TARGETING ORGANIC ANION TRANSPORTING POLYPEPTIDES IN CANCER TO IMPROVE DIAGNOSTICS AND THERAPY

By

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Abstract

Organic Anion Transporting Polypeptides (OATPs) are multispecific transport proteins that mediate the uptake of numerous endogenous and exogenous compounds into cells. Recently, OATPs have been shown to have altered expression in cancer tissue compared to their normal expression profiles. It has been proposed that OATPs can be targeted to improve cancer therapeutics. Therefore, I tested the hypothesis that expression of OATPs in cancer combined with their ability to transport cytotoxic anticancer drugs makes them potential targets for improving cancer diagnosis and therapy.

The hypothesis was tested via the following specific aims: 1) to identify and characterize OATP expression in cancer, 2) to identify novel anticancer drug substrates of OATPs, and 3) to identify novel cytotoxic compounds from plant extracts that are substrates of OATPs and can be developed into anticancer drugs that target OATP-expressing cancers.

In the first specific aim, OATPs expressed in pancreatic cancer were identified by immunohistochemical staining of pancreatic cancer tissue specimens. Completion of this specific aim identified four major OATPs expressed in pancreatic adenocarcinomas. Additionally, OATP1B3 expression was observed to be highest in low stage adenocarcinoma and absent in metastatic tissue. These results demonstrate that OATP1B3 may serve as a diagnostic marker and/or therapeutic target in early stage adenocarcinomas.

In specific aim two, novel anticancer drug substrates of OATP1B3 were identified by screening the NCI/DTP oncology drug set containing all of the FDA approved chemotherapy drugs. In this study, I determined the effect of the anticancer drugs on transport and cell viability of OATP1B3-expressing cells. Finally, I demonstrated that the anticancer drugs etoposide, oxaliplatin and plicamycin are substrates of OATP1B3. These results suggest that the mentioned cytotoxic anticancer drugs could potentially be used to treat OATP1B3-expressing cancers.

In the last specific aim, Kansas plant extracts were screened using bioassay guided fractionation and cell viability assays to isolate novel cytotoxic compounds that are substrates of OATP1B3. Given that these novel plant compounds are cytotoxic and are also transported by OATP1B3 suggests that they can be used for lead optimization studies to develop new anticancer drug entities.

This dissertation demonstrates that OATP1B3 is a potential target for mechanisms of OATP-mediated anticancer therapy. Ultimately, this knowledge can be used to utilize OATP1B3 expression in cancer as a diagnostic marker, as well as a target for cytotoxic anticancer drug therapies.

Dedication

I would like to dedicate the work herein to my parents, Naser and Iris Obaidat.

Acknowledgments

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List of Abbreviations

ABC:	ATP-binding cassette
ADME:	Absorption, distribution, metabolism and excretion
ATP:	Adenosine triphosphate
BCRP:	Breast cancer resistance protein (ABCG2)
CCK8:	Cholecystokinin-octapeptide
CHO:	Chinese hamster ovary
DHEAS:	Dehydroepiandosterone sulfate
DMSO:	Dimethyl sulfoxide
ECL:	Extracellular loop
EGCG:	Epigallocatechin gallate
HEPES:	4-(2-hydroxyethyl)-1-peperazineethanesulfonic acid
HCC:	Hepatocellular carcinoma
HTS:	High throughput screening
IC_{50}:	Half maximal inhibitory concentration
K_m:	Michaelis-Menten constant
MDR1:	Multidrug resistance protein (P-glycoprotein)
mRNA:	Messenger RNA
MRP:	Multidrug resistance associated protein
OATP/Oatp:	Organic anion transporting polypeptide
PBS:	Phosphate buffered saline
P-gp:	P-glycoprotein (MDR1)
PXR:	Pregnane X receptor
RT-PCR:	Real time polymerase chain reaction
SD:	Standard deviation

SNP: Single nucleotide polymorphism

SLC: Solute carrier

SLCO: Solute carrier family of the OATPs

TM: Transmembrane domain

V_{max}: Maximal rate of transport

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Chapter 1

Background and Significance

1.1 Overview of Transport and Drug Disposition

All human cells are enclosed by plasma membranes known as the phospholipid bilayer. The phospholipid bilayer is comprised of phospholipids, cholesterol and other molecules that make up an amphipathic structure consisting of a hydrophobic core and hydrophilic surfaces that interact with the extracellular and intracellular environments. The hydrophobic component of biological membranes acts as a barrier for certain molecules. Whereas small non-polar molecules are able to freely diffuse through the membrane, large polar molecules require membrane transporters to move them into cells. Given that most drugs are large polar compounds, they require membrane transporters for their delivery into cells where they can exert their therapeutic mechanisms.

The field of pharmacology is comprised of mainly two modules, pharmacodynamics and pharmacokinetics. Pharmacodynamics describes the effect of drugs on the body while pharmacokinetics describes the effect of the body on drugs. Pharmacokinetics includes four main processes: absorption, distribution, metabolism and excretion, often referred to by the acronym ADME. Membrane transporters play a significant role in all four of these processes. They are required for the absorption of orally applied drugs into enterocytes, for the distribution of drugs into different organs for subsequent metabolism, and for the excretion of drugs from the body. Therefore, drug transporters are crucial for the effectiveness and therapeutic outcomes of drugs. In contrast, transporters can also be responsible for the ineffectiveness of certain chemical entities because they are eliminated from the circulation and excreted too fast. Thus, for drug development, transporters need to be considered for both, efficient delivery and

undesired fast elimination. Given the multispecificity of many drug transporters it is also important to consider the potential for drug-drug interactions at the transporter level. For example inhibition of transport-mediated drug uptake and inhibition of drug excretion from the body can lead to undesirable side effects and toxicities in certain organs.

1.2 Introduction to Membrane Transporters

Multispecific drug transporters can be divided into two main classes, solute carriers (SLC) and ATP-binding cassette (ABC) transporters. SLC transporters are mainly uptake transporters and mediate transport into cells, whereas ABC transporters are considered efflux transporters and pump substrates out of cells. Within the SLC superfamily there are the organic anion transporters (OATs), the organic cation transporters (OCTs), the organic anion transporting polypeptides (OATPs) and the nucleoside transporters (ENTs, CNTs) that have been shown to transport anticancer drugs beside their specific endogenous substrates. Several of the ABC transporters are well characterized with respect to their involvement in multidrug resistance and cancer. Multidrug resistance protein (MDR, also known as P-gp), multidrug resistance-associated protein (MRPs) and breast cancer resistance protein (BCRP) are examples of proteins that belong to the ABC class of transporters. This dissertation focuses on organic anion transporting polypeptides and their potential role in cancer therapy. A major part of the introduction to this dissertation is taken from the recent review published in the 52nd edition of Annual Reviews in Pharmacology and Toxicology (Obaidat et al., 2012).

1.3 The Organic Anion Transporting Polypeptide (OATP) Superfamily

OATPs are multispecific transport proteins which mean that they can transport a wide range of structurally diverse compounds. They are expressed in a wide range of tissues in the body and are responsible for the Na+-independent uptake of large amphipathic organic anions into cells. Generally, OATP substrates are anions with molecular weights greater than 350 Daltons. However, OATP substrates are not limited to anions; they transport cationic and neutral compounds as well.

1.3.1 Nomenclature and Structure

OATPs belong to the superfamily of solute carrier transporters and are classified within the solute carrier of the OATPs (*SLCO*) gene family (Hagenbuch and Meier, 2004). There are 11 known human OATPs that are divided into six families, on the basis of a 40% amino acid sequence identity. Families are further divided into subfamilies, on the basis of a 60% amino acid sequence identity. The OATP1 family remains the best characterized among the OATP families. This family includes OATP1A2, OATP1B1, OATP1B3, and OATP1C1.The OATP2 family contains two members, OATP2A1 and OATP2B1, both of which have a narrow substrate specificity compared with other OATPs. OATP3A1, OATP5A1, and OATP6A1 are the only members in the OATP3, OATP5, and OATP6 families, respectively. The OATP4 family is composed of both OATP4A1 and OATP4C1. Orthologs of human OATPs are present in other species (referred to as Oatps). Owing to species divergence and gene duplication events, more than one rodent ortholog can correspond to a single human OATP or vice versa. The phylogenetic tree of all known human OATPs along with their rodent homologs is shown in Figure 1-1.

OATPs are predicted to have 12 transmembrane domains with intracellular amino and carboxy termini (Figure 1-2). Several structural features are important for membrane localization and transport function. For instance, many OATPs contain a PDZ consensus sequence that is thought to be important for membrane anchoring (Wang et al., 2005). The fifth extracellular loop is rich with conserved cysteines, which are thought to form sulfhydryl bonds that may be important for surface expression (Hanggi et al., 2006). Furthermore, recent structure-function studies have identified amino acids within transmembrane domain 10 of OATP1B1 and OATP1B3 that are important in substrate recognition and translocation across the membrane (Gui and Hagenbuch, 2008; Gui and Hagenbuch, 2009; Miyagawa et al., 2009).

Figure 1-1:

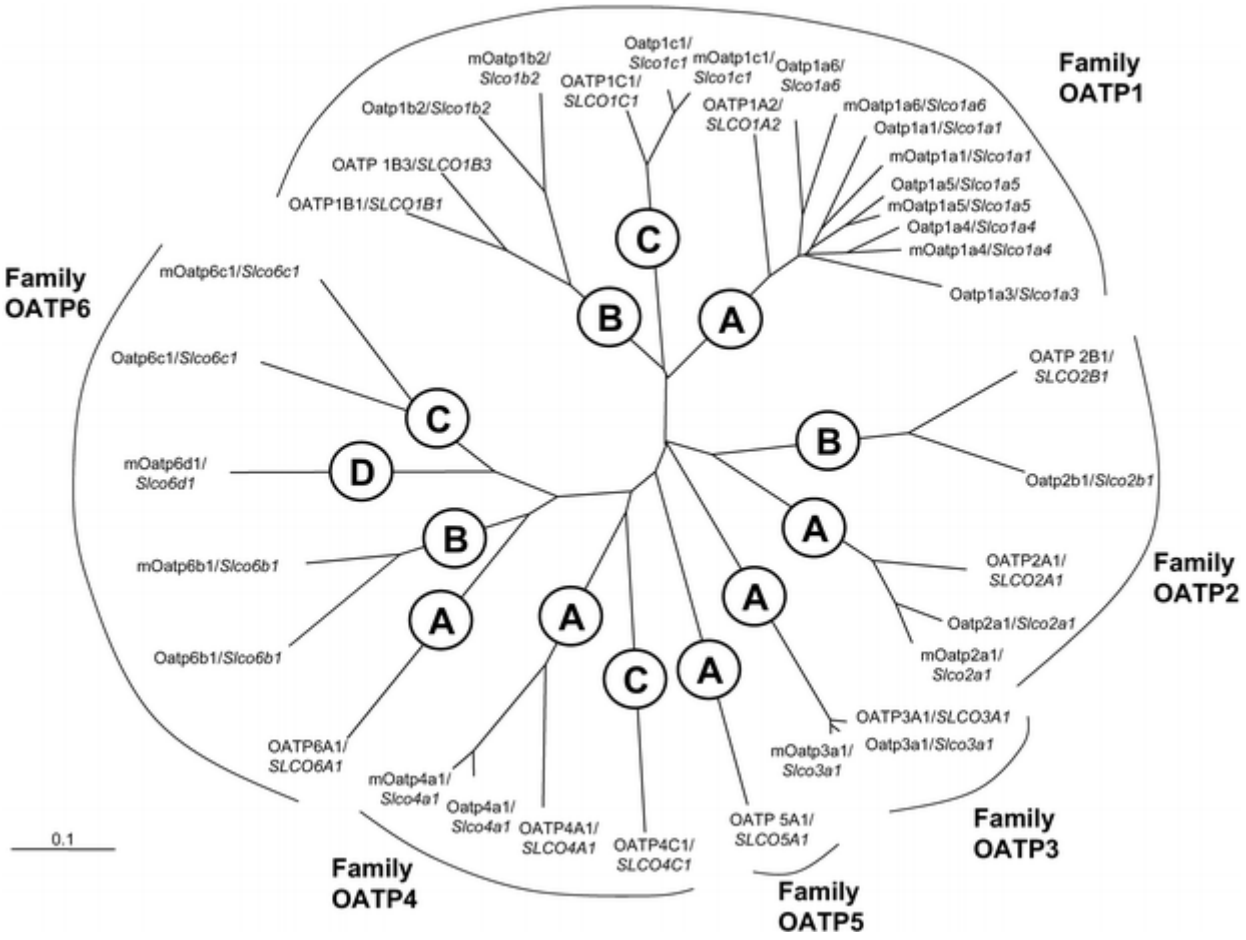

Family OATP1
Family OATP2
Family OATP3
Family OATP4
Family OATP5
Family OATP6
mOatp1b2/ Slco1b2
Oatp1c1/ Slco1c1
mOatp1c1/ Slco1c1
Oatp1a6/ Slco1a6
OATP1C1/ SLCO1C1
OATP1A2/ SLCO1A2
mOatp1a6/Slco1a6
Oatp1a1/Slco1a1
Oatp1b2/Slco1b2
OATP 1B3/SLCO1B3
mOatp1a1/Slco1a1
OATP1B1/SLCO1B1
Oatp1a5/Slco1a5
mOatp1a5/Slco1a5
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
OATP6A1/ SLCO6A1
mOatp3a1/ Slco3a1
Oatp3a1/Slco3a1
mOatp4a1/ Slco4a1
Oatp4a1/ Slco4a1
OATP4A1/ SLCO4A1
OATP4C1/ SLCO4C1
OATP 5A1/ SLCO5A1
0.1
A
B
C
D

Figure 1-1:

Phylogenetic tree of the eleven human OATPs along with their rodent homologs. OATPs with amino acid sequence identities ≥ 40% belong to the same OATP family (e.g. OATP1, OATP2, OATP3, OATP4, OATP5, OATP6). OATPs with amino acid sequence identities ≥ 60% are grouped into subfamilies and represented with a letter after the family number (e.g. OATP1A, OATP1B, OATP1C, etc.) Individual OATPs are sequentially numbered according to the chronology of their identification. The Oatp (rodent) and OATP (human) symbols represent protein, whereas Slco (rodent) and SLCO (human) represent gene symbols. Reproduced with kind permission from Springer Science+Business Media: (Hagenbuch and Meier, 2004).

Figure 1-2:

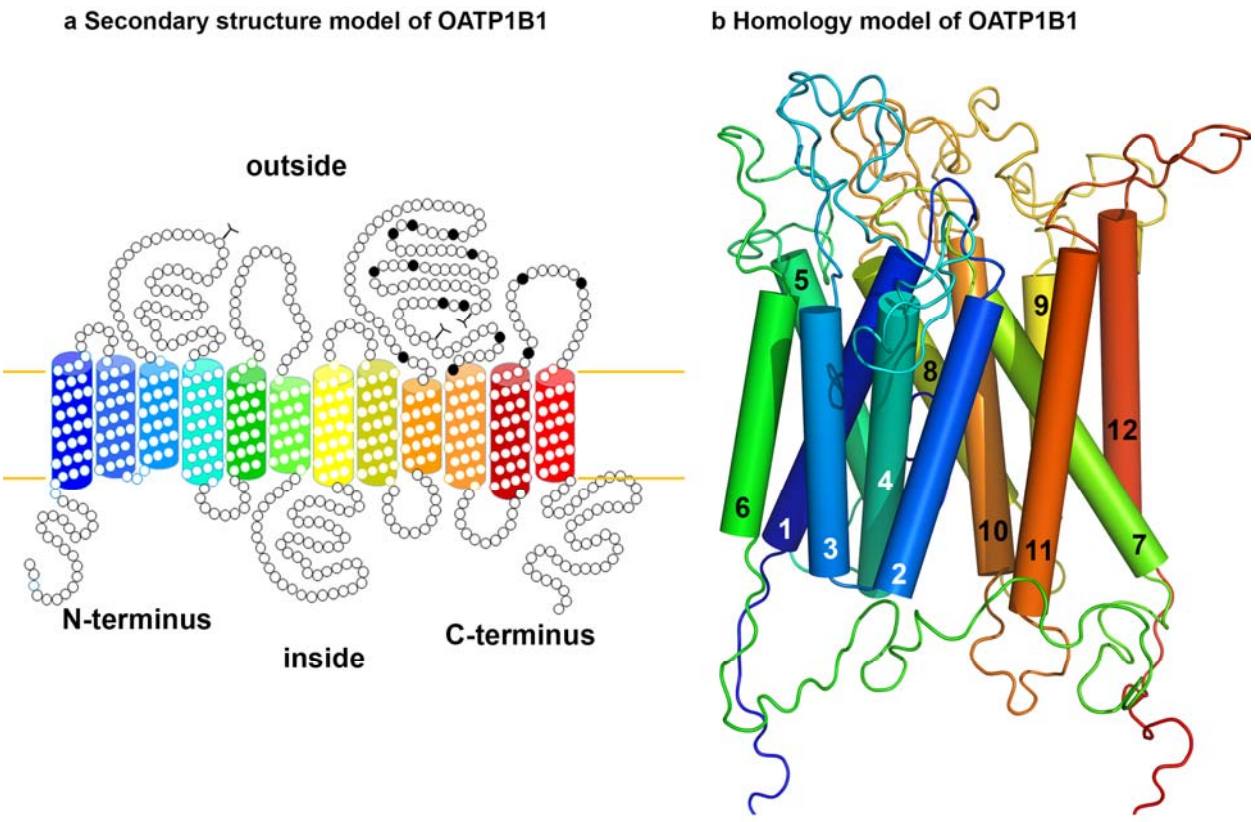

a Secondary structure model of OATP1B1
outside
N-terminus
inside
C-terminus
b Homology model of OATP1B1
1
2
3
4
5
6
7
8
9
10
11
12

Figure 1-2:

Structure of OATPs. Predicted 12 transmembrane domain (a) secondary structure and (b) homology model of OATP1B1. (a) Putative transmembrane domains were predicted based on amino acid hydrophobicity. The highly conserved cysteine residues in the loop between transmembrane domains 9 and 10 are indicated by black circles. At the C-terminal end, several OATPs have a PDZ consensus sequence. (b) The homology model for OATP1B1 was constructed on the basis of the crystal structure of the bacterial multidrug transporter EmrD.

1.3.2 Distribution of OATPs in Normal Human Tissue

Some OATPs are expressed in multiple tissues, whereas the expression of other OATPs is restricted to a single tissue. In the OATP1 family, expression of OATP1A2 mRNA is highest in the brain, followed by expression in the kidney, liver, lung, testis, and placenta, according to Northern blot analysis (Kullak-Ublick et al., 1995; Steckelbroeck et al., 2004). OATP1A2 expression was confirmed at the protein level in the blood-brain barrier (Gao et al., 2000; Lee et al., 2005); at the apical membrane of distal nephrons (Lee et al., 2005); at the apical membrane of enterocytes, where it is thought to be critical in the absorption of numerous xenobiotics (Glaeser et al., 2007); and at the apical membrane of cholangiocytes, which make up the bile duct epithelium (Lee et al., 2005). OATP1B1 and OATP1B3 are examples of tissue-specific OATPs. Expression of OATP1B1 was shown in adult and fetal liver by Northern blot analysis. This expression was confirmed at the protein level and localized to the basolateral membrane of hepatocytes throughout the liver lobule. Similarly, OATP1B3 is expressed exclusively in the liver at the basolateral membrane of hepatocytes; however, expression was much stronger in the pericentral region compared with the periportal region (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000b; Konig et al., 2000a; Abe et al., 2001). A recent study based on real-time polymerase chain reaction (RT-PCR) analysis described OATP1B3 mRNA expression in the retina; however, protein expression has not been confirmed (Okabe et al., 2008). OATP1C1 mRNA expression is highest in the brain and testis, and, because of its high affinity for T4 and reverse T3 (in the nanomolar range), OATP1C1 is thought to be a crucial thyroid hormone transporter (Pizzagalli et al., 2002; Hagenbuch, 2007; Heuer and Visser, 2009). In addition, OATP1C1 protein was localized to the basolateral membrane of the choroid plexus epithelium (Roberts et al., 2008), to the basolateral membrane of the pigmented ciliary body epithelium (Gao et al., 2005), and in leydig cells of the testis (Pizzagalli et al., 2002).

OATP2A1, which is also known as the prostaglandin transporter, is thought to be ubiquitously expressed. OATP2A1 mRNA was detected in brain, colon, heart, liver, kidney, ovary, lung, pancreas, prostate, skeletal muscle, spleen, and small intestine (Schuster, 2002). Thus far, expression of OATP2A1 at the protein level has been demonstrated in neurons in the frontal gyrus of the brain (Choi et al., 2008), in the pyloric glands of the antrum and parietal cells in the gastrointestinal tract (Mandery et al., 2010), and in the luminal and glandular epithelium of the endometrium (Kang et al., 2005). OATP2B1, the second member of the OATP2 family, is expressed in several different tissues in the body, with the highest transcript levels in the liver (Tamai et al., 2000; Kullak-Ublick et al., 2001). OATP2B1 protein expression was confirmed at the basolateral membrane of hepatocytes (Kullak-Ublick et al., 2001), at the apical membrane of enterocytes (Kobayashi et al., 2003), at the endothelium of the blood-brain barrier (Bronger et al., 2005), at the endothelial cells of the heart (Grube et al., 2006), at the myoepithelium of mammary ducts (Pizzagalli et al., 2003), and in the placenta (St-Pierre et al., 2002).

OATP3A1 is the most highly conserved OATP among all species and has two different splice variants in humans (Huber et al., 2007). In general, OATP3A1 is considered to be widely expressed and was shown to be expressed at the mRNA level in testis, brain, heart, lung, spleen, peripheral blood leukocytes, and thyroid (Adachi et al., 2003; Huber et al., 2007). At the protein level, the two splice variants were localized to different cell types or cellular membranes in various tissues. OATP3A1_v1 was localized to the germ cells of the testes, to the neuroglial cells of the frontal cortex, and to the basolateral membrane of the choroid plexus. In contrast, expression of OATP3A1_v2 was shown in Sertoli cells in the testes, at the apical membrane of the choroid plexus, and in cell bodies of the frontal cortex neurons (Huber et al., 2007). Recently, OATP3A1 protein expression was demonstrated in epithelial cells of the lactiferous

ducts in normal breast tissue (Kindla et al., 2011a). OATP4A1 is another ubiquitously expressed OATP, with highest mRNA levels in the heart and placenta, followed by levels in the lung, liver, skeletal muscle, kidney, and pancreas (Tamai et al., 2000; Fujiwara et al., 2001). Thus far, OATP4A1 protein expression has been confirmed only at the apical membrane of syncytiotrophoblasts in the placenta (Sato et al., 2003). The other OATP4 family member, OATP4C1, is expressed only in the kidney, as shown by Northern blot analysis (Mikkaichi et al., 2004), and thus is considered a kidney-specific OATP. Little is known about OATP5A1 and OATP6A1. OATP5A1 protein was recently reported to be expressed at the plasma membrane of the epithelial cells that line the lactiferous ducts in normal breast tissue (Kindla et al., 2011a), whereas OATP6A1 mRNA expression has been detected in the testes, with low levels in the spleen, brain, fetal brain, and placenta (Suzuki et al., 2003; Lee et al., 2004).

1.3.3 Expression Profile of OATPs in Cancer Tissue

Aside from their normal expression profiles, OATPs have altered expression in cancer tissues. OATP1A2 expression has been identified in gliomas, colon polyps and tumors, and cancers of the breast and bone. A study of human gliomas by RT-PCR showed OATP1A2 expression in different histological subtypes. Through the use of immunofluorescence microscopy, OATP1A2 was localized in the luminal membrane of the blood-brain barrier endothelium and in the blood-tumor barrier but not in the glioma cells (Bronger et al., 2005). Through the use of RT-PCR, OATP1A2 expression was detected in healthy colon tissue; however, expression was decreased in polyps and in colon cancer tissue (Ballestero et al., 2006). In breast cancer cell lines, RT-PCR analysis showed that expression of OATP1A2 was highest in T47-D and ZR-75-1 cells and low in MCF-7, MDA-MB-231, and MDA-MB-468 cells. OATP1A2 expression was confirmed in tissue obtained from patients with breast cancer and was localized to the cell membrane and cytoplasm of breast carcinoma cells. However,

OATP1A2 expression was not observed in non-neoplastic epithelium, stroma, and adipose tissue surrounding the carcinoma (Miki et al., 2006). These results were confirmed through the use of RT-PCR (Meyer zu Schwabedissen et al., 2008; Banerjee et al., 2012). OATP1A2 transcript levels were significantly higher in malignant breast tissue than they were in adjacent nonmalignant breast tissue, and transcripts of OATP1A2 were highest in stage I and stage IIA breast cancers (Meyer zu Schwabedissen et al., 2008). Immunofluorescence analysis confirmed OATP1A2 protein expression and demonstrated that it was restricted to the malignant cells of the breast tissue samples (Meyer zu Schwabedissen et al., 2008). In contrast, Wlcek et al. (Wlcek et al., 2008) were unable to detect significant mRNA levels of OATP1A2 in the four breast cancer cell lines MCF-7, MDA-MB-231, ZR-75-1, and MCF-10A, or in breast cancer tissue. These discrepancies might arise from differences found in cell lines cultured in different laboratories (Hayeshi et al., 2008); therefore, such results must be interpreted cautiously if the exact experimental conditions are not known. OATP1A2 transcripts were detected both in bone metastases from primary kidney cancer and in the malignant osteosarcoma cell lines HOS and MG-63 (Liedauer et al., 2009).

