IDENTIFICATION AND CHARACTERIZATION OF THE OLIGOMERIZATION AND STRUCTURAL FUNCTIONAL RELATIONSHIP OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

By

© 2017

Yuchen Zhang

B.S., Fudan University, 2011

Submitted to the graduate degree program in Pharmacology

and the Graduate Faculty of the University of

Kansas Medical Center in partial fulfillment of the requirement for the degree of

Doctor of Philosophy.

_____

Chair: Bruno Hagenbuch, Ph.D.

_____

Partha Kasturi, Ph.D.

_____

Jed Lampe, Ph.D.

_____

Gregory Reed, Ph.D.

_____

Gustavo Blanco, Ph.D.

Date Defended: 08-18-2017

The dissertation committee for Yuchen Zhang certifies that this is the approved version of the following dissertation:

IDENTIFICATION AND CHARACTERIZATION OF THE OLIGOMERIZATION AND STRUCTURAL FUNCTIONAL RELATIONSHIP OF ORGANIC ANION TRANSPORTING POLYPEPTIDE 1B3

_____

Chair: Bruno Hagenbuch, Ph.D.

Date Approved: 08-20-2017

Abstract

Many transporters are expressed at the basolateral membrane of human hepatocytes, including Organic Anion Transporting Polypeptide 1B1 (OATP1B1), OATP1B3, Organic Cation Transporter 1 (OCT1), Na+/Taurocholate Cotransporting Polypeptide (NTCP) and more. These transporters are part of the absorption system of the liver, which is responsible for the uptake of chemicals from the portal vein into hepatocytes for further metabolism and elimination. Extensive studies have characterized the function of these transporters, and results suggest that liver uptake transporters, like OATP1B3 and OATP1B1, or OATP1B3 and NTCP, have many overlapping substrates. Because these transporters seem to play an essential role in the protection of the body from xenobiotics, it is important to better understand their function and how they interact with each other. In this dissertation, I focused on one of these transporter, OATP1B3, as a model transporter to improve the understanding of liver uptake transporters. OATP1B3 is responsible for the uptake of many endogenous compounds like bile acids and hormones as well as xenobiotics, including numerous drugs. Recent studies demonstrated that some of the liver uptake transporters like OATP1B1, OCT1 and NTCP can form homo-oligomers. In the first specific aim, I evaluated the hypothesis that OATP1B3 also can form homo-oligomers. To address this aim, co-immunoprecipitation and proximity ligation assays of differently tagged OATP1B3 were performed in transiently transfected HEK293 cells. The results demonstrated that OATP1B3 indeed can form homo-oligomers. In addition, uptake assays with wild-type and non-functional OATP1B3 suggested that the OATP1B3 unit in the homo-oligomers works as individual functional unit. Besides that, by using proximity ligation assays, the interaction between OATP1B3 and OATP1B1, and between OATP1B3 and NTCP was demonstrated in HEK293 cells. Interactions between OATP1B3 and NTCP were also confirmed in frozen liver sections. In the second specific aim, I evaluated the hypothesis that OATP1B3 can form hetero-oligomers with other transporters and that these interactions can influence their expression and function. I

was able to extend the findings of hetero-oligomerization to include OCT1 using both immunoprecipitation and proximity ligation assays. Uptake assays and surface biotinylation experiments were performed with HEK293 cells co-expressing OATP1B3 and OCT1, OATP1B3 and OATP1B1, or OATP1B3 and NTCP. The results demonstrated that these interactions between OATP1B3 and the other transporters lead to changes in both, function and expression of OATP1B3 in a transporter-dependent manner. In the third specific aim, I evaluated the hypothesis that photoaffinity labeling can be used to study the binding sites and translocation pathways of OATP1B3. The known OATP1B3 substrate 8-fluorescein-cAMP (8-FcA) was used to perform photoaffinity labeling experiments with CHO Flp-In cells stably expressing His-tagged OATP1B3. The results suggested that 8-FcA can label proteins but background labeling was very high even without UV activation. Purification of OATP1B3 was also attempted to improve chances for a successful follow-up mass spectrometry analysis. Although relatively pure protein was obtained, the low yield prevented further analysis. In summary, this dissertation reveals that 1) OATP1B3 can from homo- and hetero-oligomers, and that the homo-oligomers of OATP1B3 work as separate functional units; 2) hetero-oligomerization of OATP1B3 can result in changes of function and surface expression of OATP1B3 in a transporter-dependent manner; 3) photoaffinity labeling may be a good method to study the binding sites of OATP1B3, after the purification methods have been improved.

Acknowledgements

First I would like to acknowledge the funding sources, including NIH Grant GM077336 to make the work in this dissertation possible.

I would like to thank my mentor Dr. Bruno Hagenbuch for his support. You considered me and other lab members not only your students but also your co-workers, and gave me the freedom to test my own thoughts. As a scientist, you are a role model of scientific thinking and work ethics to me, while as a mentor, you taught me to be both optimistic and skeptical about my own results. I enjoyed all the discussions or even arguments we had during these six years, because these conversations prepared me to be successful as a scientist. Your attitude when facing difficulties also impressed and motivated me. Thank you again for your encouragement, patience and support, and I will always remember your catch phrase: "Let me get a cup of coffee for this."

My committee members: Dr. Kasturi, Dr. Lampe, Dr. Reed and Dr. Blanco, I would like to give my sincere appreciation to all your time and support. Your suggestions to my projects helped me solving so many problems and speeding up my studies.

I also want to thank all of the current and the previous lab members, Melissa, Regina, Kelli, Wen, Amanda, Jessica and Miki for your friendship and support. I would like to thank all the other graduate students, faculty and staff in the department. I am grateful that I could study in such a friendly and supportive environment.

I would especially like to thank my parents and grandparents for their support all the time. Without your trust and encouragement, I could never have finished this degree.

Last but not least, I want to thank my wife Cuiwen for her support and patience. The long distance between us during these six years made me love you even more.

Table of Contents

List of tables

List of figures

List of abbreviations

8-FcA	=	8-Fluorescein-cAMP
ABC	=	ATP-binding Cassette
AhR	=	Arylhydrocarbon receptor
ASBT	=	Apical Sodium-Dependent Bile Acid Transporter
AUC	=	Area Under Curve
BBB	=	Blood-brain barrier
BCRP	=	Breast Cancer Resistance Protein
BSEP	=	Bile Salt Export Pump
cAMP	=	Cyclic adenosine monophosphate
CAR	=	Constitutive androstane receptor
CCK-8	=	cholecystokinin octapeptide
cDNA	=	complementary DNA
CHO	=	Chinese hamster ovary
CMC	=	Critical micelle concentration
CML	=	Chronic myeloid leukemia

CNS	=	Central Nervous System
DAPI	=	4',6-diamidino-2-phenylindole
DDI	=	Drug-drug interaction
DMEM	=	Dulbecco's modified eagle medium
E17βG	=	Estradiol-17β-glucuronide
E3S	=	Estrone-3-sulfate
ECL	=	Extracellular loop
EMT	=	Extraneuronal Monoamine Transporter
EV	=	Empty vector
FBS	=	Fetal bovine serum
FDA	=	Food Drug Administration
FRET	=	Fluorescence Resonance Energy Transfer
FXR	=	Farnesoid X receptor
HAT	=	Heteromeric amino acid transporter
HBV	=	Hepatitis B virus
HDV	=	Hepatitis D Virus

HEK	=	Human embryonic kidney
HEPES	=	25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGF	=	Hepatocyte growth factor
HIF-1α	=	Hypoxia inducible factor-1α
HNF1α	=	Hepatocyte Nuclear Factor 1-Alpha
HPLC	=	High-performance liquid chromatography
HUGO	=	Human Genome Organization
IBAT	=	Ileal Na^+-dependent bile acids transporter
IF	=	Immunofluorescence
Km	=	Michaelis constant
LC-MS	=	Liquid chromatography–mass spectrometry
LST	=	Liver Specific Transporter
MDR	=	Multidrug resistance protein
MPP	=	1-methyl-4-phenylpyridinium
mRNA	=	messenger ribonucleic acid
MRP	=	Multidrug Resistance associated Protein

NASH	=	Nonalcoholic steatohepatitis
NBD	=	Nucleotide-binding domains
NFE	=	Nuclear Factor Erythroid
NHE	=	Sodium-proton exchanger
Ni-NTA	=	Nickel-charged chelating ligand nitrilotriacetic acid
NKCC1	=	Na^+-K^+-$2Cl^-$- cotransporter
Nrf2	=	NFE2-related factor
NTCP	=	Na^+/Taurocholate Cotransporting Polypeptide
OAT	=	Organic Anion Transporter
OATP	=	Organic Anion Transport Polypeptide
OCT	=	Organic Cation Transporter
OCTN	=	Organic Zwitterion/Cation Transporter
OSTα/β	=	Organic Solute Transporter α/β
PBS	=	Phosphate-buffer saline
Pgp	=	P-glycoprotein
PI3K	=	Phophoinositide-3-kinase

PLA	=	Proximity Ligation Assay
SLC	=	Solute Carrier
SNP	=	single-nucleotide polymorphism
SOAT	=	Sodium-dependent organic anion transporter
TEA	=	Tetraethylammonium
TKI	=	Tyrosine Kinase Inhibitor
TMD	=	Transmembrane domain
URAT	=	Urate Anion Exchanger
UTR	=	Untranslated region
Vmax	=	Maximum transport rate

Chapter 1

Introduction

I. Membrane transporters

Membrane transporters are proteins expressed at the cell membrane and facilitate the transport of chemicals and macromolecules in and out of the cells. Since cell membranes consist of a lipid bilayer which only allows certain compounds with distinct physiochemical specificities to penetrate freely, transporters are required for cells to take up or efflux the majority of large and hydrophilic endo- and xenobiotics. The function of some ubiquitously expressed transporters is required for all cell types. For example, the Na^+/K^+ ATPase is involved in establishing and maintaining of electric-chemical gradients across the cell membranes. In contrast, the expression and function of other transporters are unique to certain cell types and such a distinct expression of transporters in only certain cell types also determines what kinds of chemicals can enter or exit these cells.

Transporters can be divided into passive and active transporters based on their energy (Koegh et al., 2016). Passive transporters move substrates along electrochemical gradients, while active transporters transfer their substrates against the gradients and consume energy. Based on how they consume energy, active transporters are divided into two groups: primary-active transporters use energy directly from ATP hydrolysis, like the ATP-binding cassette (ABC) transporters and numerous ion pumps; secondary-active transporters operate using the electrochemical gradients which were established by primary active transporters. Depending on how they use the gradients,

secondary-active transporters can be further classified into antiporters, like the Na^+/H^+ exchanger, or symporters like the Na^+/Taurocholate Cotransporting Polypeptide (NTCP).

Membrane transporters play an essential role not only in physiology but also in pharmacology. They contribute to the absorption, distribution and excretion of nutrients, signaling molecules, metabolic end products and drugs. For example, the enterohepatic circulation of bile acids is facilitated by several transporters: NTCP mediates their uptake into hepatocytes, the Bile Salt Export Pump (BSEP) secretes them into bile canaliculi, the Apical Sodium-Dependent Bile Acid Transporter (ASBT) mediates their uptake from the intestine into the enterocytes and Organic Solute Transporter α/β (OSTα/β) mediates their exit across the basolateral membrane into portal blood (Kosters and Karpen, 2008). Transporters are also important for drug development. They are major determinants of the pharmacokinetics of numerous drugs: some uptake transporters, like Organic Anion Transporting Polypeptides (OATPs), affect the bioavailability of drugs because certain OATPs remove oral drugs by rapid uptake into hepatocytes, also called first pass effect. Some efflux transporters, like the Breast Cancer Resistance Protein (BCRP) or some Multidrug Resistance associated Proteins (MRPs) influence whether certain drugs will remain inside the cells (Konig et al., 2013).

II. Liver uptake transporters

The liver is the most important organ for drug absorption, distribution, metabolism and excretion. The polarized hepatic parenchymal cells, or hepatocytes have two types of membranes, one facing the plasma and other hepatocytes, called basolateral or sinusoidal membrane, and one facing bile ductules, called apical or canalicular membrane. The majority of the uptake transporters are localized at the basolateral membrane, and are responsible for the transport of

nutrients and drugs cross the membrane. The uptake of drugs into hepatocytes is also referred as Phase 0 metabolism, because before drugs go through Phase I and II metabolisms, mediated by drug metabolizing enzymes, the majority of drugs need to be transported into hepatocytes by a set of liver uptake transporters. Transporters expressed at the basolateral membrane of hepatocytes can be divided into several major families, including *SLC10*, *SLCO*, *SLC22* and *SLC51* (Figure 1-1) (Hagenbuch and Stieger, 2013; Koepsell, 2013; Anwer and Stieger, 2014).

In this part of the introduction, I will focus on the physiological, pharmacological and pharmaceutical perspectives of some uptake transporters and their family on the basolateral membrane of hepatocytes.

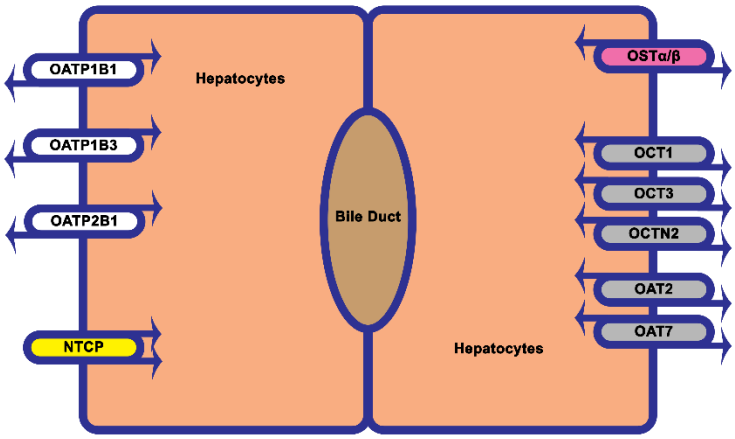

Figure 1-1: Selected SLC transporters expressed in hepatocytes.

A. The SLCO superfamily

SLCO encodes OATP membrane transporters. The superfamily was originally named *SLC21A*, but in 2004, the HUGO Gene Nomenclature Committee approved an amino acid sequence based classification system for the SLCO superfamily. A minimum of 20% identity at the amino acid level is required for a transporter to be assigned into a given SLC superfamily (Hediger et al., 2013). Within the SLCO superfamily, members with more than 40% amino acid identity belong to the same family. These families are numbered. Members with more than 60% amino acid sequence identity belong to the same subfamily, identified with a letter alphabetically after the family number (Hagenbuch and Stieger, 2013). The first human OATP, OATP1A2, was isolated and cloned in 1995 by the Meier group (Kullak-Ublick et al., 1995), and now over 300 OATPs have been identified from more than 40 species. The mammalian transporters are classified into 6 families (OATP1, OATP2, OATP3, OATP4, OATP5 and OATP6), and a total of 11 OATPs have been identified in humans (Hagenbuch and Stieger, 2013).

Human OATPs are expressed in essentially every organ in epithelial or endothelial cells. Although some OATPs are expressed ubiquitously (e.g. OATP2A1, OATP3A1), others have a restricted distribution. For example, OATP1B1 and OATP1B3 seem to be hepatocyte specific. The mRNA expression levels of OATPs do not necessarily predict protein expression levels. This is exemplified by OATP1A2, whose mRNA can be detected in most tissues. However, at the protein level OATP1A2 was only detected at the blood-brain barrier, in the brush-border membrane in the distal nephron, in cholangiocytes, in the pars plana of the ciliary body epithelium, and in syncytiotrophoblasts (Hagenbuch and Stieger, 2013).

OATPs have a broad spectrum of structurally independent substrates. General features of OATP substrates are that most of them are organic anions with a molecular weight higher than 300Da. However, exceptions exist: some OATPs can transport organic cations and neutral compounds.

For example, OATP1A2 can mediate the uptake of the cationic APD-ajmalinium (Bossuyt et al., 1996). Conjugated and unconjugated bile acids are the most extensively studied endogenous substrates of OATPs. They are substrates of OATP1A2, OATP1B1, OATP1B3, OATP2B1 and OATP4A1(Roth et al., 2012). Other endogenous substrates of OATPs include thyroid hormones, prostaglandins, and bilirubin.

In the human liver OATP1B1, OATP1B3 and OATP2B1 are expressed in hepatocytes, and OATP1A2 is expressed in cholangiocytes under normal physiological conditions.

1) OATP1B1

OATP1B1 was first identified and characterized in 1999, and was named as LST-1, OATP-C or OATP2 (Abe et al., 1999; Hsiang et al., 1999; König et al., 2000). It has since then been recognized as an important transporter for the Na^+-independent clearance of bile acids. The tissue distribution of OATP1B1 is restricted to the human liver, and more specifically, it is located throughout the liver lobule evenly at the basolateral membrane of hepatocytes.

The mRNA and protein expression of OATP1B1 is decreased in cholestatic liver disease (Zollner et al., 2001; Sticova et al., 2015). Early investigations into the transcriptional regulation of *SLCO1B1* determined that HNF1α played a predominant role. Other nuclear transcription factor, like FXR can also positively affect the *SLCO1B1* expression (Gui et al., 2008; Meyer Zu Schwabedissen et al., 2010). At the post-translational level, activation of Protein Kinase C can trigger the rapid internalization and recycling of OATP1B1, and thus decrease its expression (Kock et al., 2010; Hong et al., 2015).

Because OATP1B1 has a broad spectrum of structurally independent substrates, it is proposed that OATP1B1 has multiple translocation pathways with binding sites for the individual substrates. Endogenous substrates of OATP1B1 include both conjugated and unconjugated bile

acids, bilirubin, conjugated estradiol, and more. Unlike some other transporters that are mainly involved in the homeostasis of endogenous compounds, OATP1B1 is also very important for the transport of numerous xenobiotic substrates, which include various drugs, like the lipid lowering statins, anti-hypertensives, antibiotics, antifungals and even chemotherapeutics drugs (Table 1-1) (Roth et al., 2012; Hagenbuch and Stieger, 2013).

The broad spectrum of substrates of OATP1B1 can lead to drug-drug interactions (DDIs). For example, the immunosuppressant cyclosporine A inhibits the uptake of several statins, resulting in an increased AUC and C_{max} of the lipid lowering drugs when co-administered (Shitara, 2011). Besides DDIs, SNPs and mutations of OATP1B1 also are of significant clinical relevance. Two common ethnicity-dependent polymorphisms of OATP1B1, 521 T>C and 388 A>G, which can lead to four distinct haplotypes, are reported to influence the AUC and C_{max} of statins, affecting the result of statin therapies (Lee and Ho, 2016). A rare liver disease, Rotor syndrome, is a result of loss of OATP1B1 and OATP1B3 function, and leads to a phenotype of hyperbilirubinemia (van de Steeg et al., 2012).

Table 1-1: Brief summary of selected substrates of OATP1B1, OATP1B3, OCT1 and NTCP.

Transporter	Selected Substrates	
	Endogenous	**Xenobiotics**
OATP1B1	Bilirubin Cholate Estradiol-17β-glucuronide Leukotriene C4 Prostaglandin E2 Taurocholate	Bosentan Cyclosporine A Methotrexate Pravastatin Rifampicin Valsartan
OATP1B3	Bilirubin CCK-8 Cholate Estradiol-17β-glucuronide Taurocholate	Digoxin Epigallocatechin gallate Fexofenadine Imatinib Telmisartan Fluorescein
OCT1	agmatine cyclo(His-Pro) putrescine Salsolinol	Acyclovir ASP Metformin MPP Pentamidine TEA
NTCP	Cholate Estrone-3-sulfate Glycocholate	Chlorambucil-taurocholate Rosuvastatin Tyroxine sulfate

2) OATP1B3

OATP1B3 was cloned and described in 2000, one year after OATP1B1 (Konig et al., 2000; Abe et al., 2001). It was initially named LST-2 or OATP-8. Further studies showed that under normal physiological conditions OATP1B3 is also a liver specific membrane transporter, and that it shares most of the OATP1B1 substrates. However, compared to OATP1B1, OATP1B3 shows an increasing expression from the portal vein towards the central vein (Konig et al., 2000). OATP1B3 mRNA and protein were also detected in different cancer cells, such as colon, pancreatic and gastric cancer cell lines (Obaidat et al., 2012). Recent studies however, showed that OATP1B3 expressed in cancer represents an altered splicing variant, and thus has been named cancer type OATP1B3 (ctOATP1B3) (Thakkar et al., 2013). Based on this alternative splicing, ctOATP1B3 is missing exon 1 and part of exon 2, which results in the loss of the first 28 amino acids. Cancer type OATP1B3 is localized to the cytoplasm instead of to the cell membrane of cancer cells and has reduced transport. A recent report demonstrates that this is due to the missing 28 amino acids (Thakkar et al., 2013; Chun et al., 2017).

Similar to OATP1B1, OATP1B3 function in hepatocytes is decreased by activation of Protein Kinase C, however without affecting protein expression in the membrane (Powell et al., 2014). Furthermore, the regulation of expression of OATP1B1 and OATP1B3 is not always synchronized. For example, Hepatocyte growth factor (HGF) down-regulates the expression of OATP1B1 mRNA but not of OATP1B3 mRNA, while HNF3β only decreases the mRNA expression of OATP1B3 but not OATP1B1 (Vavricka et al., 2004). Using isolated human hepatocytes and cell models it was demonstrated that activator of the aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR), or NFE2-related factor (Nrf2) down-regulated OATP1B3 (Jigorel et al., 2006). In cancer cells, OATP1B3 expression is regulated via several different pathways. Expression of OATP1B3 can be regulated at the epigenetic level by gene

silencing involving methyl-DNA binding proteins (Imai et al., 2013). Hypoxia is also a regulator for the expression of OATP1B3 in cancer, and this effect is a combination of suppressing wtOATP1B3 and inducing ctOATP1B3 by hypoxia inducible factor-1α (HIF-1α) (Han et al., 2013).

Although OATP1B3 shares most substrates with OATP1B1, it has some unique substrates of its own, such as cholecystokinin octapeptide (CCK-8) and digoxin (Roth et al., 2012). Besides that, OATP1B1 and OATP1B3 also have their own modulators and react differently to the same stimulators. For example, clotrimazole inhibits the uptake of estradiol-17β-glucuronide (E17βG), estrone-3-sulfate (E3S) and Fluo-3 by OATP1B1, but only inhibits Fluo-3 uptake and stimulates E17βG uptake of by OATP1B3 (Gui et al., 2008).

More than 40 *SLCO1B3* polymorphisms have been identified, and most of them are found within regulatory regions like the promoter, the 5'-UTR, 3'-URT, and in introns (Boivin et al., 2010). However, two frequent polymorphisms, *SLCO1B3*-334T>G, encoding OATP1B3-S112A, and *SLCO1B3*-699G>A, encoding OATP1B3-M233I, have more than 70% allelic frequency in American and European Caucasians and can alter the uptake of endogenous compounds like bile acids, E17βG, and E3S (Letschert et al., 2004; Boivin et al., 2010). Clinical studies also suggest that the polymorphism OATP1B3-M233I can influence the pharmacokinetics of digoxin in terminal renal failure, by increasing the concentration-to-dose ratio in hemodialysis patients (Tsujimoto et al., 2008). Another study focused on chronic myeloid leukemia (CML) and indicated a correlation between an increased intracellular to plasma concentration ratio of imatinib in leukocytes and the *SLCO1B3*-334T>G polymorphism (Nambu et al., 2011).

Because both OATP1B1 and OATP1B3 are involved in the uptake of numerous drugs, and can be modulated in a substrate-dependent way, they are of clinical relevance. In the FDA draft guidance for industry regarding drug interaction studies, OATP1B1 and OATP1B3 are two of

seven transporters which are required to be tested for potential DDIs. As a potential drug target for the uptake of anti-cancer drugs, the function of ctOATP1B3 has also been studied and it was shown that uptake mediated by ctOATP1B3 is only a fraction of the uptake by wild-type OATP1B3, suggesting the it is not a good drug target to increase anti-cancer drug uptake into cancer cells (Thakkar et al., 2013; Chun et al., 2017). Although numerous reports are available regarding substrates and inhibitors of OATP1B3, it is still not clear how OATP1B3 and other OATPs facilitate the cross-membrane movement of substrates.

The first 3D model of OATP1B3 was proposed by the Mizuguchi group in 2005 (Meier-Abt et al., 2005). It is a structure-based alignment of OATP1B3 to the *E. coli* glycerol-3-phosphate transporter and lactose permease. The study of the 3D model provided a hypothesis that OATP1B3, like most other transporters, has a central pseudo twofold symmetry axis, perpendicular to the membrane plane and a central pore. Helices 1, 2, 4 and 5 of the N-terminal half and 7, 8, 10 and 11 of the C-terminal half formed the central pore, and the electrostatic analysis suggested that the putative pore is positively charged, which would be consistent with the transport of anionic substrates. Based on this model, further studies were done to reveal the structure function relationship of OATP1B3 and the OATP superfamily. Chimeras between OATP1B3 and OATP1B1 were used to study the OATP1B3 selective substrate CCK-8 (Gui and Hagenbuch, 2008). The results demonstrated that transmembrane domain 10 (TM10) is important for CCK-8 selectivity of OATP1B3, and especially that amino acids Y537, S545 and T550 are crucial for maintaining the uptake function. Similarly, replacing OATP1B1 amino acids at positions 45 in TM1, 554 in TM10 and 615 in extracellular loop 6 (ECL6) to the respective OATP1B3 amino acids led to an OATP1B1 with partial OATP1B3 function when measuring CCK-8 uptake (DeGorter et al., 2012). Besides transmembrane domains, the putative central pore also drew attention for their potential function in substrate recognition. The König group investigated all conserved positively charged lysine and arginine residues facing the predicted

central pore by site-directed mutagenesis, and showed that the positive charges at K41 and R580 are important for the function of OATP1B3 (Glaeser et al., 2010). A similar study in our lab identified R57, K361 and R580 as part of the translocation pathway of OATP1B1 (Weaver and Hagenbuch, 2010). Using a High-Throughput Screening Assay to identify inhibitors for OATP1B1 and OATP1B3, De Bruyn et al. then developed a proteochemometrics-based model to predict OATP1B1 and OATP1B3 inhibitors (De Bruyn et al., 2013). They concluded that in order to be a potent OATP1B inhibitor compounds should have high lipophilicity and a large polar surface, have anionic atoms or several hydrogen bond acceptors and there should not be any cationic atoms present.

3) Brief introduction to OATP2B1 and OATP1A2

OATP2B1 and OATP1A2 are the other two members of the functionally well-characterized OATPs in human liver. However, unlike OATP1B1 and OATP1B3, they are also expressed in other organs. The mRNA of OATP2B1 (used to be called OATP-B) was first cloned in 2001 from a brain cDNA library, and was subsequently detected in heart, placenta, lung, liver, kidney and more organs (Kullak-Ublick et al., 2001). Protein expression of OATP2B1 was then reported at the basolateral membrane of hepatocytes, and other tissues, such as the intestinal epithelial cells, syncytiotrophoblasts in the placenta, and endothelial cells of the blood-brain barrier. OATP1A2 was the first human OATP and was cloned in 1995 from a liver cDNA library (Kullak-Ublick et al., 1995). It was named OATP when first cloned, and the mRNA expression was also found in multiple organs like brain, lung, liver, kidney and testes. Further characterization revealed that the liver OATP1A2 is exclusively expressed in cholangiocytes. Because of their wide expression in many different organs, OATP2B1 and OATP1A2 seem to play important roles in xenobiotic absorption, distribution and excretion.

Like the liver-specific OATPs, OATP2B1 and OATP1A2 have a broad substrate specificity and share numerous substrates with other OATPs, for example, various bile acids, E3S and some statins (Roth et al., 2012). Besides general OATP substrates, OATP1A2 can also transport β-blockers like acebutolol, while OATP2B1 is responsible for the uptake of the antidiabetic drug glibenclamide (Satoh et al., 2005). Although the general driving force for OATPs is still not clear, extracellular pH can affect the function of OATP2B1 (Kobayashi et al., 2003; Visentin et al., 2012). Two studies have shown that OATP2B1 transport activity decreased from pH 5.0 to 7.4 (Nozawa et al., 2004), and pH 6.5 to 8.0 (Leuthold et al., 2009). However, different mechanisms of pH-sensitive transport have been proposed. The Tamai group claimed that the proton gradient can serve as the driving force of OAP2B1-mediated uptake, while the results of the Stieger group suggested that the protonation of a conserved histidine residue at ECL2 could increase the affinity of certain substrates to OATP2B1.

