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The Role of Drug Transporters in Statin-Induced Myopathy

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A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy

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Abstract

Statins are the first line therapy for treatment and prevention of cardiovascular disease. The 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors, or statins, reduce plasma cholesterol levels by inhibiting the rate-limiting step in cholesterol biosynthesis. While statins are well tolerated, up to 15% of patients develop myopathy, manifesting as muscle aches and pain and in rare cases, potentially life-threatening statin-induced rhabdomyolysis. Given that approximately 3 to 4 million Canadians are treated with statins, an estimated 500,000 patients experience associated skeletal muscle side effects that may prevent the continued treatment of hypercholesterolemia. Despite the prevalence of this side effect, little is known regarding the molecular determinants of statin myopathy. Increased systemic statin exposure is linked to risk of developing myopathy, but the role of skeletal muscle exposure and its relevance to muscle toxicity remains to be determined.

Drug transporter proteins are important determinants of drug absorption, tissue exposure and drug elimination. Statins are substrates of multiple drug transporters and require hepatic uptake to exert their cholesterol lowering effect. However, little is known about the role drug transporters have in the skeletal muscle distribution of statins and their toxicity. We aimed to identify drug transporters in skeletal muscle involved in controlling muscle exposure. We found that the uptake transporter OATP2B1 and three novel statin efflux transporters, MRP1, MRP4 and MRP5, are expressed in skeletal muscle. We demonstrate that OATP2B1 sensitizes muscle to toxicity and MRP1 attenuates toxicity of atorvastatin and rosuvastatin in an *in vitro* skeletal muscle model.

We studied the regulation and function of two transcriptional variants of OATP2B1, demonstrating that these variants had similar function but differential regulation, resulting in ubiquitous expression for OATP2B1 full length form and primarily hepatic expression for the truncated variant. We employed a novel Oatp2b1 knockout mouse model to examine the *in vivo* role of Oatp2b1 in rosuvastatin disposition. We found that Oatp2b1 does not have a significant effect on rosuvastatin pharmacokinetics but the hepatic exposure was increased in Oatp2b1 knockout mice.

Taken together, these studies further our understanding of the *in vitro* and *in vivo* involvement of drug transporters in the context of statin myopathy.

Keywords

Organic anion transporting polypeptides, multidrug resistant associated proteins, drug transporter, drug transporter regulation, statins, statin transport, statin pharmacokinetics, statin myopathy, rhabdomyolysis

Co-Authorship Statement

Chapter 3:

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MJK, and RGT designed the experiments. MJK, BLU, HEMS, CJL and BFL conducted the experiments. MJK and RGT analyzed and interpreted the data. MJK and RGT wrote the manuscript. All authors approved the final version of the manuscript.

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MJK, and RGT designed the experiments. MJK, AJG, and BFL conducted the experiments. MJK, AJG and RGT analyzed and interpreted the data. MJK and RGT wrote the manuscript.

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MJK, and RGT designed the experiments. MJK, SEM, HT, and RGT conducted the experiments. MJK and RGT analyzed and interpreted the data. MJK and RGT wrote the manuscript.

Dedication

In Memory of Hans Knauer

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Abbreviations

ABC	ATP-binding cassette
ADR	adverse drug reaction
ATP	adenosine triphosphate
AUC	area under the curve
AUMC	area under the first moment curve
BCRP	breast cancer resistance protein
BMI	body mass index
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CK	creatine kinase
CL	clearance
C_{max}	maximal concentration
CoQ10	coenzyme Q-10
CVD	cardiovascular disease
CYP	cytochrome P450
DAPI	4',6-diamidino-2-phenylindole
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E1S	estrone 3-sulfate
F	bioavailability
FBS	fetal bovine serum
FDA	Food and Drug Administration
FPP	farnesylpyrophosphate
GGPP	geranylgeranylpyrophosphate
GWAS	genome wide association study
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
HNF4α	hepatocyte nuclear factor 4α
HSMM	human skeletal muscle myoblast

IC_{50}	half maximal inhibitory concentration
IFU	infectious units
IV	intravenous
k_e	terminal rate constant
KHB	Krebs Henseleit Bicarbonate
K_m	substrate concentration producing 50% maximal velocity
KO	knockout
LC-MS/MS	liquid chromatography tandem mass spectrometry
LDL-C	low-density lipoprotein cholesterol
LogD	distribution coefficient
m/z	mass-to-charge ratio
MDR	multidrug resistance
MOI	multiplicity of infection
MRP	multidrug resistance-associated protein
MRT	mean resonance time
MTT	methylthiazolyldiphenyl-tetrazolium bromide
NDA	new drug application
NTCP	sodium-dependent taurocholate co-transporting polypeptide
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
P-gp	P-glycoprotein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PO	per os (by mouth)
qPCR	quantitative polymerase chain reaction
RCT	randomized clinical trials
RCT	randomized controlled trial
rpm	revolutions per minute
RT-PCR	real time polymerase chain reaction
SD	standard deviation

SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SLC	solute carrier
SNP	single nucleotide polymorphism
T 1/2	elimination half life
T_{max}	time of maximum concentration
TSS	transcription start site
UGT	UDP-glucuronosyltransferases
ULN	upper limit of normal
V_{max}	maximal velocity
V_{ss}	volume of distribution at steady-state
w/v	weight per volume
WT	wild type

1 INTRODUCTION

1.1 Overview of Statin Pharmacology

1.1.1 Therapeutic Indications

Cardiovascular disease (CVD), which can result in myocardial infarction or stroke, is a leading cause of death in Canada and the world, accounting for about 30% of all deaths. The economic impact of cardiovascular disease has been estimated at a cost of $22 billion annually, in direct or indirect health care expenses (Genest et al., 2009). Multiple epidemiological studies have confirmed that age, smoking status, diabetes, hypertension, and hypercholesterolemia are major risk factors for the development of CVD. Elevation of low density lipoprotein cholesterol (LDL-C) was first shown in the 1960s by the Framingham Heart Study to be a major risk factor for CVD (Kannel et al., 1961). Since then, compelling evidence from multiple randomized clinical trials (RCTs) has revealed that LDL-C reduction is effective for the treatment of cardiovascular disease. One such class of drugs used to lower LDL-C are the statins, or 3-hydroxy-3-methylglutaryl-coemzyme A (HMG-CoA) reductase inhibitors. Meta analysis of 14 large RCTs has demonstrated that a statin-mediated reduction of 1 mmol/L in LDL-C correlates with 20-25% reduction in risk for major cardiovascular events and a 12% reduction in all cause mortality (Baigent et al., 2005). This has led to wide spread use of statins as first-line therapy for primary and secondary prevention of coronary, cerebral and peripheral artery disease.

1.1.2 Mechanisms of Action

Approximately 70% of cholesterol is produced endogenously in the liver through the cholesterol biosynthetic pathway (Ikonen, 2008). The statin class of drugs acts to reduce the endogenous production of cholesterol in the liver by inhibiting hydroxyl-methylglutaryl-CoA (HMG-CoA) reductase and synthesis of mevalonate. This is the rate limiting step in the 30 step cholesterol biosynthetic pathway (Endo, 2010). The reduction in hepatic cholesterol synthesis results in the upregulation of the LDL-receptor on surface of hepatocytes and ultimately leads to a reduction in circulating LDL-C (Hafner et al., 2011). A simplified diagram showing statin inhibition of the cholesterol biosynthetic pathway is presented in Figure 1.1.

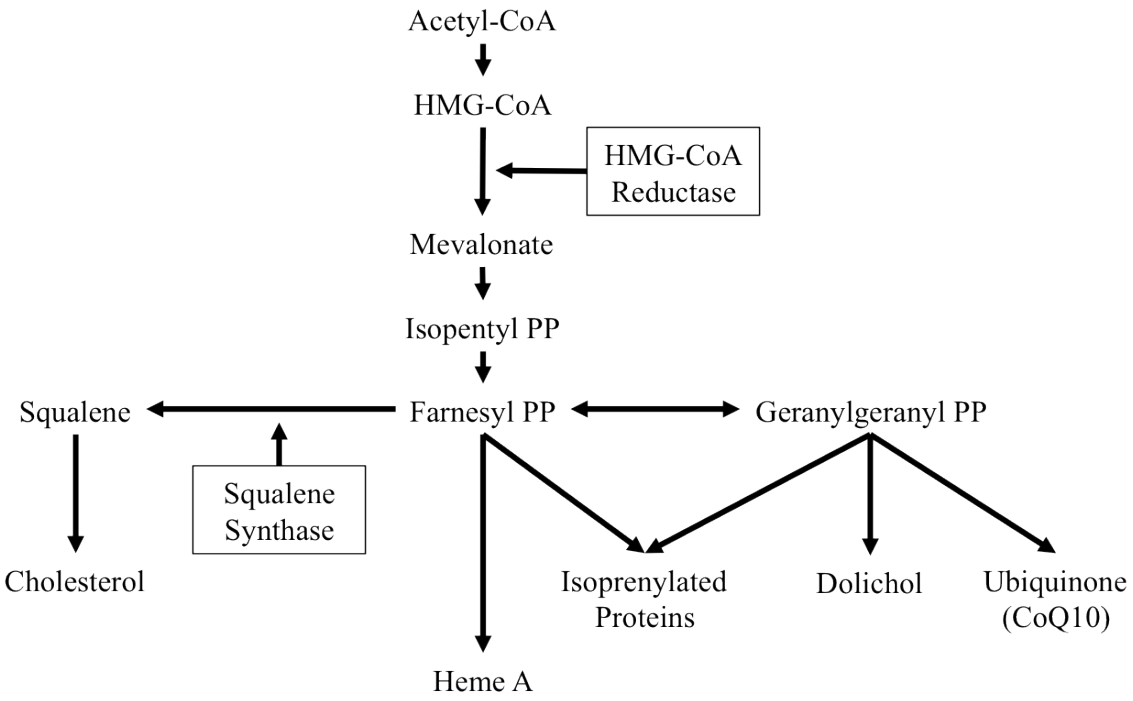

Figure 1.1 Simplified diagram of the cholesterol biosynthetic pathway.

1.1.3 Development

In the early 1970s, Dr Akira Endo discovered the first statin named mevastatin or compactin. Mevastatin was isolated from the fungus *Penicillium citrinum* while screening microbial strains for the ability to block lipid synthesis (Endo, 1992). Despite being shown to be effective at lowering LDL-C, mevastatin was never marketed over concerns of carcinogenicity in dogs (Endo, 1992). In 1979, another statin, termed lovastatin, was successfully isolated from 2 sources; *Aspergillus terrus* by Merck Research Laboratories and *Monascus ruber* by Dr Akira Endo (Endo, 2010). Lovastatin was shown to be safe and effective in early clinical trials and was approved by the U.S. Food and Drug Administration (FDA) in 1987, becoming the first marketed statin. Since then seven other statins have followed suit for the treatment of hypercholesterolemia including simvastatin, pravastatin, fluvastatin, atorvastatin, cerivastatin, rosuvastatin and pitavastatin (Fig. 1.2). While all statins share the same pharmacophore, similar to the open acid ring structure of HMG-CoA, they differ widely in their chemical attributes, dosage forms, and potency. Statins can be administered in the open β-hydroxyacid form (active) or the closed lactone form (inactive) because interconversion between forms occurs *in vivo* (Shitara and Sugiyama, 2006). The pharmacological properties of statins are summarized in Table 1.1.

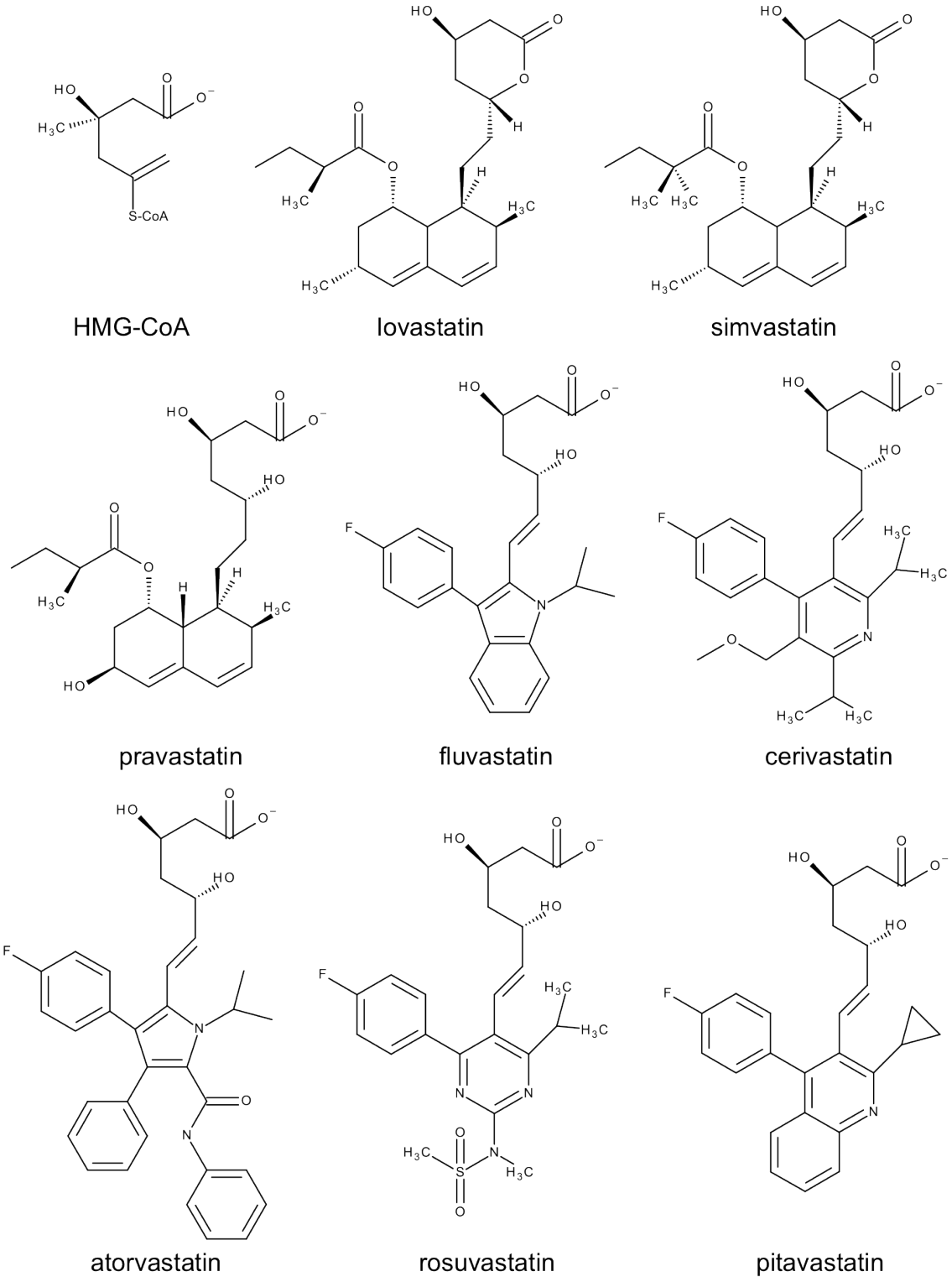

Figure 1.2 Chemical structures of HMG-CoA reductase inhibitors.

Table 1.1 Pharmacological Properties of Statins.

Statin	FDA Approval	Form	Dose	IC_{50} (nM)	LogD (pH 7.0)
Atorvastatin	1996	acid	10 - 80 mg	0.82	1.53
Cerivastatin	1997 (Withdrawn in 2001)	acid	0.2 - 0.8 mg	2.50	2.32
Fluvastatin	1993	acid	20 - 80 mg	4.80	1.75
Lovastatin	1987	lactone	10 - 80 mg	4.70	3.91 (acid 1.51)
Pitavastatin	2009	acid	1 - 4 mg	1.70	1.5
Pravastatin	1991	acid	10 - 40 mg	5.00	-0.47
Rosuvastatin	2003	acid	5 - 40 mg	0.30	-0.25 to -0.50 (pH 7.4)
Simvastatin	1991	lactone	5 - 80 mg	5.20	4.4 (acid 1.88)

IC_{50} = half maximal inhibitory concentration of HMG-CoA reductase activity in cultured rat hepatocytes; LogD = distribution coefficient

References: (Aoki et al., 1997; Shitara and Sugiyama, 2006; Yee and Wright, 2011)

1.2 Statin Pharmacokinetics

Statins are administered orally on a daily basis, but the differences in biopharmaceutical and biochemical properties results in different pharmacokinetic profiles. For several statins, extensive first pass effects limits their oral bioavailability. Metabolism by the cytochrome (CYP) P450 enzymes also plays an important role in the pharmacokinetics of some statins. Lovastatin and simvastatin are both lipophilic statins administered in their lactone forms making them easily absorbed from the intestine. However, lovastatin and simvastatin are substrates of P-glycoprotein (P-gp) and CYP3A4, which are expressed in the liver and intestine. Both P-gp and CYP3A4 play an important part in the first pass effects of these two statins leading to their low bioavailabilities (<5%) (Shitara and Sugiyama, 2006). Atorvastatin is dosed in the β-hydroxyacid form and it is also metabolized by CYP3A4. However, it is relatively more hydrophilic than lovastatin and simvastatin and requires transporters for its hepatic uptake (Lau et al., 2006; Shitara and Sugiyama, 2006). Fluvastatin is unique from the other statins, since it is primarily metabolized by CYP2C9. As a result, inhibition of CYP3A metabolism does not affect pharmacokinetics of fluvastatin. Cerivastatin also has distinct metabolism via a dual metabolic pathway involving CYP3A4 and CYP2C8 metabolism (Shitara et al., 2004). Pravastatin and rosuvastatin are hydrophilic and do not undergo any major metabolism involving CYPs. They are primarily eliminated from the body by transport mediated processes in the liver (Shitara and Sugiyama, 2006). The newer statins, like atorvastatin and rosuvastatin, have much longer biological half lives (>14.5h) compared to the other statins (<3h). Most statins are highly protein bound in plasma and are primarily eliminated in the bile except in the case of pravastatin. The pharmacokinetic properties of some statins are summarized in Table 1.2.

Table 1.2 Pharmacokinetic Properties of Statins.

	Atorvastatin	Fluvastatin	Lovastatin	Pravastatin	Rosuvastatin	Simvastatin
Pharmacokinetics						
T_{max} (h)	1.0-2.0	0.5-1.0	2.0-4.0	1.0-1.5	3.0-5.0	1.3-3.0
C_{max} (ng/mL)	27-66	448	10-20	45-55	37	10-34
Bioavailability (%)	12-14	19-29	<5	18	20	<5
Plasma Protein Binding (%)	>98	>98	>95	45-54	88	95
Metabolism						
CYP	3A4	2C9	3A4	-	Minimal: 2C9, 2C19 (minor)	3A4
UGT	1A1, 1A3					
Number of known active metabolites	2	0	3	0	1	3
$T_{1/2}$ (h)	14.5	3.0	2.0	2.0	20.0	1.4-3.0
Excretion						
Renal (%)	≤2	<6	≥10	20	10	13
Biliary (%)	70	90	83	53 (non-renal)	90	58

Values based on a 40 mg oral dose for each statin

References: (Bellosta and Corsini, 2012; Shitara and Sugiyama, 2006)

1.2.1 Drug Transporters Involved in Statin Pharmacokinetics

1.2.1.1 Overview of Drug Transporters

Transporters are membrane spanning proteins that act as gate keepers for all cells controlling the uptake and efflux of endogenous substrates such as amino acids, sugars, ions, and hormones. Compounds that are large or polar cannot pass through biological membranes by simple diffusion and rely on transporter proteins to facilitate membrane crossing. Transporters are not only limited to recognizing endogenous compounds, but some also have the potential to transport xenobiotics like drugs, dietary and environmental compounds. Drug transporters are particularly important in drug disposition because of their expression in organs such as the intestine, liver and kidney (Giacomini et al., 2010; Ho and Kim, 2005).

Drug transporters can be broadly classified into two classes, uptake and efflux transporters. Uptake transporters belong to the solute carrier superfamily (SLC) and they are responsible for the movement of substrates into cells. SLC transporters utilize facilitated diffusion, ion coupling, or ion exchange to transport substrates down or against their concentration gradients (DeGorter et al., 2012b). In contrast, efflux transporters belong to the ATP-binding cassette (ABC) superfamily and are responsible for the extrusion of compounds from cells into the extracellular environment. ABC transporters have an intracellular nucleotide binding domain that catalyzes the hydrolysis of ATP to generate energy to power the transport of substrates across membranes (Schinkel and Jonker, 2003).

The pharmacokinetics of statins are highly dependent upon membrane transporters from the ATP-binding cassette and solute carrier families. Importantly, statin response requires hepatic uptake to their intracellular target, to exert their clinical effects. Specifically, statins rely on drug transporting systems for their hepatic uptake including: organic anion transporting polypeptides (OATP) 1B1, OATP1B3, OATP2B1 and sodium-taurocholate co-transporting polypeptide (NTCP). Biliary excretion of statins involves drug transport systems on the hepatocyte canalicular membrane including P-glycoprotein (P-gp, MDR-

1, ABCB1), breast cancer resistance protein (BCRP, ABCG2) and multidrug resistance-associated protein 2 (MRP2, ABCC2).

All of the marketed statins are well absorbed in the intestine but the absorption mechanism is thought to vary from one statin to another. Lovastatin and simvastatin are lipophilic and are considered to be absorbed by passive diffusion. Both are also substrates of P-gp in the intestine, which may account partially for their low bioavailability (Sakaeda et al., 2002). The pharmacokinetics of simvastatin and lovastatin may be affected by inhibition of P-gp leading to increased bioavailability and area under the curve (AUC) (Holtzman et al., 2006; Shitara and Sugiyama, 2006). Hydrophilic statins like pravastatin are predicted to be absorbed by a transporter mediated mechanism versus passive diffusion (Tamai et al., 1995). The OATPs are thought to be responsible for the transport-mediated absorption of pravastatin (Shirasaka et al., 2010). OATP2B1 is expressed in the intestine and is capable of transporting rosuvastatin, pravastatin, pitavastatin, and atorvastatin (Grube et al., 2006; Hirano et al., 2006; Ho et al., 2006; Kobayashi et al., 2003; Nozawa et al., 2004). Transport-mediated by OATP2B1 is also reported to be pH dependent and may contribute to the intestinal absorption of statins (Kobayashi et al., 2003; Nozawa et al., 2004). The intestinal transporter OATP1A2 could also be involved in the absorption of the substrates including pitavastatin, atorvastatin and rosuvastatin (Ho et al., 2006; Knauer et al., 2010; Shirasaka et al., 2011).

In general the renal elimination of most statins, except for pravastatin, is low. The renal elimination of pravastatin accounts for about 47% of total elimination. Renal clearance of pravastatin is also much greater than glomerular filtration rate suggesting a role for transporters in pravastatin secretion (Hatanaka, 2000). Studies in rats and humans suggest a role of organic anion transporter 3 (OAT3) in the renal excretion of pravastatin (Hasegawa et al., 2002; Takeda et al., 2004). However, polymorphisms in OAT3 have not been associated with changes in pravastatin elimination or pharmacokinetics (Nishizato et al., 2003).

Some of the drug transporters involved in the pharmacokinetics of statins are discussed below and summarized in Table 1.3.