In general, the expression of OATP1B1 and OATP1B3, both specific to the liver, tends to be reduced in hepatocellular carcinomas (HCCs). OATP1B1 and OATP1B3 mRNA was undetectable or reduced in the Hep3B and HepG2 cell lines (Libra et al., 2006; Monks et al., 2007), confirming the previously reported reduced expression in HepG2 and PLC cell lines as well as in HCC tissue samples at the protein level (Cui et al., 2003; Zollner et al., 2005). Vavricka et al. (Vavricka et al., 2004) also reported reduced expression of OATP1B3 in 60% of HCC tissues compared with normal surrounding tissue. However, OATP1B1 levels were not significantly different in HCC samples compared with normal liver samples in their study. The expression of OATP1B1 and OATP1B3 in different benign liver tumors was investigated by Vander Borght and colleagues (Vander Borght et al., 2005). They showed reduced expression

of both OATPs in hepatocellular adenomas and a strong diffuse expression of both OATPs in focal nodular hyperplasia. Recently, Tsuboyama et al. (Tsuboyama et al., 2010) supported the general trend of reduced OATP1B1 and OATP1B3 expression in HCC tissue samples. In conclusion, the downregulation of OATP1B1 and OATP1B3 in HCC resembles the downregulation observed in primary cultured hepatocytes (Jigorel et al., 2005) and could be the result of dedifferentiation of the HCC cells.

Expression of the normally liver-specific OATP1B1 and OATP1B3 has also been identified in cancers of many different tissues (Abe et al., 2001). Overall, OATP1B3 is upregulated in a wide range of cancer types. Northern blot analysis showed that OATP1B3 is expressed in different gastrointestinal cancer cell lines and cancers, including the gastric cancer cell line KatoIII; the colon cancer cell lines DLD-1, MIP-101, Clone A, and CX-1; the pancreatic cancer cell lines MIA-Paca2, BXPC-1, PK-8, PK-9, and PK-45P; and the gallbladder cancer cell lines HuCCT-1, OcuchLM1, and TFK-1. Weak expression levels were also seen in the lung cancer cell line A549 and the glioblastoma cell line A172. Moreover, immunohistochemical staining of OATP1B3 was detected in gastric cancer tissue, pancreatic cancer tissue, colon cancer tissue, and a colon cancer metastatic to a lymph node (Abe et al., 2001). A recent study showed OATP1B1 expression in the breast cancer cell line MDA-MB-231 via western blot (Banerjee et al., 2012). OATP1B1 was increased in colon polyps and in colon cancer tissue at the mRNA level as compared with normal colon tissue (Ballestero et al., 2006). However, the same study could not demonstrate significant differences in OATP1B3 mRNA expression between healthy and colon cancer tissue (Ballestero et al., 2006). OATP1B3 expression was markedly increased in colorectal adenocarcinoma tissues with obvious staining in the cytoplasm as opposed to membranous expression in normal liver (Lee et al., 2008). Analysis of OATP1B3 expression across different colorectal tumor stages showed that it was highest in earlier-stage and lower-grade tumors, suggesting that OATP1B3 expression might be indicative of clinical

outcome (Lockhart et al., 2008). In non-small-cell lung cancer, OATP1B3 mRNA expression was significantly increased as compared with the nonmalignant surrounding tissue (Monks et al., 2007). OATP1B3 transcript levels were observed in prostate cancer tissue through the use of RT-PCR (Wright et al., 2011). Additionally, OATP1B3 expression was confirmed at the protein level in prostate tumor tissue but not confirmed in normal prostate or benign prostatic hyperplasia (Hamada et al., 2008). Muto et al. (Muto et al., 2007) detected OATP1B3 by using immunohistochemistry in cells of invasive ductal breast carcinoma and suggested that OATP1B3 expression could be used as a prognostic factor in breast cancer.

Thus far, the only report to show expression of OATP1C1 in cancer demonstrated OATP1C1 mRNA in several samples from osteosarcomas, in a sample from a kidney cancer metastasis, and in several specimens from aneurysmal bone cysts, which had the highest levels (Liedauer et al., 2009).

OATP2A1 mRNA was detected at high levels in bone metastases from kidney cancer (Liedauer et al., 2009); in breast cancer; and in the breast cancer cell lines MCF-7, MDA-MB-231, and ZR-75-1 (Wlcek et al., 2008). OATP2A1 mRNA expression was generally higher in malignant breast tissue compared with adjacent nonmalignant breast tissue, but the difference did not reach statistical significance (Wlcek et al., 2008). Holla and colleagues (Holla et al., 2008) showed a trend for decreased OATP2A1 expression in colorectal tumor specimens as well as in stomach, ovary, lung, and kidney tumors. They were not able to detect OATP2A1 in several colorectal cancer cell lines, including LS-174T, HCT-116, HT-29, SW-620, SW-480, HCT-15, and HCA-7, although the colorectal cancer cell line LoVo showed high expression levels of OATP2A1 mRNA and protein (Holla et al., 2008). OATP2A1 was also detected at the protein level in hepatocellular carcinoma, in cholangiocellular carcinoma, and in liver metastases from colon tumors (Wlcek et al., 2011).

OATP2B1 mRNA expression was identified in the colon adenocarcinoma cell line CX-1 (Tamai et al., 2000) and was higher in bone cysts than in osteosarcoma tissues (Liedauer et al., 2009). With respect to breast cancer, OATP2B1 expression was shown in both normal and breast tumor specimens, and expression increased with increased tumor grade (Al Sarakbi et al., 2006). This has also been studied by Wlcek et al. (Wlcek et al., 2008), who detected higher expression levels of OATP2B1 in nonmalignant specimens than in malignant breast tumors. OATP2B1 expression was also identified in human gliomas, where it was localized to endothelial cells at the blood-brain barrier and blood-tumor barrier (Bronger et al., 2005).

RT-PCR analysis showed that OATP3A1 expression was significantly higher in aneurismal bone cysts than in osteosarcomas. OATP3A1 transcripts were found (*a*) in the nonmalignant human osteoblast-like cells and bone marrow stromal cells derived from normal bone marrow and (*b*) in the osteosarcoma cell lines HOS and MG-63 (Liedauer et al., 2009). OATP3A1 expression was identified in a variety of additional cancer cell lines, including breast carcinoma (GI-101), lung carcinoma (LX-1 and GI-117), colon adenocarcinoma (CX-1 and GI-112), ovarian carcinoma (GI-102), and pancreatic adenocarcinoma (GI-103) (Tamai et al., 2000). Through RT-PCR, two independent groups showed OATP3A1 expression in the breast cancer cell line T-47D (Pizzagalli et al., 2003; Nozawa et al., 2004). OATP3A1 expression was also detected in the breast cancer cell line MCF-7 (Nozawa et al., 2005b), and recently it was detected in the membrane and cytoplasm of malignant breast tumor specimens (Kindla et al., 2011a).

OATP4A1 has an expression pattern similar to that of OATP3A1 in various breast carcinoma, lung carcinoma, colon adenocarcinoma, ovarian carcinoma, and pancreatic carcinoma cell lines (Tamai et al., 2000), as well as in the breast cancer cell lines T-47D and MCF-7 (Pizzagalli et al., 2003; Nozawa et al., 2004). OATP4A1 expression is higher in bone

cysts than in osteosarcoma tissues, with significantly higher expression in the malignant osteosarcoma cell lines HOS and MG63 as compared with the nonmalignant human osteoblast-like cells and bone marrow stromal cells (Liedauer et al., 2009). The expression of OATP4A1 was detected in normal and tumorous breast tissue (Wlcek et al., 2008). In the same study, expression of OATP4C1 and the poorly characterized OATP5A1 was also revealed in normal and cancerous breast tissue (Wlcek et al., 2008). Recently, the expression of OATP5A1 was confirmed at the membrane and in the cytoplasm of malignant breast tumor specimens (Kindla et al., 2011a). Expression of the presumably gonad-specific OATP6A1 was shown in lung cancer cell lines, lung cancer, bladder cancer, and esophageal cancer tissues (Lee et al., 2004). The tissue expression profile of all 11 human OATPs is summarized in Table 1-1.

A few studies have suggested that OATP expression in cancer could be predictive of patient survival or the success of hormone therapy (Muto et al., 2007; Hamada et al., 2008; Lockhart et al., 2008). However, because of the many inconsistent reports, it is too early to propose that the presence or absence of a certain OATP in a given cancer is predictive. Additional, larger population-based studies are required to confirm the predictive value of OATPs in different cancer types.

1.3.4 Substrate Specificity

OATPs transport a wide range of structurally unrelated compounds, including numerous endo- and xenobiotics (Hagenbuch and Gui, 2008; Konig, 2011). Their substrates are generally organic anions, but OATPs can also transport cations and neutral compounds (van Montfoort et al., 2003). Among their endogenous substrates are bile acids, conjugated steroid hormones, thyroid hormones, and cyclic and linear peptides. OATPs are important for drug disposition because their exogenous substrates include antibiotics, antidiabetic drugs, anti-inflammatory

drugs, antifungals, antivirals, antihistamines, antihypertensives, fibrates, statins, cardiac glycosides, immunosuppressants, and anticancer drugs (Hagenbuch and Gui, 2008; Konig, 2011). For a list of clinically relevant OATP substrates, including anticancer drugs, refer to Table 1-2.

Many compounds (e.g. estrone-3-sulfate, estradiol-17β-glucuronide) are transported by multiple OATPs and are commonly used as model substrates. However, there are some substrates that are specifically transported by a certain OATP; for example, cholecystokinin-octapeptide (CCK-8) is selectively transported by OATP1B3 (Ismair et al., 2001), while digoxin is mainly transported by OATP4C1 (Mikkaichi et al., 2004).

Table 1-1: Tissue Expression of OATPs in Normal and Cancer Tissues

OATP	Normal tissue expression	Cancer tissue expression
OATP1A2	Blood brain barrier Cholangiocytes Kidney Enterocytes	Expressed in bone cancer tissues and cell lines **Reduced** in colon polyps and cancer **Increased** in breast carcinoma cells and malignant breast tissue
OATP1B1	Liver	**Reduced** in Hepatocellular carcinoma
OATP1B3	Liver	**Reduced** in Hepatocellular carcinoma Expressed in: Colorectal adenocarcinoma tissues Non-small cell lung tumors Prostate cancer tissue Invasive ductal carcinoma breast cells Cell lines of stomach, colon, pancreatic, and gall bladder cancers
OATP1C1	Brain Testes Ciliary body	Expressed in bone cancers
OATP2A1	Ubiquitous	**Increased** in malignant breast tissue and liver cancers **Reduced** in tumors of bowel, stomach, ovary, lung and kidney
OATP2B1	Liver Blood-brain barrier Enterocytes Placenta Heart	**Increased** in bone cysts Altered in breast cancers
OATP3A1	Ubiquitous	Expressed in bone cancer and cancer cell lines of multiple tissues
OATP4A1	Ubiquitous	Expressed in bone cancer and cancer cell lines of multiple tissues
OATP4C1	Kidney	
OATP5A1	Lactiferous ducts in breast	Expressed in malignant breast tumors
OATP6A1	Testes	Expressed in tumors of the lung, bladder and esophagus

The discovery of altered expression of OATPs in cancer tissue has led to the recent interest in their transport of anticancer drugs, mainly by the well-characterized OATP transporters OATP1A2, OATP1B1 and OATP1B3. The multispecific OATP1A2 can transport a wide variety of compounds, including the bile acids cholate and taurocholate; the conjugated steroid hormones estrone-3-sulfate and estradiol-17β-glucuronide; and the thyroid hormones T3 and T4. Recently, OATP1A2 has been shown to transport imatinib, a drug used to treat certain types of leukemias (Hu et al., 2008), and to transport the folate antimetabolite methotrexate (Badagnani et al., 2006). OATP1B1 and OATP1B3 are thought to be important for uptake of a wide variety of xenobiotics into hepatocytes and can transport many of the aforementioned substrates, including estrone-3-sulfate and, in the case of OATP1B3, also testosterone (Hamada et al., 2008). OATP1B1 has been implicated in the transport of the camptothecin analogs gimatecan and BNP1350 (Oostendorp et al., 2009), the irinotecan metabolite 7-ethyl-10-hydroxycamptothecin (SN-38) (Nozawa et al., 2005a), the cyclin-dependent kinase inhibitor flavopiridol (Ni et al., 2010), the Her2 tyrosine kinase inhibitor CP-724,714 (Feng et al., 2009), methotrexate (Abe et al., 2001; Sasaki et al., 2004), and the bile-acid cisplatin derivative *cis*-diammine-chloro-cholylglysinate-platinum II (Briz et al., 2002). OATP1B3 transports methotrexate (Abe et al., 2001); the taxanes paclitaxel (Smith et al., 2005; Letschert et al., 2006) and docetaxel (Smith et al., 2005); and, to a lesser extent, imatinib (Hu et al., 2008). Yamaguchi and colleagues (Yamaguchi et al., 2008) have shown that OATP1B3 transports the irinotecan metabolite SN-38 but does not transport either paclitaxel or docetaxel. In addition, transport of the immunosuppressant rapamycin (sirolimus) has been demonstrated for both OATP1B1 and OATP1B3 (Oswald et al., 2010). The ability of OATPs to transport anticancer drugs as well as hormones and hormone precursors could affect the growth and survival of cancer cells. Thus, OATPs could play a role in cancer progression or response to treatment.

Table 1-2: Selected Substrates of OATPs. Anticancer drugs are highlighted in red.

OATP1A2	OATP1B1	OATP1B3	OATP1C1
Hormones and conjugates Estradiol-17β-glucuronide Estrone-3-sulfate DHEA-S Reverse triiodothyronine (rT3) Thyroxine (T4) Triiodothyronine (T3) **Prostglandins** Prostaglandin E_2 **Bile acids** Cholate Taurocholate Glycocholate Taurochenodeoxycholate Tauroursodeoxycholate **Others** DPDPE **Drugs** Acebutolol Rosuvastatin Atenolol Pitavistatin Sotolol Fexofenadine Labetalol Deltorphin II Nadolol Ciprofloxacin Talinolol Gatifloxacin Saquinavir Imatinib Darunavir Levofloxacin Ouabain Methotrexate	**Hormones and conjugates** Estradiol-17β-glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) DHEA-S **Prostglandins** Prostaglandin E_2 **Bile acids** Cholate Taurocholate Tauroursodeoxycholate **Drugs** Atorvastatin Olmesartan Lopinavir Phalloidin Darunavir Caspofungin SN-38 Pitavastatin Valsartan Cefazolin Enalapril Pravastatin Ezetimibe Cerivastatin Bosentan Rifampicin Saquinavir Rosuvastatin Fluvastatin Temocapril Gimatecan Troglitazone Methotrexate Demethylphalloin	**Hormones and conjugates** Estradiol-17β-glucuronide Estrone-3-sulfate DHEA-S **Drugs** Atresentan Dimethylphalloin Bosentan Paclitaxel Cefadroxil Docetaxel Cefazolin Methotrexate Cephalexin Imatinib Digoxin Olmesartan Enalapril Phalloidin Lopinavir Telmisartan SN-38 Fexofenadine Fluvastatin Gimatecan Pitavastatin Telmisartan Rifampicin Rosuvastatin Valsartan	**Hormones and conjugates** Estradiol-17β-glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) Reverse triiodothyronine (rT3) Thyroxine sulfate (T4S) **Others** BSP
OATP2A1	**OATP2B1**	**OATP3A1**	**OATP4A1**
Prostaglandins Prostaglandin E_1 Prostaglandin E_2 Prostaglandin $F_{2\alpha}$ Prostaglandin H_2 Prostaglandin D_2 8-iso-prostaglandin $F_{2\alpha}$ Thromboxane B_2 **Drugs** Latanoprost	**Hormones and conjugates** Estrone-3-sulfate DHEA-S Thyroxine (T4) **Prostglandins** Prostaglandin E_2 **Drugs** Atorvastatin Bosentan Ezetimibe Fluvastatin Glibenclamide Pitavastatin	**Hormones and conjugates** Thyroxine (T4) Estrone-3-sulfate **Prostglandins** Prostaglandin E_1 Prostaglandin E_2 Prostaglandin $F_{2\alpha}$ **Drugs** Deltorphin BQ-123 Benzylpenicillin **Others** Vasopressin	**Hormones and conjugates** Estradiol-17β-glucuronide Estrone-3-sulfate Thyroxine (T4) Triiodothyronine (T3) Reverse triiodothyronine (rT3) **Bile acids** **Prostglandins** **Drugs**

1.3.5 Regulation

A recent review provides a detailed summary of the current knowledge about OATP regulation (Svoboda et al., 2011a). However, there have not been extensive studies on how OATP expression is regulated within cancer cells. OATP1A2 expression has been associated with expression of the nuclear receptor PXR (pregnane X receptor) in breast carcinoma tissue and its cell lines (Miki et al., 2006). In addition, a PXR response element was identified in the OATP1A2 promoter (Meyer zu Schwabedissen et al., 2008), suggesting that PXR could play a role in the upregulation of OATP1A2 seen in breast cancers. In 2004, it was suggested that OATP1B3 expression in HCC was downregulated via transcriptional repression by hepatocyte nuclear factor 3β (HNF3β) (Vavricka et al., 2004). A more recent study showed that DNA methylation-dependent gene silencing is involved in the regulation of OATP1B3 expression in several cancer cell lines (Ichihara et al., 2010). Posttranslational regulation also could be altered in cancer cells. Although OATPs are normally expressed on cell membranes, strong cytoplasmic staining is seen for both OATP1A2 in breast cancer andOATP1B3 in colon cancer. This could result from aberrant posttranslational regulation such as altered phosphorylation, which regulates cell surface expression of human OATP2B1 (Kock et al., 2010), rat OATP1a1, and rat OATP1a4 (Guo and Klaassen, 2001; Choi et al., 2011). Owing to the limited knowledge in this area, further research is required to better understand the mechanism of OATP up- or downregulation in cancer and to determine what role, if any, it may play in cancer progression or treatment.

1.3.6 Polymorphisms and Drug Disposition

Several studies have documented single-nucleotide polymorphisms (SNPs) of OATPs that are associated with reduced or absent protein function. Several of these SNPs have been linked to altered disposition of chemotherapeutic drugs and consequently increased adverse effects, confirming the importance of OATPs in the disposition of drugs. For example, methotrexate, a substrate of OATP1A2, has been implicated in serious adverse effects seen in patients. To identify potential contributors to varying methotrexate pharmacokinetics, common OATP1A2 polymorphisms were investigated (Badagnani et al., 2006). Among the 12 OATP1A2 polymorphisms studied, four showed altered transport of methotrexate in vitro. The common I13T OATP1A2 SNP showed enhanced methotrexate uptake, whereas R168C, E172D and N278DEL variants showed significantly decreased methotrexate uptake (Badagnani et al., 2006). In addition, common polymorphisms of OATP1B1 may alter disposition of the irinotecan metabolite SN-38 and potentially contribute to the variable adverse gastrointestinal effects seen with this drug (Nozawa et al., 2005a). Uptake of SN-38 was determined in cell lines expressing three common genetic polymorphisms of OATP1B1. Compared with the wild-type OATP1B1*1a, OATP1B1*15, which has an allelic frequency of 10.3% to 15.0%, had significantly reduced SN-38 uptake, suggesting that patients with this polymorphism may have altered SN-38 pharmacokinetics (Nozawa et al., 2005a). This prediction was confirmed in vivo: Patients with the *SLCO1B1*15* polymorphism showed higher systemic exposure and lower clearance of SN-38 (Xiang et al., 2006; Takane et al., 2009). A similar study looked at the effect of three nonsynonymous SNPs of OATP1B3 on the pharmacokinetics of paclitaxel (Smith et al., 2007). However, none of the three genetic variations resulted in significantly different paclitaxel clearance or altered pharmacokinetic parameters.

Recently, van de Steeg et al. (van de Steeg et al., 2011) generated the knockout mouse model *Slco1a/1b*$^{-/-}$, which lacks all OATP1A and OATP1B family members, to study the role of OATPs in the disposition of drugs such as paclitaxel and methotrexate. The *Slco1a/1b*$^{-/-}$ mice had higher plasma concentrations of both drugs, suggesting that proteins in the OATP1A or OATP1B family are involved in their distribution. Introducing polymorphic human OATPs into these null mice could provide a powerful tool to study their effect on drug disposition (van de Steeg et al., 2011).

1.3.7 OATPs and Cancer Development

As briefly discussed above, hormones and their conjugates are substrates of many OATPs, and therefore expression of OATPs in cancer might contribute to the proliferation of androgen- and estrogen-dependent tumors. Several studies have investigated whether OATP expression could affect the growth of hormone-dependent cancers such as breast and prostate cancers.

In 2004, Nozawa et al. showed that uptake of estrone-3-sulfate into T47-D breast cancer cells led to increased cell proliferation and was mediated by a Na+-independent transport system (Nozawa et al., 2004). The authors detected OATP3A1 and OATP4A1 in these cells and suggested that these OATPs might be involved in estrone-3-sulfate uptake into these cells. In a follow-up study, estrone-3-sulfate uptake was measured into the breast cancer cell line MCF-7, and inhibition studies suggested that an OATP could be involved (Nozawa et al., 2005b). On the basis of these findings, inhibition of the estrone-3-sulfate uptake system was proposed as a potential treatment for estrogen-responsive breast cancers (Nozawa et al., 2005b). Additionally, recent studies suggested OATP1B3 as one of the transporters involved in the uptake of estrone-3-sulfate into the breast cancer cell line MCF-7 (Maeda et al., 2010). Meyer zu

Schwabedissen et al. (Meyer zu Schwabedissen et al., 2008; Banerjee et al., 2012) showed that expression of OATP1A2 in T47-D cells was regulated via the nuclear receptor PXR, and treatment of these cells with the PXR activator rifampicin resulted in both increased expression of OATP1A2 and increased proliferation of the cells. Furthermore, when these estrogen-dependent cells were treated with the potent PXR antagonist A-792611, both decreased OATP1A2 expression and decreased proliferation were observed. This led the authors to propose targeting the regulation of OATP1A2 as a potential treatment for breast cancer (Meyer zu Schwabedissen et al., 2008).

OATP1B3 protein expression in various breast carcinomas has been correlated with various pathological parameters including tumor size, recurrence, and prognosis (Muto et al., 2007). OATP1B3 expression was shown in 50% of the breast cancer specimens analyzed. Surprisingly, expression of OATP1B3 was inversely correlated with tumor size and significantly associated with decreased recurrence and good prognoses. However, this correlation was seen only in postmenopausal women. OATP1B3 has thus been hypothesized to be implicated in a hormone-dependent growth mechanism in breast cancer (Muto et al., 2007).

Prostate cancer recurrence and progression have also been correlated with the expression of OATPs. An OATP1B3-related survival advantage for certain patients was seen in androgen dependent prostate cancer (Hamada et al., 2008). Although testosterone is a substrate of wild-type OATP1B3, a variant containing two SNPs, 334G and 699A, does not transport testosterone. Patients with the 334GG/699AA haplotype had an improved overall survival over 10 years (Hamada et al., 2008). A separate study demonstrated that patients with the 334T allele of OATP1B3, which does transport testosterone, had an increased prostate cancer mortality rate (Wright et al., 2011). This study also showed that patients with the rs12422149 polymorphism in the *SLCO2B1* gene, which had previously been shown to affect

OATP2B1-mediated substrate uptake, had an increased risk of prostate-cancer-specific mortality (Wright et al., 2011). Immunofluorescence micrographs of OATP1B3 staining on different prostate cancer tissue sections demonstrated that OATP1B3 expression was primarily observed in the stroma of prostate cancer tumors and not in normal prostate tissue (Pressler et al., 2011).

OATP1B3 may also be involved in the response to anticancer chemotherapy. Overexpression of wild-type OATP1B3 in colon cancer cell lines treated with camptothecin and oxaliplatin conferred an antiapoptotic effect and reduced the transcriptional activity of p53, whereas a nonfunctional OATP1B3 did not have these effects (Lee et al., 2008).