B. The Na^+/taurocholate cotransporting polypeptide (NTCP)

NTCP belongs to the SLC10A family, which is also known as the "sodium bile salt cotransport family" (Hagenbuch and Dawson, 2004; Anwer and Stieger, 2014). This transporter gene family has 7 members but only three members have been well characterized (*SLC10A1*, *SLC10A2* and *SLC10A6*), and two of them (*SLC10A1*, encoding NTCP and *SLC10A2*, encoding the apical sodium-dependent bile salt transporter, or ASBT) are responsible for the uptake of classic bile acids into liver or intestine, while the other one (*SLC10A6*, encoding sodium-dependent organic anion transporter, or SOAT) is a transporter for sulfated bile acids. As indicated in the name, transporters in this family are all secondary active transporters, using the sodium gradient to carry out their function. NTCP and ASBT are considered as essential transporters to maintain the enterohepatic circulation of bile acids (Kosters and Karpen, 2008). ASBT reabsorbs the bile

acids from intestine through the apical membrane of enterocytes, while NTCP takes them up from plasma back into liver across the basolateral membrane of hepatocytes.

Human NTCP was first cloned in 1994, and is considered the major bile acid uptake transporter (Hagenbuch and Meier, 1994). Although both NTCP and OATPs can transport bile acids, studies using *Slco1a/1b* or *Slco1b2* knockout mice showed unchanged levels of conjugated bile acid levels but elevated levels of unconjugated bile acids in plasma, which suggest that at least in mice, conjugated bile acids are the main substrates of NTCP while unconjugated ones are substrates of OATPs (van de Steeg et al., 2010; Csanaky et al., 2011). Other than bile acids, NTCP can also transport steroid hormones, thyroid hormones and drugs like statins and micafungin (Yanni et al., 2010). In addition, drugs conjugated with bile acids, for example, chlorambucil-taurocholate (Kullak-Ublick et al., 1997), have been designed to target NTCP as the route to enter hepatocytes (Sievanen, 2007; Anwer and Stieger, 2014).

High bile acid levels can activate FXR and subsequently down-regulate the mRNA expression of NTCP via activation of SHP and inhibition of HNF1α (Kosters and Karpen, 2008). NTCP plasma membrane levels are highly regulated by several factors. Signaling pathways including cAMP, intracellular Ca^{2+}, nitric oxide, phophoinositide-3-kinase (PI3K), protein kinases and protein phosphatases can all influence the plasma membrane expression of NTCP (Anwer and Stieger, 2014). In addition, NTCP undergoes retrieval and degradation when exposed to high concentrations of cholestatic bile acids, thus reducing the uptake of bile acids and protecting hepatocytes from further increase of intracellular bile acids in cholestasis (Keitel et al., 2005).

Not much evidence of clinical relevance of NTCP has been shown so far. There are only two studies that have demonstrated that mutations or single-nucleotide polymorphism (SNP) in NTCP result in hypercholanemia (Vaz et al., 2015; Deng et al., 2016). In addition, recent studies described a novel important function of human NTCP (hNTCP). hNTCP supports the binding

and entry of hepatitis B virus (HBV) and hepatitis D virus (HDV) into hepatocytes (Yan et al., 2012; Tsukuda et al., 2017). Follow-up studies demonstrated that unlike hNTCP, mouse NTCP can only support the binding but not the entry of HBV and HDV into cells. The van de Graaf group generated an NTCP knockout mouse model and further demonstrated that in *Slc10a1*$^{-/-}$ mice, the HBV hepatic binding was completely abrogated in vivo. In support of these findings another group generated an hNTCP transgenic mouse and could restored the entry of HDV mediated by hNTCP (He et al., 2015). With these useful animal models, a new class of chemicals, like proanthocyanidin, have been developed to inhibit the binding and entry of HBV and HDV by targeting NTCP (Tsukuda et al., 2017). Besides that, an NTCP-directed inhibitor peptide (Myrcludex B), with an IC_{50} of approximately 80 pM, has also been developed and is currently in Phase II clinical trials (Schulze et al., 2010; Lempp and Urban, 2014).

C. The organic cation transporter 1 (OCT1)

OCT1 belongs to the SLC22 family, which consists of 13 functionally characterized transporter members in humans (Koepsell and Endou, 2004; Koepsell, 2013). This family contains 3 subgroups with closely related proteins, including organic cation transporters (OCTs), organic zwitterion/cation transporters (OCTNs) and organic anion transporters (OATs). The OCT subgroup has 3 members encoded by *SLC22A1-3* (OCT1-3) which transport small organic cations, weak bases and some neutral compounds. OCTs are generally considered as facilitated diffusion systems that can mediate the transport of their substrates in both directions across the plasma membrane, depending on the electrochemical gradients. The OCTN subgroup also has 3 members, encoded by *SLC22A4*, *SLC22A5* and *SLC22A16* (OCTN1, OCTN2 and OCT6, respectively). This subgroup can serve as either uniporter, antiporter or sodium-cotransporter, depending on the transported substrates. The third subgroup has 9 members, encoding OAT1-7

and 10, and URAT1. The substrates of the OAT subgroup are mostly organic anions, but with a smaller molecular weight compared to OATPs.

Human OCT1 was first cloned in 1997 and is mainly expressed at the basolateral membrane of human hepatocytes (Grundemann et al., 1994). At the protein level expression of OCT1 is highest in liver, but OCT1 is also detectable in the kidney at the basolateral membrane of S1 and S2 segments of the proximal tubules, in the lung at the luminal membrane of bronchial epithelial cells, in the brain in endothelial cells of microvessels and in the cornea, the iris-ciliar body and blood-retina barrier of the eye (Koepsell et al., 2007). Substrates of OCT1 include endogenous compounds, such as the neuromodulators histidyl-proline diketopiperazine (cyclo(His-Pro)) and salsolinol; model cations and toxins such as tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP); and drugs like the antidiabetic metformin, the antiviral acyclovir and more (Koepsell, 2013).

Malfunction of OCT1 is not currently associated with any diseases, but large interindividual differences in expression of OCT1 have been observed. Since OCT1 is a transporter for multiple drugs, the variability in OCT1 expression can lead to altered pharmacokinetics (Soodvilai et al., 2017). Besides that, many nonsynonymous SNPs have been identified, 10 of them affect the expression and 6 alter substrate selectivity (Koepsell et al., 2007). Because of its multiple substrates, DDIs are to be expected at OCT1. Tyrosine kinase inhibitors (TKI) including imatinib and gefitinib potently inhibit the uptake of metformin and could influence the clinical outcome of metformin treatment (Minematsu and Giacomini, 2011).

D. Brief introduction to other SLC transporters in hepatocytes

Besides the transporters introduced above, there are several other related uptake transporters expressed in human hepatocytes. Three other members from the SLC22 family, OCT3

(*SLC22A3*), OAT2 (*SLC22A7*) and OAT7 (*SLC22A9*) are also localized at the basolateral membrane (Roth et al., 2012). Compared to OCT1, OCT3 has a much wider expression range (Koepsell, 2013). Strong mRNA expression of OCT3 can be detected in liver, placenta, kidney and skeletal muscle. OCT3 plays an important physiological role in the CNS, where it can regulate the interstitial concentration of monoamine neurotransmitters and cationic drugs and thus control neuronal activities and behavior (Baganz et al., 2008). Because OCT3 can also transport many monoamines outside the CNS, it is also called "extraneuronal monoamine transporter" or EMT (Koepsell et al., 2007).

OAT2 is mainly expressed in the liver, but can also be detected in the kidney, in testis, ileum and uterus. Compared to OATP substrates, substrates of OAT2 are relatively small organic anions including many endogenous compounds like glutamate, purine and pyrimidine nucleobases, nucleosides and nucleotides, drugs like salicylate, erythromycin and 5-fluorouracil. Unlike OATPs, the major role of OAT2 at basolateral membrane of hepatocytes is the efflux of glutamate into sinusoids. OAT7 is exclusively expressed in the liver, and compared to other OATs it has a much narrower substrates selectivity, mainly transporting sulfate conjugates. The transport mechanism of OAT7 is also unique, compared to other OATs, because it uses short-chain fatty acids to exchange its sulfate conjugated substrates (Koepsell, 2013).

Organic solute transporters α and β (OSTα-OSTβ, *SLC51A-SLC51B*) are expressed at the basolateral membrane of hepatocytes. They represent one of the most unique transporters in the SLC family, because OSTα-OSTβ consist of the heterodimer of OSTα and OSTβ, encoded by *SLC51A* and *SLC51B*, respectively. OSTα is a 340-amino acids transporter with 7 transmembrane domains, while OSTβ has only 128 amino acids and one transmembrane domain. OSTα-OSTβ are expressed ubiquitously in the body, but the strongest expression is found in tissues involved in bile acid homeostasis like the small intestine, the kidney and the liver. It

represents a multispecific transporter with substrates including bile acids, E3S, digoxin and more. OSTα-OSTβ is part of the transporter system to maintain the enterohepatic circulation of bile acids. In enterocytes, it excretes bile acids into the portal circulation and NTCP takes them back up into hepatocytes across the basolateral membrane (Ballatori et al., 2013).

E. Introduction to oligomerization of transporters

Oligomerization of proteins is an important physiological phenomenon. Oligomeric proteins contain two or more associating polypeptide chains. From an evolutionary perspective, such oligomerization represents a potential advantage because protein oligomers can be controlled or regulated via allosteric interactions and higher-orders of complexity can be created (Ali and Imperiali, 2005). Based on a survey of crystal structures, at least 50% of the proteins in a cell are oligomeric (Goodsell and Olson, 2000; Nishi et al., 2013). Depending on the type of interaction, oligomerization of proteins results in various consequences. Many proteins require the close interaction with itself or with other proteins to be functional. For example, hemoglobin is a heterotetramer of two α and two β subunits, and the interaction between those subunits leads to a cooperative binding of oxygen. In addition, oligomerization can lead to variations in expression and function or alter cell signaling (Ali and Imperiali, 2005).

The interactions of proteins require an oligomeric interface, and in general, the residues involved in the interface are more hydrophobic and non-polarized compared to the other fraction of the protein, which will facilitate the formation of hydrophobic interactions between proteins (Ali and Imperiali, 2005). These characters of oligomerization indicate that transporters and other membrane proteins may have a higher chance of oligomerization because of the abundance of hydrophobic α-helical transmembrane domains.

ABC transporters can be classified into “full” or “half transporters”. Instead of having 2 transmembrane domains (TMD) and 2 nucleotide-binding domains (NBD), the half transporter consists of one TMD fused to one NBD. Therefore, homo- or hetero-dimerization with another half transporter is required for function (Dean et al., 2001; Wilkens, 2015). For example, Breast Cancer Resistance Protein (BCRP), encoded by *ABCG2*, forms homo-oligomers and tetrameric complexes (two BCRP dimers), and it has been suggested that BCRP function could be regulated by the dynamic association/dissociation of BCRP monomers via protein-protein interactions (Ni et al., 2010). Besides interactions within the ABC family, it has also been demonstrated that some of the ABC transporters can oligomerize with other proteins. P-glycoprotein (Pgp) or MDR1 encoded by *ABCB1*, is a drug efflux pump. It is expressed in the liver, the kidney, the intestine and at the blood-brain barrier (BBB), and also in many cancers where it confers the multidrug resistance phenotype. At the BBB, Pgp forms hetero-oligomers with caveolin-1 and caveolin-2, which colocalize in caveolae. When bovine brain capillary endothelial cells were cultured with caveolae-disrupting agents like filipin III and nystatin, the transport activity of Pgp was reduced significantly, suggesting that the formation of this oligomer modulates the function of Pgp (Jodoin et al., 2003).

OSTα-OSTβ, described above, are a great example of SLC transporters that form oligomers. For their proper function, oligomerization of the two proteins of different sizes is required (Wang et al., 2001). Another example of such hetero-oligomerization is the heteromeric amino acid transporters (HAT) that are formed between the light chains of the SLC7 family with one of the two heavy chains of the SLC3 family (Fotiadis et al., 2013). Recent studies demonstrated homo- and hetero-oligomerization of other SLC family members. These interactions did not all have functional consequences. OAT1 (*SLC21A6*) is predominantly expressed in kidney and brain and is responsible for the uptake of many environmental toxins. Several studies showed that OAT1 can form homo-oligomers (Hong et al., 2005), and when the oligomerization is interrupted by

either TM6 short peptide (Duan et al., 2011a) or mutations at the GXXXG motif (Duan et al., 2011b), the surface expression of OAT1 was reduced, suggesting that the oligomerization may be important for the correct localization of OAT1.

The oligomeric states of some liver transporters were also studied. The first study was conducted in 2008 by the Koepsell group. They used a cell free expression system to reconstitute rat OCT1, OCT2 and OAT1, then solubilized them and detected the formation of rOCT1 and rOAT1 homo-oligomers by co-immunoprecipitation (Keller et al., 2008). Another study from the same group confirmed the in vivo formation of oligomers in *X. laevis* oocytes that were injected with rOCT1 cRNA (Keller et al., 2011). In the same study, the cysteine residues in ECL1 of rOCT1 were replaced by serine residues, and these mutations prevented the oligomerization, suggesting that disulfide bonds are crucial for the dimerization. Though a decreased K_m of the uptake of tetraethyammonium$^+$ (TEA) by mutant rOCT1 was determined, similar substrate affinities of the oligomerizing and non-oligomerizing rOCT1 were obtained indicating that the altered K_m was due to the cysteine mutation instead of the "non-oligomerization". A further study confirmed this result by showing no changes after comparing K_m before and after treatment with dithiothreitol (Keller et al., 2011). Combining these studies, it was concluded that rat OCT1 can form homo-oligomers and that oligomerization does not affect the transport function.

The oligomeric state of the first human liver transporter reported was NTCP (Bijsmans et al., 2012). Based on older studies and results that showed unexpected higher bands on immunoblots, two differently tagged NTCP proteins were generated for co-immunoprecipitation and fluorescence resonance energy transfer (FRET) experiments. Indeed, the results showed that NTCP can form homo-oligomers. Additional experiments demonstrated that like rat OCT1, NTCP homo-oligomers worked as individual subunits. In this study, immunofluorescence images suggested that besides with itself, NTCP also co-localized with additional transporters of

the SLC10 family, P4 (*SLC10A4*) and SOAT (*SLC10A6*). The hetero-oligomerization with P4 influenced the expression of NTCP, and reduced the bile acid uptake function of NTCP. Besides NTCP, a recent study also demonstrated the homo-oligomerization of OATP1B1, and suggested that the GXXXG motif at position 219 and 393 could affect expression and function of OATP1B1 (Ni et al., 2017).

III. Photoaffinity Labeling

A. Overview

Photoaffinity labeling is a powerful technique for the study of protein ligand interactions. This method was first introduced by Frank Westheimer in the early 1960s (Singh et al., 1962), and was developed and evolved continuously after then. The rationale behind this method is to turn the weak binding into a solid covalent bond between the target protein and the photoreactive ligand (Smith and Collins, 2015). By modifying a ligand with a photo-reactive moiety, such as a diazirine, arylazide or benzophenone, the ligand can be activated at the appropriate wavelength of light, and the reactive intermediate reacts with almost any type of amino acid close to it and forms a covalent bond with it (Hashimoto and Hatanaka, 2008). After it was developed, photoaffinity labeling was widely applied in the field of protein interaction studies, such as ligand binding, protein-protein or protein-nucleic acid interactions (Smith and Collins, 2015). However, with the development of structural analysis techniques, such as mass spectrometry and high-performance liquid chromatography, scientists can now perform even more in-depth analysis after the labeling process (Robinette et al., 2006), and thus, currently, identifying or characterizing the ligand-binding site within the protein has become the common application of photoaffinity labeling (Smith and Collins, 2015).

The most significant strength of photoaffinity labeling is the relative tight interaction between ligand and target proteins (Hashimoto and Hatanaka, 2008). Besides that, several other benefits make photoaffinity labeling a good technique for protein interaction studies. The photoreactive moiety is relatively small, so it can be attached to proteins, nucleic acids and even small molecule drugs like salicylic acid, without significantly interfering with their original structure and function (Tian et al., 2012). Some small molecule ligands may be capable of intrinsic photoactivation, such as compounds contain sulfides, enones, dienones and halogenated moieties (Robinette et al., 2006). Photoreactive moieties can also be attached to ligands with a fluorophore or radioactive ligand which can further facilitate the detection process.

Though photoaffinity labeling is well-characterized, there are some downsides of this method. The design of a photoreactive probe is relative challenging. A number of variables can affect the outcome of the experiment, such as the stability of the probe in the dark at a range of pHs, and the strength of the newly formed bond (Smith and Collins, 2015). Besides that, the wavelength required to activate certain photoreactive moieties can also damage the biological molecules. Because of these shortcomings, the application of photoaffinity labeling is limited. Photoaffinity labeling can be a good technique to study transporters. As a membrane protein, it is relatively difficult to purify and crystalize a transporter, and thus, the structure information of transporters is very limited compared to soluble proteins. Photoaffinity labeling provides the possibility of identifying binding-sites of a transporter without crystallization. In addition, photoaffinity labeling may help to characterize the translocation pathways by analyzing the binding of ligands in the transmembrane domains.

B. Application of Photoaffinity labeling to transporters

The first identification and characterization of the rabbit ileum Na^+-dependent bile acid transporter (IBAT, or rabbit ASBT) in 1993 included photoaffinity labeling and transporter

assays (Kramer et al., 1993). Brush border membrane vesicles from 8 different segments in the small intestine were analyzed for the expression of the bile acid transport system. A diazirine-labeled bile acid derivative was used to screen membrane proteins which can bind bile acids. A 93-kDa membrane protein was identified as the potential ileal Na^+-dependent bile acid transporter because its expression correlated in the ileum with bile acid transport and because photoaffinity labeling occurred only in the presence of sodium. This protein was further investigated and named IBAT. After IBAT was cloned in 1994, Kramer's group again used photoaffinity labeling techniques to characterize the uptake function of IBAT by competing the binding site of the photoreactive bile acids with potential substrates and thus identified potential substrates or inhibitors of IBAT (Kramer et al., 1997; Kramer et al., 2001a).

Photoaffinity labeling was also used to study the binding pockets of transporters like P-glycoprotein (Pgp), also called multidrug resistance protein 1 (MDR1) (Safa, 2004). By using site-directed antibodies, the binding of photoreactive substrates could be blocked and the binding domains could then be identified (Morris et al., 1994). Combining studies of many different photoreactive substrates, the Callaghan group concluded that there are at least four binding sites in Pgp, which can be divided into transport sites and regulatory sites (Martin et al., 2000). By mutating these binding sites that were identified by photoaffinity labeling, the mechanism of drug resistance by Pgp was also investigated. Furthermore, based on photoaffinity labeling results a general pharmacophore was proposed for Pgp, which contained two hydrophobic points, three hydrogen bond acceptor points and one hydrogen bond donor (Pajeva and Wiese, 2002).

To conclude, photoaffinity labeling can be a powerful tool to identify and characterize the binding pockets of proteins. It is widely applied in the study of protein related interactions, especially in the drug discovery and development processes. The application of photoaffinity

labeling in the study of IBAT and Pgp has shown a great potential of using this technique to improve the structure function studies of transporters.

IV. Concluding remarks for the introduction

Transporters in human liver play a pivotal role in the absorption, distribution and elimination of numerous compounds. Previous results have revealed the oligomeric state of some transporters and their potential influence on the function of transporters. Besides that, photoaffinity labeling has been used as a unique technique to study the translocation mechanism of transporters. To further understand liver uptake transporters, the oligomeric state and structure function relationship of OATP1B3 needs to be studied in order to answer the following questions: **1) Does OATP1B3 form homo-oligomers and what would be its functional consequence? 2) What is the relationship among liver transporters, do they interact and are there any functional consequences of such interactions? 3) How does OATP1B3 translocate its substrates?**

V. Specific aims of this dissertation

The overall goal of my research was to study the oligomeric state and translocation pathways of OATP1B3, and the potential functional consequences. In presenting this dissertation, I will defend 3 specific aims.

Specific aim 1 evaluates the hypothesis that **OATP1B3 can form homo-oligomers and the interaction can affect the uptake function of OATP1B3**. To address this aim, two differently tagged OATP1B3 plasmids were constructed and were used for co-immunoprecipitation in co-transfected HEK293 cells. Then the proximity ligation assay was used to further confirm the

interaction between OATP1B3 and itself. Uptake assays were performed to investigate potential functional consequences. The purpose of this study was to investigate the oligomeric state of OATP1B3 and its potential functional consequences.

Specific aim 2 evaluates the hypothesis that **OATP1B3 can form hetero-oligomers and the interaction with other transporters can affect the expression and function of the involved transporters**. To investigate this aim, OATP1B3 was cotransfected into HEK293 cells with other transporters including OATP1B1, NTCP and OCT1, and the proximity ligation assay was used to detect the interaction between these transporters. Then frozen liver sections were used to confirm the interaction in human liver. After the interaction was confirmed, functional consequences and surface expression were studied by using cotransfected HEK293 cells. The purpose of this study was to determine the interaction between liver transporters and the potential functional consequences.

Specific aim 3 evaluates the hypothesis that **photoaffinity labeling can be used to study the substrate binding sites and translocation pathways of OATP1B3**. To study this aim, a working method to isolate, solubilize and purify OATP1B3 from OATP1B3 stable transfected Chinese Hamster Ovary (CHO) cells was established. After that, 8-fluorescein-cAMP was applied to intact CHO cells and UV light was used to form covalent bonds between the molecule and OATP1B3. I then attempted to isolate and purify labeled OATP1B3 for mass spectrometry analysis to identify the substrate binding sites and translocation pathways.

Chapter 2

Materials and Methods

I. Materials

Radioactive compounds, [^{3}H]estrone-3-sulfate (E3S, 54.0 Ci/mmol), [^{3}H]estradiol-17β-glucuronide (E17βG, 49.8 Ci/mmol), [^{3}H]CCK-8 (87.9 Ci/mmol) and [^{3}H]taurocholate acid (15.4 Ci/mmol) were purchased from PerkinElmer (Boston, MA). [^{3}H]1-methyl-4-phenylpyridinium (MPP^{+}) (80 Ci/mmol) was from American Radiolabeled Chemicals (St. Louis, MO), and [^{14}C]metformin (112 mCi/mmol) was from Moraveck Biochemicals (Brea, CA). Unlabeled CCK-8 was purchased from Bachem (Torrance, CA). The crosslinking reagent 3,3'-dithiobis (sulfosuccinimidyl propionate) (DTSSP) and Dynabeads® Protein G for immunoprecipitation were purchased from ThermoFisher (Waltham, MA). Duolink components for the proximity ligation assay were from Sigma-Aldrich (St. Louis, MO). Mouse anti-FLAG antibody conjugated to magnetic beads was from Sigma-Aldrich (St. Louis, MO). Primary antibody information is listed in Table 2-1. All other chemicals and reagents were of analytical grade and were readily available from commercial sources. Frozen human liver was obtained through the Cell Isolation Core in the Department of Pharmacology, Toxicology and Therapeutics at University of Kansas Medical Center, in accordance with a protocol approved by the Institutional Review Board of the University of Kansas Medical Center from patients undergoing hepatic resection procedures or from liver donors.

Table 2-1: List of primary antibodies.

Epitope	Species	Vendor	Catalog number	Dilution Ratio	
				Western Blot	Immunofluorescence
FLAG tag	Mouse	Sigma-Aldrich	F3040	1:2000	1:1000
Fluorescein	Mouse	Abcam	Ab6656	1:1000	-
HA tag	Mouse	Lifetein	LT0422	1:2000	1:500
His tag	Rabbit	Abcam	ab125262	1:1000	1:1000
His tag	Mouse	Qiagen	34670 (Tetra-His)	1:2000	1:1000
Na^+/K^+ ATPase	Mouse	Abcam	ab7671	1:2000	1:1000
NTCP	Goat	Santa Cruz	sc134461	-	1:500
OATP1B3	Mouse	Abcam	ab15442 (MDQ)	1:100	-
OATP1B3	Rabbit	Gift from Dr. Bruno Stieger	K28	1:1000	1:1000
OCT1	Mouse	Novus	NBP1-51684	1:1000	1:1000

II. Generation of OATP1B3 with a FLAG- or HA-tag at the C-terminal end

To generate OATP1B3 with either a C-terminal FLAG- or HA-tag, the open reading frame (ORF) of OATP1B3 was amplified with the following primers using the expression plasmid encoding the His-tagged OATP1B3 as a template: Forward, 5' GCTAGCTAATACGACTCACTATAGGGACCATGGAC 3'; OATP1B3-FLAG reverse, 5' GCGGCCGCTTACTTAT CGTCGTCATCCTTGTAATCGTTGGCAGCAGCATTGTCTTGCATGT 3'; OATP1B3-HA reverse, 5' GCGGCCGCTTAAGCGTAATCTGGAACATCGTATGGGTAGTTGGCAGCAGCATTGTCT TGCATGT 3'. After amplification, the ORF of OATP1B3 with either a FLAG- or an HA-tag was inserted between the *NheI* and *XhoI* sites of the pcDNA5/FRT plasmid. The resulting constructs were sequenced to verify insertion of the desired tags.

III. Cell culture and transporter expression

Chinese Hamster Ovary (CHO) Flp-In cells transfected with OATP1B3-His or pcDNA5/FRT (Empty Vector) were previously generated. Human embryonic kidney (HEK293) cells were purchased from ATCC (Manassas, VA); the cells were routinely tested for the absence of mycoplasma using the MycoAlert™ Mycoplasma Detection Kit (Lonza, Hopkinton, MA). CHO Flp-In cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 1 g/L D-glucose, 2mM L-glutamine, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, and 110mg/L sodium pyruvate supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, Utah), 50mg/ml L-proline, 100U.mL penicillin, 100mg/mL streptomycin (Invitrogen), and 500 mg/mL hygromycin (Invitrogen, Carlsbad, California) in a humidified environment at

37°C and 5% CO_2. HEK293 cells were grown in DMEM high glucose (Invitrogen, Carlsbad, CA), supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified environment at 37°C and 5% CO_2. Plating, transfection and uptake experiments on 24-well plates were performed as previously reported (Zhao et al., 2015). Transfection conditions varied among different experiments (for detailed conditions see figure legends) with constant 500ng total plasmids been transfected. The following day media was changed and uptake experiments (for time points see figure legends) were performed 24 or 48 hours after transfection. Each transfection condition was performed in triplicate.

IV. DUALmembrane screening

The DUALmembrane screening, a modified yeast two-hybrid screening, was performed by Dualsystems Biotech AG (Schlieren, Switzerland) with full-length OATP1B3 as the bait and a human adult liver cDNA library with 1.5×10^6 independent clones.

V. Immunofluorescence

Transiently transfected HEK293 cells in 4-well chambers were washed three times with phosphate-buffer saline (PBS) and fixed and permeabilized with 2% paraformaldehyde in PBS for 10 minutes in the presence or absence of 1% Triton-X 100. After another 4 washes with PBS, cells were blocked with 5% normal donkey serum for 1 hour at room temperature. Cells were then incubated at 4°C overnight with the respective primary antibodies in 1% normal donkey serum in PBS. After washing with PBS, slides were incubated with donkey anti-mouse AlexaFluor 488 (ThermoFisher) as secondary antibody, diluted 1:1000 in 0.1% PBS-Tween for 1

hour, and after a final wash, slides were mounted in Prolong Gold containing DAPI (ThermoFisher). For negative controls, the sections were incubated with secondary antibody only.

VI. Proximity ligation assay

Transiently transfected HEK293 cells in 8-well chambers were washed three times with PBS and fixed with PBS containing 2% paraformaldehyde and 1% TX-100 in PBS for 10 minutes. After 4 washes with PBS, cells were blocked with 5% normal donkey serum for 1 hour at room temperature and then incubated overnight at 4°C with respective primary antibodies (see details in figure legends). After 4 washes with PBS, slides were incubated with the respective pair of Duolink® In Situ PLA® Probes (Sigma-Aldrich) at room temperatures for 1 hour, and then washed twice for 5 minutes each with Wash Buffer A (Sigma-Aldrich). Ligation at 37°C for 30 minutes and amplification at 37°C for 100 minutes were then performed following the manufacturer's protocols. Before mounting with Duolink® In Situ Mounting Medium with DAPI (Sigma-Aldrich), cells were washed twice for 10 minutes each with Wash Buffer B and once for 1 minute with 0.01 x Wash Buffer B.

