STATIN TRANSPORT BY HEPATIC ORGANIC ANION-TRANSPORTING POLYPEPTIDES (OATPs)

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Abstract

Drug transport proteins are important determinants of drug absorption, tissue accumulation, and elimination from the body, and there is growing appreciation for the contribution of altered drug transporter function to interindividual variability in drug response. The organic anion-transporting polypeptides (OATPs/*SLCO*) are uptake transporters with broad substrate specificity. Notably, the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are commonly prescribed OATP substrates.

The OATP1B subfamily, expressed predominantly in the liver, is of particular importance to statins, which require hepatic entry to exert their low-density lipoprotein cholesterol lowering effect. We aimed to identify molecular determinants of substrate specificity in the human OATP1B subfamily in vitro, and found three regions required for transport of a non-statin substrate, cholecystokinin-8, thus improving our understanding of OATP1B transport mechanism. We employed Oatp1b2$^{-/-}$ mice to model reduced OATP1B function in humans, and observed liver-to-plasma ratios of atorvastatin and rosuvastatin were lower in Oatp1b2$^{-/-}$ mice compared with wild-type animals, further emphasizing the importance of this OATP subfamily to hepatic drug uptake.

One challenge to statin therapy is the risk for muscle toxicity associated with elevated systemic statin exposure. We assessed intraindividual variability in statin pharmacokinetics in human subjects, and found a correlation in exposure to atorvastatin and simvastatin, which are both metabolized by cytochrome P450 3A (CYP3A). In contrast, atorvastatin and simvastatin exposure were not correlated with rosuvastatin, a statin that is transported but not

significantly metabolized, thus illustrating the interplay between transport and metabolism that influences statin pharmacokinetics.

Though numerous clinical trials have investigated statin effectiveness, interindividual variability in statin pharmacokinetics in a clinical setting is not well understood. We characterized atorvastatin and rosuvastatin concentration in 299 patients at London Health Sciences Center, and observed 45-fold variability. Genetic variants in *SLCO1B1* and *ABCG2* were associated with rosuvastatin concentration. Atorvastatin concentration was associated with *SLCO1B1* variants and with 4β-hydroxycholesterol concentration, a marker of CYP3A activity. Lathosterol, a marker of HMG-CoA reductase function, was not associated with statin concentration in our population.

Taken together, these studies further our understanding of OATP function, both in vivo and in vitro, and the contribution of OATPs to pharmacokinetics and drug response.

Keywords

Organic anion-transporting polypeptides, solute carrier transporters, ATP-binding cassette transporters, transporter knockout mice, drug transporter polymorphisms, drug transporter pharmacogenetics, hepatic uptake transport, statins, statin transport, statin pharmacokinetics

Co-Authorship Statement

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MKD, RGT, and RBK designed the experiments. MKD and BFL conducted the experiments. MKD, RGT, and RBK analyzed and interpreted the data. MKD and RBK wrote the manuscript. All authors approved the final version of the manuscript.

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MKD, BLU, RGT, and RBK designed the study. MKD, BLU, and UG conducted the human study. MKD conducted the mouse experiments. MKD, RGT, and RBK analyzed and interpreted the the data. MKD and RBK wrote the manuscript. All authors approved the final version of the manuscript.

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RBK, MKD, RGT, and RAH designed the study. MKD, UIS, RAH, GKD, NS, and KM participated in patient enrolment and data collection. MKD measured the statin and sterol concentrations and performed genotyping. MKD, RBK, RAH, RGT, UIS, YHC, and GYZ analyzed and interpreted the data. MKD, RBK, and RAH wrote the manuscript.

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Abbreviations

ABC	ATP-binding cassette
ALT	alanine transaminase
ASBT	apical sodium-dependent bile acid cotransporter
ATP	adenosine triphosphate
AUC	area under the curve
BCRP	breast cancer resistance protein
BMI	body mass index
BSEP	bile-salt export pump
CCK-8	cholecystokinin-8
cDNA	complementary deoxyribonucleic acid
C_{max}	maximum concentration
CNS	central nervous system
cRNA	complementary ribonucleic acid
CRP	C-reactive protein
CSF	cerebrospinal fluid
CV	coefficient of variation
CYP	Cytochrome P450
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
ECL	extracellular loop
ED_{50}	half-maximal effective dose
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ESCC	esophageal squamous cell carcinoma

FDA	Food and Drug Administration
GWAS	genome-wide association studies
HESI	heated electrospray ionization source
HIV	human immunodeficiency virus
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IC_{50}	half maximal inhibitory concentration
ICV	intracerebroventricular
IV	intravenous
JUPITER	Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin
Ke	terminal rate constant
KHB	Krebs Henseleit Bicarbonate
K_m	substrate concentration producing 50% maximal velocity
KOH	potassium hydroxide
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL-C	low-density lipoprotein cholesterol
LogD	distribution coefficient
LST	liver-specific transporter
m/z	mass-to-charge ratio
MATE	multidrug and toxin extrusion
MDR	multidrug resistance
MFS	major facilitator superfamily
mRNA	messenger ribonucleic acid
MRP	multidrug resistance-associated protein
NSAID	nonsteroidal anti-inflammatory drug

NTCP	sodium-dependent taurocholate co-transporting polypeptide
OAT	organic anion transporter
OATP	organic anion-transporting polypeptide
OCT	organic cation transporter
OCTN	OCTN, organic cation/carnitine transporter
PBS	phosphate buffered saline
PEPT	peptide transporter
P-gp	P-glycoprotein
QSAR	quantitative structure-activity relationship
R^2	coefficient of determination
RCT	randomized controlled trial
RNA	ribonucleic acid
RPM	revolutions per minute
SC	subcutaneous
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLC	solute carrier
SNP	single nucleotide polymorphism
$t_{1/2}$	elimination half life
TM	transmembrane
t_{max}	time of maximum concentration
TMD	transmembrane domains
V_{max}	maximal velocity

1 INTRODUCTION TO TRANSPORTER PHARMACOGENETICS[1]

[1] Reproduced from: DeGorter MK and Kim RB. “Introduction to Pharmacogenomics of Drug Transporters" in *Pharmacogenomics of Human Drug Transporters: Clinical Impacts*. Ishikawa T, Kim RB and Konig J, Eds.; John Wiley and Sons Inc: Hoboken, New Jersey, 2013. This material is reproduced with permission of John Wiley & Sons, Inc.

1.1 Introduction

Understanding the molecular mechanisms and clinical relevance of interindividual variability in drug response remains an important challenge. Pharmacogenomics, the study of genetic variation in the genes that influence drug effect, can provide insight into interindividual variability and a more accurate prediction of drug response than may be obtained by relying solely on a patient's clinical information. The goal of drug transporter pharmacogenomics is to understand the impact of genetic variation on the function of transporters that interact with medications. For many drugs in clinical use, transporters are important determinants of absorption, tissue accumulation, and elimination from the body, and thereby transporters significantly influence drug efficacy and toxicity. Adverse drug reactions can result from toxicity associated with high drug concentrations and lack of efficacy can result from subtherapeutic drug exposure. By understanding the genetic basis for drug transporter activity, it will be possible to enhance a predictive approach to individualization of drug therapy.

The purpose of this chapter is to highlight the advances in transporter pharmacogenomics that have been made since polymorphisms in drug transporter genes were first described in the late 1990s (1-3). As we enter the genomic era of medicine, pharmacogenomics will inform prescribing practices to maximize drug efficacy while minimizing risk for toxicity. Given the importance of transporters to the absorption, distribution, and elimination of many drugs, there is no doubt that transporter pharmacogenomics will make significant contributions to this aim.

1.2 Overview of drug transporters

Membrane transporters have diverse and important roles in maintaining cellular homeostasis by the uptake and efflux of endogenous compounds to regulate solute and fluid balance, facilitate hormone signaling, and extrude potential toxins. Drug transport proteins are a functional subset of membrane transporters that also interact with drugs and their metabolites. Compounds that rely on carrier mechanisms tend to be polar and bulky, and less likely to pass through cell membranes by simple diffusion. Transporter substrates include numerous drugs, their hydroxylated metabolites and the glutathione, sulfate or glucuronide-conjugated products of Phase II metabolism. Transporters that are expressed in the epithelia of intestine, liver, and kidney are of particular importance for vectorial or directional movement of drugs, resulting in efficient and rapid drug absorption, distribution, metabolism, and elimination. Moreover, expression of drug transporters on the basolateral *versus* apical domain of polarized epithelial cells in organs such as the intestine and liver may also be critical for a drug to enter the tissue and interact with its target (4-5).

Membrane transporters are comprised of multiple transmembrane domains (TMDs) that form a pore in the membrane through which the substrates pass. These domains are joined by alternating intracellular and extracellular loops which, together with TMDs, facilitate substrate recognition, binding, and translocation. The functional mechanism and conformational changes required for transport are not completely understood, and remain an active area of investigation (6). Of particular interest to transporter pharmacogenomics

is the ability to predict the functional effect of novel mutations that are discovered in individual genomes.

Drug transporters belong to two major classes, the solute carrier superfamily (SLC), and the ATP-binding cassette (ABC) superfamily. In the human genome there are 350 transporters in the SLC superfamily, and 48 ABC transporters; these transporters are divided into subfamilies based on sequence homology (5). ABC transporters are distinguished by the presence of an intracellular nucleotide binding domain that catalyzes the hydrolysis of ATP to generate the energy required to transport substrates against their concentration gradient (7). In contrast, SLC transporters utilize facilitated diffusion, ion coupling or ion exchange to translocate their substrates. In some cases, transport relies on an ion gradient that is actively maintained by ABC transporters (8).

Transporter function may be influenced by multiple factors, and interindividual variability in transporter function is now recognized as a major source of variability in drug disposition and response. Drug transporters can be inhibited by numerous compounds, typically by competition for recognition and binding, resulting in unexpected pharmacokinetics of substrate drugs, and drug-drug interactions. Genetic variants may also affect transporter function, and, in recent years, the discovery of genetic variation in drug transporters has opened up an area of research in transporter pharmacogenomics (5; 9).

1.3 Overview of pharmacogenomics

The study of inherited differences in drug response dates back to observations of inherited differences in metabolism by Garrod in 1902 (10), although the field did not come into its own until the 1950s and 1960s, when Kalow first used the term pharmacogenetics to describe the emerging discipline (11). In the late 1980s, molecular advances provided a mechanistic explanation for these findings (12-13). Many early achievements in pharmacogenetics described the effect of genetic variation in cytochrome P450 (CYP) drug metabolizing enzyme genes on metabolite concentrations. Pharmacogenomics studies have benefited from having well defined phenotypes: a pharmacokinetic measure such as the plasma or urine concentration of a drug or its metabolite, or a measure of drug response, such as a change in blood pressure or heart rate. For monogenic traits, this approach has led to new insights in our understanding of the factors underlying drug disposition and response, and provided a solid foundation to study traits that are influenced by multiple genes and other clinical factors. Today, pharmacogenomics encompasses a broad spectrum of genes involved in metabolism as well as transport, and in drug targets and related pathways (14).

Genetic variants include single nucleotide polymorphisms (SNPs), which are typically present in >1% of the population, while more rare variants are considered to be genetic mutations. SNPs in the coding regions of proteins may be classified as synonymous or non-synonymous, depending on whether the amino acid sequence is altered in the variant allele. Single nucleotide polymorphisms may also come in the form of small insertions or deletions, which result in frameshift of amino acid sequence or premature truncation of

the protein, and likely a non-functional product (15). Duplication or deletion of larger regions of genomic sequence (>50bp) are classified as copy number or structural variants (16). A classic example of copy number variation comes from the field of pharmacogenomics: CYP2D6 is commonly duplicated or deleted, resulting in profound differences in the rate of metabolism of its substrate drugs in individuals with these changes (17). There is a growing appreciation for the importance of structural differences as a source of variation in the human genome, and further study of this variation as it relates to transporter genes is expected (16).

Pharmacogenomic information may be used to predict treatment outcomes and choose the best drug and its optimal dose. Pharmacogenomics may also be used to predict a patient's risk for an adverse drug reaction, including drug-drug interactions that may be more severe depending on genetic changes. At the time of writing, the United States Food and Drug Administration (FDA) listed nearly 80 drugs for which pharmacogenomic biomarkers in over 30 genes were included in some part of the label recommendations. To date, the FDA has focused on drug metabolizing enzymes and target proteins; however, transporter genes are expected to be added in the future, following the work of the International Transporter Consortium, sponsored by the FDA's Critical Path Initiative (5).

1.4 Pharmacogenomics of drug transporters

Transporter polymorphisms may increase or reduce an individual's overall exposure to a substrate, depending on the tissue expression and localization of the transporter. For example, reduced function uptake transporters on the luminal membrane of the intestine would result in reduced systemic exposure of its substrate, whereas reduced function uptake transporters on the basolateral membrane of the liver or kidney may result in increased systemic exposure if the drug in question relies on these organs for its elimination. On the other hand, reduced function of ABC efflux transporters present on the luminal membrane of the intestine will result in increased plasma concentration of the substrate drug, as less drug is returned to the intestinal lumen by the transporter. In some cases, the precise in vivo contribution of a transporter may be difficult to define, particularly if the transporter is present in multiple tissues, or has overlapping function with transporters of similar expression patterns. The extent of phenotypic variation observed will depend on how much the substrate relies on the single transporter in question, and the extent of genetic variation present in the other transporters, metabolizing enzymes, and targets that interact with the drug.

To date, the best studied transporter polymorphisms have been those in the coding regions of transporter genes. Some variants cause reduced trafficking of the transporter to the cell membrane, resulting from incorrect folding or an inability to interact with molecular chaperones, and other variants may affect substrate recognition or binding. Certain amino acid changes, particularly in substrate binding regions, have been shown to alter transport in a substrate-specific fashion, making it difficult to fully predict the effect

of a polymorphism on transport of a particular compound without testing that compound directly. Although numerous polymorphisms in transporter genes have been identified, not all polymorphisms appear to affect transporter function. One method to test the function of a SNP is to express its protein product and measure its transport function in vitro. Of 88 protein-altering variants studied in 11 SLC transporters, 14% had decreased or total loss of functional activity in in vitro assays (15). This is likely an underestimation, due to the possibility of substrate-specific differences in effect.

Analysis of large numbers of SNPs in the coding regions of transporters demonstrated that genetic diversity is significantly higher in loop domains compared with TMDs, suggesting there is selective pressure against amino acid changes in these regions (18). Polymorphisms may also occur in intronic regions, affecting splicing, or in promoter and enhancer regions, affecting RNA expression. Analysis of proximal promoter region variation showed that SLC transporter promoters are more likely to contain variants than ABC transporter promoters, and highly active promoters are more likely to contain variants than less active ones (19). Genetic diversity in transporter genes also appears to be related to ethnicity. In a study of 680 SNPs identified from samples representing five ethnic populations, only 83 SNPs were present in all five populations (18). Thus differences in transporter polymorphism frequency may account for some variability in drug response observed across ethnicities.

1.5 Techniques to study drug transporter function

The application of advances in molecular biology techniques to the study of transporters over the last twenty years has made significant contribution to our understanding of transporter biology. In vitro, transporter activity is often characterized in primary cells and in expression systems, including transiently and stably transfected cultured human cell lines, inside-out membrane vesicles, and insect cells. One challenge to studying transporters in vivo is the overlapping substrate specificity and tissue distribution of many transporters, which can lead to difficulties in the precise identification of the transporter(s) responsible for a particular effect. Knockout mouse models of transporters have proven to be useful to delineate the contribution of certain transporters to drug disposition (20). Knockout mice exist for many of the SLC and ABC transporters, and double and triple ABC transporter knockout models have been used to characterize the contribution of multiple transporters with overlapping substrate specificities (21). It is important to bear in mind that there are species-related differences in transporter expression and substrate specificity that may make it difficult to interpret and extrapolate the results obtained in mice to the human situation. The relative contribution of a given transporter in vivo has also been examined by drug-specific pharmacokinetic and pharmacodynamic studies in individuals with and without polymorphisms in the transporter gene of interest.

In the last decade, the field of genomics has developed rapidly, with the sequencing of the human genome (22-23) and subsequent efforts to determine haplotype structure by the HapMap project (24), and sequence variation by the 1000 Genomes Project (25).

Genome-wide association studies (GWAS) incorporating clinical and genetic data have been widely used to identify genetic variants that predict risk for disease and also to assess drug response or toxicity. For pharmacogenomics studies, GWAS offer to identify candidate genes unrelated to our current knowledge of drug mechanism (26).

Methods for detecting transporter polymorphisms and predicting the functional consequences of unique polymorphisms in real time will be required to use pharmacogenomics in the clinical setting. To address this need, genotyping platforms for a focused set of important pharmacogenetic genes are being developed for clinical use (27). QSAR and molecular dynamics simulations are in silico approaches that are active areas of research aimed at addressing this challenge of SNP prediction (28).

1.6 Transporter pharmacogenomics in drug discovery and development

An understanding of transporter pharmacogenomics is important for the design and development of new drugs that are safe and effective. Transporters interacting with drug candidates may be identified during the preclinical stage of drug development, taking into consideration the limitations inherent to extrapolating in vitro and animal data to predict human response. For this reason, pharmacogenomic studies in later phases of drug development and post marketing surveillance are crucial to identify potential transporter-mediated drug interactions, and individuals with transporter polymorphisms who may require dose adjustment or an alternative compound (29). The International Transporters

Consortium is a group of academic, industry, and regulatory leaders formed to create guidelines for the systematic inclusion of transporter studies in the drug development and approval process (5).

1.7 Clinical implications of transporter pharmacogenomics

As our understanding of transporter pharmacogenomics matures, and pharmacogenomics technologies are more widely adopted in the clinic, transporter genomics could be used to select an appropriate dose, or the best medication from a particular class of compounds, and identify those individuals who may be at increased risk for an adverse drug reaction. Transporters that affect drug response are numerous and diverse in their effect; key examples from the SLC and ABC superfamilies are summarized in Tables 1.1 and 1.2, respectively.

P-glycoprotein is an example of an efflux transporter that can significantly limit the accumulation of its substrates in certain tissues. The expression of P-glycoprotein at the blood brain barrier prevents the central nervous system accumulation of drugs such as protease inhibitors, and its overexpression in cancer cells is associated with a multidrug resistant phenotype (30). Genetic variants in the cation transporter OCT1 (*SLC22A1*) have been associated with reduced efficacy of metformin, an antidiabetic drug that targets the liver as its site of action (31). The organic anion-transporting polypeptide 1B1

Table 1.1 Drug transporters of the solute carrier superfamily

Transporter (Gene)	**Tissue(s) of predominant expression in humans**	**Key drug substrates**	**Key inhibitors**	**SNPs associated with drug response**	**Ref**
OCT1 *(SLC22A1)*	hepatocyte (basolateral)	metformin, oxaliplatin	quinine	multiple associated with metformin response	(32)
OCT2 *(SLC22A2)*	renal proximal tubule (basolateral)	metformin, oxaliplatin	cimetidine	none to date	(32)
MATE1 *(SLC47A1)*	hepatocyte (canalicular membrane); renal proximal tubule (luminal)	cimetidine, metformin, procainamide	cimetidine, pyrimethamine	possibly rs2289669	(32)
OAT1 *(SLC22A6)*	renal proximal tubule (basolateral)	acyclovir	probenecid, NSAIDs	none to date	(33)
OAT3 *(SLC22A8)*	renal proximal tubule (basolateral)	NSAIDs, furosemide	probenecid, NSAIDs	none to date	(33)
OATP1B1 *(SLCO1B1)*	hepatocyte (basolateral)	statins, repaglinide	rifampicin, gemfibrozil cyclosporine	c.521T>C (rs4149056)	(34)
OATP1B3 *(SLCO1B3)*	hepatocyte (basolateral)	statins, taxanes	rifampicin, cyclosporine	possibly c.334T>G (rs4149117)	(35)
OATP2B1 *(SLCO2B1)*	hepatocyte (basolateral); enterocyte (luminal)	statins, fexofenadine	cyclosporine	possibly c.935G>A (rs12422149)	(35)

Table 1.2 Drug transporters of the ATP-binding cassette superfamily

Transporter (Gene)	**Tissue(s) of predominant expression in humans**	**Key drug substrates**	**Key inhibitors**	**SNPs associated with drug response**	**Ref**
P-gp *(ABCB1)*	hepatocyte (canalicular); enterocyte (luminal); blood-brain barrier	HIV protease inhibitors, antineoplastics	cyclosporine, verapamil	possibly c.3435T>C (rs1045642)	(30)
BSEP *(ABCB11)*	hepatocyte (canalicular)	pravastatin	cyclosporine, glibenclamide	possibly p.V444A (rs2287622)	(36-37)
MRP2 *(ABCC2)*	hepatocyte (canalicular)	β-lactam antibiotics, methotrexate, multiple Phase-II conjugates	cyclosporine	none to date	(36)
MRP4 *(ABCC4)*	hepatocyte (basolateral); renal proximal tubule (luminal)	nucleoside-based antivirals, methotrexate, topotecan	dipyridamole, losartan	possibly p.E757K (rs3765534)	(38)
BCRP *(ABCG2)*	hepatocyte (canalicular); enterocyte (luminal); blood-brain barrier	statins, antineoplastics	dipyridamole, cyclosporine	c.421C>A (rs2231142)	(39)

(OATP1B1/*SLCO1B1*) polymorphism c.521T>C has been associated with increased risk for statin-induced muscle toxicity (40) and genotyping patients for this variant has been proposed to identify those at greater risk for side effects (41).

Transporter pharmacogenomics have not yet been widely used in a clinical setting. Moving forward, studies are needed to show the risk-benefit ratio of a drug is improved by pharmacogenomic testing, and some efforts are being made to determine the key components to be included in pharmacoeconomic evaluations of pharmacogenomic tests (42). As sequencing becomes more cost-efficient, the possibility of sequencing relevant genes or even genomes in a clinical setting poses a new challenge of interpreting pharmacogenomic information on an individual level (43).

Finally, it is important to bear in mind that many factors contribute to variability in drug responsiveness, including renal and hepatic function, underlying disease processes, and drug interactions. At the end of the day, a patient's actual drug response phenotype, in terms of efficacy and toxicity, is the key clinically relevant endpoint, and pharmacogenomics should be integrated with other parameters such as drug levels, biomarkers, and measures of drug response in order to provide truly personalized medicine.

1.8 Conclusion

Genetic variation in transporters contributes significantly to observed interindividual variability in drug response. In the future, systematic inclusion of drug transporter studies that assess genetic variation, whether affecting transporter function or expression, will be essential to the development of drugs that are safe and effective. There is little doubt drug transporter pharmacogenomics is expanding rapidly and new insights will continue to inform improved drug prescribing and thereby enhance the delivery of optimal medical care.

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2 DRUG TRANSPORTERS IN DRUG EFFICACY AND TOXICITY[2]

[2] Reproduced from: DeGorter MK, Xia CQ, Yang JJ, Kim RB. 2012. Drug transporters in drug efficacy and toxicity. *Annu Rev Pharmacol Toxicol* 52:249-73

2.1 Introduction

Optimizing drug efficacy and minimizing drug toxicity requires that the drug reach its target at adequate concentration, without excessive accumulation in other tissues. For many drugs in clinical use today, intracellular concentration is determined by the balance in activity of multiple uptake and efflux transporters that facilitate the drugs' movement across biological membranes. Transporters are large, membrane-bound proteins expressed in tissues throughout the body; those found in the epithelia of major organs of absorption and secretion such as liver, intestine, and kidney and in sanctuary sites such as the brain, testes, and placenta are of particular importance in drug disposition (Figure 2.1). Interindividual variation in transporter activity can arise from numerous factors, including genetic heterogeneity, certain disease processes, concomitant medications, and herbals and dietary constituents that may inhibit or induce transporter expression or activity (1-3).

Transporter function has been studied extensively in vitro through the use of cRNA-injected *Xenopus laevis* oocytes and transfected mammalian cell lines. Knockout mice and other animal models have provided significant insights into the role of transporters in vivo, particularly when multiple transporters with overlapping substrate specificities are expressed in the same tissue. However, species-related differences in transporter expression and substrate specificity are relatively common and need to be considered when the results of experiments in rodent models are being interpreted. In humans, the role of transporters in drug efficacy and toxicity has been indirectly shown by inhibition

Figure 2.1 Expression of transporters with major roles in drug efficacy or toxicity. Tissues shown are (a) human intestinal epithelia, (b) kidney proximal tubule epithelia, (c) hepatocytes and (d) brain capillary endothelial cells. Transporters discussed in the text are coloured red. NTCP, ASBT, and BSEP are bile acid transporters. PEPT1 and PEPT2 transport small peptide fragments. OCTN1 and OCTN2 transport organic cation and carnitine. Abbreviations: ASBT, apical sodium-dependent bile acid cotransporter; BCRP, breast cancer resistance protein; BSEP, bile-salt export pump; MATE, multidrug and toxin extrusion; MRP, multidrug resistance-associated protein; NTCP, sodium-dependent taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; OCTN, organic cation/carnitine transporter; PEPT, peptide transporter; P-gp, P-glycoprotein

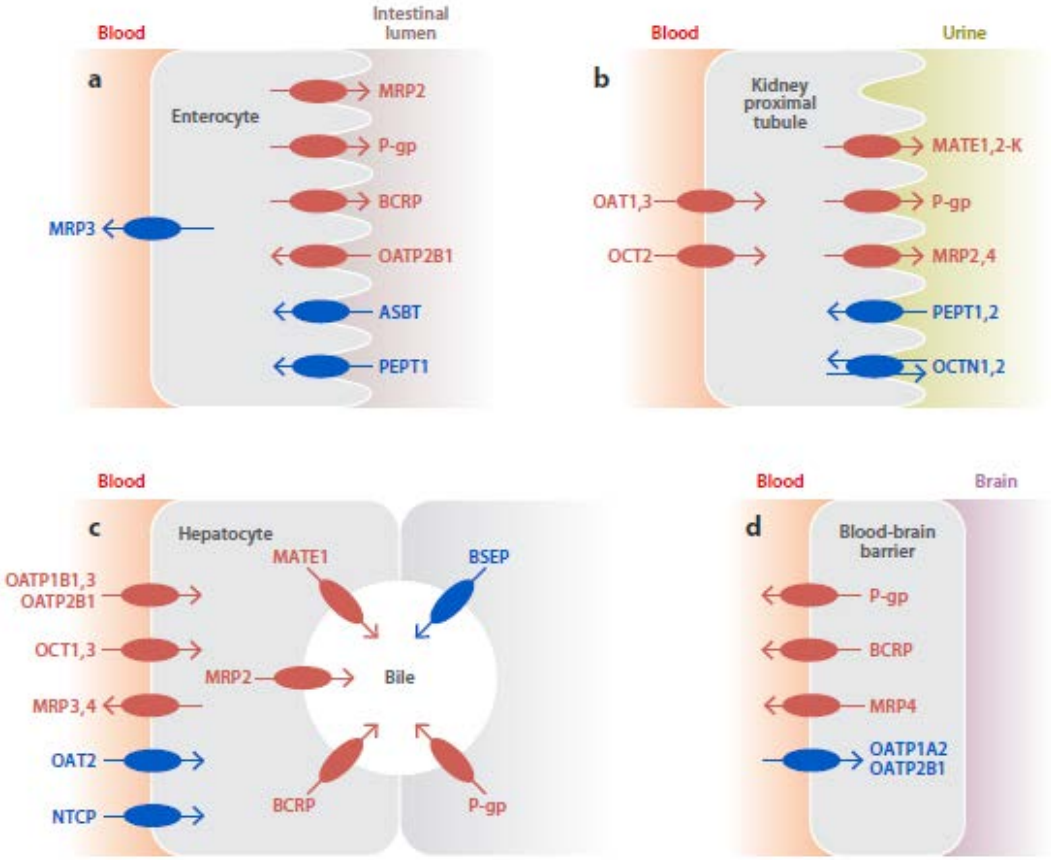

Figure 2.1 Expression of transporters with major roles in drug efficacy or toxicity

or induction studies both in healthy volunteers and in patients. Naturally occurring genetic polymorphisms cause reduced expression or function of specific transporters, an effect that is not readily achieved by pharmacological inhibitors in most cases. For this reason, studies in human subjects with genetic polymorphisms have been instrumental in defining the clinical relevance of certain transporters to drug disposition and response.

Given the critical role of transporters in mediating the pharmacokinetics of many drugs, transporter studies are an important part of the drug discovery and development process. A recent report from the International Transporter Consortium provides some guidance for the circumstances under which transporter studies may be indicated for a new molecular entity during the drug development process, with the caveat that the proposed decision structures will continue to evolve as the drug transporter field matures (1).

In this review, we focus on transporters with well-defined roles in drug efficacy and toxicity. From the solute carrier (SLC) superfamily, these include the organic cation transporters (OCTs/*SLC22A*), the multidrug and toxin extrusion transporters (MATE transporters/*SLC47A*), the organic anion transporters (OATs/*SLC22A*), and the organic anion-transporting polypeptides (OATPs/*SLCO*). Members of the ATP-binding cassette (ABC) superfamily important in drug efficacy and toxicity include P-glycoprotein (MDR1/*ABCB1*), breast cancer resistance protein (BCRP/*ABCG2*), and transporters of the multidrug resistance-associated protein (MRP/*ABCC*) family.

2.2 Uptake transporters of the solute carrier superfamily

The SLC superfamily is a large family of membrane-bound transporters that share 20-25% sequence homology. SLC transporters translocate their substrates across biological membranes through numerous mechanisms, including facilitated diffusion, ion coupling, and ion exchange, which, in some cases, is driven by an ion gradient that is maintained by active transporters of the ABC superfamily (4).

2.2.1 Organic cation transporters

Organic cation transporters (OCTs/*SLC22A*) identified in humans include OCT1 (*SLC22A1)* and OCT2 (*SLC22A2*), which are predominantly expressed on the basolateral membranes of hepatocytes and kidney proximal tubules, respectively, and OCT3 (*SLC22A3*), which is more widely expressed in tissues throughout the body. OCTs are uptake transporters that control cellular entry of small, positively charged compounds, including endogenous substrates, such as monoamine neurotransmitters and creatinine, and numerous drug substrates, including the platinum-containing antineoplastics, the antidiabetic metformin, and the histamine H2 receptor antagonist cimetidine (5-7). OCT expression is highly variable among individuals, which may be a result of genetic variants or disease processes: A study of OCT1 and OCT3 expression in 150 livers from Caucasian subjects revealed significant variation that was associated with genetic polymorphisms and cholestasis (8).