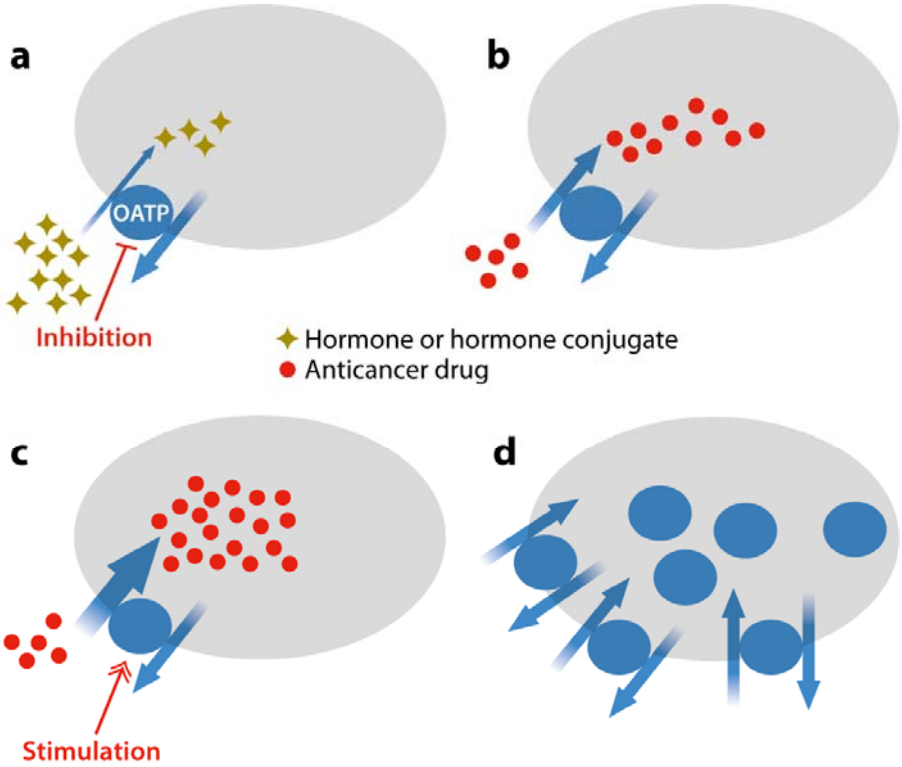
Taken together, these studies demonstrate that OATP expression in cancer can be associated with a survival advantage or disadvantage for the tumor. However, additional research is required to elucidate the underlying molecular mechanisms and to identify the transported substrates that are potentially involved before such findings will lead to improved tumor diagnostics and treatment.

1.3.8 Mechanisms of OATP-mediated Cancer Therapy

OATPs are expressed throughout the body and are generally responsible for the Na+-independent uptake of a wide range of amphipathic compounds. Expression levels of OATPs are altered in many different types of cancers, and in several cancers, these altered expression levels have been correlated with cancer stage. OATPs are capable of transporting multiple compounds that affect cancer cell growth and survival, including hormones, hormone precursors, and anticancer drugs.

Furthermore, uptake mediated by OATPs can be either inhibited or allosterically stimulated by small molecules. In addition, OATP polymorphisms have been associated with altered pharmacokinetics of anticancer drugs, altered transport of hormones, and cancer outcomes. These findings suggest that OATPs could be valuable targets for anticancer therapy in four ways: (*a*) OATP-mediated uptake of hormones, hormone conjugate, or unidentified growth promoting chemicals could be prevented with OATP-selective inhibitors (Figure 1-3*a*); (*b*) novel anticancer drugs could be developed as OATP substrates to increase their uptake into OATP-expressing cancer cells (Figure 1-3*b*); (*c*) uptake of anticancer drugs could be enhanced by allosteric stimulators (Figure 1-3*c*); and (*d*) expression of OATPs in the plasma membrane could be modulated to increase or decrease uptake of various substrates into cancer cells (Figure 1-3*d*). Further research is required to elucidate the role OATPs play in cancer development and anticancer drug transport and to determine how these uptake transporters can be rationally targeted in cancer treatment.

Figure 1-3:



Obaidat A, et al. 2012.
Annu. Rev. Pharmacol. Toxicol. 52:135–51

Figure 1-3: Mechanisms of OATP-mediated Anticancer Therapy. (a) Because OATPs can transport hormones, hormone conjugates and additional chemicals (yellow-brown diamonds), that are beneficial for cancer growth, inhibition of the uptake of these “procancer” compounds into cancer cells could have antiproliferative effects. (b) OATPs can transport known anticancer drugs (red circles). Therefore, investigators could attempt anticancer therapy by designing novel chemotherapeutics that are OATP substrates. (c) Because some OATPs can be allosterically stimulated with small molecules, uptake of anticancer drugs (red circles) could be enhanced in the presence of such stimulators. (d) The membrane expression of OATPs in cancer cells could be regulated to increase or decrease transport into these cells.

1.4 Specific Aims of this Dissertation

Despite the discovery of numerous novel anticancer chemotherapeutic drugs, the problem of multidrug resistance remains a recurrent challenge in cancer treatment (Jemal et al., 2009). One of the mechanisms that contribute to multidrug resistance is the overexpression of efflux pumps, such as MDR1 or MRP1, which decrease the intracellular concentrations of these drugs in cancer cells (Gottesman et al., 2002). Therapeutic approaches to eliminate this problem currently focus on inhibiting the function of these efflux transporters. Unfortunately the results of these strategies have so far not been very promising (Daenen et al., 2004; Pusztai et al., 2005; Friedenberg et al., 2006; Saeki et al., 2007). Thus, there is an urgent need for an alternative approach to improve drug delivery and overcome multidrug resistance in cancer treatment. Under normal physiological conditions Organic Anion Transporting Polypeptides (OATPs) are expressed in certain tissues where they are responsible for the transport of numerous endogenous compounds and xenobiotics. Recently, OATPs have been found to have altered expression profiles in cancer tissues. The function of OATPs in cancer cells has not yet been investigated in detail but because several OATPs have been shown to transport anticancer drugs, OATPs have been proposed to be potential targets to improve cancer therapy (Obaidat et al., 2012). By better understanding the expression and function of OATPs in cancer cells we may be able to improve cancer diagnostics and therapies.

The studies presented in this dissertation were designed to address the lack of understanding of how OATPs can be used as cancer biomarkers or in cancer drug delivery. Therefore, the *long term goal* of the studies presented in this dissertation is to improve cancer diagnosis and therapy. The *objective* of this dissertation was to characterize the expression of OATPs in cancer and to determine their contribution to the transport of cytotoxic chemotherapeutic drugs into cancer cells. The *central hypothesis* of this dissertation was that

expression of OATPs in cancer combined with their ability to transport cytotoxic anticancer drugs makes them potential targets for improving cancer diagnosis and therapy. I formulated this hypothesis based on our preliminary studies that showed that OATP1B3 can mediate the uptake of several anticancer drugs including etoposide and paclitaxel and that small molecules can stimulate OATP1B3 mediated uptake of model substrates. By testing this central hypothesis I have discovered novel information about OATPs in cancer and expect that this information will lead to a better understanding of the roles OATPs play in cancer and to an improved diagnosis and drug delivery via OATPs.

The central hypothesis was tested via the following specific aims:

1.4.1: Specific Aim 1: Identify and characterize cancer cells that express OATPs.

Our working hypothesis was that certain OATPs are expressed in human cancers. To test this hypothesis, we measured mRNA and protein expression of OATPs in several different cancer tissues on a tissue microarray. Expression of OATPs was further characterized in pancreatic cancer tissues of different types and stages. Completion of this specific aim identified four major OATPs upregulated in pancreatic cancer. Furthermore these studies demonstrated that expression of OATP1B3 was highest in earlier stage pancreatic adenocarcinomas, and provide a basis for utilizing OATP1B3 as a diagnostic marker and early therapeutic target.

1.4.2: Specific Aim 2: Identify and functionally characterize anticancer drug uptake mediated by OATPs.

Our working hypothesis was that OATPs can transport a variety of anticancer drugs. Anticancer drugs that interact with OATPs were identified by screening the NCI/DTP oncology drug set for 1) inhibition of OATP-mediated uptake of two model substrates and 2) for their effect on cell viability of OATP-expressing cells. Uptake of selected anticancer drugs was measured. Completion of this specific aim identified etoposide, oxaliplatin, and plicamycin as three novel anticancer drug substrates of OATP1B3.

1.4.3: Specific Aim 3: Identify cytotoxic compounds from plant extracts that can be used to develop anticancer drugs that target OATP1B3-expressing cancers.

Our working hypothesis was that Kansas plants are a source for cytotoxic OATP1B3 substrates that can be developed into novel anticancer drugs. A bioassay guided isolation approach coupled with cell viability assays identified the most potent sub-fractions of two cytotoxic Kansas plant extracts that were shown to kill cells expressing OATP1B3.

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). [^{14}C]Etoposide and [^{14}C]Oxaliplatin were purchased from American Radiolabeled Chemicals (St. Louis, MO). The approved oncology drug set II library of 89 drugs for the treatment of cancer was obtained from the NCI/DTP Open Chemical Repository (http://dtp.cancer.gov), compounds were dissolved in DMSO at 10mM concentrations in 96-well plates. Anticancer drugs for confirmatory experiments were also obtained from NCI/DTP Open Chemical Repository or purchased from Sigma (St. Louis,MO) or Toronto Research Chemicals (North Tork, Ontario).

The fresh frozen human tissues used in these studies were obtained under IRB approval of the University of Kansas Cancer Center and provided by the Biospecimen Shared Resource of the University of Kansas Cancer Center (Kansas City, KS). Quantigene Plex 2.0 System Reagents were purchased from Panomics Inc. (Fremont, CA). Fetal bovine serum was obtained from Hyclone (Logan, UT). Anti-OATP1B3, OATP2A1, OATP3A1 and OATP4A1 antibodies used in the immunoflourescence analyses were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The anti-OATP1B3 antibody used for immunohistochemical staining analyses on the tissue microarray was raised against the last 14 amino acids at the C-terminal end of OATP1B3 and was obtained from Sigma (St. Louis, MO). The pancreas tissue microarray was purchased from US Biomax (Rockville, MD). Dulbecco's modified Eagle's medium (low glucose)

was purchased from Caisson Laboratories (North Logan, UT). All other chemicals were purchased from Sigma-Aldrich or Toronto Research Chemicals.

2.2: Cell culture

Chinese Hamster Ovary (CHO) cells stably expressing OATP1B3 and wild-type CHO cells were described previously (Gui et al., 2008; Roth et al., 2011b) and were grown at 37 °C in a humidified 5% CO_2 atmosphere in Dulbecco's Modified Eagle Medium, containing 1 g/l D-glucose, 2 mM L-glutamine, 25 mM Hepes buffer and 110 mg/l sodium pyruvate, supplemented with 10% FBS (Hyclone, Logan, UT), 50 µg/ml L-proline, 100 U/ml penicillin and 100 µg/ml streptomycin (non-select medium), and for the OATP1B3 expressing cells 500 µg/ml G-418 (select medium). Cells were passaged twice a week and used up to passage 60.

2.3: Quantigene Multiplex Assays

Frozen pancreas tissue samples were homogenized with a glass-teflon tissue homogenizer in a hypotonic buffer (1mM NaCl, 5mM Tris-HCl pH 7.5) containing protease inhibitors (Complete Protease Inhibitor Cocktail, Roche, Indianapolis, IN) and mRNA expression of OATPs was determined using the QuantiGene Plex 2.0 Reagent System (Panomics Inc., Fremont, CA). Bead-based oligonucleotide probe sets specific for all eleven human OATP genes were developed by Panomics Inc. Assays were performed according to the manufacturer's protocol (Panomics Inc). Briefly, tissue homogenates were lysed with lysis buffer containing proteinase K. Samples were incubated with capture bead probe set oligonucleotides,

capture extenders, label extenders and blocking probes in a 96 well hybridization plate. The hybridization plate was sealed and incubated at 54ºC while shaking at 300 rpm for 18 hours. The hybridization plate was centrifuged at 240 x g for 1 minute and samples were transferred to a 96 well pre-wet filter plate. Samples were washed three times with the provided wash buffer and filtered at 0.5 psi. The samples were incubated with pre-amplification mixture, amplification buffer and label probe working reagent at 50ºC while shaking at 600 rpm for 1 hour. Samples were washed three times with wash buffer. Streptavidin phycoerythrin (SAPE) was added to each sample and incubated for 30 minutes at room temperature. The filter plate was vacuumed and washed three times with wash buffer. SAPE was added to resuspend samples and samples were analyzed using a Bio-Plex System Array reader with Luminex 100xMAP technology, and data were acquired using Bio-Plex Data Manager software version 5.0 (Bio-Rad Laboratories, Hercules, CA) All data were standardized to the internal control ribosomal protein L13A.

2.4: Affinity Purification of OATP1B3 Antibody

The anti-OATP1B3 antibody obtained from Sigma (St. Louis, MO) was affinity purified with an amino-link plus immobilization kit according to the manufacturer's protocol (Thermo Scientific, Rockford, IL). Briefly, 2 mg of peptide was dissolved in 3 ml of citrate-carbonate buffer (0.1 M sodium citrate, 0.05 M sodium carbonate, pH 10.0) and coupled to an equilibrated amino-link plus column (4 % beaded agarose slurry, 0.02 % sodium azide) by rocking end-over-end for 4 hours at room temperature. The column was centrifuged at 1000 x g for 1 minute to remove non-bound peptide. The column was washed twice with PBS and then a final concentration of 50 mM sodium cyanoborohydride solution was added to the column for 4 hours at room temperature. The remaining active sites were blocked by washing the column with quenching buffer for 30 minutes at room temperature (1 M Tris-HCl, 0.05 % sodium azide, pH-

7.4). Quenching buffer was removed by centrifugation at 1000 x g for 1 minute and the column was washed four times with wash solution (1 M NaCl, 0.05 % sodium azide). For affinity purification, anti-OATP1B3 antibody was added to the column and bound by rocking for 1 hour at room temperature. The column was washed four times with PBS and the antibody was eluted by centrifugation at 1000 x g for 1 minute with elution buffer (0.1 M Glycine-HCl, pH=3.0) and the sample was neutralized with neutralization buffer (1 M Tris-HCl, pH=9.0).

2.5: Immunofluorescence Staining on Fresh Frozen Tissue

Frozen pancreas tissue samples were cut into 6 µm sections with a cryostat onto positively charged slides. The slides were then fixed and permeabilized using a 2% paraformaldehyde and 1% Triton X-100 solution (pH=7.4) for 10 minutes, and blocked with 5% donkey serum in PBS for 1 hour. Sections were incubated overnight at 4 ºC with polyclonal antibodies for OATPs diluted 1:100 in 1% donkey serum in PBS. After washing three times in PBS, slides were incubated in the dark with an anti-goat AlexaFluor 594 antibody (Invitrogen, Carlsbad, CA) diluted 1:1000 in 0.1 % PBS-Tween for 1 hour at room temperature and after three washes in PBS, sections were mounted in Prolong Gold containing DAPI (Invitrogen, Carlsbad, CA). For negative controls, the sections were incubated with secondary antibody only.

2.6: Immunohistochemistry Staining on Paraffin-embedded Tissue Microarray

A tissue array slide containing 42 cases of ductal adenocarcinomas (stages 1-3), 3 adenosquamous carcinomas, 1 islet cell carcinoma, 6 metastatic carcinomas, 10 islet cell tumors, 2 hyperplasias, 10 inflammations, 20 normal tissues adjacent to cancer and 10 normal

tissues from autopsies was used for these studies. Following deparrafinization in two changes of xylene for 5 minutes, sections were rehydrated in two changes of 100 % ethanol for 5 minutes, and two changes of 95 % ethanol for 5 minutes, and deionized water for 5 minutes. Sections were quenched with 3 % hydrogen peroxide in deionized water for 10 minutes and washed in three changes of deionized water for 2 minutes. Antigen retrieval was conducted by boiling the slides in citrate buffer (pH=7.4) for 5 minutes and sub-boiled for 10 minutes, followed by cooling the sections to room temperature for 30 minutes. Sections were washed in three changes of deionized water for 2 minutes followed by 0.1 % PBS-Tween for 1 minute. Sections were blocked with 5 % normal donkey serum (Sigma) in 0.1 % PBS-Tween for 1 hour at room temperature. Sections were incubated overnight at 4ºC with affinity purified polyclonal anti-OATP1B3 antibody at a 1:30 dilution in 0.1 % PBS-Tween. After washing, slides were incubated with biotin-conjugated secondary antibody (Jackson Immuno Research Labs, West Grove, PA) in 0.1 % PBS-Tween for 30 minutes at room temperature, and the signal was detected using an ABC Elite kit (Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature. After washing in three changes of 0.1 % PBS-Tween for 2 minutes, the tissues were stained with DAB chromagen for 5 minutes, counterstained with hematoxylin for 30 seconds, washed in tap water for 2 minutes and deionized water for 2 minutes. Sections were dipped in Scott's Blue dip 12 times followed by incubating in two changes of 95 % ethanol for 5 minutes, two changes of 100 % ethanol for 5 minutes and two changes of xylene for 5 minutes. Sections were coverslipped and mounted with cytoseal (Richard-Allen scientific, Kalamazoo, MI). For negative controls, the sections were incubated with secondary antibody only.

2.7: Pathological evaluation

OATP1B3 expression was evaluated in all sections of the pancreatic cancer tissue microarray and scored in three independent evaluations within 14 days of staining. OATP1B3 expression was assessed according to the staining intensity for each specimen and graded as negative or positive staining. The percentage of staining intensity was calculated by comparing the stained specimens to total number of specimens for each category.

2.8: Transport Assays

Transport assays were performed as follows: 40,000 cells per well were seeded on 24 well plates or 8,000 cells per well were seeded on 96 well plates for 48 to 72 hours. Once cells reached confluency, non-specific gene expression was induced by replacing medium with non-select medium containing 5 mM sodium butyrate. Transport assays were performed 24 hours after induction as described previously (Gui and Hagenbuch, 2008; Roth et al., 2011b). Briefly, cells were washed three times with pre-warmed (37 °C) uptake buffer (116.4 mM NaCl, 5.3 mM KCl, 1 mM NaH_2PO_4, 0.8 mM $MgSO_4$, 5.5 mM D-glucose and 20 mM Hepes, pH adjusted to 7.4 with Trizma base) and uptake was started by adding uptake buffer containing the indicated concentration of substrate. After the indicated time period at 37 °C, uptake was stopped by removing the uptake solution and washing the cells four times with ice-cold uptake buffer. To measure uptake of radiolabeled compounds, the cell membranes were lysed with 1% Triton X-100 (dissolved in PBS) and the cell-associated radioactivity was quantified using liquid scintillation spectroscopy (Research Products International Corp., Mt. Prospect, IL). Plicamycin in cell lysates from uptake studies was quantified by liquid chromatography-tandem mass

spectrometry. Briefly, 10 µL aliquots of cell lysates lysed with 75:50 acetonitrile:water and 0.1 % formic acid were injected into a Phenomenex Luna C18 column (50 x 2.1 mm, 3.5 µ) maintained at 40° C. Analytes were eluted with 20% methanol/80% water for 1 min, followed by a 2 min linear gradient to 100% methanol, with all solvents containing 0.1% formic acid (v/v), at a flow rate of 0.3 mL/min. Retention times were 4.7 min for both plicamycin and the internal standard docetaxel. The entire eluate was introduced into the electrospray probe of the mass spectrometer and analyzed in positive ion mode. Plicamycin was detected and quantified using the transition from 1107.8 > 427.2, and the internal standard docetaxel was measured using the 808.1 > 527.1 transition. The assay was validated using spiked HeLa cell lysates, and was linear for plicamycin concentrations between 0.3 and 3000 ng/mL. The rate of uptake in each well was normalized to its total protein concentration using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA). Net OATP-mediated uptake was determined by subtracting the uptake in the OATP-expressing cells minus the uptake in the wild-type control cells.

2.9: Plant Compound Extraction and Isolation

Plant collection was performed by Kelly Kindscher from the Kansas Biological Survey at the University of Kansas, Lawrence, Kansas. Plant compound extraction and isolation was performed under the supervision of Dr. Barbara N. Timmermann, Department of Medicinal Chemistry, The University of Kansas, Lawrence, Kansas.

2.10: Cell Viability Assays

Cell viability assays were performed by the following procedure. Eight thousand cells per well were seeded onto 384-well plates and 24 hrs later were induced in non-select medium containing 5mM sodium butyrate. After another 24 hours the cells were treated with anticancer drugs or plant extracts and incubated for 48 hours. Then cell viability was measured by adding Cell Titer Glo® reagent to the cells for 10 minutes and luminescence was measured using a Bio-Tek Synergy HT microplate reader with an integration time of 1 second per well (Winooski, VT).

2.11: Calculations and Statistical Analysis

All calculations were performed using Prism 5 (GraphPad Software Inc., San Diego, CA). To determine statistical significance between groups, student's t-test was performed and $P < 0.05$ was considered significant. IC_{50} values were calculated using the one-site fit $LogIC_{50}$ equation and kinetic constants were calculated using the Michaelis-Menten equation for non-linear regression.

Chapter 3

Organic Anion Transporting Polypeptides Expressed in Pancreatic Cancer may Serve as Potential Diagnostic Markers and Therapeutic Targets for Early Stage Adenocarcinomas

3.1: Abstract

Purpose: Organic Anion Transporting Polypeptides (OATPs) are expressed in various epithelial tissues in the body. Because they can be expressed in cancers and because they can transport anticancer drugs, OATPs could be potential targets for cancer therapy. Therefore we examined their expression in human pancreatic ductal adenocarcinomas.

Methods: Expression of all eleven human OATPs was measured at the mRNA level and OATPs with highest expression were characterized at the protein level.

Results: Transcripts of *SLCO1B3*, *SLCO2A1*, *SLCO3A1* and *SLCO4A1* were detected in all the tested pancreatic tissues. OATP1B3, OATP2A1, OATP3A1 and OATP4A1 protein expression was confirmed in these tissues and expression of all four transporters increased in pancreatic adenocarcinoma compared to normal pancreas. OATP1B3 expression was highest in pancreatic hyperplasia and stage one adenocarcinomas compared to stage two and three adenocarcinomas.

Conclusion: OATP1B3, OATP2A1, OATP3A1 and OATP4A1 are up-regulated in pancreatic adenocarcinoma and could potentially be used to target anticancer drugs to pancreatic cancer. Additionally, because expression of OATP1B3 is highest in pancreatitis and stage one adenocarcinoma, which leads to pancreatic cancer, OATP1B3 is a potential marker to diagnose patients with early stage pancreatic adenocarcinomas.

3.2: Introduction

Pancreatic cancer remains one of the most difficult cancers to treat clinically. Commonly, patients remain asymptomatic until diagnosed with a late stage of pancreatic adenocarcinoma and often do not respond to surgery or conventional chemotherapy. For this reason, there is an urgent need for more effective diagnostics and therapeutics for this disease (Jemal et al., 2009; Hidalgo, 2010; Zafar and El-Rayes, 2012).

Relapse and incomplete recovery from this disease can be a result of multidrug resistance (MDR) (Szakacs et al., 2006). A very well characterized mechanism of multidrug resistance in cancer is the over-expression of efflux transporters, members of the ATP-binding cassette (ABC) transporter superfamily. Fueled by ATP, these efflux transporters pump anticancer drugs out of cancer cells leading to drug ineffectiveness and ultimately resistance (Gottesman et al., 2002). One way to overcome MDR would be to target these efflux transporters by developing selective inhibitors, also called reversal agents (Daenen et al., 2004; Pusztai et al., 2005; Friedenberg et al., 2006; Saeki et al., 2007). Unfortunately these attempts have so far not been successful and recent attention has shifted to a different class of potential therapeutic targets, namely uptake transporters.

Organic Anion Transporting Polypeptides (OATPs) are a class of uptake transporters belonging to the solute carrier family of the OATPs (*SLCO*) gene superfamily (Hagenbuch and Meier, 2004). OATPs mediate the Na^+-independent uptake of a wide range of structurally diverse endogenous and exogenous compounds including bile acids, hormone conjugates, peptides, toxins, as well as a multitude of therapeutic drugs (Hagenbuch and Gui, 2008). OATPs are expressed in the epithelia of many different tissues in the body and have altered expression profiles in various cancers. For example, the liver-specific OATP1B3, under normal

conditions, is exclusively expressed at the basolateral membrane of human hepatocytes (Konig et al., 2000a; Abe et al., 2001). However, several studies showed OATP1B3 expression in cancers of the colon (Abe et al., 2001; Cui et al., 2003; Lee et al., 2008), prostate (Hamada et al., 2008), breast (Muto et al., 2007), ovary (Svoboda et al., 2011b), lung (Monks et al., 2007) and bone (Liedauer et al., 2009).

The expression of OATPs in many different cancers combined with their ability to transport cancer drugs, suggests that OATPs could potentially serve as therapeutic targets for cancer drug delivery into cancer cells (Obaidat et al., 2012). Recently, Kounnis and colleagues studied the expression of three OATPs in pancreatic cancer (Kounnis et al., 2011). We wanted to extend this study and evaluated the expression of all eleven human OATPs in pancreatic cancer and expanded on the understanding of OATP1B3 expression at different stages of pancreatic adenocarcinomas.