1.2.2 Statin Uptake Transporters

1.2.2.1 Organic Anion Transporting Polypeptides

Organic anion transporting polypeptides (OATPs), gene family SLCO, represent a superfamily of important drug transporters that mediate the sodium independent transport of a diverse range of substrates (Hagenbuch and Gui, 2008). The OATPs are integral membrane proteins that are predicted to consist of 12 transmembrane domains and have a characteristic OATP superfamily amino acid sequence (Hagenbuch and Meier, 2003). The amino and carboxy tails of OATP proteins are located intracellularly. N-glycosylation of OATP proteins occurs on conserved amino acid regions in extracellular loops two and five (Hagenbuch and Gui, 2008). The human OATP superfamily consists of 11 members including; OATP1A2, OATP1B1, OATP1B3, OATP1C1, OATP2A1, OATP2B1, OATP3A1, OATP4A1, OATP4C1, OATP5A1, and OATP6A1. The mechanism of transport appears to be an anion exchange consisting of counter transport of endogenous intracellular substances like bicarbonate and glutathione (Hagenbuch and Gui, 2008). Statins have been identified as substrates of OATP1A2, OATP1B1, OATP1B3 and OATP2B1. These transporters are thought to facilitate the intestinal absorption, and hepatic uptake of statins.

OATP1A2 and OATP2B1 are expressed in a wide variety of tissues, while others have tissue specific expression like OATP1B1 and OATP1B3. OATP1B1 and OATP1B3 are expressed on the basolateral membrane of hepatocytes and are involved in the hepatic uptake of statins (Hirano et al., 2004; Ho et al., 2006; Kitamura et al., 2008). OATP1B1 is highly polymorphic (Niemi et al., 2011; Tirona et al., 2001) and reduced function variants have been studied for their involvement in the pharmacokinetics of statins. One of the most well characterized variants is the 521T>C (rs4149056) single nucleotide polymorphism (SNP) denoted *5. This variant results in reduced cell surface trafficking and reduced transport function (Tirona et al., 2001). Multiple pharmacokinetic studies have shown that the OATP1B1*5 variant increases the area under the curve (AUC) for many statins.

OATP2B1 is found much more widely expressed than OATP1B1 and OATP1B3 transporters in tissues that include liver, kidney, brain, intestine, colon, heart, lung, placenta, ovary, testis and skeletal muscle (Knauer et al., 2010; Kullak-Ublick et al., 2001; Tamai et al., 2000). Mutation analysis of extracellular loop five revealed that mutation of cysteine residues or deletion of the loop results in protein mistrafficking to the cell surface (Hanggi et al., 2006). Mutation of cysteine residues in extracellular loop five has revealed distinct higher molecular weight species of OAPT2B1. Crosslinking experiments with wild type OATP2B1 have revealed that homo-dimerization is possible and responsible for the higher molecular weight bands (Hanggi et al., 2006). Rapid regulation of OATP activity has been shown to occur by protein phosphorylation of OATP1B1 and OATP2B1 by PKA and PKC, respectively (Kock et al., 2010; Sun et al., 2008). Increased phosphorylation of OATP2B1, by PKC, results in transporter internalization via a clathrin-dependent pathway (Kock et al., 2010). Phosphorylation of OATPs is predicted to influence physiological and pharmacokinetic transport functions but the *in vivo* relevance remains to be determined.

Functionally, OATP2B1 has a much narrower substrate specificity compared to members of the OATP1B family (Hagenbuch and Gui, 2008). However, human OATP2B1 has been shown to possess substrate-specific pH-dependent transport properties (Kobayashi et al., 2003; Nozawa et al., 2004). This pH-dependent transport mechanism and expression on the apical membrane of enterocytes of the intestine suggests a potential role in the intestinal absorption of substrates. Based on structural modeling of OATP1B3 and OATP2B1, OATPs are thought to transport solutes across membranes by a rocker-switch type of mechanism through a positively charged central pore (Meier-Abt et al., 2005).

Pharmacogenetic variations have also been observed in OATP2B1 (*SLCO2B1*); however, only a few have had their functional impact on OATP2B1 mediated transport characterized (Konig, 2011). The *SLCO2B1* 935G>A polymorphism has been associated with reduced plasma levels of the leukotriene receptor antagonist montelukast (Mougey et al., 2009). However, recently it has been demonstrated that montelukast is not a substrate of OATP2B1 (Chu et al., 2012). The most common variant in *SLCO2B1* gene is

the 1475C>T polymorphism with an allele frequency of 30.9% in a Japanese population. The 1475C>T polymorphism shows reduced maximal transport activity (V_{max}) for estrone sulfate compared to wild type protein *in vitro* (Nozawa et al., 2002). One pharmacokinetic study in humans has shown that carriers of the *SLCO2B1* 1475C>T have a significant reduction in the fexofenadine area under the plasma concentration-time curve (AUC) compared to wild type subjects (Imanaga et al., 2011).

1.2.2.2 Organic Anion Transporters

Organic anion transporters (OATs) represent a superfamily of important drug transporters that mediate the sodium independent transport of a diverse range of substrates (VanWert et al., 2010). OATs have been shown to play important roles in mediating the renal reabsorption and secretion of drugs, xenobiotics and endogenous compounds. OAT3 is one member of this family, predominantly expressed in the renal proximal tubule cells, that likely functions as an organic anion/α-ketoglutarate exchanger (Burckhardt and Burckhardt, 2011). OAT3 facilitates the uptake of a wide range of endogenous anions and xenobiotics from the blood into tubular cells (Erdman et al., 2006). OAT3 facilitates the renal uptake of pravastatin and rosuvastatin while other statins have been shown to be inhibitors of OAT3 (Burckhardt and Burckhardt, 2011; Takeda et al., 2004; Windass et al., 2007).

1.2.2.3 Sodium Taurocholate Co-transporting Polypeptide

Sodium taurocholate co-transporting polypeptide (NTCP) is the primary uptake system for bile acids from the portal blood into the liver. NTCP has seven putative transmembrane domains with the carboxy tail in the cytoplasm (Klaassen and Aleksunes, 2010). In addition to transporting bile acids, NTCP has also been shown to facilitate the sodium dependent hepatic uptake of various statins (Choi et al., 2011; Ho et al., 2006).

1.2.3 Statin Efflux Transporters

1.2.3.1 Breast Cancer Resistance Protein

Breast cancer resistance protein (BCRP) has wide spread tissue expression. The BCRP protein has six transmembrane domains and is considered a half transporter that must homo-oligomerize in order to function. BCRP is expressed on the apical membrane of enterocytes and the canalicular membrane of hepatocytes and functions to limit oral bioavailability and facilitate biliary excretion, respectively (Meyer zu Schwabedissen and Kroemer, 2011). Atorvastatin, pravastatin, pitavastatin and rosuvastatin have all been shown to be substrates of BCRP (Hirano et al., 2005; Keskitalo et al., 2009b; Kitamura et al., 2008).

1.2.3.2 Multidrug Resistance Associated Proteins

Multidrug resistance associated proteins (MRPs) are a family of ATP dependent efflux pumps with a broad substrate specificity for both endogenous compounds and xenobiotics (Leslie et al., 2005). MRP2 is one member of this family that is capable of statin transport. MRP2 is expressed on the bile canalicular membrane and is involved in the elimination of xenobiotic and bilirubin glucuronide conjugates and statins (Kitamura et al., 2008; Kivisto et al., 2005). Other members of the MRP family have been shown to transport statins. In Chapter Three we discuss MRP1, MRP4 and MRP5 as novel statin transporters expressed in skeletal muscle.

1.2.3.3 P-glycoprotein

P-glycoprotein (MDR1, P-gp, ABCB1) is an ABC transporter that has important roles in protecting tissues from toxic xenobiotics. P-gp is expressed in the apical membrane of enterocytes, hepatocytes, and proximal tubule cells of the kidney as well as endothelial cells of the blood brain barrier (Cascorbi, 2011). P-gp is capable of transporting some statins (Dong et al., 2008; Shirasaka et al., 2011) and data suggests that P-gp affects statin pharmacokinetics by modulating statin absorption and elimination (Rodrigues, 2010).

Table 1.3 Statin Transporters.

Transporter	Transport Mode	Tissue Localization	Atorvastatin	Fluvastatin	Lovastatin	Pitavastatin	Pravastatin	Rosuvastatin	Simvastatin
P-gp	Efflux	Intestine, Liver, Kidney, Brain	Y	N	Y	Y	Y	Y	Y
MRP1	Efflux	Lung, Brain, Heart, Muscle	Y	?	?	?	?	Y	?
MRP2	Efflux	Intestine, Liver, Kidney, Brain	Y	?	?	?	Y	Y	?
MRP4	Efflux	Muscle, Kidney, Intestine	Y	?	?	?	Y	Y	?
MRP5	Efflux	Muscle, Heart, Lung, Intestine	?	?	?	?	Y	Y	?
BRCP	Efflux	Intestine, Liver, Brain	Y	Y	?	Y	Y	Y	?
BSEP	Efflux	Liver	?	Y	?	?	Y	?	?
OATP1A2	Uptake	Intestine	?	?	Y	?	Y	Y	?
OATP1B1	Uptake	Liver	Y	Y	Y	Y	Y	Y	Y
OATP1B3	Uptake	Liver	Y	Y	?	Y	Y	Y	Y
OATP2B1	Uptake	Intestine, Liver, Kidney, Brain, Muscle	Y	Y	?	Y	Y	Y	?
OAT3	Uptake	Kidney, Brain	?	Y	Y	?	Y	Y	Y
NTCP	Uptake	Liver	Y	Y	?	Y	Y	Y	Y

Y = yes substrate, N = not substrate, ? = Unknown

References: (Bellosta and Corsini, 2012; Knauer et al., 2010; Shitara and Sugiyama, 2006)

1.2.4 Ethnic Differences in Pharmacokinetics

It is well known that ethnicity may affect drug disposition and response. In the case of statins, many believe that Asians need lower statin doses because of a lower body mass index (BMI). In fact, most statins have lower recommended doses approved in Japan then those in North America (Saito et al., 2005). The Canadian Cardiovascular Society guidelines recommend that lower doses of statins be used in all Asian patients for the management and treatment of dyslipidemia (McPherson et al., 2006). The package insert for rosuvastatin recommends a starting dose of 5 mg per day in Asian subjects. This recommendation is based on pharmacokinetic studies revealing that rosuvastatin plasma exposure is about two-fold higher in Asian compared to Caucasian subjects (Tzeng et al., 2008; Wang, 2011). Lee and colleagues demonstrated a striking difference in rosuvastatin plasma exposure between Chinese, Malay, Asian-Indians and white subjects living in Singapore. They showed that Asians had 1.6 to 2.3-fold greater rosuvastatin exposure in comparison to white subjects living in the same environment (Lee et al., 2005). There is good evidence that rosuvastatin exposure is higher in Asian subjects, but similar evidence for other statins is lacking. To date, no ethnic differences have been reported in cerivastatin or atorvastatin exposure (Gandelman et al., 2012; Muck et al., 1998). Although, Asians need lower doses of rosuvastatin compared to Caucasians, this does not appear to be a class effect. Importantly, there does not appear to be differences in the efficacy or safety in Asian populations (Wang, 2011).

1.3 Statin-Associated Myopathy

Statins are generally very effective and well tolerated with a good safety profile (McKenney et al., 2006; Pasternak, 2002; Ward et al., 2007). Although statins are well tolerated, their use is associated with a number of side effects. The most common adverse effect associated with statin therapy is skeletal muscle toxicity. All statins have the ability to cause muscle toxicity but the incidence rate varies widely between statins (Thompson et al., 2003). In fact, this side effect resulted in the withdrawal of cerivastatin (Baycol)

from the market in 2001 due to an unexpectedly high incidence of life-threatening muscle toxicity (rhabdomyolysis) compared with other marketed statins.

Statin induced myopathy encompasses a broad spectrum of muscle disorders ranging from myalgia to potentially life-threatening rhabdomyolysis (Bosch et al., 2009; Harper and Jacobson, 2007; Thompson et al., 2006). Rhabdomyolysis is characterized by skeletal muscle breakdown with leakage of muscle contents leading to acute renal failure, hypocalcaemia and hyperkalaemia, which may result fatal cardiac arrhythmias (Lane and Phillips, 2003; Warren et al., 2002). Collectively, statin-induced myopathies have been defined into 3 clinical categories on the basis of clinical presentation and plasma creatine (CK) levels – myalgia, myositis, and rhabdomyolysis (Table 1.4).

The prevalence of myalgia according to meta analysis of statin RCTs is between 1.5-5% (Bays, 2006; Law and Rudnicka, 2006). The rate of statin myalgia, myopathy and rhabdomyolysis in patients treated with statins or placebo was estimated by a meta-analysis of 21 clinical trials providing 180,000 person years of follow-up. They defined myalgia, as minor muscle pain, myopathy, as muscle symptoms with an elevation in CK levels above 10 times the upper limit of normal (ULN), and rhabdomyolysis, as CK levels above 10,000 IU/L or above 10 times the ULN with an elevation in serum creatinine or requirement for hydration therapy. The estimated rate for myalgia, myopathy and rhabdomyolysis was 190, 5, and 1.6 patients per 100,000 person years respectively (Law and Rudnicka, 2006). In contrast other meta-analysis studies report that the incidence of myalgia, rhabdomyolysis, elevations in CK or statin withdrawal were not significantly different between those receiving statins or placebo (Kashani et al., 2006). Voluntary reporting of rhabdomyolysis in statin treated patients to the FDA Adverse Events Reporting System (AERS) reports the rate of rhabdomyolysis is 0.70 per 100,000 person years (Harper and Jacobson, 2007). In 2001, the FDA AERS rates for fatal rhabdomyolysis were: 0.19, 0.12, 0.043, 0.037 and 3.2 reported case per 1 million prescriptions for lovastatin, simvastatin, atorvastatin, pravastatin, and cerivastatin respectively (Staffa et al., 2002). Since there are several definitions for types of statin myopathy (Table 1.4), it has been difficult to compare the incidence of myopathy in RCTs. Importantly, the data from RCTs do not represent the real world clinical practice.

The frequency of statin myopathy is reported more frequently in clinical practices, since RCTs are not explicitly designed to assess myopathy and the patient population studied does not reflect the same population. Indeed, observational evidence estimates that skeletal muscle myopathies occur in 10-15% of patients (Bruckert et al., 2005; Buettner et al., 2008; Jacobson, 2008; Thompson et al., 2003). All statins have been implicated in causing muscle side effects, albeit at differencing frequencies. The *in vitro* rank order for statin cytotoxicity has been reported to be cerivastatin > simvastatin acid > fluvastatin > atorvastatin > lovastatin acid > pitavastatin ≫ rosuvastatin, pravastatin (Kobayashi et al., 2008). Analysis of the FDA AERS database from 1990-2002 for statin associated rhabdomyolysis reports a similar pattern to the *in vitro* cytotoxicity. Cerivastatin was the most commonly implicated statin in 57% of cases followed by simvastatin (18%), atorvastatin (12%), pravastatin (7%), lovastatin (4%) and fluvastatin (2%) (Thompson et al., 2003). The frequency of muscle symptoms associated with statins was estimated in the observational PRIMO study in patients receiving high dose statins. Muscle related symptoms were reported by 10.5% of patients. The reports of muscle symptoms were most common in patients receiving simvastatin (18.2%), followed by atorvastatin (14.9%), pravastatin (10.9%) and fluvastatin (5.1%) (Bruckert et al., 2005).

While serum biochemistry is a useful diagnostic tool for myopathy, it should be noted that there have been case reports of statin-induced muscle myopathies without elevated plasma CK (Phillips et al., 2002). Myopathy associated with statin usage can begin as early as one week into therapy but onset also can be delayed for several years. On average, myopathy is reported to occur 6 months after starting therapy (Hansen et al., 2005). The duration of myopathy lasts an average of 2.3 months after discontinuation of statin and over 50% of patients experiencing myopathy cannot tolerate another statin (Bruckert et al., 2005; Hansen et al., 2005; Thompson et al., 2006). Muscle complaints from statins are dose dependent, of diffuse origin (Ballantyne et al., 2003; Bays, 2006) and often result in the structural damage to muscle fibres which can persist even after discontinuation of therapy (Draeger et al., 2006; Mohaupt et al., 2009).

Table 1.4 Definitions of Statin-Related Myopathy

	ACC/AHA/HHLBI	NLA	FDA
Myopathy	General term referring to any disease of muscles	Complaints of myalgia (muscle pain or soreness), weakness, and/or cramps plus elevation in serum CK	CK ≥ 10 x ULN
Myalgia	Muscle aches or weakness without CK elevation	NA	NA
Myositis	Muscle symptoms with CK elevation	NA	NA
Rhabdomyolysis	Muscle symptoms with significant CK elevation (>10 x ULN) with myoglobinuria	CK > 10,000 IU/L or CK > 10 x ULN plus an elevation in serum creatinine or medical intervention with intravenous hydration	CK > 50 x ULN and evidence of organ damage, such as renal compromise

ACC/AHA/HHLBI = American College of Cardiology/American Heart Association/National Heart, Lung and Blood Institute, NLA = National Lipid Association, FDA = Food and Drug Administration
CK = creatine kinase, ULN = Upper Limit of Normal
References: (Ballantyne et al., 2003; Joy and Hegele, 2009)

1.3.1 Etiology of Statin-Associated Myopathy

The pathophysiology of statin-induced myopathy is not completely understood. Multiple pathophysiological mechanisms have been proposed to attempt to explain the relationship between statins and myotoxicity including isoprenoid depletion, depletion of ubiquinone or coenzyme Q-10 (CoQ10) synthesis, decreased or altered sarcolemma membrane cholesterol, or disturbed calcium homeostasis.

1.3.2 Depletion of Membrane Cholesterol

Initially, it was hypothesized that decreased membrane cholesterol levels lead to myocyte membrane destabilization and degeneration (Baker, 2005). This mechanism has been challenged by the results of clinical studies involving the novel squalene synthase inhibitors, which block cholesterol synthesis downstream of HMG-CoA reductase. Squalene synthase catalyzes the first committed step in the biosynthesis of cholesterol (Fig. 1.1). Inhibitors of squalene synthase selectively inhibit cholesterol synthesis and not the other non-sterol end products, and clinical studies of these drugs did not reveal indications of muscle toxicity (Do et al., 2009; Flint et al., 1997; Nishimoto et al., 2003; Seiki and Frishman, 2009).

1.3.3 Isoprenoid Depletion

The leading mechanism proposed for statin-induced myopathy involves the cellular depletion of secondary metabolic intermediates of mevalonate in the development of myotoxicity (Baker, 2005). HMG-CoA reductase inhibition by statins decreases mevalonate and cholesterol synthesis. However, cholesterol is not the only end product of this pathway and in addition to decreased cholesterol there are subsequent reductions in the levels of downstream metabolic products including isoprenoids, dolichol and ubiquinone (CoQ10) (Baker, 2005; Hanai et al., 2007; Itagaki et al., 2009; Sakamoto et al., 2007). Geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate (FPP) are

two very important isoprenoid secondary metabolic intermediates of the cholesterol synthesis pathway. GGPP and FPP are involved in the post-translational modification of an estimated 300 cellular proteins through isoprenylation. Isoprenylation involves the covalent addition of geranylgeranyl or farnesyl groups to cysteine residues at or near the C-terminus of proteins such as members of the small G protein superfamily (McTaggart, 2006). Isoprenylation allows proteins to anchor to cell membranes and in some cases is required for activation of some proteins including small GTPases such as Rho and Rab (Konstantinopoulos et al., 2007). Recently, induction of the muscle atrophy-linked protein atrogin-1 has been shown to mediate statin myotoxicity through a mechanism involving decreased isoprenylation (Cao et al., 2009; Hanai et al., 2007). A reduction in prenylation of small GTPases appears to stimulate the mitochondrial apoptotic cell death pathway by increasing levels of cytosolic calcium (Liantonio et al., 2007), calpain activation, Bax translocation to the mitochondria (Sacher et al., 2005), cytochrome C release (Kaufmann et al., 2006) and induction of pro-apoptotic caspases (Dirks and Jones, 2006; Johnson et al., 2004) (Fig 1.3). The importance of isoprenylation in statin induced myotoxicity is highlighted by the findings that supplementation of GGPP leads to attenuation of statin toxicity in cultured skeletal myotubes or isolated myofibers (Cao et al., 2009; Itagaki et al., 2009; Johnson et al., 2004; Sakamoto et al., 2007), while inactivation of Rab and RhoA has been shown to induce toxicity through inhibition of endoplasmic reticulum to golgi vesicular trafficking (Itagaki et al., 2009; Sakamoto et al., 2007; Sakamoto et al., 2011).

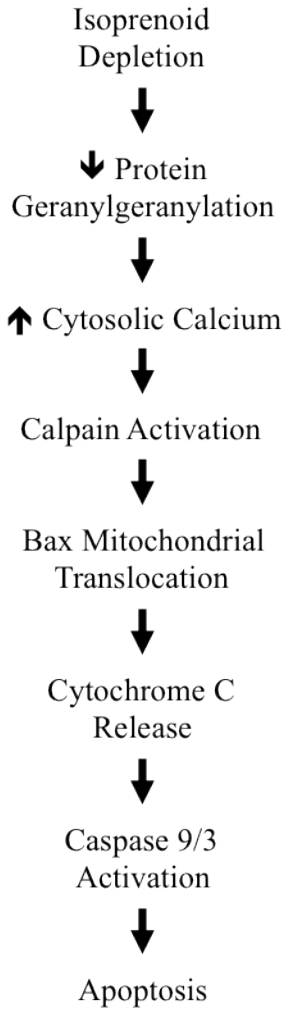

Figure 1.3 A proposed mechanism of statin induced skeletal muscle damage.

1.3.4 Mitochondrial Dysfunction and Depletion of Coenzyme Q10

CoQ10 is synthesized from mevalonate and is an essential cofactor for oxidative respiration in the mitochondria. Depletion of CoQ10 might contribute to statin myopathy because it affects oxidative phosphorylation, protects from statin-induced oxidative stress and regenerates antioxidant vitamins C and E (Marcoff and Thompson, 2007). Extensive evidence demonstrates that statins lower plasma CoQ10 levels in a drug and dose dependent manner (Folkers et al., 1990; Kawashiri et al., 2008; Mabuchi et al., 2007; Schaars and Stalenhoef, 2008). However, the reduction in plasma CoQ10 levels can be attributed to the lowering of LDL cholesterol, since it is the primary carrier of plasma CoQ10 (Laaksonen et al., 1994; Tomasetti et al., 1999). If CoQ10 concentrations are normalized for decreased LDL or total cholesterol there is no significant change in CoQ10 concentration (Marcoff and Thompson, 2007). Interestingly, the serum concentrations of CoQ10 do not consistently reflect muscle concentrations (Paiva et al., 2005; Schaars and Stalenhoef, 2008) and evidence suggests that low dose statin treatment does not affect the intramuscular CoQ10 levels (Marcoff and Thompson, 2007). However, one study suggests that even if CoQ10 is decreased in muscle, most patients have no histochemical or biochemical evidence of mitochondrial myopathy or morphologic evidence of apoptosis (Lamperti et al., 2005). Supplementation of CoQ10 during statin therapy can increase the circulating CoQ10 concentrations (Palomaki et al., 1998; Schaars and Stalenhoef, 2008) and provide protection from statin-induced oxidative stress. Compelling clinical evidence is lacking regarding the efficacy of CoQ10 treatment for statin myopathy (Caso et al., 2007; Marcoff and Thompson, 2007; Young et al., 2007); there is no evidence that supplementation has any effect on muscle CoQ10 levels (Schaars and Stalenhoef, 2008).

If reduced CoQ10 levels mediate statin-induced myopathy, there should be evidence of impaired mitochondrial function. However, only a few studies have shown indirect evidence of mitochondrial dysfunction in humans. Decreased CoQ10 was associated with mitochondrial dysfunction when measuring an increase in the blood lactate to pyruvate ratio (De Pinieux et al., 1996). This ratio is used as a marker of mitochondrial impairment because it shows a shift toward anaerobic metabolism (De Pinieux et al., 1996). Muscle

biopsies from patients with statin induced myopathy have also suggested a link between mitochondrial dysfunction and clinical symptoms despite normal CK levels (Levy and Kohlhaas, 2006; Phillips et al., 2002). One study reported that muscle pain and weakness was more prevalent in patients with mitochondrial respiratory chain defects (Vladutiu et al., 2006).