VII. Crosslinking and isolation of membrane fractions

Transiently transfected HEK293 cells and freshly isolated and plated human hepatocytes were washed with PBS and then incubated in PBS with or without 2mM DTSSP in PBS for crosslinking at 4°C for 2 hours. Crosslinking was stopped by incubating with 20mM Tris-HCl (pH 7.5) for 15 minutes. Cells were then washed with ice-cold PBS, and cold hypotonic

homogenization solution (1mM NaCl, 5mM Tris-HCl pH 7.5) with Complete Protease Inhibitor Cocktail (Roche Diagnostics Corporation, Indianapolis, IN) was added before cells were scraped off the plates. The cells were homogenized with 20 strokes using a glass-Teflon homogenizer, and the homogenates were centrifuged at 900 x g for 10 minutes. The resulting supernatants were centrifuged again at 10,000 x g for 20 minutes and the pellet containing a membrane enriched fraction was solubilized with PBS containing 0.1% NP-40. After centrifugation at 14,000 x g for 30 minutes to remove insoluble particles, the supernatant was used for immunoprecipitation experiments.

VIII. Protein purification by Ni-NTA columns

Membrane enriched fractions isolated from one 10 cm dish of CHO Flp-In cells were first solubilized in 500 μL of Lysis Buffer (150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 10mM Tris-HCl, pH=7.5) for 10 minutes on ice. After a 14,000 x g centrifugation for 30 minutes at 4 °C the supernatant was kept for purification and the pellet was discarded. Ni-NTA spin columns (Qiagen) were equilibrated twice with 500 μL of Lysis buffer and centrifuged at 900 x g to remove the Lysis Buffer. The supernatants were then loaded on to the columns and spun down at 300 x g to bind the 6 x His-tagged proteins. After binding, columns were washed twice with Lysis Buffer containing 20 mM imidazole and spun down at 300 x g, to remove non-specific proteins. Proteins with a 6 x His tag were then eluted with 100 μL of Lysis Buffer containing 50mM imidazole. The eluted proteins were stored at -20 °C before separation with SDS-PAGE and analysis by western blot.

IX. Surface biotinylation

Transiently transfected HEK293 cells in 6-well plates were washed with ice-cold PBS and then treated with 1mg/mL EZ-Link Sulfo-NHS-SS-Biotin (Thermo Fisher, Waltham, MA) in PBS for 2 hours at 4 °C. The reaction was stopped by washing with PBS containing 100mM Tris (pH = 7.5) for 15 minutes, and then the cells were lysed and harvested in Lysis Buffer (150mM NaCl, 1mM EDTA, 0.1% SDS, 1% Triton X-100, 10mM Tris-HCl, pH=7.5) with Complete Protease Inhibitor Cocktail for 10 minutes. Lysates were centrifuged at 10,000 x g for 5 minutes and the resulting supernatants were added to Streptavidin-coupled Dynabeads (Thermo Fisher) and rotated end-over-end for 2 hours at room temperature. The beads were then centrifuged at 850 x g for 2 minutes and after two washes for 5 minutes each with Lysis Buffer, cell surface proteins were recovered by an incubation with Elution Buffer (2X Laemmli buffer containing 10% β-mercaptoethanol diluted with an equal volume of Lysis Buffer containing 2X Protease Inhibitors) for 30 minutes at room temperature. After heating at 50°C for 10 minutes, cell membrane proteins were separated using SDS-PAGE followed by immunoblotting.

X. Western blot analysis

SDS-PAGE was performed with a mini-protean Tetra system from Bio-Rad. Samples were mixed with 5X Laemmli-buffer (300mM Tris-Cl pH 6.8, 10% SDS, 50% glycerol, 0.05% bromophenol blue and 12.5%β-mercaptoethanol), heated to 50°C for 10 minutes, and then separated on 4%-20% gradient SDS gels (Expedeon, San Diego, CA) for 2 hours. After SDS-PAGE, separated proteins were transferred to PVDF membranes using the Trans-Blot Turbo Transfer system from Bio-Rad. The membranes were then blocked with 5% non-fat milk in PBS for 1 hour at room temperature and incubated with the respective primary antibodies in 1% BSA

in PBS-Tween overnight at 4°C. After 3 washes with PBS-Tween, HRP-conjugated secondary antibodies were added to the membrane for 1 hour at room temperature. Protein bands were then visualized using the SuperSignal West Pico Chemiluminescent Substrate (ThermoFisher).

XI. Cell-based uptake assay

Cells plated in 24-well plates were carefully washed 3 times with prewarmed uptake buffer (for recipes of uptake buffers for different cell lines, see Table 2-2) and then incubated with 200 µL of uptake buffer containing substrate for the indicated time periods at 37°C. Uptake was stopped by removing the substrate solution and washing the cells with ice-cold uptake buffer 4 times. To quantitate uptake by the transporter, the cells were then solubilized with 300µL of 1% Triton X-100 in PBS per well and 200µL were used for liquid scintillation counting. From the remaining lysate, protein concentration was determined for each well using the BCA protein assay kit (ThermoFisher).

Table 2-2: Recipes of uptake buffers for HEK293 cells and CHO Flp-In cells.

Chemicals	HEK293 Cells	CHO Flp-in cells			
		CHO	A-Cl	KRH1	KRH2
NaCl/Choline Chloride*	142mM	116.4mM	-	118mM	125mM
KCl	5mM	5.3mM	100mM	4.75mM	4.8mM
KH_2PO_4	1mM	-	-	-	1.2mM
NaH_2PO_4	-	1mM	-	1.2mM	-
$NaHCO_3$	-	-	-	25mM	-
$MgSO_4$	1.2mM	0.8mM	-	1.2mM	1.2mM
$CaCl_2$	1.5mM	-	-	2.5mM	1.3mM
Glucose	5mM	5.5mM	-	11.12mM	5.6mM
HEPES	12.5mM	20mM	25mM	-	-
EGTA	-	-	2.5mM	-	-
IBMX	-	-	20μM	-	-

Tris buffer was used to adjust pH to 7.4 for all buffer.

*: Choline Chloride was used to replace Na^+ for the NTCP uptake study.

XII. Photoaffinity labeling

Wild-type or OATP1B3 expressing CHO Flp-In cells were plated in 10 cm dishes at 1 million cells per dish and incubated for three days with complete culture medium listed above. Dishes were then washed with 5 mL of A-Cl buffer (Table 2-2) twice to remove medium. Five mL of A-Cl buffer containing 8-fluorescein-cAMP (8-FcA) in the presence or absence of 100 μM losartan was added into the dishes, and immediately exposed under UV light (302 nm wavelength, UVP) for the indicated length of time (Figure 5-3) while control dishes without UV activation were kept in the dark. To stop the reaction, cells were washed with 5 mL ice-cold A-Cl buffer three times. Membrane enriched fractions were isolated according to the protocol mentioned above, and analyzed by SDS-PAGE and western blot.

XIII. Calculations and statistics

All calculations were performed using Prism 6 (GraphPad Software Inc., San Diego, CA). Kinetic parameters were determined within the initial linear period of uptake after correction for protein and subtraction of uptake by the control cell line. Statistical analysis was performed with GraphPad Prism 6.

Chapter 3

Organic Anion Transporting Polypeptide 1B3 can form homo- and hetero-oligomers

Published: Plos one. June 23, 2017.

I. Introduction

Organic anion transporting polypeptide (OATP) 1B3 is a multispecific drug uptake transporter belonging to the SLCO superfamily (Hagenbuch and Stieger, 2013). Under normal physiological conditions OATP1B3 and the closely related OATP1B1 are selectively expressed at the sinusoidal membrane of human hepatocytes (Obaidat et al., 2012). The two drug transporters are responsible for the uptake of numerous endogenous compounds, such as bile acids and hormones, and many xenobiotics including various drugs, into hepatocytes (Roth et al., 2012). In addition, conjugated bile acids, including taurocholate or glycocholate, are transported into human hepatocytes via Na^+/Taurocholate Cotransporting Polypeptide (NTCP) (Claro da Silva et al., 2013) which has recently also been identified as the receptor for human hepatitis B and D virus (Yan et al., 2012).

Drug uptake transporters contribute to drug metabolism like the cytochrome P450 drug-metabolizing enzymes, and both can be modulated at different levels (Svoboda et al., 2011; Stieger and Hagenbuch, 2014). The most direct way is to modulate OATP-mediated drug uptake with compounds that either inhibit or stimulate the transporter. Several studies have demonstrated that inhibition of OATP1B1 and/or OATP1B3 can lead to drug-drug-interactions (Konig et al., 2013). For example, the rifampicin - bosentan interaction that was observed in healthy subjects (van Giersbergen et al., 2007) or the interaction between bosentan and sildenafil (Burgess et al.,

2008) could both be explained by direct inhibition of OATP1B1- and OATP1B3-mediated bosentan uptake by rifampicin and sildenafil (Treiber et al., 2007). Besides inhibition, direct stimulation of OATP-mediated uptake has also been reported. OATP1B3-mediated uptake of estradiol-17β-glucuronide (E17βG) can be stimulated by clotrimazole (Gui et al., 2008) or a substituted quercetin (Zhang et al., 2013), OATP1B3-mediated uptake of estrone-3-sulfate (E3S) is stimulated by the green tea catechin epigallocatechin gallate (Roth et al., 2011), and OATP1B1- or OATP1B3-mediated uptake of pravastatin is stimulated by NSAIDS like diclofenac or ibuprofen (Kindla et al., 2011). Importantly, these stimulatory effects increased the affinities of the substrates to the OATPs, indicating allosteric modulation. Besides direct interactions with the transport protein, the function of OATPs can also be regulated at the transcriptional, translational, or even post-translational level (Powell et al., 2014; Stieger and Hagenbuch, 2014; Mayati et al., 2015). Furthermore, there is the potential for protein-protein interactions that could influence transporter function, similar as has been shown for drug-metabolizing enzymes (Kandel and Lampe, 2014). It has been demonstrated that some rat transporters expressed in hepatocytes, including NTCP (Bijsmans et al., 2012), and OCT1 can form homo-oligomers (Keller et al., 2008). In addition, a recent study showed that human OATP1B1 may form and function as oligomers (Ni et al., 2017). However, no such studies have been reported so far for human OATP1B3. Therefore, the goal of the present study was to investigate whether human OATP1B3 could form homo- or hetero-oligomers and how such oligomerization would affect transport function.

II. Results

2.1 Generation and characterization of different tagged OATP1B3

Previous results from our lab showed that two bands were detected when OATP1B3 transiently expressed in HEK293 cells was analyzed on western blots; one at around 120kDa (normal size of OATP1B3) and another one at around 250kDa (Gui and Hagenbuch, 2008). When the samples were treated with DTSSP, a crosslinker that contains a cleavable disulfide bond, the major band for OATP1B3 appeared around 250kDA and was reduced to about 120kDa in the presence of β-mercaptoethanol (Figure 3-1). These results suggested that OATP1B3 could form dimers, either with itself or with other proteins.

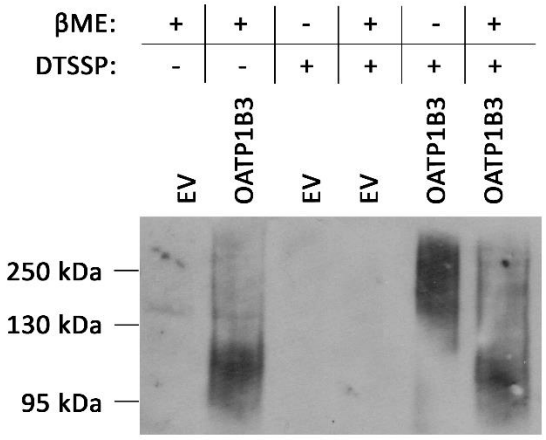

Figure 3-1: Western blot of His-tagged OATP1B3.

HEK293 cells transfected with empty vector (EV) or His-tagged OATP1B3 were treated with or without DTSSP. Whole cell lysates were separated on a 7.5% gel in the presence or absence of β-mercaptoethanol (βME) and after transfer to a PVDF membrane His-tagged OATP1B3 was detected with an anti-His antibody.

We first wanted to test the hypothesis that OATP1B3 could form homodimers and performed co-immunoprecipitation experiments using OATP1B3 constructs with different C-terminal tags. In addition to the already published OATP1B3-His (Gui and Hagenbuch, 2008) we also constructed OATP1B3-HA and OATP1B3-FLAG and characterized these constructs. Uptake of two model substrates, estradiol-17β-glucuronide (E17βG) and estrone-3-sulfate (E3S) was compared to OATP1B3-His which has the same function as wild type OATP1B3 (Gui and Hagenbuch, 2008). As can be seen in Figure 3-2A all three constructs mediated uptake of both model substrates to the same extent, suggesting that the newly constructed HA- and FLAG-tagged OATP1B3 have the same function as wild-type and His-tagged OATP1B3. In order to test whether the commercial antibodies against the different tags would work for immunofluorescence and for the Proximity Ligations Assay (PLA) we first tested them using transiently transfected HEK293 cells. Under normal conditions, the C-terminal end of OATP1B3 is on the cytoplasmic side (Abe et al., 2001) and an antibody reacting with the C-terminal end only stains cells if they have been permeabilized, e.g. with 1% Triton X-100. However, adding a tag of 6 to 9 amino acids with additional charges might change the topology of OATP1B3 at least at the C-terminal end. Therefore, we transiently transfected HEK293 cells with the three differently tagged OATP1B3 and after 48 hours incubated the fixed HEK293 cells in the absence and presence of 1% Triton X-100 before adding the respective antibodies. The results shown in Figure 3-2B demonstrate that the anti-His and anti-FLAG antibodies indeed only reacted with the transfected OATP1B3 after permeabilization with Triton X-100. However, the anti-HA antibody also resulted in positive staining in the absence of Triton X-100, suggesting that the C-terminal end of OATP1B3-HA is located at the extracellular side of the plasma membrane. None of the three antibodies used reacted with empty vector transfected HEK293 cells in the absence or presence of Triton X-100 (Figure 3-3).

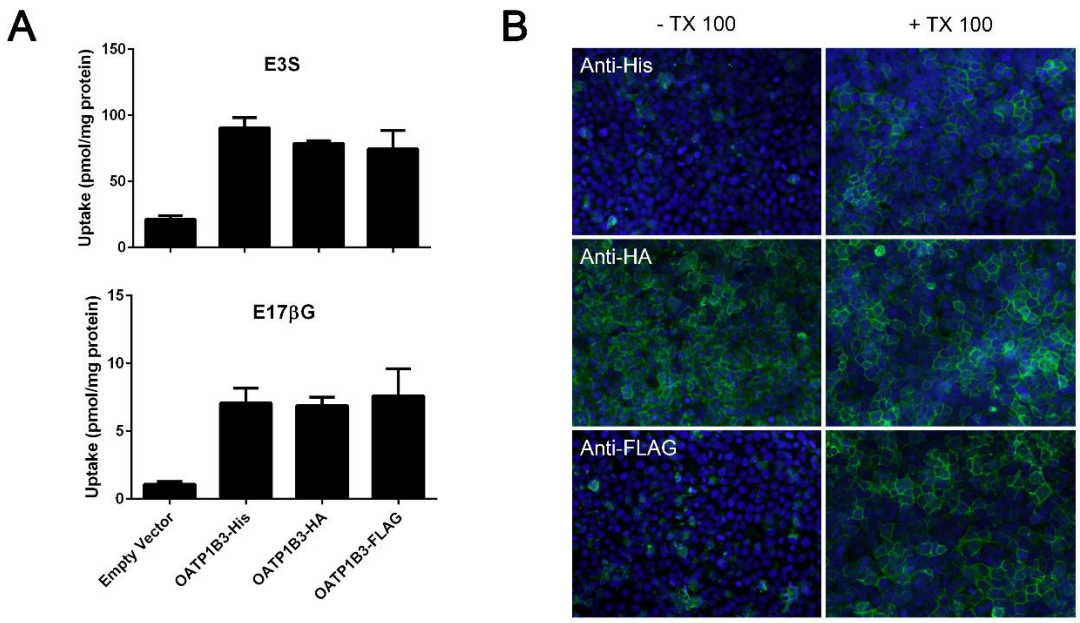

Figure 3-2: Characterization of differently tagged OATP1B3 proteins.

(A) Uptake of 10 µM estrone-3-sulfate (E3S) or 1 µM estradiol-17-β-glucuronide (E17βG) was measured at 37°C with HEK293 cells transiently transfected with empty vector or OATP1B3 with a His-, HA- or FLAG-tag for 1 minute. Values are means ± SD of three independent experiments each performed with triplicate measurements. (B) HEK293 cells transiently transfected with OATP1B3 with a His-, HA- or FLAG-tag were fixed in the absence or presence of 1% TX-100. The different OATP1B3s were detected using the respective antibodies (shown in green) and nuclei were stained by DAPI (shown in blue).

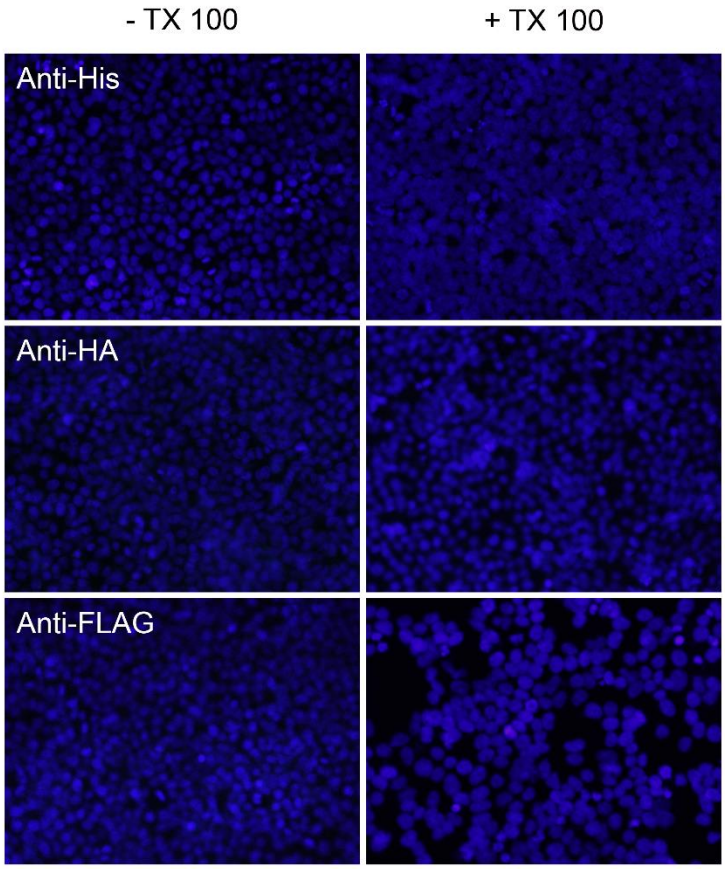

Figure 3-3: Immunofluorescence control of the three antibodies used to detect His-, HA- and FLAG-tagged OATP1B3.

HEK293 cells transiently transfected with empty vector were fixed in the absence or presence of 1% TX-100. The cells were then incubated with either anti-His, anti-HA or anti-FLAG antibodies followed by the respective secondary antibodies that should result in a green signal. Nuclei were stained by DAPI (shown in blue).

2.2 Homo-oligomerization of OATP1B3

To investigate whether OATP1B3 would be able to interact with itself, we transfected HEK293 cells with equal amounts of His- and FLAG-tagged OATP1B3 and 48 hours later we immunoprecipitated the respective OATP1B3 either with an anti-His or an anti-FLAG antibody. As can be seen in Figure 3-4A, immunoprecipitation with the anti-His antibody followed by western blotting with an anti-His antibody results in the detection of the His-tagged OATP1B3. Immunoprecipitation with the anti-FLAG antibody followed by western blotting with the anti-His antibody also allowed detection of His-tagged, and therefore co-immunoprecipitated OATP1B3. The obtained signals are much stronger after treatment with the chemical crosslinker DTSSP (Figure 3-4A, right hand side). Because the proteins were separated in the presence of β-mercaptoethanol which cleaves DTSSP, the most of the immunoprecipitated proteins are seen at the molecular weight of the monomer. When the experiments were repeated with HA- and FLAG-tagged OATP1B3 and the western blot was probed with an HA-antibody a similar result was obtained (Figure 3-4B); immunoprecipitation with the HA-antibody also co-precipitated the FLAG-tagged OATP1B3. Thus, these results demonstrated that OATP1B3 can form homo-oligomers when expressed in HEK293 cells.

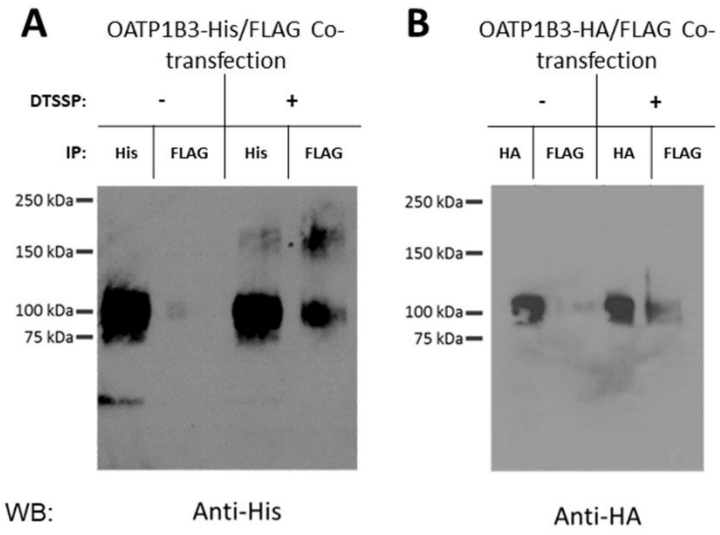

Figure 3-4: Immunoprecipitation of differently tagged OATP1B3 proteins.

Membrane fractions isolated from HEK293 cells transiently transfected with OATP1B3-His and OATP1B3-FLAG (A) or OATP1B3-HA and OATP1B3-FLAG (B) and treated with or without DTSSP were solubilized. Immunoprecipitation was performed with Anti-His or Anti-FLAG antibodies (A), or Anti-HA or Anti-FLAG antibodies (B). The eluted proteins were detected with Anti-His antibody (A) or Anti-HA antibody (B).

To confirm the co-immunoprecipitation results with an additional experimental approach and to demonstrate that OATP1B3 interacts with itself in the plasma membrane we used the Duolink PLA. In this assay the interaction or close proximity (<40 nm) of two proteins, here OATP1B3-His and OATP1B3-FLAG or OATP1B3-FLAG with another His-tagged transporter (see below) is detected by using the respective anti-His and anti-FLAG antibodies that were raised in different species (rabbit anti-His and mouse anti-FLAG). After incubation with species specific secondary antibodies that have a short DNA sequence attached, the samples are incubated with linker DNA and a ligase to form DNA circles. Subsequently these circles are amplified in the presence of fluorescent nucleotides and the signals are detected using fluorescence microscopy. In Figure 3-5, a rabbit anti-His antibody and a mouse anti-FLAG antibody were used to perform PLA. Transfection of neither His- nor FLAG-tagged OATP1B3 alone resulted in any signals, demonstrating that there was no cross-reaction between the mouse and the rabbit PLA probe. However, when OATP1B3-His and OATP1B3-FLAG were co-transfected, clear signals at the plasma membrane could be detected, which confirmed the immunoprecipitation results and further demonstrated that OATP1B3 can form homodimers or oligomers when expressed in HEK293 cells.

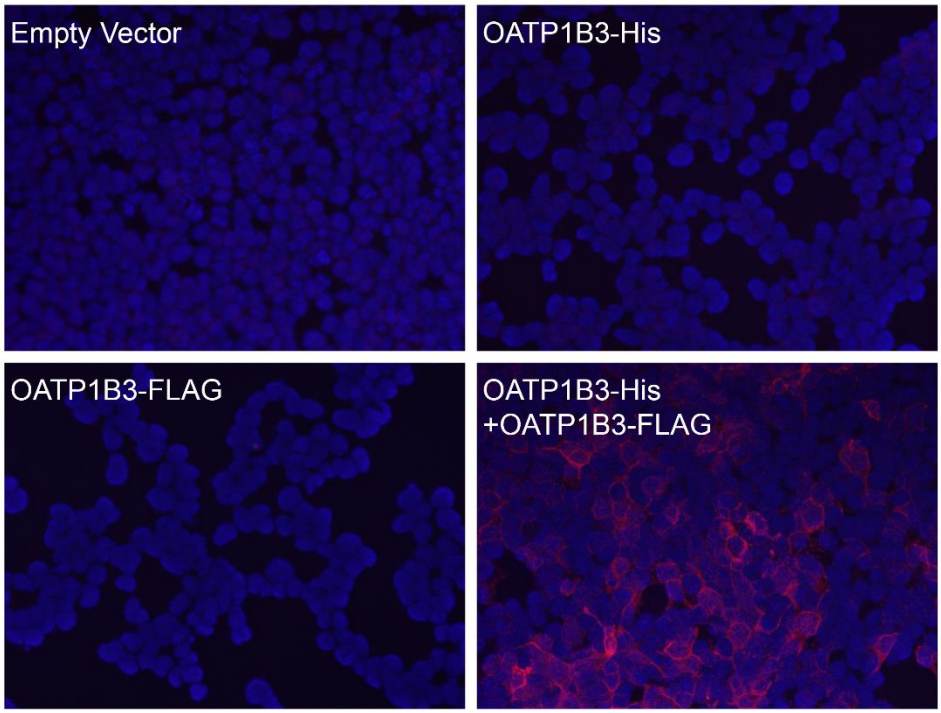

Figure 3-5: DuoLink Proximity Ligation Assay (PLA) of OATP1B3-His and OATP1B3-FLAG.

HEK293 cells were transiently transfected with empty vector, OATP1B3-His, OATP1B3-FLAG or both OATP1B3-His and OATP1B3-FLAG. After fixation, Duolink PLA was performed on all cells using a rabbit anti-His antibody in combination with a mouse anti-FLAG antibody. Co-localization signals are shown in red and nuclei in blue.

2.3 Functional influence of OATP1B3 oligomerization

Since there was abundant interaction of OATP1B3 at the plasma membrane, we further investigated whether oligomerization would affect transport activity. We used a non-transporting mutant of OATP1B3, where the lysine at position 41 was replace by a cysteine, OATP1B3-K41C-His. To ascertain that the mutant OATP1B3 is still capable of forming oligomers we repeated the co-immunoprecipitation experiment and co-transfected the His-tagged mutant with wild-type OATP1B3-FLAG. The results in Figure 3-6 demonstrate that in particular after crosslinking with DTSSP, immunoprecipitation with the His-tagged mutant OATP1B3-K41C will co-precipitate wild-type FLAG-tagged OATP1B3. We then measured uptake of the two model substrates E3S and E17βG after co-transfection and compared the results with transport obtained with single transfections of either wild-type OATP1B3-FLAG or the non-functional mutant OATP1B3-K41C-His (Figure 3-6B). If OATP1B3 oligomers would only work as a functional unit, and thus require two or more functionally active monomers, then the introduction of the mutant OATP1B3-K41C should abolish the whole unit and there should be less uptake, if any. However, uptake into HEK293 cells that were transfected with 500ng of OATP1B3-FLAG plus 500ng of OATP1B3-K41C-His (co-transfection) was 25-35% higher than uptake into HEK293 cells transfected with 500ng of OATP1B1-FLAG plus 500ng of empty vector (OATP1B3-FLAG). Uptake into HEK293 cells transfected with 500ng of OATP1B3-K41C-His plus 500ng of empty vector (OATP1B3-K41C) was as expected very low. This result indicates that each OATP1B3 in the dimer or multimer can transport on its own. However, why was transport higher after co-transfection as compared to single transfection? We transfected HEK293 cells with increasing amounts of OATP1B3 plasmid (up to 1,000 ng per well) and measured uptake. The results demonstrated that uptake after transfection with 1,000 ng was more than twice the uptake after transfection with 500ng of plasmid (Figure 3-7) suggesting that the amount of OATP1B3 at the plasma membrane was affected. Therefore, we used surface biotinylation to

determine the relative amounts of wild-type OATP1B3-FLAG and mutant OATP1B3-K41C-His at the plasma membrane after individual or co-transfection. The results shown in Figure 3-6C show that after co-transfection indeed more OATP1B3 protein was detectable at the plasma membrane, explaining the increased uptake. After correcting for this increased amount of protein expressed at the membrane (OATP1B3 at the membrane after single transfection was set to 100% after correction with Na^+/K^+ ATPase) uptake was the same for the single wild-type OATP1B3-FLAG only or the co-transfection with the nonfunctional mutant OATP1B3-K41C-His (co-transfection) (Figure 3-6D). OATP1B3-FLAG plus 500ng of pcDNA5/FRT (WT), or with 500ng of OATP1B3-FLAG plus 500ng of OATP1B3-K41C-His (Co-Transfection). Forty-eight hours later uptake of 10μM E3S or 1μM E17βG was performed at 37°C for 1 min. (C) Surface biotinylated proteins were isolated from HEK293 cells transiently transfected as in (B). Western blot was performed with Anti-His (Mutant-His) or Anti-FLAG (WT-FLAG). Na^+/K^+ ATPase was used as loading control. (D) Uptake shown in (B) was normalized by the surface expressed mutant-His or WT-FLAG protein determined in (C) after subtracting of empty-vector control uptake.