3.3: Results

3.3.1: Transcripts of OATP1B3, OATP2A1, OATP3A1 and OATP4A1 are expressed in normal human pancreas and in pancreatic adenocarcinoma tissue

To determine which OATPs are expressed in normal and cancerous human pancreatic tissue, we examined the expression of all eleven human OATPs at the messenger RNA level in seven normal pancreas tissues and four ductal adenocarcinomas or metastasis (Table 3-1) using the Quantigene Plex 2.0 reagent system. Among the eleven OATPs, we identified the expression of only four OATPs in the tested samples: OATP1B3, OATP2A1, OATP3A1 and

OATP4A1. The expression of the remaining OATPs, OATP1A2, OATP1B1, OATP1C1, OATP2B1, OATP4C1, OATP5A1 and OATP6A1 were below the detection limit for all the tested tissue samples (data not shown). Transcripts of OATP4A1 were highest in all the pancreas samples followed by OATP2A1, OATP3A1 and OATP1B3 (Figure 3-1). Transcript levels of OATP2A1 decreased in pancreatic adenocarcinomas compared to normal pancreatic tissues, however the difference was not statistically significant ($p < 0.05$). Similarly, the transcript levels of OATP1B3, OATP3A1 and OATP4A1 were not statistically significant between normal pancreas and pancreatic adenocarcinoma tissues. These results suggest that OATP1B3, OATP2A1, OATP3A1 and OATP4A1 are the main OATPs expressed in human pancreas.

Table 3-1: Pathological Staging and Characteristics of Pancreas Tissue Samples

Sample Number	Tissue Description	Histological Grade	Pathological Staging	Final Diagnosis
1360	Normal	N/A	N/A	
1613	Normal	N/A	N/A	
1671	Normal	N/A	N/A	
1675	Normal	N/A	N/A	
1878	Normal	N/A	N/A	
1369	Tumor	G_2	$pT_2\,N_0\,M_x$	Invasive moderately differentiated adenocarcinoma
1934	Tumor	G_2	$pT_3\,N_0\,M_1$	Moderately differentiated ductal adenocarcinoma
1952	Tumor	G_2	$pT_3\,N_{1b}\,M_x$	Invasive moderately differentiated ductal adenocarcinoma
1597	Metastasis to the Omentum	N/A	N/A	Metastatic adenocarcinoma to the omentum

Pathological staging is in accordance with the Tumor, Node, Metastasis (TNM) classification system from the American Joint Committee on Cancer (AJCC). T: size and/or extent of the tumor, N: extent of spread to lymph nodes, M: presence of metastasis.

Figure 3-1:

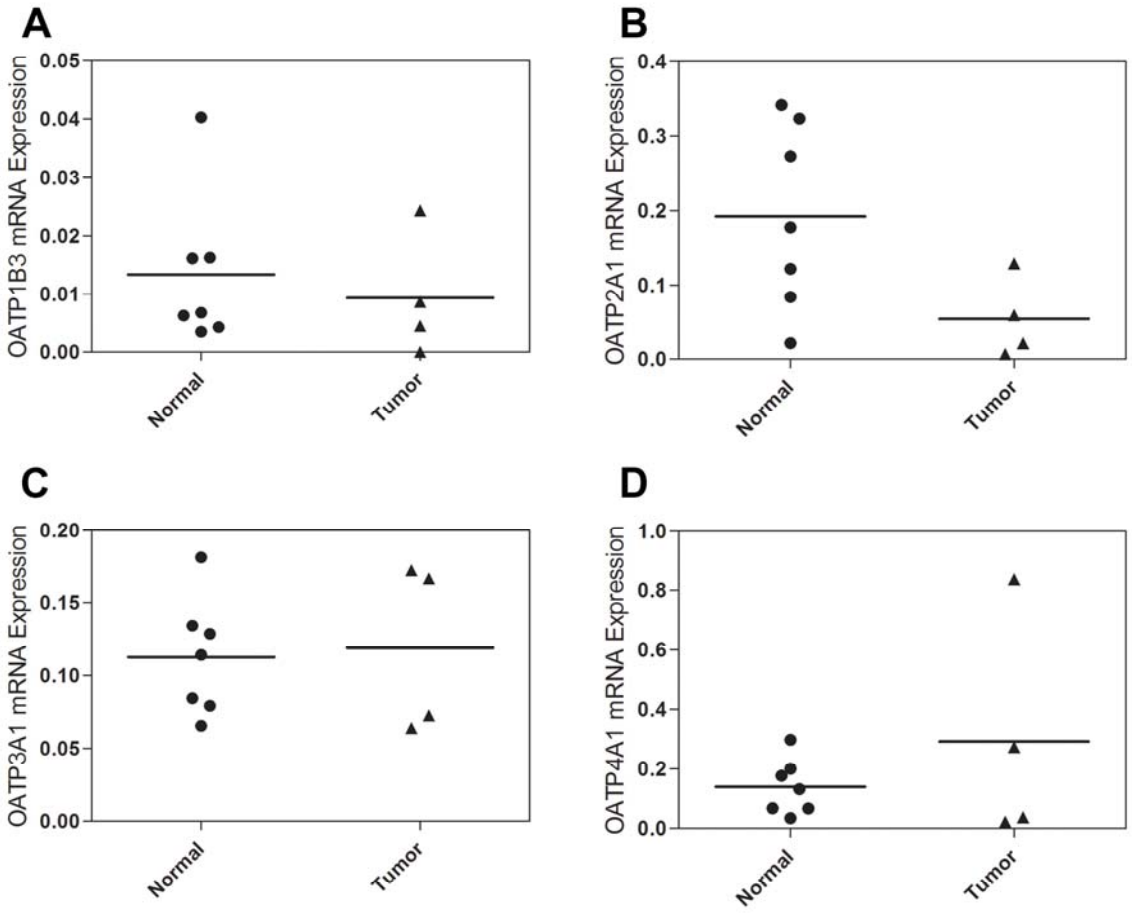

A
OATP1B3 mRNA Expression
0.05
0.04
0.03
0.02
0.01
0.00
Normal
Tumor
B
OATP2A1 mRNA Expression
0.4
0.3
0.2
0.1
0.0
Normal
Tumor
C
OATP3A1 mRNA Expression
0.20
0.15
0.10
0.05
0.00
Normal
Tumor
D
OATP4A1 mRNA Expression
1.0
0.8
0.6
0.4
0.2
0.0
Normal
Tumor

Figure 3-1: Messenger RNA expression of OATPs in normal pancreas and pancreatic tumor samples. Messenger RNA expression of A) OATP1B3, B) OATP2A1, C) OATP3A1 and D) OATP4A1 in normal pancreas and pancreas cancer tissue samples were measured by Quantigene Plex 2.0 Reagent System. Data were normalized to the housekeeping gene ribosomal protein L13A. $P < 0.05$ by unpaired t-test.

3.3.2: OATP expression is higher in pancreatic adenocarcinomas than in normal pancreas at the protein level

To determine changes in OATP protein expression in pancreatic adenocarcinoma and normal tissues we used immunofluorescence staining. Our data show expression of OATP2A1, OATP3A1 and OATP4A1 in normal pancreas and adenocarcinoma tissues (Figure 3-2). In contrast to the transcript data, OATP2A1, OATP3A1 and OATP4A1 protein expression was higher in pancreatic adenocarcinoma than in normal pancreas tissue as evidenced by more intense staining observed throughout the adenocarcinoma tumors. Thus OATP2A1, OATP3A1 and OATP4A1 protein expression is up-regulated in pancreatic adenocarcinomas.

Furthermore, we investigated the expression of OATP1B3 in normal pancreas and pancreatic adenocarcinoma. Immunofluorescence staining revealed low or absent OATP1B3 expression in normal pancreas, which was increased in pancreatic adenocarcinoma tumor specimens (Figure 3-3). OATP1B3 was expressed in the entire tumor specimen in two out of three cases and selectively in the epithelial cells that line the pancreatic ducts in one out of three cases. Ductal expression of OATP1B3 is especially visible in the pancreatic tumor sample 1952 (Figure 3-3, white arrowheads). We used normal human liver tissue as a positive control for these studies which has constitutive expression of OATP1B3. To determine the location of OATP1B3 expression in pancreatic tumors, we used co-immunofluorescence with OATP1B3 and carbonic anhydrase II (CAII), a marker for ductal epithelium. Our results indicate that OATP1B3 was localized, but not restricted, to the ductal epithelium of pancreatic adenocarcinoma (Figure 3-4). Overall, protein expression of OATP1B3, OATP2A1, OATP3A1 and OATP4A1 was low in normal human pancreas but increased in pancreatic adenocarcinoma tissue.

Figure 3-2:

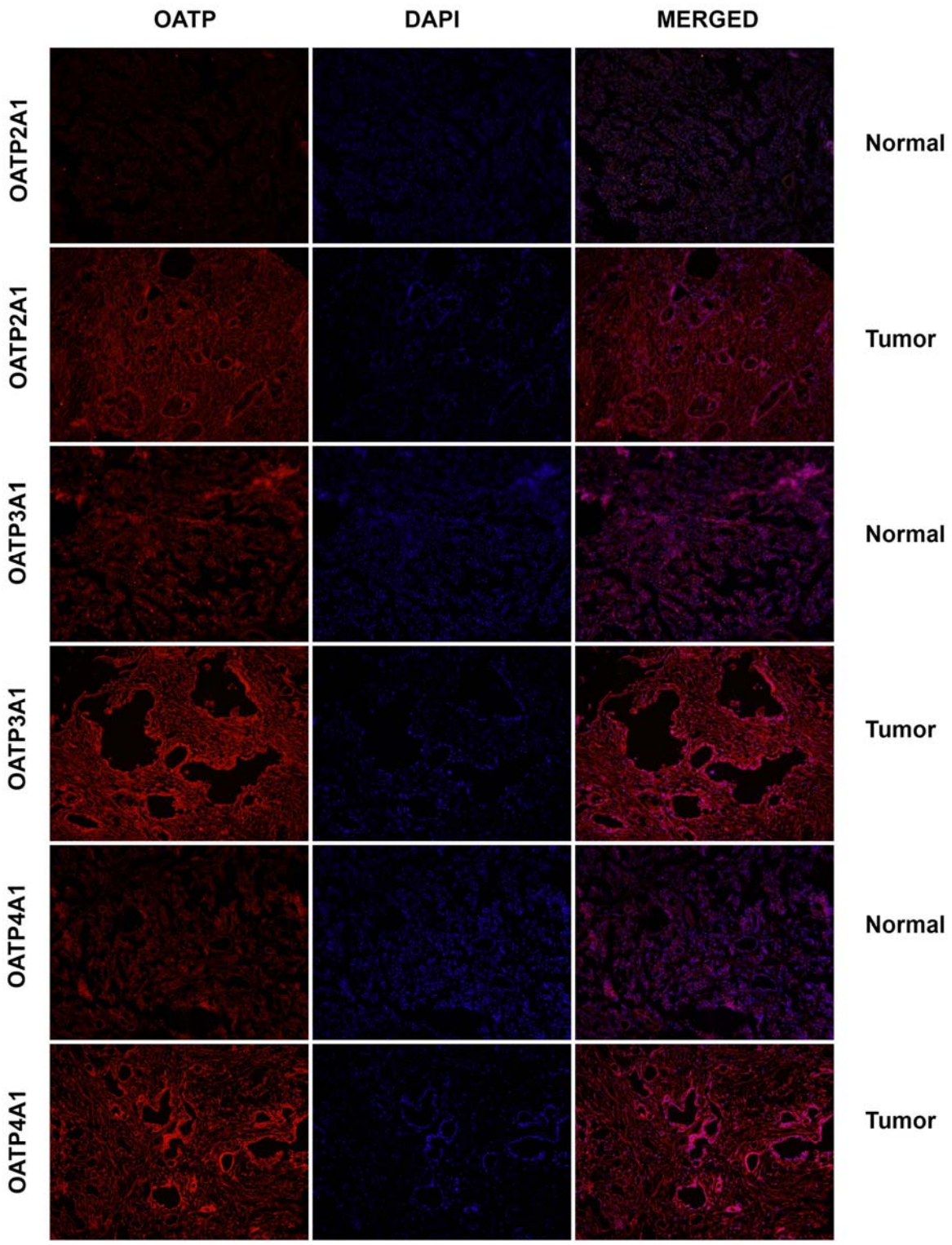

OATP
DAPI
MERGED
OATP2A1
Normal
OATP2A1
Tumor
OATP3A1
Normal
OATP3A1
Tumor
OATP4A1
Normal
OATP4A1
Tumor

Figure 3-2: Immunofluorescence staining of human pancreas tissue samples with anti-OATP antibodies. Immunofluorescence staining was conducted on frozen sections of normal and cancerous pancreatic tissues. The positive signal is visualized with red color for OATP2A1, OATP3A1, and OATP4A1. Nuclei are visualized in blue color with DAPI. All images are at 200X magnification.

Figure 3-3:

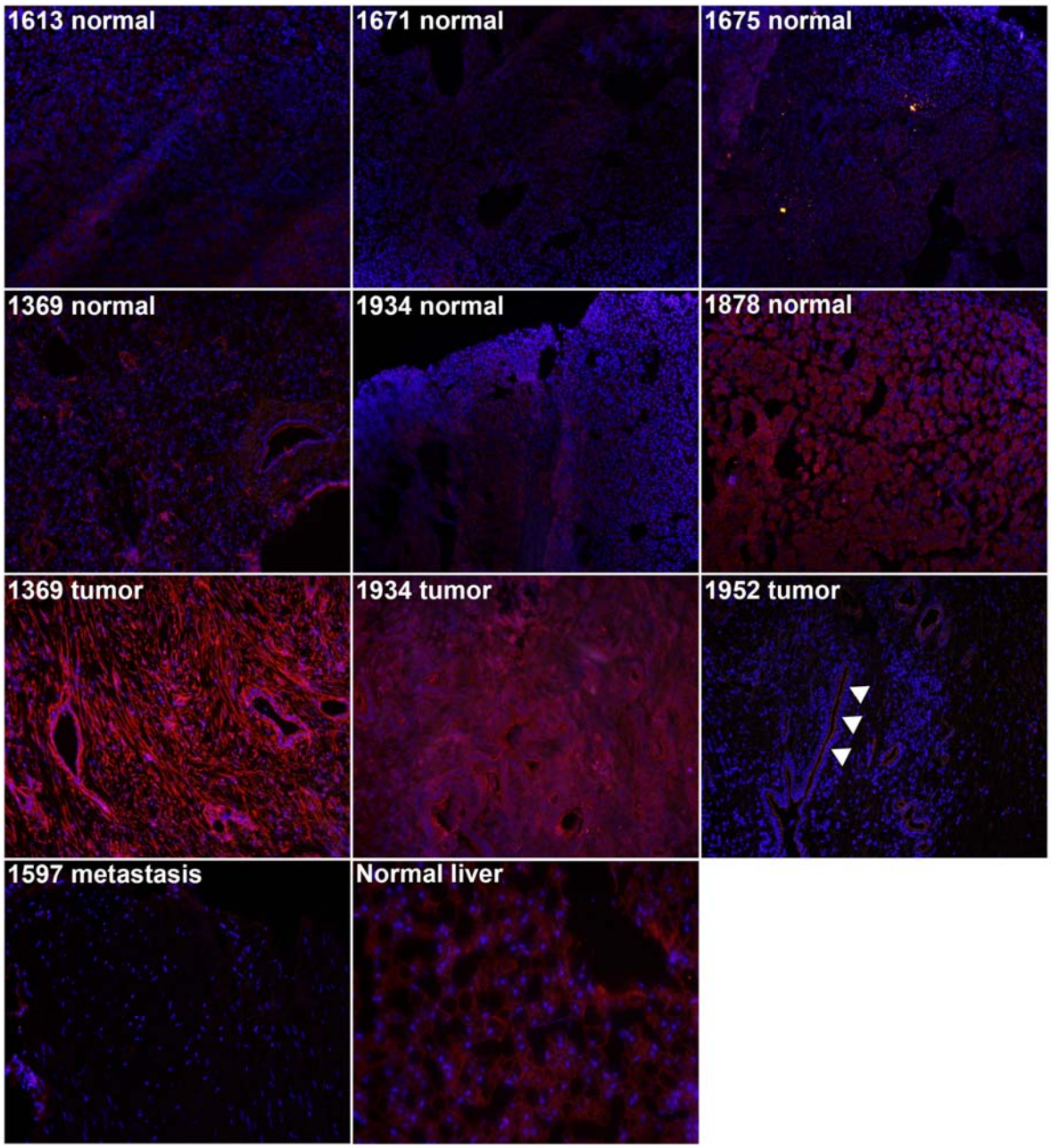

1613 normal
1671 normal
1675 normal
1369 normal
1934 normal
1878 normal
1369 tumor
1934 tumor
1952 tumor
1597 metastasis
Normal liver

Figure 3-3: Immunofluorescence staining of human pancreas tissue samples against OATP1B3. Immunofluorescence staining was conducted on frozen sections of normal and cancerous pancreatic tissues. The positive signal for OATP1B3 is visualized with red color. Nuclei are visualized in blue color with DAPI. Normal human liver was used as a positive control to confirm the specificity of the OATP1B3 polyclonal antibody and shows membranous staining in the hepatocytes. Expression of OATP1B3 in the tumor sample 1952 is restricted to the epithelial cells lining the acini (white arrowheads).All images are at 200X magnification except for the normal liver image which is a 400X magnification.

Figure 3-4:

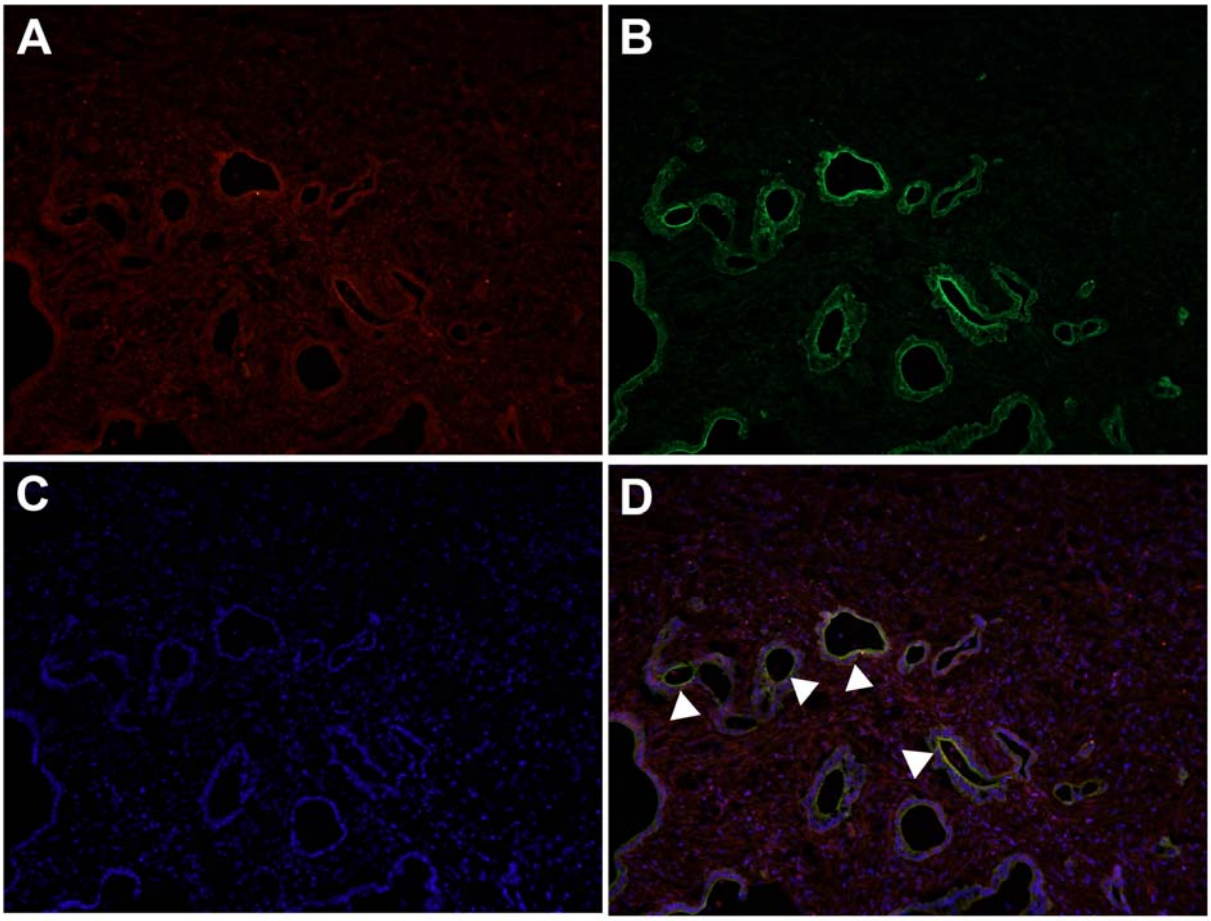


Figure 3-4: Co-localization of OATP1B3 and Carbonic Anhydrase II in human pancreatic tumor tissue. Immunofluorescence staining was conducted on frozen sections of pancreatic cancer tissue. A) Positive signal for OATP1B3 is visualized with red color; B) The ductal marker, carbonic anhydrase II (CAII), is visualized in green color; C) Nuclei are visualized in blue color with DAPI; D) The merged image shows an overlay of the nuclei, OATP1B3 and CAII for localization. White arrowheads point to the strongest co-localization of OATP1B3 and CAII. Images are at 200X magnification.

3.3.3: OATP1B3 is expressed in chronic pancreatitis and earlier stages of adenocarcinoma

We further assessed the frequency and extent of OATP1B3 expression at different stages and types of pancreatic cancer. The rationale to further evaluate the expression of OATP1B3 was that unlike other OATPs, under normal physiological conditions, OATP1B3 is selectively expressed in human hepatocytes. However, recent studies revealed OATP1B3 expression in a wide range of different cancers. Thus, given the limited OATP1B3 expression in normal pancreatic tissue, OATP1B3 could be a good marker for diagnostic purposes and a practical target for anticancer drug delivery into tumor cells. Therefore, we examined OATP1B3 expression on a pancreas cancer tissue microarray using immunohistochemical staining. The results confirm previous observations, indicating low expression of OATP1B3 in normal pancreas (10% of cases; n=10) (Figure 3-5A) and cancer adjacent normal pancreas tissue (20% of cases; n=20) (Figure 3-5B). Interestingly, OATP1B3 expression was high in hyperplasia, pancreatic inflammation and chronic pancreatitis specimens (42% of cases; n=12) (Figure 3-5C). Similarly OATP1B3 was expressed in 44% of stage one pancreatic adenocarcinoma cases (n=9) (Figure 3-5D), decreased in stage two (28% of cases; n=18) (Figure 3-5E) and stage three adenocarcinoma (13% of cases; n=15) (Figure 3-5F) and was completely absent in metastatic tissues from primary pancreatic adenocarcinoma (Figure 3-5G). Aside from pancreatic adenocarcinoma, we also evaluated OATP1B3 expression in two other types of pancreatic cancer, islet cell tumors and pancreatic adenosquamous carcinomas. OATP1B3 expression was absent in islet cell tumors (n=11) (Figure 3-5G), and, it was highly expressed in adenosquamous carcinoma (67% of cases, n=3) (Figure 3-5H). Examples of OATP1B3 expression in chronically inflamed pancreas (Figure 3-5I), in stage one (Figure 3-5J) and in stage three pancreatic adenocarcinoma (Figure 3-5K) are shown. These results reveal that

OATP1B3 is highly expressed in pancreatic hyperplasia and early stage adenocarcinomas with decreasing expression in higher stage adenocarcinomas.