1.3.5 Impairment of Calcium Homeostasis

The regulation of calcium release and uptake is critical for the normal function of muscle cells. The initial increase in intracellular calcium, mediated by an action potential, results in the opening of ryanodine receptors on the sarcoplasmic reticulum leading to muscle contraction. *In vitro* studies show that statins impair calcium homeostasis and lead to membrane depolarization (Liantonio et al., 2007; Sirvent et al., 2005). Muscle biopsies from patients with statin-induced myopathy have shown alterations in expression of genes regulating calcium homeostasis, impairment of calcium signalling and structural changes to the T-tubular system and sarcoplasmic reticulum (Draeger et al., 2010; Mohaupt et al., 2009; Sirvent et al., 2012). However, it is still unclear if these statin-induced muscle dysregulations contribute to or are a consequence of myotoxicity.

1.3.6 Patient Related Risk Factors

Many clinical factors have been shown to increase the risk of statin-induced myopathy including age, female gender, Asian descent, low body mass index, strenuous exercise, excess alcohol consumption, diet (grapefruit and other fruit juices), drugs of abuse (cocaine), untreated hypothyroidism, impaired hepatic or renal function and perioperative periods (Abd and Jacobson, 2011; Ballantyne et al., 2003; Joy and Hegele, 2009; Pasternak, 2002; Rosenson, 2004). Family history of statin induced myopathy and previous history of statin myopathy are also significant risk factors (Bruckert et al., 2005). Inherited muscle diseases like McArdle's disease and carnitine palmitoyl 2 (CPT-2) deficiencies have also been associated with the development of myopathy (Vladutiu et

al., 2006). Recently deficiency in vitamin D has been associated with statin induced myopathy and supplementation can result in the resolution of myalgia (Ahmed et al., 2009; Gupta and Thompson, 2011).

An epidemiology study recently developed a model to predict statin adverse reactions based on risk factors readily available and recorded in patient health records. The Qstatin risk score was developed from data encompassing 2.2 million patients in the UK and it has proven useful for predicting the 5 year statin risk of developing acute renal failure, cataracts, and myopathy (Collins and Altman, 2012). The Qstatin score is currently the only quantitative measure to assess myopathy risk using the following factors: statin usage, age, sex, BMI, self assigned ethnicity, treated hypertension, corticosteroid usage, diagnosis of type 1 or 2 diabetes, diagnosis of chronic liver disease, and diagnosis of hypothyroidism. The 5 year risk of developing myopathy, CK $\geq$ 4 x ULN or rhabdomyolysis, is predicted to be between 0.05% and 0.25%, depending on age and gender. Factors that increase the risk of statin induced myopathy are summarized in Table 1.5.

Table 1.5 Risk Factors for Statin-Induced Myopathy

- Increased age
- Female gender
- Low body mass index
- Asian descent
- Renal or hepatic impairment
- Hypothyroidism
- Perioperative periods
- Metabolic muscle disease
- Family history of statin induced myopathy
- Previous history of statin induced myopathy
- Vitamin D deficiency
- Strenuous exercise
- Excess alcohol consumption
- Diet - grapefruit juice
- Drugs of abuse
- Co-medications
    - Other myotoxic drugs
    - Metabolic inhibition of CYP or UGT
- Statin Characteristics
    - High statin dose
    - Lipophilicity?
    - Potential for metabolic drug interactions

1.3.7 Drug Interactions

It is well documented that myotoxicity is statin dose-dependent, and myopathy risk increases when statins are co-administered with drugs that either interact to increase plasma statin levels or themselves have propensity for muscle damage (Ballantyne et al., 2003; Huerta-Alardin et al., 2005; Jones and Davidson, 2005; Neuvonen et al., 2006). Co-administered drugs that cause increases in statin blood levels are a significant risk factor for development of myotoxicity. Many of the statins are significantly metabolized in the intestinal enterocytes and hepatocytes by the cytochrome (CYP) P450 superfamily and UDP-glucuronosyltransferases (UGT) resulting in low bioavailability (Table 1.2). Therefore, inhibition of these enzymes by co-administered medications can be associated with increased plasma statin levels (Ballantyne et al., 2003; Bottorff, 2006; Jones and Davidson, 2005). Grapefruit juice, macrolide antibiotics and azole antifungals are well known drug inhibitors of hepatic statin metabolism via CYP P450 enzymes, particularly CYP3A4, and their interaction can lead to a many-fold increase in plasma statin levels and risk of myopathy (Lilja et al., 1998; Lilja et al., 1999; Neuvonen et al., 1998; Neuvonen et al., 2006). The importance of CYP3A4 in statin metabolism is demonstrated by published reports associating more than 50% of statin induced rhabdomyolysis cases with a CYP3A4 inhibitor and possible drug interaction (Bottorff, 2006; Dreier and Endres, 2004; Omar et al., 2001; Sorokin et al., 2006). It has been estimated that 7% of individuals taking statins are co-prescribed drugs that would cause metabolic interactions (Ratz Bravo et al., 2005). One important drug interaction occurs with the often co-prescribed fibrate drug, gemfibrozil, which interacts through inhibition of statin glucuronidation (Prueksaritanont et al., 2002) and CYP2C8 mediated statin oxidation (Wang et al., 2002). Combination treatment of gemfibrozil with a statin appears to increase myopathy risk up to 50-fold compared to statin monotherapy. In fact for cerivastatin, the absolute rhabdomyolysis incidence with fibrate co-administration is an astounding 10% or a 1400-fold increase over statin monotherapy (Graham et al., 2004). However, gemfibrozil has been shown to only modestly increase the plasma levels of other statins (2 to 3-fold) (Jacobson and Zimmerman, 2006). Therefore, alterations in

systemic exposure by gemfibrozil mediated inhibition of statin metabolism do not entirely account for the enhanced risk of myopathy.

Atorvastatin is metabolized by CYP3A4 into two primary active metabolites o-hydroxyatorvastatin and p-hydroxyatorvastatin. Recently it was reported that patients with atorvastatin related myopathy had an altered metabolic profile with significantly higher plasma levels of atorvastatin lactone, o-hydroxyatorvastatin and p-hydroxyatorvastatin with no change in the atorvastatin plasma levels (Hermann et al., 2006). This demonstrates distinct metabolic profiles, in the absence of CYP3A4 inhibitors, for patients with atorvastatin induced myopathy and healthy controls. Concurrent treatment of atorvastatin and CYP3A4 inhibitors, leads to increased levels of atorvastatin lactone, and is associated with an increased risk of developing muscular side effects (Kantola et al., 1998; Omar and Wilson, 2002).

Drug interactions with statins can also involve drug transport proteins. Cyclosporine is an immunosuppressant drug and a potent inhibitor of several drug transporters including; OATP1B1, OATP1B3, OATP2B1, NTCP, MRP2, and P-gp (Chen et al., 1999; Hirano et al., 2006; Ho et al., 2006; Kajosaari et al., 2005; Rao and Scarborough, 1994; Shitara et al., 2003). The AUC of statins is increased with cyclosporine use by 2 to 25-fold without effecting the terminal half life (Neuvonen et al., 2006). This interaction could be a result of increased statin bioavailability or decreased systemic clearance through inhibition of hepatic uptake or billary efflux transporters (Neuvonen et al., 2006). Drug interactions have also been reported for protease inhibitors and statins. Simvastatin and lovastatin are contraindicated in the presence of protease inhibitors due to inhibition of CYP3A4/5. Statins undergoing minimal hepatic metabolism, like rosuvastatin and pravastatin, are also subject interactions involving the activity or expression of uptake or efflux transporters including; OATP1B1, P-gp, BCRP and, MRP2 (Bachmeier et al., 2005; Dixit et al., 2007; Kim et al., 1998). Many studies involving rosuvastatin, pravastatin and atorvastatin have reported increases in the AUC when taken concurrently with protease inhibitors (Busti et al., 2008; Kiser et al., 2008; Pham et al., 2009; Samineni et al., 2012).

1.3.8 Cerivastatin – What Can Be Learned?

Cerivastatin was withdrawn from the market in 2001 because of a pronounced increase in the risk of rhabdomyolysis and myopathy (Staffa et al., 2002). Approximately 100 deaths have been linked to cerivastatin use and rhabdomyolysis (Furberg and Pitt, 2001; Thompson et al., 2003). Approximately half of the rhabdomyolysis cases reported to the FDA were linked to a drug interaction with gemfibrozil (Psaty et al., 2004). Gemfibrozil has been shown to inhibit the major elimination pathways for cerivastatin including; drug metabolizing enzymes CYP2C8, UGT1A1, UGT1A3 and the hepatic uptake transporter OATP1B1 (Backman et al., 2002; Muck et al., 1998; Shitara et al., 2004). In one pharmacokinetic study, gemfibrozil was shown to greatly increase the AUC of cerivastatin by 559% in healthy volunteers (Backman et al., 2002).

Although the risk of muscle side effects with statins was known before, the cerivastatin experience unfortunately gave us the opportunity to study the link between statins and myopathy in many more cases. The cerivastatin experience was the first to demonstrate a clear statin dose-response relation with myopathy and a threshold effect above which myotoxicity increases significantly. Cerivastatin gave us insights into the mechanism of statin induced muscle toxicity and expanded our knowledge of risk factors. The pronounced effect of gemfibrozil on genes involved with cerivastatin suggests that genetic variants might play a major role in statin myopathy in patients who did not use gemfibrozil (Jacobson, 2006; Marciante et al., 2011).

The tragic rate of rhabdomyolysis with cerivastatin was at least 10 fold higher than other statins (Farmer, 2001). This highlighted problems in the post-marketing surveillance of pharmacologic agents with the potential for adverse effects. The cerivastatin experience significantly increased the initial awareness of safety issues for all of the statins. The FDA became much more stringent with new drug applications (NDA) for statins. Rosuvastatin, approved in 2003, initially had data on 3,903 patients in its NDA. The FDA requested more safety data from AstraZeneca, and as a result, additional studies were completed, to include 12,569 patients in the revised rosuvastatin NDA (Jacobson, 2006). From a risk benefit standpoint, if high dose statins are only marginally more efficacious at lowering LDL-C, but have even just a mild increase in muscle adverse events, then a

lower maximal dose is more appropriate dose to market (Bays, 2006; Rosenson and Bays, 2003). In the case of rosuvastatin the FDA did not approve the 80 mg dose because the lipid-lowering benefits were outweighed by the increased risks for renal toxicity and myotoxicity (Jacobson, 2006).

1.3.9 Therapeutic Approaches in the Management of Statin Intolerance

It is inevitable that some patients will discontinue statin therapy because of adverse effects. To be certain of a diagnosis of statin intolerance, it is recommended that patients undergo rechallenge with the same or lower dose of the statin after the resolution of their symptoms. In general, patients will not be able to tolerate high dose statin treatment but they may tolerate lower doses or alternate dosing regimes. The goal in patients with confirmed statin intolerance is to maintain sustained LDL-C lowering and prevention of CVD. Switching to a different statin should be considered because over 40% of patients will tolerate another statin without incident (Hansen et al., 2005). Rosuvastatin and fluvastatin have been considered preferred choices because they appear to be associated with lower risks of myopathy (Glueck et al., 2006; Stein et al., 2008). One study showed that 57% of patients intolerant to the usual dose of simvastatin were able to tolerate low dose simvastatin of 0.825 to 8.75 mg daily (Degreef et al., 2010). Studies evaluating alternate dosing regimes of rosuvastatin have also been assessed in patients with previous statin intolerance. Alternating-day dosing of rosuvastatin was tolerated in 72.5% of patients at a mean does of 5.6 mg, and LDL-C was reduced by 34.5% (Backes et al., 2008). Once weekly dosing of rosuvastatin was also studied in 10 patients with statin intolerance. This dosing regime was tolerated by 8 patients and with an average LDL-C reduction of 29% (Backes et al., 2007). Atorvastatin has also been successful in reducing LDL-C when used in alternate dosing regimes (Ferrer-Garcia et al., 2006; Juszczyk et al., 2005; Matalka et al., 2002). Other alternatives for the treatment of hypercholesterolemia not involving statins include: ezetimibe, niacin, fibrates, and bile acid sequestrants; however, the use of these agents as monotherapy only results in mild reductions of LDL-

C. Combination therapy of these agents with low dose statins or alternate statin dosing may allow statin intolerant individuals to achieve target LDL-C levels.

Other strategies have been investigated as symptomatic therapies, targeting muscle symptoms in patients. As discussed above, CoQ10 is an important cofactor in the mitochondrial electron transport chain and depletion has been considered a cause of statin myopathy. There are currently conflicting results for the use of CoQ10 in treatment of statin intolerance and pain (Caso et al., 2007; Young et al., 2007). A systemic review concluded that there was insufficient evidence to support the use of CoQ10 in treatment of pain or myopathy (Marcoff and Thompson, 2007). Vitamin D has also been suggested for treatment of statin induced myalgia. One report shows that supplementation of 50,000 U per week of vitamin D can reduce myalgia by 92%; however, vitamin D levels in patients with and without myalgia were reported to be the same (Ahmed et al., 2009; Backes et al., 2011). More evidence, such as a placebo controlled trial, is need to decide if vitamin D is of benefit for relief of statin myalgia (Gupta and Thompson, 2011).

No strategy is currently available to solely target the relief of muscle symptoms while taking statins. Management of this adverse effect generally requires a combination of a statin dose reduction, switching statins, alternate dosing regime, changing to an alternate lipid lowering agent, or combination therapy.

1.4 Statin Pharmacogenomics

There is wide variation among individuals and their response to statin efficacy and toxicity. This is partially due to differences in genes involved in statin pharmacokinetics. Ethnic and interindividual differences in plasma statin levels and area under the curve (AUC) has been reported before (Lee et al., 2005; Wang, 2011). The differences in statin pharmacokinetics has been linked to variation in the CYP P450 superfamily and the OATP superfamily of drug uptake transporters.

1.4.1 Pharmacogenomics of Statin Pharmacokinetics

The CYP superfamily is involved in the metabolism of many statins; therefore, genetic variation in these enzymes can be associated with changes in statin pharmacokinetics. CYP3A is the most prominent pathway for statin metabolism. To date, no polymorphisms in CYP3A4 have been reported to change statin pharmacokinetics (Shitara and Sugiyama, 2006). However, reports of significant variation within intestinal and hepatic expression of CYP3A4 may have profound effects on statin pharmacokinetics (Wandel et al., 2000). One polymorphism in CYP3A4 intron 6 (rs35599367, C>T) causes a significant reduction in the expression of CYP3A4. Carriers of the T allele have been shown to require lower doses of atorvastatin, simvastatin, or lovastatin for optimal lipid control compared to non-T carriers (Elens et al., 2011; Wang et al., 2011). Individuals expressing CYP3A5 have a reduced lipid lowering response to lovastatin, simvastatin and atorvastatin (Kivisto et al., 2004). Healthy individuals expressing CYP3A5 have a significantly reduced AUC and increased oral clearance over non-expressors (Kim et al., 2007). Interestingly, polymorphisms within CYP2D6, CYP2C9 and CYP2C8 have also been associated with pharmacokinetic changes in simvastatin, fluvastatin and cerivastatin, respectively (Ishikawa et al., 2004; Kirchheiner et al., 2003; Mulder et al., 2001; Vermes and Vermes, 2004).

Pharmacokinetic changes are also seen with variation in drug transporter genes. As mentioned above OATP1B1 is highly polymorphic (Niemi et al., 2011; Tirona et al., 2001) and the reduced function *SLCO1B1**5 521T>C variant has been well studied for involvement in statin pharmacokinetics. The OATP1B1*5 variant has been shown in multiple pharmacokinetic studies to increase the AUC for many statins including: atorvastatin (Lee et al., 2010; Pasanen et al., 2007), pravastatin (Ho et al., 2007; Igel et al., 2006; Maeda et al., 2006; Mwinyi et al., 2004; Niemi et al., 2006; Niemi et al., 2004; Nishizato et al., 2003), pitavastatin (Chung et al., 2005; Deng et al., 2008; Ieiri et al., 2007), rosuvastatin (Choi et al., 2008; Lee et al., 2005; Pasanen et al., 2007), and simvastatin (Pasanen et al., 2006).

The nonsynonymous *ABCG2* (BCRP) 421C>A SNP results in lower expression levels and function of the *ABCG2* efflux transporter protein in hepatocytes, enterocytes and

other tissues (Cusatis and Sparreboom, 2008). Various pharmacokinetic studies have shown that individuals carrying the 421C>A variant have increased plasma concentrations of atorvastatin, fluvastatin, simvastatin lactone and rosuvastatin (Keskitalo et al., 2009a; Keskitalo et al., 2009b; Zhang et al., 2006). Although no effect has been seen on the pharmacokinetics of pitavastatin, pravastatin or simvastatin acid (Ho et al., 2007; Ieiri et al., 2007; Keskitalo et al., 2009a). The *ABCG2* 421C>A has not been reported to effect the elimination T½ of rosuvastatin or atorvastatin suggesting that the increase in plasma statin concentration is due to enhanced absorption and increased bioavailability rather than decreased hepatic elimination (Keskitalo et al., 2009b; Zhang et al., 2006). The *ABCG2* 421 SNP has a much higher prevalence in Asian subjects compared to Caucasians (35% vs 9-15%) (Hu et al., 2011). The different frequencies of *ABCG2* polymorphisms in different ethnic groups, such as Asians, may contribute to the interethnic variability in the pharmacokinetics and pharmacodynamics of statins. However, the *ABCG2* SNPs cannot completely explain ethnic differences in statin pharmacokinetics between Asians and Caucasians (Wang, 2011).

1.4.2 Pharmacogenomics of Statin Efficacy

It is well documented that drug transporters are involved in the pharmacokinetics of statins. In theory changes to the pharmacokinetics of a drug will influence the pharmacodynamics and efficacy of the drug. To date only a few studies have looked at the genetic variation with regards to the LDL-C reduction associated with statins using both genome wide association studies (GWAS) and candidate gene approaches. The Genetics Effects On STATins (GEOSTAT-1) was a prospective genetic study in 601 patients taking simvastatin 40 mg or rosuvastatin 10 mg. Using a candidate gene approach they found an enhanced reduction in LDL-C in patients taking rosuvastatin with a *ABCG2* (BCRP) variant with reduced function (421C>A, rs2231142) (Bailey et al., 2010). The Justification for the Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin (JUPITER) study looked at the use of rosuvastatin for the primary prevention of CVD in patients without hyperlipidemia (Ridker et al., 2008). In a subsequent analysis using a GWAS approach, Chasman and colleagues identified that

ABCG2 421C>A genotype was associated with increased efficacy for LDL-C reduction with rosuvastatin. In a candidate gene analysis, they also demonstrated that variations in OATP1B1 (*SLCO1B1*) (rs4149056 and rs4363657) were associated with decreased efficacy in reducing LDL-C (Chasman et al., 2012). The SEARCH trial also found a small but significant association between SLCO1B1 SNP rs419056 and lower LDL-C reductions, in patients from the Heart Protection Study (HPS) (Link et al., 2008). One small study reported that variation in *ABCB1* (P-gp) is associated with increased simvastatin efficacy. They found that homozygous carriers of *ABCB1* polymorphisms, 1236C>T and 2677G>A or T, had an enhanced reduction in total cholesterol and LDL-C compared to non-carrier individuals (Fiegenbaum et al., 2005).

1.4.3 Pharmacogenomics of Statin Toxicity

Genetic variation in drug transporters is also associated with pharmacokinetic changes and toxicity of statins. As discussed above, genetic variation in the hepatic uptake transporter, OATP1B1, and biliary efflux transporter, BCRP, are associated with variability in statin pharmacokinetics and plasma levels. Polymorphisms in these genes lead to increased systemic exposure and possible statin myopathy.

Hepatic uptake of all statins is facilitated to some extent by OATP1B1 on the sinusoidal membrane. The OATP1B1*5 associated with increased plasma levels of a number of statins (Pasanen et al., 2007). In 2008, the SEARCH study preformed the first GWAS for statin-induced myopathy including over 300,000 polymorphisms in 85 patients with simvastatin myopathy and 90 matched drug exposed controls. They found one polymorphism in *SLCO1B1* (*5, rs4149056) to be the most robust predictor of the risk for simvastatin-induced myopathy, revealing an odds ratio of 4.5 per copy of the variant allele (Link et al., 2008). They also estimated that *SLCO1B1**5 variant increased 5 year cumulative risk for myopathy in subjects with one or two alleles by 3% and 18% respectively. The link between the *SLCO1B1*5* and simvastatin-induced myopathy has been confirmed and expanded to include atorvastatin but not pravastatin induced myopathy (Voora et al., 2009). Another study has also replicated the link between

*SLCO1B1**5 and simvastatin induced myopathy but not atorvastatin induced myopathy (Brunham et al., 2012). The Genetics of Diabetes Audit and Research (Go-DARTS) study replicated the impact of the *SLCO1B1**5 polymorphism on statin intolerance in a real world population. They suggest that at least one-third of individuals with the *SLCO1B1**5 variant are likely to suffer from side effects on high dose statins (Donnelly et al., 2011).

Simvastatin induced myopathy has also been associated with variation in the efflux transporter ABCB1 (P-gp). Significant differences in allele frequencies were observed for ABCB1 (P-gp) polymorphisms, 1236C>T, 2677G>A or T, and 3534C>T, in patients with myalgia associated with simvastatin treatment (Fiegenbaum et al., 2005). Interestingly, in 110 patients with statin induced myopathy, only 10% had a rare heterozygous mutation in a gene normally related to myopathy syndromes including McArdle disease, CPT-II deficiency, and myoadenylate deaminase deficiency (Vladutiu et al., 2006). This suggests that the genetic basis for statin myopathy may include a complex mixture of rare and common DNA polymorphisms (Mancini et al., 2011).

1.4.4 Current Guidelines for Statin Pharmacogenetics

All statins are substrates of OATP1B1 but the effect of the *SLCO1B1**5 polymorphism differs depending on the statin. The *SLCO1B1**5 has the largest effect on simvastatin exposure; the AUC of simvastatin acid increases by 221% in individuals homozygous for the *5, 521CC, genotype (Pasanen et al., 2006). The AUCs of atorvastatin, pravastatin, and rosuvastatin are increased by 144%, 57-130% and 62-119%, respectively in homozygous *5, 521CC, individuals (Wilke et al., 2012). Given that statin induced myopathy appears to be a plasma concentration dependent adverse effect, it would be advisable to avoid high statin doses in patients with this genetic polymorphism. Recommendations have been made to reduce the maxim dose of simvastatin, pitavastatin and atorvastatin by half for each copy of the *5 allele an individual carries (1 copy ½ maximal dose, 2 copies ¼ maximal dose). While rosuvastatin and pravastatin doses should also be reduced by half in carriers of the *5 allele (Niemi, 2010). The FDA

updated the simvastatin product label in 2011, recommending against the use of 80 mg simvastatin in any patient unless it has been previously tolerated for at least 12 months (Wilke et al., 2012). More recently the Clinical Pharmacogenomics Implementation Consortium recommended gene-based dosing for simvastatin based on their *SLCO1B1**5, 521T>C, genotype. They recommend the use of decision support tools to direct physicians away from using 80 mg simvastatin doses and warn providers that doses of 40 mg of simvastatin daily also has a modest increased risk of myopathy (Wilke et al., 2012). The Clinical Pharmacogenomics Implementation Consortium also point out that the wild type genotype *SLCO1B1* 521TT does not imply the absence of other potentially deleterious variants in *SLCO1B1* or elsewhere (Wilke et al., 2012). Based on the *5 allele frequency and the rate of adverse reactions on high dose simvastatin, only 30 subjects would have to be genotyped to avoid one adverse drug reaction (ADR) (Wilke et al., 2012). Since, genotyping for one SNP can be achieved very rapidly and at a low cost, *SLCO1B1* genotyping is recommended to increase the safety of high dose statin therapy (Niemi, 2010; Wilke et al., 2012).

