 0.05$).

protein predicted to be in the extracellular half of the transmembrane domain, cysteine substitutions at positions 38, 41, 45, 49 and 50 decreased activity, while substitutions in the remaining positions (39-40, 42-44, 46-48, and 51-52) did not. Interestingly, uptake of estrone-3-sulfate by the I47C mutant was significantly increased when compared to wild type. In addition, the effect on transport of estradiol-17β-glucuronide was significantly different from the effect on transport of estrone-3-sulfate for the mutants I46C and I47C.

5.3.3: Effect of MTSES Pre-treatment on Function of Cysteine-Substituted Mutants.

The effect of a given cysteine substitution on OATP1B3 transport activity might be dependent on whether the amino acid is exposed to the substrate-containing extracellular aqueous environment. To determine whether decreased transport activity was associated with solvent accessibility, we investigated whether preincubation with the membrane impermeable thiol-specific reagent sodium (2-Sulfonatoethyl)methanethiosulfonate (MTSES) affected substrate uptake. Cells expressing wild-type or mutant OATP1B3 proteins were incubated in a buffer containing 10 mM MTSES for 10 minutes at 37˚C. After washing, uptake of estrone-3-sulfate and estradiol-17β-glucuronide was measured as described above. Uptake of each substrate after incubation in MTSES is expressed as a percentage of the uptake by the same mutant after ten-minute incubations in buffer that did not contain MTSES (Figure 5-4). Four mutants that were found to be nearly nonfunctional in the previous experiment (K41C, K49C, I50C and E56C) were not tested. Proteins with cysteine substitutions at locations 29-37, which are predicted to be in the cytosolic half of the transmembrane domain, were unaffected by pre-

Figure 5-4:

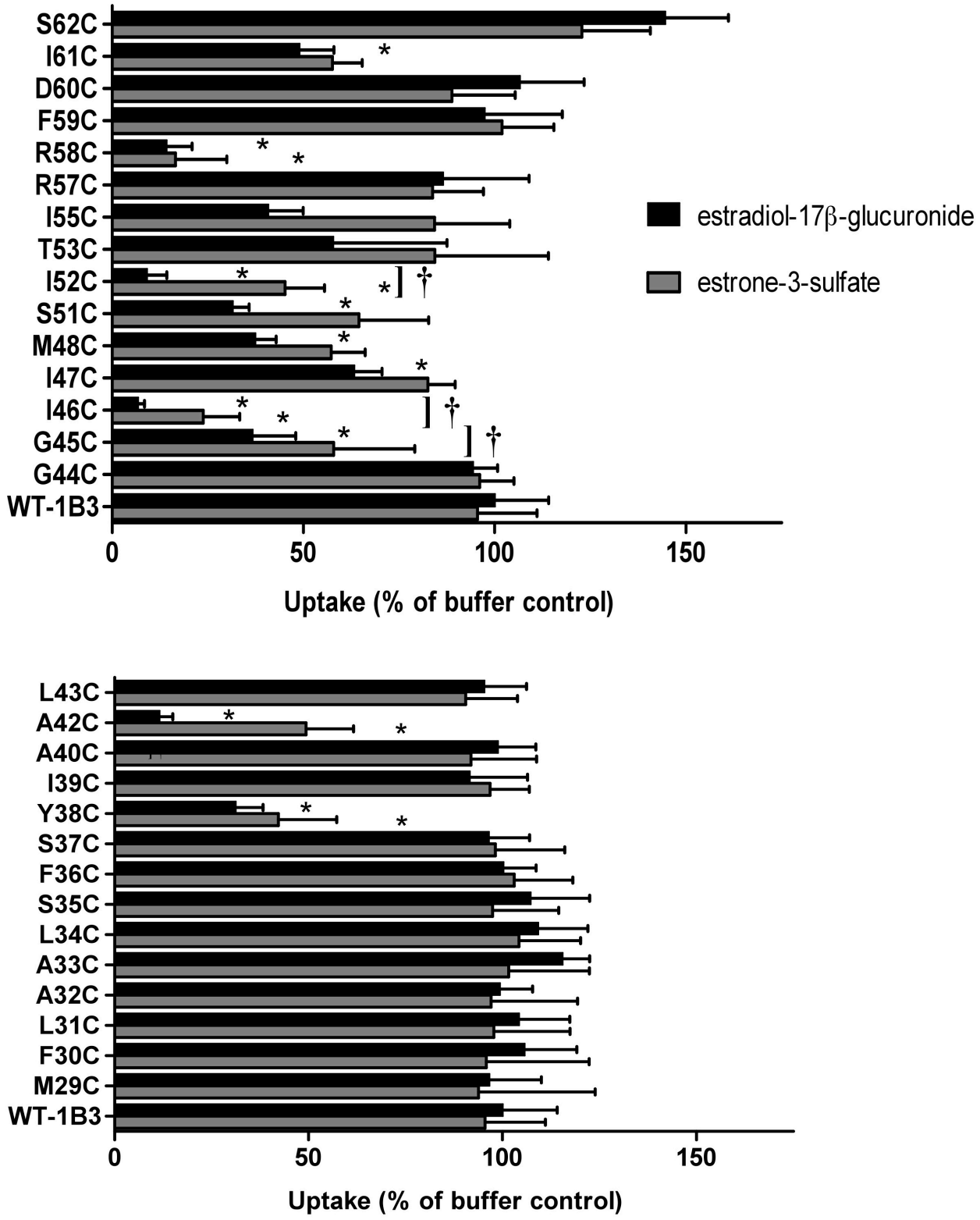

S62C
I61C
D60C
F59C
R58C
R57C
I55C
T53C
I52C
S51C
M48C
I47C
I46C
G45C
G44C
WT-1B3
estradiol-17β-glucuronide
estrone-3-sulfate
0
50
100
150
Uptake (% of buffer control)
L43C
A42C
A40C
I39C
Y38C
S37C
F36C
S35C
L34C
A33C
A32C
L31C
F30C
M29C
WT-1B3
0
50
100
150
Uptake (% of buffer control)