2.2.2 Multidrug and toxin extrusion transporters

The multidrug and toxin extrusion transporters (MATE transporters/*SLC47A*) are among the most recently identified transporters of functional importance to cation transport, although the existence of a renal efflux transport system had been known for some time (9). MATE1 (*SLC47A1*) is expressed throughout the body, but predominantly in the liver and kidneys, where it is localized to the canalicular membrane of hepatocytes and the luminal membrane of proximal tubule cells, respectively (10-11). In contrast, MATE2-K, the protein form of MATE2 (*SLC47A2*) that has been functionally characterized, is expressed specifically in the kidney proximal tubule and is localized to the luminal membranes. Many of the substrates and inhibitors of MATE transporters overlap with those of OCTs; therefore, the role of MATE transporters in mediating cation transport and drug-drug interactions in the kidney may have been underestimated in the past (5; 7).

2.2.3 Organic anion transporters

The organic anion transporters (OATs/*SLC22A*) move small organic anions against their concentration gradient using a Na+ gradient maintained by Na+/K+-ATPase. Of particular importance in drug disposition are OAT1 (*SLC22A6*), which is predominantly expressed on the basolateral membrane of proximal renal tubules, and OAT3 (*SLC22A8*), which is predominantly expressed throughout the kidney and in the choroid plexus, although both OAT1 and OAT3 are expressed in other tissues in the body. In the kidney, OAT1 and OAT3 facilitate the uptake of compounds from the blood and share a broad and partially overlapping substrate specificity. OAT substrates include steroid hormones,

biogenic amines, and drugs such as the angiotensin converting enzyme inhibitors captopril and quinaprilat, the angiotensin II receptor blocker olmesartan, and numerous antibiotics and antivirals. Many drugs in clinical use are inhibitors of OAT transport in vitro, including antibiotics, antivirals, and nonsteroidal anti-inflammatory drugs (NSAIDs) (12-13).

2.2.4 Organic anion-transporting polypeptides

The organic anion-transporting polypeptides (OATPs/*SLCO*) have a wide substrate specificity for amphipathic molecules, including endogenous compounds such as bile acids, thyroid hormones, sulfated and glucuronidated hormones, and drug substrates including rifampicin, methotrexate, antidiabetics, and statins (14-16).

2.2.4.1 Organic anion-transporting polypeptides in efficacy

Of the human OATPs, OATP1B1 (*SLCO1B1;* previously known as OATP-C, OATP2, and LST-1) has been studied most extensively, owing to the prevalence of clinically relevant polymorphisms (17). OATP1B1 is expressed exclusively on the basolateral membrane of the liver and is thought to be the driving force for hepatic uptake of statins and certain antidiabetic drugs that target the liver as their site of action. *SLCO1B1* is highly polymorphic (17-18); the most extensively characterized variant is the loss-of-function polymorphism c.521T>C (rs4149056), which has a frequency of approximately

15% in Asian and Caucasian populations. Aberrant cell surface trafficking of this allele may result in reduced hepatic uptake of OATP1B1 substrates in affected individuals.

Given that statins target the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase enzyme in the liver, it might be expected that reduced hepatic uptake by OATP1B1 would be accompanied by reduced efficacy, as the statin does not reach sufficient concentration in the liver to inhibit the enzyme effectively. This was demonstrated in studies that showed the *SLCO1B1* c.521T>C polymorphism was associated with the lipid-lowering effect of statins in healthy volunteers (19-21), and in a small group of patients (22) but the association of *SLCO1B1* c.521C>T with reduced statin efficacy has not yet been convincingly demonstrated in large patient cohorts.

In total, the influence of *SLCO1B1* polymorphisms on the pharmacokinetics of over 20 clinically used drugs has been studied (17); these drugs include fexofenadine (23), irinotecan (24-25), lopinavir (26-27), methotrexate (28), repaglinide (29-31). In addition, *SLCO1B1* c.521T>C has been associated with toxic side effects caused by the anticancer drugs irinotecan (32-33) and methotrexate (34). Not all in vitro substrates of OATP1B1 appear to be affected by OATP1B1 polymorphisms in vivo, suggesting that for certain substrates, additional transporters may compensate for loss of OATP1B1 function. For example, bosentan was described as a substrate of OATP1B1 and OATP1B3 (35); however, it does not appear that polymorphisms in either of these transporters significantly influence bosentan pharmacokinetics in vivo (36).

OATP1B3 (*SLCO1B3;* previously known as OATP8 and LST-2) is also expressed on the basolateral membrane of human hepatocytes. In addition to transporting many of the same compounds transported by OATP1B1, OATP1B3 transports taxanes and numerous small peptides. Polymorphisms in *SLCO1B3* have been identified and assessed for transport activity (37-38), although evidence for the clinical importance of these polymorphisms is less clear than for *SLCO1B1*. Genetic variants in *SLCO1B3* were not associated with paclitaxel or docetaxel pharmacokinetics in Caucasian cancer patients, despite evidence for OATP1B3-mediated transport of these drugs in vitro (39-41). *SLCO1B3* variants were, however, associated with docetaxel-induced leukopenia in Japanese cancer patients (42), and *Slco1a/1b*$^{-/-}$ knockout mice had a twofold increased exposure to paclitaxel compared with wild-type animals (43). Thus, the role of OATP1B3 in taxane transport is not fully understood, although it is interesting that OATP1B3 is overexpressed in colorectal and breast cancers, and that its transport activity may be important in drug entry to tumor cells (44).

The other OATP expressed on the basolateral membrane of human hepatocytes, OATP2B1 (*SLCO2B1;* previously known as OATP-B), is also expressed on the apical membrane of enterocytes, where it may be involved in the intestinal uptake of its substrates. Reduced plasma levels of the leukotriene receptor antagonist montelukast were associated with the nonsynonymous *SLCO2B1* polymorphism c.935G>A (rs12422149); individuals with this polymorphism also experienced less improvement in their symptoms compared with wild-type individuals (45). Reduced exposure to the OATP2B1 substrate aliskiren following ingestion of apple, orange or grapefruit juice is

postulated to result from inhibition of intestinal OATP2B1-mediated transport (46-47). For montelukast, orange juice consumption had an effect on plasma exposure for wild-type carriers but not for *SLCO2B1* c.935G>A carriers; the latter had reduced montelukast exposure regardless of treatment (48).

Oatp1b2 was the first murine Oatp transporter to be studied in a knockout mouse model and is the closest ortholog of the human OATPs expressed in the liver, OATP1B1 and OATP1B3. *Slco1b2*$^{-/-}$ mice had lower liver-to-plasma ratios of the prototypical OATP1B substrates pravastatin, lovastatin, and rifampicin compared with wild-type controls (49-50), indicating the importance of Oatp1b2 in mediating the hepatic uptake of these compounds. Reduced hepatic uptake of the toxins phalloidin and microcystin-LR in *Slco1b2*$^{-/-}$ mice resulted in protection against hepatotoxicity induced by these compounds (51). There are additional Oatps of the Oatp1a family that are expressed in mouse but not human liver, and compensation by these transporters in *Slco1b2*$^{-/-}$ mice may not fully reflect the effect of OATP1B loss in humans. *Slco1a/1b*$^{-/-}$ mice with deletion of Oatp1b2, Oatp1a1, Oatp1a4, Oatp1a5, and Oatp1a6 expression demonstrate significantly reduced hepatic concentrations and elevated plasma levels of methotrexate and fexofenadine (52), and provide a model to further elucidate the combined role of the Oatp1a and Oatp1b families in drug disposition.

2.2.4.2 Organic anion-transporting polypeptides in toxicity

Numerous studies of statin pharmacokinetics in healthy individuals have demonstrated that reduced-function *SLCO1B1* polymorphisms, particularly c.521T>C, increase the area under the curve of plasma exposure to nearly all the statins, including atorvastatin (53-54), pravastatin (55-61), pitavastatin (62-64), rosuvastatin (53; 65-66), and simvastatin acid (67) (Table 2.1). Increased systemic statin exposure is thought to be one component of risk for muscle toxicity, a side effect associated with statin use that can range from mild to life-threatening in its severity. In 2008, a genome-wide association study identified a variant in complete linkage disequilibrium with *SLCO1B1* c.521T>C to be the single best predictor of myopathy risk in individuals on high doses of simvastatin (68). Subsequently, the *SLCO1B1* c.521T>C variant was found to be a modest risk predictor for cerivastatin-induced rhabdomyolysis in a candidate gene study of 185 cases matched to controls (69). In another study, *SLCO1B1* c.521T>C was associated with severe myopathy induced by simvastatin, but not atorvastatin (70). Analysis of 509 subjects who were randomized to receive low-dose atorvastatin, simvastatin or pravastatin followed by higher doses of the same drug demonstrated an association between the same polymorphism and adverse events such as discontinuation, myalgia, or creatine kinase elevation following the dose escalation (71). Most recently, the incidence of less severe forms of statin intolerance, as manifested by adjusting the dose or switching to another statin, was associated with the *SLCO1B1* c.521T>C polymorphism in a study of more than 4,000 diabetic patients (72). Finally, OATP2B1 was identified as a statin transporter present in muscle tissue, indicating a potential role for statin entry into muscle tissue as part of the mechanism of statin-associated muscle toxicity (73).

Table 2.1 Transporter polymorphisms involved in statin pharmacokinetics and response

Statin	**Transporter polymorphism**	**Population studied**	**Experimental approach**	**Effect of polymorphism**	**Ref**
atorvastatin	*SLCO1B1* *c*.521T>C (rs4149056)	Healthy subjects	Candidate gene; full PK profile	Increased AUC and C_{max}	(53-54)
		Patients (n=509)	Candidate gene; dose escalation	Increased incidence of muscle toxicity	(71)
		Patients (25 cases, 84 controls)	Candidate gene; case-control study	No association with muscle toxicity	(70)
	ABCG2 c.421C>A (rs2231142)	Healthy subjects (n=32)	Candidate gene; full PK profile	Increased AUC	(74)
rosuvastatin	*SLCO1B1* c.521T>C	Healthy subjects	Candidate gene; full PK profile	Increased AUC and C_{max}	(53; 65-66)
	ABCG2 c.421C>A	Healthy subjects (n=32)	Candidate gene; full PK profile	Increased AUC and C_{max}	(74)
		Patients (n=386)	Candidate gene (61 genes)	Enhanced LDL-C-lowering response	(75)
		Hypercholesterolemic patients (n=305)	Candidate gene	Enhanced LDL-C-lowering response	(76)
		Myocardial infarction patients (n=601)	Candidate gene (6 genes); substudy of RCT	Enhanced LDL-C lowering response	(77)

Table 2.1 Transporter polymorphisms involved in statin pharmacokinetics and response, continued

Statin	Transporter polymorphism	Population studied	Experimental approach	Effect of polymorphism	Ref
simvastatin	*SLCO1B1* c.521T>C	Healthy subjects (n=32)	Candidate gene; full PK profile	Increased AUC and C_{max}	(67)
		Patients (85 cases, 90 controls)	Genome-wide association study; substudy of RCT	Increased incidence of muscle toxicity	(68)
		Patients (25 cases, 84 controls)	Candidate gene; case-control study	Increased incidence of muscle toxicity	(70)
		Patients (n=509)	Candidate gene; dose escalation	Increased incidence of muscle toxicity	(71)
		Diabetic patients (n=4196)	Candidate gene; Population cohort study	Increased incidence of statin intolerance	(72)

2.3 Efflux transporters of the ATP-binding cassette superfamily

ATP-binding cassette (ABC) transporters use energy from the hydrolysis of ATP to move their substrates across biological membranes and against their concentration gradients, thereby limiting cellular accumulation of their substrates. Members of this large family are identified by the presence of a highly conserved ATP-binding motif (3).

2.3.1 P-glycoprotein

P-glycoprotein (MDR1/*ABCB1*) is an ABC transporter with an important role in protecting tissues from xenobiotics. The protein was originally identified in cells selected for multidrug resistance (78) and has subsequently been studied extensively in the context of normal physiology and tumor biology (79). Of particular importance in drug disposition is the expression of P-glycoprotein in the apical membrane of enterocytes, hepatocytes, and kidney proximal tubules, and in the endothelial cells of the blood brain barrier (79).

As it became apparent that P-glycoprotein was not the only molecule capable of conferring a multidrug resistant phenotype, two other ABC transporters involved in multidrug resistance were cloned: multidrug resistance-associated protein 1 (MRP1/*ABCC1*) (80) and breast cancer resistance protein (BCRP/*ABCG2*) (81-83). Expression of these transporters, along with their functional genetic polymorphisms, has

been implicated in drug response and prognosis for numerous tumor types and chemotherapeutic agents. Many detailed reviews of ABC transporters and anticancer therapy have been published (84-85).

P-glycoprotein has broad substrate specificity for structurally divergent compounds; in general, its substrates are hydrophobic and may be cationic. Substrates of P-glycoprotein include HIV protease inhibitors, calcium channel blockers, and anticancer drugs of the vinca alkaloid, anthracycline, and taxane classes. P-glycoprotein is inhibited by numerous compounds including verapamil, ritonavir, and cyclosporine (79). A great deal of effort has been expended to identify potent and selective P-glycoprotein inhibitors that may be used to overcome multidrug resistance, but these efforts have not been as successful as hoped (86). Mouse P-glycoprotein was recently the first mammalian ABC transporter to be crystallized and characterized at a high resolution (87). The identification of substrate and inhibitor binding sites will contribute to an understanding of the mechanism of ABC transporters in general and assist the effort to design molecules that inhibit P-glycoprotein in order to overcome multidrug resistance.

The role of P-glycoprotein in reducing the absorption of xenobiotics can be directly examined by comparing oral drug exposure in Mdr1a/1b$^{-/-}$ mice with wild-type controls. This model proved to be particularly helpful in outlining the likely in vivo impact of this transporter on the observed oral bioavailability of substrate drugs such as HIV protease inhibitors, topotecan, etoposide, tacrolimus, ivermectin, and loperamide (88).

In addition to limiting oral bioavailability, the expression and function of this efflux transporter in the endothelial cells that constitute the blood brain barrier appear to be critical to limiting the central nervous system (CNS) entry of many substrate drugs, including those predicted to have brain accumulation on the basis of physicochemical properties such as lipophilicity (89). Endoxifen, the active metabolite of the estrogen receptor antagonist tamoxifen, is a newly identified P-glycoprotein substrate, with significantly higher endoxifen concentrations observed in the brains of Mdr1a/1b$^{-/-}$ mice (90-91). Expression of P-glycoprotein at the blood-brain barrier has also been implicated in anticonvulsant therapy failure, although its clinical relevance remains controversial (92). Conversely, limited CNS entry by third generation antihistamines that are P-glycoprotein substrates, such as fexofenadine, has proven to be a desirable property as it reduces the side effect of sedation (93).

For some drugs that are substrates of BCRP, P-glycoprotein alone does not fully limit CNS drug entry, and only when both transporters are absent is the magnitude of CNS drug accumulation significantly enhanced. This has been shown through the use of the Mdr1a/1b/Bcrp$^{-/-}$ mice for tyrosine kinase inhibitors such as lapatinib, imatinib, sunitinib, and tandutinib, which are substrates of P-glycoprotein and BCRP (94-97). Species differences in the brain uptake of radiolabeled P-glycoprotein substrates have been observed by positron emission tomography, and although the mechanisms for these differences are not well understood, they may be a consideration for animal studies conducted in preclinical drug development (98).

ABCB1 is highly polymorphic, however, the in vivo role of these polymorphisms has not been consistently demonstrated. To date, hundreds of studies in genotype-defined subjects have been conducted with numerous P-glycoprotein substrates, and the results have been mixed (79; 99). The *ABCB1* c.3435T>C (rs1045642) variant in particular has received a great deal of attention but the data are conflicting. These inconsistent findings may result from different experimental conditions, inadequate sample sizes, or heterogeneity of the sample population studied. Many substrates that are used as probes for transporter function are also substrates for drug metabolizing enzymes or other transporters. For example, transport studies with cyclosporine and tacrolimus may be complicated by the involvement of CYP3A metabolism, and, in addition to being transported by P-glycoprotein, fexofenadine is also a substrate of OATPs (100). Thus, metabolism and transport by proteins other than P-glycoprotein may contribute significantly to the observed variability in drug disposition. Future studies from current resequencing efforts with larger sample sizes and more detailed genetic information may help clarify the influence of genetic polymorphisms in *ABCB1*.

2.3.2 Breast cancer resistance protein

Breast cancer resistance protein (BCRP/*ABCG2*) is expressed on the luminal membrane of enterocytes, with greatest expression observed in the duodenum; it is important for limiting the oral bioavailability of its substrates (101). BCRP is also expressed on the canalicular membrane of hepatocytes, where it is involved in facilitating biliary excretion, and found in sanctuary sites such as the blood-brain barrier, placenta, and

testes. BCRP substrates include numerous anticancer agents, such as the topoisomerase II inhibitor etoposide, the camptothecin derivatives topotecan and irinotecan, and the tyrosine kinase inhibitors imatinib and gefitinib. Other substrates of BCRP include statins, antibiotics, numerous environmental toxins, and endogenous substrates such as conjugated steroid hormones, folates, and uric acid (102-104).

Bcrp1$^{-/-}$ mice have been useful in elucidating the relative contribution of Bcrp1 to drug absorption, distribution, and excretion in tissues where other ABC transporters with overlapping function may be present. The first in vivo evidence for another transporter active along with P-glycoprotein was the observation that the oral bioavailability of topotecan, a shared P-glycoprotein and Bcrp1 substrate, was significantly increased when the Bcrp1 and P-glycoprotein inhibitor GF120916 was coadministered with topotecan to *Mdr1a/1b*$^{-/-}$ mice (105). Since these early results, many studies in Bcrp1$^{-/-}$ mice have been conducted in order to better elucidate the role of BCRP in drug penetration of the CNS and in oral bioavailability (106).

Comparison of single ABC transporter gene knockout mice with multiple ABC transporter gene knockout mice may be useful in understanding the overlapping functions of BCRP and P-glycoprotein with members of the MRPs, as demonstrated by studies of methotrexate pharmacokinetics in double and triple knockout animals. For example, plasma concentration of the toxic metabolite 7-hydroxymethotrexate was not significantly different in Bcrp1$^{-/-}$ mice, but 6.2-fold increased in Mrp2$^{-/-}$ mice, and 12.4-fold increased in Mrp2;Bcrp1$^{-/-}$ mice compared with wild-type animals. These results

indicate that both Mrp2 and Bcrp1 are important determinants of methotrexate distribution but that Mrp2 is better able than Bcrp1 to compensate for the loss of the other transporter (107). Triple knockout Mrp2;Mrp3;Bcrp1$^{-/-}$ mice retained 67% of an intravenous dose of methotrexate in their livers 1 h after administration compared with wild-type mice that had only 7% of the dose remaining. These results highlight the overlapping functional roles of Mrp2, Mrp3, and Bcrp1 in biliary excretion of toxic metabolites (108).

BCRP is expressed in lactating mammary glands and has a demonstrated role in active efflux of xenobiotics into milk. Levels of topotecan, the H_2 blocker cimetidine, and the antibiotic nitrofurantoin, as well as the dietary carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), were significantly lower in milk from Bcrp1$^{-/-}$ mice than from wild-type mice (109-110). The involvement of BCRP in the secretion of toxic compounds into breast milk is counterintuitive for a transporter that otherwise plays a protective role, and avoidance of BCRP substrates may be a consideration for nursing women. BCRP was demonstrated to concentrate riboflavin (vitamin B2) into breast milk, an observation that may provide some insight into its physiological function in the mammary gland (111).

Reduced-function polymorphisms in ABCG2 have been identified, and from the known function and location of BCRP, they would be expected to increase the bioavailability of BCRP substrates, owing to reduced efflux from enterocytes and reduced biliary excretion. Exposure to sulfasalazine was significantly increased in healthy volunteers

with one or more *ABCG2* variants following oral administration of the drug (112-113). These findings were consistent with increased oral bioavailability and reduced excretion of sulfasalazine in Bcrp1$^{-/-}$ mice (114). These results raised the possibility of using sulfasalazine as an in vivo probe of BCRP activity, an especially attractive tool given that expression of both mRNA and protein is highly variable in human intestinal samples and that this variation is independent of common genetic variants (112; 115). However, a recent pharmacokinetic study of sulfasalazine in 36 healthy volunteers failed to reproduce these results, because the presence of the *ABCG2* c.421C>A (rs2231142) polymorphism or coadministration of the BCRP inhibitor pantoprozole showed no effect on sulfasalazine plasma exposure or maximum plasma concentrations (116). Thus, more work is needed to validate the utility of sulfasalazine as an in vivo probe of BCRP activity.

The total exposure to atorvastatin and rosuvastatin is higher in individuals with the *ABCG2* c.421T>C polymorphism (74; 117), consistent with reduced biliary excretion of rosuvastatin in Bcrp1$^{-/-}$ mice (118) (Table 2.1). Conversely, pitavastatin pharmacokinetics were not influenced by the *ABCG2* c.421C>A polymorphism in healthy volunteers (63), despite the involvement of Bcrp1 in biliary excretion of pitavastatin in mice (119). Another study linked *ABCG2* polymorphism to the pharmacokinetics of fluvastatin and simvastatin lactone, but not to the pharmacokinetics of pravastatin or simvastatin acid (120). BCRP appears to be particularly important for the distribution of rosuvastatin, as multiple studies have now associated reduced-function *ABCG2* polymorphisms with increased lipid-lowering response to rosuvastatin therapy in patients (75-77), presumably

a result of increased exposure to rosuvastatin, which mimics the effect of increasing the statin dose.

The role of BCRP in cancer treatment efficacy and prognosis has been widely studied owing to the vast number of antineoplastic drugs that are substrates for this efflux transporter. Indeed, *ABCG2* polymorphisms have been associated with increased exposure and/or risk for toxicity for numerous anticancer drugs in clinical use. For example, reduced function BCRP variants were associated with higher area under the curve and maximum concentration values of the tyrosine kinase inhibitor erlotinib; higher trough erlotinib levels were associated with skin rash (121). Expression of BCRP in cancer cells is generally associated with poor prognosis; however, this association has not been demonstrated for all tumor types. In particular, BCRP expression has been linked to poor prognosis in acute myeloid leukemia in adults and children (122-123) and to poor prognosis in esophageal squamous cell carcinoma (ESCC) (124). Whether the association of BCRP with reduced survival is a result of increased BCRP-mediated efflux of anticancer drugs or a marker of more complex biology is not fully understood. BCRP is expressed in stem cells, and indeed, some discrepancy in findings may be related to the relative composition of the subtypes of cells in the tissue samples obtained. For a more comprehensive review of the role of BCRP in anticancer drug efficacy, toxicity, and overall prognosis, refer to recent comprehensive reviews (125-126).

2.3.3 Multidrug resistance-associated proteins

Of the multidrug resistance-associated protein (MRP) family of ABC transporters, MRP1 (*ABCC1*), MRP2 (*ABCC2*), and MRP4 (*ABCC4*) have been most widely studied in the context of drug response and toxicity. In some cancers, their expression may be associated with poor overall prognosis or response to therapy (85).

2.3.3.1 Multidrug resistance-associated protein 1 in efficacy and toxicity

MRP1 is expressed in tissues throughout the body, including the lung, testis, kidney, cardiac and skeletal muscle, and placenta. As described above, overexpression of MRP1 in cancer cells is associated with multidrug resistance (127). Like P-glycoprotein, MRP1 is capable of transporting structurally diverse compounds. Endogenous substrates of MRP1 include oxidized glutathione, cysteinyl leukotrienes, glucuronide and sulfate conjugates, and drug substrates including anthracyclines, vinca alkaloids, and antivirals. Mice lacking Mrp1 demonstrate increased sensitivity to the topoisomerase II inhibitor etoposide (128-129). Functional *ABCC1* polymorphisms have been described (130), but to date, *ABCC1* variants have not been associated with striking changes in drug response.

2.3.3.2 Multidrug resistance-associated protein 2 in efficacy and toxicity

MRP2 is expressed on the canalicular membrane of the hepatocyte and on the apical membrane of proximal renal tubule endothelial cells (131). MRP2 transports a wide range of glutathione, sulfate, and glucuronide-conjugated endo- and xenobiotics. Genetic mutations in MRP2 cause Dubin-Johnson syndrome, a disease characterized by hyperbilirubinemia resulting from reduced transport of conjugated bilirubin into bile (132). Polymorphisms in *ABCC2* have been associated with higher plasma concentrations of some MRP2 substrates (133). The gastrointestinal toxicity associated with the use of some drugs, such as NSAIDs and antibiotics, may result from enterohepatic recirculation of these compounds and their metabolites that is driven, in large part, by MRP2 in the bile canaliculi (134).

2.3.3.3 Multidrug resistance-associated protein 4 in efficacy and toxicity

MRP4 is located on the basolateral membrane of hepatocytes and choroid plexus epithelium, and the apical membrane of renal proximal tubule cells and brain capillary endothelium (135). Localization of MRP4 to the basolateral or apical membrane, depending on the polarized cell type, is associated with the expression of the adaptor protein NHERF1 (136). Substrates of MRP4 include numerous endogenous compounds involved in cellular signaling, such as cyclic nucleotides, eicosanoids, urate, and conjugated steroids, as well as folate, bile acids, and glutathione. Drug substrates of

MRP4 include cephalosporin antibiotics, nucleotide analog reverse transcriptase inhibitors, and cytotoxic agents such as methotrexate and 6-mercaptopurine (135).

A SNP in *ABCC4* (c.G2269A, rs3765534) caused disrupted membrane localization and reduced MRP4 activity, and it was hypothesized to increase sensitivity to thiopurine-induced myelosuppression as a result of thiopurine metabolite accumulation in hematopoietic cells (137). Polymorphisms in *ABCC4* were reported to be associated with side effects and survival in childhood acute lymphoblastic leukemia patients treated with methotrexate (138); however, the same genotypes did not show any influence on the event-free survival in adult acute lymphoblastic leukemia patients receiving methotrexate (139).

2.4 Conclusions and future perspectives

The past decade has seen remarkable progress in the field of drug transporters, not only in terms of functional characterization and substrate specificity but also in elucidating the important role that transporters play in the disposition and efficacy of drugs in clinical use. Drug interactions that target uptake or efflux transporters can often result in unexpected systemic exposure and, in some cases, organ specific toxicity. Interestingly, the same processes that can result in higher tissue drug accumulation can also be utilized to produce a desirable therapeutic effect, as exemplified by the statin class of lipid-lowering drugs that utilize liver-specific uptake transporters to target hepatic HMG-CoA reductase. The next decade holds even greater promise of new discoveries relating to

drug transporters. Indeed, as we approach the personal genomics era, the field of drug transporter pharmacogenomics will no doubt prove to be integral to the delivery of personalized medicine. In addition, the systematic inclusion of drug transporter studies in the drug discovery and development process will result in drugs with greater efficacy and reduced side effects.

Finally, the efforts of dedicated drug transporter researchers over the past half century have resulted in a paradigm shift in our understanding of how drugs are handled by the body. What was once thought to be predictable, on the basis of simple physicochemical properties, has given way to our current recognition of the important role that drug transporters play in all aspects of drug absorption, tissue distribution, and elimination. Indeed, drug transporter research has matured and proven to be remarkably significant to human health and optimal therapeutics.

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3 INTRODUCTION TO STATIN PHARMACOLOGY

3.1 Therapeutic Indication

Cardiovascular disease is the leading cause of death in Canada. The burden of the disease to our society is tremendous: in addition to claiming 30% of lives, cardiovascular disease costs an estimated 22 billion dollars annually in direct and indirect health care expenses and lost productivity (1). Elevated low-density lipoprotein cholesterol (LDL-C) is a major risk factor in the development of cardiovascular disease. For many individuals, LDL-C is a modifiable risk factor that can be reduced by a combination of lifestyle modifications and drug therapy. One class of drugs of considerable benefit is the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins. Meta-analysis of statin trials indicates that a reduction of 1 mmol/L in LDL-C correlates with a 20 to 25% reduction in risk for a major cardiovascular event in patients at high risk for cardiovascular disease (2). In Canada, current guidelines for the treatment of dyslipidemia recommend a target LDL-C of less than 2 mmol/L, or greater than 50% reduction of pre-treatment LDL-C. For most patients, this goal is achievable by statin monotherapy (1).

3.2 Mechanism of Action

Statins exert their pharmacological effect by inhibiting HMG-CoA reductase, an enzyme that catalyzes the rate-limiting step of hepatic cholesterol synthesis, which is the production of mevalonate (3). Reduced cholesterol synthesis results in the up-regulation of the LDL-receptor at the surface of the hepatocyte and consequently, a reduction in plasma LDL-C. A schematic of the statin inhibition pathway is presented in Figure 3.1.

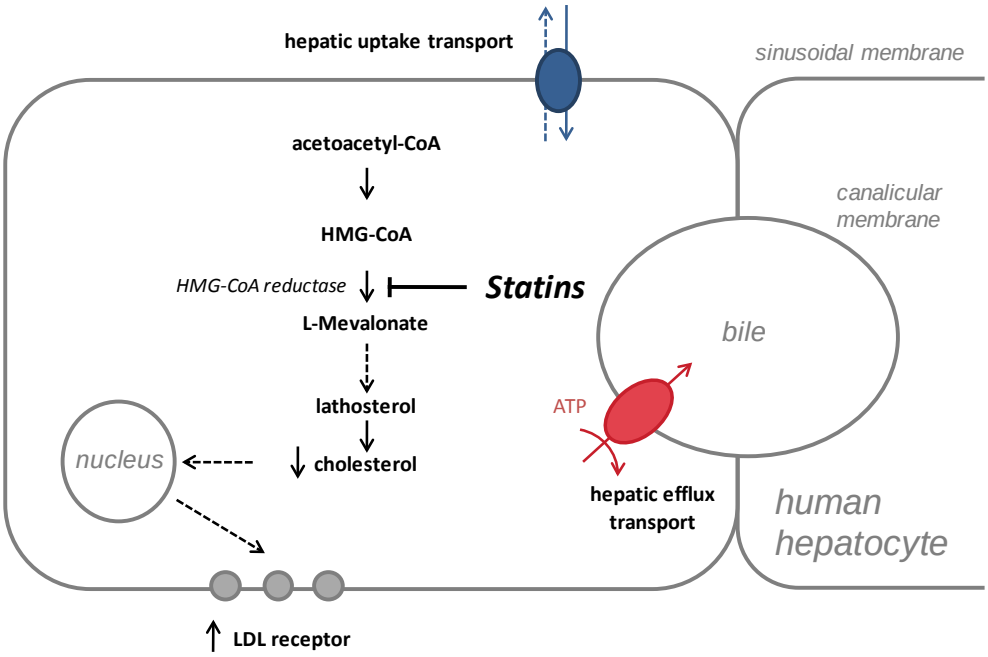

Figure 3.1 Hepatic cholesterol synthesis pathway and mechanism of statin-mediated reduction in LDL-C.