Table 1.6 Transporter Polymorphisms Involved in Statin Pharmacokinetics and Response

Statin	Transporter poly-morphism	Population	Effect on PK	Effect on Response	Ref
Atorvastatin	*SLCO1B1*5* 521T>C	Healthy Participants (n=32)	144% increase in AUC		(Pasanen et al., 2007)
		Healthy Participants (n=290)	123% increase in AUC		(Lee et al., 2010)
		Patients (n=509)		increased muscle toxicity, OR 2.7	(Voora et al., 2009)
		Patients (n=25, 85 controls)		no association with muscle toxicity	(Brunham et al., 2012)
	ABCG2 421C>A	Healthy Participants (n=32)	72% increase in AUC		(Keskitalo et al., 2009b)
Cerivastatin	*SLCO1B1*5* 521T>C	Patients (n=185, 732 control)		increased muscle toxicity, OR 1.89	(Marciante et al., 2011)
Fluvastatin	*SLCO1B1*5* 521T>C	Healthy Participants (n=32)	no effect on AUC		(Niemi et al., 2006)
	ABCG2 421C>A	Healthy Participants (n=32)	97% increase in AUC		(Keskitalo et al., 2009a)
Pitavastatin	*SLCO1B1*15* 388A>G + 521T>C	Healthy Participants (n=11)	162% increase in AUC		(Deng et al., 2008)
		Healthy Participants (n=24)	76% increase in AUC		(Chung et al., 2005)
		Healthy Participants (n=38)	209% increase in AUC		(Ieiri et al., 2007)

	ABCG2 421C>A	Healthy Participants (n=38)	no effect		(Ieiri et al., 2007)
Pravastatin	*SLCO1B1*5* 521T>C	Healthy Participants (n=41)	106% increase in AUC		(Niemi et al., 2004)
		Healthy Participants (n=32)	232% increase in AUC		(Niemi et al., 2006)
		Healthy Participants (n=107)	149% increase in AUC		(Ho et al., 2007)
		Pediatric Patients (n=32)	62% decreased AUC	lower LDL cholesterol reduction	(Hedman et al., 2006)
		Patients (n=45)		lower total cholesterol reduction	(Zhang et al., 2007)
		Patients (n=66)		lower total cholesterol reduction	(Tachibana-Iimori et al., 2004)
		Healthy Participants (n=30)	42% increase in AUC		(Mwinyi et al., 2004)
	*SLCO1B1*15* 388A>G + 521T>C	Healthy Participants (n=38)	98% increase in AUC		(Deng et al., 2008)
		Healthy Participants (n=41)	93% increase in AUC		(Niemi et al., 2004)
		Healthy Participants (n=107)	92% increase in AUC		(Ho et al., 2007)
		Healthy Participants (n=16)	110% increase in AUC		(Igel et al., 2006)
	ABCG2 421C>A	Healthy Participants (n=32)	no effect		(Keskitalo et al., 2009a)
		Healthy Participants (n=107)	no effect		(Ho et al., 2007)

Rosuvastatin	*SLCO1B1*5* 521T>C	Healthy Participants (n=142)	117% increase in AUC		(Lee et al., 2005)
		Healthy Participants (n=32)	65% increase in AUC		(Pasanen et al., 2007)
		Healthy Participants (n=30)	72% increase in AUC		(Choi et al., 2008)
	ABCG2 421C>A	Healthy Participants (n=32)	144% increase in AUC		(Keskitalo et al., 2009b)
		Healthy Participants (n=32)	76%% increase in AUC		(Zhang et al., 2006)
		Patients (n=6989)		enhanced LDL cholesterol reduction	(Chasman et al., 2012)
		Myocardial Infarction Patients (n=601)		enhanced LDL cholesterol reduction	(Bailey et al., 2010)
Simvastatin acid	*SLCO1B1*5* 521T>C	Healthy Participants (n=32)	221% increase in AUC		(Pasanen et al., 2006)
Simvastatin	*SLCO1B1*5* 521T>C	Patients (n=25, 84 control)		increased muscle toxicity	(Brunham et al., 2012)
		Patients (n=509)		increased muscle toxicity	(Voora et al., 2009)
		Diabetic Patients (N=4196)		increased statin intolerance	(Donnelly et al., 2011)
		Patients (n=85, 90 control)		increased muscle toxicity	(Link et al., 2008)
	ABCG2 421C>A	Healthy Participants (n=32)	111% increase in AUC		(Keskitalo et al., 2009a)

ABCB1 1236C>T	Patients (n=116)	lower LDL-C reduction, increased muscle toxicity	(Fiegenbaum et al., 2005)
ABCB1 2677G>A or T	Patients (n=116)	lower LDL-C reduction, increased muscle toxicity	(Fiegenbaum et al., 2005)
ABCB1 3534C>T	Patients (n=116)	increased muscle toxicity	(Fiegenbaum et al., 2005)

1.5 Drug Transporters and Statin Exposure and Myopathy

Clearly, the evidence suggests that increased statin plasma exposure causes an increased risk of muscle adverse effects and myopathy. Therefore, drug interactions or genetic polymorphisms that lead to increased statin exposure cause higher risk of developing myopathy. Most classical drug interactions involve inhibition of metabolism but drug transporters can also be involved. Most of the statins are administered in their active ionized β-hydroxyacid, which are less membrane permeable at physiological pH (Neuvonen et al., 2006). Statins also have a wide variability in LogD values for the active hydroxyacid form ranging from hydrophilic (pravastatin) to hydrophobic (atorvastatin) (Table 1.1). It is often suggested that hydrophobic statins have an increased risk of muscle injury compared with hydrophilic statins because of the greater permeability to cross myocyte membranes (Hamelin and Turgeon, 1998; Masters et al., 1995; Reijneveld et al., 1996). However, there is no reported change in the relative incidence of myotoxicity associated with statin lipophilicity (Thompson et al., 2003) suggesting that passive diffusion into muscle fibres does not completely determine the risk for toxicity. Furthermore, studies on the tissue distribution of statins show preferential accumulation in the liver and exclusion from skeletal muscle, suggesting involvement of drug transporters controlling systemic and tissue exposure (Duggan et al., 1989; Madsen et al., 2008). Most of the attention has been given to transporters the in small intestine, kidney and liver and their involvement in statin absorption and elimination (Tirona, 2005). In contrast, very little study has been done on statin transporters within skeletal muscle and their influence on myotoxic side effects of statins.

The strongest evidence for the involvement of drug transporters in statin myopathy relates to the hepatic uptake transporter OATP1B1. The study pharmacogenetics of OATP1B1 and statins has emphasized a role for OATP1B1 in controlling statin hepatic uptake and systemic exposure and the corresponding increase in muscle side effects. Drug interactions with OATP1B1 drug transport activity also presents another mechanism for transporter involvement in statin myopathy. Inhibition of OATP1B1 mediated statin uptake into the liver by gemfibrozil has been shown to increase the

plasma exposure of pravastatin (Kyrklund et al., 2003), cerivastatin (Shitara et al., 2004), and rosuvastatin (Schneck et al., 2004). Importantly, OATP1B1 is not expressed in skeletal muscle and cannot explain changes in muscle exposure that are thought to occur in myopathy.

OATP2B1 is another member of this uptake transporter family capable of transporting rosuvastatin and atorvastatin (Grube et al., 2006; Ho et al., 2006). OATP2B1 is widely expressed in the heart, kidney, intestine, liver, and placenta in comparison to the hepatic statin transporter OATP1B1 (Klaassen and Aleksunes, 2010) and expected to be responsible for controlling statin entry in to extrahepatic tissues. Studies in rats have suggested that drug transporters in muscle such as Oatp2b1 and Mrp1 may contribute to the skeletal muscle toxicity (Dorajoo et al., 2008; Sakamoto et al., 2008). Taken together evidence suggests that drug transporters have important roles in governing statin systemic exposure and that they may be involved in controlling muscle exposure and risk for myopathy.

1.6 Animal Models of Statin Myopathy and Drug Transport

The administration of statins induces myopathy in various preclinical animal models such as: guinea pigs, mice, rats, rabbits, dogs, pigs and monkeys (Bergman et al., 2003; Fukami et al., 1993; Madsen et al., 2008; Meador and Huey, 2011; Nakahara et al., 1998; Reijneveld et al., 1996; Sidaway et al., 2009; von Keutz and Schluter, 1998; Westwood et al., 2005; Westwood et al., 2008). However, the doses of statins required to induce myopathy in these species is very high when compared to therapeutic doses used in patients. Statin induced myopathy with elevations in serum CK has been demonstrated in the rat after repeated dosing for 10-14 days with lovastatin, pravastatin, simvastatin, cerivastatin, and rosuvastatin (Sidaway et al., 2009; Smith et al., 1991). The guinea pig has been suggested as a better model to study the safety margins of statins because of similarities in lipid metabolism compared with the human versus rat (Madsen et al., 2008).

The advent of drug transporter knockout mouse models has made studying the *in vivo* role of drug transporters in statin disposition possible. Many studies have examined statin pharmacokinetics in knockout models of Oatp1b2, the murine ortholog of human OATP1B1 and OATP1B3. Increased systemic exposure and decreased hepatic uptake have been reported for lovastatin, pravastatin, atorvastatin, and rosuvastatin (Chen et al., 2008; Degorter et al., 2012a; Zaher et al., 2008). However, there is a lack of data looking at muscle levels of statins in knockout mice. One study has looked at the toxicity associated with chronic pravastatin exposure in Oatp1a/1b knockout mice (Iusuf et al., 2012). Despite highly increased plasma concentrations Oatp1a/1b knockout mice, only one in five mice showed muscle lesions along the vertebral column. Oatp1a/1b knockout mice had significant increased CK after 15 days, but not 30 or 60 days, of treatment suggesting only transient muscle lesions in Oatp1a/1b knockout mice (Iusuf et al., 2012). Since, pravastatin has a lower risk of causing myopathy compared with other statins, more studies are needed to examine myopathy of other statins in transporter knockout mice.

Studies in rats and guinea pigs have cast doubt on the importance of drug uptake transporters. Muscle to plasma ratios of statins are much less than unity, and suggest that muscle is protected from statin accumulation (Madsen et al., 2008; Sidaway et al., 2009). Efflux transporters may be involved in maintaining low statin muscle concentrations and protecting muscle from toxicity. Co-administration of probenecid, a nonspecific Mrp inhibitor, and rosuvastatin in rats resulted in increased skeletal muscle toxicity (Dorajoo et al., 2008).

1.7 Summary

Statins are highly effective at lowering LDL-C and the treatment of cardiovascular disease. However, the risk of skeletal muscle toxicities may lead to the discontinuation of therapy and poor outcomes from CVD. It is well documented that statin skeletal muscle toxicities are dependent upon drug exposure. Statins are heavily dependent upon drug transporters for controlling their hepatic uptake and efficacy for reducing LDL-C. Drug

interaction and pharmacogenetic studies have demonstrated that drug transporters have a major role in controlling the statin system exposure and risk of statin myopathy.

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2 HYPOTHESES AND SPECIFIC AIMS

2.1 Focus of the Thesis

Given that at least 3-4 million Canadians are treated with statins, an estimated 500,000 patients experience the associated skeletal muscle side effects. These muscle side effects often prevent the continued treatment of hypercholesterolemia and prevention of CVD, making statin-induced muscle myopathies a significant health concern to Canadians.

It is well documented that statin myotoxicity is dose dependent and that increases in statin blood levels are a significant risk factor for the development of myotoxicity. As discussed above, the determinants of systemic statin exposure, such as the amount of drug absorbed and the duration and magnitude of exposure, play significant roles in the development of myopathy. However, statins cause toxicity directly in isolated myofibres *in vitro*, indicating that myopathy *in vivo* results from a direct effect on muscle and not an indirect action of statins on other tissues (Sakamoto et al., 2008). It is reasonable to assume that skeletal muscle fibre concentration of statins is equally as important as the plasma concentration, since many individuals are exposed to high statin blood levels but do not develop myopathy (Jacobson, 2006). The overall exposure of skeletal muscle to statins is partially determined by the systemic exposure and pharmacokinetic processes controlling absorption and elimination, occurring in the small intestine, liver and kidney. However, the process of statin distribution into skeletal muscle and how this relates to the muscle damaging effects has not been well studied. **My overall hypothesis is that the statin level in the skeletal muscle itself is a key determinant of myotoxicity.** This thesis focuses on drug transporting proteins in the skeletal muscle that govern the entry and exit of statins into muscle, regulating their exposure and toxic side effects.

2.2 Specific Aim 1

To determine the spectrum, localization and function of statin transporters expressed in human skeletal muscle.

Although the exact mechanism of myotoxicity is unknown, statins must gain entry into myocytes to exert the adverse effect of myotoxicity. Statins are large amphipathic compounds that have difficulty crossing biological membranes. Drug transporter proteins are extremely important in statin pharmacokinetics and are involved in their absorption, hepatic distribution and elimination. Drug transporters are involved in controlling tissue distribution of many different compounds in the body. Biological barriers like the placenta and blood brain barrier use drug transporters to selectively control entry of compounds into tissues. Skeletal muscle has been shown to express transporters involved in the uptake of amino acids, ions and glucose but little is known about expression of known drug transporters. In Chapter Three, we sought to identify the expression of statin transporters in skeletal muscle and determine their impact on statin exposure and toxicity *in vitro*.

We hypothesized that the functional expression of statin uptake and efflux transporters modulate the toxic response to skeletal muscle statin exposure. To test this hypothesis, we screened skeletal muscle cDNA for the expression of known drug transporters. We tested the ability of the skeletal muscle drug transporters to transport atorvastatin and rosuvastatin. Adenoviral modulation of drug transporters in primary human skeletal muscle cells was used to determine changes in toxicity. We describe in Chapter Three that the uptake transporter OATP2B1 and three novel statin efflux transporters, MRP1, MRP4 and MRP5, are found in skeletal muscle. We demonstrate that OATP2B1 sensitizes muscle to toxicity and MRP1 attenuates toxicity of atorvastatin and rosuvastatin in an *in vitro* skeletal muscle model.

2.3 Specific Aim 2

To examine the regulation and function of OATP2B1 transcription start site variants.

OATP2B1 is ubiquitously expressed in various tissues in the body like, small intestine, liver, brain, kidney and skeletal muscle. Many statins are also known to be substrates of OATP2B1 and due to the expression pattern we believe that OATP2B1 is involved in the bioavailability and extrahepatic distribution of statins including skeletal muscle exposure. Recently, it has been shown that differential promoter usage in tissues results in expression of five OATP2B1 transcriptional start site variants which utilize distinct first exons but share common subsequent exons (Pomari et al., 2009). These variants are expected to encode either a full length or shortened protein lacking 22 amino acids from the N-terminus. To date, there is an absence of information regarding the relative expression of the OATP2B1 transporter variants in key tissues responsible for drug absorption, elimination and skeletal muscle distribution. Moreover, the transport competency of the short OATP2B1 protein variant has not previously been demonstrated. In Chapter Four, we sought to examine the function and regulation of OATP2B1 transcription start site variants.

We hypothesized that OATP2B1 full length and short variant proteins would have differential tissue expression or transport function. To test this hypothesis we used absolute quantitative polymerase chain reaction to find that the full length variant is the major form expressed in duodenum but the short variant predominates in liver. The full length OATP2B1 protein variant is predominantly expressed in skeletal muscle. Function of the OATP2B1 variants were assessed using a transient heterologous cell expression system, we found that the transport activities of the short OATP2B1 variant towards substrates estrone sulfate and rosuvastatin are similar to the well-characterized full length variant. Transcriptional activity screening of the liver enriched OATP2B1 variant promoter identified hepatocyte nuclear factor 4 alpha (HNF4α) as a novel transacting factor. With a combination of *in silico* screening, promoter mutation in cell-based reporter assays and chromatin immunoprecipitation studies, we identified a

functional HNF4α binding site close to the transcription start site. We conclude that the major OATP2B1 protein form in liver is transport competent and its hepatic expression is regulated by HNF4α.

2.4 Specific Aim 3

To investigate the contribution of Oatp2b1 to the pharmacokinetics and tissue distribution of rosuvastatin in Oatp2b1 knockout mice.

Drug transporter knockout (KO) mouse models are useful in providing insight into the *in vivo* contribution that transporters have on drug distribution. Members of the Oatp1a and Oatp1b families have been successfully knocked out in a few different mouse models (Chen et al., 2008; van de Steeg et al., 2010; Zaher et al., 2008). These models have shown the importance of Oatp1a and 1b transporters in the pharmacokinetics and pharmacodynamics of some substrates. Oatp2b1 is the murine ortholog of the human OATP2B1 transporter and the effect of Oatp2b1 deletion on the substrate pharmacokinetics has not been previously described. In Chapter Five, we describe for the first time an Oatp2b1 KO mouse. This novel Oatp2b1 KO mouse provided the opportunity to investigate the role of Oatp2b1 in statin pharmacokinetics and distribution into skeletal muscle.

We hypothesize that Oatp2b1 is involved in the absorption, hepatic uptake and skeletal muscle distribution of rosuvastatin. To test this hypothesis we characterized the pharmacokinetics of rosuvastatin in Oatp2b1 KO mice after oral and intravenous administration. As described in Chapter Five, we demonstrate that Oatp2b1 does not affect the systemic exposure of rosuvastatin after oral and intravenous dosing. The extrahepatic distribution of rosuvastatin in Oatp2b1 KO mice is similar to wild type animals. However, we found that Oatp2b1 KO had an increased rosuvastatin liver to plasma ratio compared to wild-type mice. Importantly, we did not observe a significant difference in the skeletal muscle distribution of rosuvastatin.

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3 HUMAN SKELETAL MUSCLE DRUG TRANSPORTERS DETERMINE LOCAL EXPOSURE AND TOXICITY OF STATINS[1]

[1] Reproduced (adapted) with permission from: Knauer MJ, Urquhart BL, Meyer zu Schwabedissen HE, Schwarz UI, Lemke CJ, Leake BF, Kim RB and Tirona RG (2010) Human skeletal muscle drug transporters determine local exposure and toxicity of statins. *Circ Res* **106**(2): 297-306.

3.1 Introduction

3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, statins, are highly effective drugs for the treatment of hypercholesterolemia, a major risk factor of cardiovascular disease. Statins inhibit the synthesis of mevalonate, the rate-limiting step in cholesterol biosynthesis (Endo, 1992; Jacobson, 2008). While statins are generally well tolerated (Pasternak, 2002), skeletal muscle side-effects are a common complaint among those treated. One such side-effect, myalgia, which is defined as muscle aches or weakness in the absence of blood creatine kinase (CK) elevation, occurs in 5-15% of statin-treated patients (Bruckert et al., 2005; Buettner et al., 2008; Draeger et al., 2006; Evans and Rees, 2002; Jacobson, 2008; Thompson et al., 2003). In rare cases, potentially life-threatening statin-induced rhabdomyolysis may occur; a condition characterized by acute muscle damage resulting in pronounced elevation in CK levels and renal failure (Thompson et al., 2006).

The pathophysiology of statin-induced myopathy is not completely understood. The leading mechanism suggests a role for cellular depletion of secondary metabolic intermediates of mevalonate in the development of statin-induced myotoxicity (Baker, 2005). In addition to decreased cholesterol synthesis, HMG-CoA reductase inhibition by statins causes a commensurate reduction in the levels of downstream metabolic products including isoprenoids, dolichol and ubiquinone (coenzymeQ10, CoQ10) (Baker, 2005; Hanai et al., 2007; Itagaki et al., 2009; Sakamoto et al., 2007). Among these are the isoprenoid secondary metabolic intermediates geranylgeranylpyrophosphate (GGPP) and farnesylpyrophosphate that are involved in protein isoprenylation and activation of small GTPases such as Rho and Rab. An important role for diminished isoprenylation in the mechanism of statin myotoxicity is mediated by induction of the muscle atrophy-linked protein atrogin-1 (Hanai et al., 2007). This is highlighted by the findings that supplementation of GGPP to cultured skeletal myotubes or isolated myofibers treated with statins leads to attenuation of toxicity (Cao et al., 2009; Itagaki et al., 2009; Johnson et al., 2004; Sakamoto et al., 2007), while inactivation of a Rab and RhoA induces toxicity (Itagaki et al., 2009; Sakamoto et al., 2007). Decreased geranylgeranylation of small GTPases by statins appears to stimulate the mitochondrial apoptotic cell death

pathway in skeletal myotubes (Itagaki et al., 2009; Johnson et al., 2004). In addition to isoprenoids, CoQ10 levels in plasma (De Pinieux et al., 1996) and skeletal muscle (Lamperti et al., 2005) are decreased with statin treatment. Although depletion of CoQ10 is thought to affect oxidative phosphorylation and protection from statin-induced oxidative stress, compelling clinical evidence is lacking regarding the efficacy of CoQ10 treatment of statin myopathy (Marcoff and Thompson, 2007). There is recent evidence to indicate that fatty acid oxidation is perturbed in cultured myotubes of statin intolerant (myalgic) patients (Phillips et al., 2009), a finding that differs from patients with rhabdomyolysis (Phillips and Haas, 2008). Such data suggest that the mechanisms of statin toxicity are different between those affected with myalgia and rhabdomyolysis.

It is well documented that myotoxicity is statin dose-dependent, and myopathy risk increases when statins are co-administered with drugs that either interact to increase plasma statin levels or themselves have propensity for muscle damage (Ballantyne et al., 2003; Huerta-Alardin et al., 2005; Jones and Davidson, 2005; Neuvonen et al., 2006). Indeed, macrolide antibiotics and azole antifungals are well known drug inhibitors of hepatic statin metabolism via cytochrome P450 enzymes (CYP), dramatically increasing plasma statin levels (Ballantyne et al., 2003; Neuvonen et al., 1998; Neuvonen et al., 2006). Moreover, inhibition of statin liver uptake (transport) mediated by multiple members of the organic anion transporting polypeptide (OATP) family by drugs such as gemfibrozil, cyclosporine A and rifampin can elevate drug levels (Kyrklund et al., 2003; Neuvonen et al., 2006; Noe et al., 2007; Schneck et al., 2004; Shitara et al., 2004). Furthermore, we have previously reported that a common genetic polymorphism resulting in a single nucleotide difference in the *SLCO1B1* gene encoding hepatic OATP1B1 (521C>T, V174A; rs4149056) (Tirona et al., 2001) is associated with increased plasma levels of a number of statins (Pasanen et al., 2007). In a genome-wide association study, the rs4149056 polymorphism in *SLCO1B1* was found to be the most robust predictor of the risk for simvastatin-induced myopathy (Link et al., 2008). In addition, efflux transporters in liver that mediate secretion of statins into bile could play a role in risk for statin myopathy. For example, genetic variation in the statin biliary efflux transporters, multidrug resistance-associated protein 2 (MRP2) and breast cancer

resistance protein (BCRP), are associated with variability in pravastatin and rosuvastatin plasma levels, respectively (Hirano et al., 2005; Ieiri et al., 2007; Kitamura et al., 2008).