Figure 3-5:

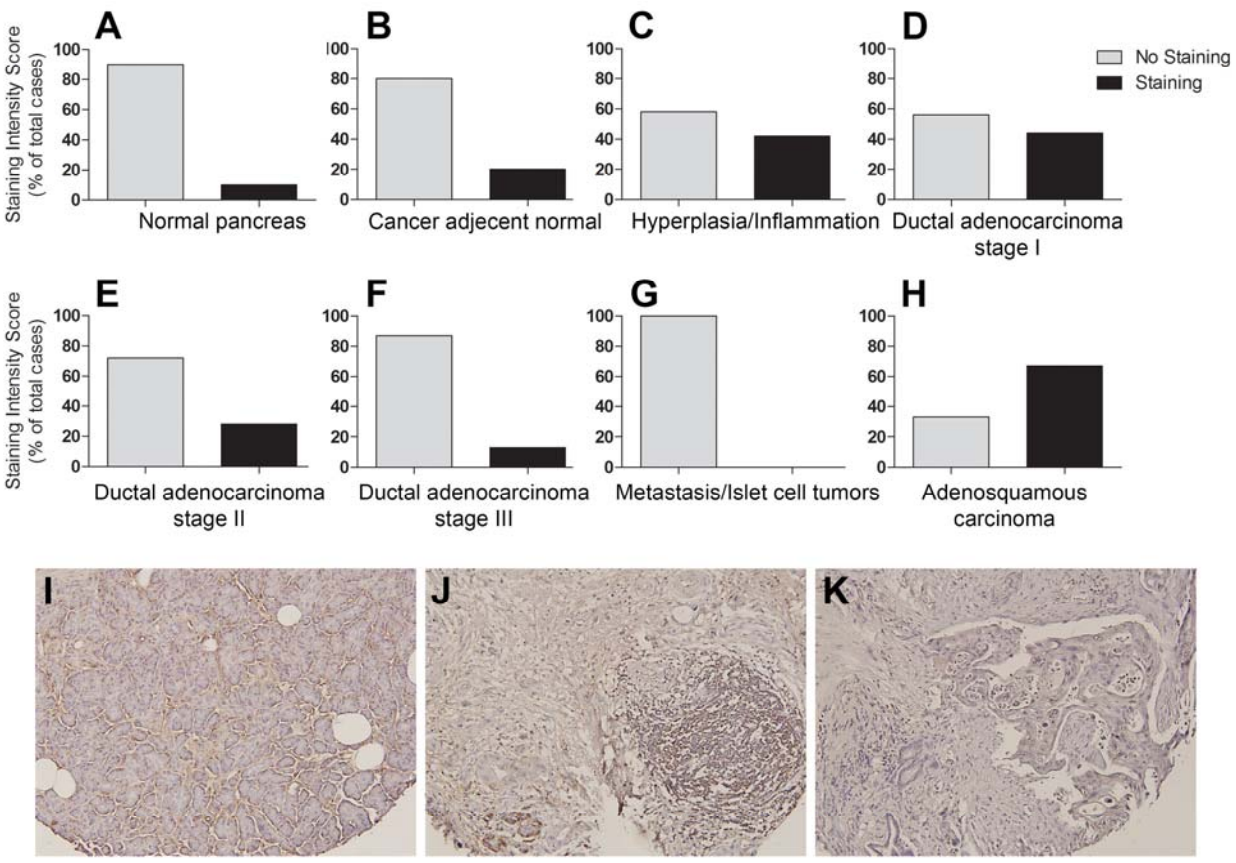

A
B
C
D
E
F
G
H
I
J
K
Staining Intensity Score (% of total cases)
No Staining
Staining
Normal pancreas
Cancer adjecent normal
Hyperplasia/Inflammation
Ductal adenocarcinoma stage I
Ductal adenocarcinoma stage II
Ductal adenocarcinoma stage III
Metastasis/Islet cell tumors
Adenosquamous carcinoma

Figure 3-5: Immunohistochemical staining of OATP1B3 on a pancreas tissue microarray. Tissue arrays containing paraffin-embedded sections were stained for OATP1B3 using immunohistochemistry. Staining intensity from the tissue microarray was scored as negative or positive staining. Percentage was calculated by the number of stained specimens compared to total number of specimens in A) normal pancreatic tissue, B) normal pancreas adjacent to cancer tissue, C) hyperplasia and/or inflammation, D) ductal adenocarcinoma stage one, E) ductal adenocarcinoma stage two, F) ductal adenocarcinoma stage three, G) metastatic tumor from pancreas and/or islet cell tumors, and H) pancreatic adenosquamous carcinoma. Representative photomicrographs of I) chronic pancreatitis, J) ductal adenocarcinoma stage one, and K) ductal adenocarcinoma stage three.

3.4: Discussion

Pancreatic cancer remains a difficult cancer to treat clinically with multidrug resistance as one of the leading causes of relapse in these patients (Jemal et al., 2009). The present study addressed the question of which major OATPs are expressed in pancreatic adenocarcinoma and to what extent OATP1B3 would be expressed in the different types and stages of pancreatic cancer. The results suggest that certain OATPs can potentially be used as a target to treat early stage pancreatic adenocarcinomas with cytotoxic chemotherapeutic drugs that are substrates of these OATPs. In addition, the results indicate that OATP1B3 may serve as a potential biomarker for the development of early stage pancreatic adenocarcinoma.

Our first set of results demonstrates that the four major OATPs expressed in pancreatic adenocarcinoma include OATP1B3, OATP2A1, OATP3A1 and OATP4A1. Although expression of the four OATPs at the messenger RNA level was not statistically significant between normal pancreas and pancreatic adenocarcinoma, we found that OATP1B3, OATP2A1, OATP3A1 and OATP4A1 expression at the protein level was increased in adenocarcinoma compared to normal tissue as evidenced by immunofluorescence staining. Although mRNA expression levels can be useful for preliminary assessment of OATP expression in tissues, it remains important to assess the expression of OATPs at their functional protein level.

OATP2A1 mainly functions in the transport of prostaglandins and is expressed ubiquitously throughout the body. Initial studies demonstrated that at the mRNA level OATP2A1 is expressed in many organs including normal pancreas (Schuster, 2002). Follow-up studies showed OATP2A1 to be up-regulated in numerous different cancers as well as in malignant breast cancer tissue compared with its adjacent nonmalignant breast tissue (Wlcek et al., 2008). Both OATP3A1 and OATP4A1 are also ubiquitously expressed in a variety of different tissues in

the body. Several reports indicate that both OATPs also share similar expression patterns in cancer and are expressed in the breast cancer cell lines MCF-7 and T-47D (Pizzagalli et al., 2003; Nozawa et al., 2004), in malignant breast tumor tissue (Kindla et al., 2011b), in malignant osteosarcoma cell lines (Liedauer et al., 2009) and in a variety of gastrointestinal cancer cell lines as well as in the pancreatic cancer cell line GI-103 (Tamai et al., 2000). Due to the ability of many OATPs to transport hormones and their conjugates, it has been hypothesized that the expression of OATPs in cancer tissue could potentially contribute to the proliferation of hormone-dependent cancers (Nozawa et al., 2004; Hamada et al., 2008). Thus, one potential therapeutic approach would be to develop OATP inhibitors to improve overall survival for patients with OATP-expressing cancers (Nozawa et al., 2005b; Obaidat et al., 2012). Another potential approach for OATP-mediated cancer therapy would be the targeting of OATP-expressing cancers with cytotoxic cancer drugs that are substrates of these OATPs (Obaidat et al., 2012). However, so far there are no reports available yet that demonstrate transport of cancer drugs by OATP2A1, OATP3A1 or OATP4A1.

OATP1B3 did get increased attention recently, owing to its expression in a wide variety of cancers while its expression under normal physiological conditions is exclusive to the liver. Combined with this expression pattern, its ability to transport several cancer drugs, namely methotrexate (Abe et al., 2001; Sasaki et al., 2004), paclitaxel, docetaxel (Smith et al., 2005; Letschert et al., 2006) and imatinib (Hu et al., 2008) makes OATP1B3 a more appropriate target for the delivery of anticancer drugs that mainly affect rapidly dividing cells. In 2001, Abe et al. were the first to demonstrate OATP1B3 expression in several pancreatic cancer cell lines by northern blot analysis. They also confirmed OATP1B3 protein expression in a single case of pancreatic cancer using immunohistochemical staining (Abe et al., 2001). Recently Kounnis et al. evaluated the expression of the three well characterized OATP1A2, OATP1B1 and OATP1B3 in two pancreatic cancer cell lines and in several biopsies of pancreatic cancer tissue

(Kounnis et al., 2011). They concluded that all three OATPs are expressed in pancreatic cancer, but some discrepancies remain due to the use of antibodies with overlapping specificities or antibodies that have not been validated. In the present study, we confirmed their finding that OATP1B3 is expressed in pancreatic cancer tissue using a different OATP1B3 specific antibody. Furthermore, we were among the first to show that expression of OATP1B3 differs in different tumor types and stages of pancreatic cancer. Interestingly, our results showed the highest OATP1B3 expression in tissues that have pathological risk factors for the development of pancreatic cancer including tissues with mild and chronic hyperplasia and inflammation. In addition, OATP1B3 was expressed at highest levels in stage one followed by stage two pancreatic adenocarcinoma with little to no staining in stage three pancreatic adenocarcinoma or metastatic tissue. These results are in accordance with findings reported for the expression of OATP1B3 in colorectal cancer stages where OATP1B3 expression was highest in lower stage colorectal tumors (Lockhart et al., 2008). Another study demonstrated an increased trend of OATP1B3 expression in precancerous colon polyps (Ballestero et al., 2006). In yet another study, OATP1B3 expression was highest in stage one and two breast carcinoma compared to stage three breast carcinoma (Muto et al., 2007). Collectively, these data suggest that OATP1B3 may be used as a potential diagnostic marker in early stage cancer detection.

In future studies it would be interesting to investigate the regulation of OATP1B3 expression during the development of pancreatic ductal adenocarcinoma. Such studies should examine which signaling pathways regulate OATP1B3 expression during acinar to ductal metaplasia (ADM), the initial process of pancreatic adenocarcinoma development, where pancreatic acinar cells with chronic inflammation undergo morphogenesis into abnormal cells with ductal phenotypes (Reichert and Rustgi, 2011) and might identify additional therapeutic targets.

In conclusion, OATPs may serve as potential diagnostic markers and therapeutic targets to improve pancreatic cancer therapy. In particular targeting OATP1B3 which has been shown to transport several anticancer drugs could be as useful as targeting efflux transporters in cancer therapy. Furthermore, our findings are the basis for further investigation of OATP1B3 regulation in pancreatic cancer and for further evaluation of OATP1B3 as a diagnostic marker for early cancer detection.

Chapter 4

The Anticancer Drugs Etoposide, Oxaliplatin and Plicamycin are Substrates of OATP1B3

4.1: Abstract

Organic anion transporting polypeptide 1B3 (OATP1B3) is a liver-specific transport protein that has a very diverse substrate specificity. Because of its ability to transport a multitude of structurally diverse xenobiotics, OATP1B3 plays a significant role in drug disposition and is considered a site for drug-drug interactions. Additionally, based on recent evidence that it is expressed in different cancer tissues OATP1B3 was suggested to be a potential therapeutic target for cancer drug delivery. In this study, we examined the interactions of 89 FDA approved oncology drugs on the function of OATP1B3 and compared cell viability of wild-type with OATP1B3-expressing Chinese Hamster Ovary (CHO) cells in the presence of these drugs. Uptake of two model substrates estradiol-17β-glucuronide and estrone-3-sulfate was inhibited by several of the anticancer drugs. However, the results of the inhibition kinetics were not predictive for known substrates. Therefore, the more direct readout of increased cytotoxicity for OATP1B3-expressing cells as compared to wild-type cells was used and bleomycin, etoposide, oxaliplatin, plicamycin and thioguanine were identified as the most potent cytotoxic compounds. Furthermore, by measuring uptake into OATP1B3-expressing cells etoposide, oxaliplatin and plicamycin could be demonstrated to be substrates of OATP1B3. These results suggest that OATP1B3-mediated transport may be involved in the disposition and hepatotoxicity of some of these anticancer drugs.

4.2: Introduction

Cancer is one of the leading causes of death and based on projections, cancer burden will continue to increase (Boyle and Levin, 2008). Many patients with non-localized or metastatic cancer often times do not respond well to conventional chemotherapy. Several of these cases may be attributed to multidrug resistance whereby an increased efflux of chemotherapy drugs from the cancer cells via efflux transporters leads to ineffective therapeutic responses (Szakacs et al., 2006). Research aimed at inhibiting these efflux transporters has shown little promise in overcoming this clinical problem. Consequently, different therapeutic approaches are sought to improve anticancer drug uptake into cancer cells. Members of the Organic Anion Transporting Polypeptide (OATP) superfamily of uptake transporters have been shown to be up-regulated in numerous different cancer types and are considered as potential therapeutic targets for anticancer drug delivery into cancer cells.

OATPs are multispecific transport proteins that mediate the Na^+-independent uptake of large amphipathic organic anions into cells. They form a superfamily with eleven known human OATPs that are expressed in a wide range of epithelial tissues in the body (Hagenbuch and Meier, 2003). A majority of OATP family members are ubiquitously expressed. However, certain OATPs have restricted expression to a specific tissue type. OATP expression and polymorphisms have been associated with altered bioavailability of drugs, stressing the importance of these transporters in drug disposition and ultimately therapy effectiveness. Additionally, studies have shown single nucleotide polymorphisms (SNPs) of OATPs that have been associated with altered drug disposition of several anticancer drugs. Subsequent studies demonstrated the ability of OATPs to transport anticancer drugs. OATP1B3 has been implicated in the transport of rapamycin, SN-38, imatinib, methotrexate, paclitaxel and docetaxel (Abe et al.,

2001; Smith et al., 2005; Letschert et al., 2006; Hu et al., 2008; Yamaguchi et al., 2008; Oswald et al., 2010).

Because of the up-regulated expression of OATPs in cancer cells and their ability to transport anticancer drugs, there has been an increased interest in these transporters as potential targets for anticancer drug therapy. Therefore, a potential mechanism of OATP-mediated anticancer therapy could be through treating OATP-expressing cancers with known anticancer drug substrates. Evidence that OATP-mediated transport can be stimulated via small molecules presents another potential mechanism for increasing drug delivery into cancer cells (Gui et al., 2008; Roth et al., 2011a).

OATP1B3 is an example of a tissue-specific OATP whereby its expression is restricted to the liver, specifically at the basolateral membrane of hepatocytes (Abe et al., 1999; Hsiang et al., 1999; Konig et al., 2000b). OATP1B3 is thought to play a role in the clearance of drugs from blood into hepatocytes for further metabolism and excretion. Although under normal physiological conditions OATP1B3 is expressed exclusively in the liver, its expression has been shown to be down-regulated in hepatocellular carcinomas (HCC), possibly owing to the dedifferentiation of HCC cells (Cui et al., 2003; Vavricka et al., 2004; Zollner et al., 2005). Despite the decreased expression in HCC cells, OATP1B3 has been shown to be up-regulated in a wide variety of different cancers including gastro-intestinal cancers (Abe et al., 2001), colorectal cancer (Lee et al., 2008), prostate cancer (Hamada et al., 2008), breast cancer (Muto et al., 2007) and non-small cell lung cancer (Monks et al., 2007). As a result, OATP1B3 is considered the most attractive candidate for drug targeting into cancer cells. In this study we aimed at identifying novel anticancer drugs substrates of OATP1B3 by screening 89 compounds of the NCI/DTP approved oncology drug set.

4.3: Results

4.3.1 Effect of the NCI/DTP oncology drug set on OATP1B3-mediated uptake

To determine the effects of the oncology drug set on OATP1B3-mediated transport, we measured uptake of 1 μM estradiol-17β-glucuronide into OATP1B3-expressing or wild-type CHO cells in the absence or presence of 100 μM anticancer drug under initial linear rate conditions. Rifampicin, a known inhibitor of OATP-mediated transport, served as a positive control in these experiments. Anticancer drugs with greater than 50% inhibition of OATP1B3-mediated estradiol-17β-glucuronide uptake included rapamycin, teniposide, actinomycin D, vinorelbine, megestrol, tretinoin, docetaxel, celecoxib, mitoxantrone, gefitinib, dasatinib, imatinib, plicamycin, vinblastine, vincristine and ixabepilone (Figure 4-1). Of interest, OATP1B3-mediated uptake of estradiol-17β-glucuronide was unaffected by many anticancer drugs but strongly stimulated by irinotecan and vorinostat. Based on previous studies that demonstrated substrate-dependent effects of compounds on OATP-mediated transport (Gui et al., 2008; Roth et al., 2011a; Roth et al., 2011b), we determined the effects of the oncology drug set on the uptake of an additional model substrate of OATP1B3, estrone-3-sulfate (0.1 μM). A majority of the anticancer drugs inhibited estrone-3-sulfate uptake whereas only letrozole and sorafenib stimulated estrone-3-sulfate uptake (Figure 4-2). We confirmed concentration-dependent inhibition of four anticancer drugs from the original screen (Figure 4-3) and determined IC_{50} values for actinomycin D, celecoxib, rapamycin and vinblastine to be 26.0 ± 0.2, 32.4 ± 0.3, 2.4 ± 0.2 and 28.1 ± 0.2 μM, respectively. To determine the mechanism(s) of substrate-dependent effects of these anticancer drug inhibitors on OATP1B3-mediated uptake we preformed inhibition kinetics of the two model substrates, estradiol-17β-glucuronide (Figure 4-4) and estrone-3-sulfate (Figure 4-5). Inhibition of OATP1B3-mediated estradiol-17β-glucuronide uptake was non-competitive for rapamycin (Figure 4-4B), teniposide (Figure 4-4C) and

vinblastine (Figure 4-4D), All three compounds reduced the V_{max}, whereas they had no effect on the K_m value (Table 4-1). Actinomycin D, however, competitively inhibited OATP1B3-mediated uptake of estradiol-17β-glucuronide by increasing the K_m value from 12.8 ± 4.8 to 21.3 ± 8.5 μM while the V_{max} value was not affected (213.9 ± 29.5 and 211.4 ± 38.7 pmol/mg*min) (Figure 4-4A; Table 4-1). Interestingly, inhibition of OATP1B3-mediated estrone-3-sulfate was non-competitive by actinomycin D (Figure 4-5A), teniposide (Figure 4-5C) and vinblastine (Figure 4-5 D), whereas rapamycin inhibition was competitive (Figure 4-5B; Table 4-1).

Figure 4-1:

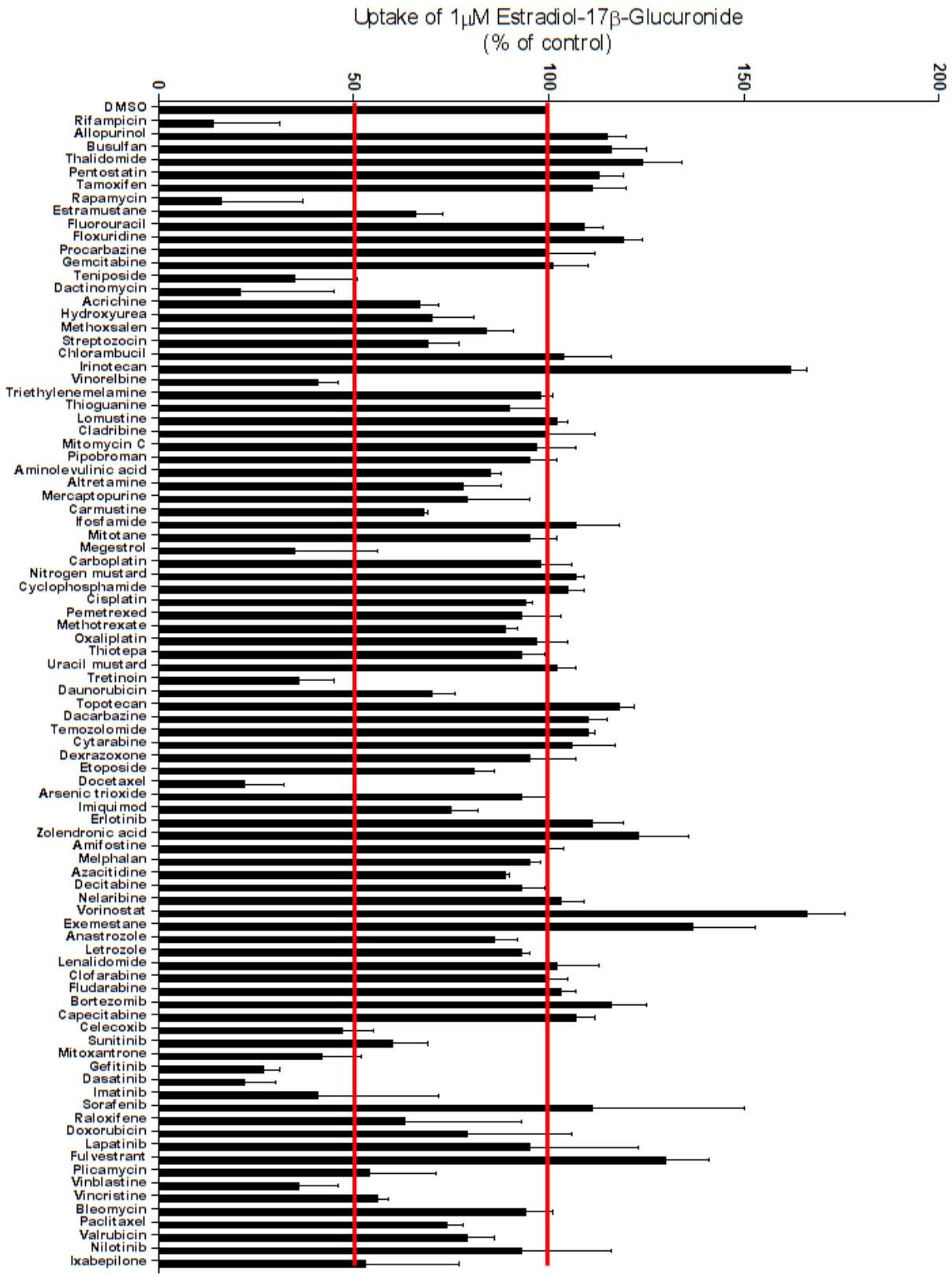

Uptake of 1μM Estradiol-17β-Glucuronide
(% of control)
0
50
100
150
200
DMSO
Rifampicin
Allopurinol
Busulfan
Thalidomide
Pentostatin
Tamoxifen
Rapamycin
Estramustane
Fluorouracil
Floxuridine
Procarbazine
Gemcitabine
Teniposide
Dactinomycin
Acrichine
Hydroxyurea
Methoxsalen
Streptozocin
Chlorambucil
Irinotecan
Vinorelbine
Triethylenemelamine
Thioguanine
Lomustine
Cladribine
Mitomycin C
Pipobroman
Aminolevulinic acid
Altretamine
Mercaptopurine
Carmustine
Ifosfamide
Mitotane
Megestrol
Carboplatin
Nitrogen mustard
Cyclophosphamide
Cisplatin
Pemetrexed
Methotrexate
Oxaliplatin
Thiotepa
Uracil mustard
Tretinoin
Daunorubicin
Topotecan
Dacarbazine
Temozolomide
Cytarabine
Dexrazoxone
Etoposide
Docetaxel
Arsenic trioxide
Imiquimod
Erlotinib
Zolendronic acid
Amifostine
Melphalan
Azacitidine
Decitabine
Nelaribine
Vorinostat
Exemestane
Anastrozole
Letrozole
Lenalidomide
Clofarabine
Fludarabine
Bortezomib
Capecitabine
Celecoxib
Sunitinib
Mitoxantrone
Gefitinib
Dasatinib
Imatinib
Sorafenib
Raloxifene
Doxorubicin
Lapatinib
Fulvestrant
Plicamycin
Vinblastine
Vincristine
Bleomycin
Paclitaxel
Valrubicin
Nilotinib
Ixabepilone

Figure 4-1: Effect of anticancer drugs from the NCI/DTP oncology drug set on OATP1B3-mediated estradiol-17β-glucuronide transport. Wild-type CHO cells and OATP1B3-expressing CHO cells were incubated with 1 μM [^{3}H]estradiol-17β-glucuronide in the presence of 100 μM of anticancer drug or the vehicle control (1% DMSO) at 37ºC for 2 minutes. Uptake into wild-type cells was subtracted from uptake in OATP1B3-expressing cells to determine OATP1B3-mediated uptake. Values are given as percentage of control and each value is the mean ± SD of triplicate determinations in a single experiment.