To investigate whether dimerization/oligomerization with the mutant OATP1B3 would affect transport kinetics, we performed concentration dependent uptake measurements with E17βG in the absence and presence of clotrimazole, a known OATP1B3 stimulator. As can be seen in Table 3-1, there was no difference in the K_m values for the monomer as compared to the dimer/oligomer. We would have expected that the V_{max} value would be decreased by about 50 % because only 50% of the functional OATP1B3 was transfected. However, the decrease was only 40% which might be due to the small and statistically not significant signal seen with the mutant alone (Figure 3-6B). In addition, similar to our previous report (Gui et al., 2008), the addition of clotrimazole increased the affinity for both the monomer as well as for the dimer/oligomer,

suggesting that the allosteric stimulation is not affected by the dimer/oligomer formation and that OATP1B3 works as two or more “monomers”.

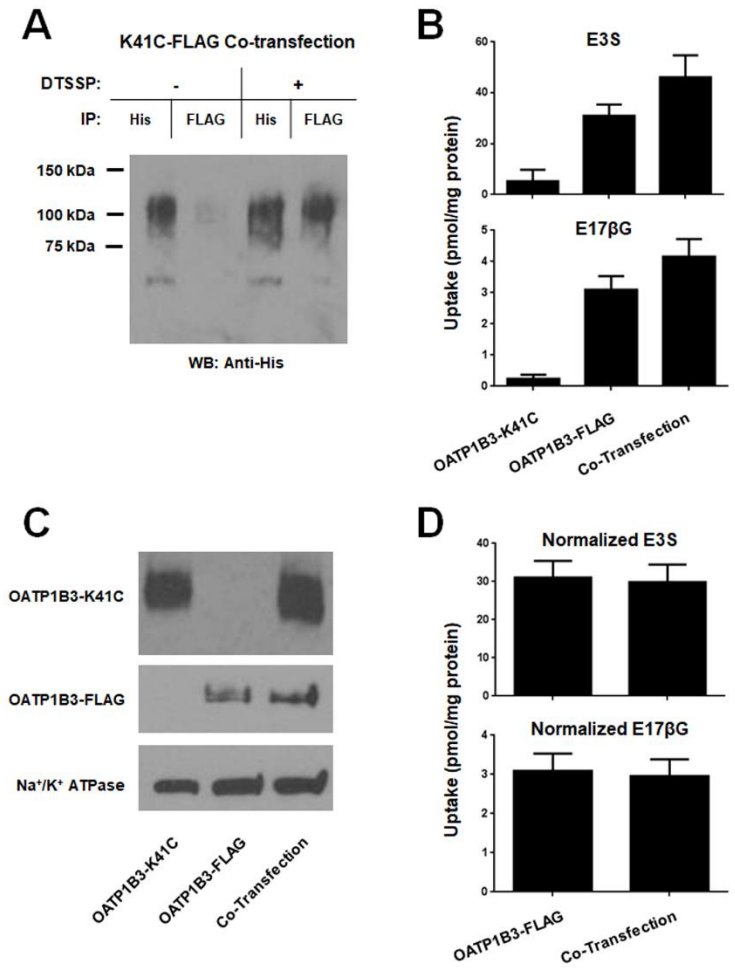

Figure 3-6: Effect of co-transfection of a-non-functional OATP1B3 mutant on function and oligomerization.

(A) Membrane fractions isolated from HEK293 cells transiently transfected with OATP1B3-FLAG and OATP1B3-K41C-His and treated with or without DTSSP were solubilized. Immunoprecipitation was performed with Anti-His or Anti-FLAG antibodies and the eluted proteins were detected with Anti-His antibody. (B) HEK293 cells were transiently transfected with 500ng of OATP1B3-K41C-His plus 500ng of pcDNA5/FRT (Mutant), with 500ng of

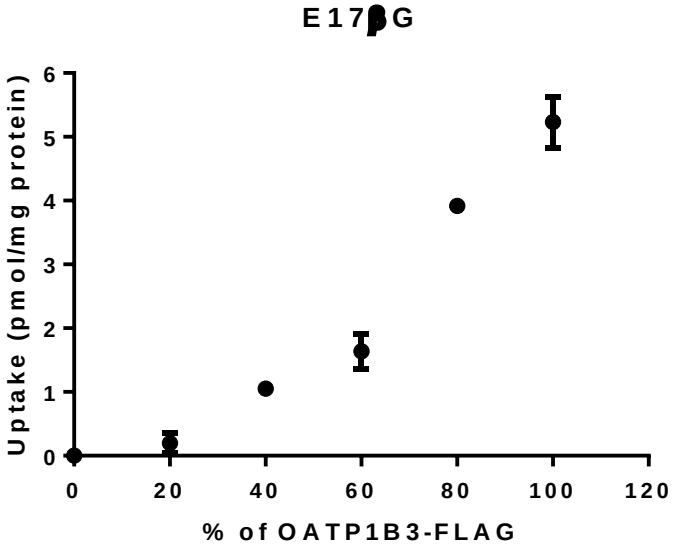

Figure 3-7: Effect of co-transfection of different ratios of empty vector and OATP1B3-FLAG.

Uptake of 1 μM estradiol-17β-glucuronide (E17βG) was measured at 37°C with HEK293 cells transiently transfected with a total amount of 1000 ng of cDNA for 1 minute. Mean ± SD of the percentage of OATP1B3-FLAG cDNA is indicated on the x-axis.

Table 3-1: Effect of clotrimazole on kinetics of wild-type OATP1B3 in the absence and presence of the non-transporting OATP1B3-K41C.

Transporter	E17βG		E17βG + 30μM clotrimazole	
	K_m (μM)	V_{max} (pmol/mg protein x min^{-1})	K_m (μM)	V_{max} (pmol/mg protein x min^{-1})
WT-OATP1B3	7.1 ± 0.9	78 ± 3.3	3.3 ± 1.0	107 ± 8.3
WT-OATP1B3 + OATP1B3-K41C	5.5 ± 1.7	48 ± 4.5	3.6 ± 1.3	68 ± 6.5

HEK293 cells were transfected with 500ng of His-tagged OATP1B3 (WT-OATP1B3) or 250ng of His-tagged OATP1B3 plus 250ng of His-tagged OATP1B3-K41C. Kinetic parameters were determined based on the 30 secs uptake of 1, 5, 10, 20, 30 and 50μM E17βG in the absence or presence of 30μM clotrimazole.

2.4 Hetero-oligomerization between OATP1B3 and other transporters

Given that OATP1B3 can form homodimers/oligomers, we asked the question whether OATP1B3 would also interact with other transporters also expressed in the basolateral membrane of human hepatocytes, such as OATP1B1 an NTCP. We performed Duolink PLA experiments using HEK293 cells that were transiently transfected with OATP1B3-FLAG and His-tagged OATP1B1 or NTCP. As a negative control we used His-tagged ASBT which is not expressed in human hepatocytes. As shown in Figure 3-8 on the left side, all the His-tagged proteins were expressed and could be detected by immunofluorescence (IF) with an anti-His antibody. Figure 3-8 also demonstrates (right side) that the co-expression of OATP1B3 with either OATP1B1 or with NTCP resulted in good PLA signals at the plasma membrane, suggesting the formation of hetero-oligomers of the two respective proteins. Importantly, the fact that co-expression of OATP1B3 with ASBT did not result in any signals demonstrates that the proximity of the proteins that yielded positive signals was not just due to overexpression of the transporters in HEK293 cells.

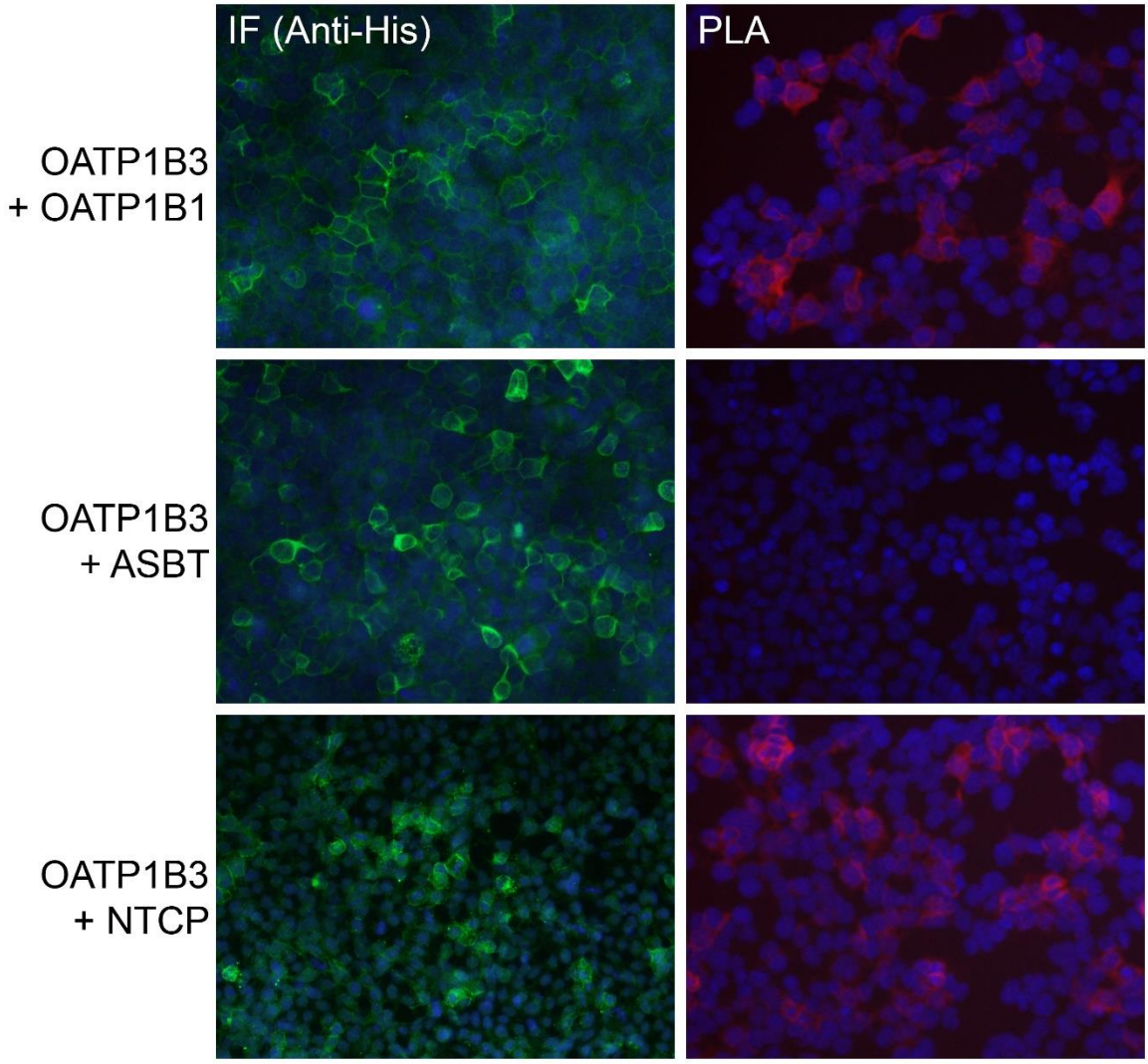

Figure 3-8: DuoLink PLA of OATP1B3-FLAG and other His-tagged transporters expressed in HEK293 cells.

HEK293 cells were transiently transfected with His-tagged OATP1B1, ASBT or NTCP only or with these His-tagged transporters plus OATP1B3-FLAG. After 48 hours, cells were fixed and processed for immunofluorescence (IF) (signals in green) or Duolink PLA (signals in red). Nuclear staining is shown in blue.

To further confirm that OATP1B3 and NTCP also interact in human liver and not only in transfected HEK293 cells, we performed PLA experiments using frozen human liver tissues. Figure 3-9 shows that compared to the OATP1B3 negative control, positive PLA signals were obtained for the OATP1B3 positive control as well as for the OATP1B3-NTCP interaction, confirming that OATP1B3 and NTCP also interact in human liver.

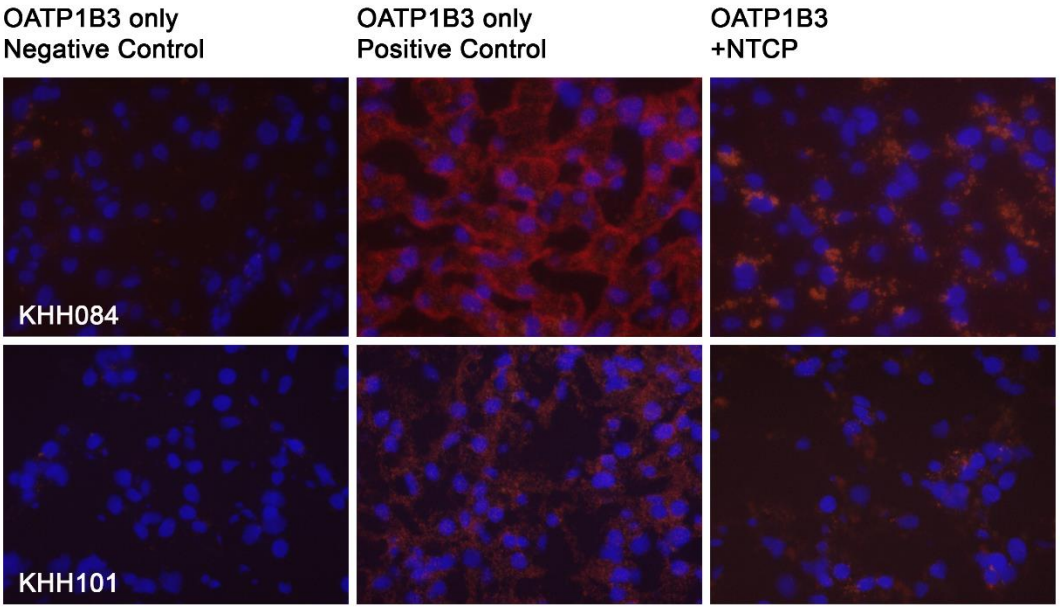

Figure 3-9: Co-localization of OATP1B3 with NTCP in human liver.

Frozen human liver sections were used to perform Duolink PLA. For the OATP1B3 negative control an anti-rabbit minus and an anti-mouse plus PLA antibody were used; for the OATP1B3 positive control, an anti-rabbit minus was combined with an anti-rabbit plus PLA antibody. To co-localize OATP1B3 with NTCP, a goat anti-NTCP antibody was combined with the rabbit anti-OATP1B3 antibody. Co-localization is shown in red, nuclei are stained with DAPI and are shown in blue. KHH084: 59 year old male; KHH101: 57 year old female.

III. Discussion

In this study, we demonstrated that the important hepatocellular drug transporter OATP1B3 can form homo-dimers or -oligomers. In these dimers/oligomers an individual OATP1B3 seems to be the functional unit because co-transfections with a nonfunctional OATP1B3-K41C mutant did not affect uptake of E3S and E17βG. Furthermore, we also established that OATP1B3 can form hetero-dimers/oligomers in HEK293 cells with other hepatocellular transporters like OATP1B1 and NTCP, and we verified the interaction with NTCP using frozen human liver sections.

Several lines of evidence support our findings. Co-immunoprecipitation experiments confirmed a physical interaction between OATP1B3-His and OATP1B3-FLAG (Figure 3-4), while the Proximity Ligation Assay verified a close association of the two differently tagged OATP1B3 proteins (Figure 3-5). Besides that, our data also show that the C-terminal end of OATP1B3 is not involved in dimerization/oligomerization because both His-tagged OATP1B3 with the C-terminal end on the cytoplasmic side of the membrane as well as HA-tagged OATP1B3 with the C-terminal end on the extracellular side of the membrane (Figure 3-2B) were able to co-immunoprecipitate FLAG-tagged OATP1B3 (Figure 3-4B). At the functional level, our results show that a non-functional unit (OATP1B3-K41C) in the homo-dimer/oligomer does not affect normal substrate transport of OATP1B3, nor does it affect the modulation of transport by clotrimazole. These results suggest that each unit within the dimer/oligomer works as an independent functional unit, which is similar to previous findings regarding NTCP (Bijsmans et al., 2012). However, our co-transfection studies with the non-functional mutant did not yield any structural information with respect to potentially involved amino acid residues or transmembrane domains. A recent report suggested that GXXXG motifs present in OATP1B1 are involved in the oligomerization of OATP1B1 (Ni et al., 2017). The three GXXXG motifs in OATP1B1 are completely conserved in OATP1B3 and therefore, it is possible that G393 in the third GXXXG

motif indeed is involved in the homo-oligomerization. Given that there are also three GXXXG motifs in NTCP, it is possible that these motifs play also a role in hetero-oligomerization or that they are important in intramolecular interactions and stabilize proper folding (Teese and Langosch, 2015). To evaluate these possibilities future experiments are required, including e.g. mutational analysis where the glycine residues are replaced by other amino acid residues besides alanine, and determining the effect these mutations have on plasma membrane localization of the proteins.

Using the proximity ligation assay in transiently transfected HEK293 cells we also demonstrated that OATP1B3 interacts with OATP1B1 and with NTCP, two additional transporters expressed at the basolateral membrane of human hepatocytes. Furthermore, we confirmed the interaction between OATP1B3 and NTCP on frozen human liver sections. Assuming that these interactions between the two different transport proteins are direct protein-protein interactions we can speculate that the two proteins might affect each other's function. This could have consequences for potential drug-drug interactions studies (see below). In contrast, if the two proteins do not directly interact and are just expressed in the same micro-domains, then OATP1B3 and NTCP might be regulated in a similar manner, at least at the post-translational level. Again, additional experiments are required to characterize these options in detail. The functional consequences of these interactions will have to be evaluated in future studies. The proximity ligation assay only demonstrates that two proteins are closer than 40nm but does not prove a direct physical interaction between the two proteins. Thus, additional experiments will be required to investigate whether the identified interaction partners, OATP1B1 and NTCP, are directly interacting with OATP1B3 or whether they reside together in the same membrane micro-domains. So far, we only demonstrated direct interaction between His-tagged OATP1B3 and FLAG-tagged OATP1B3, proving direct homo-oligomerization, while similar experiments with OATP1B1 and NTCP are still missing.

Previous western blot data suggested such homo- or hetero-dimerization/oligomerization for OATP1B3 (Gui and Hagenbuch, 2008), for OATP1B1 (Gui and Hagenbuch, 2009), as well as for other solute carriers including ASBT (Kramer et al., 2001a), Na^+-K^+-$2Cl^-$ cotransporter (NKCC1) (Moore-Hoon and Turner, 2000), *SLC10A5* (Fernandes et al., 2007), and *SLC10A7* (Godoy et al., 2007). Furthermore, it was demonstrated that NTCP can homo- and heterodimerize with other members of the SLC10A family (Bijsmans et al., 2012). Thus, it is very likely that many solute carriers work as functional dimers/oligomers and could potentially be located in the same membrane micro-domains.

The fact that OATP1B3 can hetero-oligomerize with other transporters suggests that these transporters potentially influence each other's function and even might be regulated in a similar manner at the protein level.

Most OATPs show increased function at an acidic pH value (Leuthold et al., 2009; Martinez-Becerra et al., 2011). While this seems to be an advantage for an intestinal transporter one could argue that it should not affect a transporter expressed at the basolateral membrane of hepatocytes. However, co-localization in membrane micro-domains or direct interactions with a Na^+/H^+-exchanger could lead to an acidic microenvironment which could increase OATP function. Furthermore, if indeed the microenvironment generated around these transporter micro-domains influences the physiological or pharmacological function of the involved drug transporters, drug-drug interaction studies should be performed with cell lines that express multiple transporters rather than just a single transporter. Recent findings with members of the drug metabolizing enzymes of the cytochrome P450 family suggest that in vitro systems with more than a single transporter are not only more physiological but might indeed demonstrate that that the different transporters affect each other's function. The activity of CYP2C9 was modified by co-expression with different amounts of CYP3A4 (Ramsden et al., 2014). In analogy, OATP1B3 activity could

be modulated by any of the interacting transporters and vice versa and as a consequence, the functional importance of OATP1B3 interaction with other transporters will have to be evaluated.

In summary, we have shown that the multi-specific liver transporter OATP1B3 can form homo-oligomers and can interact with other transporters in liver including OATP1B1 and NTCP. We also demonstrated that the homo-oligomers work as two or more individual functional subunits. Our findings are important because they suggest the existence of transporter micro-domains containing OATPs, and as a consequence functional transporter studies might have to be modified to include co-expression systems in future studies.

Chapter 4

Hetero-oligomerization affects Organic Anion Transporting Polypeptide 1B3 (OATP1B3) expression and function

I. Introduction

Uptake of endo- and xenobiotics into human hepatocytes is mediated by several drug uptake transporters that are expressed at the sinusoidal membrane, including organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3 (Hagenbuch and Stieger, 2013), the organic cation transporter 1 (OCT1) (Koepsell, 2013) as well as the Na^+/taurocholate cotransporting polypeptide (NTCP) (Claro da Silva et al., 2013). OATP1B1 and OATP1B3 transport mainly organic anions but also neutral compounds, and have partial overlapping substrate selectivities (Konig, 2011; Hagenbuch and Stieger, 2013). Among the transported substrates are endobiotics like bile acids, bilirubin, steroid hormone metabolites, thyroid hormones, glutathione, inflammatory mediators and coproporphyrins (Stieger and Hagenbuch, 2014; Bednarczyk and Boiselle, 2015), as well as numerous drugs (Roth et al., 2012).

Several model substrates, including estradiol-17β-glucuronide, bromosulfophthalein and cholecystokinin-8 (CCK-8) have been used to characterize OATP1B3 function (Hagenbuch and Stieger, 2013), but among them, only CCK-8 is OATP1B3 selective (Ismair et al., 2001). OCT1 is also a multispecific transporter and mainly mediates the uptake of organic cations and some neutral compounds, including the neurotransmitters serotonin, dopamine and norepinephrine, as well as acetylcholine (Koepsell et al., 2007; Boxberger et al., 2014), and numerous drugs including metformin, protease inhibitors and anti-cancer agents (Nies et al., 2011; Koepsell,

2013). Besides metformin, the model substrates tetraethylammonium (TEA) and 1-methyl-4-phenylpyridinium (MPP^+) are often used to characterize OCT1 transport (Gorboulev et al., 1997; Zhang et al., 1997) and given that these are cationic, they are not substrates of OATPs. NTCP is important for the uptake of bile acids into hepatocytes to maintain their enterohepatic circulation but can also transport several drugs, including statins (Claro da Silva et al., 2013). It has also been identified as the receptor for the hepatitis virus B and D (Yan et al., 2014).

We recently demonstrated that besides homo-oligomers, OATP1B3 can form hetero-oligomers with the two liver transporters OATP1B1 and NTCP (Zhang et al., 2017). In addition, a DUALmembrane screening (Stagljar et al., 1998), a modified yeast two-hybrid screening, identified OCT1 as a likely interaction partner. However, the functional consequences of these hetero-oligomers remain unknown. Because the FDA guidelines for the study of transporter-mediated drug-drug interactions (DDIs) include OATP1B3, OATP1B1, NTCP and OCT1 as potential targets for DDIs, it is important to understand the influence of the hetero-oligomerization of OATP1B3 with these three transporters. Therefore, in the present study we tested whether OATP1B3 and OCT1 indeed interact with each other, and we characterized the functional consequences of co-expression of OATP1B3 with OCT1, OATP1B1, or NTCP using HEK293 cells.

II. Results

2.1 OATP1B3 and OCT1 interact in human hepatocytes.

In order to investigate whether OATP1B3 would interact with other human liver proteins, Dualsystems AG (Schlieren, Switzerland) performed a DUALmembrane screening with full-length OATP1B3 as the bait. Screening a human adult liver cDNA library resulted in the isolation of 51 cDNAs that were further analyzed and classified. Nineteen of these cDNAs were very likely unspecific interactors because they were found in 20-50% of the screenings, independent of the bait. Among the other 32 that were considered to be screen-specific interactors organic cation transporter 1 (OCT1), encoded by *SLC22A1*, was found three times. Given that OCT1, like OATP1B3, is a drug uptake transporter expressed at the basolateral membrane of human hepatocytes, we further investigated this potential interaction by performing co-immunoprecipitation experiments. As can be seen in Figure 4-1A, OCT1 could be detected with an OCT1-specific antibody on a western blot of proteins that were immunoprecipitated from solubilized human hepatocytes with an anti-OATP1B3 antibody. The signal at approximately 75 kDa was stronger in the presence of the crosslinker DTSSP. To further confirm this OATP1B3-OCT1 interaction in human liver, we performed proximity ligation assays on frozen sections of human livers. The proximity ligation assay detects interacting proteins that are less than 40nm apart (Soderberg et al., 2006) using two antibodies raised in different species. If these two proteins are less than 40nm apart, the assay will result in a red signal. We previously used this assay to demonstrate OATP1B3-NTCP interactions in frozen human liver slices (Zhang et al., 2017). When we incubated frozen human liver sections only with the monoclonal antibody recognizing OCT1, no red signal was generated (Figure 4-1B; OCT1). However, when the monoclonal anti-OCT1 antibody was used together with the polyclonal anti-OATP1B3 antibody

(K28), interactions between the two transporters were evident by the red signals (Figure 4-1B; OATP1B3 + OCT1).

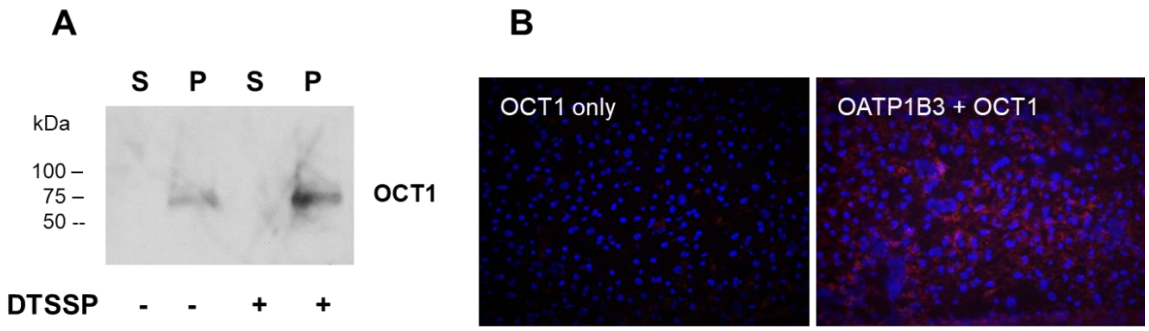

Figure 4-1: OATP1B3 and OCT1 interact in human hepatocytes.

Interaction of OATP1B3 with OCT1 is demonstrated by (A) co-immunoprecipitation and (B) with a proximity ligation assay. (A) Solubilized proteins from freshly isolated and plated human hepatocytes were incubated with an anti-OATP1B3 antibody in the presence or absence of the crosslinking agent DTSSP. After precipitating the anti-OATP1B3 antibodies with protein A beads, the resulting supernatants (S) and pellets (P) were separated by SDS-PAGE and after transfer to a PVDF membrane incubated with an anti-OCT1 antibody. (B) Frozen human liver sections were incubated either with only an anti-OCT1 antibody or with a combination of anti-OCT1 and anti-OATP1B3 antibodies. After incubation with the respective secondary antibodies and the ligation reaction mix, interactions were detected in red. The blue color represents DAPI staining of the nuclei.