Despite that the currently marketed statins have varying physicochemical characteristics, membrane transporters that act to facilitate drug uptake or efflux in tissues appear to have significant influence on the pharmacokinetics of most statins. This is evidenced by marked changes in plasma drug levels following transporter inhibition or through their attendant genetic polymorphisms (Neuvonen et al., 2008; Shitara and Sugiyama, 2006). Considerable attention has been given to drug transporters in the small intestine, kidney and liver, which affect systemic exposure to statins. However, there is a paucity of studies that have examined statin transporters within skeletal muscle and their influence on myotoxic side effects of statins, despite that this has been considered conceptually (Thompson et al., 2003). Given that plasma drug levels do not entirely predict risk for statin myopathy (Jacobson, 2006), we hypothesize that factors which control local skeletal muscle statin concentrations, such as muscle transporters, may be more relevant. Specifically, we propose that the interplay between statin uptake versus efflux transporters modulates the response to skeletal muscle statin exposure.

In this study, we identify drug transporters in human skeletal muscle that affect the distribution of two prototypical lipophilic and hydrophilic statins, atorvastatin and rosuvastatin. We demonstrate that the uptake transporter, OATP2B1, and the efflux transporters, MRP1, MRP4 and MRP5 are expressed in skeletal muscle and are capable of transporting atorvastatin and rosuvastatin. Importantly, we show that by affecting drug transporter activity in a model of human skeletal muscle, statin toxicity can be modulated.

3.2 Methods

3.2.1 Reagents

[^{3}H] atorvastatin (5 Ci/mmol, 99% radiochemical purity) and [^{3}H] rosuvastatin (5 mCi/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H] rosuvastatin (79 Ci/mmol, 97.1% radiochemical purity) and unlabeled rosuvastatin were also kindly provided by Dr. Yi Wang (AstraZeneca, Wilmington, DE). Unlabeled atorvastatin, cerivastatin and gemfibrozil-glucuronide were obtained from Toronto Research Chemicals (North York, ON). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

3.2.2 Plasmids

Expression plasmids for OATP1A2, OATP1B1, OATP1B3, OATP2B1, rOatp1b2, BCRP and P-gp are described elsewhere (Kim et al., 2001; Tirona et al., 2003; Urquhart et al., 2008). MRP2 and MRP4 cDNA was provided by Dr. R.H. Ho (Vanderbilt University) and Dr. J.D. Schuetz (St. Jude Children's Research Hospital), respectively. MRP1 cDNA was obtained by PCR, using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), from a cDNA library of human skeletal muscle cDNA using oligonucleotide primers 5'-ACCGCCATGGCGCTCCGGGGCTTCTGCAGC-3' and 5'-GTCTATACGGTCGTCTGGGGCTCACACCAA -3'. Similarly, OAT3 was amplified from human kidney using the primers: 5'-AGTGCCATGGCCTTCTCGGAGATCCTGG-3' and 5'-GTTGTCCTCAGCTGGAGCCCAGGCCTGG-3'. MRP5 cDNA was amplified in two parts from skeletal muscle with the following primers: 5'-CTCCACTCAGAGAAGATGAAGGATATCGAC-3' and 5'-CCACATAAGCGAAGGTTCCACTGATTGCAA-3'; 5'-AAACCTCTCTCATTTCAGCCATTTTAGGCC-3' and 5'-GGGTGTAGATCTAACAGGGAGGAGTCAGCCCTT-3'. Full-length MRP5 was assembled from the two parts by ligation at the *Xba* I site. The PCR products for MRP1, MRP5 and OAT3 were cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen).

3.2.3 Adenoviral vectors

Adenoviral vectors containing LacZ, OATP2B1, or MRP1 were generated in pAD/CMV/V5-DEST using the ViraPower Adenoviral Expression System (Invitrogen, Carlsbad, CA). The titer of the virus preparations were measured by infection of HEK293 cells on 24-well plates with limiting dilutions of the viral stock using Adeno-X Rapid Titer Procedure (Clontech, Mountian View, CA).

3.2.4 Gene Expression Analysis

The mRNA expression of OATP2B1, MRP1, MRP2, MRP4, MRP5, and breast cancer resistance protein (BCRP) were measured by SYBR green quantitative real-time PCR with an ABI Prism 7700 sequence detection system (Applied Biosystems). Human skeletal muscle total RNA was sourced from BioChain (Hayward, CA). Total RNA was extracted from cultured cells using Trizol reagent (Invitrogen) and RNA quality and quantity was determined using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems) according to manufacturer's instruction and 30 ng of cDNA was used in each PCR reaction. The sequences of primers used for quantitative PCR are listed in Supplemental Table C1 (Appendix C). The amount of the transporter was normalized to 18S-rRNA and relative expression was determined using the $\Delta\Delta C_T$ method.

3.2.5 Immunoblot Analysis.

Primary human skeletal muscle myoblast (HSMM) cells were harvested in 5 mmol/L Tris-HCl (pH 7.4) flash frozen in liquid nitrogen and homogenized to obtain total cellular protein. Normal human skeletal protein lysate was purchased from BioChain (Hayward,CA). Protein samples were separated by SDS-PAGE and transferred to NuPAGE nitrocellulose membrane Western blotting system (Invitrogen, Carlsbad, CA).

HeLa cells overexpressing OATP2B1, MRP1, MRP4 or MRP5 using a vtf7 vaccinia virus method, described below, was used as positive control. Membranes were probed with custom-made rabbit polyclonal OATP2B1 antibody based on a C-terminus epitope (CSPAVEQQLLVSGPGKKPEDSRV), MRPr1 (Alexis Biochemicals), MRP4 M_4I-80 (Kamiya), or MRP5 H-100 (Santa Cruz). Anti-rat and rabbit horseradish peroxidase–labeled antibodies (Bio-Rad, Hercules, CA) were used as the secondary. The immobilized secondary antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and KODAK ImageStation 4000 MM (Mandel).

3.2.6 Immunohistochemistry

Paraffin-embedded sections from normal human skeletal muscle tissue (5 μm) were obtained from BioChain. The tissue sections were deparaffinized in xylol and rehydrated with graded solutions of ethanol/water. For heat-induced epitope retrieval, the tissue sections were boiled in citrate buffer (10 mmol/L, pH 6.0). After washing twice in ice-cold PBS, the slides were blocked with 2% fetal bovine serum (FBS) – phosphate buffered saline (PBS). Thereafter, the slides were incubated with diluted anti–OATP2B1, MRPr1, MRP4 M_4I-80 or MRP5 H-100 in a humidified atmosphere for 2h at room temperature. After several washing steps with PBS, the sections were incubated with the fluorescent-labeled secondary antibody (Invitrogen). After washing the slides with PBS, the tissue was mounted in antifading mounting medium containing 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories). Images were obtained by confocal fluorescence microscopy. As negative control, the primary antibody was omitted.

3.2.7 Cell Culture

Primary human skeletal muscle myoblast (HSMM) cells were obtained from Lonza (Walkersville, MD). HSMM cells were cultured in SkBM-2 medium (Lonza) according to the manufacturer's instructions at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were plated onto 12-well plates for transport studies and 96-well plates for

toxicity assays at a density of ~1 × 10^6 cells/mL. When the HSMM cells achieved ~70% confluence in SkBM-2 growth medium, cells were cultured in differentiation medium, DMEM-F12 (Lonza) supplemented with 2% horse serum (Invitrogen) for one week. The resulting differentiated cells were then cultured in SkBM-2 growth medium for two weeks before experiments.

3.2.8 Identification of Statin Transporters

HeLa cells (human cervical cancer cell line) were grown in 12-well plates (approximately 0.8 × 10^6 cells/well) and infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to absorb for 30 minutes. Cells were transfected with 1 μg of transporter cDNA or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. Double transfection experiments of both uptake and efflux transporters used 1 μg total transporter cDNA in a 1:4 ratio of uptake to efflux. The parental plasmid lacking insert was used in place of uptake or efflux transporter cDNA in the single transfections. Transport was evaluated using labeled substrate as previously reported (Cvetkovic et al., 1999). Drug accumulation was determined at selected time intervals by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

3.2.9 Statin Accumulation and Toxicity in Skeletal Muscle Cells

HSMM cells were transduced with the adenoviruses containing the transporter coding region in SkBM-2 medium containing a total of 1 × 10^7 infectious units per mL (IFU/mL). For single transporter overexpression, cells were transduced with 5 × 10^6 IFU/mL of Ad-MRP1 or Ad-OATP2B1 as well as 5 × 10^6 IFU/mL of Ad-LacZ, whereas for overexpression of two transporters, 5 × 10^6 IFU/mL of each adenovirus was used. After 24h, cells were treated with various concentrations of atorvastatin or rosuvastatin for 48-72h. Cellular viability was evaluated by methylthiazolyldiphenyl-tetrazolium

bromide (MTT) assay (see below), measurement of ATP content using CellTiter-Glo Luminescent Cell Viability Assay (Promega Biosciences, San Luis Obispo, CA) and assessment of caspase 3 and 7 activation using Caspase-Glo 3/7 Assay (Promega).

3.2.9.1 MTT Assay

The MTT assay was performed on HSMM cells in 96-well plates with approximately 1×10^5 cells per well. The cells were treated with rosuvastatin and atorvastatin 24h after transduction with adenovirus. After the cells were incubated for 72h, 25 µL of 5 mg/mL MTT in PBS was added to each well. The plates were incubated for 4 h at 37°C and the formazan formed was dissolved in 50 µL DMSO. The background absorbance at 670 nm was subtracted from the absorbance at 560 nm to obtain the raw absorbance data.

3.2.10 Statistical Analysis

Statistical differences between group parameters were determined by 1-way ANOVA or 2-way ANOVA, using Bonferroni's multiple comparison test, as appropriate (GraphPad Software Inc, San Diego, CA). A *P* value of <0.05 was considered statistically significant.

3.3 Results

3.3.1 Identification Of Statin Transporters In Skeletal Muscle

Little is known about the expression of drug transporters in human skeletal muscle. Therefore, we screened a cDNA library of human skeletal muscle for expression of a wide variety of drug transporters including OATPs, organic anion transporters (OATs), organic cation transporters (OCTs), MRPs, breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp). The known statin uptake transporters such as OATP1B1, OATP1B3, OAT1, and OAT3 (Burckhardt and Burckhardt, 2003; Hirano et al., 2004; Ho et al., 2006; Lau et al., 2007; Sasaki et al., 2002; Takeda et al., 2004) as well as efflux transporters such as MRP2, P-gp and BCRP (Hirano et al., 2005; Huang et al., 2006; Kivisto et al., 2005; Matsushima et al., 2005; Sasaki et al., 2002) were not detected in skeletal muscle (not shown). However, we detected mRNA expression of the known statin uptake transporter OATP2B1 (Grube et al., 2006; Ho et al., 2006) (Fig. 3.1A). The mRNA level of OATP2B1 was highly detectable in skeletal muscle, although it is significantly lower than tissues with very high expression (liver, kidney and small intestine). Given that MRP2 transports statins, the expression of other members of this efflux transporter family was examined revealing high expression of MRP1, MRP4 and MRP5 in skeletal muscle. Quantitative mRNA analysis and Western blot confirmed the expression of these drug transporters in human skeletal muscle (Fig. 3.1A and B). Immunolocalization of OATP2B1, MRP1, MRP4 and MRP5 in normal human skeletal muscle by confocal microscopy demonstrated that each was expressed on the sarcolemmal membrane (Fig. 3.1C).

We then determined whether the transporters identified in skeletal muscle were capable of statin transport using a recombinant (vtf-7) vaccinia virus overexpression system in a human cervical cancer cell line (HeLa) (Cvetkovic et al., 1999). As we previously described, OATP2B1 is capable of rosuvastatin transport (Ho et al., 2006) and here we confirm that atorvastatin is also a transport substrate (Fig. 3.2) (Grube et al., 2006; Niessen et al., 2009). Indeed, in this model, OATP2B1 increases the cellular accumulation of rosuvastatin and atorvastatin by 2-fold. While OATP2B1 is the relevant

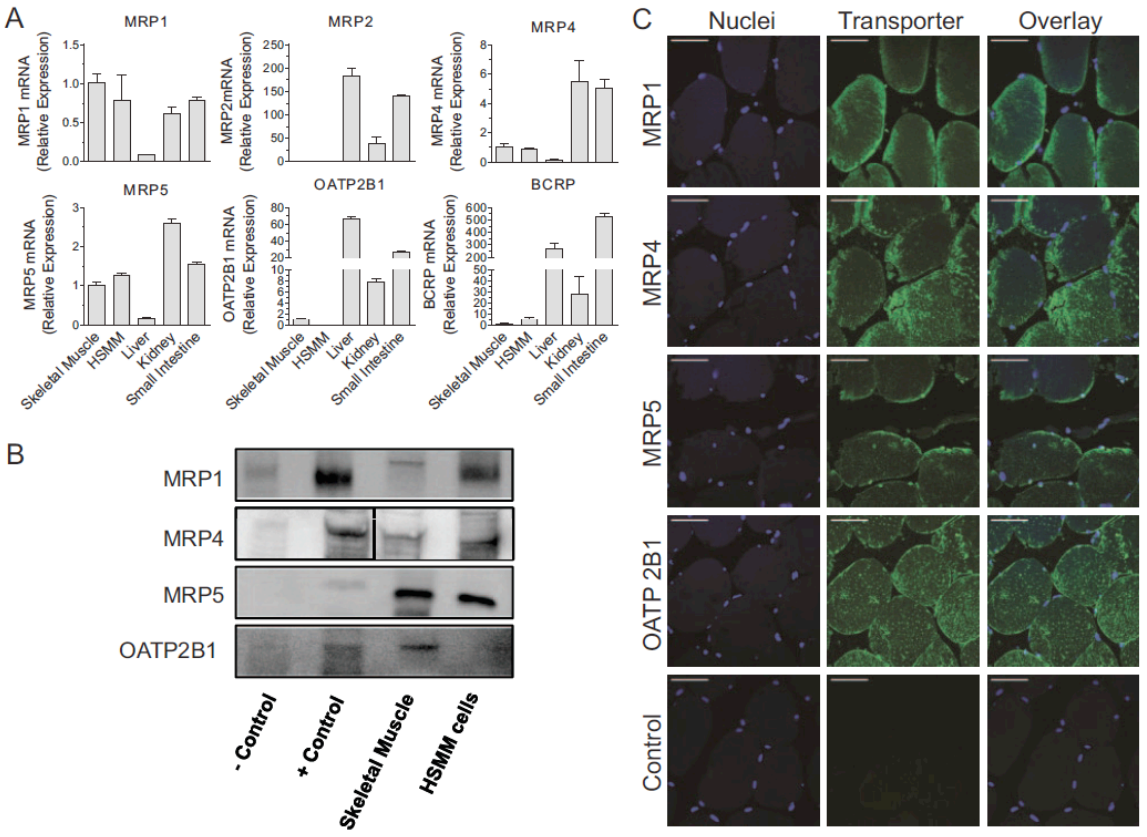

Figure 3.1 Expression of uptake and efflux transporters in various human tissues and HSMM cells.

(A) Relative gene expression of MRP1, MRP2, MRP4, MRP5, BCRP, and OATP2B1 in a range of human tissues and cultured HSMM cells. Expression was normalized to expression in human skeletal muscle. (B) Protein expression of MRP1, MRP4, MRP5, and OATP2B1 in human skeletal muscle and cultured HSMM cells by Western blot. Lysates from HeLa cells transfected with blank vector or transporter cDNA served as negative and positive controls, respectively. (C) Cellular localization of MRP1, MRP4, MRP5, and OATP2B1 in normal human skeletal muscle was determined by immunofluorescence confocal microscopy. Transporters are shown in green while nuclei are shown in blue. Scale bar = 50μm.

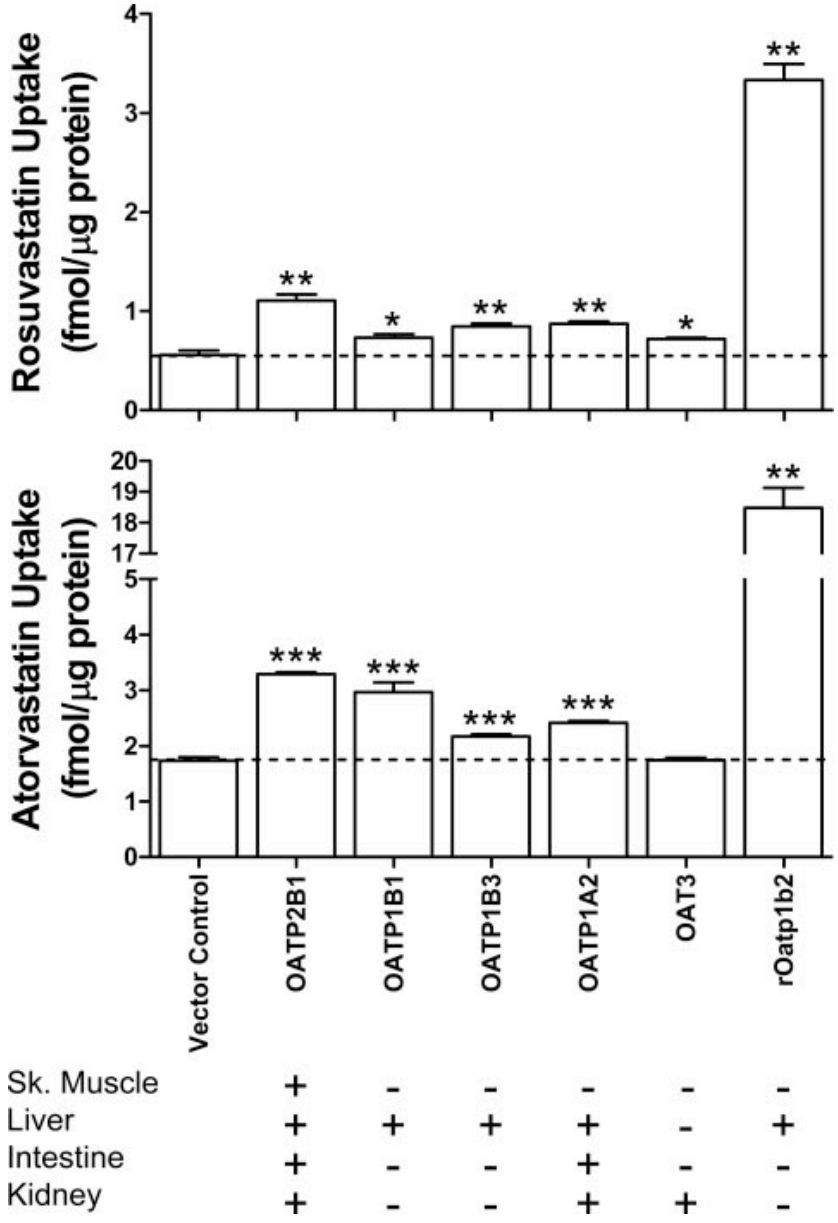

Figure 3.2 Intracellular accumulation of statins in HeLa cells transiently transfected with various uptake drug transporters.

Intracellular accumulation of [^{3}H]rosuvastatin and [^{3}H]atorvastatin in cells expressing OATP2B1, OATP1B1, OATP1B3, OATP1A2, OAT3 and rOatp1b2. Results are presented as percent of vector control ± SEM (n=3). *p<0.05, **p<0.01, ***p<0.001 compared to vector control. The presence (+) or absence (-) of transporter expression in various tissues is shown below.

transporter for uptake of statins into skeletal muscle, we present new and confirming data that other OATPs (1B1, 1B3 and 1A2) transport both atorvastatin and rosuvastatin (Fig. 3.2). It should be noted that the differences in statin transport between OATP2B1 and other transporters as shown in this model (Fig. 3.2) likely do not reflect the relative statin uptake efficiencies *in vivo*, since transporter expression was not normalized and the absolute expression of these transporters in different tissues is undetermined.

Drug interactions involving inhibition of the major liver OATPs (1B1 and 1B3) are associated with elevated plasma statin levels. Similarly, OATP2B1 is susceptible to inhibition by co-administered medications (Sai et al., 2006). Here, we show that stimulated intracellular accumulation of atorvastatin and rosuvastatin by OATP2B1 is attenuated after co-incubation with cerivastatin, gemfibrozil, gemfibrozil-glucuronide, fenofibrate, rifampin and glyburide (Fig. 3.3). Interestingly incubation with cyclosporine A caused a significant reduction in rosuvastatin accumulation but increased atorvastatin levels (Fig. 3.3).

The transport efficiency of human OATPs compared to rodent OATPs, is lower in this experimental system (Ho et al., 2006). For instance, the rat ortholog of the human transporters OATP1B1 and OATP1B3, rOatp1b2, appeared capable of mediating a significantly greater accumulation of rosuvastatin and atorvastatin into cells using this technique, in relation to human OATP2B1 (Fig. 3.2). For this reason, we used rOatp1b2 as the model transporter to maintain statin uptake into cells thereby allowing for the identification of pertinent transporters capable of statin efflux (see below).

Although highly expressed in skeletal muscle, it was not known whether MRP1, MRP4 or MRP5 transported statins. Hence, HeLa cells were double transfected with the uptake transporter, rOatp1b2, and various efflux transporters. In this system, modulation of cellular retention of atorvastatin or rosuvastatin served as an indicator for efflux transport activity. Indeed, we confirm that rosuvastatin and atorvastatin are transported by MRP2, P-gp, and BCRP using this double transporter (uptake/efflux) transfection system (Fig 3.4). Rosuvastatin retention in cells expressing rOatp1b2 together with MRP1, MRP4 or MRP5 was lower (77, 80 and 27% lower, respectively) than cells expressing rOatp1b2,

alone (Fig. 3.4). This was also true for atorvastatin when MRP1 and MRP4 (63 and 47% lower, respectively) but not MRP5 were double transfected with rOatp1b2. These findings demonstrate that MRP1, MRP4, and MRP5 are novel statin transporters.

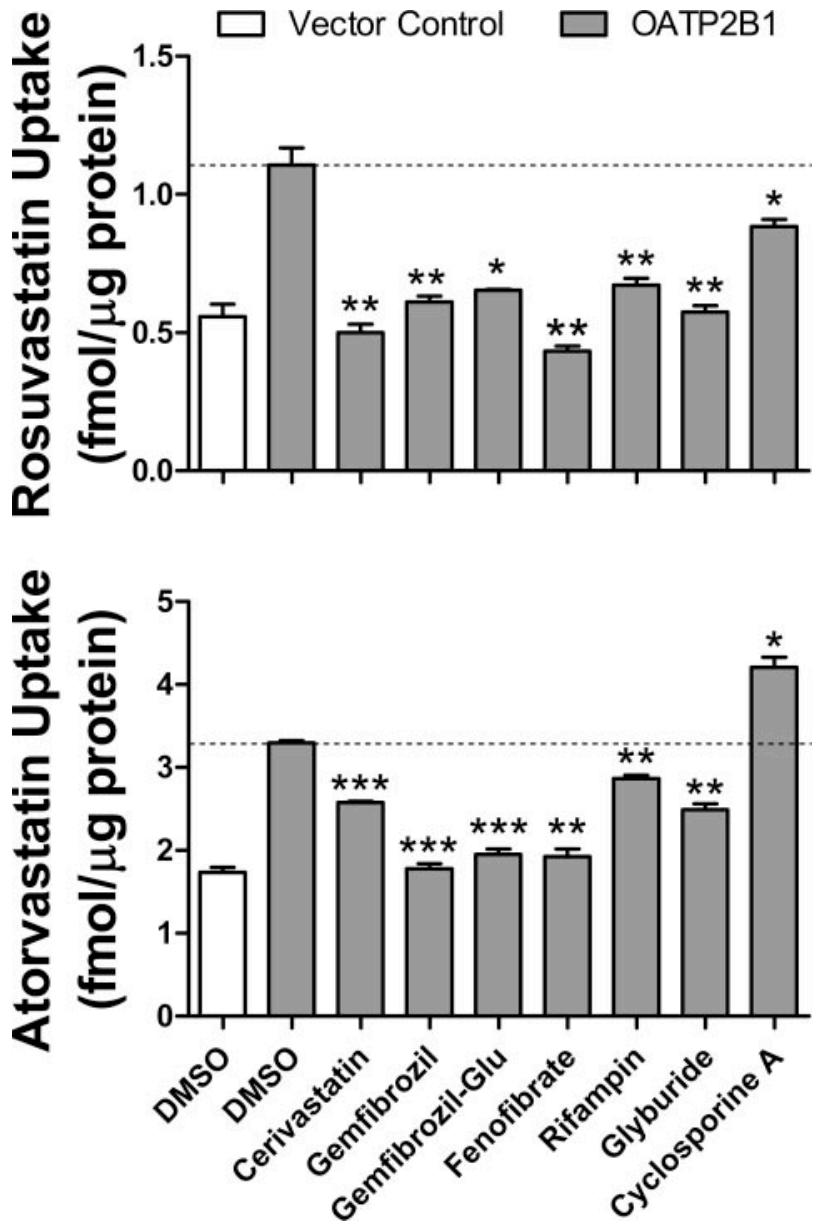

Figure 3.3 Chemical inhibition of statin uptake by OATP2B1 in transiently transfected HeLa cells.