Figure 5-4:

Effect of MTSES pre-incubation on the function of cysteine-substituted mutants. HEK293 cells transiently expressing wild-type or cysteine mutated OATP1B3 were incubated with uptake buffer containing 10 mM MTSES for 10 min at 37˚C, briefly washed, then incubated with 0.1μM 3H-estrone-3-sulfate (E3S) or 3H-estradiol-17β-glucuronide (E17β) for 5 minutes at 37˚C. After subtracting the values obtained in cells transfected with the empty vector, values were expressed as a percentage of the buffer control. Each value is the mean ± SEM of three independent experiments. Asterisks represent significant differences ($P < 0.05$) from buffer controls.

incubation with MTSES. Significant reductions in transport was seen after MTSES treatment when cysteines were substituted for amino acids at positions 38, 42, 45 – 52, 58, and 61 (Figure 5-4, asterisks). The effect of MTSES on transport activity was significantly different between the two substrates tested for mutants A42C, G45C, I46C, and I52C (Figure 5-4, daggers).

5.3.4: Accessibility of Substituted Cysteines to Aqueous Environment.

Cells expressing wild-type or mutant OATP1B3 proteins were incubated in PBS containing maleimide-PEG_2-biotin (0.5mg/ml) for 1 hour at 4˚C. Labeled proteins were then affinity purified and subjected to western blot anlysis. To ensure equal levels of transfection efficiency for each mutant, OATP1B3 protein in the total cleared lysate was also quantified (data not shown). As shown in Figure 5-5, cysteines substituted at positions 29-37 were not labeled by maleimide-PEG_2-biotin, indicating that they are inaccessible from the extracellular environment. All cysteine substitutions from positions 52-62 are strongly labeled, supporting their predicted localization in the extracellular loop. In addition, several amino acids in the presumptive extracellular half of TM1 were also consistently labeled: Y38C, K41C, A42C, I46C, and M48C.

Figure 5-5:

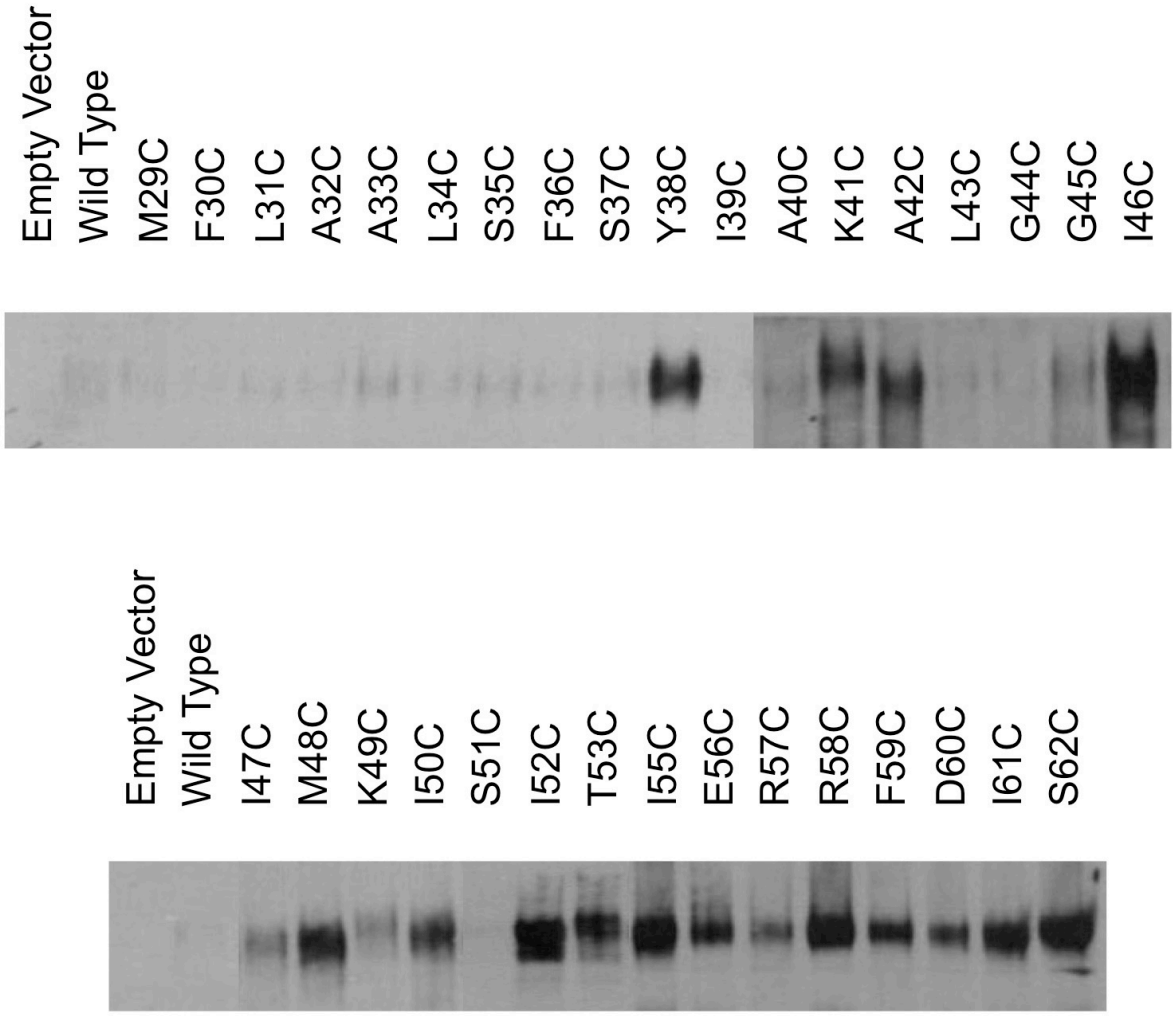

Empty Vector
Wild Type
M29C
F30C
L31C
A32C
A33C
L34C
S35C
F36C
S37C
Y38C
I39C
A40C
K41C
A42C
L43C
G44C
G45C
I46C
Empty Vector
Wild Type
I47C
M48C
K49C
I50C
S51C
I52C
T53C
I55C
E56C
R57C
R58C
F59C
D60C
I61C
S62C

Figure 5-5:

Accessibility of substituted cysteines to aqueous environment. HEK293 cells transiently expressing wild-type or cysteine mutated OATP1B3 were incubated with the membrane-impermeable, cysteine-specific reagent maleimide-PEG_2-biotin for 1 hour at 4˚C, and biotinylated proteins were affinity purified with Neutravidin. Western blots were incubated with monoclonal anti-his antibodies. Shown are representative blots of at least three independent experiments.

5.4: Discussion

OATP1B3 is exclusively expressed on the basolateral membrane of hepatocytes under normal physiological conditions, and plays a significant role in the first-pass clearance of drugs and other xenobiotics from the portal blood. Recently, it has also been shown that OATP1B3 is expressed in multiple cancer cell lines and tissues, where it could be involved in the uptake of growth-stimulating hormone conjugates or in anticancer drugs. To identify potential adverse drug-drug interactions, or to design anticancer drugs that can take advantage of OATP1B3 expression in cancer cells, we need to have a better understanding of how OATP1B3 recognizes and transports substrates. Previous studies have indicated that the first transmembrane domain (TM1) of OATP1B3 may form part of the substrate translocation pathway. In this study, we used cysteine-scanning mutagenesis to demonstrate that TM1 and the first extracellular loop (ECL1) are important for substrate transport.

It is standard to use a cysteine-less template for all cysteine-scanning mutagenesis studies to prevent labeling of native cysteines. However, previous experiments have indicated that the cysteines present in OATPs are important for surface expression and/or function. A study on OATP2B1 showed that the 10 conserved cysteines in the 5th extracellular loop all formed disulfide bonds. Removing this extracellular loop or mutating any of the cysteines into alanine resulted in a protein with limited to no surface expression, indicating that the disulfide bonds formed by these cysteines may be essential for correct protein folding (Hanggi et al., 2006). Wild-type OATP1B3 contains 19 cysteine residues, including the ten highly conserved cysteines studied on OATP2B1. Three cysteines are predicted to be on the intracellular termini

(C24 and C691) or in a transmembrane domain (C101), and therefore are not expected to be accessible to the membrane impermeable reagents (black filled circles in Figure 5-1). The remaining six cysteines are predicted to be on extracellular loops, with 2 in the second extracellular loop, 1 in the large 5th extracellular loop (which has 11 total), and 3 in the final (6th) extracellular loop. Despite the presence of these cysteines, wild-type OATP1B3 was both unaffected by the MTSES pretreatment and not labeled by maleimide-PEG_2-biotin (Figures 5-4 and 5-5). This suggests that all cysteines found in extracellular loops are involved in disulfide bonds or are otherwise unable to react with the maleimide reagent. As it was non-reactive to the reagents used, we used the wild-type OATP1B3 as the template for all experiments.

We used a three-dimensional homology model of OATP1B3 (Gui and Hagenbuch, 2008) to identify thirty-four amino acids (M29 to S62) predicted to form the first transmembrane domain and extracellular loop of OATP1B3. Each amino acid was individually mutated to cysteine, with the exception of Q54, which could not be propagated in bacteria when mutated to cysteine. When transiently expressed in HEK-293 cells, each of the cysteine mutants was expressed on the cell membrane at levels comparable to wild-type OATP1B3 (Figure 5-2). This suggests that this region of OATP1B3 is not involved in transport to the cell surface or anchoring within the membrane bilayer. In addition, one can conclude that any alterations of substrate transport in the mutant proteins are caused by changes in the protein function, rather than by altered levels of surface expression.