2.2 OATP1B3 and OCT1 interact in HEK293 cells.

To characterize the functional consequences of the OATP1B3-OCT1 interactions we wanted to use transiently transfected HEK293 cells. Therefore, we first tested whether these protein-protein interactions could also be demonstrated with the proximity ligation assay when both transporters were expressed in HEK293 cells. The results in Figure 4-2A show that OATP1B3 and OCT1 also interact when expressed in HEK293 cells. As a negative control we transfected OATP1B3 together with ASBT, a transporter which is expressed in cholangiocytes and enterocytes. The lack of a signal (Figure 4-2B) demonstrates that the interaction of OATP1B3 with OCT1 in HEK293 cells is not due to unspecific overexpression of any membrane protein but due to specific interactions between these two transport proteins.

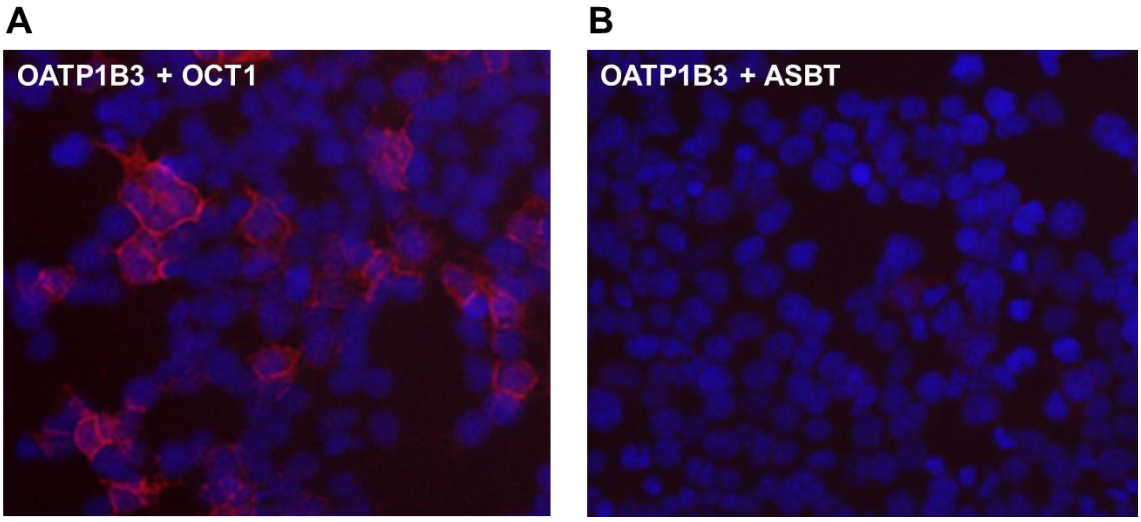

Figure 4-2: OATP1B3 and OCT1 interact in transiently transfected HEK293 cells.

Interaction of OATP1B3 with OCT1 is demonstrated using the proximity ligation assay. HEK293 cells were transfected with (A) OATP1B3-His and OCT1 plasmids (1:1), or (B) OATP1B3-FLAG and ASBT-His plasmids (1:1). Twenty-four hours after transfection HEK293 cells were fixed and incubated with a combination of corresponding antibodies. After incubation with the respective secondary antibodies and the ligation reaction mix, interactions can be detected in red. The blue color represents DAPI staining of the nuclei.

2.3 Plasma membrane expression of OATP1B3 is affected by hetero-oligomerization in HEK293 cells.

After the demonstration of interaction between OATP1B3 and OCT1 in HEK293 cells, we asked the question whether expression at the plasma membrane would be affected by co-expression of OATP1B3 with OCT1. Because we recently demonstrated that OATP1B3 can also interact with OATP1B1 and NTCP (Zhang et al., 2017), we also looked at the effect of co-expression of OATP1B3 with these two transporters. OATP1B3 was transfected in HEK293 cells either alone (co-transfected with empty vector) or together with OCT1, or OATP1B1, or NTCP, and membrane proteins were labeled and isolated using the biotin-streptavidin system. Plasma membrane expression of OATP1B3, OCT1, OATP1B1, and NTCP was then quantified by SDS-PAGE and western blot. As can be seen in Figure 4-3A, no OATP1B3 was visible when only empty vector or OCT1 were transfected. When OATP1B3 was transfected together with empty vector more biotinylated OATP1B3 was detected and thus more OATP1B3 was expressed at the plasma membrane than when OATP1B3 was co-expressed with OCT1. After quantification and normalization for the Na^+/K^+ATPase signal, we calculated that co-expression of OCT1 reduced the OATP1B3 signal to about 20% (Figure 4-3B). In contrast, expression levels for OCT1 co-transfected with OATP1B3 did not change significantly as compared to co-transfection with empty vector (Figure 4-3A, B). Interestingly, OATP1B3 protein expression was increased significantly when co-expressed with either OATP1B1 or NTCP (Figure 4-3C). Quantification and normalization with the Na^+/K^+ ATPase signal revealed an about 4.5fold and 2.5fold increase in plasma membrane expression when co-expressed with OATP1B1 and NTCP, respectively, while expression of OATP1B1 and NTCP was not affected (Figure 4-3D). Given that only transporters expressed at the surface of HEK293 cells can transport their substrates, these surface expression levels have to be taken into consideration for functional studies in HEK293 cells.

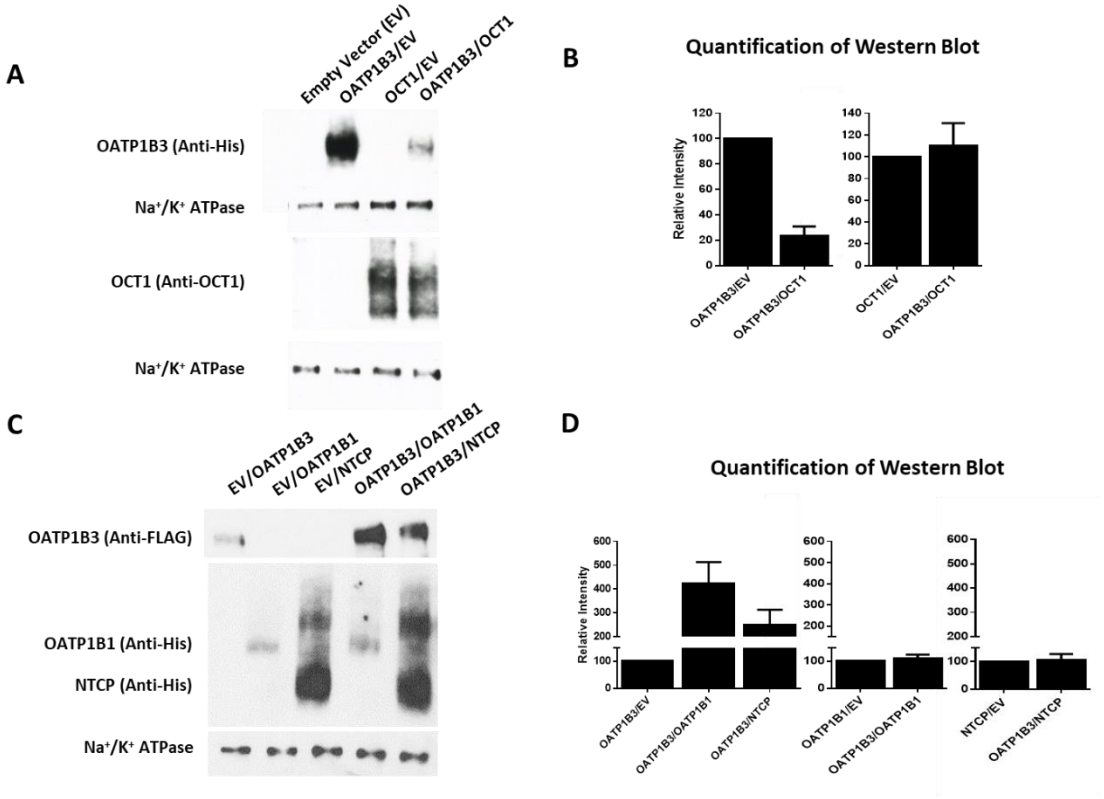

Figure 4-3: Plasma membrane expression of OATP1B3 and interacting transporters when co-expressed in HEK293 cells.

HEK293 cells were transfected with empty vector (EV), empty vector and OATP1B3-His (1:1), empty vector and OCT1 (1:1), or OATP1B3-His and OCT1 (1:1). Since both OATP1B1 and NTCP contain a His tag inserted at the C-terminal end, the same transfection conditions were used but OATP1B3-His was replace by a OATP1B3-FLAG plasmid. Twenty-four hours (for OCT1), or 48 hours (for OATP1B1 and NTCP) after transfection, surface biotinylation experiments were performed and membrane proteins were harvested and analyzed by western blot. A and C: Membrane proteins were detected using the indicated antibodies, or a Na^+/K^+-ATPase antibody as loading control. B and D: Relative intensity of western blot bands were

quantified and compared based on three independent experiments; mean ± SD are shown for the co-transfections.

2.4 Hetero-oligomerization in HEK293 cells affects OATP1B3-mediated CCK-8 transport.

To determine whether the interaction between OATP1B3 and OCT1 or OATP1B1 or NTCP also affects OATP1B3 transport function, we compared uptake of the OATP1B3 specific substrate CCK-8 into HEK293 cells transfected with OATP1B3 and empty vector, or OATP1B3 and the respective interacting transporters. Twenty-four hours (for co-transfection with OCT1), or 48 hours (for co-transfection with OATP1B1 or NTCP) after transfection uptake of 1 µM ^{3}H-CCK-8 was measured for 1 minute at 37 °C. As can be seen in Figure 4-4A, uptake of CCK-8 was detected in OATP1B3 expressing HEK293 cells but was absent in cells transfected with empty vector, OCT1, OATP1B1 or NTCP. When OATP1B3 was co-expressed with OCT1 uptake was reduced to 50% (Figure 4-4A, top). However, when normalized for plasma membrane expressed OATP1B3 this 50% reduction of transport was actually a 2.7fold stimulation (Figure 4-4B, top). Thus, although the initial observation indicated decreased OATP1B3 transport, co-expression with OCT1 actually stimulated CCK-8 uptake. Interestingly, an opposite effect was observed with OATP1B1 or NTCP. Before normalization, uptake of CCK-8 by OATP1B3 was not significantly affected by co-expression with both, OATP1B1 or NTCP (Figure 4-4A, middle and bottom). However, after normalization for membrane expressed OATP1B3, uptake of CCK-8 was reduced dramatically (Figure 4-4B, middle and bottom). We next determined the initial linear portion of CCK-8 uptake for OATP1B3 expressed alone and co-expressed with the three different transporters to be able to characterize kinetics. Based on these experiments, we performed CCK-8 uptake experiments with increasing CCK-8 concentrations (0.1 to 50 µM) at 1 minute. As shown

in Figure 4-5A (summarized in Table 4-1), 24 hours after transfection, saturable CCK-8 uptake with a K_m value of 10.9 ± 1.9 μM and a V_{max} value of 166 ± 10 pmoles/mg protein x min could be calculated for OATP1B3. When OCT1 was co-expressed and the values were normalized for plasma membrane expression of OATP1B3 the K_m value did not change but the V_{max} increased to 253 ± 10 pmoles/mg x min. Fourty-eight hours after transfection, the K_m value remained the same when OATP1B3 expressed with or without OATP1B1 or NTCP, but the V_{max} decreased significantly (Figure 4-5).

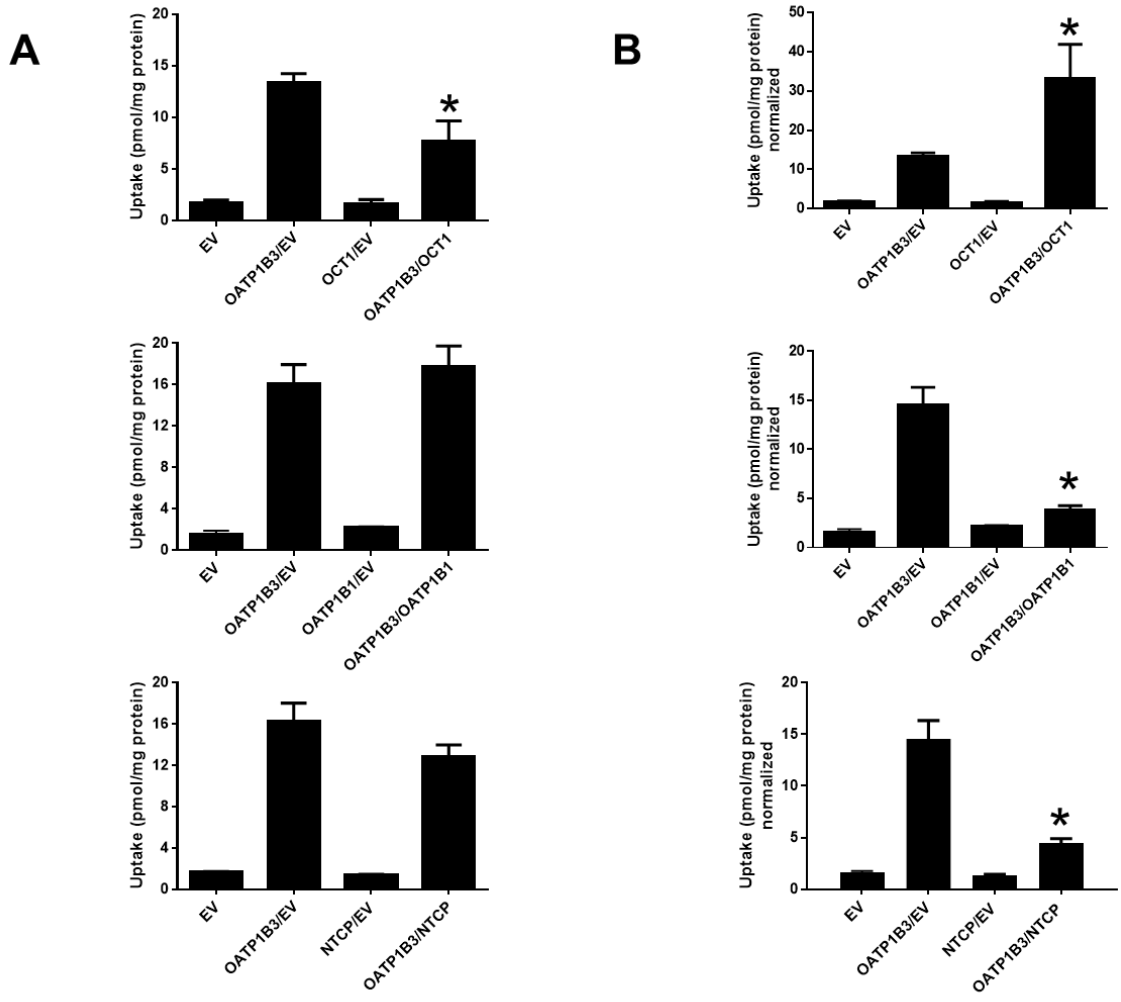

Figure 4-4: Functional consequences for OATP1B3 when co-expressed with OCT1, OATP1B1 or NTCP.

Uptake experiments with 1µM ^{3}H-CCK-8 were performed 24-hours (OCT1) or 48-hours (OATP1B1 and NTCP) after HEK293 cells were transiently transfected with an equivalent amount of cDNA with the indicated combination of plasmids or empty vector (EV). Uptake was terminated after 1 minute and normalized by total protein concentration (A) or surface expressed protein (B). The means ± SD of three experiments (n=3) are shown; an asterisk indicates a $p < 0.05$ level of significant difference from the respective OATP1B3/EV control.

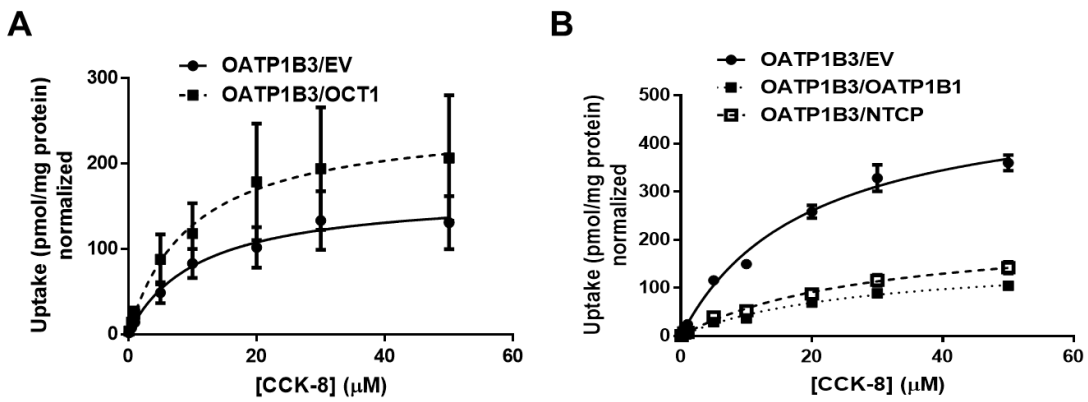

Figure 4-5: Effects of co-expression of interacting transporters on the kinetics of OATP1B3-mediated cholecystokinin-8 (CCK-8) uptake.

Uptake of increasing concentrations of ^{3}H-CCK-8 (0.1 - 50 μM) was measured at 37°C for 1 minute in HEK293 cells transiently transfected with 500ng of empty vector, or 250ng of empty vector mixed with 250ng of OATP1B3 (OATP1B3/EV), or 250ng of OATP1B3 mixed with 250ng of respective interacting transporters. Uptake was normalized by total and plasma membrane expressed protein. After subtracting the values obtained with empty vector transfected cells, net OATP1B3-mediated uptake was fitted to the Michaelis–Menten equation to obtain K_m and V_{max} values. Means ± S.E. of 7 (A: for interaction with OCT1) or 3 (B: for interaction with either OATP1B1 or NTCP) individual experiments each performed with triplicate determinations are given. Circles and a solid line represent OATP1B3/EV transfected cells while squares and a dashed line represent OATP1B3/interacting transporter transfected cells.

Table 4-1: Kinetic parameters for OATP1B3-mediated uptake of CCK-8 in single- or co-transfected HEK293 cells.

Transfection Condition	K_m (µM)	V_{max} (pmol/mg protein x min)
OATP1B3/EV (24 hours)	10.9 ± 1.9	166 ± 10
OATP1B3/OCT1 (24 hours)	9.8 ± 1.1	253 ± 10*
OATP1B3/EV	16.9 ± 3.2	477 ± 36
OATP1B3/OATP1B1	27.4 ± 3.6*	164 ± 10*
OATP1B3/NTCP	29.2 ± 7.2*	224 ± 27*

EV: empty vector; * $p < 0.05$ when compared to the respective OATP1B3/EV control

2.5 Hetero-oligomerization in HEK293 cells does not seem to affect the function of OCT1, OATP1B1 and NTCP.

Finally, to investigate whether the function of the OATP1B3 partner proteins would be affected by the co-expression, we measured uptake of representative model substrates for OCT1 (metformin), OATP1B1 (low concentration of estrone-3-sulfate) and NTCP (taurocholate) after co-transfecting HEK293 cells. The results in Figure 4-6 demonstrate that the function of neither of the three interacting transport proteins was affected by co-expression with OCT1.

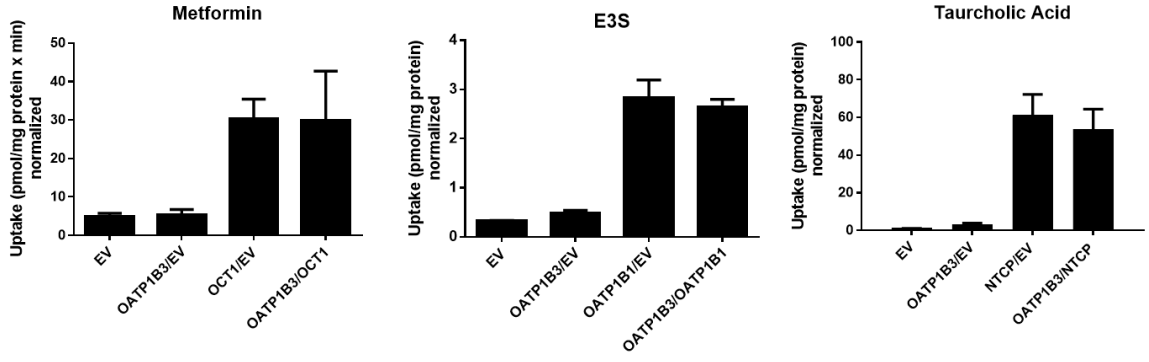

Figure 4-6: Functional consequences for interacting transporters when co-expressed with OATP1B3.

Five-minute uptake of 5µM ^{14}C-metformin (for OCT1), 1 minute uptake of 0.1µM E3S (for OATP1B1) or 1µM TCA (for NTCP) were measured 24-hours (for OCT1), or 48-hours (for OATP1B1 and NTCP) after HEK293 cells were transiently transfected with an equivalent amount of cDNA with indicated combinations. Substrate-mediated uptake was normalized by total and plasma membrane expressed protein concentrations. Means ± SD are given for three experiments (n=3) each performed in triplicates.

III. Discussion

In the present study we could demonstrate, using co-immunoprecipitation and proximity ligation assays, that OATP1B3 and OCT1 interact with each other in human hepatocytes as well as when expressed in HEK293 cells. Co-expression of OCT1 with OATP1B3 in HEK293 cells reduced OATP1B3 protein expression at the plasma membrane as compared to when OATP1B3 was expressed alone. In addition, we could show that these protein-protein interactions affected the function of OATP1B3 in a transporter-dependent manner. Furthermore, OATP1B3 expression at the plasma membrane was increased when co-expressed with OATP1B1 or NTCP.

Under normal physiological conditions OATP1B3 is exclusively expressed at the basolateral membrane of human hepatocytes with highest expression levels around the central vein (König et al., 2000; Cui et al., 2003). A similar lobular expression was also reported for mouse OCT1 (Chen et al., 2014). Although we do not yet know the mechanisms by which the OCT1-OATP1B3 protein-interaction results in down-regulated OATP1B3 protein, a similar protein-protein interaction between the corresponding two proteins in mice, mouse OCT1 and mouse OATP1B2, respectively, could be responsible for the differential regulation in a mouse model of nonalcoholic steatohepatitis (NASH) due to a methionine- and choline-deficient diet. In these animals OATP1B2 is down-regulated (Clarke et al., 2014a) while OCT1 expression goes up as compared to healthy controls (Clarke et al., 2015). Interestingly, OATP1B3 protein expression was much lower in NASH livers when compared to normal livers (Clarke et al., 2014b) but there is no information available yet on OCT1 expression under the same conditions in these mice. Nevertheless, our findings support the speculation that changes at the protein level of OCT1, either due to disease or due to polymorphisms, can affect the expression of OATP1B3 most likely through post-translational regulation.

Uptake of the OATP1B3-specific substrate CCK-8 increased when OATP1B3 was co-expressed with OCT1 (Figure 4-4B). This increased transport was due to an increased V_{max} with a constant K_m value (Figure 4-5 and Table 4-1). The V_{max} can increase either due to increased transporter expression at the plasma membrane or due to an increased transporter turnover rate. Because we normalized the results for plasma membrane expression of OATP1B3, our data demonstrate that the turnover rate was increased when OATP1B3 was co-expressed with OCT1.

Interestingly, a completely different effect was observed when OATP1B3 was co-expressed with OATP1B1 or NTCP. Unlike OCT1, OATP1B1 and NTCP have an even distribution throughout the liver lobule, which could potentially explain the reversed effect on OATP1B3 compared to OCT1.

We have demonstrated that co-expression of OATP1B3 with other transporters can affect its function. Thus, because in human hepatocytes both transporters can be expressed in the same hepatocyte, this co-expression likely affects the physiological function of OATP1B3 and/or its ability to clear drugs. When characterizing and predicting drug-drug interactions researchers normally use HEK293 or CHO cells that express a single transporter (Hirano et al., 2006; Noe et al., 2007; Gui et al., 2008). However, given that these drug transporters might be affected by co-expressed transporters, these single expression systems do not really reflect the situation in human hepatocytes and results obtained in the single transfection systems could be misleading. Experiments with several drugs, e.g. statins or sartans should be conducted in the future in the co-expression systems to confirm that the single transporter expression system is a good enough system or to demonstrate that multiple transporter expression systems should be used instead of or at least in combination with the single transporter systems.

The presented results demonstrate that the expression of OATP1B3 at the protein level and in addition at the plasma membrane is affected by co-expression with OCT1, OATP1B1 or NTCP

(Figure 4-3). We have preliminary data suggesting that the expression levels obtained in our experiments are affected by the ratios of the transfected plasmids. Furthermore, the total protein expression levels of both proteins are two to four-fold higher than the respective levels in human hepatocytes. Thus, for an ideal simulation system, the respective protein levels will have to be adjusted to reflect average protein levels in human hepatocytes.

Regulation of OATP1B3 and other OATPs has mainly been studied at the transcriptional level (reviewed in (Hagenbuch and Stieger, 2013)). Limited information is available for post-translational regulation via protein kinase C activation (Guo and Klaassen, 2001; Kock et al., 2010; Powell et al., 2014; Hong et al., 2015) and several mainly rodent OATPs have been reported to interact with adapter proteins of the PDZ family (summarized in (Stieger and Hagenbuch, 2014)). Similar interactions could occur with additional transporters in the basolateral membrane of human hepatocytes involving other OATPs, OCTs and NTCP. The fact that co-expressed transporters interact might suggest that they also could be co-regulated at the post-translational level. Thus, further experiments need to be conducted to investigate whether this indeed is the case.

The interaction between carbonic anhydrase II and the epithelial sodium-proton exchanger NHE3 results in increased activity of NHE3 (Krishnan et al., 2015). In the case of carbonic anhydrase and NHE3, functional active carbonic anhydrase is required. Thus, we will need to test in the future how the observed functional consequences are modified when transporters with inactivating or stimulating polymorphisms are co-expressed.

Whether these effects are mediated through direct protein-protein interactions or via integration of the transporters into lipid raft domains as has been shown for rat NTCP (Molina et al., 2008), for rat OCTN2 (Czeredys et al., 2013), and for rat OAT3 (Srimaroeng et al., 2013) remains to be determine in future experiments.

In summary, we have shown that the hepatocellular drug transporters OATP1B3 and OCT1 interact with each other and that this interaction can increase OATP1B3-mediated uptake of CCK-8, but reduce the plasma membrane expression level of OATP1B3. The interaction between OATP1B3 and OATP1B1, or NTCP led to an opposite effect, decreasing the function of OATP1B3 but increasing its plasma membrane expression level. These findings are important because they suggest that the clearance of endogenous compounds as well as of drugs can be affected by co-expressed proteins and thus that the characterization of these drug transporters in single transfected recombinant systems might well lead to over- or underestimation of the effects of drug-drug interactions. Therefore, we should consider test systems that co-express the involved transporters for such characterizations.

Chapter 5

Identification of binding sites and translocation pathway of OATP1B3 by photoaffinity labeling

I. Introduction

OATP1B3 is a membrane transporter with 12 transmembrane domains. It is expressed at the basolateral membrane of human hepatocytes. Along with other transporters, like OATP1B1, OCT1 and NTCP, OATP1B3 transports many endogenous compounds and xenobiotics (Stieger and Hagenbuch, 2014). Well-known OATP1B3 substrates include unconjugated and conjugated bile acids, estradiol conjugates, and drugs like statins and digoxin (Roth et al., 2012). Because of the broad spectrum of structurally-independent substrates, many efforts have been made to identify a pharmacophore of OATP1B3. The Mizuguchi group generated the first 3D model of OATP1B3, based on which they hypothesized a central pseudo twofold symmetry axis, perpendicular to the membrane plane and a central pore in OATP1B3 (Meier-Abt et al., 2005). The central pore was proposed to consist of helices 1, 2 4, 5, 7, 8, 10 and 11. After that, further studies of the OATP1B3 binding pockets have been performed to investigate the structure function relationship of OATP1B3. For example, by site-directed mutagenesis, the positively charged amino acids, K41 and R580 in the predicted central pore have been identified as important amino acids to maintain the function of OATP1B3 (Glaeser et al., 2010; Weaver and Hagenbuch, 2010). Similar studies revealed that R57, K361 and R580 in OATP1B1 are part of the translocation pathway (Weaver and Hagenbuch, 2010). However, several studies with different OATPs suggested that the binding sites for different substrates may overlap with each other and not be identical (Sugiyama et al., 2002; Grube et al., 2006; Noe et al., 2007; Gui et al.,

2010), which makes the identification of the binding sites and translocation pathways challenging.