Intracellular accumulation of [^{3}H]rosuvastatin and [^{3}H]atorvastatin in cells expressing OATP2B1 (grey bars) co-treated with cerivastatin, gemfibrozil, gemfibrozil-glucuronide, fenofibrate, rifampin, glyburide or cyclosporine A at 100 μM. Results are presented as fmol per μg of protein ± SEM (n=3). *$p<0.05$, **$p<0.01$, and ***$p<0.001$ compared to OATP2B1 DMSO treatment.

Figure 3.4 Intracellular accumulation of statins in HeLa cells transiently transfected with rOatp1b2 and various efflux drug transporters. Intracellular accumulation of [^{3}H]rosuvastatin and [^{3}H]atorvastatin in HeLa cells expressing rOatp1b2 and/or MRP1, MRP2, MRP4, MRP5, P-gp, or BCRP. Results are presented as percent of rOatp1b2 mediated uptake ± SEM (n=3 to 8). ***$p<0.001$ compared to vector control and efflux transporter alone. †††$p<0.001$ compared with rOatp1b2 mediated uptake. The presence (+) or absence (-) of transporter expression in various tissues is shown below.

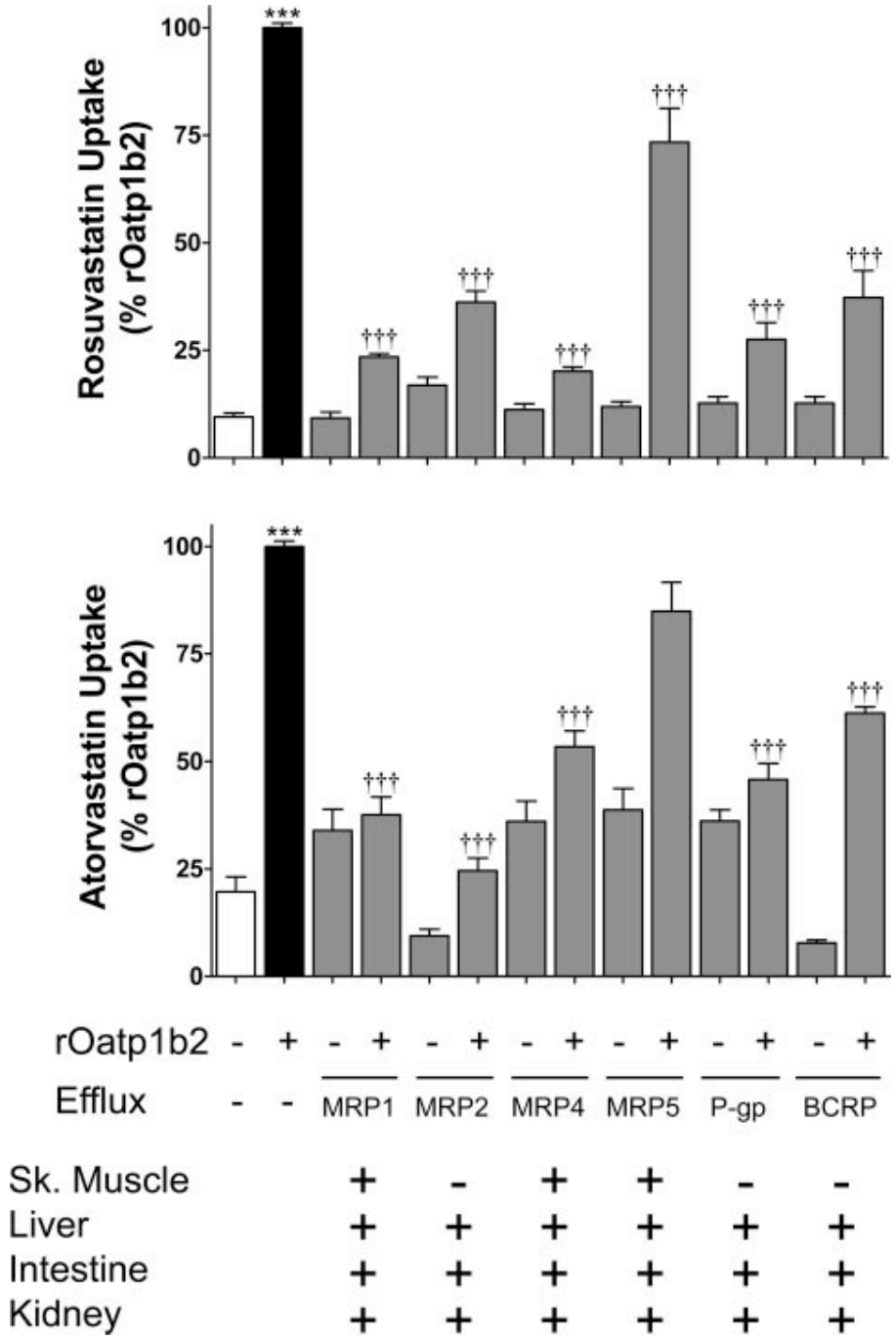

Figure 3.4 Intracellular accumulation of statins in HeLa cells transiently transfected with rOatp1b2 and various efflux drug transporters.

3.3.2 Statin Disposition In An *In Vitro* Model Of Human Skeletal Muscle

To evaluate the role of statin transport in toxicity, we employed differentiated, primary human skeletal muscle myoblasts (HSMM) as an *in vitro* model. First, we assessed whether HSMM cells expressed statin efflux transporters and found that MRP1, MRP4, and MRP5 are constitutively expressed (Fig. 3.1A and B) while MRP2, BCRP and P-gp are absent. Cellular localization studies revealed that some MRP1 is expressed on the cell surface of HSMM cells, although significant levels of the transporter are found in intracellular spaces. By contrast, MRP4 and MRP5 are not found on the plasma membrane, but localize within the golgi in HSMM cells (Fig. 3.5A). To test whether functional MRP activity is present in HSMM cells, statin accumulation was examined after chemical inhibition of efflux transport. When HSMM cells were co-incubated with known MRP inhibitors MK-571 (Gekeler et al., 1995), dipyridamole (Curtin and Turner, 1999), quercetin (Walgren et al., 2000), or verapamil (Goh et al., 2002) there was a significant increase in cellular retention for both atorvastatin and rosuvastatin when compared to cells treated with vehicle (Fig. 3.5B). Together with membrane localization studies, the results indicate that HSMM cells natively express functional MRP1 transporter whose activity determines the intracellular accumulation of statins. Co-incubation with cerivastatin also significantly increased the cellular retention of atorvastatin and rosuvastatin in HSMM cells (Fig. 3.5B), suggesting that this statin interacts with and is a possible substrate of MRPs. With respect to statin uptake, none of the known statin uptake transporters were expressed in HSMM cells and importantly, OATP2B1 is not expressed despite that it is present *in vivo* (Fig. 3.1).

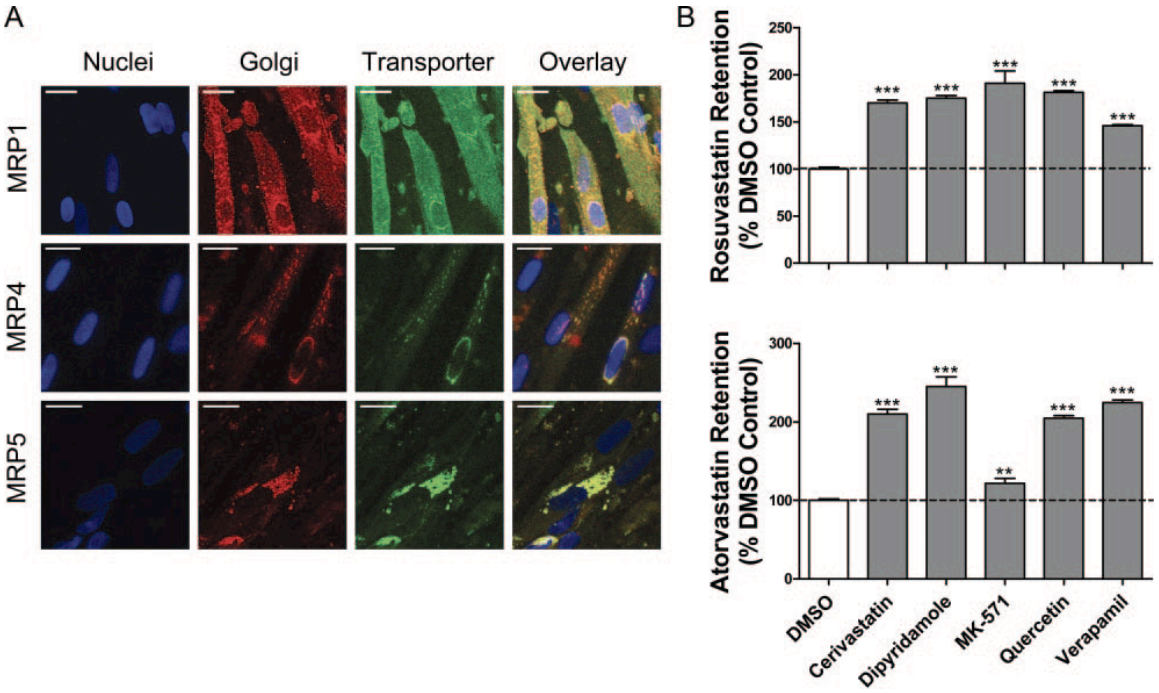

Figure 3.5 Statin accumulation in HSMM cells after co-treated with MRP inhibitors.

(A) Immunofluorescence localization of MRP1, MRP4 and MRP5 (green), golgi (red) and nuclei (blue) in differentiated HSMM cells using confocal microscopy. Scale bar = 20μm. (B) Intracellular accumulation of [^{3}H]rosuvastatin and [^{3}H]atorvastatin after 30 mins in HSMM cells co-treated with or without 100 μM cerivastatin or the non-specific MRP inhibitors dipyridamole, MK-571, quercetin, and verapamil at 100 μM. Results are presented as percent of DMSO control ± SEM (n=3-4), **p<0.01, ***p<0.001.

3.3.3 Role Of Transporters In Statin Toxicity In An *In Vitro* Model Of Human Skeletal Muscle

We next examined the effect of transporters on skeletal muscle exposure and toxicity of statins. In this experiment, we overexpressed OATP2B1 and MRP1 in HSMM cells using adenoviral vectors (Ad-OATP2B1 and Ad-MRP1, respectively). Examination by confocal microscopy demonstrated robust overexpression of these transporters in HSMM cells (Fig. 3.6). There was significant plasma membrane expression of MRP1, whereas for OATP2B1 there was some transporter on the cell membrane, but the majority was confined intracellularly.

Adenoviral over-expression of OATP2B1 in HSMM cells caused a significant increase in the cellular retention of both atorvastatin and rosuvastatin over 60 min (Fig. 3.6B). Upon transduction with Ad-OATP2B1 and Ad-MRP1, the cellular retention of both rosuvastatin and atorvastatin were significantly attenuated compared to Ad-OATP2B1 alone. Transduction with Ad-MRP1 alone did not significantly reduce the levels of rosuvastatin in HSMM cells; however, there was a trend towards reduced atorvastatin accumulation. Consequently, the effect of statin efflux transporters on intracellular statin levels is not pronounced in the absence of influx transporters. These results indicate that overexpression of OATP2B1 and MRP1 leads to changes in intracellular statin levels in an HSMM cell model.

Preliminary studies were performed to define the time- and concentration-dependency for atorvastatin and rosuvastatin cytotoxicity in HSMM cells. Cell viability, as evaluated by intracellular ATP levels, declined after 5 days of statin treatment. At comparable concentrations (e.g. 100 μM), atorvastatin was more toxic than rosuvastatin (Fig. 3.7A). The cytotoxicity of atorvastatin and rosuvastatin in HSMM cells after adenoviral-mediated transporter gene delivery was assessed by two measures of mitochondrial function, ATP content and MTT reduction to formazan (Fig. 3.7B-D), as well as activation of Caspases 3/7, a marker of apoptosis induction (Fig. 3.7D). Transduction of HSMM cells with Ad-OATP2B1 sensitized HSMM cells to atorvastatin toxicity as demonstrated by signals from all three toxicity end-points (Fig. 3.7B-D). There was a

similar trend for rosuvastatin toxicity but only the increased activity of caspases 3/7 reached statistical significance (Fig. 3.7D). This result is likely because overexpressed OATP2B1 was not well localized on the plasma membrane, leading to modest elevation of intracellular statin concentrations. Similarly, when HSMM cells were transduced with Ad-MRP1 alone, there was a lack of effects on statin cytotoxicity, consistent with the absence of significant changes in intracellular statin accumulation. However, when cells were transduced with both Ad-OATP2B1 and Ad-MRP1, there was protection against toxicity by both statins when compared to HSMM cells transduced with Ad-OATP2B1 alone to viabilities similar to those cells transduced with Ad-MRP1 alone (Fig. 3.7B-7D). Taken together, these results indicate that OATP2B1 expression promotes statin toxicity while MRP1 is cytoprotective in human skeletal muscle cells.

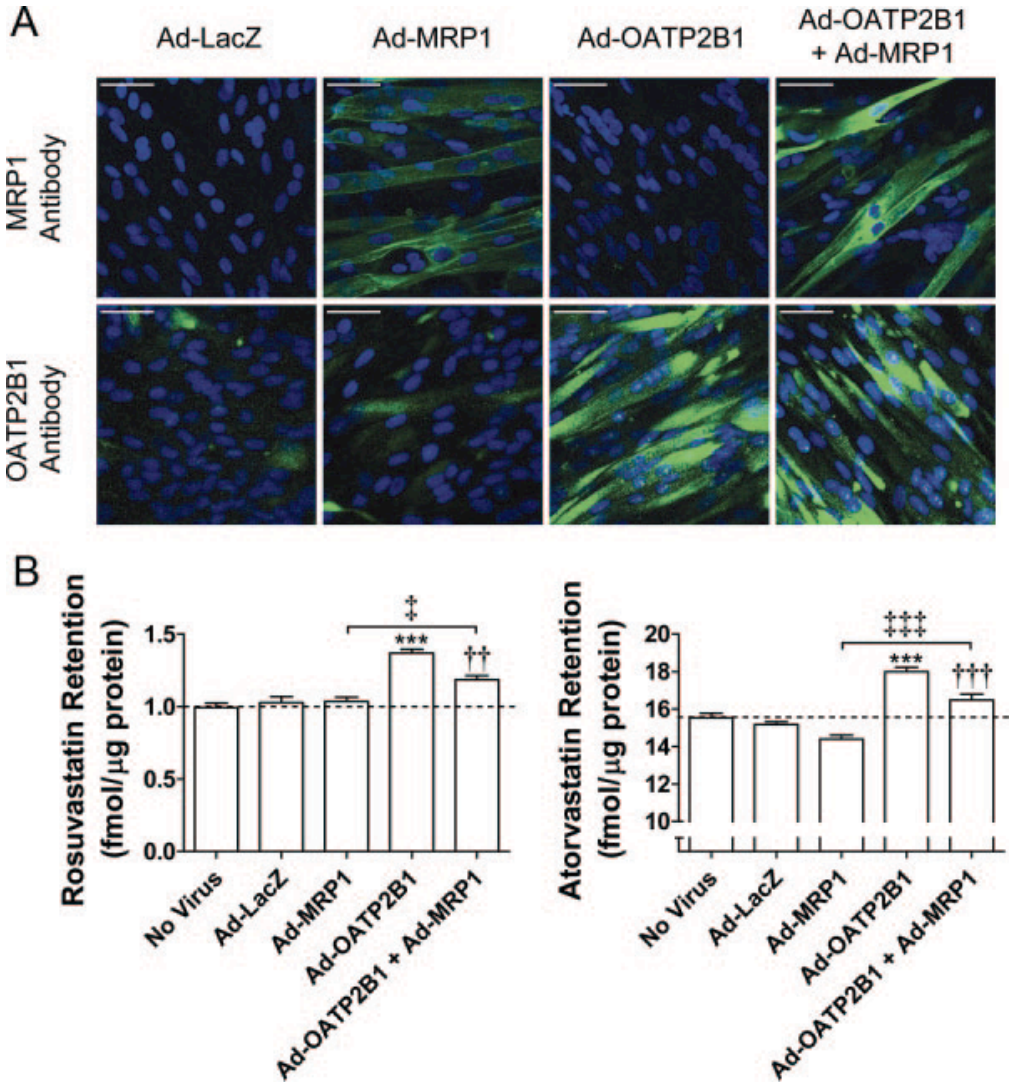

Figure 3.6 Statin accumulation in HSMM cells after adenoviral transduction of MRP1 and OATP2B1.

(A) Immunofluorescence localization of MRP1 or OATP2B1 (green) and nuclei (blue) in HSMM cells after adenoviral infection with Ad-LacZ, Ad-MRP1, Ad-OATP2B1, or Ad-OATP2B1 and Ad-MRP1 in differentiated HSMM cells using confocal microscopy. Scale bar = 50µm. (B) Intracellular accumulation of [^{3}H]rosuvastatin and [^{3}H]atorvastatin in HSMM cells after adenoviral over-expression of OATP2B1 and MRP1. Results are presented as fmol per µg of protein ± SEM (n=4). ***p<0.001 compared with no virus, Ad-LacZ or Ad-MRP1. ††p<0.01, †††p<0.001 compared with Ad-OATP2B1. ‡p<0.05, ‡‡‡p<0.001 compared with Ad-MRP1.

Figure 3.7 Statin cytotoxicity in HSMM cells after adenoviral transduction of MRP1 and OATP2B1. (A) Dose-dependent, cellular viability of HSMM cells after treatment with atorvastatin and rosuvastatin for 5 days measured by intracellular ATP concentration. Results are presented as percent of DMSO control ± SEM (two independent experiments with 3 determinations per experiment). (B) Cellular viability of HSMM cells after treatment with atorvastatin and rosuvastatin for 72h measured by intracellular ATP concentration. Results are presented as percent of DMSO control ± SEM (n=5). (C) Cellular viability of HSMM cells after treatment with atorvastatin and rosuvastatin for 72h measured by formazan formation using a MTT assay. Results are presented as percent of DMSO control ± SEM (n=4). (D) Induction of apoptosis in HSMM cells after treatment with atorvastatin and rosuvastatin for 48h measured by activation of caspase 3 and 7. Results are presented as percent of DMSO control ± SEM (n=4), *$p<0.05$, **$p<0.01$, and ***$p<0.001$.

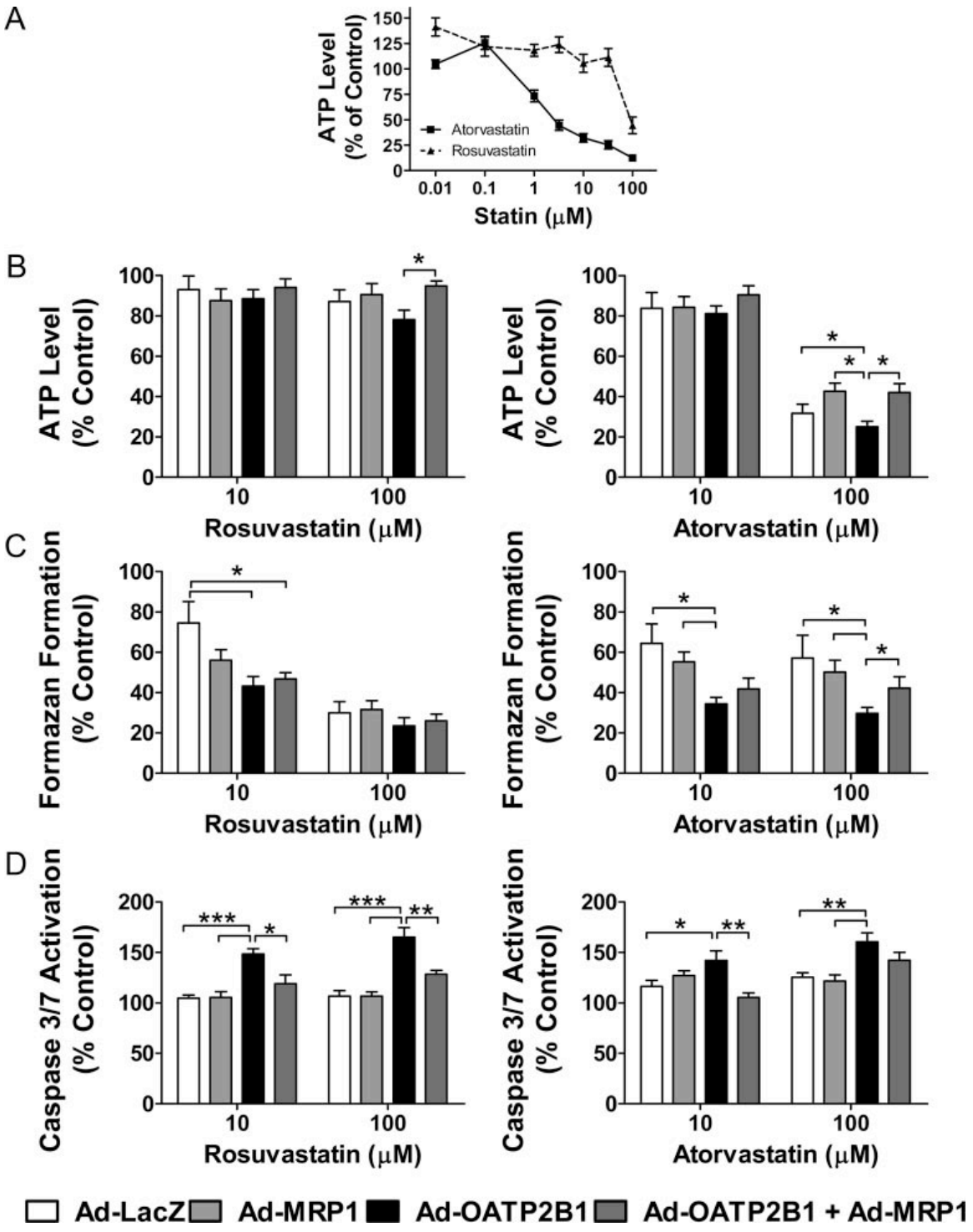

Figure 3.7 Statin cytotoxicity in HSMM cells after adenoviral transduction of MRP1 and OATP2B1.