Studies have suggested that OATP1B3 has distinct binding sites for different substrates. The affinity of OATP1B3 for estrone-3-sulfate is significantly increased in the presence of

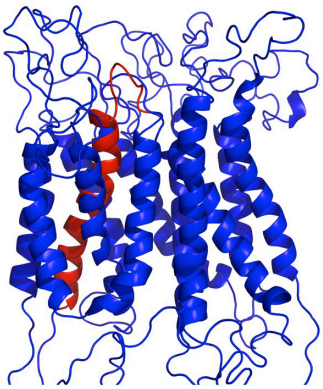
epigallocatechin-gallate (Roth et al., 2011b) or quercetin 3-*O*-α-L-arabinopyranosyl(12) α-L-rhamnopyranoside (Roth et al, 2011a), while neither compound affects the affinity for estradiol-17β-glucuronide. Similarly, rosiglitazone inhibits OATP1B3-mediated uptake of bromosulfophthalein while stimulating uptake of pravastatin (Bachmakov et al, 2008), and clotrimazone stimulates transport of estradiol-17β-glucuronide by OATP1B3, but has little effect on the uptake of estrone-3-sulfate by the same transporter (Gui et al., 2008). To account for the possibility that TM1 and ECL1 could play different roles in the transport of different substrates, function of the cysteine mutants was determined by measuring transport of two model substrates: estradiol-17β-glucuronide and estrone-3-sulfate. Thirteen of the thirty-three mutants had significantly reduced transport of at least one substrate. Mutants F30C and G45C had significantly reduced transport of estradiol-17β-glucuronide but not of estrone-3-sulfate; however, there was no statistical difference when comparing uptake between the two substrates. OATP1B3 transports estradiol-17β-glucuronide with a higher affinity (K_m = 5 μM to 25 μM) than it does estrone-3-sulfate (K_m = 60 μM to 100 μM), resulting in a much higher signal-to-noise ratio for estradiol-17β-glucuronide at the substrate concentration tested (0.1 μM). It is likely that the lower signal for estrone-3-sulfate uptake is responsible for the lack of significance seen with these two mutants, as it results in higher standard deviations. However, there were statistically significant differences between the uptake of estradiol-17β-glucuronide and estrone-3-sulfate by the mutants I46C and I47C. While estrone-3-sulfate uptake by I47C was significantly increased when compared to wild-type OATP1B3, uptake of estradiol-17β-glucuronide was unaffected by the mutation. The opposite results were caused by the 146C mutation: uptake of estrone-3-sulfate was unaffected or slightly inhibited, while transport of estradiol-17β-glucuronide was increased, although this did not reach statistical significance. In

general, MTSES pretreatment also had a greater inhibitory effect on estradiol-17β-glucuronide uptake than it did on estrone-3-sulfate uptake. Four mutants, A42C, G45C, I46C and I52C, had significantly greater loss of transport activity for estradiol-17β-glucuronide than for estrone-3-sulfate after incubation with MTSES. These data support earlier findings that the essential interactions between OATP1B3 and its substrates vary by substrate, and could indicate that the first transmembrane domain and extracellular loop of OATP1B3 have more significant interactions with estradiol-17β-glucuronide than with estrone-3-sulfate. Additional experiments are necessary to determine whether these substrate-specific results are caused by alterations in the substrate affinity (apparent K_m) or in the rate of translocation (V_{max}).

The three-dimensional homology model of OATP1B3 (Gui and Hagenbuch, 2008) is shown in Figure 5-6. Amino acids from M29 to S62, making up the first transmembrane domain and extracellular loop, are shown in green. This model suggests that the extracellular half of the transmembrane domain forms part of the central pore. However, the intracellular half is oriented towards the membrane bilayer, and may be partially covered by transmembrane domain IV. With the exception of F30C, which showed a 50% reduction in function, mutation of the amino acids predicted to be in the intracellular half of transmembrane domain I did not result in altered transport activity (M29 – S37). Similarly, none of these mutants had altered transport after preincubation with MTSES, nor were any labeled by maleimide-PEG_2-biotin. These results suggest that the intracellular half of TM1 do not form significant interactions with substrate, and that they are inaccessible from the extracellular medium, as predicted by the homology model. Transport was significantly reduced by cysteine mutagenesis of residues Y38, K41, G45 and K49 (Figure 5-3), while the intervening mutants retained function. These four residues make up

Figure 5-6:

A.



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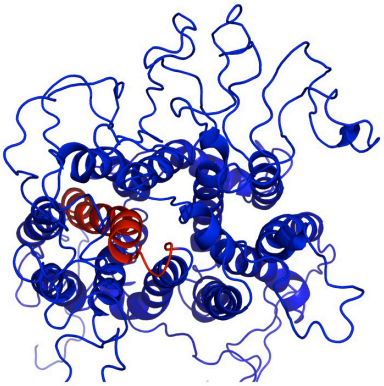

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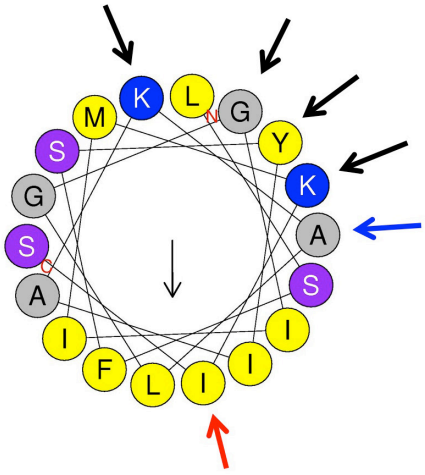