Photoaffinity labeling is a technique first applied in the early 1960s (Singh et al., 1962). When it was developed, it was a powerful tool to study the protein-ligand binding. This technique is based on a photoreactive moiety that is either part of the ligand or attached to it. The labelled ligand will only be activated upon exposure to a certain wavelength of light, and form a highly active intermediate, which can bind to any amino acid near it (Hashimoto and Hatanaka, 2008). Thus, the binding between protein and ligand is a covalent bond, which allows harsh conditions during the purification/analysis. With the improvement of modern techniques, photoaffinity labeling has been widely used in the study of protein-protein, protein-nucleotide and protein-small molecule interaction (Smith and Collins, 2015). In combination with LC-MS technologies, photoaffinity labeling can be used to identify the ligand binding sites of proteins (Robinette et al., 2006). Over the last 3 decades, photoaffinity labeling combined with MS spectrometry has been applied to study several transporters. For example, the rabbit ileal Na^+-dependent bile acid transporter (IBAT) was identified and characterized using photoaffinity labeling of rabbit brush border membrane vesicles (Kramer et al., 1993). The same group later used a radioactive diazirine-labeled bile acid derivative to characterize the substrate binding site of this transporter (Kramer et al., 1997; Kramer et al., 2001b). The binding pockets of P-glycoprotein (Pgp) were also investigated by a series of studies using photoaffinity labeling (Morris et al., 1994; Safa, 2004; Chanmahasathien et al., 2011). By mutating the binding sites proposed by photoaffinity labeling, the mechanism of drug resistance effect of Pgp was investigated, and a pharmacophore was proposed based on these studies (Pajeva and Wiese, 2002; Pleban et al., 2005; Globisch et al., 2008).

Based on this information, we hypothesized that by using photoaffinity labeling technology, the binding sites and translocation pathways of OATP1B3 can also be investigated.

II. Results

2.1 8-Fluorescein-cAMP (8-FcA) is a substrate of OATP1B3

8-Fluorescein-cAMP (8-FcA) (Figure 5-1) is a substrate of OATP1B3 (Bednarczyk, 2010). 8-FcA was also used as a photoreactive compound in the study of cGMP-dependent signaling pathways in bovine sperm (Borchert, 2005).

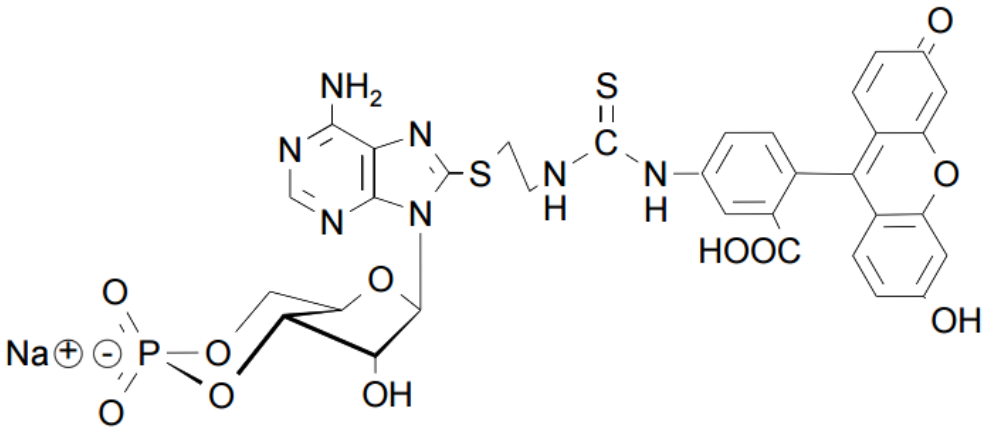

Figure 5-1: Structure of 8-fluorescein-cAMP.

To confirm 8-FcA as a substrate of OATP1B3 in our cell system, uptake of 8-FcA was measured at different time points in wild-type, OATP1B1, or OATP1B3 stable transfected CHO cells. The result showed that 8-FcA is a potent substrate of OATP1B3, with a linear uptake up to 10 mins (Figure 5-2A). After that, the kinetics of 8-FcA uptake by OATP1B3 were also characterized, to determine a suitable concentration for the labelling studies (Figure 5-2B). The result suggested a K_m value of 2.3 μM, which is comparable to the published K_m of 1.8 μM (Bednarczyk, 2010), and therefore we used 5 μM 8-FcA for the following studies. According to a literature search, at least three different uptake buffers were used for transporter-related photoaffinity labeling experiments (A-Cl, KRH1, and KRH2; Table 2-2) (Middendorff et al., 2002; Lapinsky et al., 2009), and a comparison of these buffers with our regular uptake buffer was done to confirm transport activity under different buffer systems. The results shown in Figure 5-2C demonstrate that the uptake of 8-FcA with the A-Cl buffer showed slightly better signals compared to the other buffer systems. Besides that, the A-Cl buffer was also originally used for the photoaffinity labeling of 8-FcA (Borchert, 2005). Based on these findings, the A-Cl buffer was used for the photoaffinity labeling experiments in this study.

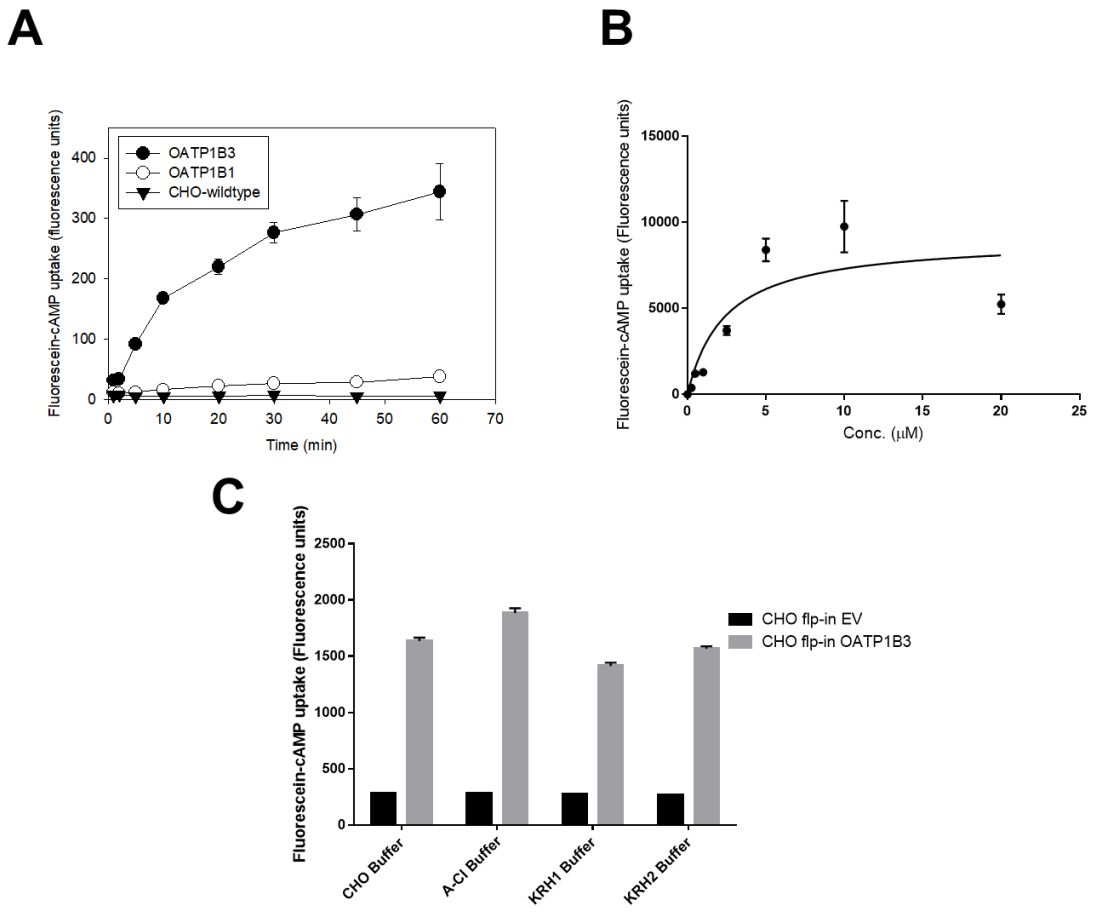

Figure 5-2: Characterization of 8-FcA as a substrate of OATP1B3.

(A) Uptake of 5 µM 8-FcA was measured at 37 °C with wild-type (closed triangles), OATP1B1 (open circles) or OATP1B3 (closed circles) expressing CHO cells on 96-well plates. Values are means ± SD of quadruplicate determinations in a single experiment. (B) Kinetics of 8-FcA was measured at 37 °C for 10 minutes with wild-type and OATP1B3 expressing CHO Flp-In cells on 24-well plates. The uptake by wild-type cells was subtracted from OATP1B3 expressing cells. Values are means ± SD of triplicate determinations in a single experiment. (C) Uptake of 1 µM

8-FcA was measured at 37 °C for 2 mins with wild-type and OATP1B3 expressing CHO Flp-In cells on 24 well plates. Values are means ± SD of triplicate determinations in a single experiment.

2.2 Labeling with fluorescein-cAMP

To test the hypothesis that 8-FcA can be used to label OATP1B3 in live cells, a time dependent labeling experiment was performed first. CHO Flp-In cells stably expressing OATP1B3-His were plated in 10 cm dishes. Once confluent, they were incubated with 5 μM 8-FcA. Immediately after the 8-FcA solution was added to the cells, a 302 nm wavelength UV light was used to activate 8-FcA for 3, 6, 9 or 12 mins. The cells were then washed with ice-cold PBS to remove the unbound 8-FcA and the labeling was stopped. Membrane proteins were then extracted from the cells and used for immunoblotting. The western blot probed with an anti-fluorescein antibody (Figure 5-3A) showed that with increasing time of UV exposure, the binding of 8-FcA to proteins increased up to 9 minutes. However, at 12 minutes exposure, the signals decreased, which may be due to the internalization or degradation of proteins after UV exposure. Therefore, we selected 6 minutes of UV exposure for the following studies. The photoaffinity labeling experiments were performed in both OATP1B3-expressing CHO Flp-In cells and wild-type control CHO Flp-In cells. Losartan, a substrate and strong inhibitor of OATP1B3, was used to compete for the binding of 8-FcA. The western blot result showed that in both cell types, 8-FcA labeled many proteins with or without UV activation (Figure 5-3B). However, when cells were co-incubated with 8-FcA and 100 μM losartan, the signals obtained with the anti-fluorescein antibody were reduced in both wild-type and OATP1B3 expressing cells (Figure 5-3B).

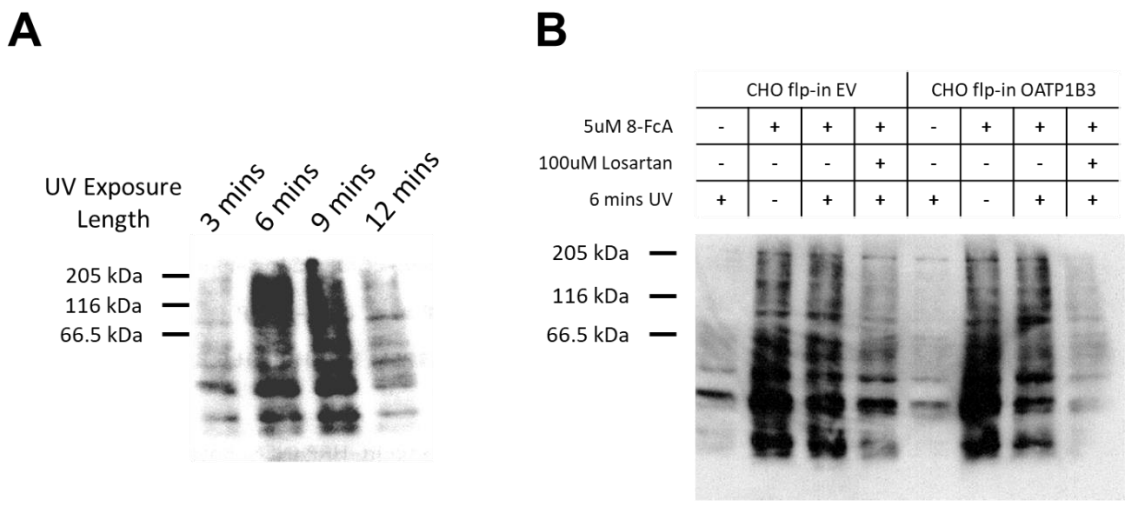

Figure 5-3: Photoaffinity labeling of OATP1B3 by 8-fluorescein-cAMP.

(A) Photoaffinity labeling of 5 μM 8-FcA was performed with 3, 6, 9 and 12 minutes exposure to UV light at a wavelength of 302nm with OATP1B3 expressing CHO Flp-In cells. (B) Photoaffinity labeling with 5 μM 8-FcA with 6 minutes UV exposure in the presence or absence of 100 μM losartan of empty vector or OATP1B3 expressing CHO Flp-In cells. Membrane fractions were extracted and separated by SDS-PAGE. Anti-fluorescein antibody was used for western blot detection.

2.3 Purification of OATP1B3

Based on the high background observed in the 8-FcA labeling experiments, we concluded that a good method to purify OATP1B3 was required after labeling. Since OATP1B3 stably expressed in CHO Flp-In cells has a His tag at the C-terminal end, a Ni-NTA column was used initially to purify the OATP1B3-His from membrane proteins. Solubilized membrane proteins were loaded on the Ni-NTA column and eluted with 50mM imidazole. Then, each fraction was separated by SDS-PAGE and His-tagged proteins were detected with an anti-His antibody on a western blot (Figure 5-4A). Although OATP1B3-His bound to the column efficiently, it was washed off at a low imidazole concentration (50 mM, compare to recommended 200 mM). This low binding affinity led to a poor purity of OATP1B3 in the eluted fractions (Data not shown). Therefore, we tested purification of OATP1B3-His by immunoprecipitation. To increase the expression of OATP1B3, instead of using CHO Flp-In cells, HEK293 cells were transiently transfected with empty vector or OATP1B3-His. Membrane proteins were extracted and solubilized with 0.1% NP-40, and then immunoprecipitated with anti-His antibody and magnetic protein G beads. The silver stained gel shown in Figure 5-4B demonstrates that after immunoprecipitation, the purity of OATP1B3 is relatively high, and the other proteins detected in the eluted fraction can be cut off without affecting the OATP1B3 band. However, the yield of immunoprecipitation was low and most of the OATP1B3-His did not bind to the beads and remained in the flow through fraction.

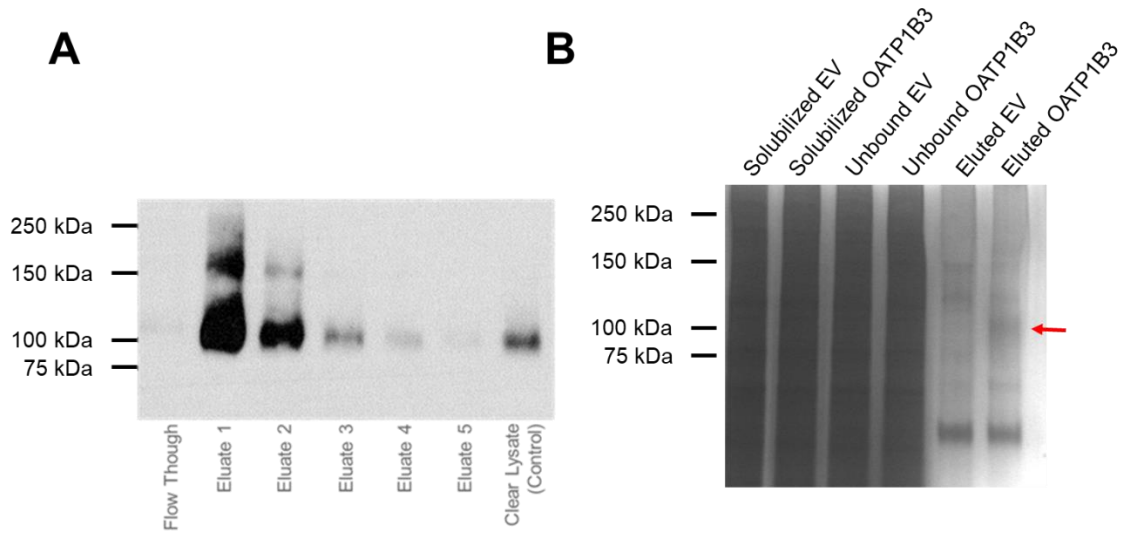

Figure 5-4: Purification of OATP1B3.

(A) Membrane proteins from OATP1B3 expressing CHO Flp-In cells were solubilized in lysis buffer containing 1% Triton X-100, and purified with Ni-NTA columns. After loading, the columns were washed with lysis buffer containing 20 mM imidazole and OATP1B3 was eluted with lysis buffer containing 50mM imidazole 5 times. Fractions were detected with Anti-His antibody on western blot. (B) Membrane proteins from empty vector or OATP1B3-His transiently transfected HEK293 cells in a full 10 cm dish were solubilized in PBS contain 0.1% NP-40. Immunoprecipitation was performed with 2 μg Anti-His antibody and 100 μL magnetic protein G beads. Silver staining was performed with the Pierce silver staining kit.

III. Discussion

In the present study, we confirmed that in our expression system the photoreactive compound, 8-fluorescein-cAMP indeed is a substrate of OATP1B3 and therefore might be used for photoaffinity labeling of OATP1B3. However, we do not know whether specific labeling of OATP1B3 is possible because of the significant background labeling. We then tried to purify OATP1B3 to improve the chances for successful labeling of OATP1B3. Unfortunately, although a small amount of OATP1B3 can be pulled down by immunoprecipitation, the efficiency of the pull-down experiment was not high enough for follow-up labeling steps.

Although the major aim of this study was not completed, several conclusions can be drawn and improvements can be suggested for future experiments. First of all, the increasing signals in the time dependency for the photoaffinity experiments suggest that the protocol for photoaffinity labeling on live cells works. However, 8-FcA also bound non-specifically to many other proteins even without UV activation (Figure 5-3B). This suggests that 8-FcA may not be stable enough under normal condition and therefore most likely is not suitable for these experiments with OATP1B3. The application of photoaffinity labeling with live cells is usually aimed at finding potential targets for photoreactive compounds, and since cAMP is a highly reactive substrate for many cell signaling pathways (Yan et al., 2016), it is expected to interact with a lot of different proteins inside and outside the cells. Although co-incubation with losartan decreased 8-FcA labeling (Figure 5-3B), it is likely that this effect was due to unspecific quenching in the presence of this high concentration of losartan. Other photolabile substrates which are more stable before UV activation should be tested with the same or a very similar labeling protocol.

We have compared several different affinity purification methods to improve the efficiency of OATP1B3 pull-down. His-tagged OATP1B3 can be isolated using Ni-NTA columns. However, the interaction between the His tag and the column beads was not strong enough and resulted in a

high background binding. When the Ni-NTA beads were washed with increasing imidazole-containing elution buffer, OATP1B3-His was eluted off in the fraction with a rather low imidazole concentration (50mM), which resulted in a very low purity of OATP1B3. In the experiments shown in Figure 5-4A the membrane proteins were solubilized with 1% TX-100. However, lower concentrations and milder types of detergent, including Tween 20 and NP40, were also tested to increase the binding strength between OATP1B3-His and the Ni-NTA column. No significant difference was observed. Immunoprecipitation of wild-type OATP1B3, i.e. without a His tag, with a specific polyclonal antibody recognizing OATP1B3, significantly increased the purity but also affected the efficiency in a negative way. The immunoprecipitation assays described in this chapter were performed by first incubating the extracted proteins with the antibody and then adding protein G. This order of combining antibody first can enhance the binding between OATP1B3 and the antibody, but the binding between the relative large complexes of OATP1B3 and antibody with protein G beads will be competed for by free antibodies in the solution. To avoid this competition and to improve the efficiency, antibody conjugated protein G beads should be generated first and the extracted proteins should be added later to the pre-formed antibody-protein G mix.

In conclusion, the present study confirmed that fluorescein-cAMP is a substrate of OATP1B3 and that it can be activated by UV light. Purification of OATP1B3 should be possible, but experimental conditions need to be further improved for a better efficiency.

Chapter 6

Summary and overall discussion

Drug transporters at the basolateral membrane of human hepatocytes form an efficient but complex system to take up endogenous compounds and xenobiotics into the liver. Some of these transporters, like OATP1B1, OATP1B3, OCT1 and NTCP, are listed in the FDA drafted guideline for industry and are suggested to be tested for potential DDIs (International Transporter et al., 2010). OATP1B3, as an important member of this uptake system, has a broad spectrum of structurally independent substrates, including both conjugated and unconjugated bile acids, hormones and drugs like statins, anti-hypertensives and antibiotics (Stieger and Hagenbuch, 2014). Among them, only a small portion of the substrates are transported selectively by OATP1B3, for example, CCK-8 and telmisartan (Roth et al., 2012; Zhu et al., 2014), while most of the substrates are shared by other transporters from different families. This makes the *in vivo* study of individual transporters challenging. Even knocking out a single transporter in a mouse model might not yield the desired results because other transporters expressed in the same cells could compensate for the lost function. Therefore, in the case of OATPs the Schinkel group knocked out a large cluster of DNA that contains the genes for *Slco1a1, Slco1a3, Slco1a4, Slco1a5, Slco1a6,* and *Slco1b2* encoding OATP1A1, OATP1A4 and OATP1B2 that are expressed in hepatocytes (van de Steeg et al., 2012). A humanized OATP1B1/3 mouse model has been generated based on this cluster knockout mouse and has facilitated the study of the contribution of OATP1B1 and/or OATP1B3 to the pharmacokinetics of statins and antitumor drugs (Salphati et al., 2014; Durmus et al., 2016).

Currently, transporters are typically characterized using cell-based assays. A single transporter expressing cell line is constructed and uptake experiments are performed to demonstrate the function of the expressed transporter (De Bruyn et al., 2013; Bednarczyk and Boiselle, 2015; Zhao et al., 2015). This is an efficient method to determine the substrates or stimulators of transporters, and it can provide information for drug development. For example, the DDIs between grape fruit juice and fexofenadine were discovered using such cell models and the results eventually influenced the clinical use (Dresser et al., 2002; Bailey, 2010). However, the results generated by using an isolated *in vitro* study may not always reflect the situation *in vivo*. To better predict the *in vivo* conditions, more complex cell models are required. Studies have shown that some of the drug transporters at the basolateral membrane of human hepatocytes can form homo-oligomers, including OATP1B1, OCT1, OAT1 and NTCP (Keller et al., 2008; Duan et al., 2011b; Bijsmans et al., 2012; Ni et al., 2017). However, it seems that most of the interactions between themselves do not lead to any functional consequences. In contrast to the homo-oligomerization of these transporters, little was known regarding the co-expression of drug transporters and the functional consequences prior to this dissertation. With this background, we investigated the homo- and hetero-oligomerization of OATP1B3 and its potential consequences in the first and second part of this dissertation.

Besides the oligomerization status, the study of structure function relationship of OATP1B3 can also help understanding the mechanism of transport and predicting potential substrates and DDIs of OATP1B3. Photoaffinity labeling is a technique which has been previously used to determine the binding and regulatory sites of other transporters, including Pgp (Safa, 2004). With the development of HPLC and MS, the analysis after the photoaffinity labeling can be even more precise and in depth (Robinette et al., 2006). Photoaffinity labeling in conjunction with LC/MS analysis can rapidly reveal the accurate binding sites of ligands to the receptors. After labeling, subsequent proteolytic digestion of labelled protein and MS-based sequencing can identify the

ligand-binding region and provide valuable information regarding the structure function relationship. In support of the structure function relationship results generated by photoaffinity labeling, a general pharmacophore for Pgp was proposed (Pajeva and Wiese, 2002), which facilitated drug design and development by providing potential markers as Pgp substrates (Li et al., 2007). Using 8-fluorescein-cAMP, we tried to investigate the binding sites and translocation pathways of OATP1B3 by using the photoaffinity labeling technique in Chapter 5.

In Chapter 3, I evaluated the hypothesis that OATP1B3 can form homo-oligomers, and that the interaction of OATP1B3 with itself may lead to functional consequences. Drug transporters such as OATP1B1, OCT1, OAT1 and NTCP can all form homo-oligomers, which suggested that OATP1B3 likely can also interact with itself. As demonstrated in Chapter 3, OATP1B3 indeed can form homo-oligomers, and we proved it with several lines of evidence. Co-immunoprecipitation experiments confirmed a physical interaction between the His- and the FLAG-tagged OATP1B3, while the PLA demonstrated the close association between the two differently tagged OATP1B3. Interestingly, I also observed an interaction between the His- and the HA-tagged OATP1B3, although the C-terminal end of OATP1B3-HA, unlike wild-type or His-tagged OATP1B3, is accessible at the extracellular side of the membrane. This result suggested that the C-terminal end of OATP1B3 is not exclusively involved in the interaction. Then, I investigated the consequences of OATP1B3 homo-oligomers, by co-expressing wildtype OATP1B3 with a non-functional mutant OATP1B3. Two transporter models have been proposed by the van de Graaf group for NTCP homo-oligomers (Bijsmans et al., 2012). In the first one the subunits in the oligomers work as one functional unit, so a mutant subunit would completely block the function of this oligomer. In the other model the subunits in the oligomers work as individual functional units and a mutant subunit would not influence the other subunits. My results demonstrated that the individual subunits work independently from each other and thus the functional unit model is the transport model for OATP1B3 homo-oligomers. This suggested that

the homo-oligomerization does not influence the function of OATP1B3. Though the mechanism of oligomerization is not the focus of this dissertation, a recent study of OATP1B1 homo-oligomerization may give some suggestions for OATP1B3 homo-oligomers (Ni et al., 2017). This study demonstrated that the three GXXXG motifs in OATP1B1 are involved in the function or formation of homo-oligomers. Because the three motifs are completely conserved in OATP1B3 it is well possible that they are involved in the oligomerization.

Since many of the transporters expressed at the basolateral membrane of human hepatocytes can form homo-oligomers and NTCP can also interact with other transporters in the SLC10 family (Bijsmans et al., 2012), we speculated that uptake transporters in hepatocytes could be co-localized in the same membrane micro-domains and their interactions could lead to functional consequences. In Chapters 3 and 4 I investigated part of this hypothesis by focusing at the hetero-oligomerization of OATP1B3 and its functional consequences. As a starting point, a DUALmembrane screening using OATP1B3 as a bait was performed to investigate potential interaction partners. The DUALmembrane screening is a modified yeast two hybrid screening assay designed for detecting the interaction between membrane proteins. Instead of using a soluble fraction of membrane proteins for the regular yeast two hybrid screening, the DUALmembrane screening can use the full-length, membrane-anchored protein as the bait. By using this screening method, OCT1 was suggested to have the highest potential as an interaction partner of OATP1B3. I then demonstrated the interaction of OATP1B3 and OCT1 by performing co-immunoprecipitation experiments using primary human hepatocytes as the starting material. After that, HEK293 cells were used as a cell-based model to further study interactions between OATP1B3 and other transporters. The Proximity Ligation Assay showed that OATP1B3 can interact not only with OCT1, but also with OATP1B1 and NTCP. In addition, I could further verify the interaction with OCT1 and NTCP in frozen human liver sections. However, because of the lack of a pair of OATP1B1 and OATP1B3 specific antibodies from two different species, the

interaction between OATP1B3 and OATP1B1 could not be studied in frozen liver sections. The observed interaction between OATP1B3 and OCT1 or NTCP in frozen liver section supported the hypothesis that there are transporter micro domains in the basolateral membrane of human hepatocytes. To investigate this hypothesis, co-localization of these transporters with lipid raft markers like caveolins should be investigated.