3.4 Discussion

Drug transporters have recently been implicated in statin-induced myopathy. However, those that have been previously considered have been the transporters located in the liver and small intestine, which are largely responsible for controlling plasma statin concentrations. Indeed, genetic polymorphisms in the hepatic statin uptake transporter, OATP1B1, leading to reduced transport function are associated with a dramatic increase in risk for simvastatin-related myopathy (Link et al., 2008). Moreover, inhibition of both OATP1B1 and liver glucuronidation activity by concomitant treatment with the antilipidemic drug, gemfibrozil, causes elevation of cerivastatin plasma concentrations conferring greater predisposition to rhabdomyolysis (Prueksaritanont et al., 2002; Shitara et al., 2004). Although high plasma statin level is thought to be a risk factor, it does not entirely predict myopathy. In fact, there are individuals who exhibit high statin plasma levels but do not develop myopathy, suggesting that other factors including skeletal muscle fiber statin concentration may have an impact on side-effect risk (Jacobson, 2006). Despite the recognition that drug transporters control intracellular statin concentrations, the relevant transporters in human skeletal muscle have long been over-looked.

In this report we identified drug transporters in human skeletal muscle capable of transporting statins. Previous reports have shown that OATP2B1 is a high affinity uptake transporter for both atorvastatin and rosuvastatin (Grube et al., 2006; Ho et al., 2006). OATP2B1 is expressed on the apical and basolateral membranes of enterocytes and hepatocytes, respectively, and contributes to the oral absorption and hepatic distribution of statins. In addition, OATP2B1 is localized on the plasma membrane of cardiac endothelial cells (Grube et al., 2006), as well as in platelets (Niessen et al., 2009), where it is thought to be involved in the pleiotropic cardiovascular effects of statins. Here, we show for the first time, OATP2B1 is similarly expressed on human skeletal muscle sarcolemmal membrane. These findings are consistent with a report that suggested the presence of Oatp1a4 and Oatp2b1 in rat skeletal myofibers at the mRNA level (Sakamoto et al., 2008). However, demonstration of rat Oatp1a4 and Oatp2b1 protein expression in muscle was not confirmed, nor were data presented to show that these transporters

mediate statin uptake (Sakamoto et al., 2008). Despite that direct measurement of statin accumulation was not monitored, co-treatment of rat skeletal myofibers with the OATP inhibitor, estrone sulfate, afforded protection against the toxicity of the hydrophilic and lipophilic statins, pravastatin and fluvastatin, respectively (Sakamoto et al., 2008).

The known statin efflux transporters, namely P-gp, MRP2 and BCRP are not expressed in human skeletal muscle (Fig. 3.1A). However, isoforms of the MRP transporter family such as MRP1, MRP4 and MRP5 are highly expressed in skeletal muscle, though previous to this report, their capacity for statin efflux was unknown. Here, we demonstrate that the three human skeletal muscle MRPs (MRP1, MRP4, and MRP5) transport rosuvastatin and/or atorvastatin. These transporters are expressed on the sarcolemmal membrane of muscle fibers, indicating a protective role against intracellular statin accumulation. There is wide substrate overlap among MRPs (Leslie et al., 2005) and this is certainly also the case for statins which are transported by the skeletal muscle MRPs, albeit at differing efficiencies. Recently, a role for rat Mrp1 in statin-induced myopathy has been suggested in studies that demonstrate precipitation of rosuvastatin-mediated skeletal muscle toxicity in rats co-treated with the MRP inhibitor, probenecid (Dorajoo et al., 2008). Interpretation of these findings remains difficult for a number of reasons including a lack of demonstration that rat Mrp1 transports rosuvastatin, absence of Mrp1 expression data in tissues such as skeletal muscle, and a deficiency of information regarding differences in plasma and tissue concentrations of rosuvastatin after probenecid cotreatment (Dorajoo et al., 2008).

The dynamic interplay between uptake and efflux transporter activities likely controls muscle fibre statin concentrations, which determines susceptibility to toxicity. We have shown that the toxicity of rosuvastatin and atorvastatin in primary human skeletal muscle cells is dependent on the achieved intracellular drug concentrations. This is highlighted by the findings that reduction of cellular statin accumulation by MRP1 overexpression in cultured skeletal muscle cells heterologously expressing OATP2B1 (Fig. 3.6B), afforded cytoprotection against statin exposure (Fig. 3.7C and D). In the guinea pig, skeletal muscle concentrations of rosuvastatin and atorvastatin are less than 10% of that found in plasma, suggesting that the balance is tipped towards higher efflux than uptake activity

(Madsen et al., 2008). In our evaluation of the literature, the plasma-to-skeletal muscle concentration ratio of statins in humans is not known, but this value will undoubtedly be dependent on the relative expression and intrinsic activities of the attendant uptake (OATP2B1) and efflux (MRP1, MRP4, MRP5) transporters. The current results would also suggest that drug-statin interactions occurring not only at the level of the hepatocyte cell membrane, but also in skeletal muscle fibers could contribute to myopathy. Certainly, a number of clinically used drugs are substrates/inhibitors of the here identified skeletal muscle statin transporters (Fig. 3 and 5B) (Bakos and Homolya, 2007; Borst et al., 2007). Our data suggest that skeletal muscle statin uptake by OATP2B1 can be inhibited by concomitantly administered drugs such as gemfibrozil, fenofibrate and glyburide (Fig. 3.3). This finding could be considered contradictory to the increased risk of statin myopathy in patients co-treated with gemfibrozil. However, it should be mentioned that the gemfibrozil inhibits hepatic statin clearance to increase systemic statin exposure and there remains the possibility that inhibition of skeletal muscle efflux transport could offset the protection provided by OATP2B1 inhibition. Indeed, we demonstrate that statin efflux can be blocked by concurrent treatment of HSMM cells with known inhibitors of MRPs (Fig. 3.5B). Moreover, one must consider not only pharmacokinetic but also pharmacodynamic interactions in extrapolating the current transport inhibition findings to myopathy risk.

There are limitations with the differentiated HSMM cell model for the study of statin toxicity. Firstly, although MRP1, MRP4 and MRP5 are constitutively expressed in HSMM cells, only MRP1 localized to the plasma membrane (Fig. 3.5A). This is in contrast to immunofluorescence data that show these MRPs are expressed on the sarcolemmal membrane of intact skeletal muscle fibers (Fig. 3.1C). It is for this reason we are only able to assess the effect of MRP1 but not MRP4 or MRP5 on cytoprotection against statins in this model of skeletal muscle. Furthermore, HSMM cells do not natively express OATP2B1 as is found *in vivo*, hence we required viral gene delivery to assess the role of uptake transport on statin toxicity. That OATP2B1 is not expressed in HSMM cells compares well with other skeletal muscle genes that we have found at very low levels in relation to intact skeletal muscle including CK-M isoform (Supplemental Figure C1, Appendix C). Therefore, in interpreting the current toxicity findings it should be

considered that this *in vitro* model of skeletal muscle differs phenotypically to muscle fibres *in vivo*.

In conclusion, statin transporters are present in human skeletal muscle, which control intracellular drug exposure. We propose a role for OATP2B1 in sensitizing skeletal muscle cells to statin toxicity and that the novel statin efflux transporters, MRP1, MRP4 and MRP5 protect muscle from toxicity. The dynamic functional interplay between these uptake and efflux transporters *in vivo* likely determines risk for statin-induced myopathy.

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4 TRANSPORT FUNCTION AND TRANSCRIPTIONAL REGULATION OF A LIVER-ENRICHED HUMAN ORGANIC ANION TRANSPORTING POLYPEPTIDE 2B1 TRANSCRIPTIONAL START SITE VARIANT[2]

[2] The material in this chapter is based on a manuscript in preparation: Knauer MJ, Girdwood AJ, Leake BF, Ho RH, Kim RB, Tirona RG. Transport Function and Transcriptional Regulation of a Liver-Enriched Human Organic Anion Transporting Polypeptide 2B1 Transcriptional Start Site Variant (2012).

4.1 Introduction

The Organic Anion Transporting Polypeptides (OATPs, gene symbol solute carrier family SLCO) are a superfamily of transmembrane proteins. The SLCO genes encode 12-transmembrane domain proteins capable of transporting a wide variety of amphipathic substrates in a sodium independent manner. Different OATPs have partially overlapping substrates and differing tissue expression patterns. For example, OATP1B1 and OATP1B3 and are primarily expressed on the basolateral membrane of hepatocytes, whereas others, like OATP2B1, have a much broader expression pattern (Hagenbuch and Meier, 2004). OATP2B1 is expressed in liver, placenta, small intestine, kidney, brain, skin, heart, platelets, and skeletal muscle (Hagenbuch and Meier, 2004; Knauer et al., 2010; Kullak-Ublick et al., 2001; Niessen et al., 2009; Tamai et al., 2000). A wide variety of drugs are transport substrates of OATP2B1 including: benzylpenicillin (Tamai et al., 2000), bosentan (Treiber et al., 2007), ezetimibe-glucuronide (Oswald et al., 2008), fexofenadine (Nozawa et al., 2004a), glibenclamide (Satoh et al., 2005), troglitazone (Nozawa et al., 2004b), atorvastatin (Grube et al., 2006), fluvastatin (Kopplow et al., 2005), pitavastatin (Shirasaka et al., 2011), pravastatin (Shirasaka et al., 2010), and rosuvastatin (Ho et al., 2006) as well as steroid sulfate conjugates like estrone sulfate (E1S) (Tamai et al., 2001). OATP2B1 has also been implicated in the pH dependent absorption of multiple drugs across the human intestine epithelial cells (Kobayashi et al., 2003; Nozawa et al., 2004a). OATP2B1, like OATP1B1 and OATP1B3, is thought to mediate the sodium independent uptake of amphiphilic organic anions into the liver (Aoki et al., 2009). Given expression in placenta and mammary gland, OATP2B1 has been connected to the uptake and supply of precursor molecules for steroid synthesis (Ugele et al., 2003). Previously, we have described a potential role for OATP2B1 in the skeletal muscle uptake and sensitization to statin toxicity (Knauer et al., 2010).

Originally cloned from human brain, OATP2B1 is predicted to be a 709 amino acid protein (Kullak-Ublick et al., 2001; Tamai et al., 2000). Gene regulation analysis using promoter reporters in intestinal and liver cells revealed that the constitutive expression of the originally cloned OATP2B1 protein variant was regulated by the Sp1 transcription factor (Maeda et al., 2006). Recently, Pomari and colleagues have shown that differential

promoter usage in tissues results in the expression of several OATP2B1 transcription start site (TSS) variants (Fig. 4.1) (Pomari et al., 2009). These OATP2B1 variants utilize 5 distinct first exons and associated promoters but share common subsequent exons (exons 2 to 14). One TSS variant represents the original full length (709 amino acids) protein with translation start site within this first exon (termed exon 1b). The other variants arising from transcription initiation at exons 1a, 1c, 1d and 1e are expected to produce a shorter protein (687 amino acids) than the 1b variant, as translation is predicted to start in exon 2. Hence, the short OATP2B1 protein variants lack 22 amino acids from the N-terminus in comparison to the original full length form. To date, there is an absence of information regarding the relative expression of the OATP2B1 transporter variants in key tissues responsible for drug absorption and elimination. Moreover, the transport competency of the short OATP2B1 protein has not previously been demonstrated. In this report, we find that the short OATP2B1 transporter variant is the predominant form expressed in human liver while the major form in small intestine is the full length version. The liver enrichment of the short OATP2B1 variant was partly due to transcriptional regulation by hepatocyte nuclear factor 4α (HNF4α). Importantly, we demonstrate the short OATP2B1 protein variant has comparable transport activity to the full length form.

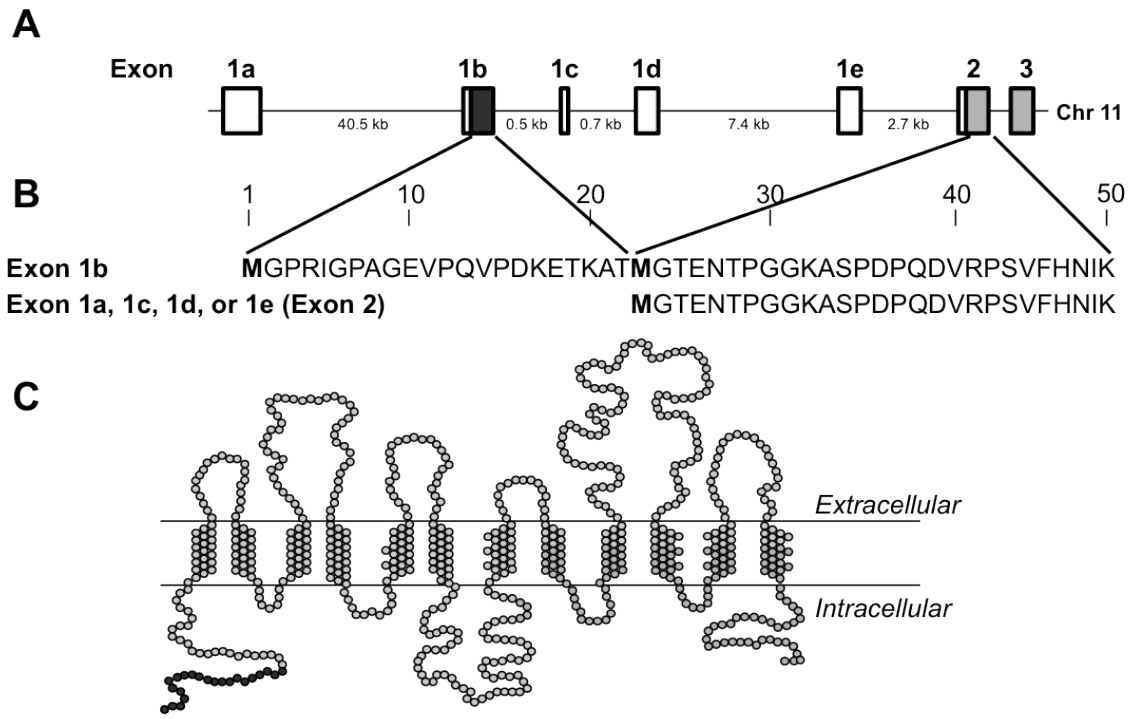

Figure 4.1 OATP2B1 Transcription start site variants.

(A) Exon/intron structure of the beginning of the *SLCO2B1* gene. Exons are shown as boxes and introns as lines. The grey shading indicated the translated regions of the exons. (B) The N-terminal amino acid sequence of the OATP2B1 protein encoded using different translation start sites encoded in exon 1b or exon 2. (C) Topology of OATP2B1, with the black shows the 22 amino acids that are missing in the truncated variant.

4.2 Materials and Methods

4.2.1 Reagents

[^{3}H] esterone 3-sulfate (54.26 Ci/mmol, >97% radiochemical purity) was purchased from PerkinElmer (Woodbridge, ON, Canada) and [^{3}H] rosuvastatin (5 Ci/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Unlabeled rosuvastatin was obtained from Toronto Research Chemicals (North York, ON). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

4.2.2 Cell Culture

Human cervical adenocarcinoma HeLa (American Type Culture Collection, ATCC, Manassas, VA), human hepatocellular carcinoma HepG2 (ATCC), human colorectal adenocarcinoma Caco-2 (ATCC) and human hepatocellular carcinoma Huh-7 (Japanese Collection of Research Bioresources; http://cellbank.nihs.go.jp) cells were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were plated onto 12-well plates for transport studies and 24-well plates for dual luciferase reporter assays at a density of ~1×10^6 cells/mL.

4.2.3 Transporter Expression Plasmids

The expression plasmid for the full length (1b) OATP2B1 (OATP2B1-FL) is described elsewhere (Tirona et al., 2003a). The cDNA of the short variant of OATP2B1 1e (OATP2B1-Short) was obtained by PCR, using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), from a human liver cDNA library using oligonucleotide primers (Table 4.1) and ligated into pEF6/V5-His-TOPO vector (Invitrogen).

4.2.4 Adenoviral vectors

An adenoviral vector containing OATP2B1-Short was generated using the ViraPower Adenoviral Expression System (Invitrogen) as previously described for OATP2B1-FL (Knauer et al., 2010).

4.2.5 *SLCO2B1* luciferase reporter plasmids

The 5' region of the *SLCO2B1* full length promoter (1b exon) and truncated promoter (1e exon) were PCR amplified with Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN) from human genomic DNA using primers listed in Table 4.1. The resulting *SLCO2B1* 1b promoter PCR product (-2000 to +1 bp) was digested with *Kpn* I and *Xho* I then ligated into pGL3-Basic (Promega, Madison, WI). The *SLCO2B1* 1e promoter amplicon was cloned into pCR2.1, digested with *Hind III* and *Xho I* then ligated into pGL3-Basic. Disruption of the DR1-1 site in the *SLCO2B1* 1e promoter was done by site-directed mutagenesis (QuickChange, Stratagene, La Jolla, CA) using primers listed in Table 4.1.

4.2.6 Gene Expression Analysis

The absolute mRNA expression of each OATP2B1 TSS variant, was determined by SYBR green quantitative real-time PCR (RT-PCR), with an ABI Prism 7700 sequence detection system (Applied Biosystems, Carlsbad, CA). Total RNA from a cohort of 23 healthy human livers and 17 intestinal samples were obtained from healthy individuals undergoing diagnostic esophagogastroduodenoscopy was described elsewhere (Ho et al., 2006; Urquhart et al., 2010). Human skeletal muscle, kidney, placenta and brain total RNA was sourced from BioChain (Hayward, CA). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems, Carlsbad, CA) according to manufacturer's instruction and 40 ng of cDNA was used in each PCR reaction. Primer sequences for quantitative PCR were obtained from Pomari et al., (2009) and are listed in Table 4.1. Standard curves of the OATP2B1 TSS variant amplicons cloned into pCR2.1 TOPO vector (Invitrogen) were generated for absolute determination of copy number. Expression was normalized to the copy number of 18S-rRNA.

Table 4.1 Primer Sequences

Primer	Sequence	Type
SLCO2B1 1e Fw	5′-GAGGCTGGGATTGAAGCTTCAGGGAGAGCC-3′	Cloning
SLCO2B1 1e Rv	5′-CAAGACAGCTCACACTCGGGAATCCTCTGG-3′	Cloning
SLCO2B1 1b 2kb pro Fw	5′-ATCAATCAGATAAACTTAGCCAGACAAGC-3′	Cloning
SLCO2B1 1b 2kb pro Rv	5′-CATGACTGCTGGAGTGCAGTGAGCTCC-3′	Cloning
SLCO2B1 1e 84	5′-TCCCTGAAGCTTCAATCCCAGCCTTTTCTG-3′	Cloning
SLCO2B1 1e -200XhoI	5′-ATTTCTCGAGAAGGTTTGCTGTCAGCGCGTCAGCAG-3′	Cloning
SLCO2B1 1e -500XhoI	5′-ATTTCTCGAGGCCCCTGGAGCCACCTGGCCTACCTGG-3′	Cloning
SLCO2B1 1e -1000XhoI	5′-ATTTCTCGAGACCCAGGTCTGAGGCCTTAAAGCC-3′	Cloning
SLCO2B1 1e -2250XhoI	5′-ATTTCTCGAGTGGAGGGGTTTACCTGGAGGAAGACC-3′	Cloning
OATP2B1 - R	5′-CACTGTGGAGATGGAGCTC-3′	qPCR
OATP2B1 - 1a	5′-CCTGATAAACTTCATGATGGAG-3′	qPCR
OATP2B1 - 1b	5′-GGCTGGAGCTCACTGCAC-3′	qPCR
OATP2B1 - 1c	5′-GCACACAGGAGGTCGGAG-3′	qPCR
OATP2B1 - 1d	5′-ACTGCAGTACGGCAGGAAG-3′	qPCR
OATP2B1 - 1e	5′-TGGGATTGAAGCTTCAGGGAG-3′	qPCR
OATP2B1 1e DR1-1 Del	5′-CCAGAGGCACAGGCTGTGGACTCCCTCCACAAACAG CCATATCTC-3′	Mutagenesis
OATP2B1 1e DR1-1 Mut	5′-CCAGAGGCACAGGCTGTGGAGTTTACCATCCACAAA CAGCCATATCTC-3′	Mutagenesis

4.2.7 Immunoblot and Immunohistochemistry

HeLa cells were transduced with adenovirus encoding LacZ (control), OATP2B1-FL or OATP2B1-Short. Protein samples were separated by SDS-PAGE and transferred to NuPAGE nitrocellulose membrane using the XCell *SureLock* Western blotting system (Invitrogen, Carlsbad, CA). Membranes were probed with custom-made rabbit polyclonal OATP2B1 antibody based on a C-terminus epitope (CSPAVEQQLLVSGPGKKPEDSRV) (Invitrogen). Anti-rabbit horseradish peroxidase–labeled antibody (Bio-Rad, Hercules, CA) was used as the secondary. The immobilized secondary antibody was detected using the ECL Plus Western Blotting Detection System (GE Healthcare) and KODAK ImageStation 4000 MM (Mandel). HeLa cells were transduced with adenovirus encoding LacZ (control), OATP2B1-FL or OATP2B1-Short on culture slides and were stained as previously described (Knauer et al., 2010). The custom-made rabbit polyclonal OATP2B1 antibody was used to localize transporter expression and anti-rabbit Alexa Fluor® 488 (Invitrogen) was used as the secondary antibody. Images were obtained by confocal fluorescence microscopy. For the negative control, the primary antibody was omitted.

4.2.8 Transport Studies

For transport kinetic experiments, HeLa cells (human cervical cancer cell line) were grown in 12-well plates (approximately 0.8×10^6 cells/well) and infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to absorb for 30 minutes. Cells were transfected with 1 μg of transporter cDNA or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. To examine pH dependent transport of OATP2B1, HeLa cells were transduced with the adenoviruses containing the full length or short OATP2B1 variant transporter coding region in DMEM medium at a multiplicity of infection (MOI) of 100 and incubated at 37°C for 48h. Transport was evaluated in Opti-MEM using labeled substrate as previously reported (Cvetkovic et al., 1999). Drug accumulation was determined after 5 min for rosuvastatin and 10 min for

estrone 3-sulfate by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry. Transport studies with varying pH environments were preformed in Krebs-Heinseleit bicarbonate Buffer (KHB) using rosuvastatin (100 nM) and estrone 3-sulfate (100 nM) as previously reported (Urquhart et al., 2010).

4.2.9 Luciferase Reporter Assays

Reporter gene constructs were transfected into HeLa, HepG2, Huh-7 or Caco-2 cells using Lipofectamine 2000 (Invitrogen) according to the manufacture's protocol. Cells were plated into 24 wells plates at approximately 0.6×10^6 cells per well for 24h before transfection. Each well was transfected with 250 ng of reporter construct and 2.5 ng of pRL-CMV vector (Promega) as an internal control in 400 μL Opti-MEM. After 24h the media was changed to DMEM with 10% FBS, then cells were incubated for 24h at 37°C. Cells were then rinsed with PBS and harvested using passive lysis buffer (Promega). Luciferase activity was measured in cell extracts using Dual-Luciferase reporter assay (Promega) and detected using a Flouroskan Ascent FL luminometer (Thermo Scientific, Hudson, NH). Relative luciferase activity is reported as the ratio of firefly/*Renilla* luciferase activities. Nuclear receptor expression vectors were prepared as previously described (Tirona et al., 2003b) and in certain instances, 250 or 500 ng/well of each nuclear receptor expression plasmid or blank vector control plasmid were used for reporter assays.