Figure 5-6:

Three-dimensional homology model of OATP1B3 (Gui and Hagenbuch, 2008). A side view is shown in (A) and the extracellular view is shown in (B). Residues that were individually mutated to cysteines are illustrated in green while the remaining residues are blue. The helical wheel analysis (C) was generated with HeliQuest (http://heliquest.ipmc.cnrs.fr/) (Gautier et al., 2008). The internal black arrow represents the total hydrophobic moment; external arrows are described in the text.

one face of a transmembrane domain α-helix as predicted by helical wheel analysis (Figure 5-6C, black arrows), and are predicted to face the central pore according to the three-dimensional homology model. These results suggest that the extracellular portion of transmembrane domain I forms part of the substrate translocation pathway, and that the residues facing the central pore make interactions that are important for substrate recognition and transport. While transport by the I50C construct is also drastically reduced, this residue is located on the opposite and hydrophobic face of the predicted α-helix (Figure 5-6C, red arrow). As this amino acid is near the extracellular end of the predicted transmembrane domain, it could be that the protein is coming out of the α-helix formation. Cysteine substitution at residues 41, 49, 50 and 56 caused near abrogation of transport activity; therefore, these mutants could not be tested for the effect of MTSES pre-treatment on function. MTSES pre-treatment did reduce transport by the Y38C and G45C proteins, as well as A42C, which share the same face of the α-helix (Figure 5-6C, blue arrow). Within this region, cysteines substituted for Y38, K41, and A42 were all labeled by maleimide-PEG_2-biotin, while the intervening residues could not be labeled. This also supports the homology model, which predicts that these amino acids line the central pore of the protein, and thus could be accessible from the extracellular media. Intervening residues, which were neither labeled by maleimide-PEG_2-biotin nor affected by MTSES incubation, are predicted to face away from the central pore and toward the other transmembrane domains, and therefore would be protected from the extracellular environment.

Within and near the predicted extracellular loop, the effect of cysteine substitution is more variable. Almost all cysteines inserted at positions 48 through 62 were labeled by maleimide-PEG_2-biotin (Figure 5-5), demonstrating that these residues are located near the

extracellular surface of the protein and therefore are accessible from the extracellular environment. Mutants I47C and K49C were labeled less strongly than the other mutant proteins, which suggests that their location at the predicted transition from the transmembrane domain to the extracellular loop could interfere with the interaction between the inserted cysteine and maleimide-PEG_2-biotin. Similarly, mutant S51C, which had reduced function after pre-incubation with MTSES, was not labeled by maleimide PEG_2-biotin. This could indicate that this residue faces another part of the OATP1B3 protein, with just enough intervening space for the small, negatively charged MTSES to interact with the inserted cysteine, while remaining inaccessible to the significantly larger, neutral maleimide compound. Numerous residues within the predicted extracellular loop appear to form significant interactions with substrates, as all cysteine substitutions from positions 53 to 60 caused significant reductions (>50%) in transport activity. The reduced function of these mutants makes it difficult to determine whether there is an additional effect of MTSES pre-treatment; however, only the R58C mutant had significantly reduced function after incubation with MTSES (Figure 5-4). The importance of the residues in ECL1 for full function suggests that the extracellular domains of OATP1B3 play a role in directing substrates into the central translocation pore.

The results of this study indicate that the extracellular half of TM1 of OATP1B3 forms part of the open pore of the protein, as indicated by the homology models. Amino acids that face the pore are important for function, and modifying these with MTSES causes a further decrease in function. Amino acids on the extracellular loop appear to play a varying role in directing substrate into the pore.

Chapter 6

Summary and Discussion

6.1: Significance

Organic anion transporting polypeptides (OATPs) are multispecific transporters that mediate the uptake of a broad range of drugs and other xenobiotics into cells. OATP1B1 and OATP1B3 are exclusively expressed at the basolateral membrane of hepatocytes under normal phsyiological conditions. With this localization, these proteins are involved in the first-pass clearance of drugs from the portal blood into the liver, and thus play an important role in determining the pharmacokinetic profile of many drug substrates. Polymorphisms in these transporters have been associated with altered pharmacokinetics of many drug substrates, such as statins and antidiabetic agents, demonstrating their importance to drug disposition. Drug-drug or drug-food interactions at OATPs can also alter the pharmacokinetic profiles of these or other substrates, leading to decreased efficacy or increased toxicity. In addition, OATP1B3, which transports numerous anticancer drugs, is expressed in a wide variety of cancer tissues and cell lines. Altering OATP1B3-mediated transport by co-administration with small molecules could be used to target anticancer drugs to the OATP1B3-expressing cancer tissues.

Due to the limited structural information available for OATPs, little is known about the mechanism of substrate binding and translocation. Subsequently, little is known about the mechanism of drug-drug interactions or drug-food interactions that occur at OATPs. Without a detailed understanding of these mechanisms, it will remain impossible to predict potentially

hazardous OATP-mediated drug-drug or drug-food interactions, or potentially beneficial interactions with small molecules. The studies presented in this dissertation were designed to address this lack of understanding by characterizing the mechanism of drug-drug interactions at OATP1B1 and OATP1B3. To do this, I formulated the central hypothesis that OATP1B1 and OATP1B3 have overlapping but distinct binding sites, which are affected in substrate-dependent ways. This hypothesis was based on recent studies that suggest these liver-specific OATPs contain multiple substrate binding sites. Experiments in our laboratory have demonstrated that mutations in OATPs have varied effects on the transport of different substrates, and that small molecules can stimulate, inhibit, or have no effect on OATP1B1 or OATP1B3 function, depending upon the substrate transported. This hypothesis was tested via two specific aims: the identification and characterization of plant compounds that have substrate-dependent effects on OATP1B-mediated transport, and the identification of regions of OATP1B3 that are involved in the recognition and translocation of individual model substrates.