Figure 4-2:

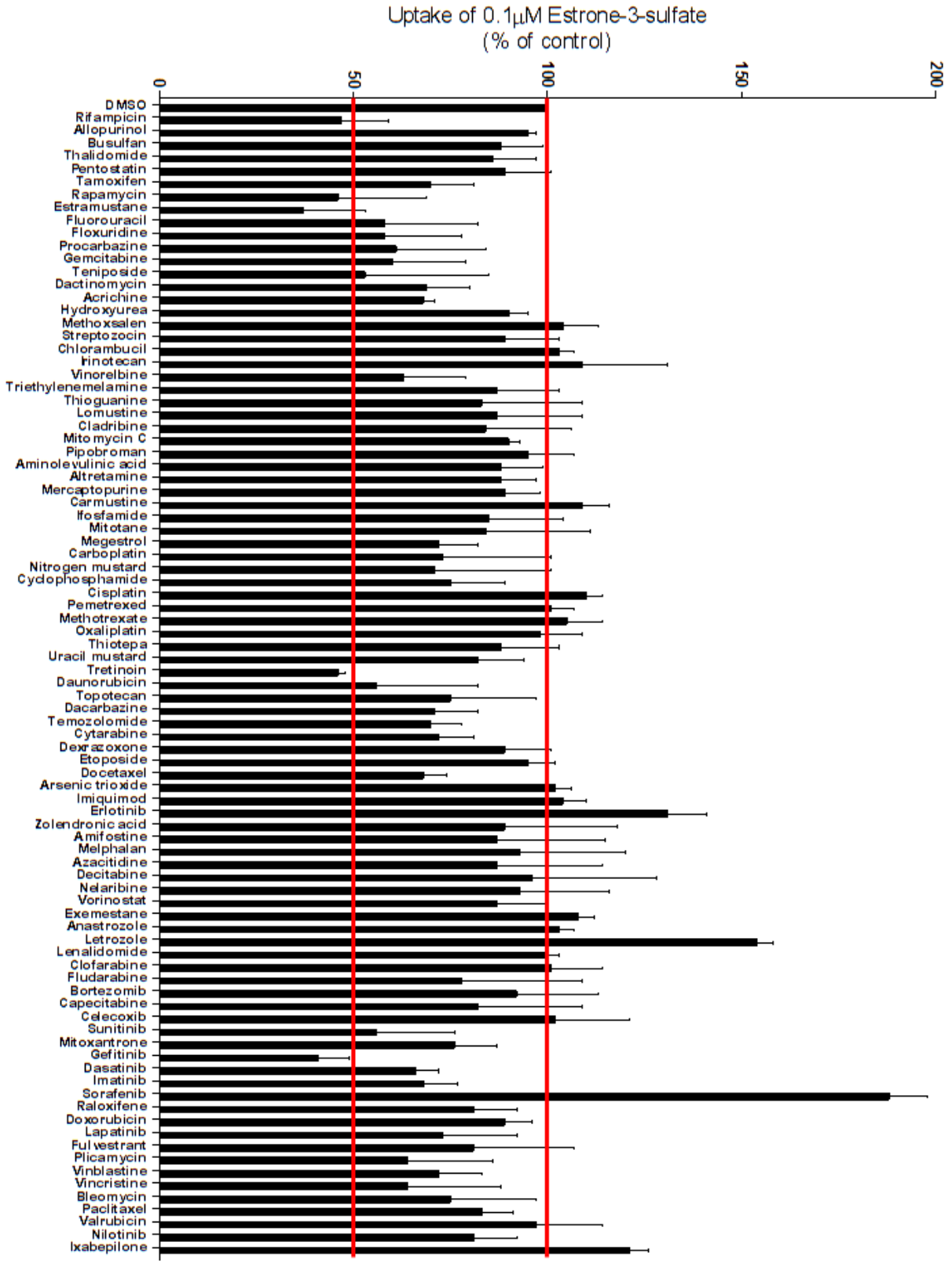

Uptake of 0.1μM Estrone-3-sulfate
(% of control)
0
50
100
150
200
DMSO
Rifampicin
Allopurinol
Busulfan
Thalidomide
Pentostatin
Tamoxifen
Rapamycin
Estramustane
Fluorouracil
Floxuridine
Procarbazine
Gemcitabine
Teniposide
Dactinomycin
Acrichine
Hydroxyurea
Methoxsalen
Streptozocin
Chlorambucil
Irinotecan
Vinorelbine
Triethylenemelamine
Thioguanine
Lomustine
Cladribine
Mitomycin C
Pipobroman
Aminolevulinic acid
Altretamine
Mercaptopurine
Carmustine
Ifosfamide
Mitotane
Megestrol
Carboplatin
Nitrogen mustard
Cyclophosphamide
Cisplatin
Pemetrexed
Methotrexate
Oxaliplatin
Thiotepa
Uracil mustard
Tretinoin
Daunorubicin
Topotecan
Dacarbazine
Temozolomide
Cytarabine
Dexrazoxone
Etoposide
Docetaxel
Arsenic trioxide
Imiquimod
Erlotinib
Zolendronic acid
Amifostine
Melphalan
Azacitidine
Decitabine
Nelaribine
Vorinostat
Exemestane
Anastrozole
Letrozole
Lenalidomide
Clofarabine
Fludarabine
Bortezomib
Capecitabine
Celecoxib
Sunitinib
Mitoxantrone
Gefitinib
Dasatinib
Imatinib
Sorafenib
Raloxifene
Doxorubicin
Lapatinib
Fulvestrant
Plicamycin
Vinblastine
Vincristine
Bleomycin
Paclitaxel
Valrubicin
Nilotinib
Ixabepilone

Figure 4-2: Effect of anticancer drugs from the NCI/DTP oncology drug set on OATP1B3-mediated estrone-3-sulfate transport. Wild-type CHO cells and OATP1B3-expressing CHO cells were incubated with 0.1 μM [^{3}H]estrone-3-sulfate in the presence of 100 μM of anticancer drug or the vehicle control (1% DMSO) at 37ºC for 2 minutes. Uptake into wild-type cells was subtracted from uptake in OATP1B3-expressing cells to determine OATP1B3-mediated uptake. Values are given as percentage of control and each value is the mean ± SD of triplicate determinations in a single experiment.

Figure 4-3:

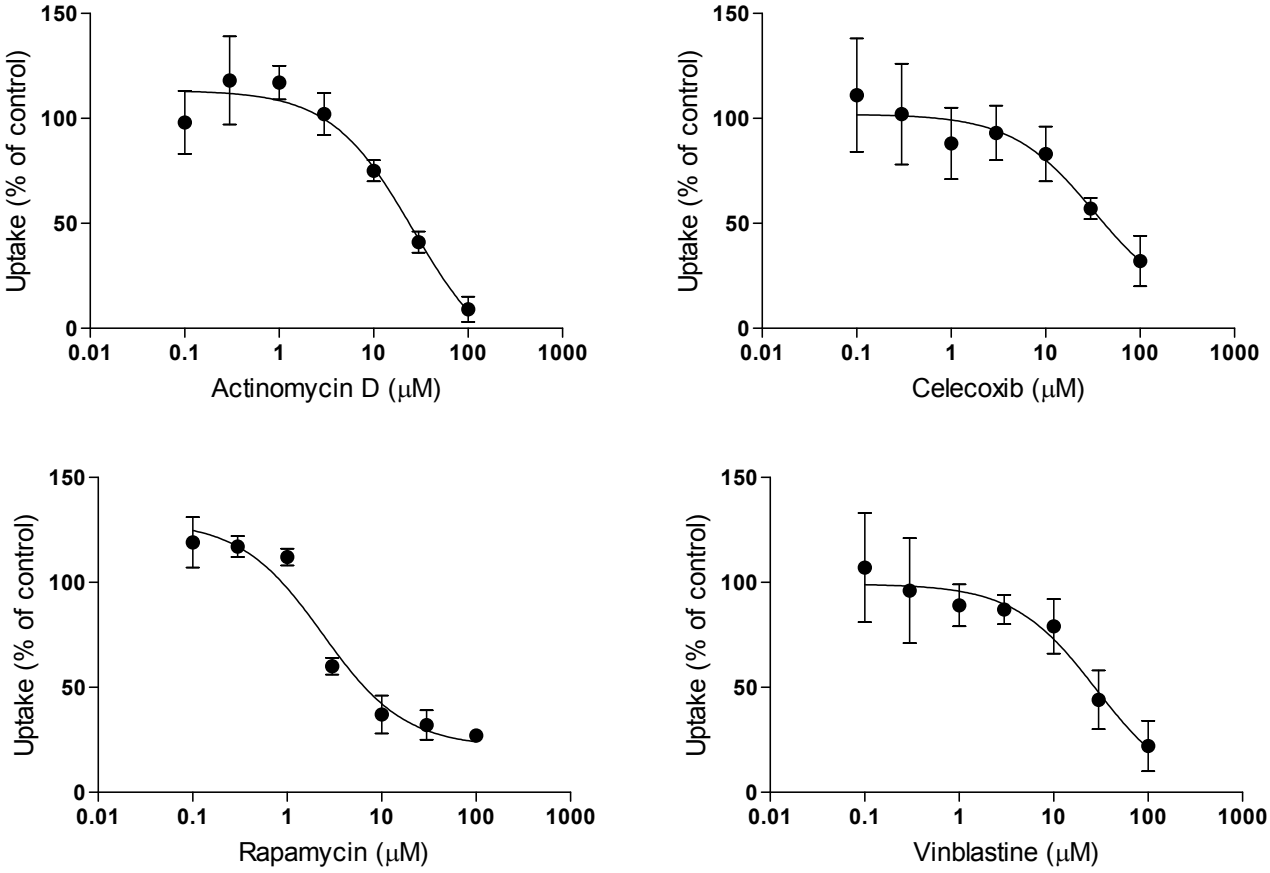

Uptake (% of control)
150
100
50
0
0.01
0.1
1
10
100
1000
Actinomycin D (μM)
Celecoxib (μM)
Rapamycin (μM)
Vinblastine (μM)

Figure 4-3: Concentration-dependent effect of actinomycin D, celecoxib, rapamycin and vinblastine on OATP1B3-mediated estradiol-17β-glucuronide uptake. Cells were coincubated with 1 μM [^{3}H]estradiol-17β-glucuronide and increasing concentrations of actinomycin D, celecoxib, rapamycin and vinblastine for 1 minute. Net OATP1B3-mediated uptake was expressed as percentage of control after subtracting values obtained from wild-type cells from values obtained from OATP1B3-expressing cells. Values are means ± SD of three independent experiments preformed in triplicate.

Figure 4-4:

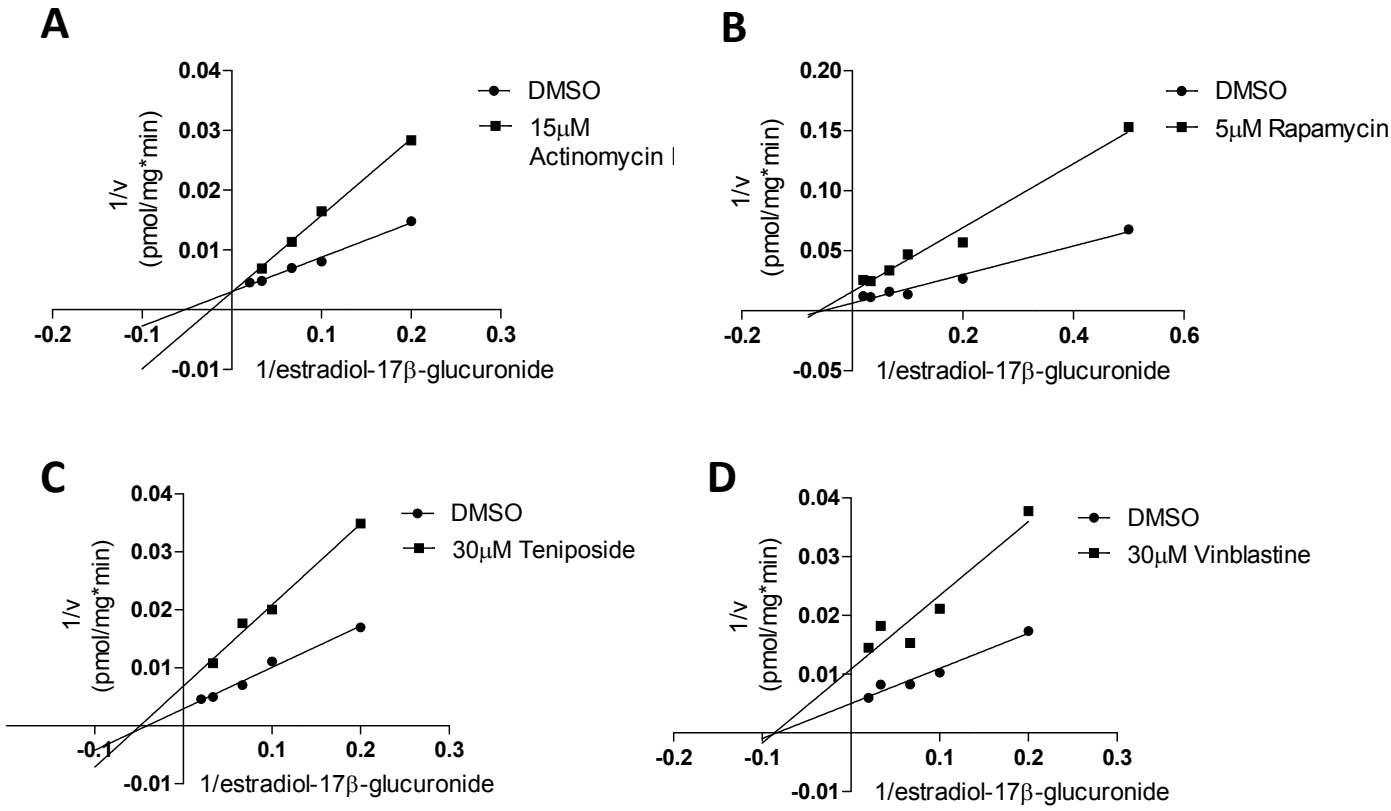

A
0.04
0.03
0.02
0.01
-0.01
1/v
(pmol/mg*min)
-0.2
-0.1
0.1
0.2
0.3
1/estradiol-17β-glucuronide
DMSO
15μM
Actinomycin
B
0.20
0.15
0.10
0.05
-0.05
1/v
(pmol/mg*min)
-0.2
0.2
0.4
0.6
1/estradiol-17β-glucuronide
DMSO
5μM Rapamycin
C
0.04
0.03
0.02
0.01
-0.01
1/v
(pmol/mg*min)
-0.1
0.1
0.2
0.3
1/estradiol-17β-glucuronide
DMSO
30μM Teniposide
D
0.04
0.03
0.02
0.01
-0.01
1/v
(pmol/mg*min)
-0.2
-0.1
0.1
0.2
0.3
1/estradiol-17β-glucuronide
DMSO
30μM Vinblastine

Figure 4-4: Effect of actinomycin D, rapaymcin, teniposide and vinblastine on OATP1B3-mediated uptake of estradiol-17β-glucuronide. Cells were incubated with increasing concentrations of estradiol-17β-glucuronide in the presence of A) 15 μM actinomycin D B) 5 μM rapamycin C) 30 μM teniposide and D) 30 μM vinblastine or vehicle control (1% DMSO) for 20 seconds. Net OATP1B3 uptake was measured by subtracting values obtained from wild-type cells and values were plotted by linear regression Dixon plots to determine K_m and V_{max} values. Values are means ± SD of one or two independent experiments preformed in triplicate.

Figure 4-5:

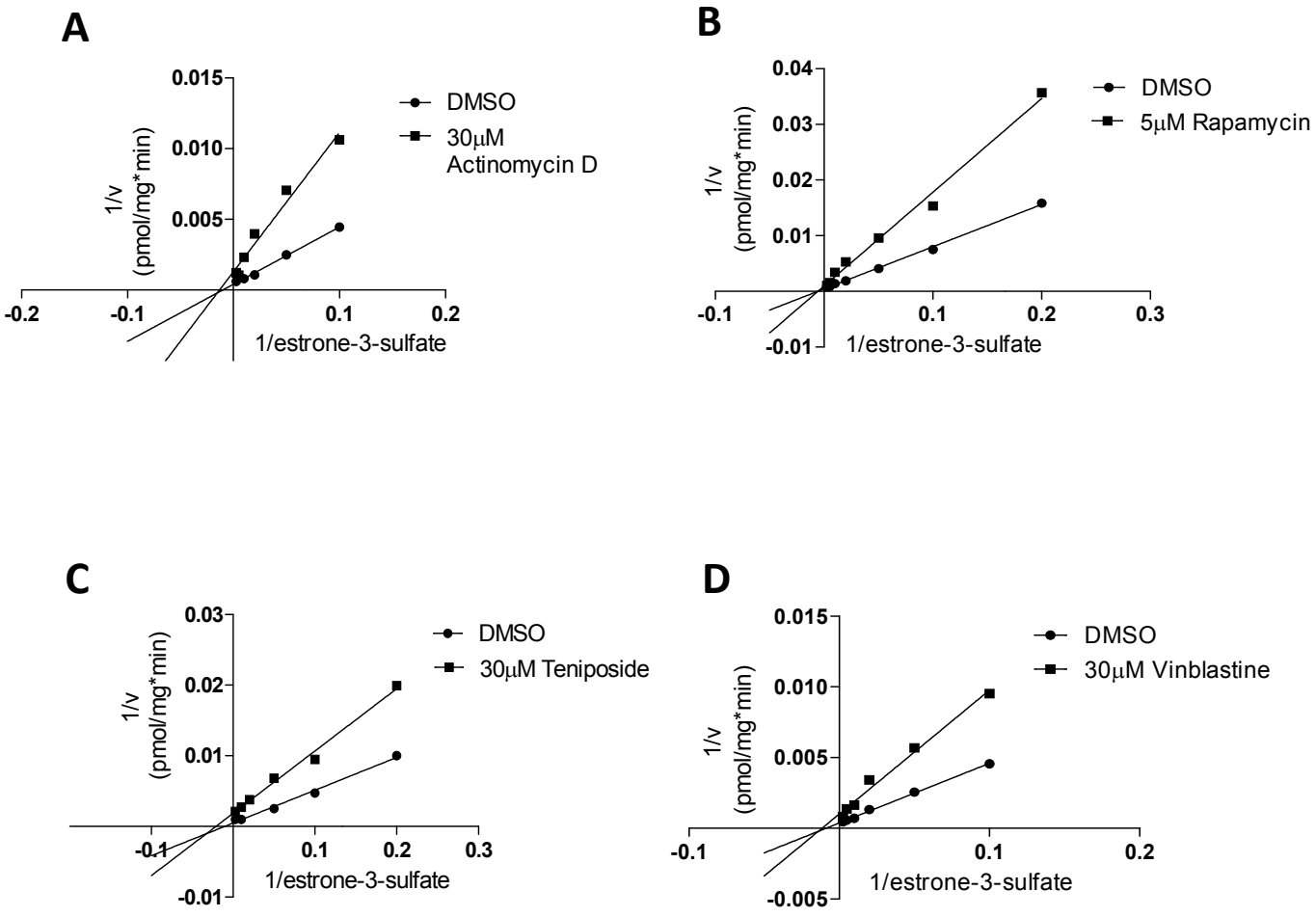

A
0.015
0.010
0.005
1/v
(pmol/mg*min)
-0.2
-0.1
0.1
0.2
1/estrone-3-sulfate
DMSO
30μM
Actinomycin D
B
0.04
0.03
0.02
0.01
1/v
(pmol/mg*min)
-0.1
0.1
0.2
0.3
-0.01
1/estrone-3-sulfate
DMSO
5μM Rapamycin
C
0.03
0.02
0.01
1/v
(pmol/mg*min)
-0.1
0.1
0.2
0.3
-0.01
1/estrone-3-sulfate
DMSO
30μM Teniposide
D
0.015
0.010
0.005
1/v
(pmol/mg*min)
-0.1
0.1
0.2
-0.005
1/estrone-3-sulfate
DMSO
30μM Vinblastine

Figure 4-5: Effect of actinomycin D, rapaymcin, teniposide and vinblastine on OATP1B3-mediated uptake of estrone-3-sulfate. Cells were incubated with increasing concentrations of estrone-3-sulfate in the presence of A) 30 μM actinomycin D B) 5 μM rapamycin C) 30 μM teniposide and D) 30 μM vinblastine or vehicle control (1% DMSO) for 20 seconds. Net OATP1B3 uptake was measured by subtracting values obtained from wild-type cells and values were plotted by linear regression Dixon plots to determine K_m and V_{max} values. Values are means ± SD of one or two independent experiments preformed in triplicate.

Table 4-1: Kinetics of OATP1B3-mediated transport in the absence and presence of anticancer drug inhibitors.

Estradiol-17β-glucuronide	**K_m (μM)**	**V_{max} (pmol/mg*min)**	**Inhibition Type**
DMSO	12.8 ± 4.8	213.9 ± 29.5	
5μM Rapamycin	11.0 ± 2.8	50.5 ± 4.7	Non-competitive
15μM Actinomycin D	21.3 ± 8.5	211.4 ± 38.7	Competitive
30μM Teniposide	7.0 ± 4.4	84.2 ± 14.1	Non-competitive
30μM Vinblastine	7.0 ± 3.0	77.9 ± 10.2	Non-competitive
Estrone-3-sulfate	**K_m (μM)**	**V_{max} (pmol/mg*min)**	**Inhibition Type**
DMSO	68.5 ± 18.5	1839 ± 171.1	
5μM Rapamycin	549.3 ± 157.8	2241 ± 424.3	Competitive
15μM Actinomycin D	143.4 ± 80.0	1244 ± 297.5	Non-competitive
30μM Teniposide	39.7 ± 14.5	465.1 ± 49.5	Non-competitive
30μM Vinblastine	202.4 ± 57.0	1543 ± 208.6	Non-competitive

4.3.2 Effect of anticancer drugs on the cell viability of OATP1B3-expressing cells

Because rapamycin is a known substrate of OATP1B3 but exhibited non-competitive inhibition, we concluded that inhibition kinetics would not allow to predict potential substrates of OATP1B3 and we decided to measure the effects of the NCI/DTP oncology drug set on the cell viability of OATP1B3-expressing cells on 384-well plates. The rationale was that if a certain drug would be more cytotoxic to OATP1B3-expressing cell as compared to wild-type cells, this drug had to be a substrate of OATP1B3. The most potent anticancer drugs for OATP1B3-expressing CHO cells were bleomycin, etoposide, oxaliplatin, plicamycin and thioguanine (Figure 4-6) that exhibited greater cytotoxicity to the OATP1B3-expressing cells than wild-type cells. The potency of the anticancer drugs in the cell viability screen was based on their respective IC_{50} values comparing OATP1B3-expressing cells to wild-type cells (Table 4-2). Plicamycin was the most potent with an IC_{50} value of 0.2 μM for OATP1B3-expressing cells and an IC_{50} of 3.5 μM for wild-type cells. Oxaliplatin which had a nearly 10 fold lower IC_{50} value for OATP1B3-expressing cells compared to wild-type cells, was followed by bleomycin, thioguanine and etoposide with IC_{50} values that were 8.3, 5.9 and 3.6 fold lower for OATP1B3-expressing cells.

Figure 4-6:

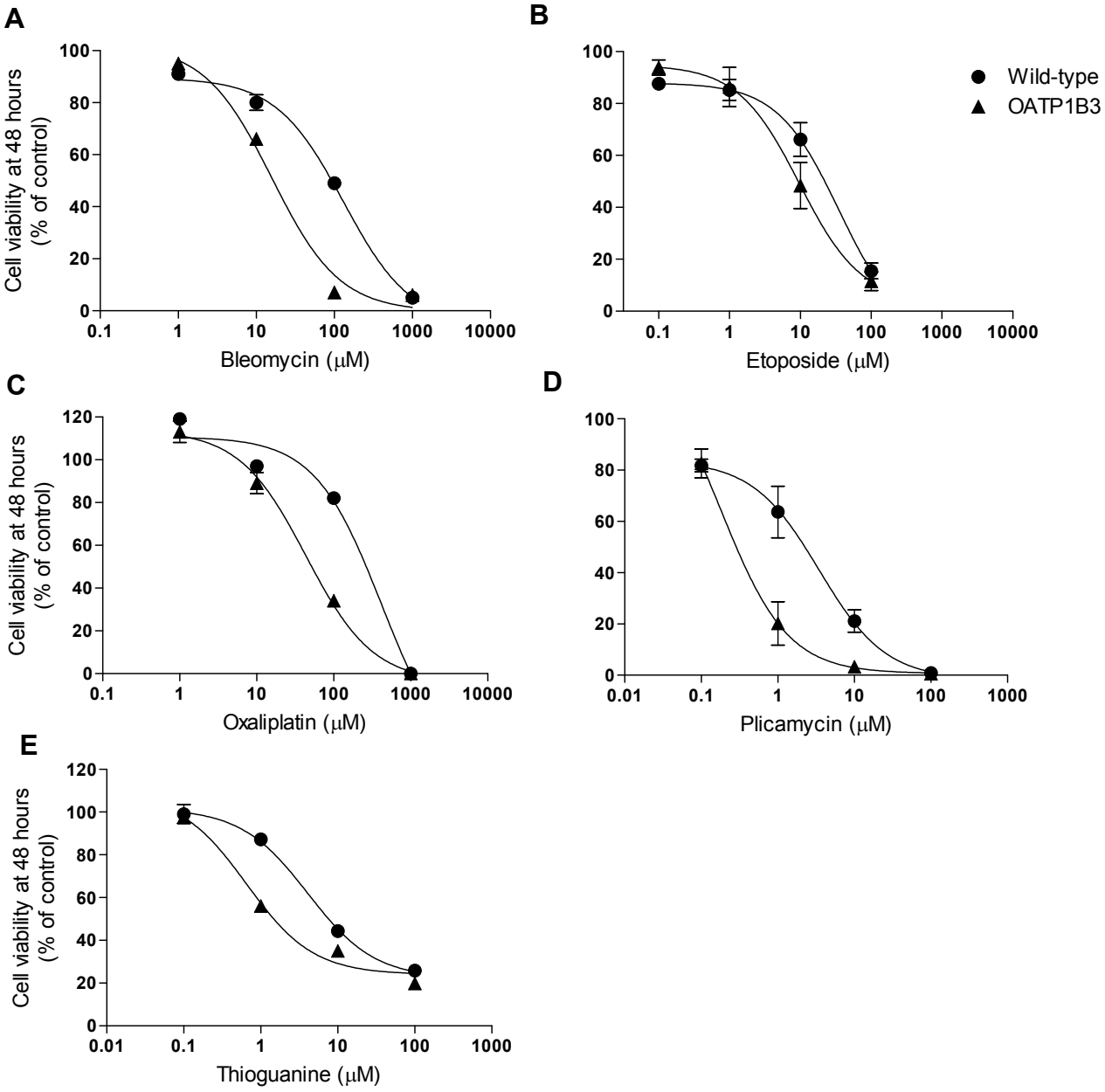

A
B
C
D
E
Cell viability at 48 hours (% of control)
Bleomycin (μM)
Etoposide (μM)
Oxaliplatin (μM)
Plicamycin (μM)
Thioguanine (μM)
Wild-type
OATP1B3

Figure 4-6: Effect of anticancer drugs on cell viability of wild-type and OATP1B3-expressing CHO cells. Cell viability was measured with increasing concentrations of A) bleomycin, B) etoposide, C) oxaliplatin, D) plicamycin and E) thioguanine for 48 hours on 384-well plates of wild-type (circles) and OATP1B3- expressing (triangles) CHO cells. Values are given as percent of control and are means ± SD of at least three independent experiments.