Statins inhibit HMG-CoA reductase, the rate-limiting step in cholesterol synthesis. Reduced hepatic cholesterol results in the upregulation of the LDL receptor at the membrane of the hepatocyte. Increased hepatic uptake reduces plasma LDL-C.

The statin pharmacophore, which competitively binds to the active site of HMG-CoA reductase, is similar to HMG-CoA; it is the open, acid form of this ring structure that is active. The newer synthetic statins, such as atorvastatin and rosuvastatin, are administered in the acid form, whereas older statins like simvastatin are administered in the closed, lactone form. Interconversion between the two forms occurs in vivo; this may be an enzyme mediated process or occur spontaneously (4). The pharmacological properties of selected statins are summarized in Table 3.1.

Clinically, statin response is measured by reduction in LDL-C. The plasma concentration of lathosterol, a late intermediate in the endogenous cholesterol synthesis pathway, can be used to indicate the rate of endogenous cholesterol synthesis, and thus, the efficacy of statin treatment (5).

3.3 Statin Pharmacokinetics

Statins are typically administered daily by an oral dose, and are sometimes used in combination with other lipid-modifying therapies such as ezetimibe, fibrates or niacin, depending on the clinical need to achieve target lipid concentration. Statins are metabolized by members of the cytochrome P450 (CYP) enzymes, and undergo mainly biliary excretion. The oral bioavailability of statins is limited by extensive first-pass effect, owing to metabolism in the gut and high efficiency of portal extraction by the liver (4; 6). The pharmacokinetic properties of selected statins are summarized in Table 3.2.

Table 3.1 Pharmacological properties of statins

statin	**form**	**dose**	**IC_{50} [nM]**	**LogD (pH 7.0)**
atorvastatin	acid	10-80 mg/day	0.82	1.53
fluvastatin	acid	20-80 mg/day	4.8	1.75
lovastatin	lactone	10-80 mg/day	4.7	3.91 (acid -0.51)
pravastatin	acid	10-80 mg/day	5.0	-0.47
rosuvastatin	acid	5-40 mg/day	0.30	-0.25 to -0.50*
simvastatin	lactone	5-80 mg/day	5.2	4.4 (acid: 1.88)

IC_{50}, half-maximal inhibitory concentration of HMG-CoA reductase activity in primary cultured rat hepatocytes; Log*D*, distribution coefficient; *pH 7.4. References: (4; 6)

Table 3.2 Pharmacokinetic properties of statins

statin	t_{max} (h)	C_{max} (ng/mL)	$t_{1/2}$ (h)	bioavail-ability (%)	metabolism	transporter substrate?
Atorvastatin (acid)	1-2	13-67 (40 mg)	8-21	12	CYP3A4	yes
fluvastatin	0.5-1	448 (40 mg)	0.5-2.3	19-29	CYP2C9	yes
Lovastatin (lactone)	4	3 (40 mg)	2.5	5	CYP3A4	yes
Pravastatin (acid)	1	45-66 (40 mg)	2	18	Not by CYP enzymes	yes
Rosuvastatin (acid)	5	19 (40 mg)	17-20	20	CYP2C9 & CYP2C19 (both minor)	yes
Simvastatin (lactone)	1-4	6.9 (40 mg)	3	<5	CYP3A4	yes

C_{max}, maximum concentration, t_{max}, time of maximum concentration $t_{1/2}$, elimination half-life. References: (4; 6)

The amphipathic chemical structure of statins limits their passage through biological membranes by simple diffusion, and thus statins rely on active transport processes to enter cells. Consequently, the balance of expression and activity of uptake and efflux transporters in a particular tissue will determine the selectivity of a statin for that tissue.

Given their site of action in the liver, statins require hepatic uptake in order to exert their effect. Statin rely on uptake transporters present on the basolateral membrane of the liver, including organic anion-transporting polypeptides (OATPs) OATP1B1 (*SLCO1B1)*, OATP1B3 (*SLCO1B3*), and OATP2B1 (*SLCO2B1*), and the sodium-taurocholate co-transporting polypeptide (NTCP/*SLC10A1*). Statins are transported into bile by ATP-binding cassette (ABC) efflux transporters on the canalicular membrane of the hepatocyte, including P-glycoprotein (MDR1/*ABCB1*), breast cancer resistance protein (BCRP/*ABCG2*), and multidrug resistance-associated protein 2 (MRP2/*ABCC2*). Statin transporters in liver are presented in Figure 3.2.

Numerous drug-drug interactions mediated by transporters and enzymes involved in statin distribution and metabolism have been reported. In particular, fibrates are thought to increase statin exposure by inhibiting the OATP family of transporters (7). There are other clinical factors that may influence statin pharmacokinetics. For example, decreased hepatic function results in reduced statin clearance, and increased risk for statin-induced toxicity. Age and gender have been demonstrated to affect the pharmacokinetics of some, but not all, of the statins. Finally, ethnicity can influence statin pharmacokinetics.

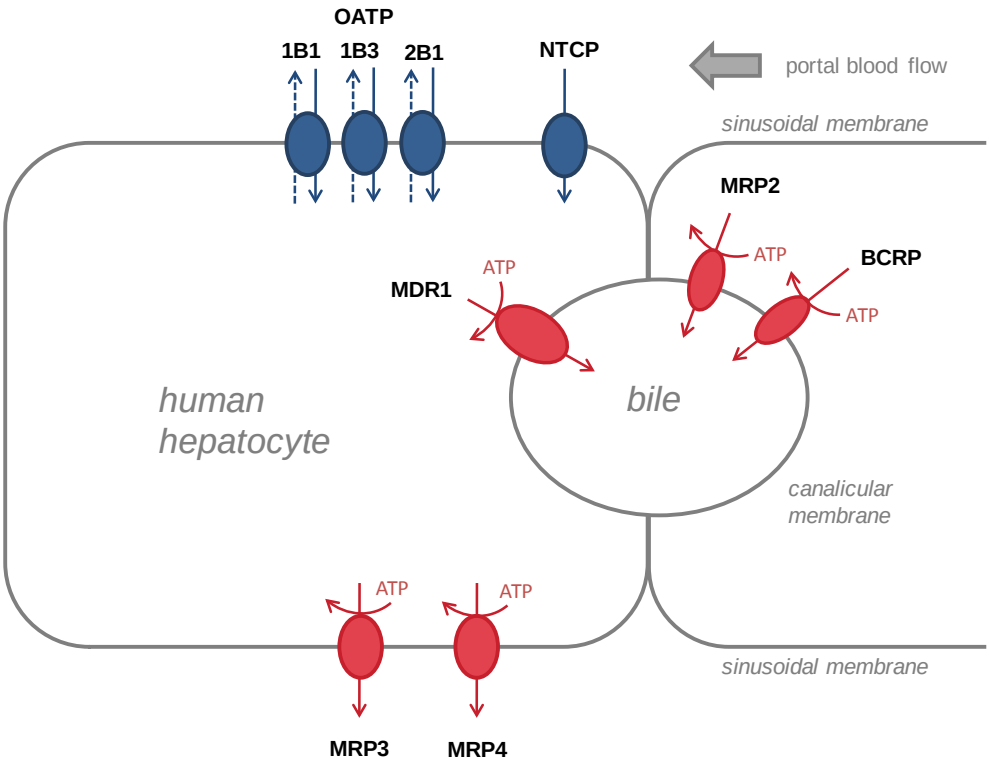

Figure 3.2 Statin transporters expressed in the human hepatocyte

BCRP, breast cancer resistance protein; MDR, multidrug resistance; MRP, multidrug resistance-associated protein; NTCP, Na+-dependent taurocholate co-transporting polypeptide; OATP, organic anion-transporting polypeptide

Statin clearance is lower in Asians than Caucasians (8), and as a result, statins are approved for half the dose in Asian countries compared with North America and Europe. The mechanism for this effect remains unclear.

The pharmacokinetics of the most commonly prescribed statins in Canada, atorvastatin, rosuvastatin, and simvastatin, are described in more detail below.

3.3.1 Atorvastatin pharmacokinetics

Atorvastatin is metabolized by CYP3A4 and CYP3A5, into two major hydroxylated metabolites, ortho-hydroxy atorvastatin and para-hydroxy atorvastatin. Both metabolites are active inhibitors of HMG-CoA reductase (9). Atorvastatin is a relatively hydrophilic statin, and is transported by OATPs and ABC transporters. The renal elimination of atorvastatin is less than 1% (9). Early pharmacokinetic studies demonstrated that the mean area under the curve (AUC) of atorvastatin was approximately 23% higher in elderly patients compared with young adults; it was approximately 11% lower in women than men (10).

3.3.2 Rosuvastatin pharmacokinetics

Rosuvastatin does not undergo extensive metabolism, and is excreted 70% unchanged (8). It is the most hydrophilic of the statins currently in use, and thus it relies on transport to traverse biological membranes. Rosuvastatin is also the most potent of the statins. The

renal elimination of rosuvastatin is 10 - 30% (6; 8). There appeared to be no significant effect of age or gender on the pharmacokinetics of rosuvastatin (11).

3.3.3 Simvastatin pharmacokinetics

Like atorvastatin, simvastatin is metabolized by CYP3A4 and CYP3A5 (12). Simvastatin is administered in its lactone form, and is converted into its active acid form in vivo. Simvastatin acid inhibits OATP1B1 transport in vitro, and in healthy individuals, *SLCO1B1* polymorphisms appear to influence simvastatin pharmacokinetics more than any other statin tested (13). Exposure to simvastatin was higher in elderly individuals and in women compared with men (14).

3.4 Potential for Adverse Side Effects

The major challenge to statin therapy is the considerable risk for adverse side effects, most often muscle pain or weakness, presenting with or without creatine kinase elevation. Up to 10% of individuals will experience these side effects at some point during therapy, requiring dose adjustment, switching to another compound in the statin class, or eliminating the possibility of statin use altogether (15). In addition there is the risk, in less than 1% of individuals, of developing serious adverse reactions, including a life-threatening form of muscle damage, rhabdomyolysis (16). This is a significant concern given that over 3 million Canadians take statins to lower LDL-C.

The mechanisms by which statins produce muscle-related side effects are unclear. Statin transporters expressed in human skeletal muscle include OATP2B1 (*SLCO2B1*), MRP1 (*ABCC1*), MRP4 (*ABCC4*), and MRP5 (*ABCC5*) (17). Muscle-related adverse effects are often associated with increased statin dose and higher systemic statin exposure (18); however, there is considerable interindividual variation in the pharmacokinetic and pharmacodynamic profile of statins, making it very challenging to predict which individuals will suffer from these unintended effects.

3.5 Statin Pharmacogenetics

As described in Chapter Two, polymorphisms in statin transporters have been associated with altered statin pharmacokinetics. Some of these polymorphisms have also been associated with clinical outcomes. In particular, the *SLCO1B1* polymorphism c.521T>C has been associated with simvastatin-induced muscle toxicity (18). The reduced function *ABCG2* variant c.421C>A has been associated with increased lipid-lowering effect from rosuvastatin therapy (19-21). Single nucleotide polymorphisms in other genes related to cholesterol and lipoprotein homeostasis have also been associated with variability in statin response, however, the proportion of the pharmacodynamic variation attributed to these polymorphisms remains small (22-24).

3.6 The Future of Statin Use

In recent years, considerable attention has been given to the pleiotropic effects of statins, which include improved endothelial function and plaque stability, and reduced vascular inflammation (25). The large and highly publicized trial, Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER), published in 2008, examined the role of the inflammatory marker C-reactive protein (CRP) and cardiovascular risk (26). The JUPITER trial provided new evidence to suggest statins may prevent cardiovascular events in individuals with elevated CRP but normal cholesterol levels. These results, if confirmed, will significantly increase the number of individuals for whom statins are indicated. The growth in statin users expected in the coming years, resulting from such expanded indications combined with an aging population, means that understanding statin pharmacokinetics, pharmacodynamics, and transporter involvement will continue to be an important endeavour.

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4 SPECIFIC AIMS AND HYPOTHESES

4.1 Specific Aim 1

To identify the molecular determinants of CCK-8 transport by OATP1B transporters.

The unique localization of the organic anion-transporting polypeptide (OATP/*SLCO*) 1B subfamily of transporters to the basolateral membrane of hepatocytes makes these transporters desirable targets for drugs requiring hepatic entry for elimination or to exert their effect. An understanding of the structural determinants of OATP function may prove to be useful to the drug design process, and to predict the effect of novel polymorphisms. OATP1B1 and OATP1B3 share nearly 80% sequence identity, and transport many of the same drugs and endogenous substrates; however, there are notable exceptions, such as cholecystokinin-8 (CCK-8), a gastrointestinal peptide hormone that is transported by OATP1B3 but not by OATP1B1. The molecular determinants of CCK-8 transport by OATP1B transporters are not well understood.

We hypothesized that mutation of key divergent amino acid residues in OATP1B1 to the corresponding sequence of OATP1B3 would confer CCK-8 transport to OATP1B1. To generate this hypothesis, we created a library of OATP1B1 and OATP1B3 chimeras and characterized the transport of CCK-8 by these chimeras to identify three regions of OATP1B3 involved in CCK-8 transport. We utilized site-directed mutagenesis to alter individual amino acids in OATP1B1 to the corresponding sequence in OATP1B3, and identified the amino acids with the greatest potential to confer CCK-8 transport. As described in Chapter Five, we showed that three distinct

regions of OATP1B3 are involved in CCK-8 transport. Three mutations to OATP1B1 (A45G, L545S, T615I), one in each of the three regions, were required to confer CCK-8 transport activity to OATP1B1.

4.2 Specific Aim 2

To investigate the contribution of Oatp1b2 to the hepatic uptake of atorvastatin, rosuvastatin, and simvastatin in Oatp1b2$^{-/-}$ (*Slco1b2*$^{-/-}$) mice.

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, reduce low-density lipoprotein cholesterol (LDL-C) by inhibiting the rate-limiting step of cholesterol synthesis. To exert this effect, statins require transport-facilitated entry into the hepatocyte, the primary site of cholesterol synthesis. Oatp1b2 is the murine ortholog of the human transporters OATP1B1 and OATP1B3; the Oatp1b2$^{-/-}$ mouse thus provides an in vivo model of the effect of loss-of-function OATP1B1 (*SLCO1B1*) or OATP1B3 (*SLCO1B3*) polymorphisms. More background on the strengths and limitations of using solute carrier knockout mice to assess in vivo solute carrier function can be found in Appendix C. The effect of Oatp1b2 deletion on the pharmacokinetics of some statins has been previously described, however, the commonly prescribed statins, atorvastatin and rosuvastatin, have not been reported.

We hypothesized that Oatp1b2 is involved in the hepatic uptake of atorvastatin, rosuvastatin, and simvastatin. To test this hypothesis, we characterized the liver and plasma concentrations of atorvastatin, rosuvastatin, and simvastatin in Oatp1b2$^{-/-}$ mice after a tail vein injection of each compound. As described in Chapter Six, the liver-to-plasma ratios of atorvastatin and rosuvastatin but not simvastatin were significantly lower in Oatp1b2$^{-/-}$ mice compared with wild-type animals. The results indicate that Oatp1b2 is critical for the hepatic uptake of atorvastatin and rosuvastatin.

4.3 Specific Aim 3

To characterize the intraindividual variability in pharmacokinetics of atorvastatin, rosuvastatin, and simvastatin in healthy volunteers.

Statin use is associated with risk of muscle pain or injury, an adverse effect that is associated with increased systemic statin exposure. Statin switching can be an effective strategy to avoid toxicity and is often used in clinical practice (1); however, the intraindividual variability in statin pharmacokinetics has not been well described.

We hypothesized that the relative exposure to atorvastatin, rosuvastatin, and simvastatin in the same individual would be different. That is, those individuals with the highest areas under the curve (AUCs) for one statin would not be the same individuals with the highest AUCs for another statin. To test this hypothesis, we

administered each of the three drugs to eleven healthy subjects, and calculated the AUC of each statin over ten hours. As described in Chapter Six, the AUCs of atorvastatin and simvastatin were correlated in this group, but the AUC of rosuvastatin was not predictive of atorvastatin or simvastatin exposure.

4.4 Specific Aim 4

To characterize the contribution of statin transporter polymorphisms to the interindividual variability of statin pharmacokinetics in patients.

The *SLCO1B1* polymorphism c.521T>C has been identified as the single best predictor of simvastatin-induced muscle toxicity in a genome-wide association study (2); the mechanism of this effect is thought to be related to increased systemic exposure resulting from reduced hepatic uptake. In addition, a polymorphism in breast cancer resistance protein (BCRP/*ABCG2*), c.421C>A, has been associated with improved response to statin therapy. This is postulated to result from increased systemic exposure resulting from reduced statin efflux from the enterocyte into the lumen and from the hepatocyte into the bile. Despite numerous large statin trials performed to date, to our knowledge, these studies have not measured statin concentration.

We hypothesized that genetic variation in drug metabolizing enzymes and transporters contributes to interindividual variability in statin pharmacokinetics in

patients. To test this hypothesis, we measured statin concentration in patients undergoing routine clinical care at London Health Sciences Center, and genotyped these patients for single nucleotide polymorphisms (SNPs) in genes involved in statin pharmacokinetics. As described in Chapter Seven, we observed up to 45-fold interindividual variability in circulating plasma statin concentration, among patients on the same dose. Rosuvastatin concentration was associated with *ABCG2* and *SLCO1B1* polymorphisms, while only *SLCO1B1* polymorphisms were associated with atorvastatin level. Atorvastatin concentration was also associated with 4β-hydroxycholesterol concentration, a marker of CYP3A activity.

4.5 Specific Aim 5

To characterize the association of lathosterol concentration with statin concentration and transporter polymorphisms in patients on statin therapy.

Given the importance of the liver to the excretion of statins, higher plasma level may reflect reduced hepatic uptake. Thus patients with reduced *SLCO1B1* function may be expected to have reduced LDL-C lowering response, despite higher statin level. Lathosterol is a late intermediate of cholesterol synthesis that may be used as a specific measure of the extent of HMG-CoA reductase inhibition.

We hypothesized that lathosterol concentration would be correlated with statin concentration and transporter polymorphisms in patients on statin therapy. To test this hypothesis, we measured lathosterol and total cholesterol concentration in our patient population to assess HMG-CoA reductase inhibition. As described in Chapter Seven, lathosterol concentration was not associated with statin concentration or *SLCO1B1* polymorphism. Instead, lathosterol concentration was associated with total cholesterol and ezetimibe use.

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5 INTERACTION OF THREE REGIO-SPECIFIC AMINO ACID RESIDUES IS REQUIRED FOR OATP1B1 GAIN OF OATP1B3 SUBSTRATE SPECIFICITY[3]

[3] Reprinted with permission from: DeGorter MK, Ho RH, Leake BF, Tirona RG, Kim RB. 2012. Interaction of Three Regiospecific Amino Acid Residues Is Required for OATP1B1 Gain of OATP1B3 Substrate Specificity. *Mol Pharm* 9:986-95. Copyright 2012 American Chemical Society.

5.1 Introduction

The organic anion-transporting polypeptides (OATPs; gene symbol solute carrier family *SLCO*) form a superfamily of transmembrane proteins involved in the transport of a variety of amphipathic substrates across the plasma membrane in a sodium-independent manner. To date, over 80 members of the OATP superfamily in 13 different species have been identified by the presence of the OATP superfamily signature D-X-RW-(I,V)-GAWW-X-G-(F,L)-L. The two members of the human subfamily OATP1B, OATP1B1 (previously known as OATP-C, liver-specific transporter 1 (LST-1), or OATP2; gene symbol *SLCO1B1*, previously *SLC21A6*) and OATP1B3 (previously known as LST-2, or OATP8; gene symbol *SLCO1B3*, previously *SLC21A8*), share 80% sequence identity (1). Their expression is predominantly observed on the basolateral membrane of hepatocytes, where they mediate the hepatic uptake of substrates from the portal blood (2-5). Not surprisingly, OATP1B1 and OATP1B3 share a broad substrate specificity, and are capable of transporting bile salts, steroid conjugates, the thyroid hormones T3 and T4, eicosanoids, cyclic peptides, bromosulfophthalein, the natural toxins phalloidin and microcystin-LR as well as numerous drugs, such as methotrexate, rifampin, and many of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statin) family of compounds (1; 6-8).

Reports of mice with deletion of Oatp1b2 (*Slco1b2)*, the closest murine ortholog to OATP1B1 and OATP1B3, described altered pharmacokinetic profiles of prototypical OATP1B1 substrates pravastatin and rifampin (9), as well as protection from hepatotoxicity induced by phalloidin and microcystin-LR (10). The clinical relevance of

OATP1B1 to hepatic elimination is also evidenced by the profound effect of single nucleotide polymorphisms (SNPs) on the observed pharmacokinetic profile of drug substrates (7). Remarkably, a previously identified SNP in *SLCO1B1* has been shown to be the single most important predictor of statin-induced muscle myopathy, a relatively rare but potentially fatal side effect of statin therapy (11-12).

Despite their remarkable sequence similarity and overlapping substrate specificity, there are some notable differences in the compounds transported by OATP1B1 and OATP1B3. For example, OATP1B3 transports the gastrointestinal peptide hormone cholecystokinin-8 (CCK-8), which is not a substrate of OATP1B1 (1; 13-14). Conversely, OATP1B1 transports the steroid conjugate estrone sulfate while OATP1B3 does not show appreciable transport activity. Accordingly, the wide and overlapping but not identical substrate specificity of OATP1B1 and OATP1B3, combined with their significant sequence homology, suggests that there may be key sequence differences that confer isoform-specific divergence in substrate specificity.

Previously, transmembrane (TM) helices eight and nine were identified as important for estrone sulfate and estradiol-17β-glucuronide transport by OATP1B1 (15), and the mutation of four residues in TM10, Leu^{545}, Phe^{546}, Leu^{550} and Ser^{554}, resulted in complete loss of estrone sulfate transport (16). Conserved, positively charged amino acids in other areas of OATP1B1 also appear to be important for estrone sulfate and estradiol-17β-glucuronide transport (17). With respect to OATP1B3, previous studies have indicated a role for TM10 in mediating CCK-8 transport (18). Similar to the case of OATP1B1,

conserved, positively charged amino acids in OATP1B3 appear to be important for transport of sulphobromophthalein (BSP), pravastatin, and taurocholate (19-20).

Given the importance of OATP1B1 in hepatic drug uptake, the molecular basis for substrate specificity needs to be defined to more fully understand the in vivo distribution of its substrates, and to aid in the rational design of drugs targeting the liver as their site of action. In the present work, we employed a strategy of random chimeragenesis to obtain insight to specific regions involved in CCK-8 transport. Our results indicate that amino acid residues in three distinct regions of the transporter are required to enable CCK-8 transport by OATP1B3. Importantly, we were able to confer CCK-8 transport by OATP1B1 through targeted mutagenesis of amino acids in the regions noted to be important for CCK-8 transport by OATP1B3.

5.2 Experimental Section

5.2.1 Materials

[^{3}H]-CCK-8(L-aspartyl-L-tryosyl-L-methionylgylcyl-L-tryptophyl-L-methionyl-L-aspartyl-L-phenylalaninamide hydrogen sulfate ester; 93 Ci/mmol, >97% purity) was purchased from GE Healthcare (Buckinghamshire, UK), [^{3}H]-estrone sulfate (57.3 Ci/mmol, >97% purity) from PerkinElmer (Boston, MA), and [^{3}H]-atorvastatin (5 Ci/mmol, >97% purity) from American Radiolabeled Chemicals (St Louis, MO). Unlabeled estrone sulfate was from Sigma-Aldrich (St. Louis, MO), atorvastatin was

from Toronto Research Chemicals (North York, Canada), and cholecystokinin-8 was from Bachem Bioscience (King of Prussia, PA).

5.2.2 OATP1B chimera plasmid construct

The master plasmids for chimeragenesis were created by inserting the coding sequence of OATP1B1 into a previously described pEF6/V5-His TOPO plasmid containing OATP1B3 (21). Two master plasmids with the transporters in a tandem head-to-tail arrangement were created: OATP1B1-1B3 and OATP1B3-1B1. OATP1B1 was released from pEF6 (12) by PCR using the Phusion High Fidelity PCR kit (New England Biolabs, Ipswich, MA), with primers that introduced restriction enzyme sites to allow insertion of OATP1B1 into the multiple cloning regions of pEF6-OATP1B3. For OATP1B1-1B3, OATP1B1 was released using the forward primer 5'-ggatccacta gtccagtgtg gtggaattgc ccttgatatc tatatttcaa-3' and the reverse primer 5'-tctagac**a**c**t** ag**t**ggccg**tt** a**ac**gtgctg**c** atat**g**tgcag aattgccctt ttaacaatgt-3', with nucleotides mutated to add *Hpa*I, *Nde*I and *Spe*I restriction sites in bold. The resulting fragment was ligated into pEF-OATP1B3 using *Spe*I and the orientation of the fragment was confirmed by restriction digest (Figure 5.1A). For OATP1B3-1B1, the forward primer 5' – gtccagtg**c**g g**cc**g**c**attgc c**a**tt**t**a**a**atc tatatttcaa ccatggacca – 3' to add *Not*I and *Swa*I sites and reverse primer 5' – gccactgt gctggatatc t**ct**agaattg ccctttaac aatgtgt – 3' to add *Xba*I sites were used. The resulting fragment was ligated into pEF-OATP1B3 using *Not*I and *Xba*I (Figure 5.1B). The resulting master plasmids OATP1B1-1B3 and OATP1B3-1B1 were linearized by *Hpa*I

Figure 5.1 Cloning strategy for OATP1B chimeragenesis. (A) The expression plasmid pEF-OATP1B1-1B3 was created by ligating cDNA coding for OATP1B1 into pEF6-OATP1B3 at the *Spe*I restriction site. The unique restriction sites *Nde*I and *Hpa*I allow for linearization of the plasmid prior to transformation and homologous recombination in *E. coli*. (B) The expression plasmid pEF-OATP1B3-1B1 was created by ligating OATP1B1 into pEF6-OATP1B3 between *Not*I and *Xba*I restriction sites in the multiple cloning region. The unique restriction sites *Not*I and *Swa*I allow for linearization of the plasmid prior to transformation and homologous recombination in *E. coli*.

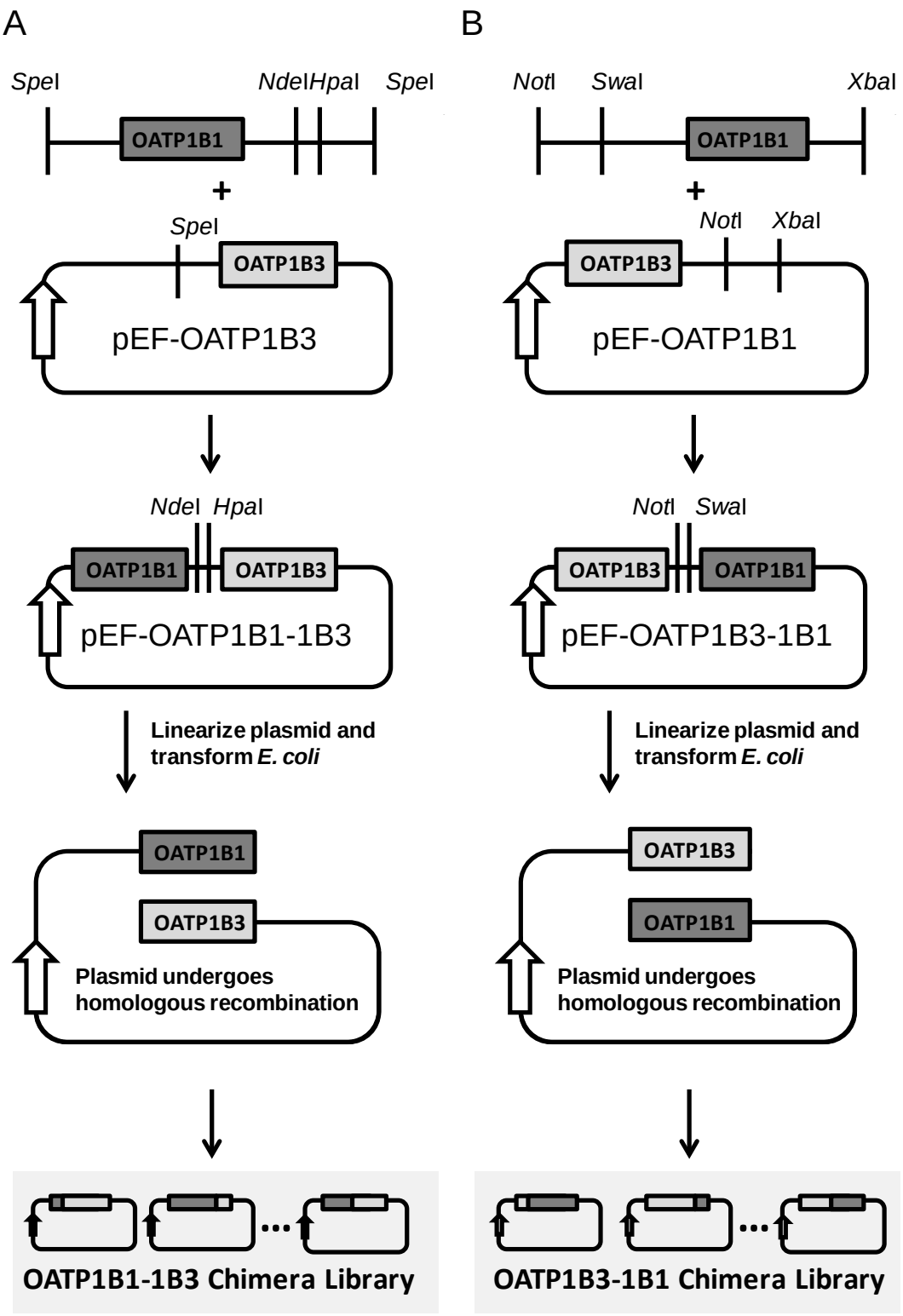


Figure 5.1 Cloning strategy for OATP1B chimeragenesis

and *Nde*I, and *Not*I and *Swa*I, respectively, and inserted into TOP10 *Escherichia coli* (Invitrogen, Carlsbad, CA). Restriction fragments resulting from digesting the ensuing plasmids with *Spe*I and *Xba*I were used to select those plasmids containing a monomeric OATP1B sequence. OATP1B1-specific restriction enzymes were used to estimate the approximate location of the junction between OATP1B1 and OATP1B3; sequencing determined the exact location of the junction.