6.2: Specific Aim 1

In the first specific aim, I screened a small library of plant extracts to identify plant compounds that have substrate-dependent effects on OATP-mediated transport. Initial screens identified two plant extracts that had similar substrate-dependent effects on OATP1B3-mediated transport: *Camellia sinensis* and *Rollinia emarginata* Schlecht (Annonaceae). These plant extracts were fractionated using a bioassay-guided approach, and the active compounds were identified. The substrate-dependent effects on OATP1B3-mediated transport were then

characterized by determining how the kinetic parameters of substrate transport were altered in the presence of the modifying compound.

The leaves and leaf buds of *Camellia sinensis* are used in making tea. Initial fractionation of this plant extract demonstrated that the substrate dependent stimulation of OATP1B3-mediated transport caused by the whole plant extract was due to the fraction containing catechins. The catechins found in tea include epicatechin (EC), epigallocatechin (EGC), epicatechin gallate (ECG) and epigallocatechin gallate (EGCG). Green tea is characterized by very high concentrations of catechins, and is a very commonly consumed beverage that has received much attention for its reputed health benefits. The most predominant catechin in green tea, EGCG, is widely considered responsible for many of the beneficial actions of green tea. Due to this, catechin and EGCG supplements are also widely used. The widespread consumption of green tea or catechin-containing supplements makes the possibility of drug-food interactions very high. Therefore, in addition to characterizing the substrate-dependent alterations of OATP1B3-mediated transport, I studied the effect of all four catechins on transport mediated by the four OATPs expressed at the apical membrane of enterocytes (OATP1A2 and OATP2B1) or the basolateral membrane of hepatocytes (OATP1B1, OATP1B3, and OATP2B1).

This study showed that, while EC and EGC have little effect on OATPs, the larger gallated catechins (ECG and EGCG) do significantly alter transport by all four OATPs investigated. Both compounds inhibit transport of estrone-3-sulfate by OATP1A2, OATP1B1, and OATP2B1 at physiologically relevant concentrations, indicating a significant risk of adverse drug-food interactions. EGCG was identified as the compound responsible for the substrate-

dependent effects on OATP1B3 caused by *Camellia sinensis* extract in the initial screening. EGCG stimulates transport of estrone-3-sulfate, inhibits transport of Fluo-3, and has no effect on transport of estradiol-17β-glucuronide. The stimulation of estrone-3-sulfate transport was shown to be caused by an increase in substrate affinity (reduced K_m), although the maximal rate of transport (V_{max}) was decreased for both estrone-3-sulfate and Fluo-3. Therefore, EGCG non-competitively inhibits transport of multiple substrates by OATP1B3, but only alters the affinity for certain substrates. This strongly indicates that estrone-3-sulfate does not share a single binding site on OATP1B3 with either estradiol-17β-glucuronide or Fluo-3.

As a final step in this study, I tested whether ECG or EGCG were substrates of the four OATPs expressed in the intestine or liver. Although there was no significant transport of either catechin by OATP1B1 or OATP2B1, both catechins were substrates of OATP1A2 and OATP1B3. The uptake was characterized, and it was found that the Michaelis-Menten constants (K_m) were within the range of physiological exposure, indicating that OATP-mediated uptake into enterocytes or hepatocytes could be responsible for the absorption and first-pass clearance of catechins. The identification of EGCG as a substrate of OATP1B3 further complicates the analysis of its ability to stimulate OATP1B3-mediated transport of certain substrates. It is not uncommon for a substrate to increase transport of another substrate through trans-stimulation. However, this type of stimulation is characterized by an increased maximal rate of transport. As EGCG caused a decrease in the maximal rate of transport, trans-stimulation cannot be responsible for the increased uptake of estrone-3-sulfate. Rather, it seems likely that the binding of EGCG to OATP1B3 causes a conformational change in the estrone-3-sulfate binding site that increases its affinity for estrone-3-sulfate, indicating that the EGCG and estrone-3-sulfate

binding sites are distinct. In addition, the inhibitory effect of EGCG on Fluo-3 uptake was found to be non-competitive, indicating that EGCG also has a distinct binding site from Fluo-3. This part of my first specific aim therefore strongly suggests that OATP1B3 has at least three distinct substrate binding sites, and that these sites may be altered in different ways by the same compound.

In the second part of my first specific aim, I identified the compounds in *Rollinia emarginata* Schlecht (Annonaceae) that are responsible for substrate-dependent effects on OATP1B1 or OATP1B3-mediated transport. Two compounds, ursolic acid and oleanolic acid, inhibited OATP1B3-mediated uptake of estrone-3-sulfate while having no effect on estradiol-17β-glucuronide uptake. Unfortunately, OATP1B3 has relatively low affinity for estrone-3-sulfate (K_m = 60 – 100 μM), and transport was not inhibited by either compound at substrate concentrations over 25 μM. This made it impossible to identify any effect on the kinetic characteristics of estrone-3-sulfate transport. As both compounds inhibit estrone-3-sulfate uptake only at low substrate concentrations, and have no effect on the maximal rate of transport, it seems likely that this inhibition is competitive. If this is the case, the identification of competitive inhibitors of OATP1B3-mediated estrone-3-sulfate transport that have no effect on estradiol-17β-glucuronide transport would be further evidence that the two substrates have distinct binding sites on OATP1B3. However, this is merely speculative, as both compounds were too weak of inhibitors to be fully characterized.