I then wanted to study the influence of the hetero-oligomerization on function by measuring the uptake of model substrate of both OATP1B3 and the respective interacting transporters. The results showed that when co-expressed with OATP1B3, there is no functional effect on OCT1, OATP1B1 and NTCP. However, OATP1B3 is affected in a transporter-dependent way. When co-expressed in HEK293 cells with OCT1, the uptake of the OATP1B3 model substrate CCK-8 decreased significantly, but was not affected when OATP1B3 was co-expressed with OATP1B1 or NTCP. I also performed surface biotinylation experiments to determine the surface expression change due to the co-expression. Interestingly, although there is no effect on the expression level of the interacting transporter, the OATP1B3 expression level is affected by co-expression in a transporter-dependent way. When co-expressed with OCT1, the plasma membrane expression of OATP1B3 decreased dramatically, while co-expression with OATP1B1 or NTCP led to an increase of OATP1B3 surface expression. I then normalized the uptake result based on surface expression and the apparent decrease of OATP1B3 function due to the OCT1 interaction was actually a stimulation while interaction with OATP1B1 and NTCP led to a reduction of OATP1B3 function. Normalized kinetics suggested that modulation of OATP1B3 uptake by OCT1 only changed the V_{max} but not the K_m of CCK-8 uptake, which suggested that oligomerization affected the turnover rate. Interactions with OATP1B1 or NTCP however, affected both the K_m and the V_{max} values. These results showed that the interacting partners can influence both the expression and function of OATP1B3, in a transporter-dependent way. Although no direct *in vivo* result can prove my findings under normal physiological condition, the

corresponding transporters in mice, mouse OATP1B2 and mouse OCT1, showed a similar pattern. In the mouse model of nonalcoholic steatohepatitis (NASH), the mouse OATP1B2 was down-regulated while the mouse OCT1 expression level went up (Clarke et al., 2014a; Clarke et al., 2015). To test this hypothesis, the OCT1 knockout mouse model could be used to investigate the potential expression level change of OATP1B2. Pharmacokinetic parameters of an OATP1B2 specific substrate should be collected from both wild-type and OCT1 knockout mice, which could demonstrate a potential functional influence of OCT1 for OATP1B2 function *in vivo*.

Combining the findings from these two chapters, we can conclude that OATP1B3 can from homo- and hetero-oligomers, and that the hetero-oligomerization of OATP1B3 with OCT1, OATP1B1 and NTCP can lead to changes in expression and function. There are several potential physiological and pharmacological implications of these findings. First, these results could be explained by assuming that the drug transporters at the basolateral membrane of human hepatocytes are co-localized in the same micro domains. Some transporters expressed in other membranes are co-localized in membrane micro domains. For example, in the endothelial cells of the blood brain barrier, Pgp is localized in caveolae and can interact with caveolin-1(Jodoin et al., 2003). Rat MRP2 also co-localizes with caveolin-1 in the canalicular membrane of rat hepatocytes (Ismair et al., 2009). Therefore, transporters expressed at the basolateral membrane of human hepatocytes could also be expressed in the same micro domain and be regulated together. Second, the co-localization of OATP1B3 and OATP1B1, NTCP, and OCT1 and potentially other transporters could lead to a change of the micro environment and influence the function of certain transporters. For example, most OATPs work better at an acidic pH (Leuthold et al., 2009) but the plasma pH in the liver is at 7.3-7.4 which would not affect OATP function. However, if an OATP would be co-localized with the Na^+/H^+ exchanger or other proton extruding transporters or pumps, the local environment could become more acidic and could enhance the

transport activity of OATPs. Third, the change in OATP1B3 plasma membrane expression, when co-expressed with OCT1, OATP1B1 or NTCP suggests a post-translational modulation. Unlike OATP1B1 and NTCP who are evenly distributed throughout the liver lobule, expression of OATP1B3 is lowest around the portal field (zone 1) and increases toward the central vein (zone 3) (König et al., 2000). Although the lobular distribution of human OCT1 is not clear, the expression of mouse OCT1 also increases toward the central vein (Chen et al., 2014). Assuming that the situation is similar in human liver this might explain the difference in the effects of co-expression of OATP1B3 with OCT1 as compared to OATP1B1 and NTCP. To investigate this hypothesis, the lobular distribution of mouse OATP1B2 needs to be compared between wild-type and OCT1 knockout mice and the same comparison could be performed with NTCP knockout mice. Last but not least, the significant modulation of OATP1B3 transport activity when co-expressed with OCT1, OATP1B1 and NTCP suggests that the *in vitro - in vivo* extrapolation of transporter activity could be misleading. The effects of a certain transporter to the pharmacokinetic parameters of a drug is usually extrapolated from K_m and V_{max} values that are obtained from cell-based uptake assays, which are normally conducted using cell lines expressing a single transporter. If my findings indeed represent the *in vivo* situation, then cell lines expressing single transporters over- or underestimate V_{max} values, which can result in dramatic differences of the pharmacokinetics parameters. Thus, in the future co-expression systems might have to be used to get better predictions.

In chapter 5, the hypothesis that photoaffinity labeling can be used to investigate the binding pocket and translocation pathways of OATP1B3 was not successfully tested because of the technical problems I encountered. Before a proteomics mass spectrometry approach can be used to verify the binding of a photolabile compound, purified OATP1B3 in high microgram quantities need to be isolated. By using immunoprecipitation, I was able to pull down OATP1B3 with high purity, but the efficiency was not high enough to generate enough material for the follow-up

analysis. One reason for this problem is that the detergent serves as a double-edged sword in this case. As a highly hydrophobic transmembrane protein, the solubilization of OATP1B3 requires the help of detergents. However, a high concentration of detergent can significantly reduce the antigen-antibody binding efficiency required for immunoprecipitation. To find a balanced type and concentration of detergent is very time consuming. Unfortunately, time for my study was running out. I tested several detergents, including TX-100, Tween 20 and NP40, at a variety of concentrations. Although I was able to pull down a small amount of OATP1B3 in the presence of 0.1% NP-40, I lost most of the protein during solubilization and immunoprecipitation. There are several directions that should be followed up to improve the efficiency of purification. First of all, other non-ionic detergents need to be tested. Typically, a detergent with a higher critical micelle concentration (CMC) can solubilize more membrane protein, but a detergent with a lower CMC can minimize the influence to the antibody-antigen complex formation and thus increase the pull down efficiency. Second, a suitable epitope can be added to the protein to increase the strength of the antibody-antigen interaction. Although several copies of tags, for example two or three His tags at the C-terminal end, can increase the binding affinity, the structural and functional consequences introduced by the larger size and more significantly the electrochemical properties might become a problem. Besides that, the ratio between antibody and amount of solubilized protein can also influence the pull-down efficiency. Overloading the assay with free antibody can reduce the efficiency because these unused antibodies can compete for agarose-protein G with the antibody-antigen complexes, while overloading the assay with solubilized protein can increase the non-specific binding.

Overall, in this dissertation, I investigated the oligomerization of OATP1B3 and its potential consequences. First, I demonstrated that OATP1B3 can form homo-oligomers in HEK293 cells.

The follow-up studies showed that the subunits in the homo-oligomers worked as individual functional units. Then, I also demonstrated that OATP1B3 can interact with OCT1, OATP1B1 and NTCP in HEK293 cells, and further verified the interaction between OATP1B3 with OCT1 and NTCP in frozen human liver sections. The functional consequences have also been studied in HEK293 cells and showed a transporter-dependent effect on both, expression and function of OATP1B3, but not on the other interacting transporters. Based on my findings, I proposed that the current single transporter expressing cell model may lead to incorrect *in vitro to in vivo* extrapolation, and that a multi-transporter expression system may better mimic the normal physiological basolateral membrane of human hepatocytes. In addition, I established a preliminary method for the purification of OATP1B3. However, the method still requires a lot of work to increase the efficiency.

Chapter 7

Future directions

In general, the study of liver drug transporters is challenging, especially *in vivo* studies, because of the overlapping substrate and inhibitor specificity among the different transporters. Though some human transgenic mice or single transporter knockout mouse models were generated (van de Steeg et al., 2013; Chen et al., 2014), there are still limitations. The most significant shortcoming is the redundancy of several transporters for one compound which can compensate the effects of knockout or knockin of a specific transporter (Cheng et al., 2016). Besides that, murine hepatic transporters like OATP1A1, OATP1A4 and OATP1B2 are not orthologs of individual human liver transporters like OATP1B1 and OATP1B3. There is no OATP1A family member expressed under normal conditions in human hepatocytes but there are two members in mouse liver. Similarly, the two human OATP1B1 and OATP1B3 have only a single mouse ortholog, OATP1B2 (Hagenbuch and Meier, 2004). Thus, the distribution and function of these transporters cannot clearly be correlated to each other between the two species. Because of these difficulties of using small animal models, cell-based studies have been developed to determine the function or individual function of specific transporters (Cheng et al., 2016). However, similar to all other *in vitro* studies, although the cell-based assays provide excellent data for each transporter, the respective transport activity *in vivo* remains unknown. As a consequence, the *in vitro* to *in vivo* extrapolation which plays a key role in clinical transporter studies can sometimes be proven wrong (Cheng et al., 2016), which suggests that the cell model may not reliably reflect the physiological condition. In this dissertation, I investigated and discussed the homo- and hetero-oligomerization of OATP1B3 and its functional consequences. Based on my findings, it may be necessary to consider the functional variations due to the interactions among transporters

when extrapolating from *in vitro* to *in vivo* models. To better address these potential consequences for extrapolations, several directions could be taken based on this dissertation.

For the homo-oligomerization of OATP1B3, I showed the formation and functional model of OATP1B3 homo-oligomers. However, the mechanism of oligomerization was not investigated. Other homo-oligomerization studies of transporters, including OATP1B1 (Ni et al., 2017) and OAT1 (Duan et al., 2011b), suggested that the GXXXG motifs could affect the formation of oligomers or the function of transporters. It is well possible that OATP1B3 shares the same mechanism, since the GXXXG motifs in OATP1B1 are completely conserved in OATP1B3. To study a possible involvement of a GXXXG motif for OATP1B3 oligomerization, site-directed mutagenesis of the different GXXXG motifs could be performed. Since GXXXG motifs have been proven to be involved in both correct oligomerization and protein folding of many transmembrane proteins (Teese and Langosch, 2015), several outcomes of these experiments are expected. The first potential outcome is that the surface expression of mutant OATP1B3 remains the same but the oligomerization is affected. This outcome would show that the mutated GXXXG motif is involved in the process of OATP1B3 oligomerization. Another potential outcome is that the surface expression of OATP1B3 is either increased or decreased. After normalization for surface-expressed OATP1B3, the effect on oligomerization of OATP1B3 by the mutated GXXXG could then be measured. However, if no effect on OATP1B3 surface expression and oligomerization can be observed after site-directed mutagenesis of GXXXG motifs, several other motifs or amino acids could be investigated to at least find residues important for oligomerization, including (small)XXX(small) motifs in the TMD, or cysteines in the ECL. Once the mechanism of OATP1B3 homo-oligomerization is understood, we can further manipulate the formation of OATP1B3 oligomers to study the functional influence of oligomerization. For example, comparison of the expression and function between oligomerized

and individual OATP1B3 proteins could explain the cooperative effects when oligomers are formed.

The study of hetero-oligomerization of OATP1B3 was the primary focus of this dissertation. However, several questions remain unsolved. For example, the interaction between OATP1B1 and OATP1B3 could so far not be confirmed in frozen liver sections because no suitable OATP1B1 and OATP1B3 specific antibodies are available. Since a rabbit OATP1B3 specific antibody is available in our lab, the problem can be solved by generating a monoclonal OATP1B1 specific antibody. As an alternative to the conventional production of monoclonal antibodies, antibody Phage display technology could be used. By screening a library of bacteriophages expressing a variable chain with cells expressing the target protein (OATP1B1) and with wild type cells, the phages expressing a specific variable chain could be selected. After panning and extracting, the corresponding plasmid for this variable chain can then be purified and used right away or the specific variable chain could be inserted into a full-length antibody plasmid and expressed by bacteria.

The functional consequences of hetero-oligomerizations need to be further investigated in primary human hepatocytes by knocking out the interacting partners. Based on the findings in this dissertation, that the presence of OCT1 reduces the apparent uptake function of OATP1B3, knocking out OCT1 in human hepatocyte should stimulate the uptake of CCK-8 by OATP1B3. Although studies have shown that during the *in vitro* cultivation of human hepatocytes the uptake function of OATP1B3 increased (Zhu et al., 2014), it is not known how OCT1 function changes and whether it would affect OATP1B3, but it is well possible that the gain of OATP1B3 function is due to the loss of OCT1. To test this hypothesis, scrambled siRNA and OCT1 specific siRNA should be transfected into human hepatocytes. The expression level and function of OCT1 and OATP1B3 could then be monitored by performing uptake assays with their specific substrates

like MPP and CCK-8. If the functional consequences demonstrated *in vitro* indeed would be recapitulated *in vivo*, an increase of OATP1B3 function should be correlated with the decrease of OCT1 function. Besides that, to generate a reliable hetero-oligomerization cell model for functional studies that resembles more the normal physiological conditions, the ratio of OATP1B3 and its interacting partners need to be adjusted. Preliminary data from our lab suggested that the ratio of surface-expressed OATP1B3 and OCT1 can be adjusted based on the ratio of respective plasmids transfected into the HEK293 cells. Combined with the published data of OATP1B3 and OCT1 expression ratio in human hepatocytes (Peng et al., 2015), different ratios of OATP1B3 and OCT1 plasmids can be co-transfected into cells, and have to be followed up with mass spectrometry to determine the respective protein quantities. After adjustments according to the expression levels, a cell model with an accurate ratio between OATP1B3 and OCT1 should be achieved. Functionally, interactions between OATP1B3 and its partners can lead to a change in transport activity by altering the turnover rate of OATP1B3. Our findings that indeed the V_{max} was increased after correction of surface expression clearly demonstrates that the interaction itself can lead to functional consequences. However, it is well possible that the transport activity of OATP1B3 can be affected by the functional change of interacting partners, such as activation, inhibition and mutation. This hypothesis can be investigated by treating the co-expressing cells with OATP1B3 substrates in the presence or absence of specific substrates or inhibitors for the interacting partners. For example, uptake of CCK-8 could be measured in cells expressing OATP1B3 in the presence or absence of OCT1 and treated with or without cimetidine. Preliminary data in our lab demonstrated that cimetidine is not an OATP1B3 inhibitor when OATP1B3 is expressed in HEK293 cells alone. However, the outcome of this experiment could show that when OCT1 is co-expressed, cimetidine stimulates or inhibits the uptake of CCK-8 by OATP1B3 or does not affect it at all.

Since the effect of co-expression of OATP1B3 with OCT1, OATP1B1 or NTCP is not the same, the interaction among three or even more transporters can be very complex but it is nevertheless important to understand it in order to delineate the physiological conditions from a cell model. To conclude, a challenging but ideal liver transporter-related cell model should have all human hepatic drug transporters expressed at their physiological expression ratios.

All the experiments and strategies mentioned above will lead to a better understanding of the interactions and functional consequences among human hepatic drug transporters, and thus contribute to an improved *in vitro* to *in vivo* extrapolation. Eventually these findings should allow to improve the physiological-based pharmacokinetic models in predicting transporter-related effects.

Though the low efficiency of purification of OATP1B3 has prevented the successful completion of the binding site and translocation pathway study, I believe that the photoaffinity labeling is still a good method to study the structure function relationship of OATP1B3. Since the purification of OATP1B3 by immunoprecipitation has been proven very challenging, besides optimization of the protocol, an alternative way to isolate OATP1B3 should be considered. For example, two-dimensional electrophoresis may help to separate the OATP1B3 away from the massive amount of other proteins and help to isolate OATP1B3. During the optimizing of OATP1B3 purification, the generation of a radiolabeled or fluorescent photoreactive substrate for OATP1B3 would be helpful to develop a good protocol for photoaffinity labeling. These strategies may allow to improve the chances of successful labeling and purification of OATP1B3.

Reference list

Abe T, Kakyo M, Tokui T, Nakagomi R, Nishio T, Nakai D, Nomura H, Unno M, Suzuki M, Naitoh T, Matsuno S, and Yawo H (1999) Identification of a novel gene family encoding human liver-specific organic anion transporter LST-1. *The Journal of biological chemistry* **274:**17159-17163.

Abe T, Unno M, Onogawa T, Tokui T, Kondo TN, Nakagomi R, Adachi H, Fujiwara K, Okabe M, Suzuki T, Nunoki K, Sato E, Kakyo M, Nishio T, Sugita J, Asano N, Tanemoto M, Seki M, Date F, Ono K, Kondo Y, Shiiba K, Suzuki M, Ohtani H, Shimosegawa T, Iinuma K, Nagura H, Ito S, and Matsuno S (2001) LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology* **120:**1689-1699.

Ali MH and Imperiali B (2005) Protein oligomerization: how and why. *Bioorganic & medicinal chemistry* **13:**5013-5020.

Anwer MS and Stieger B (2014) Sodium-dependent bile salt transporters of the SLC10A transporter family: more than solute transporters. *Pflugers Archiv : European journal of physiology* **466:**77-89.

Baganz NL, Horton RE, Calderon AS, Owens WA, Munn JL, Watts LT, Koldzic-Zivanovic N, Jeske NA, Koek W, Toney GM, and Daws LC (2008) Organic cation transporter 3: Keeping the brake on extracellular serotonin in serotonin-transporter-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* **105:**18976-18981.

Bailey DG (2010) Fruit juice inhibition of uptake transport: a new type of food-drug interaction. *British journal of clinical pharmacology* **70:**645-655.

Ballatori N, Christian WV, Wheeler SG, and Hammond CL (2013) The heteromeric organic solute transporter, OSTalpha-OSTbeta/SLC51: a transporter for steroid-derived molecules. *Molecular aspects of medicine* **34:**683-692.

Bednarczyk D (2010) Fluorescence-based assays for the assessment of drug interaction with the human transporters OATP1B1 and OATP1B3. *Analytical biochemistry* **405:**50-58.

Bednarczyk D and Boiselle C (2015) Organic anion transporting polypeptide (OATP)-mediated transport of coproporphyrins I and III. *Xenobiotica; the fate of foreign compounds in biological systems***:**1-10.

Bijsmans IT, Bouwmeester RA, Geyer J, Faber KN, and van de Graaf SF (2012) Homo- and hetero-dimeric architecture of the human liver Na(+)-dependent taurocholate co-transporting protein. *The Biochemical journal* **441:**1007-1015.

Boivin AA, Cardinal H, Barama A, Pichette V, Hebert MJ, and Roger M (2010) Organic anion transporting polypeptide 1B1 (OATP1B1) and OATP1B3: genetic variability and haplotype analysis in white Canadians. *Drug metabolism and pharmacokinetics* **25:**508-515.

Borchert O (2005) cGMP-abhängige Signalwege in Spermatozoen des Rindes., in: *Fachbereich Medizin*, pp 85, Universität Hamburg.

Bossuyt X, Muller M, and Meier PJ (1996) Multispecific amphipathic substrate transport by an organic anion transporter of human liver. *Journal of hepatology* **25:**733-738.

Boxberger KH, Hagenbuch B, and Lampe JN (2014) Common drugs inhibit human organic cation transporter 1 (OCT1)-mediated neurotransmitter uptake. *Drug metabolism and disposition: the biological fate of chemicals* **42:**990-995.

Burgess G, Hoogkamer H, Collings L, and Dingemanse J (2008) Mutual pharmacokinetic interactions between steady-state bosentan and sildenafil. *European journal of clinical pharmacology* **64:**43-50.

Chanmahasathien W, Ohnuma S, Ambudkar SV, and Limtrakul P (2011) Biochemical mechanism of modulation of human P-glycoprotein by stemofoline. *Planta medica* **77:**1990-1995.

Chen L, Shu Y, Liang X, Chen EC, Yee SW, Zur AA, Li S, Xu L, Keshari KR, Lin MJ, Chien HC, Zhang Y, Morrissey KM, Liu J, Ostrem J, Younger NS, Kurhanewicz J, Shokat KM, Ashrafi K, and Giacomini KM (2014) OCT1 is a high-capacity thiamine transporter that regulates hepatic steatosis and is a target of metformin. *Proceedings of the National Academy of Sciences of the United States of America* **111:**9983-9988.

Cheng Y, El-Kattan A, Zhang Y, Ray AS, and Lai Y (2016) Involvement of Drug Transporters in Organ Toxicity: The Fundamental Basis of Drug Discovery and Development. *Chemical research in toxicology* **29:**545-563.

Chun SE, Thakkar N, Oh Y, Park JE, Han S, Ryoo G, Hahn H, Maeng SH, Lim YR, Han BW, and Lee W (2017) The N-terminal region of organic anion transporting polypeptide 1B3 (OATP1B3) plays an essential role in regulating its plasma membrane trafficking. *Biochemical pharmacology* **131:**98-105.

Clarke JD, Dzierlenga AL, Nelson NR, Li H, Werts S, Goedken MJ, and Cherrington NJ (2015) Mechanism of Altered Metformin Distribution in Nonalcoholic Steatohepatitis. *Diabetes* **64:**3305-3313.

Clarke JD, Hardwick RN, Lake AD, Canet MJ, and Cherrington NJ (2014a) Experimental nonalcoholic steatohepatitis increases exposure to simvastatin hydroxy acid by decreasing hepatic organic anion transporting polypeptide expression. *J Pharmacol Exp Ther* **348:**452-458.

Clarke JD, Hardwick RN, Lake AD, Lickteig AJ, Goedken MJ, Klaassen CD, and Cherrington NJ (2014b) Synergistic interaction between genetics and disease on pravastatin disposition. *Journal of hepatology* **61:**139-147.

Claro da Silva T, Polli JE, and Swaan PW (2013) The solute carrier family 10 (SLC10): beyond bile acid transport. *Molecular aspects of medicine* **34:**252-269.

Csanaky IL, Lu H, Zhang Y, Ogura K, Choudhuri S, and Klaassen CD (2011) Organic anion-transporting polypeptide 1b2 (Oatp1b2) is important for the hepatic uptake of unconjugated bile acids: Studies in Oatp1b2-null mice. *Hepatology* **53:**272-281.

Cui Y, Konig J, Nies AT, Pfannschmidt M, Hergt M, Franke WW, Alt W, Moll R, and Keppler D (2003) Detection of the human organic anion transporters SLC21A6 (OATP2) and SLC21A8 (OATP8) in liver and hepatocellular carcinoma. *Lab Invest* **83:**527-538.

Czeredys M, Samluk L, Michalec K, Tulodziecka K, Skowronek K, and Nalecz KA (2013) Caveolin-1--a novel interacting partner of organic cation/carnitine transporter (Octn2): effect of protein kinase C on this interaction in rat astrocytes. *PloS one* **8:**e82105.

De Bruyn T, van Westen GJ, Ijzerman AP, Stieger B, de Witte P, Augustijns PF, and Annaert PP (2013) Structure-based identification of OATP1B1/3 inhibitors. *Molecular pharmacology* **83:**1257-1267.

Dean M, Hamon Y, and Chimini G (2001) The human ATP-binding cassette (ABC) transporter superfamily. *Journal of lipid research* **42:**1007-1017.

DeGorter MK, Ho RH, Leake BF, Tirona RG, and Kim RB (2012) Interaction of three regiospecific amino acid residues is required for OATP1B1 gain of OATP1B3 substrate specificity. *Molecular pharmaceutics* **9:**986-995.

Deng M, Mao M, Guo L, Chen FP, Wen WR, and Song YZ (2016) Clinical and molecular study of a pediatric patient with sodium taurocholate cotransporting polypeptide deficiency. *Experimental and therapeutic medicine* **12:**3294-3300.

Dresser GK, Bailey DG, Leake BF, Schwarz UI, Dawson PA, Freeman DJ, and Kim RB (2002) Fruit juices inhibit organic anion transporting polypeptide-mediated drug uptake to

decrease the oral availability of fexofenadine. *Clinical pharmacology and therapeutics* **71:**11-20.
Duan P, Li S, and You G (2011a) Transmembrane peptide as potent inhibitor of oligomerization and function of human organic anion transporter 1. *Molecular pharmacology* **79:**569-574.
Duan P, Wu J, and You G (2011b) Mutational analysis of the role of GXXXG motif in the function of human organic anion transporter 1 (hOAT1). *International journal of biochemistry and molecular biology* **2:**1-7.
Durmus S, van Hoppe S, and Schinkel AH (2016) The impact of Organic Anion-Transporting Polypeptides (OATPs) on disposition and toxicity of antitumor drugs: Insights from knockout and humanized mice. *Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy* **27:**72-88.
Fernandes CF, Godoy JR, Doring B, Cavalcanti MC, Bergmann M, Petzinger E, and Geyer J (2007) The novel putative bile acid transporter SLC10A5 is highly expressed in liver and kidney. *Biochemical and biophysical research communications* **361:**26-32.
Fotiadis D, Kanai Y, and Palacin M (2013) The SLC3 and SLC7 families of amino acid transporters. *Molecular aspects of medicine* **34:**139-158.
Glaeser H, Mandery K, Sticht H, Fromm MF, and Konig J (2010) Relevance of conserved lysine and arginine residues in transmembrane helices for the transport activity of organic anion transporting polypeptide 1B3. *British journal of pharmacology* **159:**698-708.
Globisch C, Pajeva IK, and Wiese M (2008) Identification of putative binding sites of P-glycoprotein based on its homology model. *ChemMedChem* **3:**280-295.
Godoy JR, Fernandes C, Doring B, Beuerlein K, Petzinger E, and Geyer J (2007) Molecular and phylogenetic characterization of a novel putative membrane transporter (SLC10A7), conserved in vertebrates and bacteria. *European journal of cell biology* **86:**445-460.
Goodsell DS and Olson AJ (2000) Structural symmetry and protein function. *Annual review of biophysics and biomolecular structure* **29:**105-153.
Gorboulev V, Ulzheimer JC, Akhoundova A, UlzheimerTeuber I, Karbach U, Quester S, Baumann C, Lang F, Busch AE, and Koepsell H (1997) Cloning and characterization of two human polyspecific organic cation transporters. *DNA and Cell Biology* **16:**871-881.
Grube M, Kock K, Karner S, Reuther S, Ritter CA, Jedlitschky G, and Kroemer HK (2006) Modification of OATP2B1-mediated transport by steroid hormones. *Molecular pharmacology* **70:**1735-1741.
Grundemann D, Gorboulev V, Gambaryan S, Veyhl M, and Koepsell H (1994) Drug excretion mediated by a new prototype of polyspecific transporter. *Nature* **372:**549-552.
Gui C and Hagenbuch B (2008) Amino acid residues in transmembrane domain 10 of organic anion transporting polypeptide 1B3 are critical for cholecystokinin octapeptide transport. *Biochemistry* **47:**9090-9097.
Gui C and Hagenbuch B (2009) Role of transmembrane domain 10 for the function of organic anion transporting polypeptide 1B1. *Protein Sci* **18:**2298-2306.
Gui C, Miao Y, Thompson L, Wahlgren B, Mock M, Stieger B, and Hagenbuch B (2008) Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. *European journal of pharmacology* **584:**57-65.
Gui C, Obaidat A, Chaguturu R, and Hagenbuch B (2010) Development of a cell-based high-throughput assay to screen for inhibitors of organic anion transporting polypeptides 1B1 and 1B3. *Current chemical genomics* **4:**1-8.
Guo GL and Klaassen CD (2001) Protein kinase c suppresses rat organic anion transporting polypeptide 1- and 2-mediated uptake. *Journal of Pharmacology and Experimental Therapeutics* **299:**551-557.
Hagenbuch B and Dawson P (2004) The sodium bile salt cotransport family SLC10. *Pflugers Archiv : European journal of physiology* **447:**566-570.

Hagenbuch B and Meier PJ (1994) Molecular cloning, chromosomal localization, and functional characterization of a human liver Na+/bile acid cotransporter. *The Journal of clinical investigation* **93:**1326-1331.

Hagenbuch B and Meier PJ (2004) Organic anion transporting polypeptides of the OATP/ SLC21 family: phylogenetic classification as OATP/ SLCO superfamily, new nomenclature and molecular/functional properties. *Pflugers Archiv : European journal of physiology* **447:**653-665.

Hagenbuch B and Stieger B (2013) The SLCO (former SLC21) superfamily of transporters. *Molecular aspects of medicine* **34:**396-412.