Table 4-2: Effect of Anticancer Drugs on the Cell Viability of OATP1B3-expressing CHO cells.

	Wild-type	OATP1B3	
	IC_{50} (μM)	IC_{50} (μM)	WT/OATP1B3
Bleomycin	131 ± 0.1	15.8 ± 0.2	8.3
Etoposide	34.8 ± 0.2	9.7 ± 0.2	3.6
Oxaliplatin	421 ± 0.5	43.8 ± 0.1	9.6
Plicamycin	3.5 ± 0.2	0.2 ± 0.3	17.5
Thioguanine	4.1 ± 0.1	0.7 ± 0.1	5.9

4.3.3 OATP1B3 transports etoposide, oxaliplatin and plicamycin

The anticancer drugs that were more cytotoxic for OATP1B3-expressing cells compared to wild-type cells had to gain access to the cytoplasm via OATP1B3-mediated uptake and are therefore substrate of OATP1B3. Because etoposide and oxaliplatin were readily available radiolabeled, we tested whether these two anticancer drugs were transported by OATP1B3 over a time period of two hours. We measured accumulation of 1 μM oxaliplatin or 0.6 μM etoposide in the OATP1B3-expressing cells and the control cells at different incubation times (Figure 4-7 A and B). As clearly seen in Figure 4-7, uptake of radiolabeled etoposide and oxaliplatin was higher in OATP1B3-expressing cells compared to wild-type at every time point measured. Uptake of both etoposide and oxaliplatin increased with time and was linear for at least 40 seconds and 2 hours, respectively. Additionally, we are currently optimizing an LC/MS-MS method that should allow us to confirm that plicamycin is a substrate of OATP1B3.

Figure 4-7:

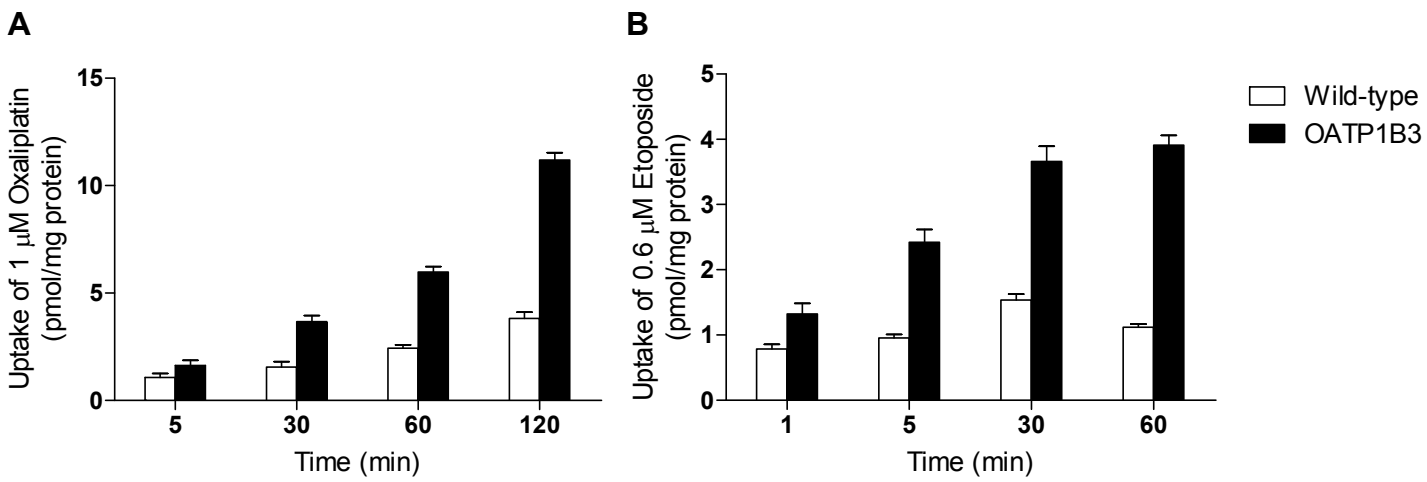

A
B
Uptake of 1 μM Oxaliplatin (pmol/mg protein)
15
10
5
0
5
30
60
120
Time (min)
Uptake of 0.6 μM Etoposide (pmol/mg protein)
5
4
3
2
1
0
1
5
30
60
Time (min)
Wild-type
OATP1B3

Figure 4-7: Time-dependent uptake of etoposide, oxaliplatin by OATP1B3.

Uptake of A) 1 μM oxaliplatin and B) 0.6 μM etoposide was measured at increasing time points on 24-well plates in wild-type (white bars) and OATP1B3-expressing (black bars) CHO cells. Values are given as pmol/mg protein and are means ± SD of triplicate determinations in a single experiment.

4.4: Discussion

In the present study we identified five known anticancer drugs as substrates of OATP1B3 using a cytotoxicity assay that compared the toxicity of the drugs in OATP1B3-expressing CHO cells to wild-type CHO cells. Bleomycin, etoposide, oxaliplatin, plicamycin and thioguanine were all more cytotoxic in OATP1B3-expressing cells and we therefore conclude that these five anticancer drugs are transported into the CHO cells via OATP1B3. Two of these drugs are readily available as radiolabeled compounds and we could confirm that ^{3}H-etoposide and ^{14}C-oxaliplatin are taken up into OATP1B3-expressing cells more efficiently than into wild-type CHO cells in a time dependent manner.

A recent study suggested that etoposide uptake into the liver would occur primarily via OATP1B1 and OATP2B1 with very little transport by OATP1B3 using stable transfected HEK293 cells (Fahrmayr et al., 2012). Our results are in agreement with that study because uptake was slow and increased over one hour (Figure 4-7). Furthermore, another study demonstrated that both mouse and human OCTN2 are able to transport etoposide (Hu et al., 2012). The fact that etoposide is a substrate of several OATPs and of OCTN2 demonstrates that there are overlaps in the substrate specificities of members of the OATP and the OCT families. This suggestion is further supported by our finding that oxaliplatin is a substrate for OATP1B3 because previous studies demonstrated that members of the OCT family including OCTN1, OCTN2, OCT1, OCT2 and OCT3 (Ciarimboli et al., 2005; Yonezawa et al., 2005; Zhang et al., 2006; Filipski et al., 2008; Filipski et al., 2009; Burger et al., 2010; Ciarimboli et al., 2010; Jong et al., 2011) are involved in oxaliplatin transport.

Thioguanine which has been suggested to be a substrate of CNT3 and ENT2 (Fotoohi et al., 2006) seems to be a substrate of OATP1B3 in our assay. This is one of the first documented

overlaps in the substrate specificities of nucleoside transporters and OATPs and further demonstrates that the different xenobiotic transporters are quite promiscuous and have overlapping substrate specificities that go beyond the respective families.

It is important to note that although etoposide and oxaliplatin had no effect on OATP1B3-mediated estrone-3-sulfate and estradiol-17β-glucuronide uptake, they are nevertheless substrates of OATP1B3. This can be explained by differences in the affinities of the different substrates to the transporter or to the fact that they use different substrate binding or translocation pathways and confirm that substrates of OATPs are not necessarily inhibitors of OATP-mediated uptake of all substrates.

Plicamycin, an antitumor antibiotic used to treat testicular cancer and hypercalcemia, is considered the most hepatotoxic chemotherapeutic drug used in the clinic and is therefore rarely used in treatment regimens (Kennedy, 1970; King and Perry, 2001). Our finding that plicamycin is transported by OATP1B3 can explain the disposition of plicamcyin into the liver and thus OATP1B3-mediated uptake of plicamycin into hepatocytes can be considered a mechanism that contributes to its hepatotoxicity in patients treated with this drug.

Our interaction results demonstrate that the majority of the anticancer drugs in the oncology drug set did not affect OATP1B3-mediated substrate uptake while several compounds inhibited, and only four compounds stimulated uptake. Furthermore, we found that the effects were substrate dependent, especially evident for the stimulation of OATP1B3-mediated uptake. While irinotecan and vorinostat stimulated uptake of estradiol-17β-glucuronide, they had no effect on estrone-3-sulfate transport. Similar, letrozole and sorafenib stimulated estrone-3-sulfate uptake but had no effect on estradiol-17β-glucuronide uptake. These results confirm that it is important to test multiple substrates of OATP1B3 when screening for inhibitors and supports

findings from other studies that demonstrated similar evidence of multiple substrate binding sites in OATPs (Gui et al., 2008; Roth et al., 2011a; Roth et al., 2011b).

In conclusion, we have presented strong evidence that bleomycin, etoposide, oxaliplatin, plicamycin and thioguanine are substrates of OATP1B3 and supported these findings with uptake studies involving radiolabeled substrates for etoposide and oxaliplatin. Our data suggest that OATP1B3-mediated transport of plicamycin is a potential mechanism contributing to its hepatotoxicity seen in patients. We also demonstrated that the effect of anticancer drugs on OATP1B3-mediated transport is substrate dependent confirming previous reports from our and from other groups. Because OATP1B3 is expressed in several cancers and because OATP1B3 can transport anticancer drugs, our results suggest that anticancer therapy could potentially be improved by using OATP1B3 as a potential target for the delivery of anticancer drug substrates into OATP1B3-expressing cancers.

Chapter 5

Identification of Cytotoxic Kansas Plant Compounds that are Substrates of Organic Anion Transporting Polypeptide 1B3

5.1: Abstract

Organic Anion Transporting Polypeptide 1B3 (OATP1B3) is a multispecific membrane transport protein that mediates the uptake of numerous drugs and xenobiotics. Normally, OATP1B3 is exclusively expressed in hepatocytes but recent studies have shown that it is also expressed in several different cancers. Cytotoxic compounds that are transported by OATP1B3 could potentially be used to target OATP1B3-expressing cancer cells. Therefore, in this study, we used bioassay-guided fractionation to identify novel cytotoxic compounds from Kansas plant extracts. Crude extracts of Kansas plants were screened in a cytotoxicity assay for their ability to selectively kill OATP1B3-expressing cells. After two rounds of screening we have identified two butanol sub-fractions of the related plants *Rhus aromatica* and *Rhus glabra* that contain cytotoxic compounds that are substrates of OATP1B3. Through further fractionation-screening assays, we plan to isolate pure compounds that are responsible for the cytotoxicity seen in the OATP1B3-expressing cells. Thus far, these results demonstrate that the established fractionation and cytotoxicity screening assay is a good tool for the identification of novel plant-based cytotoxic OATP1B3 substrates and could provide lead compounds for the development of novel anticancer drugs.

5.2: Introduction

Organic anion transporting polypeptides (OATPs) are multispecific drug transporters that mediate the uptake of numerous endogenous and exogenous compounds into cells. OATP1B3, a member of this superfamily of drug transporters, is normally exclusively expressed at the basolateral membrane of human hepatocytes (Hagenbuch and Meier, 2004). Recent studies have demonstrated altered expression of OATP1B3 in numerous different cancer tissues (Obaidat et al., 2012). For example, OATP1B3 expression has been shown to be decreased in hepatocellular carcinoma (Vavricka et al., 2004; Vander Borght et al., 2005; Tsuboyama et al., 2010), while OATP1B3 expression is increased in colon (Lee et al., 2008), prostate (Hamada et al., 2008), breast (Muto et al., 2007) and even bone cancer (Liedauer et al., 2009). Among the multitude of substrates transported by OATP1B3 are the anticancer drugs methotrexate (Abe et al., 2001), paclitaxel, docetaxel (Smith et al., 2005) and imatinib (Hu et al., 2008). Based on the up-regulated expression of OATP1B3 in several cancers and based on its ability to transport anticancer drugs, OATP1B3 has been suggested as a potential therapeutic target for the transport of anticancer drugs into cancer cells.

One way to improve OATP1B3-mediated anticancer therapy would be the use of small molecule stimulators of OATP1B3-mediated anticancer drug transport to increase the uptake of cytotoxic anticancer drugs into cancer cells. Another way could be based on the identification of novel anticancer drugs that are substrates of OATP1B3 and could be used to treat OATP1B3-expressing cancers (Obaidat et al., 2012). Cytotoxic compounds isolated from plants have been previously used to develop anticancer drug treatments. Several anti-microtubule agents including vinblastine and vincristine that were isolated from the plant *Vinca rosea L (Johnson et al., 1963)* or paclitaxel and docetaxel that were isolated from the bark of a Pacific yew tree (*Taxus brevifolia*) (Pazdur et al., 1993) are currently used clinical anticancer drugs for the

treatment of multiple different cancers. Thus, plants are a rich source for the identification of novel anticancer drugs that can be used in the clinic. A previous study using bioassay-guided fractionation isolated and characterized modulators of OATPs from an extract from the plant *Rollinia emarginata* Schlecht (Roth et al., 2011a). In this study, we used a similar approach using cytotoxicity assays as guidance for the fractionation assays to screen a library of Kansas plants for novel cytotoxic substrates of OATP1B3 that potentially could be used as lead compounds to develop anticancer drugs.

5.3: Results

5.3.1: The green tea compound epigallocatechin gallate is toxic for OATP-expressing CHO cells

The most abundant component of green tea, epigallocatechin gallate (EGCG), has been shown to have anti-cancer effects. A recent study identified EGCG as a substrate of OATP1B3 (Roth et al., 2011b). Therefore, we tested whether expression of OATP1B3 would confer toxicity to CHO cells exposed to increasing concentrations of EGCG. As can be seen in Figure 5-1, the viability of CHO cells was dramatically decreased if they expressed either OATP1B1 or OATP1B3. The calculated IC_{50} values were 271 ± 1 μM for wild-type CHO cells, 7.7 ± 0.1 μM for OATP1B1- and 3.1 ±0.1 μM for OATP1B3-expressing CHO cells. Therefore, we used EGCG as a positive control for the screening of the plant extracts.

5.3.2: Identification of Kansas plants with effects on the cell viability of OATP1B3-expressing CHO cells

To identify Kansas plants that are cytotoxic to OATP1B3-expressing cells, we compared the cell viability of OATP1B3-expressing to wild-type CHO cells in the absence or presence of a number of Kansas plant extracts. As can be seen in Figure 5-2, there were plant extracts that had hardly any effect on both CHO wild-type and OATP1B3-expressing cells (e.g. V001 – V012). There were also extracts that killed wild-type and OATP1B3-expressing CHO cells completely (e.g. V361 – V365) and there were extracts like V021, V345, V570, V576, V579, V581 and V585 that were preferentially cytotoxic to OATP1B3-expressing cells.
To confirm the preferentially OATP1B3 cytotoxic plant extracts, we measured cell viability of wild-type and OATP1B3-expressing cells after 48 hrs of incubation in the presence of increasing concentrations of the extracts. We confirmed that the butanol fractions of *Rhus aromatica* (V345) and *Rhus glabra* (V585) were preferentially cytotoxic to OATP1B3-expressing CHO cells (Figure 5-3).

5.3.3: Sub-fractions of *Rhus aromatica* and *Rhus glabra* contain potent cytotoxic substrates of OATP1B3

To identify cytotoxic components of the butanol fractions of *Rhus aromatica* and *Rhus glabra*, the extracts were further separated according to their hydrophobicity using MCI GEL® CHP20P resin and increasing concentrations of methanol. Five fractions of *Rhus aromatica* and six fractions for *Rhus glabra* were tested using the same cytotoxicity assay and the results are summarized in Figure 5-4. Most of the tested sub-fractions exhibited preferential cytotoxicity for the OATP1B3-expressing cells. *Rhus aromatica* sub-fraction 4 and *Rhus glabra* sub-fractions 2 and 4 were the most potent cytotoxic fractions with IC_{50} values of 9 µg/mL, 2.6 µg/mL and 3

μg/mL, respectively (Table 5-1). These three sub-fractions will be further sub-fractionated by a combination of gel filtration, thin-layer chromatography and HPLC to identify pure compounds that will be characterized using the same cytotoxicity assay.

Figure 5-1:

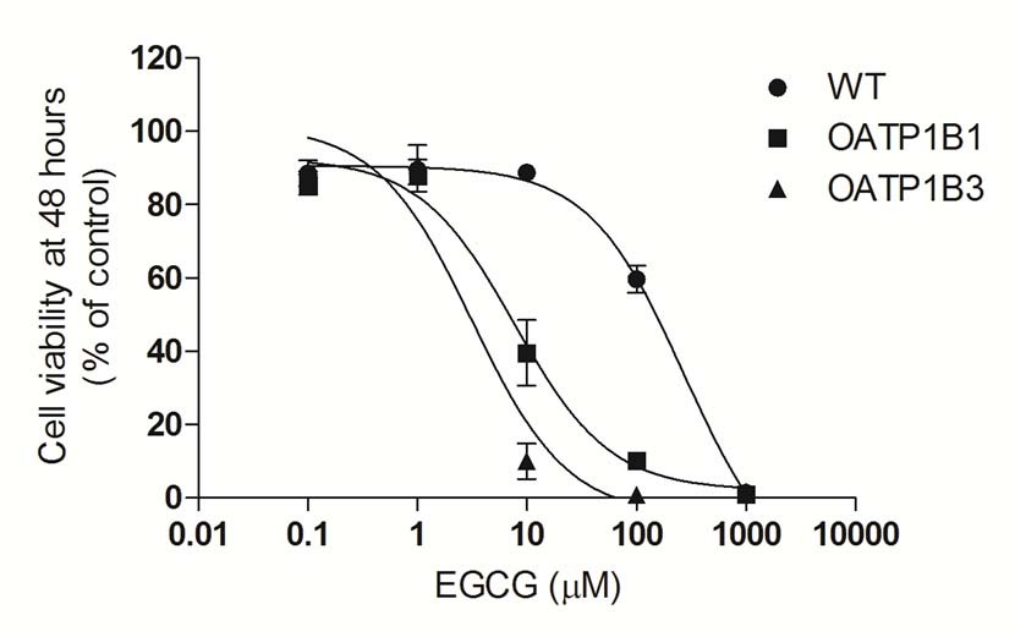
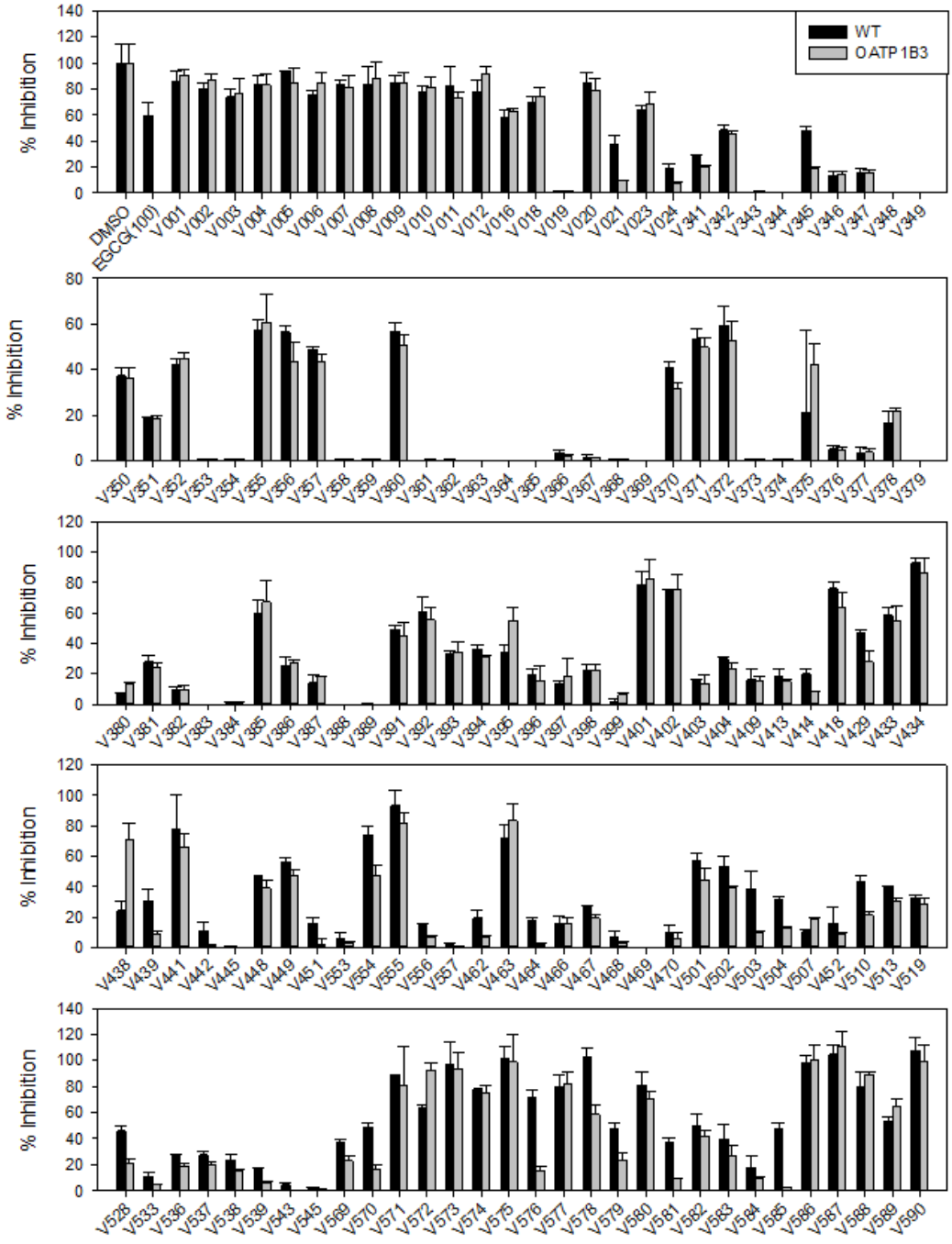

Cell viability at 48 hours
(% of control)
120
100
80
60
40
20
0
0.01
0.1
1
10
100
1000
10000
EGCG (μM)
WT
OATP1B1
OATP1B3

Figure 5-1: Cell viability assay of wild-type and OATP-expressing CHO cells in the absence and presence of EGCG. Cell viability of wild-type CHO cells (circles), OATP1B1- (squares) or OATP1B3-expressing CHO cells (triangles) was measured after a 48 hour incubation in the absence or presence of increasing concentrations of EGCG. Values obtained are given as percent of control and are means ± SD of triplicate determinations of four to six experiments.

Figure 5-2:


WT
OATP1B3
% Inhibition
DMSO
EGCG(100)
V001
V002
V003
V004
V005
V006
V007
V008
V009
V010
V011
V012
V016
V018
V019
V020
V021
V023
V024
V341
V342
V343
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V545
V569
V570
V571
V572
V573
V574
V575
V576
V577
V578
V579
V580
V581
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V583
V584
V585
V586
V587
V588
V589
V590

Figure 5-2: Initial cell viability screen of wild-type and OATP1B3-expressing CHO cells in the presence of selected plant extracts of the V series. Cell viability of wild-type CHO cells (black bars) and OATP1B3-expressing CHO cells (grey bars) was measured at 48 hours in the absence or presence of 50 µg/ml of plant extract. Values obtained are given as percent of control and are means ± SD of triplicate determinations of one experiment.

Figure 5-3:

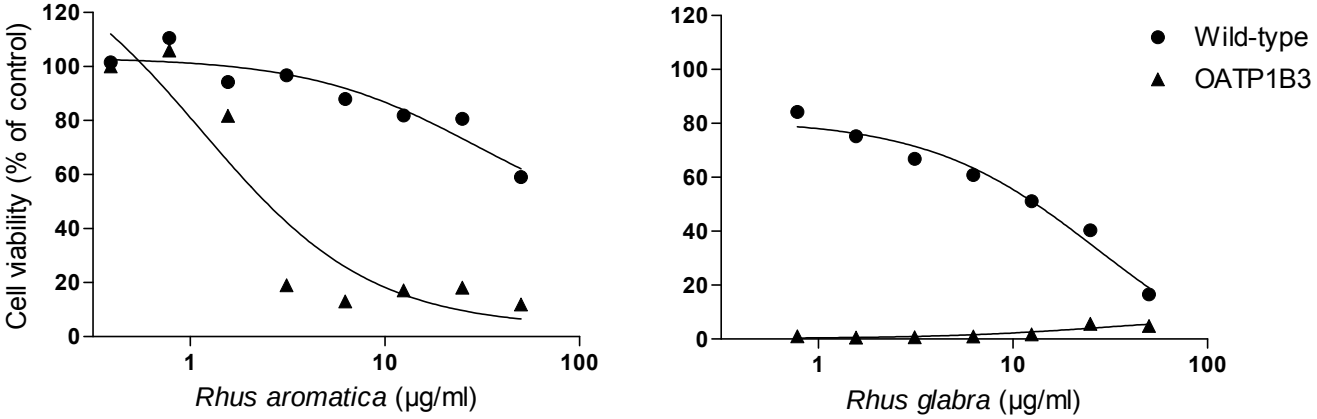
Cell viability (% of control)
120
100
80
60
40
20
0
1
10
100
Rhus aromatica (µg/ml)
Rhus glabra (µg/ml)
Wild-type
OATP1B3

Figure 5-3: Cell viability of wild-type and OATP1B3-expressing CHO cells in the presence of the butanol extracts of *Rhus aromatica* (V345) and *Rhus* glabra (V585). Cell viability of wild-type CHO cells (circles) and OATP1B3-expressing CHO cells (triangles) was measured at 48 hours in the presence of increasing concentrations of plant extract. Values obtained are given as percent of control and are means ± SD of three independent experiments.