5.2.3 Site-directed mutagenesis

Single, double and triple point mutations were introduced into the coding sequence of pEF6-OATP1B1 and pEF6-OATP1B3 using the QuikChange Multi Site-directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. The primers used are summarized in Table 1 of Supplementary Information I. The presence of all mutations was confirmed by sequencing.

5.2.4 Transient transfection and transport assay

HeLa cells were plated in 12-well plates at 2.5 x 10^5 cells/well, to be transfected the next day. Transporters were expressed using a transient heterologous expression system as previously described (12). Briefly, 750 ng of cDNA was added per well as measured by PicoGreen Assay (Invitrogen) with Lipofectin (Invitrogen) in Opti-MEM (Lonza, Walkersville, MD). Sixteen hours later, the cells were washed in prewarmed Opti-MEM (CCK-8) or Krebs Henseleit Bicarbonate (KHB) buffer (estrone sulfate and atorvastatin;

1.2 mM $MgSO_4$, 0.96 mM KH_2PO_4, 4.83 mM KCl, 118 mM NaCl, 1.53 mM $CaCl_2$, 23.8 mM $NaHCO_3$, 12.5 mM HEPES, 5 mM glucose, pH 7.4), then dosed with 400 µL of Opti-MEM or KHB buffer containing radiolabeled substrates and varying concentrations of unlabeled compounds, and incubated at 37 °C. Chimeric transporters and OATP1B1 mutants screened for CCK-8 transport activity (2 nM) were incubated for 30 min. Kinetics experiments measuring uptake by the triple mutants were conducted at 10 min, within the linear uptake phase of CCK-8 by OATP1B3. Calculations assume that CCK-8 is not extensively metabolized during the initial 10 min of uptake. Estrone sulfate uptake (100 nM) was measured after 5 min incubation, atorvastatin uptake (75 nM) was measured after 10 min. Cells were washed three times in ice-cold PBS, and harvested in 500 µL of 1% SDS, and radioactivity was measured by scintillation counting. Specific uptake was determined by subtracting uptake by vector-transfected control from the total measured. Percent OATP1B3 uptake was calculated by dividing the specific uptake of a chimeric or mutated transporter by the specific uptake of CCK-8 by wild-type OATP1B3 during the same experiment. Statistical determination of differences was by analysis of variance, with Dunnett's multiple comparison test, and Student's *t* test as appropriate. The kinetic parameters K_m and V_{max} were calculated by Michaelis-Menten nonlinear curve fitting (GraphPad Prism, San Diego, CA).

5.2.5 Cell surface expression and immunoblots

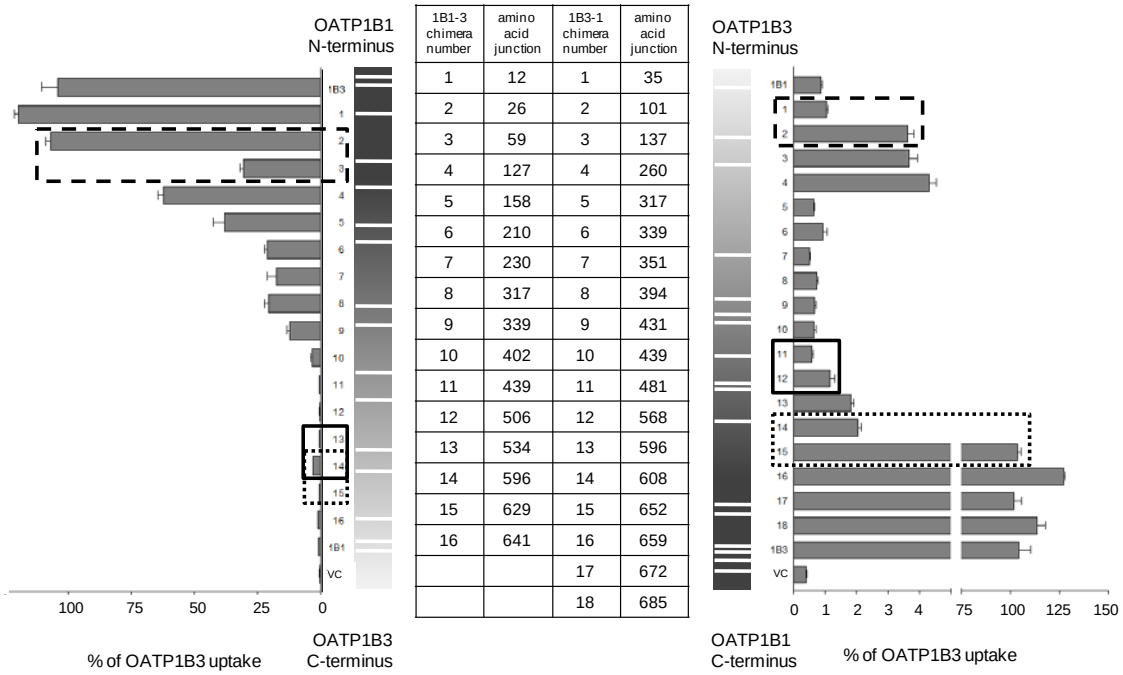
Cell surface biotinylation was carried out as previously described (12) to determine the extent of cell surface trafficking of heterologously expressed transporters. Briefly, Hela

cells (~ 8 x 10^5 cells/well) were transfected as described for transport experiments. Sixteen hours post-transfection, the cells were washed in ice-cold PBS-Ca^{2+}/Mg^{2+} (138 mM $NaCl_2$, 2.7mM KCl, 1.5 mM KH_2PO_4, 1mM $MgCl_2$, 0.1 mM $CaCl_2$, pH 7.3) and treated with sulfo-N-hydroxysuccinimide-SS-biotin (Thermo Scientific, Rockford, IL). The cells were washed with ice-cold PBS-Ca^{2+}/Mg^{2+} containing 100 mM glycine and disrupted with lysis buffer (10 nM Tris base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, pH 7.4) containing protease inhibitors (Complete, Roche Applied Science, Indianapolis, IN). Following centrifugation, 140 µL of streptavidin-agarose beads (Thermo Scientific, Rockford, IL) were added to 600 µL of cell lysate, and incubated for one hour at room temperature. Beads were washed four times with ice-cold lysis buffer, and biotinylated proteins were released from the beads by adding Laemmli buffer. Biotinylated (cell surface-expressed) fractions and total cell lysates (25µL) were subjected to Western blotting analysis for detection of OATP1B1 or OATP1B3 by specific polyclonal antibodies as previously described (12). The intracellular, endoplasmic reticulum-resident protein calnexin was probed as a loading control (1:2000 dilution, StressGen, Victoria, British Columbia, Canada). Densitometry analysis was performed using ImageJ (http://imagej.nih.gov/ij/).

5.3 Results

5.3.1 [^{3}H]-CCK-8 uptake by transfected cells expressing OATP1B1-1B3 and OATP1B3-1B1 chimeras identifies regions in TM1, TM10 and extracellular loop 6 (ECL6) involved in CCK-8 transport.

A library of OATP1B1-1B3 and OATP1B3-1B1 chimeric expression constructs was generated using homologous recombination of linear DNA by *E. coli.* Sequencing of the constructs indicated that the chimeric junctions were well distributed throughout the coding sequence (Figure 5.2). Screening of the chimeras for transport of CCK-8 identified three regions of interest defined by the overlap of sequences causing altered transport activity in both sets of chimeras. A substantial decrease in CCK-8 transport by OATP1B1-1B3 chimeras with junctions at Gly^{26} and Phe^{59} combined with a modest increase by. OATP1B3-1B1 chimeras with junctions at Ser^{35} and Cys^{101} forms a region of interest between Ser^{35} and Phe^{59}. This region is located close to the predicted extracellular boundary of transmembrane helix 1 (TM1). A second region of interest is formed by a change in transport activity in OATP1B1-1B3 chimeras with junctions at Phe^{534} and Asp^{596}, and OATP1B3-1B1 chimeras with junctions at Tyr^{481} and Lys^{568}, creating a region of interest between Phe^{534} and Lys^{568}. A third region is formed by the overlap of a region responsible for a significant gain in OATP1B3-1B1 transport in chimeras with junctions at Gly^{608} and Gln^{652} with a small but detectable decrease in CCK-8 transport in OATP1B1-1B3 chimeras with junctions at Asp^{596} and Ser^{629}. This region, defined by Gly^{608} and Ser^{629}, is located in a portion of the predicted extracellular loop (ECL) 6 close



1B1-3 chimera number	amino acid junction	1B3-1 chimera number	amino acid junction
1	12	1	35
2	26	2	101
3	59	3	137
4	127	4	260
5	158	5	317
6	210	6	339
7	230	7	351
8	317	8	394
9	339	9	431
10	402	10	439
11	439	11	481
12	506	12	568
13	534	13	596
14	596	14	608
15	629	15	652
16	641	16	659
		17	672
		18	685

Figure 5.2 Identification of regions involved in CCK-8 transport

OATP1B1-1B3 and OATP1B3-1B1 chimeric constructs were generated by random chimeragenesis, and sequencing determined the exact location of the junction as indicated. In total, 16 OATP1B1-1B3 chimeras and 18 OATP1B3-1B1 chimeras were expressed in HeLa cells and assayed for CCK-8 transport activity. Regions of interest in TM1 (hatched line), TM10 (solid line) and ECL6 (dotted) formed by overlap of regions exhibiting changes in [^{3}H]-CCK-8 transport in both sets of chimeras were identified for further investigation by site-directed mutagenesis. Values are expressed as means ± SEM of n = 5 from at least two independent experiments

to TM12. Within these regions, seven nonconserved amino acids in both TM1 and ECL6 and twelve nonconserved residues in TM10 were mutated in OATP1B1 to the corresponding residue in OATP1B3.

5.3.2 Site-directed mutagenesis of non-conserved residues indicates amino acids at positions 45 in TM1, 545 in TM10 and 615 in ECL 6 near TM12 contribute to CCK-8 transport.

OATP1B1 mutants of nonconserved residues in OATP1B3 located in the region of TM1 defined by the chimeras were created by site-directed mutagenesis and screened for CCK-8 transport (Figure 5.3). Of the seven mutants created, L36F, F38Y, T42A, A45G, S50I, I53T and H54Q, the OATP1B1 mutant A45G showed the greatest transport activity, at 0.8% of wild-type OATP1B3 CCK-8 transport, compared with 0.3% activity normally observed for wild-type OATP1B1 (Figure 5.3B; $p < 0.01$). The corresponding mutation of OATP1B3 to the OATP1B1 residue, OATP1B3 G45A, exhibited approximately a 35% decrease in CCK-8 transport compared to wild-type OATP1B3 (Figure 5.3E; $p < 0.001$).

In the region of TM10 defined by the chimeric transporters, a total of 12 OATP1B1 mutants were created: Y535F, F536I, F537Y, L543I, L545S, F546L, L550T, S554T, H555F, V556I, M557L, and I559T (Figure 5.3D). Of the mutations in this region, OATP1B1 L545S exhibited the highest level of CCK-8 transport, at 0.8% of wild-type OATP1B3 ($p < 0.01$). Approximately 16% of wild-type CCK-8 transport was observed by the corresponding mutation OATP1B3 S545L (Figure 5.3E; $p < 0.001$).

Figure 5.3 Uptake of [^{3}H]-CCK-8 by cells expressing OATP1B1 and OATP1B3 mutants. (A) Schematic of OATP1B1, including positions of residues mutated in this study. OATP1B1 topology is as predicted by TMPred (22). Uptake of [^{3}H]-CCK-8 by cells transfected with OATP1B1 mutants in TM1 (B), ECL6 (C), TM10 (D) and OATP1B3 mutants (E) is expressed as % of wild-type OATP1B3 uptake ± SEM, n=4 from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ relative to wild-type OATP1B1 (B-D) and OATP1B3 (E). Amino acid sequence alignment of OATP1B1 and OATP1B3 in regions of TM1, ECL6 and TM10 formed by overlapping areas of interest as identified by [^{3}H]-CCK-8 uptake by the chimeras.

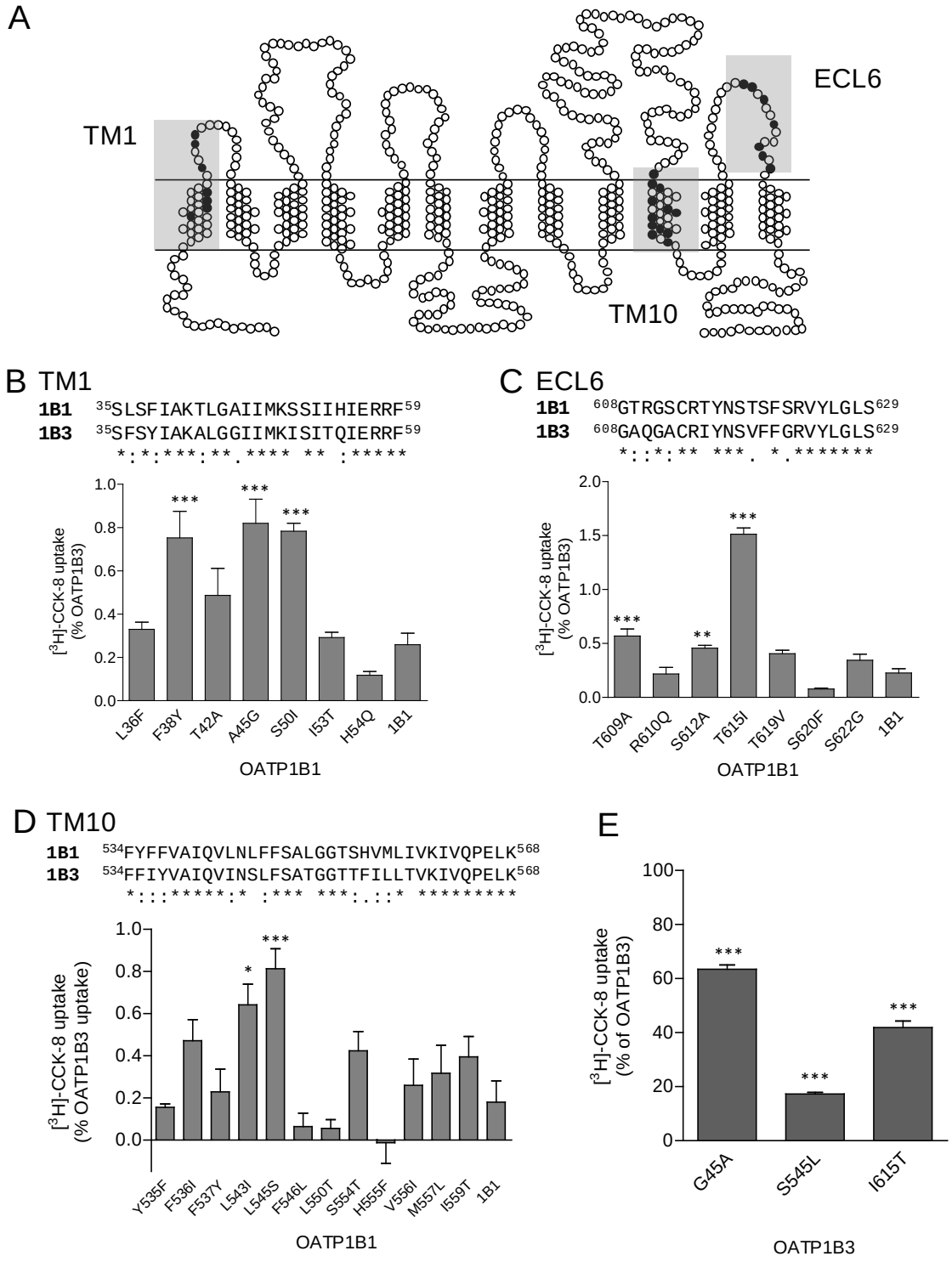


Figure 5.3 Uptake of [^{3}H]-CCK-8 by cells expressing OATP1B1 and OATP1B3 mutants

Finally, of seven OATP1B1 mutants in ECL6, T609A, R610Q, S612A, T615I, T619V, S620F, and S622G, the OATP1B1 mutant T615I exhibited the greatest increase in CCK-8 transport over wild-type OATP1B1 transport, to 1.5% of OATP1B3 transport activity ($p < 0.001$). The corresponding mutation, OATP1B3 I615T, showed a close to 55% decrease in transport activity (Figure 5.3E; $p < 0.001$).

To investigate the potential for interactions between two or more regions identified by the chimeric transporters to be involved in CCK-8 transport, the double mutants OATP1B1 A45G/L545S, OATP1B1 L545S/T615I, and OATP1B1 A45G/T615I were constructed. The double mutants exhibited 3.9, 2.2, and 2.9 % of OATP1B3 CCK-8 uptake, respectively (Figure 5.4A). Similarly, the double mutants OATP1B3 G45A/S545L, OATP1B3 S545L/I615T, and OATP1B3 G45A/I615T exhibited a marked, but not total, loss of CCK-8 transport activity (Figure 5.4C).

In contrast, when a mutation from each of the three regions identified by the chimeric transporters was combined in the triple mutant OATP1B1 A45G/L545S/T615I, a profound gain of CCK-8 transport activity was observed, corresponding to 16% of wild-type OATP1B3 CCK-8 uptake (Figure 5.4B; $p < 0.001$). The corresponding triple mutant OATP1B3 G45A/S545L/I615T exhibited almost complete abrogation of CCK-8 transport (Figure 5.4D; $p < 0.001$).

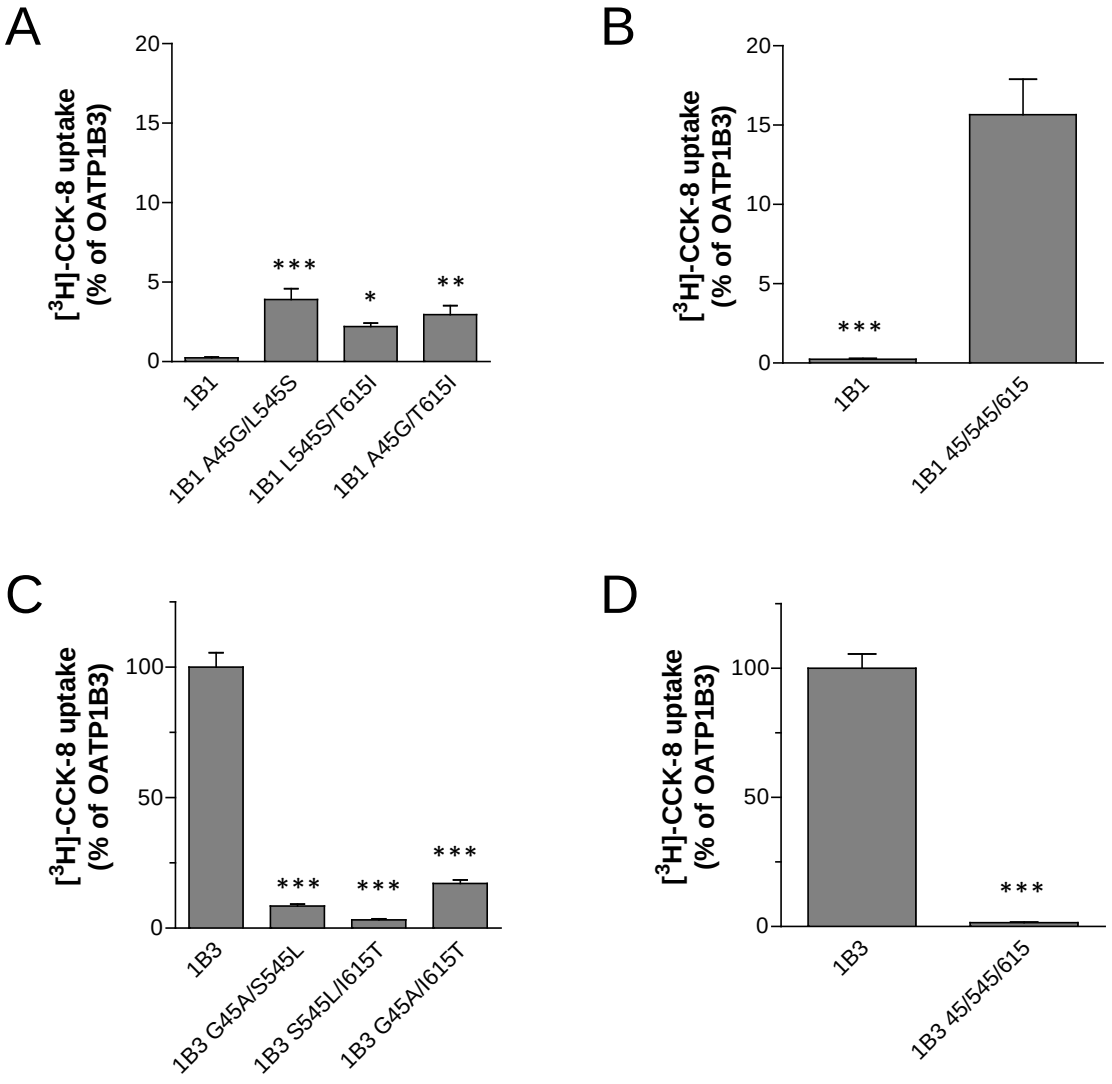


Figure 5.4 Uptake of [^{3}H]-CCK-8 by cells expressing OATP1B1 double and triple mutants, and OATP1B3 double and triple mutants

Values are expressed as mean % of OATP1B3 wild-type uptake ± SEM, n=4-5 from two independent experiments. ** $p < 0.01$, *** $p < 0.001$ relative to wild-type OATP1B1 (A, B) and OATP1B3 (C, D).

Cell surface biotinylation studies were conducted to examine whether the observed changes in transport activity were related to the cell surface expression of the transporter. Western blot analysis indicates that there is no significant difference in cell surface expression in those OATP1B1 mutants with altered CCK-8 transport activity (Figure 5.5A, C), suggesting that the increase in CCK-8 transport activity observed is due to altered substrate recognition or transport capacity and does not appear to be a result of changed levels of cell surface expressed transporter. On the other hand, reduced cell surface expression of OATP1B3 G45A/S545L/I615T may partially account for the loss of CCK-8 transport, though it is important to note that the mutant cell surface expression is approximately 40% of wild-type, suggesting that the protein is not capable of CCK-8 transport (Figure 5.5D).

Figure 5.5 Immunoblot of biotinylated fractions of OATP1B1 and OATP1B3 mutants. Total protein lysates (biotinylated and nonbiotinylated fractions) from Hela cells transfected with OATP1B1 (A) or OATP1B3 (B) mutants were subjected to SDS-PAGE, transferred to nitrocellulose and blotted with anti-OATP1B1 or anti-OATP1B3 antibody, respectively. Cell surface lysates (biotinylated fractions) from Hela cells transfected with OATP1B1 (C) or OATP1B3 (D) were similarly probed.

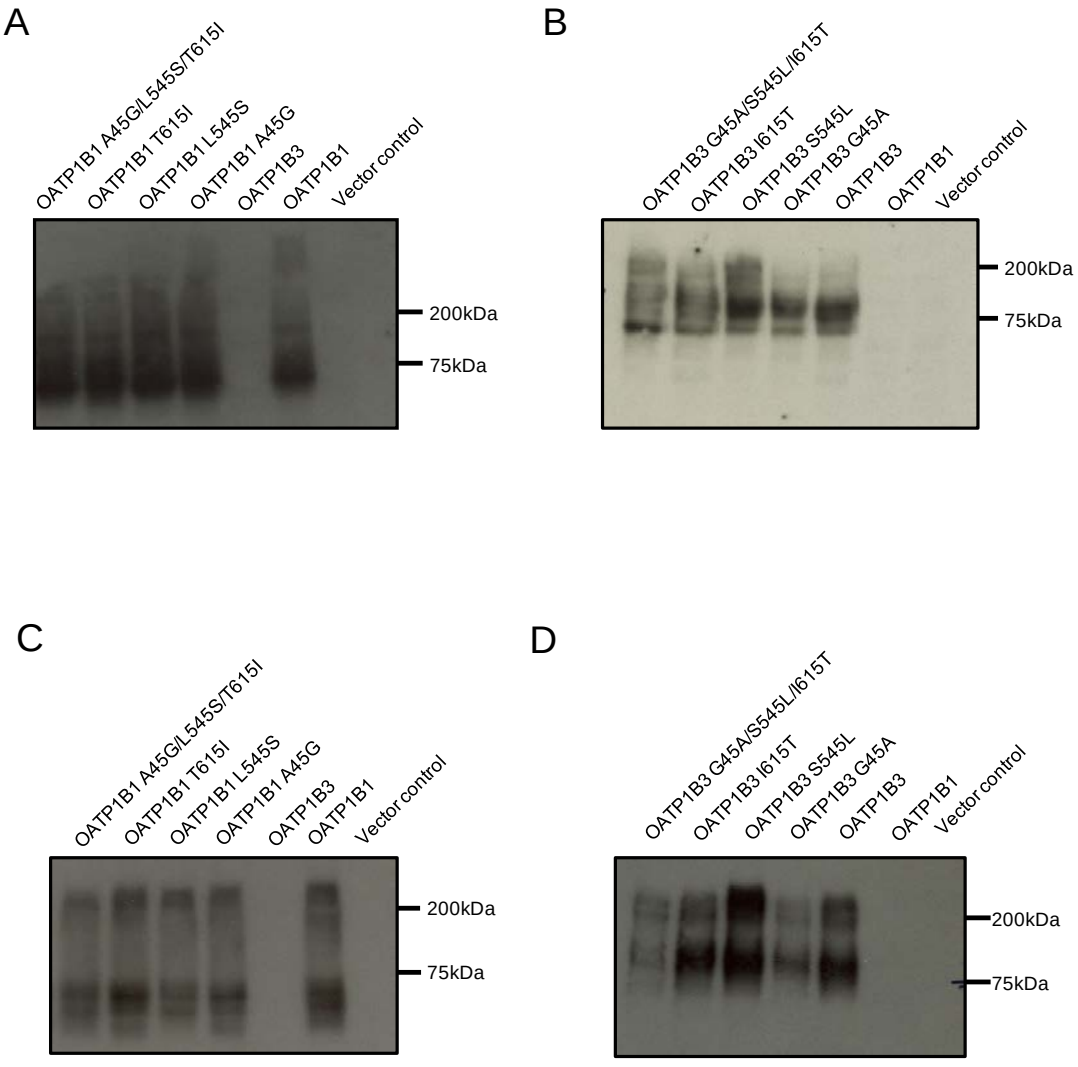


Figure 5.5 Immunoblot of biotinylated fractions of OATP1B1 and OATP1B3 mutants

5.3.3 Characterization of CCK-8 transport kinetics of OATP1B1 triple mutant and wild-type OATP1B3.

The kinetics of CCK-8 uptake by the OATP1B1 triple mutant A45G/L545S/T615I compared to wild-type OATP1B3 were examined by measuring [^{3}H]-CCK-8 uptake after 10 minutes in the presence of unlabeled CCK-8 varying in concentration from 2 nM to 100 µM. Results indicate a higher K_m (15.4 ± 4.2 µM vs. 6.5 ± 2.0 µM) and a lower V_{max} (0.020 ± 0.0018 nmol/mg protein/min vs. 0.064 ± 0.0049 nmol/mg protein/min) for the OATP1B1 triple mutant compared to wild-type OATP1B3 uptake (Figure 5.6). V_{max} and K_m for the OATP1B1-mediated uptake of CCK-8 were undeterminable. Intrinsic clearance values (V_{max}/K_m) were lower in the OATP1B1 triple mutant (1.2 µl/mg protein/min) compared to wild-type OATP1B3 (9.8 µl/mg protein/min), due to the lower V_{max} and higher K_m of the OATP1B1 triple mutant.

5.3.4 Transport of other OATP1B substrates by OATP1B1 and OATP1B3 triple mutants.

The OATP1B1-specific substrate estrone sulfate was not transported by wild-type OATP1B3 or the OATP1B3 triple mutant G45A/S545L/I615T (Figure 5.7A). Transport of estrone sulfate by the OATP1B1 triple mutant A45G/L545S/T615I was reduced to approximately 50% of uptake by wild-type OATP1B1. Transport of the shared OATP1B substrate atorvastatin was modestly increased by OATP1B1 A45G/L545S/T615I and modestly reduced by OATP1B3 G45A/S545L/I615T, compared to the cases of their respective wild-type transporters (Figure 5.7B).

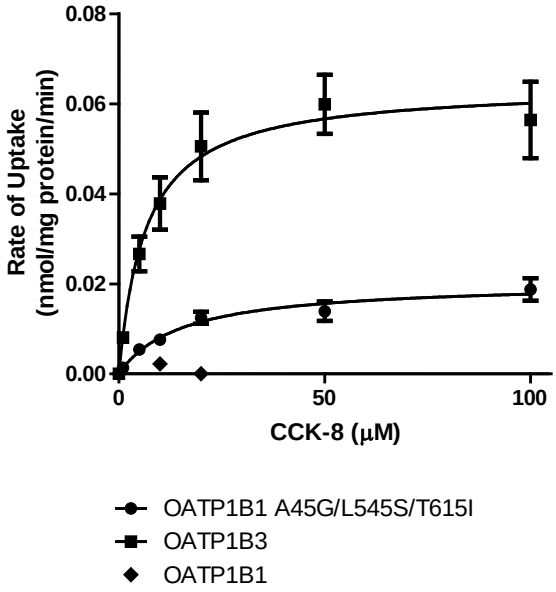


Figure 5.6 Concentration-dependent uptake of CCK-8 by Hela cells transfected with wild-type OATP1B3 and OATP1B1 A45G/L545S/T615I

Values are expressed as means ± SEM, n = 4 from two independent experiments . Kinetic parameters were obtained by non-linear curve fitting.