Han S, Kim K, Thakkar N, Kim D, and Lee W (2013) Role of hypoxia inducible factor-1alpha in the regulation of the cancer-specific variant of organic anion transporting polypeptide 1B3 (OATP1B3), in colon and pancreatic cancer. *Biochemical pharmacology* **86:**816-823.

Hashimoto M and Hatanaka Y (2008) Recent Progress in Diazirine-Based Photoaffinity Labeling. *European Journal of Organic Chemistry* **2008:**2513-2523.

He W, Ren B, Mao F, Jing Z, Li Y, Liu Y, Peng B, Yan H, Qi Y, Sun Y, Guo JT, Sui J, Wang F, and Li W (2015) Hepatitis D Virus Infection of Mice Expressing Human Sodium Taurocholate Co-transporting Polypeptide. *PLoS pathogens* **11:**e1004840.

Hediger MA, Clemencon B, Burrier RE, and Bruford EA (2013) The ABCs of membrane transporters in health and disease (SLC series): introduction. *Molecular aspects of medicine* **34:**95-107.

Hirano M, Maeda K, Shitara Y, and Sugiyama Y (2006) Drug-drug interaction between pitavastatin and various drugs via OATP1B1. *Drug metabolism and disposition: the biological fate of chemicals* **34:**1229-1236.

Hong M, Hong W, Ni C, Huang J, and Zhou C (2015) Protein kinase C affects the internalization and recycling of organic anion transporting polypeptide 1B1. *Biochimica et biophysica acta* **1848:**2022-2030.

Hong M, Xu W, Yoshida T, Tanaka K, Wolff DJ, Zhou F, Inouye M, and You G (2005) Human organic anion transporter hOAT1 forms homooligomers. *The Journal of biological chemistry* **280:**32285-32290.

Hsiang B, Zhu Y, Wang Z, Wu Y, Sasseville V, Yang WP, and Kirchgessner TG (1999) A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *The Journal of biological chemistry* **274:**37161-37168.

Imai S, Kikuchi R, Tsuruya Y, Naoi S, Nishida S, Kusuhara H, and Sugiyama Y (2013) Epigenetic regulation of organic anion transporting polypeptide 1B3 in cancer cell lines. *Pharmaceutical research* **30:**2880-2890.

International Transporter C, Giacomini KM, Huang SM, Tweedie DJ, Benet LZ, Brouwer KL, Chu X, Dahlin A, Evers R, Fischer V, Hillgren KM, Hoffmaster KA, Ishikawa T, Keppler D, Kim RB, Lee CA, Niemi M, Polli JW, Sugiyama Y, Swaan PW, Ware JA, Wright SH, Yee SW, Zamek-Gliszczynski MJ, and Zhang L (2010) Membrane transporters in drug development. *Nature reviews Drug discovery* **9:**215-236.

Ismair MG, Hausler S, Stuermer CA, Guyot C, Meier PJ, Roth J, and Stieger B (2009) ABC-transporters are localized in caveolin-1-positive and reggie-1-negative and reggie-2-negative microdomains of the canalicular membrane in rat hepatocytes. *Hepatology* **49:**1673-1682.

Ismair MG, Stieger B, Cattori V, Hagenbuch B, Fried M, Meier PJ, and Kullak-Ublick GA (2001) Hepatic uptake of cholecystikinin octapeptide by organic anion-transporting

polypeptides OATP4 and OATP8 of rat and human liver. *Gastroenterology* **121:**1185-1190.

Jigorel E, Le Vee M, Boursier-Neyret C, Parmentier Y, and Fardel O (2006) Differential regulation of sinusoidal and canalicular hepatic drug transporter expression by xenobiotics activating drug-sensing receptors in primary human hepatocytes. *Drug metabolism and disposition: the biological fate of chemicals* **34:**1756-1763.

Jodoin J, Demeule M, Fenart L, Cecchelli R, Farmer S, Linton KJ, Higgins CF, and Beliveau R (2003) P-glycoprotein in blood-brain barrier endothelial cells: interaction and oligomerization with caveolins. *Journal of neurochemistry* **87:**1010-1023.

Kandel SE and Lampe JN (2014) Role of protein-protein interactions in cytochrome P450-mediated drug metabolism and toxicity. *Chemical research in toxicology* **27:**1474-1486.

Keitel V, Burdelski M, Warskulat U, Kuhlkamp T, Keppler D, Haussinger D, and Kubitz R (2005) Expression and localization of hepatobiliary transport proteins in progressive familial intrahepatic cholestasis. *Hepatology* **41:**1160-1172.

Keller T, Egenberger B, Gorboulev V, Bernhard F, Uzelac Z, Gorbunov D, Wirth C, Koppatz S, Dotsch V, Hunte C, Sitte HH, and Koepsell H (2011) The large extracellular loop of organic cation transporter 1 influences substrate affinity and is pivotal for oligomerization. *The Journal of biological chemistry* **286:**37874-37886.

Keller T, Schwarz D, Bernhard F, Dotsch V, Hunte C, Gorboulev V, and Koepsell H (2008) Cell free expression and functional reconstitution of eukaryotic drug transporters. *Biochemistry* **47:**4552-4564.

Kindla J, Muller F, Mieth M, Fromm MF, and Konig J (2011) Influence of non-steroidal anti-inflammatory drugs on organic anion transporting polypeptide (OATP) 1B1- and OATP1B3-mediated drug transport. *Drug metabolism and disposition: the biological fate of chemicals* **39:**1047-1053.

Kobayashi D, Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2003) Involvement of human organic anion transporting polypeptide OATP-B (SLC21A9) in pH-dependent transport across intestinal apical membrane. *J Pharmacol Exp Ther* **306:**703-708.

Kock K, Koenen A, Giese B, Fraunholz M, May K, Siegmund W, Hammer E, Volker U, Jedlitschky G, Kroemer HK, and Grube M (2010) Rapid modulation of the organic anion transporting polypeptide 2B1 (OATP2B1, SLCO2B1) function by protein kinase C-mediated internalization. *The Journal of biological chemistry* **285:**11336-11347.

Koegh J, Hagenbuch B, Rynn C, Stieger B, and Nicholls G (2016) Chapter 1 Membrane Transporters: Fundamentals, Function and Their Role in ADME, in Drug Transporters: Volume 1: Role and Importance in ADME and Drug Development. *The Royal Society of Chemistry* **1:**1-56.

Koepsell H (2013) The SLC22 family with transporters of organic cations, anions and zwitterions. *Molecular aspects of medicine* **34:**413-435.

Koepsell H and Endou H (2004) The SLC22 drug transporter family. *Pflugers Archiv : European journal of physiology* **447:**666-676.

Koepsell H, Lips K, and Volk C (2007) Polyspecific organic cation transporters: structure, function, physiological roles, and biopharmaceutical implications. *Pharmaceutical research* **24:**1227-1251.

Konig J (2011) Uptake Transporters of the Human OATP Family : Molecular Characteristics, Substrates, Their Role in Drug-Drug Interactions, and Functional Consequences of Polymorphisms. *Handb Exp Pharmacol* **201:**1-28.

Konig J, Cui Y, Nies AT, and Keppler D (2000) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *The Journal of biological chemistry* **275:**23161-23168.

König J, Cui Y, Nies AT, and Keppler D (2000) Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide. *J Biol Chem* **275:**23161-23168.
Konig J, Muller F, and Fromm MF (2013) Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacological reviews* **65:**944-966.
Kosters A and Karpen SJ (2008) Bile acid transporters in health and disease. *Xenobiotica; the fate of foreign compounds in biological systems* **38:**1043-1071.
Kramer W, Girbig F, Glombik H, Corsiero D, Stengelin S, and Weyland C (2001a) Identification of a ligand-binding site in the Na+/bile acid cotransporting protein from rabbit ileum. *The Journal of biological chemistry* **276:**36020-36027.
Kramer W, Girbig F, Gutjahr U, Kowalewski S, Jouvenal K, Muller G, Tripier D, and Wess G (1993) Intestinal bile acid absorption. Na(+)-dependent bile acid transport activity in rabbit small intestine correlates with the coexpression of an integral 93-kDa and a peripheral 14-kDa bile acid-binding membrane protein along the duodenum-ileum axis. *The Journal of biological chemistry* **268:**18035-18046.
Kramer W, Sauber K, Baringhaus KH, Kurz M, Stengelin S, Lange G, Corsiero D, Girbig F, Konig W, and Weyland C (2001b) Identification of the bile acid-binding site of the ileal lipid-binding protein by photoaffinity labeling, matrix-assisted laser desorption ionization-mass spectrometry, and NMR structure. *The Journal of biological chemistry* **276:**7291-7301.
Kramer W, Wess G, Bewersdorf U, Corsiero D, Girbig F, Weyland C, Stengelin S, Enhsen A, Bock K, Kleine H, Le Dreau MA, and Schafer HL (1997) Topological photoaffinity labeling of the rabbit ileal Na+/bile-salt-cotransport system. *European journal of biochemistry* **249:**456-464.
Krishnan D, Liu L, Wiebe SA, Casey JR, Cordat E, and Alexander RT (2015) Carbonic anhydrase II binds to and increases the activity of the epithelial sodium-proton exchanger, NHE3. *Am J Physiol Renal Physiol* **309:**F383-392.
Kullak-Ublick GA, Glasa J, Boker C, Oswald M, Grutzner U, Hagenbuch B, Stieger B, Meier PJ, Beuers U, Kramer W, Wess G, and Paumgartner G (1997) Chlorambucil-taurocholate is transported by bile acid carriers expressed in human hepatocellular carcinomas. *Gastroenterology* **113:**1295-1305.
Kullak-Ublick GA, Hagenbuch B, Stieger B, Schteingart CD, Hofmann AF, Wolkoff AW, and Meier PJ (1995) Molecular and functional characterization of an organic anion transporting polypeptide cloned from human liver. *Gastroenterology* **109:**1274-1282.
Kullak-Ublick GA, Ismair MG, Stieger B, Landmann L, Huber R, Pizzagalli F, Fattinger K, Meier PJ, and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120:**525-533.
Lapinsky DJ, Aggarwal S, Huang Y, Surratt CK, Lever JR, Foster JD, and Vaughan RA (2009) A novel photoaffinity ligand for the dopamine transporter based on pyrovalerone. *Bioorganic & medicinal chemistry* **17:**3770-3774.
Lee HH and Ho RH (2016) Interindividual and interethnic variability in drug disposition: polymorphisms in organic anion transporting polypeptide 1B1 (OATP1B1; SLCO1B1). *British journal of clinical pharmacology* **83**:1176-1184.
Lempp FA and Urban S (2014) Inhibitors of hepatitis B virus attachment and entry. *Intervirology* **57:**151-157.
Letschert K, Keppler D, and Konig J (2004) Mutations in the SLCO1B3 gene affecting the substrate specificity of the hepatocellular uptake transporter OATP1B3 (OATP8). *Pharmacogenetics* **14:**441-452.

Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, and Stieger B (2009) Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. *American journal of physiology Cell physiology* **296:**C570-582.

Li WX, Li L, Eksterowicz J, Ling XB, and Cardozo M (2007) Significance analysis and multiple pharmacophore models for differentiating P-glycoprotein substrates. *Journal of chemical information and modeling* **47:**2429-2438.

Martin C, Berridge G, Higgins CF, Mistry P, Charlton P, and Callaghan R (2000) Communication between multiple drug binding sites on P-glycoprotein. *Molecular pharmacology* **58:**624-632.

Martinez-Becerra P, Briz O, Romero MR, Macias RI, Perez MJ, Sancho-Mateo C, Lostao MP, Fernandez-Abalos JM, and Marin JJ (2011) Further characterization of the electrogenicity and pH sensitivity of the human organic anion-transporting polypeptides OATP1B1 and OATP1B3. *Molecular pharmacology* **79:**596-607.

Mayati A, Le Vee M, Moreau A, Jouan E, Bucher S, Stieger B, Denizot C, Parmentier Y, and Fardel O (2015) Protein kinase C-dependent regulation of human hepatic drug transporter expression. *Biochemical pharmacology* **98:**703-717.

Meier-Abt F, Mokrab Y, and Mizuguchi K (2005) Organic anion transporting polypeptides of the OATP/SLCO superfamily: identification of new members in nonmammalian species, comparative modeling and a potential transport mode. *The Journal of membrane biology* **208:**213-227.

Meyer Zu Schwabedissen HE, Bottcher K, Chaudhry A, Kroemer HK, Schuetz EG, and Kim RB (2010) Liver X receptor alpha and farnesoid X receptor are major transcriptional regulators of OATP1B1. *Hepatology* **52:**1797-1807.

Middendorff R, Muller D, Mewe M, Mukhopadhyay AK, Holstein AF, and Davidoff MS (2002) The tunica albuginea of the human testis is characterized by complex contraction and relaxation activities regulated by cyclic GMP. *The Journal of clinical endocrinology and metabolism* **87:**3486-3499.

Minematsu T and Giacomini KM (2011) Interactions of tyrosine kinase inhibitors with organic cation transporters and multidrug and toxic compound extrusion proteins. *Molecular cancer therapeutics* **10:**531-539.

Molina H, Azocar L, Ananthanarayanan M, Arrese M, and Miquel JF (2008) Localization of the Sodium-Taurocholate cotransporting polypeptide in membrane rafts and modulation of its activity by cholesterol in vitro. *Biochimica et biophysica acta* **1778:**1283-1291.

Moore-Hoon ML and Turner RJ (2000) The structural unit of the secretory Na+-K+-2Cl- cotransporter (NKCC1) is a homodimer. *Biochemistry* **39:**3718-3724.

Morris DI, Greenberger LM, Bruggemann EP, Cardarelli C, Gottesman MM, Pastan I, and Seamon KB (1994) Localization of the forskolin labeling sites to both halves of P-glycoprotein: similarity of the sites labeled by forskolin and prazosin. *Molecular pharmacology* **46:**329-337.

Nambu T, Hamada A, Nakashima R, Yuki M, Kawaguchi T, Mitsuya H, and Saito H (2011) Association of SLCO1B3 polymorphism with intracellular accumulation of imatinib in leukocytes in patients with chronic myeloid leukemia. *Biological & pharmaceutical bulletin* **34:**114-119.

Ni C, Yu X, Fang Z, Huang J, and Hong M (2017) Oligomerization Study of Human Organic Anion Transporting Polypeptide 1B1. *Molecular pharmaceutics* **14:**359-367.

Ni Z, Bikadi Z, Rosenberg MF, and Mao Q (2010) Structure and function of the human breast cancer resistance protein (BCRP/ABCG2). *Current drug metabolism* **11:**603-617.

Nies AT, Koepsell H, Damme K, and Schwab M (2011) Organic cation transporters (OCTs, MATEs), in vitro and in vivo evidence for the importance in drug therapy. *Handb Exp Pharmacol***:**105-167.

Nishi H, Hashimoto K, Madej T, and Panchenko AR (2013) Evolutionary, physicochemical, and functional mechanisms of protein homooligomerization. *Progress in molecular biology and translational science* **117:**3-24.

Noe J, Portmann R, Brun ME, and Funk C (2007) Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. *Drug metabolism and disposition: the biological fate of chemicals* **35:**1308-1314.

Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* **308:**438-445.

Obaidat A, Roth M, and Hagenbuch B (2012) The expression and function of organic anion transporting polypeptides in normal tissues and in cancer. *Annual review of pharmacology and toxicology* **52:**135-151.

Pajeva IK and Wiese M (2002) Pharmacophore model of drugs involved in P-glycoprotein multidrug resistance: explanation of structural variety (hypothesis). *Journal of medicinal chemistry* **45:**5671-5686.

Peng KW, Bacon J, Zheng M, Guo Y, and Wang MZ (2015) Ethnic variability in the expression of hepatic drug transporters: absolute quantification by an optimized targeted quantitative proteomic approach. *Drug metabolism and disposition: the biological fate of chemicals* **43:**1045-1055.

Pleban K, Kopp S, Csaszar E, Peer M, Hrebicek T, Rizzi A, Ecker GF, and Chiba P (2005) P-glycoprotein substrate binding domains are located at the transmembrane domain/transmembrane domain interfaces: a combined photoaffinity labeling-protein homology modeling approach. *Molecular pharmacology* **67:**365-374.

Powell J, Farasyn T, Kock K, Meng X, Pahwa S, Brouwer KL, and Yue W (2014) Novel mechanism of impaired function of organic anion-transporting polypeptide 1B3 in human hepatocytes: post-translational regulation of OATP1B3 by protein kinase C activation. *Drug metabolism and disposition: the biological fate of chemicals* **42:**1964-1970.

Ramsden D, Tweedie DJ, Chan TS, and Tracy TS (2014) Altered CYP2C9 activity following modulation of CYP3A4 levels in human hepatocytes: an example of protein-protein interactions. *Drug metabolism and disposition: the biological fate of chemicals* **42:**1940-1946.

Robinette D, Neamati N, Tomer KB, and Borchers CH (2006) Photoaffinity labeling combined with mass spectrometric approaches as a tool for structural proteomics. *Expert review of proteomics* **3:**399-408.

Roth M, Obaidat A, and Hagenbuch B (2012) OATPs, OATs and OCTs: the organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *British journal of pharmacology* **165:**1260-1287.

Roth M, Timmermann BN, and Hagenbuch B (2011) Interactions of green tea catechins with organic anion-transporting polypeptides. *Drug metabolism and disposition: the biological fate of chemicals* **39:**920-926.

Safa AR (2004) Identification and characterization of the binding sites of P-glycoprotein for multidrug resistance-related drugs and modulators. *Current medicinal chemistry Anti-cancer agents* **4:**1-17.

Salphati L, Chu X, Chen L, Prasad B, Dallas S, Evers R, Mamaril-Fishman D, Geier EG, Kehler J, Kunta J, Mezler M, Laplanche L, Pang J, Rode A, Soars MG, Unadkat JD, van Waterschoot RA, Yabut J, Schinkel AH, and Scheer N (2014) Evaluation of organic anion transporting polypeptide 1B1 and 1B3 humanized mice as a translational model to study the pharmacokinetics of statins. *Drug metabolism and disposition: the biological fate of chemicals* **42:**1301-1313.

Satoh H, Yamashita F, Tsujimoto M, Murakami H, Koyabu N, Ohtani H, and Sawada Y (2005) Citrus juices inhibit the function of human organic anion-transporting polypeptide OATP-B. *Drug metabolism and disposition: the biological fate of chemicals* **33:**518-523.

Schulze A, Schieck A, Ni Y, Mier W, and Urban S (2010) Fine mapping of pre-S sequence requirements for hepatitis B virus large envelope protein-mediated receptor interaction. *Journal of virology* **84:**1989-2000.

Shitara Y (2011) Clinical importance of OATP1B1 and OATP1B3 in drug-drug interactions. *Drug metabolism and pharmacokinetics* **26:**220-227.

Sievanen E (2007) Exploitation of bile acid transport systems in prodrug design. *Molecules* **12:**1859-1889.

Singh A, Thornton ER, and Westheimer FH (1962) The photolysis of diazoacetylchymotrypsin. *The Journal of biological chemistry* **237:**3006-3008.

Smith E and Collins I (2015) Photoaffinity labeling in target- and binding-site identification. *Future medicinal chemistry* **7:**159-183.

Soderberg O, Gullberg M, Jarvius M, Ridderstrale K, Leuchowius KJ, Jarvius J, Wester K, Hydbring P, Bahram F, Larsson LG, and Landegren U (2006) Direct observation of individual endogenous protein complexes in situ by proximity ligation. *Nat Methods* **3:**995-1000.

Soodvilai S, Soodvilai S, Chatsudthipong V, Ngawhirunpat T, Rojanarata T, and Opanasopit P (2017) Interaction of pharmaceutical excipients with organic cation transporters. *International journal of pharmaceutics* **520:**14-20.

Srimaroeng C, Cecile JP, Walden R, and Pritchard JB (2013) Regulation of renal organic anion transporter 3 (SLC22A8) expression and function by the integrity of lipid raft domains and their associated cytoskeleton. *Cell Physiol Biochem* **31:**565-578.

Stagljar I, Korostensky C, Johnsson N, and Te Heesen S (1998) A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. *Proc Natl Acad Sci USA* **95:**5187-5192.

Sticova E, Lodererova A, van de Steeg E, Frankova S, Kollar M, Lanska V, Kotalova R, Dedic T, Schinkel AH, and Jirsa M (2015) Down-regulation of OATP1B proteins correlates with hyperbilirubinemia in advanced cholestasis. *International journal of clinical and experimental pathology* **8:**5252-5262.

Stieger B and Hagenbuch B (2014) Organic anion-transporting polypeptides. *Curr Top Membr* **73:**205-232.

Sugiyama D, Kusuhara H, Shitara Y, Abe T, and Sugiyama Y (2002) Effect of 17 beta-estradiol-D-17 beta-glucuronide on the rat organic anion transporting polypeptide 2-mediated transport differs depending on substrates. *Drug metabolism and disposition: the biological fate of chemicals* **30:**220-223.

Svoboda M, Riha J, Wlcek K, Jaeger W, and Thalhammer T (2011) Organic anion transporting polypeptides (OATPs): regulation of expression and function. *Current drug metabolism* **12:**139-153.

Teese MG and Langosch D (2015) Role of GxxxG Motifs in Transmembrane Domain Interactions. *Biochemistry* **54:**5125-5135.

Thakkar N, Kim K, Jang ER, Han S, Kim K, Kim D, Merchant N, Lockhart AC, and Lee W (2013) A cancer-specific variant of the SLCO1B3 gene encodes a novel human organic anion transporting polypeptide 1B3 (OATP1B3) localized mainly in the cytoplasm of colon and pancreatic cancer cells. *Molecular pharmaceutics* **10:**406-416.

Tian M, von Dahl CC, Liu PP, Friso G, van Wijk KJ, and Klessig DF (2012) The combined use of photoaffinity labeling and surface plasmon resonance-based technology identifies multiple salicylic acid-binding proteins. *The Plant journal : for cell and molecular biology* **72:**1027-1038.

Treiber A, Schneiter R, Hausler S, and Stieger B (2007) Bosentan is a substrate of human OATP1B1 and OATP1B3: inhibition of hepatic uptake as the common mechanism of its interactions with cyclosporin A, rifampicin, and sildenafil. *Drug metabolism and disposition: the biological fate of chemicals* **35:**1400-1407.

Tsujimoto M, Dan Y, Hirata S, Ohtani H, and Sawada Y (2008) Influence of SLCO1B3 gene polymorphism on the pharmacokinetics of digoxin in terminal renal failure. *Drug metabolism and pharmacokinetics* **23:**406-411.

Tsukuda S, Watashi K, Hojima T, Isogawa M, Iwamoto M, Omagari K, Suzuki R, Aizaki H, Kojima S, Sugiyama M, Saito A, Tanaka Y, Mizokami M, Sureau C, and Wakita T (2017) A new class of hepatitis B and D virus entry inhibitors, proanthocyanidin and its analogs, that directly act on the viral large surface proteins. *Hepatology* **65:**1104-1116.

van de Steeg E, Stranecky V, Hartmannova H, Noskova L, Hrebicek M, Wagenaar E, van Esch A, de Waart DR, Oude Elferink RP, Kenworthy KE, Sticova E, al-Edreesi M, Knisely AS, Kmoch S, Jirsa M, and Schinkel AH (2012) Complete OATP1B1 and OATP1B3 deficiency causes human Rotor syndrome by interrupting conjugated bilirubin reuptake into the liver. *The Journal of clinical investigation* **122:**519-528.

van de Steeg E, van Esch A, Wagenaar E, Kenworthy KE, and Schinkel AH (2013) Influence of human OATP1B1, OATP1B3, and OATP1A2 on the pharmacokinetics of methotrexate and paclitaxel in humanized transgenic mice. *Clin Cancer Res* **19:**821-832.

van de Steeg E, Wagenaar E, van der Kruijssen CM, Burggraaff JE, de Waart DR, Elferink RP, Kenworthy KE, and Schinkel AH (2010) Organic anion transporting polypeptide 1a/1b-knockout mice provide insights into hepatic handling of bilirubin, bile acids, and drugs. *The Journal of clinical investigation* **120:**2942-2952.

van Giersbergen PL, Treiber A, Schneiter R, Dietrich H, and Dingemanse J (2007) Inhibitory and inductive effects of rifampin on the pharmacokinetics of bosentan in healthy subjects. *Clinical pharmacology and therapeutics* **81:**414-419.

Vavricka SR, Jung D, Fried M, Grutzner U, Meier PJ, and Kullak-Ublick GA (2004) The human organic anion transporting polypeptide 8 (SLCO1B3) gene is transcriptionally repressed by hepatocyte nuclear factor 3beta in hepatocellular carcinoma. *Journal of hepatology* **40:**212-218.

Vaz FM, Paulusma CC, Huidekoper H, de Ru M, Lim C, Koster J, Ho-Mok K, Bootsma AH, Groen AK, Schaap FG, Oude Elferink RP, Waterham HR, and Wanders RJ (2015) Sodium taurocholate cotransporting polypeptide (SLC10A1) deficiency: conjugated hypercholanemia without a clear clinical phenotype. *Hepatology* **61:**260-267.

Visentin M, Chang MH, Romero MF, Zhao R, and Goldman ID (2012) Substrate- and pH-specific antifolate transport mediated by organic anion-transporting polypeptide 2B1 (OATP2B1-SLCO2B1). *Molecular pharmacology* **81:**134-142.

Wang W, Seward DJ, Li L, Boyer JL, and Ballatori N (2001) Expression cloning of two genes that together mediate organic solute and steroid transport in the liver of a marine vertebrate. *Proceedings of the National Academy of Sciences of the United States of America* **98:**9431-9436.

Weaver YM and Hagenbuch B (2010) Several conserved positively charged amino acids in OATP1B1 are involved in binding or translocation of different substrates. *The Journal of membrane biology* **236:**279-290.

Wilkens S (2015) Structure and mechanism of ABC transporters. *F1000prime reports* **7:**14.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, and Li W (2012) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* **1:**e00049.

Yan H, Zhong G, Xu G, He W, Jing Z, Gao Z, Huang Y, Qi Y, Peng B, Wang H, Fu L, Song M, Chen P, Gao W, Ren B, Sun Y, Cai T, Feng X, Sui J, and Li W (2014) Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *eLife* **3**.

Yan K, Gao LN, Cui YL, Zhang Y, and Zhou X (2016) The cyclic AMP signaling pathway: Exploring targets for successful drug discovery (Review). *Molecular medicine reports* **13:**3715-3723.

Yanni SB, Augustijns PF, Benjamin DK, Jr., Brouwer KL, Thakker DR, and Annaert PP (2010) In vitro investigation of the hepatobiliary disposition mechanisms of the antifungal agent micafungin in humans and rats. *Drug metabolism and disposition: the biological fate of chemicals* **38:**1848-1856.

Zhang L, Dresser MJ, Gray AT, Yost SC, Terashita S, and Giacomini KM (1997) Cloning and functional expression of a human liver organic cation transporter. *Molecular pharmacology* **51:**913-921.

Zhang Y, Boxberger KH, and Hagenbuch B (2017) Organic anion transporting polypeptide 1B3 can form homo- and hetero-oligomers. *PloS one* **12(6):e0180257**.

Zhang Y, Hays A, Noblett A, Thapa M, Hua DH, and Hagenbuch B (2013) Transport by OATP1B1 and OATP1B3 enhances the cytotoxicity of epigallocatechin 3-O-gallate and several quercetin derivatives. *Journal of natural products* **76:**368-373.

Zhao W, Zitzow JD, Ehresman DJ, Chang SC, Butenhoff JL, Forster J, and Hagenbuch B (2015) Na+/Taurocholate Cotransporting Polypeptide and Apical Sodium-Dependent Bile Acid Transporter Are Involved in the Disposition of Perfluoroalkyl Sulfonates in Humans and Rats. *Toxicol Sci* **146:**363-373.

Zhu Q, Xia H, Xia CQ, Yang Q, Doshi U, Li AP, and Liao M (2014) Culture duration-, donor-, and medium-dependent changes in OATP1B3-mediated telmisartan uptake in human hepatocytes. *Drug metabolism letters* **7:**117-125.

Zollner G, Fickert P, Zenz R, Fuchsbichler A, Stumptner C, Kenner L, Ferenci P, Stauber RE, Krejs GJ, Denk H, Zatloukal K, and Trauner M (2001) Hepatobiliary transporter expression in percutaneous liver biopsies of patients with cholestatic liver diseases. *Hepatology* **33:**633-646.