Figure 5-4:

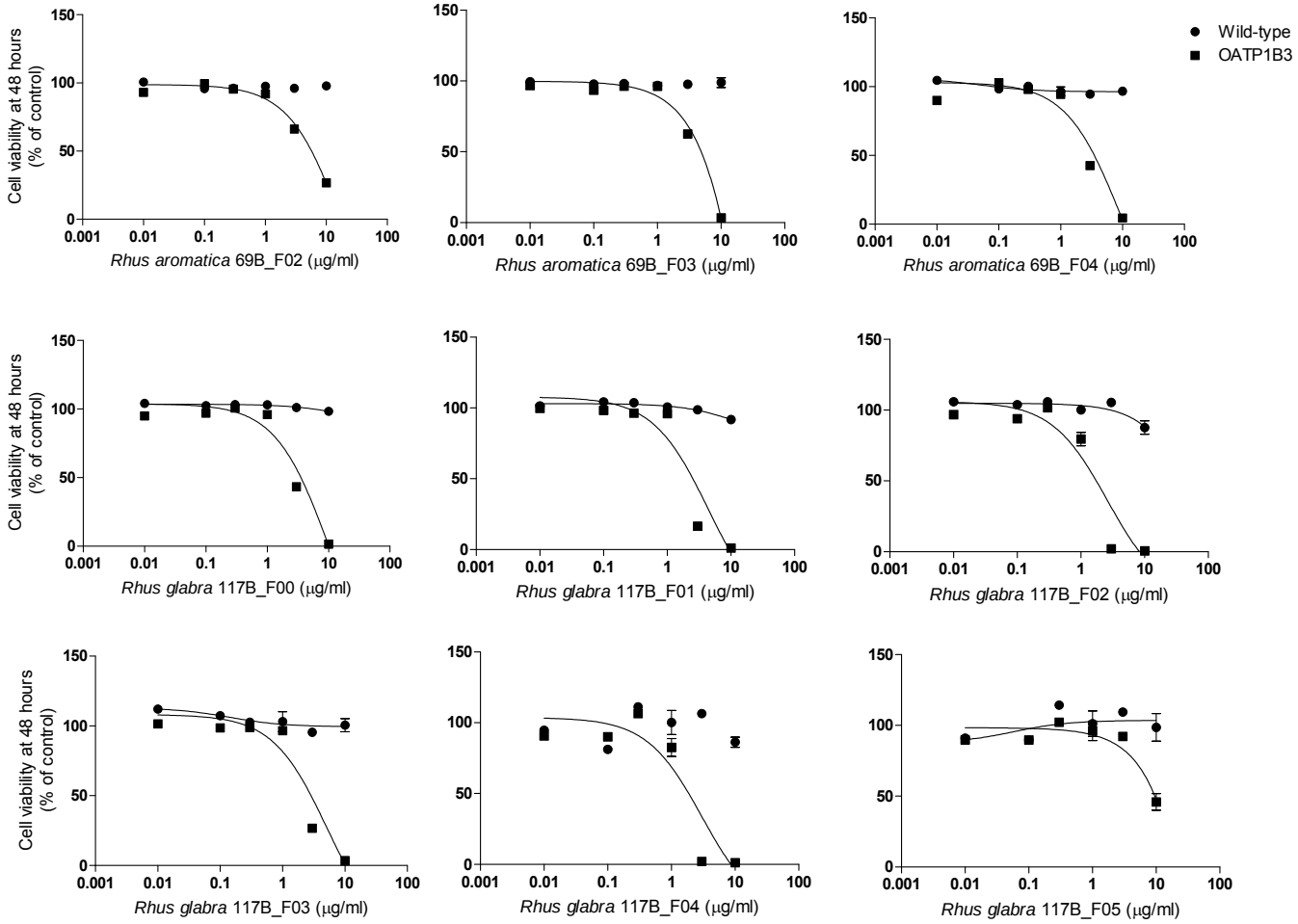
Wild-type
OATP1B3
Cell viability at 48 hours (% of control)
150
100
50
0
0.001
0.01
0.1
1
10
100
Rhus aromatica 69B_F02 (μg/ml)
Rhus aromatica 69B_F03 (μg/ml)
Rhus aromatica 69B_F04 (μg/ml)
Rhus glabra 117B_F00 (μg/ml)
Rhus glabra 117B_F01 (μg/ml)
Rhus glabra 117B_F02 (μg/ml)
Rhus glabra 117B_F03 (μg/ml)
Rhus glabra 117B_F04 (μg/ml)
Rhus glabra 117B_F05 (μg/ml)

Figure 5-4: Cell viability of wild-type and OATP1B3-expressing CHO cells in the presence of sub-fractions of *Rhus aromatica* and *Rhus glabra*. Cell viability of wild-type CHO cells (circles) and OATP1B3-expressing CHO cells (squares) was measured at 48 hours in the presence of increasing concentrations of plant extract. Values obtained are given as percent of control and are means ± SD of two independent experiments.

Table 5-1: Effect of Sub-fractions from Kansas Plant Extracts on the Cell Viability of Wild-type and OATP1B3-expressing CHO cells

Plant Extract	Wild-type IC_{50} (µg/mL)	OATP1B3 IC_{50} (µg/mL)
Rhus aromatica (69B-F00)	--	--
Rhus aromatica (69B-F01)	--	--
Rhus aromatica (69B-F02)	--	18.8
Rhus aromatica (69B-F03)	--	86.2
Rhus aromatica (69B-F04)	--	9
Rhus glabra (117B_F00)	--	10.2
Rhus glabra (117B_F01)	--	4.4
Rhus glabra (117B_F02)	--	2.6
Rhus glabra (117B_F03)	--	5.1
Rhus glabra (117B_F04)	--	3
Rhus glabra (117B_F05)	--	--

5.4: Discussion

Recent studies showing the expression of OATP1B3 in different cancers suggests its potential use as a therapeutic target for the transport of cytotoxic anticancer drugs into cancer cells. In this study, we used a bioassay-guided approach along with cytotoxicity assays to identify novel cytotoxic compounds from Kansas plants that are substrates of OATP1B3. We demonstrated that a few butanol fractions from the plants *Rhus aromatica* and *Rhus glabra* preferentially kill OATP1B3-expressing cells compared with wild-type cells. We plan to continue this study by further sub-fractionating the most potent Rhus aromatica and Rhus glabra butanol fractions until we identify pure compounds from both plants that are responsible for the cytotoxicity of OATP1B3-expressing cells. These results demonstrate that Kansas plants are a good source for identifying cytotoxic OATP1B3 substrates that can eventually be developed into novel anticancer drugs used in the clinic.

Chapter 6

Summary and Discussion of Dissertation

6.1: Significance

Organic anion transporting polypeptides (OATPs) are multispecific transporters that mediate the uptake of numerous endogenous and exogenous compounds into cells. Some OATPs can be expressed in the epithelium of many different tissues in the body and the expression of some OATPs can be restricted to a single tissue. OATP1B3 is an example of a tissue-specific OATP that is exclusively expressed in the liver, specifically at the basolateral membrane of human hepatocytes. Aside from their normal expression profiles, expression of OATPs has also been shown to be up- or down-regulated in diseased tissues including numerous different cancers. Because OATPs are responsible for the transport of a wide range of compounds their contribution to the disposition of drugs in the body is important. Consequently, polymorphisms in these transporters can alter pharmacokinetics of drugs and lead to ineffective therapy or even to toxicities. Because OATPs are expressed in different cancers and they are able to transport anticancer drugs, they are considered potential targets for cancer diagnostics and therapy.

Due to the limited information regarding OATPs and cancer, little is known about the role they play in cancer and about the potential uses to improve cancer therapy. Furthermore, without a detailed understanding of the expression and function of OATPs in cancer cells, it will be difficult to utilize OATPs as therapeutic targets. The studies presented in this dissertation were designed to address this lack of understanding by characterizing OATP expression in cancer and anticancer drug transport by OATP1B3. To do this I formulated the central hypothesis that expression of OATPs in cancer combined with their ability to transport cytotoxic

anticancer drugs makes them potential targets for improving cancer diagnosis and therapy. This hypothesis was based on recent studies that demonstrated altered expression of OATPs in different cancers and on the ability of OATPs to transport some anticancer drugs. Additionally, experiments in our laboratory have demonstrated that small molecules can allosterically stimulate OATP-mediated transport. I tested this hypothesis via three specific aims: 1) identification and characterization of cancer cells that express OATPs, 2) identification and functional characterization of anticancer drug uptake mediated by OATPs and 3) identification of cytotoxic compounds from plant extracts that can be developed into anticancer drugs that target OATP-expressing cancers.

6.2: Specific Aim 1

In the first specific aim, I examined the expression of OATPs in human pancreatic ductal adenocarcinoma tissues. Initial experiments using a quantigene multiplex assay lead to the detection of transcripts of four OATPs in all the tested pancreatic tissues. Expression of these OATPs was further confirmed at the protein level using immunoflourescence staining. Expression of OATP1B3 was then characterized on a tissue microarray containing several different types and stages of pancreatic cancer.

Pancreatic cancer commonly develops drug resistance and remains to be a difficult cancer to treat. Efflux transporters have been major targets in cancer therapy. However, recently uptake transporters have gained interest as targets to improve cancer therapy. Initial experiments revealed expression of four OATPs in pancreatic adenocarcinoma at both messenger RNA and protein level: OATP1B3, OATP2A1, OATP3A1 and OATP4A1. My results revealed that expression of OATP1B3, OATP2A1, OATP3A1 and OATP4A1 was increased in pancreatic adenocarcinoma compared to normal pancreas tissue. OATP2A1, OATP3A1 and

OATP4A1, under normal conditions, are expressed in a multitude of different tissues in the body. However, OATP1B3 is exclusively expressed in the liver suggesting that upon treatment with anticancer drug substrates of OATP1B3, other normal tissues that do not express OATP1B3 would not be affected by these toxic drugs. Therefore, OATP1B3 is clearly a good target for anticancer therapy using drugs that kill rapidly dividing cells. Thus, OATP1B3 expression was further characterized in different stages and types of pancreatic cancer.

Because expression of OATP2A1, OATP3A1 and OATP4A1 increases from normal pancreas tissue to pancreatic adenocarcinoma tissues, these OATPs may serve as potential therapeutic targets for the delivery of anticancer drugs into pancreatic cancer tumors. However, none of these three OATPs has been shown to transport any anticancer drugs. OATP1B3 was also detected in pancreatic adenocarcinoma, however, there was little to no OATP1B3 expression in normal pancreas tissue. Because OATP1B3 has been shown to transport anticancer drugs like methotrexate, docetaxel and paclitaxel, it is a better candidate for anticancer therapy than the other three OATPs.

As a final step in this aim, I examined OATP1B3 expression at different stages and types of pancreatic cancer. The highest expression of OATP1B3 was detected in pancreatic tissues with mild and chronic inflammation, followed by stage one and stage two pancreatic adenocarcinomas. OATP1B3 expression was low in stage three pancreatic adenocarcinomas and absent in metastatic pancreatic cancer. These data suggest that OATP1B3 may serve as a biomarker for detection of early stage pancreatic cancer and also represents a potential target for the transport of anticancer drugs early on in pancreatic adenocarcinoma tumor cells. Future studies should investigate whether circulating OATP1B3 can be detected in pateints with early stage pancreatic cancer.

6.3: Specific Aim 2

As the first specific aim indicated that OATP1B3 is the best target for anticancer therapy, in the second specific aim I tested the hypothesis that OATP1B3 can transport anticancer drugs that are used in the clinic. The NCI/DTP anticancer drug library, comprised of 89 FDA approved anticancer drugs, was screened for substrates of OATP1B3. Initial experiments demonstrated that several anticancer drugs inhibit OATP1B3-mediated transport of the two model substrates estradiol-17β-glucuronide and estrone-3-sulfate. Inhibition of model substrate transport indicated that the anticancer drugs interact with OATP1B3 transport and therefore could be potential substrates. Therefore, we further confirmed inhibition of OATP1B3-mediated uptake and determined the mechanism of OATP1B3 inhibition of the four anticancer drugs, actinomycin D, rapamycin, teniposide and vinblastine. Our results showed that actinomycin D competitively inhibited OATP1B3-mediated transport of estradiol-17β-glucuronide, but non-competitively inhibited OATP1B3-mediated transport of estrone-3-sulfate. This suggests that anticancer drug inhibition of OATP1B3-mediated transport is substrate-dependent. These findings corroborated that OATP1B3 does indeed have multiple binding sites or translocation pathways for the different substrates it transports. However, because inhibition kinetics were not consistent and because even the known substrate rapamycin exhibited non-competitive inhibition it was impossible to predict novel substrates of OATP1B3 based on inhibition kinetics. It is important to note that an inhibitor is not necessarily a substrate of OATP1B3, even if it inhibits in a competitive way, and a compound that does not inhibit should not be ruled out as a potential substrate for OATP1B3. Therefore, we established a different and unique approach to identify novel OATP1B3 substrates from the oncology drug set using a cell viability screen.

Using this cell viability approach where drug toxicity was compared between wild-type and OATP1B3-expressing cells we could demonstrated that the five anticancer drugs bleomycin,

etoposide, plicamycin, oxaliplatin and thioguanine indeed preferentially killed OATP1B3-expressing cells as compared to wild-type cells. These results strongly suggest that these anticancer drugs are transported by OATP1B3 into the cells to facilitate cell killing. We could confirm that etoposide and oxaliplatin are indeed substrates of OATP1B3 using radiolabeled substrates and thus confirmed the validity of the novel approach of screening for cytotoxic OATP1B3 substrates through the cell viability assay. The results of this specific aim confirmed that OATP1B3 can transport additional anticancer drugs and suggests that OATP1B3-expressing cancers could potentially be treated with these cytotoxic anticancer drug substrates of OATP1B3.

6.4: Specific Aim 3

In the final specific aim, we started with Kansas plant extracts and wanted to identify and isolate cytotoxic substrates of OATP1B3 that can potentially be developed into novel anticancer drugs for the treatment of OATP1B3-expressing cancers. In this study we used cell viability assays that would identify cell extracts or sub-fractions if they contained cytotoxic substrates of OATP1B3 to screen a series of Kansas plant extracts. Butanol fractions of *Rhus aromatica* and *Rhus glabra* were identified as plant extracts with cytotoxic effects on OATP1B3-expressing CHO cells. Both extracts were further sub-fractionated and the fractions were again tested for their effect on OATP1B3-dependent cytotoxicity. Subfraction four from *Rhus aromatica* and subfractions two and four from *Rhus glabra* exhibited the most potent cytotoxic effects and will be further fractionated until pure cytotoxic substrates are identified. We expect that this study will identify novel cytotoxic OATP1B3 substrates that could be lead compounds for the development of novel therapeutic chemicals for the treatment of OATP1B3-expressing cancers. Such lead compounds would be optimized with the help of medicinal chemists from the University of Kansas in Lawrence and structure function relationship studies will allow us to

identify structural components that are required for OATP1B3 substrates. The long term goal of these studies is to design novel cytotoxic compounds that are OATP1B3 substrates for the treatment of OATP1B3-expressing cancers.

6.5: Future Directions

The studies in my dissertation demonstrated that OATPs are up-regulated in pancreatic adenocarcinoma, are able to transport several anticancer drugs and are able to transport cytotoxic plant compounds. Knowledge of the expression and function of multispecific drug transporters, such as OATPs, in cancer has the potential to improve drug targeting and delivery into cancer cells that exhibit drug resistance. Characterizing the expression and function of OATPs in cancer is of particular interest, as this could be harnessed to use different approaches for anticancer therapy. Four approaches can be taken for OATP-mediated anticancer therapy: (a) OATP-mediated uptake of hormones, hormone conjugates, or unidentified growth promoting chemicals could be prevented with OATP-selective inhibitors, (b) novel anticancer drugs could be developed as OATP substrates to increase their uptake into OATP-expressing cancer cells, (c) uptake of anticancer drugs could be enhanced by allosteric stimulators and (d) expression of OATPs in the plasma membrane could be modulated to increase or decrease uptake of various substrates into cancer cells and expression of OATPs could even be used as biomarkers in cancer development.

The first specific aim identified four major OATPs with increased expression in pancreatic adenocarcinoma and specifically showed OATP1B3 expression in early stages of pancreatic adenocarcinoma. By identifying OATPs that are expressed in pancreatic cancer, it should be possible to screen for anticancer drugs that are substrates of these OATPs. Due to

the selective expression of OATP1B3 in the liver, under normal physiological conditions, and its ability to transport a few anticancer drugs we further investigated the expression of OATP1B3 at different stages and types of pancreatic cancer and the results suggested that OATP1B3 was the best candidate for anticancer therapy and improved diagnostics. The second specific aim identified five anticancer drugs, bleomycin, etoposide, oxaliplatin, plicamycin and thioguanine, as substrates of OATP1B3. These findings suggest that anyone of these five anticancer drugs could be used to treat OATP1B3-expressing cancers. Finally, in the third specific aim, we identified two Kansas plants, *Rhus aromatica* and *Rhus glabra*, as plants that contain cytotoxic compounds that are substrates of OATP1B3 and potentially can be developed into novel anticancer drugs for the treatment of OATP1B3-expressing cancers. Collectively, these studies suggest that OATPs can be used as potential targets for anticancer therapy and diagnostics and propose additional studies than can lead to therapies that can be used in the clinic to improve future outcomes for cancer patients.

As previously mentioned, one of the approaches that can be taken to improve anticancer therapy by using OATPs is the development of inhibitors for OATPs to prevent the proliferation and development of hormone-dependent cancers. Given that OATPs can transport hormones and their conjugates, one school of thought considers OATPs as contributors to cancer development for hormone-dependent cancers specifically for breast and prostate cancer. Therefore, future studies to identify novel OATP inhibitors would be useful to inhibit the uptake of hormones. High-throughput screening techniques can be employed in identifying specific OATP inhibitors (Gui et al., 2010). Development of such high-throughput screens is beneficial for screening large libraries of small molecules in short periods of time. Similarly, high-throughput screens can be developed to identify stimulators of OATP1B3-mediated anticancer drug transport. However, it is important to note that modulation of OATP-mediated transport is

substrate dependent and this should be taken into consideration when designing such experiments.

Some OATPs are expressed in many different tissues in the body; their expression in these tissues can contribute to the distribution of drugs and xenobiotics into the tissues that harbor their expression. The studies of this dissertation suggest OATP1B3 as the more practical candidate for OATP-mediated anticancer therapy for the reason that aside from the expression of OATP1B3 in cancer tissues it is normally exclusively expressed in the liver. Therefore, upon systemic treatment with anticancer drug substrates of OATP1B3, distribution of these anticancer drugs will theoretically be targeted to the cancer cells and the liver. It is important to consider that OATP1B3-mediated anticancer therapy can lead to hepatotoxicity. However, if the anticancer drugs that are used for treatment target rapidly-dividing cells they should not affect the liver since it is not a rapidly dividing organ. Additionally, the liver is considered the ultimate detoxifying organ in the body and expresses numerous detoxifying enzymes that metabolize drugs and prepare them for excretion from the body, whereas cancer cells lack such enzymes. It is also important to understand that efflux transporters like MDR1 and MRPs of the ABC family of transporters are highly up-regulated in cancer cells. The rapid activity of these efflux transporters could negate the stimulation of OATP-mediated transport by rapidly pumping out the anticancer drugs from the cancer cells. One approach to resolve this would be to identify a small molecule that stimulates OATP1B3-mediated anticancer uptake but also inhibits MDR-1-mediated anticancer efflux. An example of such a small molecule is the green tea component epigallocatechin gallate (EGCG), which also has anti-proliferative properties. EGCG stimulates OATP1B3-mediated estrone-3-sulfate transport, has no effect on estradiol-17β-glucuronide uptake and inhibits Fluo-3 uptake (Roth et al., 2011b), while it also has been shown to inhibit MDR-1 (Mei et al., 2004). Thus, EGCG may serve as a candidate for OATP-mediated

anticancer therapy if it can stimulate OATP1B3-mediated anticancer drug uptake and inhibit MDR-1 efflux of the same anticancer drug.

In the studies of this dissertation, we used an in-vitro stable expression model of OATP1B3-expressing Chinese hamster ovary (CHO) cell lines. Our stable expression cell line was beneficial in identifying novel anticancer compounds transported by OATP1B3. However, in order to complete these studies, it is important to repeat some experiments in a more appropriate model using cancer cell lines that express OATP1B3. Several studies have shown OATP1B3 expression in numerous different cancer cell lines at the mRNA level. Although these studies are helpful as initial experiments, it is important to identify expression of OATP1B3 at the protein level. To our knowledge, OATP1B3 expression at the protein level has only been shown in very few cancer cell lines, one of which is the colorectal cancer cell line HCT-8 (Lee et al., 2008). Additionally, it is important to consider the differences between established cell lines and tumor tissues. Differences acquired by the cultured cancer cell lines cause them to not completely resemble their respective tumor tissues that they are modeled for. This was especially evidenced in the study done by Gillet et al. where they compared established cancer cell lines to clinical samples and showed that the cell lines have more resemblance to each other regardless of the tissue of origin than to the clinical samples they are supposed to model (Gillet et al., 2011). Therefore, the more appropriate model system to pursue while considering OATP-mediated cancer therapy would be primary tumor models as they more closely mimic tumor cells.

Although in-vitro studies can be helpful to investigate many aspects of cancer biology, it is important to confirm these observations using in-vivo cell models that take into consideration systemic effects of experiments. Gene duplication events and species divergence caused human OATPs to have multiple orthologs compared to rodent Oatps. For example, Oatp1b2 is

the mouse ortholog of human OATP1B1 and OATP1B3. When considering OATP-mediated anticancer therapy, it is important to consider Oatp1b2 when preforming in-vivo experiments using mouse models. Therefore, experiments remain to be done that determine whether Oatp1b2 transport is modulated the same way as OATP1B3. One way to get around these experiments is the use of transgenic knockout mouse models to study the role of OATPs in anticancer drug disposition (van de Steeg et al., 2009; van de Steeg et al., 2011). Another beneficial in-vivo model system includes the use of nude mice. Experiments should be done where nude mice are implanted with OATP1B3-expressing cancer cells and treated with cytotoxic anticancer drugs that are OATP1B3 substrates. Taken together, there are several considerations that need to be taken when choosing the appropriate model system for these experiments.

Recent studies identified a novel OATP1B3 isoform in colon and lung tumor tissues. The isoform identified, named ct-OATP1B3 (cancer type OATP1B3), has a novel transcription start site and an alternative promoter region (Nagai et al., 2012) which results in a splice variant of OATP1B3 that lacks the amino terminus and the first transmembrane domain. Because this study did not investigate whether ct-OATP1B3 is functional, it is imperative that we assess the function of ct-OATP1B3 before moving forward with studies of OATP1B3-mediated anticancer therapy.

In conclusion, the studies in this dissertation suggest that OATPs are potentially targets for improving diagnostics and therapy in cancer. Further research is necessary to determine the exact role of OATPs in cancer and to elucidate how they can be used in anticancer therapy.

References

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Appendices:

Appendix I: Citations of published papers

Hays, A., Apte, U. and B. Hagenbuch. Organic anion transporting polypeptides are expressed in pancreatic cancer and may serve as potential diagnostic markers and therapeutic targets for early stage adenocarcinomas [Submitted, under review]

De Graan, A.M., Lancaster, C.S., **Obaidat, A.**, Hagenbuch, B., Elens, L., Friberg, L.E., de Bruijn, P., Hu, S., Gibson, A.A., Bruun, G.H., Mikkelsen, T.S., Walker, A.L., Du, G., Loos, W.J., van Schaik, R.H.N., Baker, S.D., Mathijssen, R.H.J. and A. Sparreboom (2012) Influence of Polymorphic OATP1B-Type Carriers on the Disposition of Docetaxel. *Clin Cancer Res* June 18 [Epub ahead of print]

Roth, M., **Obaidat, A.** and B. Hagenbuch (2012) OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br J Pharmacol*. **165**: 1260-87.

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Obaidat, A., Weiss, J., Wahlgren, B., Manam, R.R., Macherla, V., Chao, T., Palladino, M., Lloyd, G.K., Potts, B., Enna, S.J., Neuteboom, S. and B. Hagenbuch (2011) Proteasome regulator marizomib (NPI-0052) exhibits prolonged inhibition, attenuated efflux, and greater cytotoxicity than its reversible analogs. *J Pharmacol Exp Ther*. **337**: 479-486.

Gui, C., **Obaidat, A.**, Chagaturu, R. and B. Hagenbuch (2010) A cell-based high-throughput assay to identify inhibitors of organic anion transporting polypeptides 1B1 and 1B3. *Curr Chem Genomics*. **4**: 1-8.

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