Figure 5.7 Transport of other OATP1B substrates. (A) Uptake of estrone sulfate by OATP1B1, OATP1B1 A45G/L545S/T615I, OATP1B3 and OATP1B3 G45A/S545L/I615T (B) Uptake of atorvastatin by OATP1B1, OATP1B1 A45G/L545S/T615I, OATP1B3 and OATP1B3 G45A/S545L/I615T. Values are expressed as means ± SEM, n=4 from two independent experiments. * $p < 0.05$ compared to wild-type OATP1B1.

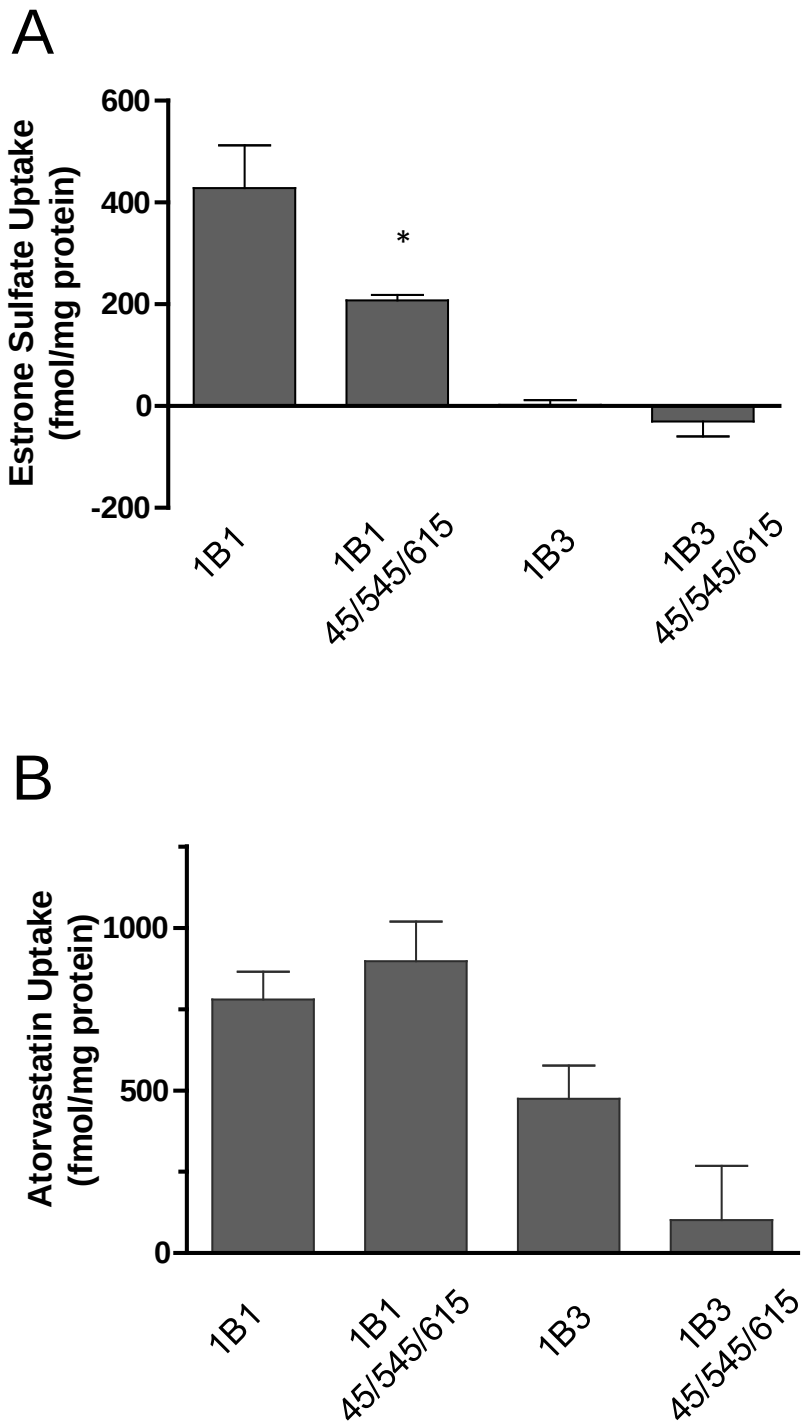


Figure 5.7 Transport of other OATP1B substrates

5.4 Discussion

The molecular basis for the substrate specificity and transport activity of the OATP superfamily is not well understood, despite the fact that OATP transporters are increasingly recognized as important determinants of interindividual variation in response to many drugs in clinical use (7). Transport by the OATPs appears to be mediated by a Na^+-independent and electroneutral process, but the precise details of the transport mechanism, including the identity of the counterion, remain to be elucidated. Recently, it has been demonstrated that the activity of many OATP transporters is stimulated by low extracellular pH (22-23). Hydropathy analysis of OATP/Oatp sequences indicates that members of the superfamily form 12 TMs with intracellular amino- and carboxy termini, an arrangement that was shown experimentally for the murine transporter Oatp1a1 (24). The OATPs have in common a large predicted ECL5 between TMs 9 and 10; characterization of conserved cysteine residues in ECL5 of OATP2B1 indicates these residues are involved in membrane trafficking and transport function (25). Other conserved features include N-glycosylation sites in ECLs 2 and 5, and the superfamily signature that designates the OATP family, found at the border between ECL3 and TM6 (26).

CCK-8 is a gastrointestinal peptide hormone released postprandially in response to nutrients in the gut, and it is involved in delaying gastric emptying, as well as stimulating pancreatic enzyme secretion, gall bladder contraction and intestinal motility (27). Interestingly, CCK-8 appears to be transported by OATP1B3 but not by the closely

related OATP1B1. The main goal of our current study was to identify key regions or amino acid residues which could confer gain of CCK-8 transport function to OATP1B1.

We first noted that chimeras from each set sharing the same junction close to the middle of the transporter (Val^{339}), OATP1B3-1B1-6, and OATP1B1-1B3-9, demonstrated a modest gain or significant loss of wild-type OATP1B3 CCK-8 transport, respectively, compared to adjacent chimeras (Figure 5.2). OATP1B1-1B3-9 demonstrated higher CCK-8 transport activity than OATP1B3-1B1-6 (Figure 5.2), consistent with other reports that the C-terminal portion of OATP1B3 is more important for CCK-8 transport than the N-terminal portion (18). Systematic comparison of the individual chimeric transporter function suggested that amino acids in TM1, TM10, and ECL6 may be important for CCK-8 transport. Given the two negatively charged aspartic acid residues in CCK-8, it might be expected that there exists some critical interaction with positively charged residues in the transporter, especially given the importance of positively charged residues to OATP1B3 transport of other substrates (19-20). However, none of the OATP1B1 mutations made were to a positively charged residue in OATP1B3. Three nonconserved positively charged residues in OATP1B1 were substituted for an uncharged residue at the corresponding position in OATP1B3, however, none of the three variants, H54Q, H555F or R610Q, showed any significant increase in CCK-8 transport (Figure 5.3B, C and D). In total, five aromatic residues, Phe^{38}, Tyr^{535}, Phe^{536}, Phe^{537}, and Phe^{546} in OATP1B1 fell within the regions identified and were mutated to the corresponding residue in OATP1B3. At three of these positions, the mutation was a semiconserved mutation to a different aromatic residue (F38Y, Y535F, and F537Y). In

three other positions, nonaromatic residues in OATP1B1 were converted to aromatic side chains: L36F, H555F, and S620F. The absence of any significant gain of function in any of these OATP1B1 mutations suggests that these residues may not be involved in the CCK-8 transport cycle.

The mutation of an alanine at position 45 to glycine in TM1 of OATP1B1 resulted in an increase in CCK-8 transport. It is possible that this may be attributed to the loss of bulk of the methyl group difference between these two side chains, a consideration particularly given that CCK-8 is a relatively large substrate. Mutation of OATP1B1 from a leucine to a serine at position 545 adds a hydroxyl group, in addition to reducing the bulk associated with the side chain, while the mutation of threonine at position 615 to isoleucine results in a loss of a hydroxyl group. This suggests a possible role for the interaction of a hydroxyl group with CCK-8 in a way that either promotes or prevents CCK-8 transport. It is also possible that the mutations noted alter protein conformation in a way that affects substrate specificity without directly interacting with CCK-8.

A previous report that utilized a TM domain swapping strategy indicated the importance of TM10 in CCK-8 transport by OATP1B3 (18). It should be noted that the study by Gui and Hagenbuch focused on the substitution of individual transmembrane spanning domains in OATP1B3 with the corresponding TMs of OATP1B1. Accordingly, their study was designed to detect a loss of CCK-8 transport due to the presence of an OATP1B1-specific TM region. In the current study, we pursued a chimeragenesis approach to generate a library of both OATP1B1-1B3 and OATP1B3-1B1 monomer

sized chimeras to identify chimeric junctions that revealed a gain and corresponding loss of function, without an a priori bias regarding the overall importance of TM regions versus intracellular or extracellular loops in the transporter. Although our data confirms TM10 is a key region for CCK-8 interaction, mutation of a single residue in TM10 is not sufficient to impart a true gain of OATP1B1-mediated CCK-8 transport (Figure 5.3D). Indeed, our current data reveals that the synergistic interaction with two additional domains is essential. Although it should be noted that the overall activity of the OATP1B1 45/545/615 triple mutant was lower than that of the wild-type OATP1B3, it is remarkable that three targeted amino acid substitutions changed OATP1B1 from complete inability to transport CCK-8 to attaining near 15% of OATP1B3 activity (Figure 5.4B). Conversely, substitution of amino acids at those positions in OATP1B3 to the corresponding residue in OATP1B1 resulted in the near complete loss of OATP1B3-mediated CCK-8 uptake (Figure 5.4D), despite the fact that this mutant is expressed on the cell surface, albeit at lower levels than those for wild-type OATP1B3 (Figure 5.5D).

The three key amino acid residues noted for CCK-8 gain of substrate specificity do not appear to confer the opposite effect, that is, OATP1B3 gain of function for an OATP1B1-specific substrate such as estrone sulfate (Figure 5.7A). Similarly, there was not a readily discernible effect of the three amino acid residues on the transport of the shared (OATP1B1 and OATP1B3) substrate and commonly prescribed statin, atorvastatin (Figure 5.7B,C). Therefore, it seems the amino acids we have identified in this study, though key residues with respect to CCK-8 transport, are not essential to conferring OATP1B1-specific or OATP1B1 and 1B3 shared substrate specificity. This is consistent

with a recent report that TM8 and TM9 of OATP1B1 are involved in the transport of its steroid conjugate substrates estrone sulfate and estradiol glucuronide (15).

As is the case for other transporters, little structural data for the OATPs exists as a result of challenges associated with the purification and crystallization of large membrane-bound structures. A homology model for OATP1B3 based on the crystal structures of the glycerol-3-phosphate transporter and lactose permease from *E. coli* has been reported (28-30). More recently, the structure of the multidrug transporter EmrD from *E. coli* has been used to model OATP1B3 (18; 31). There appears to be significant structural conservation in the major facilitator superfamily (MFS) of transporters (32), so these crystal structures from bacteria may serve as models to interpret data arising from functional characterization of the distantly related OATPs. In each of the three MFS structures mentioned, it appears that both TM1 and TM10 partially form the pore of the transporter, consistent with the biochemical data presented here to suggest that these regions are involved in CCK-8 transport (Figure 5.8).

This study is important to the drug development process for a number of reasons. The exclusive expression of OATP1B1 on the basolateral membrane of hepatocytes makes it an attractive target for drugs requiring entry into the liver to exert their effect. Species differences in OATP expression in the liver and other organs adds complexity to studying human OATPs and mean that in vitro and in silico approaches may prove useful in predicting the in vivo activity of human OATPs. In addition to species differences in

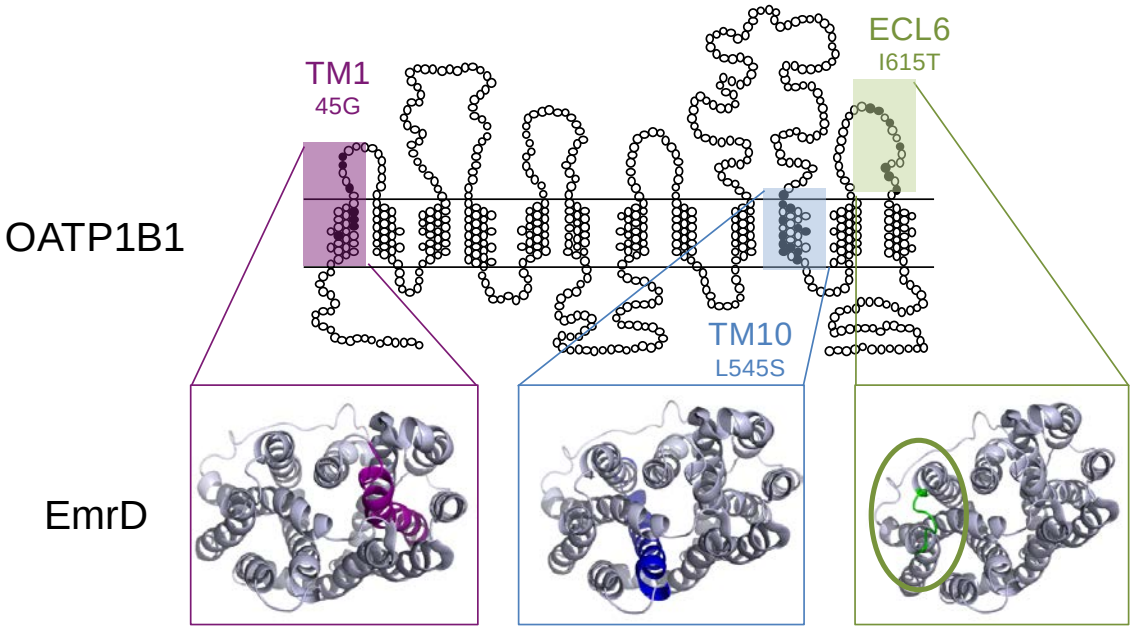


Figure 5.8 Regions involved in CCK-8 transport by OATP1B1 and OATP1B3, mapped to the crystal structure of the multidrug resistance protein EmrD from E. coli

Similar results were obtained for other bacterial protein structures of the major facilitator superfamily, the glycerol-3-phosphate transporter and lactose permease from *E. coli* (30-31). Coordinates for the crystal structures were obtained from the Protein Data Bank (www.pdb.org; PDB IDs 2GFP, 1PW4, and 1PV6 respectively). Sequences were aligned using ClustalW with default settings and manually optimized with respect to secondary structure as predicted by TMPred (33). Figure images were created in Pymol (www.pymol.org).

substrate specificity, there are zonal differences in OATP expression within a given tissue. In particular, OATP1B1 is expressed in hepatocytes throughout the liver, while OATP1B3 is expressed primarily in the perivenous hepatocytes, indicating there are important differences in transporter regulation (34). Thus, targeting one OATP over another has the potential to result in even greater tissue specificity.

Finally, given the importance of OATPs to the cellular uptake of many drugs, it will be useful to have the ability to predict the functional effect of novel polymorphisms in OATPs that will be discovered as whole genome sequencing expands into clinical applications. At the time of this writing, there was only one reported case of a naturally occurring polymorphism in any of the same positions as the 26 mutations in OATP1B1 presented here, Leu543Trp (rs72661137). It remains difficult to predict the precise effect of a polymorphism in OATP1B1; however, there was a modest increase in CCK-8 transport by the Leu543Ile mutant in our study (Figure 5.3D). Given this, combined with its proximity to the Leu545 residue identified here to be important for CCK-8 transport, we believe it is not unreasonable to expect that Leu543 polymorphisms may alter OATP1B1 function, and further studies of this polymorphism may be warranted.

In conclusion, hepatic uptake transporters are increasingly recognized as important determinants of drug disposition and response. Accordingly, substrate recognition by OATP1B1 and OATP1B3 may be an important consideration for predicting potential transporter mediated drug interactions and rational drug design. Indeed, the substrate specificity of OATP1B1 and OATP1B3 may also provide valuable information for

enhancing the liver-to-plasma ratio in the design of compounds targeted to the liver. This report is the first to identify three amino acids, 45 (TM1), 545 (TM10), and 615 (ECL6), in distinct regions of the transporter interact to confer gain of function of transport of CCK-8 by OATP1B1. This data contributes new insight to our understanding of substrate specificity in these important hepatic transporters.

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6 DISPOSITION OF ATORVASTATIN, ROSUVASTATIN, AND SIMVASTATIN IN OATP1B2$^{-/-}$ MICE AND INTRAINDIVIDUAL VARIABILITY IN HUMAN SUBJECTS[4]

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6.1 Introduction

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are widely prescribed to reduce cardiovascular disease risk. There is considerable interindividual variation in statin pharmacokinetics and response arising from variability in both drug transport and metabolism (1). Patients are often switched between statins to achieve greater efficacy or reduce side effects. However, predicting a patient's response to other statins on the market, whether to lower cholesterol or to avoid adverse events, remains a difficult clinical problem.

Statins exert their effect through targeted accumulation in liver, which is mediated by hepatic uptake transporters of the organic anion-transporting polypeptide (OATP; gene symbol *SLCO*) family as well as sodium-dependent taurocholate cotransporting polypeptide (NTCP) and by efflux transporters of the ATP-binding cassette (ABC) family, located on the basolateral and canalicular membranes of the liver, respectively (2). In addition, atorvastatin and simvastatin share a common pathway of metabolism by CYP3A (3-4), whereas rosuvastatin is not subject to significant metabolism (5-6). Hence, it is likely there are both similarities and differences in the relative interplay between specific metabolism and transport pathways and their contribution to overall drug exposure among the various statins.

Slco1b2$^{-/-}$ mice have proven to be a useful in vivo model for predicting the role of two important liver-expressed OATPs in humans, OATP1B1 and OATP1B3. Previously, we showed that hepatic uptake and clearance of the prototypical OATP1B substrate,

pravastatin, were significantly reduced in Oatp1b2$^{-/-}$ mice compared with wild-type (7). In another study, differences between wild-type and knockout were observed for lovastatin, but not simvastatin acid or cerivastatin, indicating that the transporter is critical for some but not all members of the statin class (8). However, the extent of hepatic uptake for the most widely prescribed statins, atorvastatin and rosuvastatin, in this animal model has not been described.

Our aim was to better understand the in vivo relevance of OATP1B transporters to the commonly prescribed statins, atorvastatin, rosuvastatin, and simvastatin, by measuring their disposition in *Slco1b2*$^{-/-}$ mice. In addition, we addressed the role of metabolism vs transport and intraindividual variability by comparing atorvastatin, rosuvastatin, and simvastatin pharmacokinetics in healthy subjects given all three statins.

6.2 Methods

6.2.1 Mouse pharmacokinetic study design

Slco1b2$^{-/-}$ mice were created as previously described (7). Male *Slco1b2*$^{-/-}$ and wild-type mice (8-16 weeks old; 4-6 per group) were dosed 1 mg/kg atorvastatin, rosuvastatin or simvastatin (1% DMSO in water) by tail vein injection. After 30 minutes, animals were euthanized by isoflurane; blood was collected into EDTA-containing tubes by cardiac puncture and livers were excised, blotted, and weighed. Plasma was obtained by centrifugation of blood (14,000 rpm for 20 minutes at 4 °C) and all tissues were stored at -80 °C until analyzed by LC-MS/MS as described below. The study protocol was

approved by the Animal Use Subcommittee of the University of Western Ontario, London, Canada.

6.2.2 Human pharmacokinetic study design

Four females and seven males, with a mean ± SD age of 33 ± 13 years and body mass index 23.7 ± 5.0, participated in the study. All participants provided written informed consent. The study was approved by the Research Ethics Board of the University of Western Ontario, London, Canada.

In an open, randomized, crossover design, participants were administered single oral doses of 20 mg of atorvastatin, 10 mg of rosuvastatin, or 20 mg of simvastatin, with a washout period of at least one week. One male subject did not complete the atorvastatin arm of the study. Participants were not taking any prescription medications, with the exception of oral contraceptives. Pregnancy tests were administered on each study day for all female participants. Subjects were not permitted to take over-the-counter medications or supplements for one week prior to and during the study period.

Statins were administered following an overnight fast of at least nine hours. A standardized meal was served four hours after the dose, and a standardized afternoon snack was served seven hours after the dose. Blood was collected 0, 0.5, 1, 1.5, 2, 3, 4, 5, 6, 7, 8, and 10 hours after dose and immediately placed on ice and separated within 20

minutes. Plasma samples were stored at -80 °C until analysis of statin concentration by LC-MS/MS.

6.2.3 Determination of statin concentration

Concentration of atorvastatin, rosuvastatin, or simvastatin acid in plasma was determined by a TSQ Vantage triple-quadrupole mass spectrometer (Thermo Scientific, Pittsburgh, PA, USA) attached to a TLX2 high performance liquid chromatography system (Thermo Scientific). All chemical standards were purchased from Toronto Research Chemicals (North York, Canada). Internal standards were atorvastatin-d5 for atorvastatin and simvastatin acid, and rosuvastatin-d6 for rosuvastatin.

Liver samples were homogenized 1:1 (w/v) in 0.05% formic acid and standard curves created using blank liver homogenates. Plasma and liver samples (100 µL) were precipitated in 300 µL acetonitrile containing internal standard, and centrifuged for 20 minutes at 14,000 rpm at 4 °C. The supernatant was diluted 1:1 in 0.05% formic acid. A 50 µL aliquot was injected onto a Hypersil GOLD C18 column (50 x 3 mm, 5 µm particle size; Thermo Scientific), and the analytes were separated using 0.05% formic acid and acetonitrile starting at a ratio of 70:30 with a gradient to a ratio of 10:90. Detection was performed by a HESI II probe (Thermo Scientific) in positive mode for atorvastatin and rosuvastatin, and negative mode for simvastatin acid, using transitions m/z 559.2 to 440.4 for atorvastatin, 564.1 to 255.2 for atorvastatin-d5 (positive mode), 562.3 to 402.2 for atorvastatin-d5 (negative mode), 482.1 to 258.2 for rosuvastatin, 488.0

to 264.3 for d-rosuvastatin, and 435.0 to 319.1 for simvastatin acid. The ranges of statin quantification were 1 to 500 ng/mL in liver and 1 to 100 ng/mL in plasma. The interday coefficients of variation (CV%) were 5.8%, 8.9% and 14.8% for atorvastatin, rosuvastatin and simvastatin, respectively. The bias was between 2.3% and 4.9% at 2.5 ng/ml, and between 2.4% and 3.2% at 10 ng/ml for each of the statins measured.

6.2.4 Pharmacokinetics and statistical analysis

For the mouse study, liver-to-plasma ratios were calculated by dividing liver concentration by plasma concentration at 30 minutes, and *Slco1b2*$^{-/-}$ vs wild-type values were compared by Mann-Whitney *U* test. For the human study, area under the curve from 0 to 10 hours ($AUC_{0\text{-}10h}$) was calculated by the linear trapezoid rule, and $AUC_{0\text{-}10h}$ for the three different statins was compared pair-wise using Spearman correlation. To calculate area under the curve from 0 hours to infinity ($AUC_{0\text{-}\infty}$), the residual area was calculated by dividing the final concentration by the terminal rate constant (K_e), and added to $AUC_{0\text{-}10}$. Statistical analyses were performed using GraphPad Prism 5 (La Jolla, CA, USA).

6.3 Results

6.3.1 Atorvastatin, rosuvastatin, and simvastatin liver-to-plasma ratios in *Slco1b2*$^{-/-}$ mice

The plasma concentration of simvastatin acid was significantly higher in wild-type compared with *Slco1b2*$^{-/-}$ mice (P = 0.03; Figure 6.1g). The liver concentration of atorvastatin was significantly higher in wild-type compared with *Slco1b2*$^{-/-}$ mice (P = 0.002; Figure 6.1b). The liver-to-plasma concentration ratio of a given compound is a sensitive marker of its dependence on hepatic transport processes. Liver-to-plasma concentration ratios of atorvastatin and rosuvastatin were 2.7-fold (P = 0.002; Figure 6.1c) and 1.9-fold (P = 0.03; Figure 6.1f) higher, respectively, in wild-type compared with *Slco1b2*$^{-/-}$ mice, 30 minutes after a single tail vein injection of 1 mg/kg. Liver-to-plasma ratios of simvastatin acid were not significantly different in *Slco1b2*$^{-/-}$ mice compared with wild-type (P = 0.49; Figure 6.1i).

6.3.2 Intraindividual variability in atorvastatin, rosuvastatin, and simvastatin pharmacokinetics in humans

Pharmacokinetic parameters following oral administration of 20 mg of atorvastatin, 10 mg of rosuvastatin, and 20 mg of simvastatin for each participant are summarized in Table 6.1.

Figure 6.1 Plasma, liver, and liver-to-plasma ratios of atorvastatin (a-c), rosuvastatin (d-f) and simvastatin (g-i) in *Slco1b2*$^{-/-}$ mice compared to wild-type controls, 30 minutes after a 1mg/kg intravenous dose. *, $p < 0.05$; **, $p < 0.01$

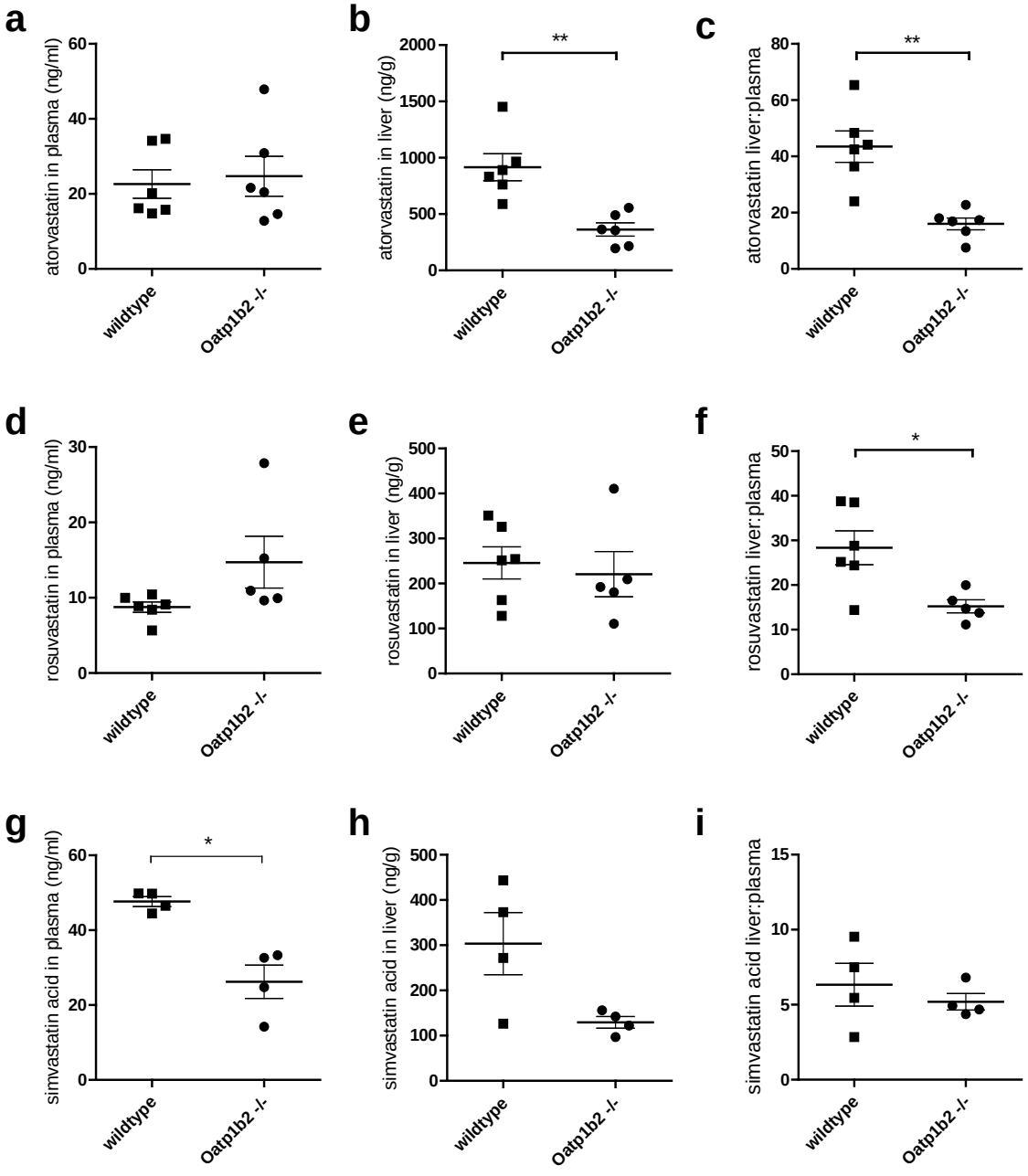

Figure 6.1 Plasma, liver, and liver-to-plasma ratios of atorvastatin, rosuvastatin, and simvastatin in *Slco1b2*$^{-/-}$ mice

Table 6.1 $AUC_{0\text{-}10h}$, $AUC_{0\text{-}\infty}$, C_{max} and t_{max} of atorvastatin, rosuvastatin, and simvastatin in healthy human subjects following oral doses of 20 mg, 10 mg, or 20 mg, respectively

	Atorvastatin				**Rosuvastatin**				**Simvastatin**			
Subject	**$AUC_{0\text{-}10}$ (ng•h/ml)**	**$AUC_{0\text{-}\infty}$ (ng•h/ml)**	**C_{max} (ng/ml)**	**t_{max} (h)**	**$AUC_{0\text{-}10}$ (ng•h/ml)**	**$AUC_{0\text{-}\infty}$ (ng•h/ml)**	**C_{max} (ng/ml)**	**t_{max} (h)**	**$AUC_{0\text{-}10}$ (ng•h/ml)**	**$AUC_{0\text{-}\infty}$ (ng•h/ml)**	**C_{max} (ng/ml)**	**t_{max} (h)**
1	36.2	49.6	5.4	5	27.0	28.4	4.1	3	18.9	22.3	3.7	2
2	20.5	43.3	5.7	0.5	24.6	31.9	4.3	5	7.56	9.7	1.9	1
3	50.6	82.5	23.3	0.5	54.5	73.0	8.4	5	25.4	26.8	11.5	0.5
4	25.9	40.8	9.4	0.5	6.44	8.9	1.0	5	12.8	14.9	4.2	1.5
5	44.7	118.1	11.3	1	26.0	42.0	4.1	4	16.2	21.4	3.5	1
6	39.0	55.5	6.0	0.5	61.7	76.6	9.2	2	65.6	175.3	12.8	3
7	27.3	39.7	5.9	1.5	32.9	48.7	4.8	5	6.80	10.4	1.1	3
8	37.1	47.4	6.8	1	27.0	37.0	4.6	2	18.1	31.8	3.4	2
9	NA	NA	NA	NA	22.8	29.6	3.2	5	12.1	12.7	2.8	1
10	18.1	22.5	7.6	0.5	34.2	76.0	5.7	3	7.43	9.3	1.7	0.5
11	45.3	71.6	7.8	1.5	25.2	25.2	4.3	5	15.8	19.1	3.7	2
	34.5 ± 11.1	57.1 ± 27.2	8.9 ± 5.4	0.75 (0.5-5)	31.1 ± 15.2	43.4 ± 22.8	4.9 ± 2.3	5 (2-5)	18.8 ± 16.5	32.2 ± 48.0	4.6 ± 3.9	1.5 (0.5-3)

$AUC_{0\text{-}10}$, area under the curve from 0 hours to 10 hours; $AUC_{0\text{-}\infty}$, area under the curve from 0 hours to infinity; C_{max}, peak plasma concentration; T_{max}, time of peak plasma concentration. Summary data are given as mean ± SD, except T_{max} , which is given as median and range.