The other compound identified from *Rollinia emarginata* Schlecht (Annonaceae) had very similar effects to EGCG on OATP1B3-mediated transport. While uptake of estradiol-17β-

glucuronide was unaffected by EGCG, this transport was inhibited by quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (Compound 6). As was seen with EGCG inhibition of Fluo-3 transport, this inhibition was non-competitive, causing a decrease in the maximal rate of transport without affecting the substrate affinity. As was the case with EGCG, Compound 6 also stimulated estrone-3-sulfate uptake by OATP1B3 at low substrate concentrations due to increased substrate affinity. At high substrate concentrations, estrone-3-sulfate transport was non-competitively inhibited. Inclusion of 50 μM EGCG or Compound 6 increased the affinity for estrone-3-sulfate by five- to ten-fold. However, the maximal rate of transport was decreased to less than 20% in the presence of EGCG, while it was only reduced by half in the presence of Compound 6.

Both parts of my first specific aim clearly demonstrate that OATP1B3 has overlapping but distinct binding sites, which are affected in substrate-dependent ways. OATP1B3 transport of estradiol-17β-glucuronide is inhibited by ursolic acid, oleanolic acid, and 8-*trans-p*-coumaroyloxy-α-terpineol, while the uptake of estrone-3-sulfate is unaffected by these compounds, indicating that the binding sites on OATP1B3 for estradiol-17β-glucuronide and estrone-3-sulfate are distinct. OATP1B3 transport of estradiol-17β-glucuronide, estrone-3-sulfate, and Fluo-3 is altered in different ways by both Compound 6 and EGCG, which is also an OATP1B3 substrate, indicating the presence of at least three distinct binding sites on this transporter protein.

6.3: Specific Aim 2

As the first specific aim indicated that OATP1B3 does have distinct binding sites for estradiol-17β-glucuronide and estrone-3-sulfate, in the second specific aim I tested the hypothesis that the first transmembrane domain (TM) and extracellular loop (ECL) of OATP1B3 are involved in the binding and translocation of these two substrates. Thirty-three amino acids predicted to form this region of the protein were individually mutated to cysteines, and mutant proteins were transiently expressed in HEK293 cells. All of the mutant proteins were fully expressed on the surface of the cells, indicating that any changes in transport activity were due to alterations in substrate binding or translocation. Many of the mutant proteins showed altered functionality, due both to the mutation itself and to alteration of the inserted protein with the cysteine-specific reagent sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES).

Mutating two amino acids, I46 and I47, to cysteines resulted in substrate-dependent alterations in transporter function. Uptake of estrone-3-sulfate was significantly increased by the I47C mutation when compared to the wild-type protein; however, uptake of estradiol-17β-glucuronide remained unchanged. Whereas uptake of estrone-3-sulfate was the same when mediated by either wild-type OATP1B3 or the I46C mutant, uptake of estradiol-17β-glucuronide was increased. Similarly, after incubation with the cysteine-modifying MTSES, four of the mutants (A42C, G45C, I46C and I52C) had significantly greater loss of transport activity for estradiol-17β-glucuronide than for estrone-3-sulfate.

Analysis of the functional effect of mutations, both before and after MTSES modification, and of the extracellular accessibility of the mutations, indicates that the first transmembrane domain of OATP1B3 forms part of the external portion of the substrate translocation pore. The substrate-dependent effects of cysteine insertion suggest that the interactions between TM1 and estradiol-17β-glucuronide are different than the interactions between TM1 and estrone-3-sulfate.

6.4: Future Directions

The studies in my first specific aim demonstrated that OATP1B3 activity can be stimulated, inhibited, or unaffected by the same compound, depending on the substrate transported. Substrate-selective inhibition or stimulation has the potential to improve drug therapies without the possible attendant toxicity of altering transport of endogenous substrates. Identifying the mechanism of substrate-dependent stimulation is of particular interest, as this could be harnessed to increase OATP1B3-mediated uptake of anticancer drugs into OATP1B3-expressing cancer cells. Two approaches can be taken to investigate the mechanism of this stimulation: identification of the structural requirements for stimulating compounds, and determination of the regions of OATP1B3 that are responsible for interacting with the stimulators.

The first specific aim identified plant compounds as a rich source of OATP modulating compounds, and showed that the approach of bioassay guided fractionation is an efficient way to isolate stimulators of OATP1B3-mediated transport. By using this approach to screen the large

library of plant extracts at the Department of Medicinal Chemistry, University of Kansas, it should be possible to identify a significant number of compounds that have this stimulating effect. Preliminary studies have already identified a compound from *Asclepias syriaca*, 3-*O*-α-L-xylopyranosyl(1→2)β-D-galactopyranoside, that stimulates estrone-3-sulfate uptake by OATP1B3. As with EGCG and quercetin 3-*O*-α-L-arabinopyranosyl (1→2) α-L-rhamnopyranoside (Compound 6), uptake of estrone-3-sulfate is stimulated at low substrate concentrations due to an increased substrate affinity, whereas the maximal rate of transport is inhibited. The screening has also identified many structurally related compounds that do not stimulate OATP1B3-mediated uptake of estrone-3-sulfate. Once enough compounds have been identified, a comparative structural analysis of the stimulating and non-stimulating compounds will identify the structural components necessary for this stimulation.