In the same subject, $AUC_{0\text{-}10h}$ ranks of atorvastatin and simvastatin acid were significantly related (Spearman r = 0.68; P = 0.035; Figure 6.2e). Rosuvastatin exposure was not predictive of atorvastatin or simvastatin acid exposure (Figure 6.2d-f). Similar results were obtained using $AUC_{0\text{-}\infty}$. There was no significant association in peak plasma concentration (C_{max}) rank between any two of the three compounds in the same individual (Table 6.1).

6.3.3 Correlation of single time point concentrations with $AUC_{0\text{-}10h}$

To determine the single time point measurement that best predicts $AUC_{0\text{-}10h}$, a linear regression between concentration at each time point and $AUC_{0\text{-}10h}$ was performed. For atorvastatin, rosuvastatin, and simvastatin, the concentrations most significantly correlated with $AUC_{0\text{-}10h}$ were those at 3, 5, and 4 hours, respectively (Figure 6.3).

The r^2 value for simvastatin $AUC_{0\text{-}10h}$ vs simvastatin concentration at 4 hours is 0.7516 (P = 0.0012) if the single individual with high simvastatin concentration is excluded from the analysis.

Figure 6.2 Plasma concentrations of atorvastatin (a), rosuvastatin (b) or simvastatin (c) in healthy human subjects following oral doses of 20 mg, 10 mg, or 20 mg, respectively. $AUC_{0\text{-}10}$ parameters of atorvastatin vs rosuvastatin (d), atorvastatin vs simvastatin (e) and simvastatin vs rosuvastatin (f) in the same individual.

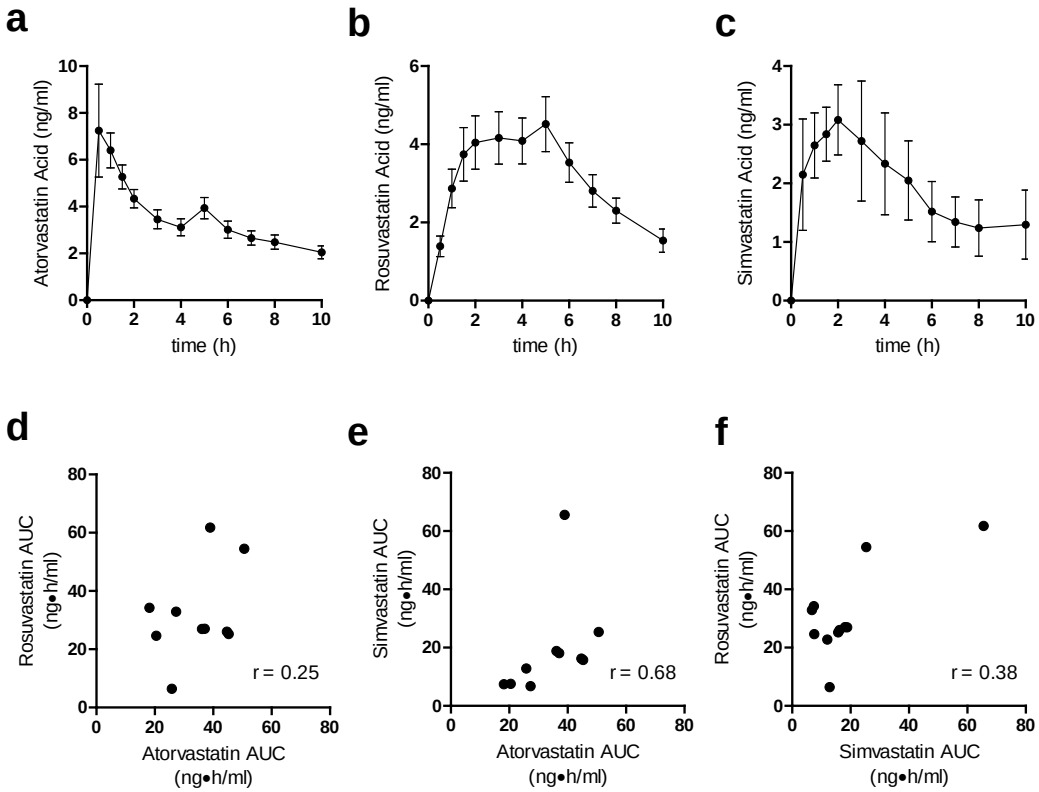

Figure 6.2 Plasma concentrations of atorvastatin, rosuvastatin, or simvastatin in healthy human subjects

Figure 6.3 Correlation between $AUC_{0\text{-}10h}$ and single time points from the pharmacokinetic profile of atorvastatin (a), rosuvastatin (b) and simvastatin (c). The time points that are most highly correlated with $AUC_{0\text{-}10h}$ are shown for each drug.

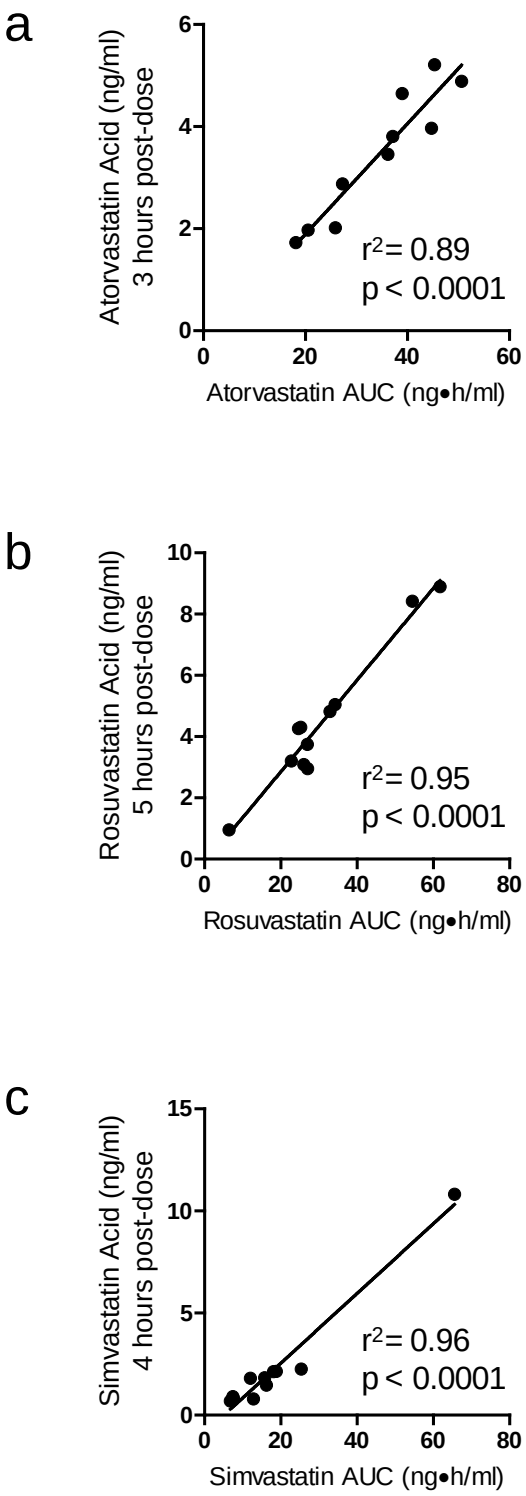

Figure 6.3 Correlation between $AUC_{0\text{-}10h}$ and single time points from the pharmacokinetic profiles of atorvastatin, rosuvastatin, and simvastatin

6.4 Discussion

In this study, we determined the liver-to-plasma ratios of atorvastatin, rosuvastatin and simvastatin using the recently created *Slco1b2*$^{-/-}$ mice. The active acid forms of statins inhibit HMG-CoA reductase; thus, their facilitated entry into liver tissue is required to exert their lipid-lowering effect. In isolation, statin concentrations in the liver and plasma are controlled by complex processes that remain unclear; however, the impact of uptake transport is most sensitive to detection by the tissue-to-plasma concentration ratio. From our data in *Slco1b2*$^{-/-}$ mice, the protein encoded by this gene, Oatp1b2, appears critical to the hepatic uptake of atorvastatin and rosuvastatin but not simvastatin acid.

Previous studies have demonstrated a role for Oatp1b2 in the disposition of pravastatin and lovastatin but not cerivastatin or simvastatin acid (7-8). We administered simvastatin to the mice in its lactone form, as it is prescribed to humans, in contrast to the previous study in which simvastatin acid was both administered and measured (8). These findings are interesting given that the genetic variant in *SLCO1B1* (c.521T>C, rs4149056) appears to have the greatest effect on the pharmacokinetics of simvastatin acid over other statins (9).

Although the statins were administered intravenously to the mice, and not orally as administered to humans, in this case the route of administration is not expected to confound the liver-specific contribution of Oatp1b2 to statin transport. The magnitude of the difference in liver concentration compared with plasma concentration, however, may be sensitive to statin concentration in the portal circulation. Liver-to-plasma ratio of

pravastatin administered subcutaneously was reduced at a higher dose of pravastatin (32 µg/h) compared with a lower dose (8 µg/h) in both *Slco1b2*$^{-/-}$ and wild-type mice, due to saturation of uptake transport (7).

In mice, unlike humans, several members of the Oatp1a family are expressed on the basolateral membrane domain of hepatocytes (10-11). Thus, Oatp1a activity in mice lacking Oatp1b2 may underestimate the effect of loss of OATP1B function in humans. A knockout mouse model with targeted deletion of Oatp1a1, Oatp1a4, Oatp1a5, Oatp1a6 and Oatp1b2 has recently been reported (12), and further characterization of statin disposition in these animals may reveal the extent to which Oatp1a transporters contribute to hepatic statin uptake in mice. However, although Oatp1b2 is liver-specific in its expression, members of the Oatp1a family are known to be expressed in other organs including the intestine, and thus would complicate the overall interpretation of such data in terms of loss of intestinal absorption vs hepatic uptake.

In this study, we characterized the intraindividual variation in the pharmacokinetics of a single oral dose of 20 mg of atorvastatin, 10 mg of rosuvastatin, and 20 mg of simvastatin administered at least one week apart. We observed a significant correlation between atorvastatin and simvastatin $AUC_{0\text{-}10h}$ but no correlation between the AUC of rosuvastatin and atorvastatin, or the AUC of rosuvastatin and simvastatin. There was no correlation in C_{max} between any two of the three statins studied. A previous study reported a significant correlation between the AUC of atorvastatin and simvastatin, and to

a lesser extent, the AUC of atorvastatin and rosuvastatin and the AUC of simvastatin and rosuvastatin (13).

Taken together, the data in humans and mice reinforce the growing appreciation for the differences in disposition of the various statins in vivo and that the disposition profile of a statin may not always predict that of another. In humans, atorvastatin and simvastatin appear to share common mechanisms of elimination through CYP3A4 (3-4; 14). A minor role for CYP2C8 in simvastatin metabolism has been described (14). Rosuvastatin, in contrast, is not subject to metabolism by CYP3A (5). The major rosuvastatin metabolite, N-desmethyl-rosuvastatin, is formed by CYP2C9 and CYP2C19 (6). Rosuvastatin is the most hydrophilic of the statins used in this study, and although some CYPs are involved, the extent of metabolism is modest as 70% of rosuvastatin is eliminated unchanged (15).

All of the statins studied here are substrates of uptake and efflux transporters. We and others have shown that rosuvastatin is a substrate of the uptake carriers OATP1B1, OATP1B3, OATP2B1, OATP1A2, and NTCP as well as the efflux transporters P-glycoprotein (MDR1/*ABCB1*), breast cancer resistance protein (BCRP/*ABCG2*), and multidrug resistance-associated protein 2 (MRP2/*ABCC2*) (16-17). In vitro, simvastatin was not transported by OATP1B1 (18), although simvastatin acid inhibits OATP1B1 transport (19-20). However, genetic variations in *SLCO1B1* in healthy subject studies in vivo appear to have the greatest effect on simvastatin compared with any other statin tested (9; 21). Both simvastatin and atorvastatin also interact with P-glycoprotein in vitro (20; 22).

Statin intolerance is an important and frequently observed issue in clinical practice, with up to 10% of individuals reporting muscle pain and weakness associated with statin use. In rare cases, a life-threatening form of muscle damage, rhabdomyolysis, may occur (23). Switching between statins, to increase efficacy or avoid toxicity, occurs relatively frequently in clinical practice: in a recent study of routine care of more than 4,000 diabetic patients, the reported rate of statin switching was approximately 20% (24). The data presented here provide some explanation for why switching statins may work for some individuals in the clinical setting. Indeed, the lack of a strong association between exposures to different members of the statin class indicates there may be other viable options for individuals with statin intolerance. Certainly, there are few data regarding intraindividual variation in statin pharmacokinetics and whether those individuals with the highest exposure to one statin have the highest exposures across all the statins. An important finding from the current study is that there may be some predictability between statins that undergo CYP3A metabolism as well as transport, such as atorvastatin and simvastatin, but such a correlation is lost when considering statins such as rosuvastatin, which is mainly subject to transport.

Finally, we identified a single time point blood sampling for the measurement of atorvastatin, rosuvastatin, and simvastatin that best correlates with the overall $AUC_{0\text{-}10h}$, thereby streamlining the design of future studies of statin pharmacokinetics, particularly those involving patients where simplicity and predictability are essential. In the way that one measurement of midazolam has been successfully used to predict total exposure to

midazolam and CYP3A activity (25), a single statin measurement will be a cost effective and less invasive method of phenotyping for statin disposition.

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7 CLINICAL AND PHARMACOGENETIC PREDICTORS OF CIRCULATING ATORVASTATIN AND ROSUVASTATIN CONCENTRATION IN PATIENTS[5]

[5] The material in this chapter is based on a manuscript in preparation: DeGorter MK, Tirona RG, Schwarz UI, Choi Y, Myers K, Suskin N, Zou GY, Dresser GK, Hegele RA, Kim RB. Clinical and pharmacogenetic predictors of circulating atorvastatin and rosuvastatin concentration in patients. 2012.

7.1 Introduction

The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, are commonly prescribed and proven to be highly effective in reducing cardiovascular event risk by lowering plasma concentration of low-density lipoprotein cholesterol (LDL-C) (1). Not surprisingly, nearly 10% of adults residing in developed countries are currently taking statins and it is predicted the number will grow as the populations of such countries continue to age and maintain unhealthy lifestyles (2). A significant barrier to statin therapy is muscle toxicity which is associated with elevated systemic drug exposure. Up to 10% of individuals will experience statin-induced muscle pain or weakness, and in rare cases, life-threatening rhabdomyolysis occurs (3-5). Currently, we do not fully understand the drug exposure necessary for optimal statin therapy, making it difficult to predict an individual's dose requirement to maximize LDL-C lowering, while minimizing the risk for muscle injury.

Remarkably few data are available regarding interpatient variability in plasma statin level, especially considering the number of large multicentre clinical trials of cardiovascular outcomes with statins performed to date. Until recently, drug metabolizing enzymes such as cytochrome P450 enzymes (CYPs) were considered to be the major determinants of statin disposition. However, studies from our laboratory and many others clearly suggest that statins, particularly the pharmacologically active acid forms of statins, are highly dependent on drug transporter proteins for their disposition and efficacy (Figure 7.1) (6-7).

Figure 7.1 Statin transporters in the hepatocyte and enterocyte. Transport mechanisms are required for statin absorption in the intestine, entry into the liver, and elimination through bile. The primary site of action for the statins is the hepatocyte, where the drugs inhibit 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the enzyme catalyzing the rate-limiting step of cholesterol synthesis. Uptake transporters mediating statin absorption and hepatic entry are members of the organic anion transporting polypeptide (OATP) family (gene symbol *SLCO*), while statin efflux is mediated by members of the ATP-binding cassette (ABC) family of transporters. Transporters present in the enterocyte include the uptake transporter OATP2B1 (*SLCO2B1*) and the efflux transporters encoded by the genes *ABCG2* and *ABCB1*. In the hepatocyte, statin uptake is mediated by transporters expressed on the basolateral membrane, including OATP1B1 (*SLCO1B1*), OATP1B3 (*SLCO1B3*), and OATP2B1. Statins are effluxed into the bile by ABC transporters on the canalicular membrane, including *ABCB1, ABCC2,* and *ABCG2*.

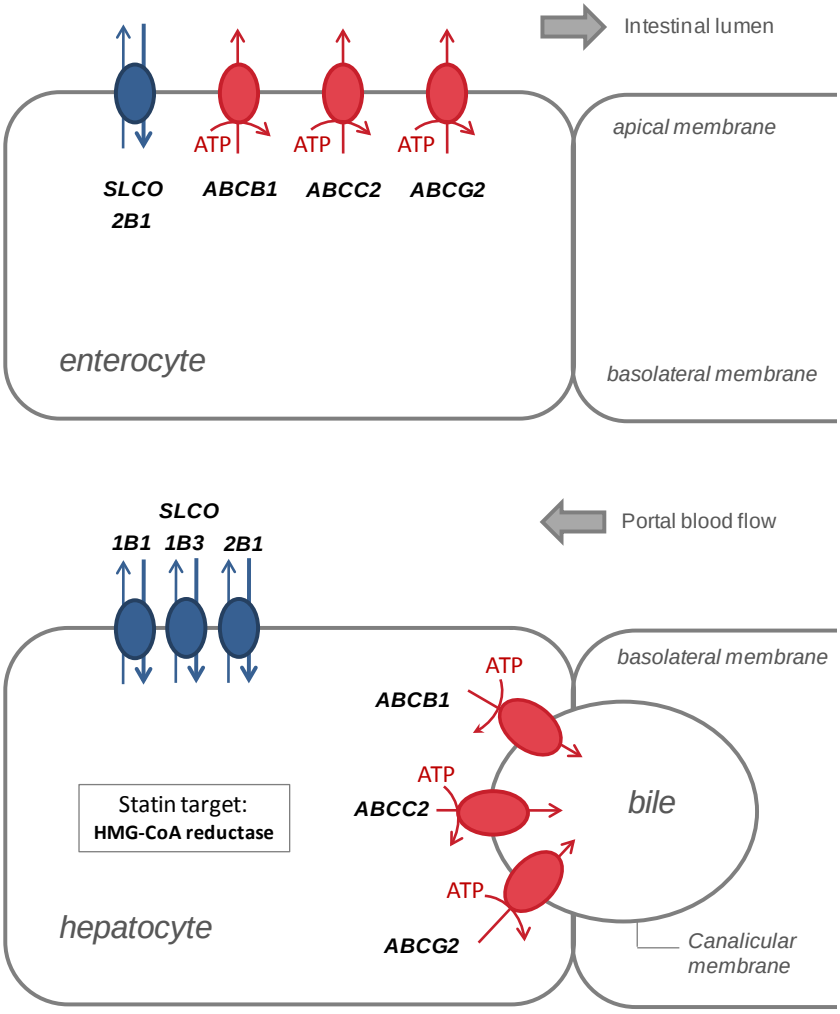

Figure 7.1 Statin transporters in the hepatocyte and the enterocyte

The relationship between drug transporter polymorphisms and plasma statin concentration in the real world clinical situation is not presently understood. Thus, our objective was to characterize in a patient population the interindividual variability in statin concentration and the relative importance of uptake and efflux transporter polymorphisms and clinical variables to plasma statin concentration. We measured 4β-hydroxycholesterol concentration as a marker of CYP3A metabolic activity in vivo. In addition, we measured lathosterol concentration to assess the efficacy of statin-mediated inhibition of endogenous cholesterol synthesis, and its relationship to statin concentration. Taken together, these data describe the relative contribution of transport genetics and metabolism to the explainable interindividual variability in statin pharmacokinetics and response.

7.2 Methods

7.2.1 Study population

We prospectively invited outpatients at London Health Sciences Center (London, Canada) aged 18 years and older who were taking a stable dose of atorvastatin or rosuvastatin to participate. The study was conducted between August 2009 and May 2011. A single venous 8 mL blood sample was drawn into EDTA-containing tubes, and placed immediately on ice. Samples were centrifuged 2,000 x g for 10 minutes; plasma was collected and stored at -80 °C until further analysis. Genomic DNA was isolated from blood samples using the Gentra Puregene extraction kit (Qiagen, Alameda, CA, USA). A detailed medical history was obtained, and the time the individual last took their

oral statin dose was recorded. Ethnicity was self-reported. LDL-C response was defined by attainment of LDL-C target values according to the 2009 Canadian Lipid Guidelines (1) and by the clinical judgment of the treating physician. All subjects provided informed written consent. The study protocol was approved by the Research Ethics Board of the University of Western Ontario (London, Canada).

7.2.2 Determination of plasma statin concentration

All chemical and deuterated standards were obtained from Toronto Research Chemicals (North York, Canada). Plasma aliquots of 100 µL were precipitated in 300 µL acetonitrile containing internal standard d5-atorvastatin or d6-rosuvastatin, and centrifuged at 14,000 rpm for 20 minutes at 4 °C. The supernatant was diluted 1:1 in 0.05% formic acid. Analytes were separated using mobile phases 0.05% formic acid in water and 0.05% formic acid in acetonitrile, starting at a ratio of 70:30, with a gradient to ratio of 10:90. Concentrations of rosuvastatin and atorvastatin were measured with by liquid chromatography-mass spectrometry (LCMS) instrumentation and transitions as previously described (8).

7.2.3 Determination of lathosterol and 4β-hydroxycholesterol concentrations

Sterol concentrations were measured according to published methods for LCMS (9-10). Lathosterol, 4β-hydroxycholesterol, and 4β-hydroxycholesterol-d7 were obtained from

Avanti Polar Lipids (Alabaster, Alabama), and lathosterol-d4 was obtained from CDN Isotopes (Pointe-Claire, Canada). All other chemicals for were obtained from Sigma-Aldrich (St. Louis, MO). Standard curves ranging from 0-50 µg/mL lathosterol were prepared in 1% fatty acid-free bovine serum albumin in phosphate-buffered saline. Aliquots of 50 µL of plasma or standard curve were saponified in 1 mL of 1M KOH in ethanol for 1 hour at 37 °C. The samples were extracted twice, in 750 µL of hexanes each time. After evaporation at 80 °C to dryness, a mixture of the following derivatization reagents was added to each sample: 15 mg 2-methyl-6-nitrobenzoic anhydride, 4.5 mg 4-dimethylaminopyridine, 12 mg picolinic acid, 225 µL pyridine, and 30 µL triethylamine. Samples were incubated with the derivatization reagents at 80 °C for 1 hour, extracted in 1 mL of hexanes, and evaporated at 80 °C to dryness. Samples were reconstituted in 20 µL 0.9% NaCl and 80 µL water; 20 µL of sample was injected on an Eclipse Plus C18 column (1.8 µm pore size; 2.1 x 100mm; Agilent Technologies, Mississauga, Canada) attached to an Agilent 1290 Infinity ultra high pressure liquid chromatography system (Agilent Technologies) coupled with a TSQ Quantum triple-quadrupole mass spectrometer (Thermo Scientific). Analytes were separated and eluted with a gradient from 80% to 98% methanol:acetonitrile (1:1). The transition used for lathosterol was m/z 555.3 to 513.8. The transition used for 4β-hydroxycholesterol was m/z 635.4 to 146.5. Interday variability was less than 25% for lathosterol and less than 30% for 4β-hydroxycholesterol, at relevant concentrations.

7.2.4 Determination of total cholesterol

Total cholesterol was measured by the enzymatic colorimetric method, using the Cholesterol E kit from Wako (Richmond, VA). Samples were measured in triplicate using the microplate procedure, according to manufacturer's directions.

7.2.5 Genotyping

Genotype was determined by TaqMan assay (Applied Biosystems, Foster City, CA) for uptake transporter polymorphisms *SLCO1B1* c.388A>G (rs2306283); *SLCO1B1* c.521T>C (rs4149056); *SLCO1B3* c.699G>A (rs7311358); *SLCO2B1* c.935G>A (rs12422149), and efflux transporter polymorphisms *ABCB1* c.3435C>T (rs1045642); *ABCC2* c.1249G>A (rs2273697); and *ABCG2* c.421C>A (rs2231142). For the atorvastatin group, polymorphisms in the drug metabolizing enzymes CYP3A4 (rs35599367) and CYP3A5 (rs776746) were assessed.

7.2.6 Statistical analysis

Statistical analysis was completed using the statistical software R (11). Differences in statin concentration with respect to each dose group were assessed by Tukey's multiple comparisons tests. For multiple linear regression analysis, statin concentration was log-transformed to adjust for right-skew. Only those patients with blood sampling times after the t_{max} of the statin were included (1.5 hours and 4.0 hours for atorvastatin and rosuvastatin, respectively (8)). Different genetic models–dominant, co-dominant,

recessive, and additive models–were considered for each transporter polymorphism and the model that best described the fit with log-transformed statin concentration or lathosterol concentration was chosen. Each polymorphism was assessed for association with log statin concentration with a cut-off p-value of 0.20 for further inclusion in the multiple linear regression model. *SLCO1B1* c.521T>C and c.388A>G, and *ABCG2* c.421C>A, were included in the model as additive models. All models were adjusted for age, gender, body mass index (BMI), ethnicity, statin dose, and hours from last dose. Of these variables, age, dose, and time from last dose were statistically significant. Next, the number of concomitant medications or presence of the specific medications ezetimibe, niacin, and fibrate were assessed for their contribution to the model and retained if $p < 0.20$. 4β-hydroxycholesterol values were similarly introduced into the model. In the final model, only those variables with $p < 0.05$ were retained. For each final model, analysis of variance was performed, and the explainable variability was derived from all variables excluding dose and time from last dose.

Maximum doses predicted to result in atorvastatin or rosuvastatin concentrations less than the 90^{th} percentile were calculated based on our linear regression models. The 90^{th} percentile was defined by the atorvastatin or rosuvastatin concentrations measured in our population and adjusted for the time of the blood sampling. Predicted concentration was calculated for a hypothetical Caucasian patient of our average population height and weight, and in the case of atorvastatin, 4β-hydroxycholesterol concentration. The difference between concentrations predicted for male and female patients were divided equally. Age was rounded to the nearest 5-year interval.

7.3 Results

7.3.1 Patient characteristics

The patients' baseline characteristics are summarized in Table 7.1. In total, 299 patients were enrolled in the study, with 134 taking atorvastatin and 165 patients on rosuvastatin therapy. Of these patients, 3 taking rosuvastatin and 6 taking atorvastatin had undetectable statin levels, and were excluded from further analysis. Two patients taking rosuvastatin were excluded from lathosterol-related analysis, due to inability to measure lathosterol or total cholesterol.

7.3.2 Rosuvastatin concentration

We observed up to 45-fold variability in plasma rosuvastatin concentration among individuals on the same dose (Figure 7.2A). In patients taking 5, 10, 20, or 40 mg rosuvastatin daily, mean plasma concentration of rosuvastatin was 1.6 ng/mL (SD 1.8), 3.5 ng/mL (2.9), 6.3 ng/mL (5.3), and 9.8 ng/mL (8.6), respectively. There was a significant difference in plasma rosuvastatin concentration between those taking 5 mg vs. 20 mg ($p < 0.01$) and 40 mg ($p < 0.0001$); 10 mg vs. 20 mg ($p < 0.05$) and 40 mg ($p < 0.001$); and 20 mg vs. 40 mg ($p < 0.05$; Figure 7.2A).

Table 7.1 Population characteristics

	Atorvastatin	Rosuvastatin
Number of patients	134	165
Male	83 (61.9%)	115 (69.7%)
Age at enrolment (years)	58.8 (12.9)	57.0 (12.7)
Caucasian	113 (83.7%)	143 (86.7%)
Body mass index (kg/m^2)	29.0 (5.2)	30.1 (6.8)
Number of concomitant medications	4.9 (3.1)	4.7 (3.1)
Statin dose (mg/kg)	0.45 (0.31)	0.22 (0.15)
5 mg	--	24 (14.5%)
10 mg	22 (16.4%)	52 (31.5%)
20 mg	30 (22.4%)	47 (28.4%)
40 mg	58 (43.2%)	38 (23.0%)
80 mg	23 (17.1%)	--
Other	1 (0.7%)	4 (2.4%)
Hours from last dose	12.9 (5.0)	11.5 (5.3)
4β-hydroxycholesterol (ng/mL)	22.0 (14.1)	18.7 (11.9)
Lathosterol (μg/mL)	3.9 (2.1)	3.4 (2.2)
Minor allelic frequency		
ABCG2 c.421A	25/268 (9.3%)	36/330 (10.9%)
SLCO1B1 c.388G	119/268 (44.4%)	145/330 (43.9%)
SLCO1B1 c.521C	30/268 (11.2%)	61/330 (18.5%)

Data are number (%) or mean (S.D.)