A transporter-centered approach can also be used to identify the mechanism of OATP1B3 stimulation. As transport of estrone-3-sulfate by OATP1B1 is not stimulated by the OATP1B3-stimulating compounds, chimeras between these two closely related proteins can be used to identifying the regions of OATP1B3 that are involved in this stimulation. Our laboratory has previously generated chimeras that contain a single transmembrane domain of OATP1B1 inserted into OATP1B3, and chimeras that contain single transmembrane domains of OATP1B3 inserted into OATP1B1. By measuring estrone-3-sulfate uptake by these chimeric proteins in the absence and presence of OATP1B3-stimulating compounds, we may be able to identify regions of OATP1B3 that directly interact with the stimulators. Once regions have been defined, individual amino acids involved in the interaction can be identified through site-directed mutagenesis experiments.

My second specific aim identified the first transmembrane domain and extracellular loop as being involved in substrate-specific interactions with estradiol-17β-glucuronide and estrone-3-sulfate. This study resulted in many interesting observations that could be followed up with further studies. As mentioned, substitution of residues I46 and I47 with cysteines resulted in substrate-specific alterations, with I46C showing increased uptake of estradiol-17β-glucuronide and I47C having increased uptake of estrone-3-sulfate (Figure 5-3). Analysis of the kinetic parameters of substrate uptake by these two mutants could reveal whether this increased uptake is due to increased substrate affinity or increased turnover rate. If the effects are caused by altered substrate affinity, this would suggest that the two substrates bind to slightly different regions of TM1 in OATP1B3.

This study also confirmed previous research showing that positive charges located within the substrate translocation pore are important for OATP-mediated transport of model substrates. Uptake of both substrates was almost completely abrogated by substitution of cysteine for the positively charged K41. Mutating this residue to the neutral amino acids alanine or glutamine resulted in similarly non-functional proteins. However, mutating K41 to another positively charged amino acid, arginine, restored full transport activity, indicating that a positive charge in this location is essential for OATP1B3 function. Similar results were shown in a recent publication (Mandery et al., 2011). The importance of positive charges was also demonstrated by the R58C mutation. Substituting cysteine for the positively charged arginine caused a 50% reduction in function (Figure 5-3). Modifying this amino acid with the negatively charged MTSES resulted in a nearly non-functional protein, which retained only 15% of the already reduced function (Figure 5-4). However, modifying the R58C protein with the positively charged

reagent [2-(trimethylammonium)ethyl] methanethiosulfonate bromide (MTSET) increased the uptake of both substrates by 50 to 100% (data not shown). This demonstrates that positive charges both within the substrate translocation pore and in the extracellular domain are important for recognition and translocation of anionic model substrates. Again, determination of the kinetic characteristics of these mutants would help define the mechanism of these interactions. Additionally, it would be valuable to learn whether the importance of these positive charges is restricted to the transport of anionic substrates, or whether the same results would be seen when measuring uptake of neutral or cationic OATP1B3 substrates.

Finally, many of the amino acids found to be important for substrate binding and/or translocation were labeled by maleimide-PEG_2-biotin (Figure 5-5). If these amino acids do interact with the substrates, incubating the mutant proteins with substrate should protect the inserted cysteine from maleimide-PEG_2-biotin labeling. Preliminary experiments suggest that this does occur for the mutants A42C, I46C and M48C. However, this only occurs after a pre-incubation with estradiol-17β-glucuronide, not after incubation with estrone-3-sulfate. Confirmation of these results would indicate that binding of estradiol-17β-glucuronide to OATP1B3 either directly covers this region of the first transmembrane domain, or causes a conformational change that prevents maleimide-PEG_2-biotin from interacting with the inserted cysteines. As estrone-3-sulfate pre-incubation does not produce the same effect, this would be evidence that the two substrates either interact with different protein regions, or result in different conformational changes of the protein.

Further studies as discussed above are essential to determine the mechanism of the substrate-dependent effects on OATP1B3-mediated transport. This increased understanding will improve our ability to predict potentially hazardous OATP-mediated drug-drug or drug-food interactions prior to the occurrence of adverse events. Additionally this knowledge may allow for the manipulation of drug-drug or drug-small molecule interactions in a way that would improve drug pharmacokinetics or target anticancer drugs to cancer cells.

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Appendices:

Appendix I: Citations of Publication

Roth M, Obaidat A, Hagenbuch B. OATPs, OATs and OCTs: The organic anion and cation transporters of the *SLCO* and *SLC22A* gene superfamilies. [Submitted]

Roth M*, Araya J*, Timmermann BN, Hagenbuch B (2011) Isolation of modulators of the liver specific Organic Anion Transporting Polypeptides (OATPs) 1B1 and 1B3 from *Rollinia emarginata* Schlecht (Annonaceae). *J Pharmacol Exp Ther* Aug 16 [Epub ahead of print]

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