Figure 7.2 Atorvastatin (A) plasma concentration in patients taking 10, 20, 40, or 80 mg daily, and rosuvastatin (B) plasma concentration in patients taking 5, 10, 20, or 40 mg daily. Blood samples were collected within 0 to 24 hours of the last oral dose. Levels are presented as box and whisker plots with the whiskers depicting the 5^{th} and 95^{th} percentile; means are depicted by +. *$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

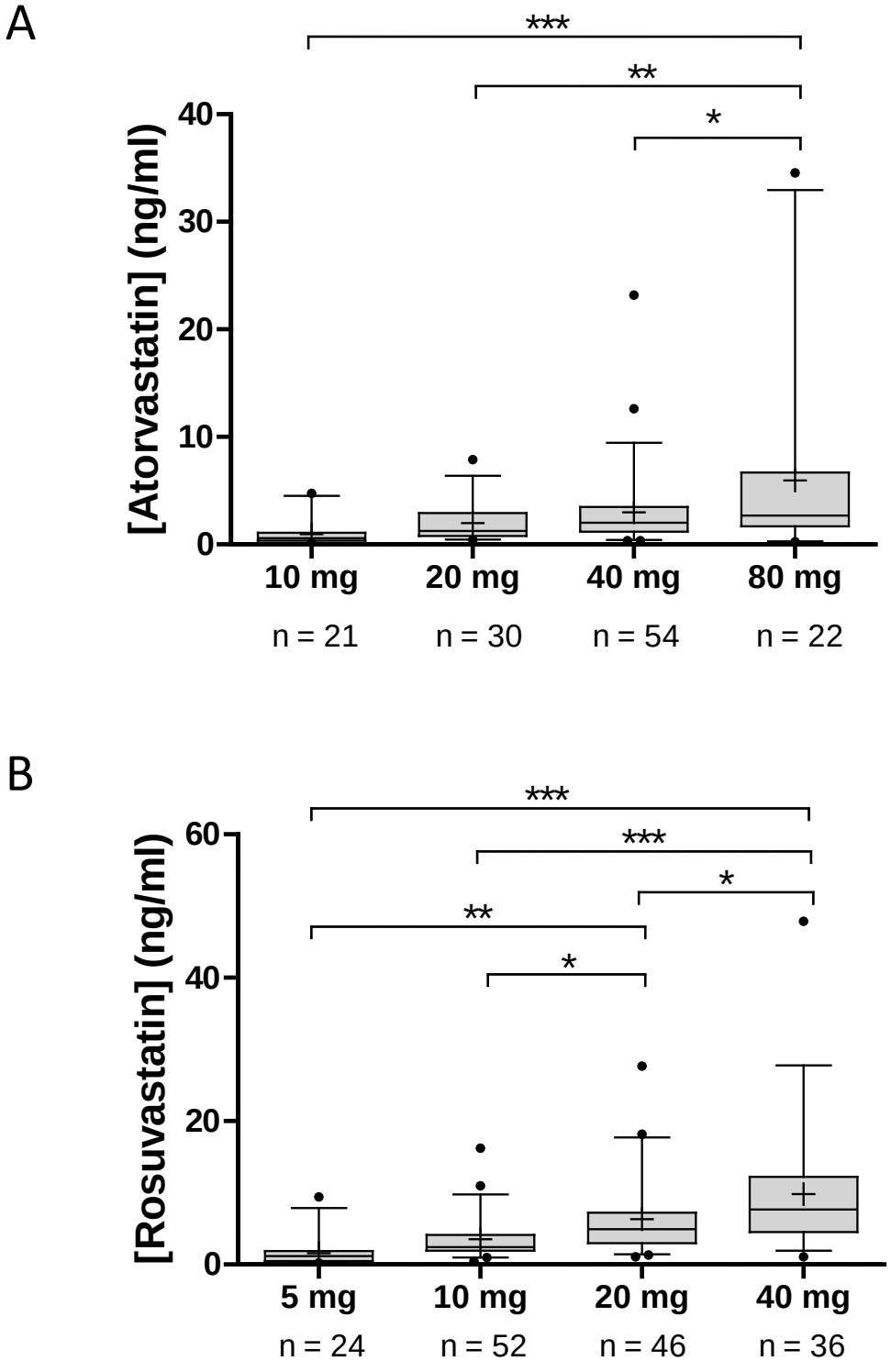

Figure 7.2 Plasma atorvastatin and rosuvastatin concentrations in patients

In order to assess the association of clinical and pharmacogenetic variables to the rosuvastatin levels observed, we performed multiple linear regression analysis. Only those patients with blood drawn at least four hours after their last oral dose were included in this analysis (n = 130). Multiple linear regression analysis indicated that plasma rosuvastatin concentration was higher in individuals with the reduced function hepatic uptake transporter allele *SLCO1B1* c.521C ($p < 0.0001$), and the reduced function efflux transporter polymorphism *ABCG2* c.421A ($p < 0.05$). Age also contributed to plasma rosuvastatin level ($p < 0.01$) (Table 7.2). The adjusted R^2 value of the model was 0.56. Polymorphisms in transporter genes *SLCO1B1* and *ABCG2* contributed to 88% of the explainable variability.

7.3.3 Atorvastatin concentration

Similar to rosuvastatin, we observed 45-fold or higher variability between patients on the same daily atorvastatin dose (Figure 7.2B). In patients taking 10, 20, 40, or 80 mg atorvastatin daily, mean plasma concentration of atorvastatin was 0.9 ng/mL (SD 1.0), 2.0 ng/mL (1.7), 3.0 ng/mL (3.5), and 6.0 ng/mL (8.2), respectively. There was a significant difference in plasma atorvastatin concentration between those taking 10 mg, 20 mg, or 40 mg vs. 80 mg ($p < 0.001$, $p < 0.01$, and $p < 0.05$, respectively; Figure 7.2B).

Multiple linear regression analysis indicated that plasma atorvastatin concentration was higher in individuals with the *SLCO1B1* c.521C allele ($p < 0.05$) but lower in those

Table 7.2 Rosuvastatin linear regression coefficients (n = 130)

	Variable	Effect (B)	p value
1	Age (yr)	0.012	0.005
2	*SLCO1B1* c.521T>C	0.413	4.48e-05
3	*ABCG2* c.421C>A	0.310	0.02

Adjusted for gender, ethnicity, BMI, dose, and time from last dose. Dose and time from last dose were also significant in this model ($p < 2e^{-16}$ and $p = 0.0003$, respectively)

Table 7.3 Atorvastatin linear regression coefficients (n = 128)

	Variable	Effect (B)	p value
1	Age (yr)	0.018	0.002
2	4β-hydroxycholesterol	-0.015	0.006
3	*SLCO1B1* c.521T>C	0.339	0.020
4	*SLCO1B1* c.388A>G	-0.278	0.009

Adjusted for gender, ethnicity, BMI, dose, and time from last dose. Dose and time from last dose were significant in this model ($p = 1.01e^{-9}$ and $p = 2.44e^{-9}$, respectively)

Table 7.4 Lathosterol linear regression coefficients

	Variable	Effect (B)	p value
Atorvastatin-treated patients (n = 128)			
1	Atorvastatin dose (mg)	-0.02	0.009
2	Total cholesterol (mmol/L)	0.23	0.032
3	Ezetimibe use	0.96	0.012
Rosuvastatin-treated patients (n = 128)			
1	Total cholesterol (mmol/L)	0.54	$7.62e^{-6}$
2	Ezetimibe use	1.70	$2.22e^{-10}$

Adjusted for gender, age, ethnicity, and BMI.

7.3.5 LDL-C lowering response to rosuvastatin

Despite the lack of association between lathosterol level and statin level, some insight can be gained from this rare opportunity to examine lipid-lowering response in combination with plasma statin concentration. At our institution, individuals who fail to reach their LDL-C target with high dose atorvastatin are typically switched to high dose rosuvastatin, a more potent compound. Thus we examined the rosuvastatin acid concentration of those patients taking 40 mg rosuvastatin as no higher dose or more potent statin is available. We included patients with blood taken 9 to 24 hours post-dose, to be within the linear range of statin elimination and to minimize the variability associated with the peak statin absorption. In patients taking 40 mg rosuvastatin daily who are not at target LDL-C (n = 12), the mean plasma concentration was 9.183 ng/mL (SD 1.6; 13.6 hours post dose) compared with a mean plasma concentration of 7.497 ng/mL (SD 1.8; 13.7 hours post dose) for those who were at target (n = 13); the difference between the two groups was not statistically significant (p = 0.45). There was also a trend toward decreased lathosterol level in those individuals at target compared with those not at target (3.4 µg/mL (SD 0.49) vs. 4.7 µg/mL (0.45), p = 0.065). Notably, there is a higher proportion of *SLCO1B1* c.521T>C variants in the non-responders (8 of 12 patients are *SLCO1B1* c.521CT heterozygotes) vs. responders (3 of 13 heterozygotes).

7.4 Discussion

This study investigated the relationship between common drug transporter polymorphisms and plasma concentrations of atorvastatin and rosuvastatin in a real world

population. We found a marked, 45-fold interpatient variability in observed plasma level, especially at the higher doses. In our clinical situation, where statin dose has been titrated to effect, statin transporter polymorphisms are associated with a detectable change in statin level. Indeed, nearly 90% of the explainable variability in rosuvastatin concentration can be accounted for by two reduced function transporter polymorphisms, in the uptake transporter *SLCO1B1* and the efflux transporter *ABCG2*. In contrast, explainable variability in atorvastatin level is almost equally divided between two polymorphisms in *SLCO1B1*, and the activity of CYP3A as measured by 4β-hydroxycholesterol concentration.

Indeed, genetic polymorphisms in transport proteins contribute to interindividual variation in exposure to a number of drugs, including the statins (12-14). In 2001, our group first identified functionally relevant single nucleotide polymorphisms (SNPs) in a hepatic statin uptake transporter, organic anion-transporting polypeptide 1B1 (OATP1B1/ gene *SLCO1B1* (previously known as OATP-C or OATP2)) (15). Healthy subjects harboring particular *SLCO1B1* SNPs had higher plasma concentrations of such statins as atorvastatin, rosuvastatin, simvastatin, pravastatin, and pitavastatin (16-19). Importantly, a genome-wide analysis revealed an association between susceptibility to biochemical myopathy (primarily elevated serum creatine kinase concentration) on high-dose simvastatin and a common reduced-function variant in *SLCO1B1*, namely c.521T>C (rs4149056) (20), which we had earlier described (15). *SLCO1B1* c.521T>C has also been associated with reduced LDL-C lowering response to rosuvastatin therapy (21).

The other *SLCO1B1* polymorphism genotyped, c.388A>G (rs2306283), appears, in vitro, to have activity equivalent to the reference sequence (15), and has been shown in some, but not all, healthy volunteer studies to be associated with a trend towards lower plasma atorvastatin level (19; 22). The presence of this common polymorphism would be predicted to be beneficial for statin therapy as atorvastatin would be taken up more avidly into liver, in turn lowering circulating plasma statin level while increasing hepatic concentration and inhibition of HMG-CoA reductase. Interestingly, the SEARCH study showed a link between this SNP and reduced risk for simvastatin-associated myopathy (20).

Polymorphisms in the ATP-binding cassette (ABC) efflux transporter *ABCG2* have been associated with higher rosuvastatin concentration in healthy volunteers (23) and recently, with improved lipid lowering response in Korean subjects (24) and Caucasians (21; 25). The effect of reduced activity *ABCG2* polymorphism on rosuvastatin concentration suggests increased statin exposure is the mechanism resulting in the augmented lipid lowering response observed by other studies. *ABCG*2 polymorphisms are much more prevalent in the Asian population and these frequency differences may account for some of the ethnicity-dependent differences observed in studies of healthy volunteers of Asian ethnicity (22). Moreover, in Asian countries such as Japan, the maximum approved dose of rosuvastatin is 20 mg/day compared with 40 mg/day in North American and European countries. Since the increase in rosuvastatin exposure is not strictly related to environment (22), physicians in North America and Europe treating patients of Asian

descent should be particularly aware that the maximum recommended dose of 40 mg/day may not be appropriate.

It has long been recognized that there is significant interindividual variation in CYP3A activity; however, the genetic basis for this variability has remained elusive. 4β-hydroxycholesterol is produced by CYP3A enzymes from cholesterol, and has been proposed to be a marker of CYP3A activity in vivo (26-27). In our population, 4β-hydroxycholesterol level was associated with atorvastatin but not rosuvastatin concentration. Thus individuals with low 4β-hydroxycholesterol and CYP3A activity may be at risk for higher atorvastatin concentration, and those with high levels may have lower atorvastatin concentration but still have therapeutic benefit, as the hydroxylated atorvastatin metabolites formed by CYP3A are active in the inhibition of HMG-CoA reductase. Numerous drug interaction studies have described increased risk of adverse events resulting from the concomitant use of CYP3A inhibitors and statins metabolized by CYP3A, particularly atorvastatin and simvastatin. The US Food and Drug Administration recommendations advocate for a reduced dose of these statins if moderate CYP3A inhibitors are prescribed, and for some potent CYP3A inhibitors, contraindicate their use entirely (28).

Finally, our study identified age as a significant factor in predicting the concentrations of atorvastatin and rosuvastatin in patients. Age has been recognized as a clinical risk factor for statin-induced muscle toxicity (3; 29). In early pharmacokinetic studies, age was associated with increased exposure to atorvastatin (30), but not rosuvastatin (31).

Rosuvastatin clearance, however, is partially mediated by tubular secretion in the kidney, thus the reduced renal function associated with advanced age may account for this effect (32). Older patients are also more likely to take more medications, though the number of comedications was not a significant predictor of atorvastatin or rosuvastatin concentrations in our population.

Lathosterol is a late intermediate in cholesterol synthesis that can be used to measure the efficacy of statin-mediated HMG-CoA reductase inhibition (33-34). In our population, plasma concentrations of atorvastatin and rosuvastatin did not correlate with lathosterol levels. This suggests statin concentration in the liver, not the plasma, is the most important factor in determining the inhibition of HMG-CoA reductase. In our population, we did not observe an association between *ABCG2* c.421C>A polymorphism and lathosterol concentration. This may arise from the fact that patients in our study were titrated to the dose required to achieve recommended LDL-C lowering. Ezetimibe is a cholesterol absorption inhibitor that has been previously associated with lathosterol level (35); here we observed that lathosterol level is increased even in patients taking statins, which limit lathosterol synthesis by inhibiting HMG-CoA reductase. This observation indicates the balance of cholesterol absorption and synthesis is tightly regulated and suggests there may be a limit to the amount that cholesterol can be reduced.

Here we present the range of atorvastatin and rosuvastatin concentrations in a patient population, providing a framework by which to assess normal variability in statin concentration, and to identify the relationship between statin exposure and common statin

transporter polymorphisms. In Figure 7.3, we describe maximum atorvastatin and rosuvastatin doses that, based on a patient's age and transporter genotype, are predicted to result in plasma concentration that remains lower than the 90th percentile, thus reducing the risk for statin-induced muscle toxicity. While several groups have called for transporter genetics-guided statin dosing (6; 20; 24-25; 36) to our knowledge, this study is the first to propose guidelines based on interindividual differences in statin concentration. These guidelines provide a maximum starting dose in order to reduce the risk for high plasma statin concentration. Among those patients that fail to attain target plasma LDL-C, these guidelines suggest a maximum dose for up-titration, and indicate an appropriate maximum dose if switching between atorvastatin and rosuvastatin. Controlled, randomized trials are required to determine whether statin myopathy is reduced if statins are prescribed using this approach. In summary, this initial report of prospectively assessed plasma statin level and transporter genotypes in a patient care setting creates a framework for individualized statin selection and dosing.

Figure 7.3 Rosuvastatin and atorvastatin dosing decision support algorithm. Doses are the maximum doses that result in a predicted rosuvastatin or atorvastatin concentration that is less than the 90th percentile. In patients taking atorvastatin, dose should be lowered if the patient is taking a CYP3A4 inhibitor, including an antifungal, macrolide antibiotic, or HIV protease inhibitor. The OATP inhibitors cyclosporine and gemfibrozil have also been associated with risk for statin-induced muscle toxicity; a dose reduction should be considered for both atorvastatin and rosuvastatin if cyclosporine or gemfibrozil are also prescribed.

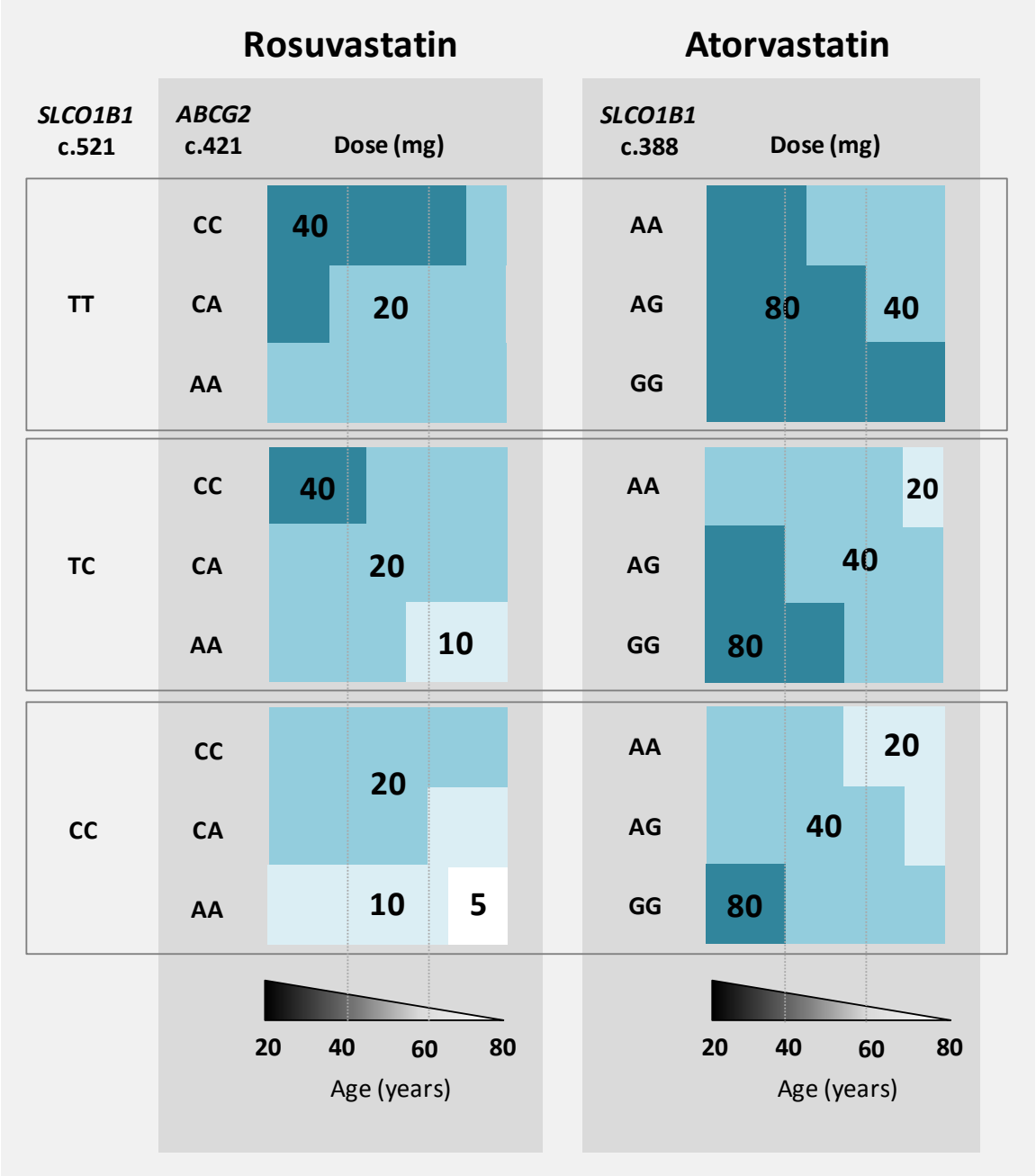

Figure 7.3 Rosuvastatin and atorvastatin decision support algorithm

7.5 References

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8 DISCUSSION AND CONCLUSIONS

8.1 Summary and Discussion

8.1.1 Chapter Five

The aim of this chapter was to identify the molecular determinants of cholecystokinin-8 (CCK-8) transport by the organic anion-transporting polypeptide (OATP/*SLCO*) 1B subfamily. We hypothesized that mutation of three divergent amino acids in OATP1B1 to the corresponding sequence of OATP1B3 would confer CCK-8 transport to OATP1B1. These mutations were A45G, L545S, and T615I, in transmembrane helix (TM) 1, TM10, and extracellular loop 6, respectively. A triple mutation of all three residues in OATP1B1 led to a profound gain of CCK-8 transport, from the less than 1% of OATP1B3 transport that is observed for wild-type OATP1B1, to over 15% of OATP1B3 transport, indicating that a synergistic interaction between all three domains of the transporter is essential for OATP1B1-mediated CCK-8 transport. From our studies, it appears that the amino acids we identified are not essential for the transport of the OATP1B substrates estrone sulfate and atorvastatin. In a report published after this one, TM2 was identified as an important determinant of estrone sulfate uptake by OATP1B1 (1); this TM was not identified in our experiments as a region that is important for the transport of CCK-8.

8.1.2 Chapter Six

In this chapter, we hypothesized that Oatp1b2 is involved in the hepatic uptake of atorvastatin, rosuvastatin, and simvastatin in mice. We observed that Oatp1b2$^{-/-}$ mice had significantly lower liver-to-plasma ratios of atorvastatin and rosuvastatin than wild-type

mice, indicating that Oatp1b2 is important for the hepatic uptake of atorvastatin and rosuvastatin in these animals. In contrast, we did not observe a significant difference in the liver-to-plasma ratio of simvastatin acid. This was unexpected, given the importance of OATP1B1 polymorphisms to simvastatin pharmacokinetics in humans (2). This observation could be a result of species-related differences in transporter function; it is possible that simvastatin acid is not a good substrate for Oatp1b2.

In this chapter we also investigated the intraindividual variability in the pharmacokinetics of atorvastatin, rosuvastatin, and simvastatin. We hypothesized that exposure to atorvastatin, rosuvastatin, and simvastatin would be different in the same individual, as measured by rank order of area under the curve (AUC) for each of the compounds. Indeed, we did not observe a correlation between rosuvastatin AUC and atorvastatin or simvastatin AUC. In contrast, the AUCs of atorvastatin and simvastatin were predictive of each other; that is, those individuals with the highest exposure to atorvastatin or simvastatin were likely to have a relatively high exposure to the other drug. These results suggest that simvastatin and atorvastatin share an important mechanism of elimination, likely related to their common CYP3A4-mediated metabolism.

8.1.3 Chapter Seven

In Chapter Seven, we hypothesized that polymorphisms in drug transporters and metabolizing enzymes would be associated with atorvastatin and rosuvastatin concentrations in patients. Indeed, we observed an association between *SLCO1B1*

c.521T>C and *ABCG2* c.421C>A, and rosuvastatin concentration. In patients taking atorvastatin, we observed an association between atorvastatin concentration and *SLCO1B1* c.388A>G and *SLCO1B1* c.521T>C. The effect of the *SLCO1B1* c.388A>G polymorphism was in the opposite direction to the effect of *SLCO1B1* c.521T>C, indicating there may be a protective effect of the c.388A>G polymorphism in those individuals with both variants. In addition, atorvastatin level was associated with 4β-hydroxycholesterol concentration, an in vivo marker of CYP3A activity.

We also hypothesized that lathosterol level would be associated with statin level and with statin transporter polymorphisms that are associated with statin level. We did not observe a correlation between lathosterol concentration and statin concentration or transporter polymorphisms in our patients. Instead, lathosterol concentration was associated with total cholesterol and ezetimibe use. In the group taking atorvastatin, lathosterol concentration was only modestly associated with atorvastatin dose. It is important to consider that plasma level does not necessarily reflect liver concentration, and it is the liver concentration that determines the extent of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibition, and thus, lipid-lowering effect. The patients in this study were closely monitored for their lipid-lowering response to statin use, with their statin dose titrated to effect; particularly for rosuvastatin, lathosterol concentration between the dosing groups was not significantly different, even as the statin concentration between these doses varied widely.

8.2 Therapeutic Implications

To our knowledge, this is the first data set of its kind to prospectively measure and describe the range of atorvastatin and rosuvastatin concentrations in patients on well-tolerated therapy. These reference values of atorvastatin and rosuvastatin concentrations in a large population of statin-treated patients may be valuable to assess the likelihood, in patients who present with symptoms of an adverse statin response, of a statin pharmacokinetic-mediated origin.

In addition, we showed that *SLCO1B1* and *ABCG2* transporter polymorphisms affect statin concentration in patients receiving statin therapy. The association of *SLCO1B1* c.521T>C with increased atorvastatin and rosuvastatin concentrations supports the hypothesis that increased plasma statin concentration is responsible for increased risk for muscle toxicity in patients harbouring this variant (3). The association of *ABCG2* c.421C>A to increased rosuvastatin concentration is consistent with reports of improved cholesterol-lowering response in patients who carry this reduced-function allele (4-6).

That there is heterogeneity in the factors affecting the pharmacokinetics of members of the statin class is demonstrated by the results of both the clinical study and the study of statin pharmacokinetics in healthy subjects. The data provide some explanation for why switching between statins can be an effective strategy to avoid side effects that might otherwise limit statin use. Taken together, the results of these studies suggest there may be benefit to genetics-based personalization of statin therapy. In Chapter Seven, we propose a statin dose selection algorithm that incorporates our findings in a format that is

practical for use in a clinical setting. We hope this will contribute to the ongoing dialogue regarding genetics-based statin dosing to minimize risk for statin-induced toxicity while maximizing cholesterol-lowering efficacy.

8.3 Future directions

We hypothesize that genetics-based statin dosing would be beneficial to reduce statin-induced side effects and minimize dose switching in a statin-treated population. The genetics-based dosing algorithm proposed in Chapter Seven could be tested by randomizing patients to be prescribed a statin with or without the algorithm to prospectively assess the benefit, if any, of genetics-guided statin dosing.

In addition, it is expected that there are many rare genetic variants with functional consequences that have yet to be fully described (7). A recent report identified total loss of function *SLCO1B1* and *SLCO1B3* alleles that cause Rotor Syndrome, a condition characterized by conjugated hyperbilirubinemia (8). Although this condition is exceedingly rare, it is likely that these individuals would be at significant risk of adverse reactions from statins and other drugs that utilize hepatic OATP-mediated transport, even at low doses of these drugs (8). As whole genome sequencing becomes more widespread, the identification of novel polymorphisms in transporter genes will become more common. There is a need to be able to predict the effect of novel transporter polymorphisms to personalize the selection and dosing of drugs that utilize transport

mechanisms. Molecular studies of transport function, such as our chimera study, provide functional validation of the roles of particular amino acids and may be useful when combined with additional molecular biology experiments and other in silico modeling approaches.

8.4 Conclusions

There is growing recognition of the role of drug transport proteins in drug absorption and distribution, and that interindividual variability in transport activity can lead to differences in pharmacokinetics and drug response. The focus of this thesis was the OATPs, an important family of uptake transporters with broad expression and substrate specify, and in particular, on two hepatic OATPs, OATP1B1 and OATP1B3. We chose to study these transporters primarily in the context of the HMG-CoA reductase inhibitors, or statins. These drugs are widely prescribed and highly effective, yet pose relatively common risk for adverse side effects. Importantly, statins are substrates of numerous transporters, including the OATPs.

The first section of this thesis focused on the molecular determinants of OATP1B transporter function, an understanding of which is important in the design of drugs, like statins, that target the liver as their site of action. The second part of the thesis showed the importance of the murine ortholog of the OATP1B family, Oatp1b2, to hepatic statin uptake in mice. Next, we examined the role of transport vs. metabolism in the pharmacokinetics of statins in healthy subjects, and ability to predict exposure to one

statin based on exposure to another. In this study, we showed that metabolism, which affects different statins to varying extents, is still an important contributor to statin pharmacokinetics, even as we better appreciate the importance of transport. Finally, we studied the influence of transporter polymorphisms on statin concentration in statin-treated patients. We observed that polymorphisms in *SLCO1B1* and *ABCG2* were associated with statin concentration but not statin response in our population. Taken together, these studies provide insight into the in vitro and in vivo function of OATPs, and improve our ability to predict variability in the pharmacokinetics of and response to drugs that are transport substrates.

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Appendices

Appendix A: Ethics Approval

Western

January 12, 2009

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2013

Dear Dr. Kim:

Your Animal Use Protocol form entitled:
Pharmacokinetic analysis of OATP1B substrates in Oatp1b2 knockout and wildtype mice
Funding Agency CIHE - Grant #MOP-89753

has been approved by the University Council on Animal Care. This approval is valid from **Jan. 12th, 2009 to Jasnuary 31, 2010.** The protocol number for this project is **#2008-123**.

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

ANIMALS APPROVED FOR 4 Years

Species	Strain	Other Detail	Pain Level	Animal # Total for 4 Years
Mouse	DBA/lacJ Wildtype	6 weeks-1 yr.	B	280
Mouse	Oatp1b2-/-	6 weeks-1 yr. (designed by Jeff Stock at Pfizer Groton Laboratories and provided by Charles River Laboratories, Wilmington; not commercially available)	B	280

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. Approved Protocol - R. Kim, S. Lemay, W. Lagerwerf
Approval Letter - R. Kim, S. Lemay, W. Lagerwerf

Office of Research Ethics

The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim

Review Number: 12646 **Review Date:** September 12, 2006 **Revision Number:**

Protocol Title: The effect of gender, ethnicity and genetic composition on the disposition of rosuvastatin and simvastatin in healthy subjects

Department and Institution: Medicine, London Health Sciences Centre

Sponsor: NIH-NATIONAL INSTITUTE OF HEALTH

Ethics Approval Date: October 23, 2006 **Expiry Date:** September 30, 2007

Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent, study advertisement

Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted full board approval to the above named research study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

This approval shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;

b) all adverse and unexpected experiences or events that are both serious and unexpected;

c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald

Deputy Chair: Susan Hoddinott

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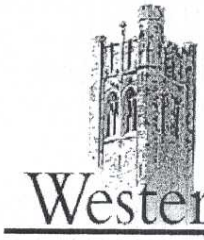

Office of Research Ethics

The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim

Review Number: 12646 **Revision Number:** 2

Review Date: October 16, 2007 **Review Level:** Expedited

Protocol Title: The effect of gender, ethnicity and genetic composition on the disposition of rosuvastatin and simvastatin in healthy subjects

Department and Institution: Medicine, London Health Sciences Centre

Sponsor: NIH-NATIONAL INSTITUTE OF HEALTH

Ethics Approval Date: October 25, 2007 **Expiry Date:** September 30, 2009

Documents Reviewed and Approved: Addition of a study drug, revised inclusion/exclusion criteria and revised letter of information & consent form

Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. John W. McDonald

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Office of Research Ethics

The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim
Review Number: 15586 **Review Level:** Full Board
Review Date: October 21, 2008
Protocol Title: Pharmacogenetics and drug response
Department and Institution: Medicine-Dept of, London Health Sciences Centre
Sponsor:
Ethics Approval Date: December 09, 2008 **Expiry Date:** November 30, 2012
Documents Reviewed and Approved: UWO Protocol, Letter of Information and Consent (v.6 Nov 2008)
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert

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Office of Research Ethics

The University of Western Ontario

Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. R.B. Kim

Review Number: 15586 **Revision Number:** 1

Review Date: March 13, 2009 **Review Level:** Expedited

Protocol Title: Pharmacogenetics and drug response

Department and Institution: Medicine-Dept of, London Health Sciences Centre

Sponsor:

Ethics Approval Date: March 24, 2009 **Expiry Date:** November 30, 2012

Documents Reviewed and Approved: UWO Protocol, Additional Co-Investigators, Letter of Information and Consent (ver. Jan 7/09)

Documents Received for Information:

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Appendix C: Use of transgenic and knockout mouse models to assess solute carrier transporter function[6]

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Introduction

Proteins of the solute carrier (*SLC*) superfamily are expressed in tissues throughout the body and mediate the transport of a diverse array of endogenous and exogenous substrates across biological membranes. Not surprisingly, of particular importance to drug disposition are solute carriers found in the barrier epithelia of the liver, kidney, gut, and brain. In these organs, intracellular concentration of compounds is determined by the activity of transporters expressed on both basolateral and apical membranes and the balance between uptake and efflux transport. In many cases, a single substrate may be transported by a number of proteins, including solute carriers as well as ATP-binding cassette (ABC) transporters.

The interplay between uptake and efflux transport can be a determining factor in drug-induced organ injury. In the liver, toxicity may ensue when loss of efflux transport on the canalicular domain of hepatocytes results in intracellular retention of a compound, thereby causing cellular injury, and failure of solute transporters on the basolateral membrane may result in increased systemic exposure and off-target toxicity. Similarly, inhibition of transport processes in the kidney can lead to increased systemic exposure or to nephrotoxicity resulting from reduced secretion. We now know that transporter function may be influenced by pharmacological inhibitors and functional genetic polymorphisms, as well as variations in expression and activation of regulatory proteins that govern transporter expression. All these have the potential to result in unexpected transporter-mediated drug interactions and interindividual variability in drug response.

Although in vitro cell- and tissue-based systems to test transporter function are useful and well-established, in vivo models have the advantage of providing insight into the contribution of transporters within the whole-system context of multiple transporters, metabolizing enzymes, inhibitors, protein binding, and blood flow. After significant effort, there has recently been progress in using the mouse as a predictive model for studying transporters of relevance to human pharmacokinetics and drug response (Table C.1).

Here we focus on murine models of solute carriers with established roles in drug disposition: the organic anion-transporting polypeptides (OATP/*SLCO*), organic anion transporters (OAT/*SLC22A*), organic cation transporters (OCT/*SLC22A*), and multidrug and toxin extrusion transporters (MATE/*SLC47A*).

Oatp1a and Oatp1b families

Endogenous substrates of OATPs include bile acids and steroid and thyroid hormones, and drug substrates include methotrexate, rifampicin, and statins. OATPs expressed on the basolateral membrane of human hepatocytes, including OATP1B1, OATP1B3, and OATP2B1, are of particular importance in the context of drugs such as statins, which target the liver as their site of action and undergo biliary excretion. In mice, Oatp1a1, Oatp1a4, Oatp1b2, and Oatp2b1 are expressed in liver (1-2) (Figure C.1).

Table C.1 Solute carrier knockout mouse models of drug transport

Gene **Protein**	**Localization**	**Probe drug Dose & route**	**Pharmacokinetic or pharmacodynamic effect of gene deletion**	**Ref**
Slco1b2 Oatp1b2	Hepatocyte (basolateral membrane)	Rifampin 1mg/kg IV	1.7-fold increase in plasma AUC 2.5-fold decrease in liver AUC	(3)
		Rifampin 8µg/h for 24h SC infusion	1.9-fold increase in steady-state plasma concentration 4-fold decrease in steady-state liver concentration	(3)
		Pravastatin 8µg/h for 24h SC infusion	1.8-fold increase in steady-state plasma concentration 1.8-fold decrease in steady-state liver concentration	(3)
		Pravastatin 32µg/h for 24h SC infusion	1.8-fold increase in steady-state plasma concentration 1.9-fold decrease in steady-state liver concentration	(3)
		Phalloidin 2.5mg/kg IP	Oatp1b2$^{-/-}$ mice protected from hepatoxicity: no change in ALT level or histology	(4)
		Microcystin-LR 120µg/kg IP	6 of 6 Oatp1b2$^{-/-}$ mice survived, compared with 3 of 6 wild-type mice	(4)
		Rifampicin 3mg/kg SC	4-fold decrease in liver-to-plasma ratio at 0.5h and 2h	(5)
		Rifamycin SV 3mg/kg SC	No significant change in liver-to-plasma ratio at 0.5h and 2h	(5)
		Cerivastatin 3mg/kg SC	No significant change in liver-to-plasma ratio at 0.5h and 2h	(5)
		Lovastatin acid 3mg/kg SC	1.5-fold decrease in liver-to-plasma ratio at 0.5h and 2h	(5)
		Pravastatin 3mg/kg SC	2.5-fold increase in liver-to-plasma ratio at 2h	(5)
		Simvastatin acid 3mg/kg SC	No significant change in liver-to-plasma ratio at 0.5h and 2h	(5)

Table C.1 continued

Gene **Protein**	**Localization**	**Probe drug Dose & route**	**Pharmacokinetic or pharmacodynamic effect of gene deletion**	**Ref**
Slco1a/1b Oatp1a/1b	Liver, kidney, small intestine, brain, testes	Methotrexate 10mg/kg IV	4.8-fold increase in plasma AUC	(6)
		Methotrexate 10mg/kg oral	3.8-fold increase in plasma AUC	(6)
		Fexofenadine 1mg/kg IV	3.3-fold increase in plasma AUC	(6)
		Fexofenadine 1mg/kg oral	4.6-fold increase in plasma AUC	(6)
		Paclitaxel 10mg/kg IV	2-fold increase in plasma AUC 2-fold decrease in liver AUC	(7)
Slc22a6 Oat1	Kidney (basolateral); Choroid plexus (apical)	Furosemide 0.1-10mg/kg IV	4-fold increase in ED_{50}	(8-9)
		Bendroflume-thiazide 0.003-1mg/kg IV	3-fold increase in ED_{50}	(9)
Slc22a8 Oat3	Kidney (basolateral); Choroid plexus (apical)	Furosemide 0.1-10mg/kg IV	3-fold increase in ED_{50}	(9)
		Bendroflume-thiazide 0.003-1mg/kg IV	2-fold increase in ED_{50}	(9)
		Penicillin G 1.87µg/kg IV	2-fold and 3-fold increase in plasma AUC in male and female mice, respectively	(10)
		Ciprofloxacin 5mg/kg IV	1.25-fold increase in plasma AUC	(11)
		Methotrexate 1.7mg/kg IV	Reduced clearance in female $Oat3^{-/-}$ mice	(12)
		Ro 64-0802 (Oseltamivir) 1mM intracerebral injection	3-fold increase in brain concentration after 2h	(13)

Table C.1 continued

Gene **Protein**	**Localization**	**Probe drug Dose & route**	**Pharmacokinetic or pharmacodynamic effect of gene deletion**	**Ref**
Slc22a1 Oct1	Kidney, liver, intestine (basolateral)	Metformin 5mg/kg IV; 150mg/h/kg IV infusion; 50mg/kg IP (5 days)	30-fold reduction in liver concentration after 10 min; protection from metformin-induced lactic acidosis; no effect on fasting plasma glucose levels	(14);(15); (16)
		Cisplatin 10mg/kg IP	No significant change	(17)
Slc22a2 Oct2	Kidney (basolateral)	Cisplatin 10mg/kg IP	No significant change	(17)
Slc22a1/2 Oct1/2		Cisplatin 10mg/kg IP	2-fold decrease in total urinary excretion, no change in plasma AUC	(17)
Slc47a1 Mate1a/1b	Kidney (apical)	Metformin 5mg/kg IV	2-fold increase in plasma AUC after 60 min	(18)
		Cisplatin 0.5mg/kg IV; 15mg/kg IP	Significant increase in plasma and renal concentration after 1 h; Increase in nephrotoxicity after 3 days	(19)
		Cephalexin 5mg/kg IV	1.5-fold increase in plasma AUC	(20)

Abbreviations used: ALT, alanine transaminase; AUC, area under the curve; CSF, cerebrospinal fluid; ED_{50}, half-maximal effective dose; ICV, intracerebroventicular; IV, intravenous; SC, subcutaneous.

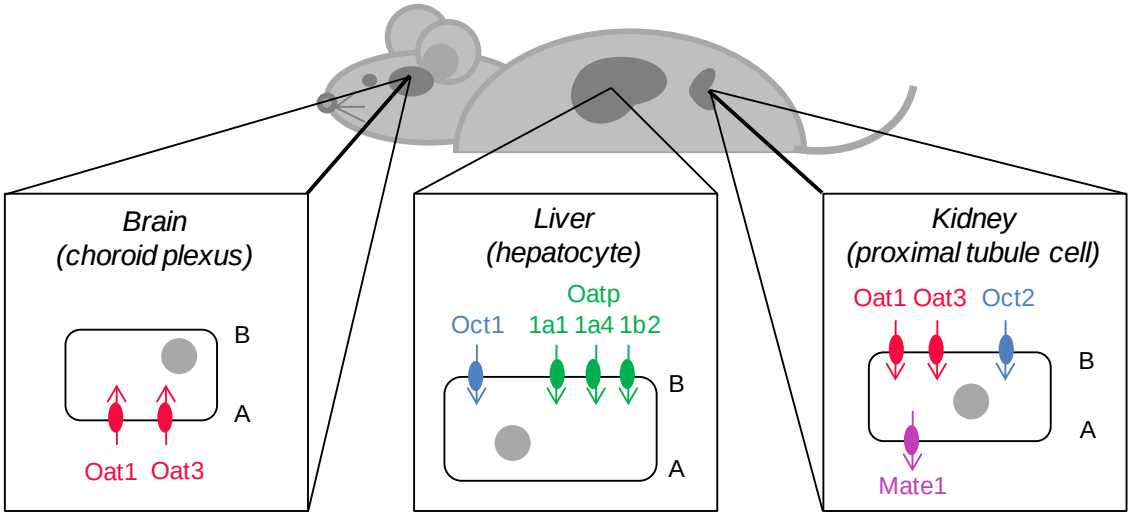

Figure C.1 Localization of expression of solute carrier transporters demonstrated to be relevant to drug disposition in knockout mice

Oat3 is also involved in drug transport at the blood brain barrier. In human hepatocytes, OATP1B1, OATP1B3, and OATP2B1 are predominantly expressed. A, apical; B, basolateral; Mate, multidrug and toxin extrusion transporter; Oat, organic anion transporter; OATP, organic anion-transporting polypeptide; Oct, organic cation transporter.

The first Oatp studied in a knockout mouse model was Oatp1b2 (*Slco1b2*) (3-5). The liver-to-plasma ratios of the prototypical OATP1B substrates pravastatin and rifampin were lower in *Slco1b2*$^{-/-}$ mice as compared with wild-type, indicating that Oatp1b2 is an important determinant of the hepatic clearance of its substrates (3). Furthermore, *Slco1b2*$^{-/-}$ mice had significantly lower liver-to-plasma ratios of lovastatin, but not cerivastatin or simvastatin acid, suggesting that, in vivo, the extent of Oatp1b2 involvement in hepatic statin uptake is variable (5). In addition, *Slco1b2*$^{-/-}$ mice were protected from hepatoxicity induced by phalloidin and microcystin-LR, indicating the critical role for Oatp1b2 in facilitating the entry of toxins into the liver (4).

The lack of straightforward murine orthologs of the human OATP1B subfamily expressed in liver limits the extent to which observations in mice may be extrapolated to humans. In order to overcome this challenge, a transgenic mouse model expressing human OATP1B1 was created, with the mice being fed a semisynthetic diet to downregulate endogenous *Slco* genes. Methotrexate exposure was 1.5-fold lower in OATP1B1-expressing mice than in wild-type ones, indicating a potential role for OATP1B1 in methotrexate elimination in vivo, and demonstrating the utility of a humanized transporter model (21).

More recently, the same group generated a *Slco1a/1b*$^{-/-}$ model by targeted chromosomal locus deletion, eliminating expression of Oatp1a1, Oatp1a4, Oatp1a5, Oatp1a6, and Oatp1b2. Increased plasma levels of glucuronide-conjugated bilirubin and unconjugated bile acids were observed in *Slco1a/1b*$^{-/-}$ mice, indicating the importance of these

transporter subfamilies to bilirubin and bile acid homeostasis. The *Slco1a/1b*$^{-/-}$ mice demonstrated significantly reduced hepatic uptake and elevated systemic exposure to methotrexate and fexofenadine after intravenous or oral administration of these compounds (6). Systemic exposure of paclitaxel, a relatively hydrophobic anticancer drug, was increased twofold in *Slco1a/1b*$^{-/-}$ mice relative to wild-type animals, thereby indicating that Oatps are important to this class of anticancer agents (7), and confirming in vitro study results suggesting that OATPs are capable of transporting taxanes (1).

With regard to the murine Oatp models that are currently available, there are some caveats to bear in mind. Indeed, although the *Slco1b2*$^{-/-}$ mouse eliminates the murine ortholog of the most functionally important OATPs in human liver—OATP1B1 and OATP1B3—the presence of other murine-specific Oatps of the 1a family may mask the true effect of loss of OATP1B function in humans, particularly in view of the data showing that the OATP1A family is expressed in human cholangiocytes, but not in hepatocytes (1). The OATP1B1 humanized model has the advantage of expressing the human OATP1B1 sequence; however, the genetic background of the mouse still contains fully functional Oatps. Moreover, such a model will not recapitulate the role of regulatory proteins such as LXRα and FXR, which are key nuclear receptors governing the expression of OATP1B1 (22). The *Slco1a/1b*$^{-/-}$ mice eliminate compensation by other Oatps in the liver; however, loss of Oatp function in other tissues may influence overall drug disposition and response. Also, current murine Oatp models do not address the impact of reduced-function OATP polymorphisms, such as the relatively common human OATP1B1 variant rs4149056, associated with increased plasma statin concentration and

risk for statin-induced muscle toxicity (1). A transgenic model expressing human OATP variants may prove to be a desirable model system for predicting the in vivo role of SNPs in OATP transporters, particularly for drugs in development.

Oat1 and Oat3

Oat1 (*Slc22a6*) and Oat3 (*Slc22a8*) are expressed on the basolateral membrane of proximal tubule cells in the kidney and the apical membrane of the choroid plexus (Figure C.1). Oat substrates include steroid hormones and biogenic amines and their metabolites, as well as various drugs including anticancer agents, antibiotics, antivirals, and antihypertensives (23). Consistent with predominant Oat expression in the kidney, most substrates are renally excreted.

Oat1$^{-/-}$ and Oat3$^{-/-}$ mice have provided new insights into the in vivo relevance of these transporters in the renal elimination of shared substrates. Kidney slices from Oat3$^{-/-}$ mice revealed that the transport of taurocholate, estrone sulfate, and the prototypical anion para-aminohippurate (PAH) was reduced, whereas no change in transport activity was seen in slices taken from the liver, an organ in which Oat3 is not expressed (24). Evidence for the localization of Oat3 to the apical membrane of the choroid plexus was provided by reduced fluorescein accumulation in Oat3$^{-/-}$ mice (24). Subsequently, results from studies in Oat1$^{-/-}$ mice established Oat1 as being the key transporter of PAH, with evidence of significant loss of transport from renal slices and in vivo clearance estimates (8). Analysis of 60 endogenous anions in Oat1$^{-/-}$ and wild-type mice identified a number of compounds that may rely on Oat1 for renal secretion (8).

More recently, Oat1$^{-/-}$ and Oat3$^{-/-}$ mice have been studied with respect to the pharmacokinetics of their shared drug substrates. Plasma clearance of the antibiotic penicillin G was reduced by one-half in male and two-thirds in female Oat3$^{-/-}$ mice (10). Systemic exposure of the carboxyfluoroquinolone antibiotic ciprofloxacin, commonly used to treat urinary tract infection, was elevated as compared with wild-type animals and was related to a measurable change in clearance in female mice but not in male mice (11). Gender-related differences in methotrexate clearance were also observed (12).

Other recognized Oat drug substrates are loop and thiazide diuretics, given that Oats probably facilitate secretion to their site of action in proximal tubules. Administration of furosemide and bendroflumethiazide to Oat1$^{-/-}$ and Oat3$^{-/-}$ mice resulted in a rightward shift of the dose-naturesis curve, indicating that Oat1 and Oat3 are involved in diuretic secretion in mice, and neither transporter is able to fully compensate for the loss of the other in vivo (8-9).

Finally, the active form of the anti-influenza drug oseltamivir, Ro64-0802, showed higher concentration levels in the brains of Oat3$^{-/-}$ mice after injection into the cerebrum (13). Oat3 is expressed on the abluminal membrane of the blood-brain barrier, facilitating uptake of compounds from the brain. It should be noted that when the compound was administered subcutaneously, the brain-to-plasma ratio of concentration levels did not differ between Oat3$^{-/-}$ and wild-type mice. The exact mechanism(s) underlying these differences remain undefined.

Oct1 and Oct2

Oct1 (*Slc22a1*) is expressed on the basolateral membranes of hepatic, renal, and intestinal epithelia in mice, whereas in humans it is found predominantly in the liver. Oct2 (*Slc22a2*) is expressed on the basolateral membranes of renal proximal tubule cells in humans and rodents (Figure C.1). Oct substrates include monoamine neurotransmitters, the antidiabetic drug metformin, and the prototypical cation tetraethylammonium (TEA) (2).

In $Oct1^{-/-}$ mice, TEA accumulation in liver was reduced sixfold relative to the wild-type animals, and a compensatory increase in renal excretion indicated that other transporters in renal tissue are capable of TEA transport (25). In $Oct2^{-/-}$ mice, TEA pharmacokinetics were similar to those in wild-type animals; however, in $Oct1/2^{-/-}$ mice, renal secretion of TEA was entirely absent (26). Taken together, these results indicate that, at least in mice, Oct1 and Oct2 act together in cation secretion, and loss of function in both transporters may have consequences for disposition of drugs that utilize this carrier system. As noted, in humans, OCT1 expression is limited to the liver; therefore, such an overlap in renal cation secretion is less likely.

Recognition of the clinical significance of OCTs has resulted from studies of metformin, a widely prescribed antidiabetic with variability in clinical efficacy. As compared with wild-type controls, the accumulation of metformin in the livers of $Oct1^{-/-}$ mice was 30-fold less, and intestinal distribution was also reduced, whereas there were no differences with respect to accumulation in the kidney and renal excretion (14). The liver was

identified as the primary organ responsible for metformin-induced lactic acidosis because Oct1$^{-/-}$ mice were seen to be protected from this potentially lethal side effect (15). Further, hepatic uptake of metformin was associated with therapeutic response because wild-type mice showed significantly reduced fasting plasma glucose concentrations whereas Oct1$^{-/-}$ mice did not (16). Consistent with these findings, functional genetic polymorphism in OCT1 resulted in reduced metformin response among genotype-defined healthy human volunteers (16).

OCTs also appear to be important in the organ-specific toxicity of cisplatin, an anticancer drug whose use is often limited by serious side effects, including nephrotoxicity and hearing loss. After administration of cisplatin, there was no significant change in the pharmacokinetics in Oct1$^{-/-}$ or Oct2$^{-/-}$ mice as compared with wild-type controls; however, Oct1/2$^{-/-}$ mice exhibited reduced urinary excretion of cisplatin and reduced nephrotoxicity (17). In another study, Oct1/2$^{-/-}$ mice showed no evidence of cisplatin-induced ototoxicity because of the absence of Oct2 expression in murine cochlear hair cells (27). Furthermore, the OCT2 polymorphism rs316019 was associated with reduced risk of renal toxicity in patients on cisplatin therapy (17). These results suggest that decreased activity of OCT2 may reduce the risk for substrate drug-induced nephro- and ototoxicity. They also raise the possibility of preventing tissue injury by using OCT2-specific inhibitors in patients undergoing treatment with cisplatin.

Mate1

The contribution of the MATE family to cation and zwitterion transport has become appreciated more recently. Mate1 is expressed in the brush border membrane of renal proximal tubules, facilitating excretion of substrates into the renal tubule (Figure C.1). As compared with their wild-type counterparts, Mate1$^{-/-}$ (*Slc47a1*) mice exhibited a twofold increase in systemic exposure to metformin resulting from reduced renal clearance of the drug (18). Cisplatin-induced nephrotoxicity as well as increased plasma and renal concentrations were observed in Mate1$^{-/-}$ mice as compared with wild-type (19). These findings indicate that, in addition to uptake by OCT2, the rate of cisplatin efflux by MATE1 is another determinant of renal toxicity. Finally, reduced renal clearance of the zwitterionic cephalosporin cephalexin was observed in Mate1$^{-/-}$ mice relative to wild-type mice (20). Therefore, in humans, the interplay between OCT2 and MATE1 probably affects the net renal secretion of shared drug substrates.

Insight into endogenous roles for solute carriers

In addition to playing a critical role in the distribution of drugs and other xenobiotics, solute carriers have important endogenous roles related to the tissue-specific transport of signaling molecules. Significant insights into the physiological functions of solute carriers have been gleaned from solute carrier knockout mice. However, a full account of these findings is beyond the scope of this article.

Conclusions

In the future, humanized models, tissue-specific knockouts of solute carriers, and combination knockouts of solute and ABC transporters will provide further insights into the relevance of transporters in vivo. Overall, transporter knockout models are a valuable tool to assess the relative contribution of an individual transporter to drug disposition and response and in some cases, to the risk for organ-specific toxicity. Such models have value both in preclinical studies and to gain better insight into the pharmacokinetics of drugs in current use.

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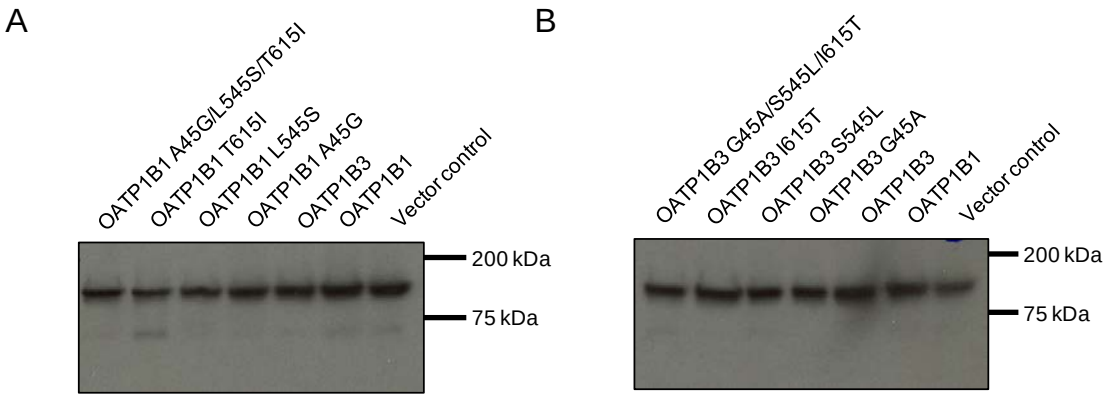
Appendix D: Supplementary Information I

Supplementary Information Table 1.1
Primers used for site-directed mutagenesis of OATP1B1 and OATP1B3.

Mutation	Sequence
OATP1B1_L36F	5'-GGCAGCTCTGTCA**T**TCAGCTTTATTGCTAAGAC-3'
OATP1B1_F38Y	5'-GCTCTGTCACTCAGCT**A**TATTGCTAAGACACTAGG-3'
OATP1B1_T42A	5'-CACTCAGCTTTATTGCTAAG**G**CACTAGGTGC-3'
OATP1B1_A45G	5'-GCTAAGACACTAGGTG**G**AATTATTATGAAAAGTTCCATC-3'
OATP1B1_S50I	5'-GGTGCAATTATTATGAAAA**T**TTCCATCATTCATATAGAACGG-3'
OATP1B1_I53T	5'-GGTGCAATTATTATGAAAAGTTCCATCA**C**TCATATAGAACGGAG-3'
OATP1B1_H54Q	5'-GAAAAGTTCCATCATTCA**A**ATAGAACGGAGATTTGAG -3'
OATP1B1_Y535F	5'-GATGCTTGTACAAGGAAATTTT**T**CTTTTTTGTTGCAATACAAGTC-3'
OATP1B1_F536I	5'-GATGCTTGTACAAGGAAATTTTAC**A**T**C**TTTGTTGCAATACAAGTCTTG-3'
OATP1B1_F537Y	5'-GCTTGTACAAGGAAATTTTACTTTT**A**TGTTGCAATACAAGTCTTG-3'
OATP1B1_L543I	5'-GTTGCAATACAAGTC**A**T**A**AATTTATTTTTCTCTGCACTTGGAGGC-3'
OATP1B1_L545S	5'-GTTGCAATACAAGTCTTGAATT**CT**TTTTTCTCTGCACTTGGAGGC-3'
OATP1B1_F546L	5'-CAATACAAGTCTTGAATTTATT**G**TTCTCTGCACTTGGAGGC-3'
OATP1B1_L550T	5'-GTCTTGAATTTATTTTCTCTGCA**ACA**GGAGGCACCTCACATGTC-3'
OATP1B1_S554T	5'-GCACTTGGAGGCACC**A**CACATGTCATGCTG-3'
OATP1B1_H555F	5'-GCACTTGGAGGCACCTCA**TT**TGTCATGCTGATTG-3'
OATP1B1_V556I	5'-CACTTGGAGGCACCTCACAT**A**TCATGCTGATTG-3'
OATP1B1_M557L	5'-GGCACCTCACATGTC**T**TGCTGATTGTTAAAATTGTTC-3'
OATP1B1_I559T	5'-GCACCTCACATGTCATGCTGA**C**TGTTAAAATTGTTCAACC-3'
OATP1B1_T609A	5'-CAACAACTGTGGC**G**CACGTGGGTCATGTAG-3'
OATP1B1_R610Q	5'-CACCAACAACTGTGGCACAC**AA**GGGTCATGTAGG-3'
OATP1B1_S612A	5'-CTGTGGCACACGTGGG**G**C**T**TGTAGGACATATAATTCC-3'
OATP1B1_T615I	5'-CGTGGGTCATGTAGGA**T**ATATAATTCCACATCATTTTCAAGGG-3'
OATP1B1_T619V	5'-GTGGGTCATGTAGGACATATAATTCC**GT**ATCATTTTCAAGGGTC-3'
OATP1B1_S620F	5'-GTAGGACATATAATTCCACAT**TT**TTTTCAAGGGTCTACTTGGGC-3'
OATP1B1_S622G	5'-GGACATATAATTCCACATCATTT**GG**AAGGGTCTACTTGGGCTTG-3'
OATP1B3_G45A	5'-GCTAAAGCACTAGGTG**C**AATCATTATGAAAATTTCCATC-3'
OATP1B3_S545L	5'-GCAATTCAAGTCATAAACTTATTGTTCTCTGCAACAGGAGG-3'
OATP1B3_I615T	5'-GCACAAGGAGCTTGTAGGA**C**ATATAATTCCGTATTTTTTGG-3'



Supplementary Information Figure 1.1
Western blot of calnexin loading control for OATP1B1 and OATP1B3 mutants are shown in (A) and (B), respectively.

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Publications:

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Invited Oral Presentations:

"Statin transport by hepatic organic anion-transporting polypeptides." Physiology and Pharmacology Seminar Series, University of Western Ontario, London, Ontario, January 16, 2012.

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"Disposition of atorvastatin, rosuvastatin and simvastatin in Oatp1b2$^{-/-}$ mice and intraindividual variability in human subjects." **DeGorter MK**, Urquhart BL, Tirona RG, Kim RB. Canadian Society of Pharmacology and Therapeutics Annual Meeting, Montreal, Quebec. May 26, 2011.

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Poster Presentations:

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"Effect of organic anion transporting polypeptide 1B1 (OATP1B1/*SLCO1B1*) polymorphisms on plasma concentrations of atorvastatin and its metabolites in patients." **DeGorter MK**, Schwarz UI, Tirona RG, Kim RB. Canadian Society of Pharmacology and Therapeutics Annual Meeting, Saskatoon, Saskatchewan, June 1, 2009.

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"Identification of key domains that confer human OATP1B1 and OATP1B3 transporter substrate specificity." **DeGorter MK**, Tirona RG, Kim RB. IXth World Congress on Clinical Pharmacology and Therapeutics, Quebec City, Quebec, July 28, 2008.