4.2.10 Chromatin Immunoprecipitation (ChIP)

For DNA cross-linking and chromatin immunoprecipitation, the EZ ChIP Assay (Millipore, Billerica, MA) was used according to the manufacturer's instructions. Briefly, HepG2, Huh-7, HeLa and Caco-2 cells were cultured in 10-cm dishes. DNA was cross-linked, sheared by sonication (Virsonic 100, Virtis, Gardiner, NY), and then chromatin was incubated with 2 μg anti-HNF4α C-19 (sc-6556; Santa Cruz Biotechnology, Santa

Cruz, CA) antibody overnight at 4°C. Binding of HNF4α was determined by qPCR (Table 4.2 for primer sequences). Western Blot of total cell lysates was preformed using anti-HNF4α C-19 (sc-6556; Santa Cruz Biotechnology) (1:1000) and anti-actin C-11 (sc-1615; Santa Cruz Biotechnology) (1:200).

4.2.11 Statistical Analysis

Statistical differences between group parameters was determined by unpaired Student's T-test, 1-way ANOVA, or 2-way ANOVA, using Bonferroni's multiple comparison test, as appropriate (GraphPad Software Inc, San Diego, CA). A p value of <0.05 was considered statistically significant.

Table 4.2 Primer Sequences used for qPCR after ChIP

Primer	Sequence	Distance to exon 1e TSS (bp)	Amplicon Size (bp)
ChIP 2B1 1e F1	5′-AGAGAGCAAGGGCCACCTAT-3′	2152	125
ChIP 2B1 1e R1	5′-TATAGGGACCTCAGCCACCA-3′		
ChIP 2B1 1e F2	5′-TCTGACTCCCAGGCTCAAGT-3′	1622	125
ChIP 2B1 1e R2	5′-GCAACATGGTGAAACCTCAT-3′		
ChIP 2B1 1e F3	5′-GCCTTTCCCACAGAGAACAG-3′	903	123
ChIP 2B1 1e R3	5′-GCCCTTCATGGGTAGAGTCA-3′		
ChIP 2B1 1e F4	5′-CTCTCAGAACCCGGAGGAAT-3′	77	124
ChIP 2B1 1e R4	5′-TCAAAGCCTTCCTGGGAGTA-3′		

4.3 Results

4.3.1 Tissue Expression of OATP2B1 TSS Variants

We preformed variant-specific quantitative RT-PCR to determine the absolute expression of the OATP2B1 variants in tissues important in drug disposition, namely the duodenum and liver. While transcripts for the OATP2B1 1b, 1d and 1e variants were readily observed; we were not able to detect OATP2B1 1a and 1c transcripts in these tissues (not shown). In duodenum, the OATP2B1 variant 1b, encoding the full length protein variant was the most abundant transcript found (Fig. 4.2). On average, OATP2B1 1d and 1e transcripts were 10-fold lower than 1b transcripts in the intestine. In liver, OATP2B1 1e transcript (short variant) was the predominant form followed by 1b and 1d transcripts. It is notable that for each transcript, the absolute expressions of OATP2B1 1b and 1d were similar when comparing duodenum versus liver levels. The clear exception was for the OATP2B1 1e transcript, whose expression was 100-fold greater in liver than in duodenum. We confirmed that the OATP2B1 1e transcript detected by qPCR arose from an mRNA encoding the short protein variant by long range PCR using liver cDNA template, cloning of the amplicon and sequencing.

Variant-specific quantitative RT-PCR of OATP2B1 variants was also preformed in samples of kidney, brain, placenta, and skeletal muscle. Like liver and intestine, transcripts for OATP2B1 1b, 1d, and 1e were observed, but OATP2B1 1a and 1c transcripts were not detected in these tissues (Table 4.3). Similar to duodenum, the OATP2B1 1b variant was the most abundant transcript found in kidney, brain, placenta and skeletal muscle. Again we noted, the absolute expressions of OATP2B1 1b transcripts (full length form) in kidney, placenta and skeletal muscle were similar when compared to duodenum and liver levels. Of the tissues analyzed, brain shows highest expression of the OATP2B1 1b transcript, encoding the full length protein. On average, OATP2B1 1d and 1e transcripts were over 10 to 100-fold lower than 1b transcripts in the brain, placenta and skeletal muscle, exhibiting a comparable expression pattern to that of intestine. However, in kidney the expression of the OATP2B1 1e transcript was 10-fold higher than duodenum, brain, placenta, and skeletal muscle and only 2 fold lower then

the OATP2B1 1b transcript. These findings demonstrate 3 different expression patterns of OATP2B1 protein variants. Liver primarily expresses the shorter OATP2B1 protein, kidney expresses similar levels of full length and short OATP2B1, while the other tissues analyzed predominantly express full length OATP2B1.

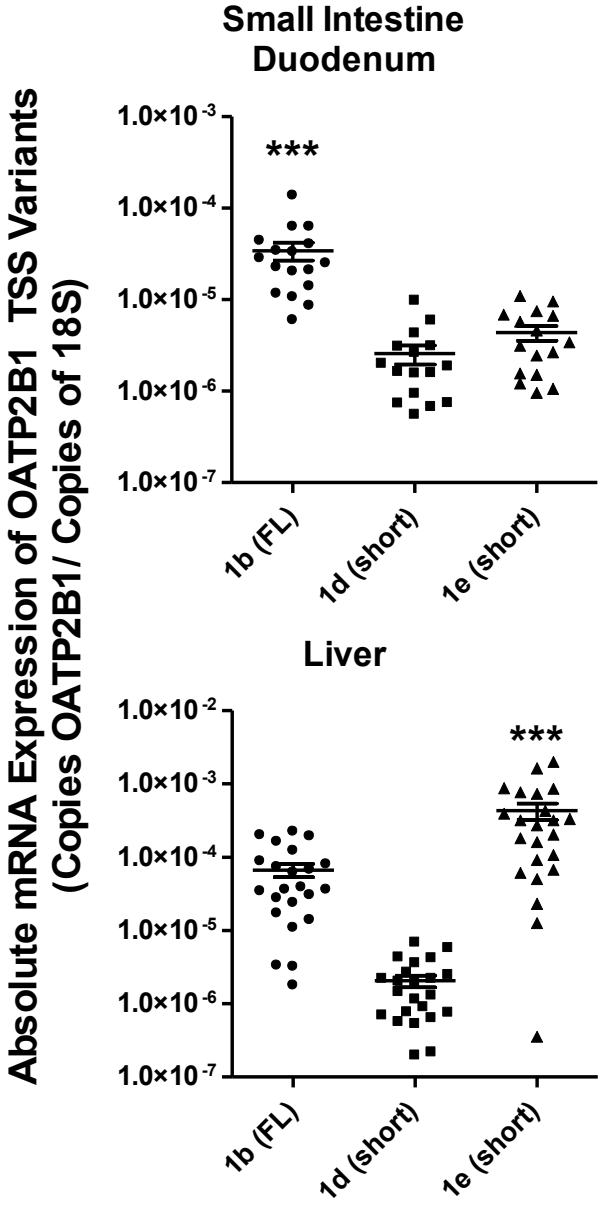

Figure 4.2 Tissue specific expression and quantitation of the alternative OATP2B1 first exons in 23 liver and 17 intestinal samples.

Absolute quantification was done by qPCR using sense primers for the specific first exon and an antisense primer located within exon 3. ***$p<0.001$

Table 4.3 Absolute mRNA Expression of OATP2B1 TSS Variants.

	Absolute mRNA Expression (Copies OATP2B1/Copies of 18S)		
Tissue	**1b (FL)**	**1d (short)**	**1e (short)**
Kidney	2.06×10^{-5}	7.42×10^{-8}	8.59×10^{-6}
Brain	1.59×10^{-4}	1.21×10^{-7}	1.45×10^{-7}
Placenta	1.45×10^{-5}	ND	2.24×10^{-7}
Sk. Muscle	2.84×10^{-5}	3.43×10^{-7}	1.32×10^{-6}

ND - not detected

4.3.2 Transport Function of OATP2B1-Short

Many groups have studied the transport function of OATP2B1-FL but to our knowledge the transport function of the short variant has not been previously assessed. Here, we examined the transport function of the OATP2B1-Short variant in comparison to OATP2B1-FL using two substrates: estrone 3-sulfate and rosuvastatin. Both OATP2B1-FL and OATP2B1-Short show time- and concentration-dependent uptake kinetics for estrone 3-sulfate and rosuvastatin (Fig. 4.3C-F). The apparent affinities (K_m) for both substrates were not significantly different between OATP2B1-FL and OATP2B1-Short (Table 4.4). The maximal velocity (V_{max}) of the OATP2B1-Short was slightly higher for both rosuvastatin and estrone 3-sulfate; however, the differences did not reach statistical significance (Table 4.4). Transport clearance (CL) of the OATP2B1-Short was also slightly higher for both rosuvastatin and estrone 3-sulfate but it also did not reach statistical significance. Heterologous expression of OATP2B1-FL and OATP2B1-Short in HeLa cells, using recombinant adenoviruses and protein detection with an OATP2B1 antibody directed towards a common C-terminus epitope, showed that both variants products are expressed at comparable levels and traffic to the cell surface (Fig 4.3A and B). HeLa cells show some protein expression of OATP2B1 by immunoblot (Fig. 4.3A) and RT-PCR analysis suggests that HeLa cells natively express low levels of the full length OATP2B1 (data not shown). These findings indicate that the *SLCO2B1* 1e promoter transcript produces a short transporter variant with similar transport activity as the previously characterized full length OATP2B1 protein.

We further examined the pH dependent transport of the OATP2B1-Short variant since previous studies have demonstrated OATP2B1-FL transports certain substrates in a pH dependent manner (Kobayashi et al., 2003; Nozawa et al., 2004a). For both the full length and the short OATP2B1 variants, we observed that rosuvastatin transport was stimulated with decreasing pH (Fig 4.3G). Rosuvastatin transport by OATP2B1-Short is significantly greater than OATP2B1-FL at low pH (5.5 and 6.0) (Fig. 4.3G). In contrast, estrone 3-sulfate transport activities for both OATP2B1-FL and OATP2B1-Short do not appear to be pH-dependent (Fig. 4.3H). These results suggest that amino acids in the

predicted intracellular N-terminus of OATP2B1 modulate substrate-specific, pH-dependent membrane transport.

Figure 4.3 Function of OATP2B1 transcription start site variants. (A) Western blot showing expression of OATP2B1 variants in HeLa cells after adenoviral overexpression. (B) Cellular localization of full length or truncated variant OATP2B1 in HeLa cells after adenoviral overexpression. OATP2B1 shown in green and the nucleus in blue. Intracellular accumulation of [^{3}H]rosuvastatin (C,E) or [^{3}H]estrone 3-sulfate (D,F) in HeLa cells transiently transfected with OATP2B1-FL (full-length) or OATP2B1-Short (truncated). Concentration-dependent transport of [^{3}H]rosuvastatin (E) and [^{3}H]estrone 3-sulfate (F) by OATP2B1-FL (full-length) or OATP2B1-Short (truncated). pH dependent transport of [^{3}H]rosuvastatin (G) or [^{3}H]estrone 3-sulfate (H) in HeLa cells transduced with OATP2B1-FL (full length) or OATP2B1-Short (truncated) adenovirus. Results are presented fmol per μg protein per min ± SEM (n=3). * $p<0.05$

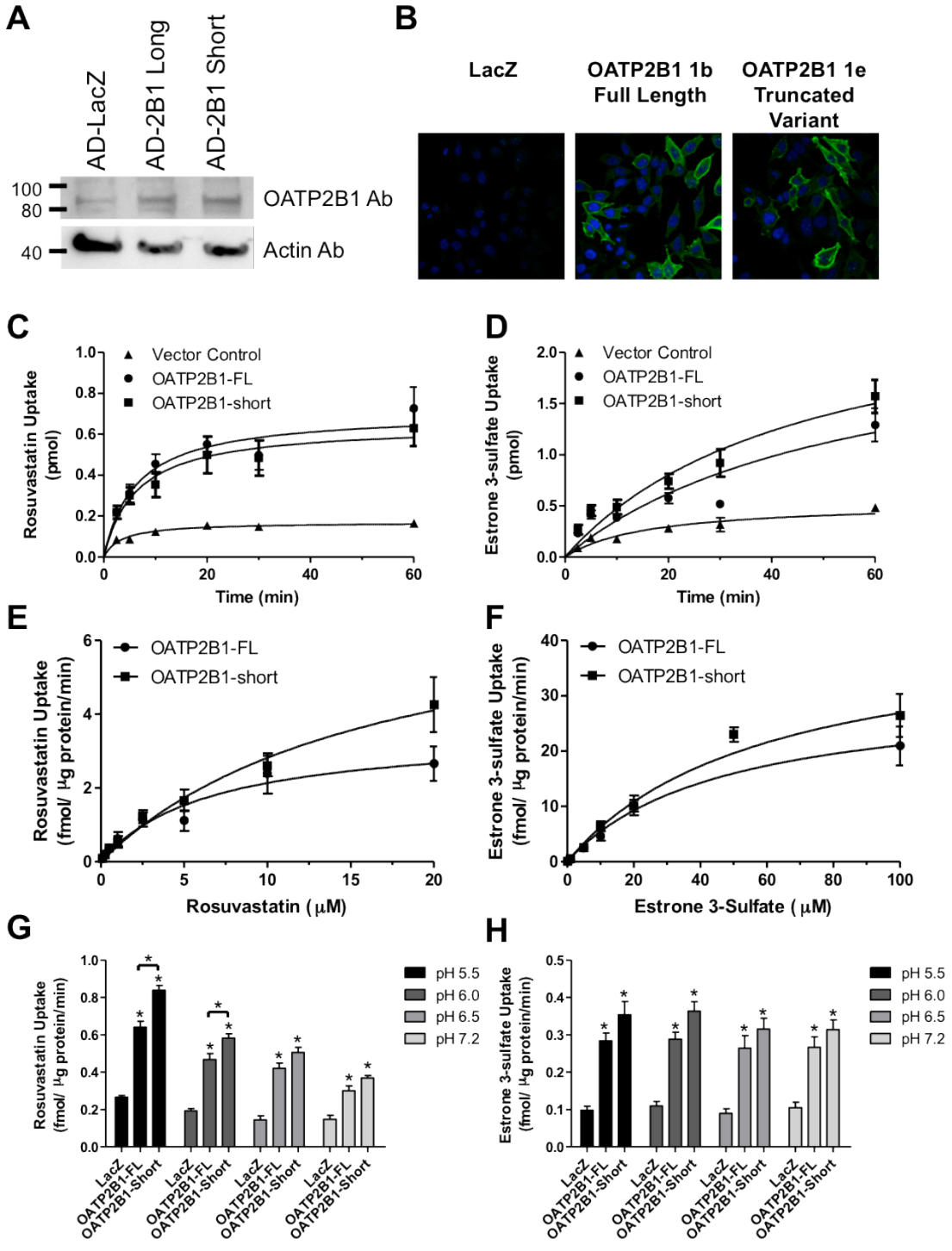

Figure 4.3 Function of OATP2B1 transcription start site variants.

Table 4.4 Transport kinetics of OATP2B1 transcription start site variants.

	V_{max} ± SD (fmol/µg protein/min)	K_m ± SD (µM)	CL ± SD V_{max}/K_m (µL/µg protein/min)
Rosuvastatin			
OATP2B1-FL	3.4 ± 1.6	7.1 ± 3.1	530.0 ± 349.9
OATP2B1-Short	4.9 ± 3.4	8.4 ± 5.5	653.9 ± 216.7
Estrone 3-sulfate			
OATP2B1-FL	31.1 ± 13.4	47.2 ± 9.4	660.3 ± 296.1
OATP2B1-Short	43.2 ± 19.0	57.7 ± 14.9	722.7 ± 160.1

4.3.3 Transactivator Screen of the *SLCO2B1* 1e Promoter

The full length form of OATP2B1 appears to be ubiquitously expressed in many tissues and the constitutive expression of OATP2B1-FL is under regulatory control by the Sp1 transcription factor (Maeda et al., 2006). However, regulatory control mechanisms of the other OATP2B1 TSS variants have not been previously assessed. We have focused on the OATP2B1 1e TSS variant because it shows liver specific expression unlike the OATP2B1 1b variant found ubiquitously (Pomari et al., 2009) (Fig. 4.2 and Table 4.3). The *SLCO2B1* 1e promoter was cloned from genomic DNA (-2250 to +78) and ligated into pGL3-basic. The +1 position was considered to be the transcription start site of exon 1e described by Pomari and colleagues (GeneBank no. FM209054) (Pomari et al., 2009). We screened the ability of a variety of nuclear receptors (HNF4α, constitutive androstane receptor, liver X receptor, pregnane X receptor, small heterodimer partner 1, peroxisome proliferator activated receptors α and γ, liver receptor homolog 1 and vitamin D receptor) to activate the *SLCO2B1* 1e promoter using co-transfection and luciferase reporter assays in HeLa cells (Fig. 4.4A). Of the nuclear receptors analyzed, we found that HNF4α strongly transactivated the *SLCO2B1* 1e (-2250/+78) promoter. Interestingly, we found that HNF4α did not transactivate the *SLCO2B1* 1b promoter (Fig. 4.4B).

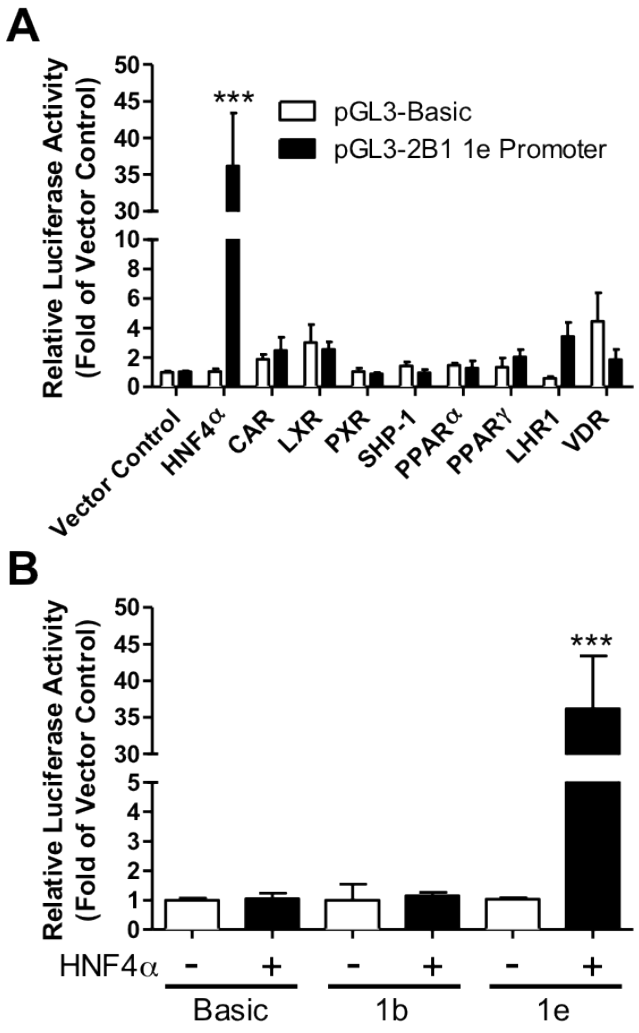

Figure 4.4 Nuclear receptor screen for transactivation of the *SLCO2B1* 1e promoter responsible for producing the truncated protein variant.

(A) Dual luciferase reporter assay in HeLa cells transfected with nuclear receptor expression plasmids and *SLCO2B1* 1e promoter (-2250 to +78) or control pGL3-basic vector to determine if they were able to transactivate the *SLCO2B1* 1e promoter. (B) Transcription activation of the *SLCO2B1* 1e promoter and not the 1b promoter in HeLa cells transfected with HNF4α and *SLCO2B1* 1b (-2250/+78) or 1e (-2000/+1) promoter constructs. Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM. ***$p<0.001$

4.3.4 Deletion-Mutation Analysis of *SLCO2B1* 1e Promoter Activity

Reporter assays were preformed in liver (HepG2 and Huh-7), colon (Caco-2) and cervical carcinoma (HeLa) cell lines to evaluate the roles of cellular milieu and *cis*-acting factors to *SLCO2B1* 1e promoter activity. For this purpose, *SLCO2B1* 1e promoter constructs were created by sequentially deleting the 5' upstream segments (-2250 to +78, -1000 to +78, -500 to +78 and -200 to +78). We observed reporter activity in three cell lines (HepG2, Huh-7 and Caco-2) for all *SLCO2B1* 1e deletion constructs (Fig. 4.5A). In comparison, *SLCO2B1* 1b reporter activity was significantly lower than of *SLCO2b1* 1e. The pattern of reporter activity was similar for all cell lines except for HeLa cells, which displayed no significant activity for all reporter constructs. Reporter activity decreased upon the deletion of -2250 to -1000 region, suggesting the presence of positive regulatory sequences. While deletion of the -1000 to -500 revealed a large increase in reporter activity in three cell lines (HepG2, Huh-7 and Caco-2) suggesting that the deleted segment contains negative regulatory sequences. Even the shortest construct (-200/+78) retained reporter activity in HepG2, Huh-7 and Caco-2 suggesting the presence of an important positive regulatory sequence in this region.

Given that OATP2B1 le transcripts are prominently expressed in liver and that *SLCO2B1* 1e luciferase reporter activities were higher in hepatic and intestinal cell lines in comparison to cervical carcinoma cells, we suspected a role for liver enriched transcription factors such as HNF4α in regulating gene expression. Hence, we performed *in silico* analysis of transcription factor binding sites within the 2.5Kb fragment of the *SLCO2B1* 1e gene promoter using the NUBIscan algorithm (Podvinec et al., 2002). Several direct repeats with one base pair spacing (DR1), known to be a potential binding site for HNF4α (Bolotin et al., 2010), were predicted which we have termed DR1-1 (AGGGCAaAGTCCA) located at position -17 to -4, a DR1-2 (AGGCCTcAGACCT) located at -954 to -941 and DR1-3 (AGAGCAaGGGCCA) located at -2149 to -2136. On the basis that the -200/+78 construct had retained significant activity in liver and intestinal cells, we hypothesized that the DR1-1 site would represent a functional HNF4α binding site. Guided by the position weight matrix for HNF4α-binding sequence motifs (Bolotin et al., 2010), the DR1-1 site was mutated at three key base pairs (Fig. 4.5B).

Furthermore, we deleted three base pairs in DR1-1 to disrupt the HNF4α motif spacing (Fig. 4.5B). Reporter assays were preformed in HeLa cells transfected with HNF4α and the DR1-1 mutation constructs (Fig. 4.5C). It is notable that in contrast to HepG2, Huh-7 and Caco-2 cells, HeLa cells do not express native HNF4α (Fig. 4.5D). Disruption of the DR1-1 response element by mutation or deletion significantly decreased the transcriptional activation of the *SLCO2B1* 1e promoter by HNF4α, suggesting that the DR1-1 site is a functional HNF4α binding site (Fig. 4.5C). These studies demonstrate that the *SLCO2B1* 1e promoter contains a functional HNF4α-binding sequence near the 1e transcription start site.

4.3.5 HNF4α Binding to *SLCO2B1* 1e Promoter

To confirm that HNF4α binds to the DR1-1 response element, chromatin immunoprecipitation (ChIP) was preformed with a HNF4α antibody in HeLa, HepG2, Huh-7 and Caco-2 cells. HNF4α-bound DNA was used as template for real-time PCR using primers designed to cover sequences along the *SLCO2B1* 1e promoter covering 2.2 kb upstream and the three predicted DR1 response elements (Fig. 4.6A). The analysis revealed significant binding of HNF4α to the proximal region of the *SLCO2B1* 1e promoter near the DR1-1 (-4 bp) response element in HepG2, Huh-7 and Caco-2 cells but not HeLa cells (Fig. 4.6B). Analysis of the other DR1 response elements does not demonstrate significant HNF4α binding in HepG2, Huh-7 and Caco-2 cells. HeLa cells do not natively express HNF4α, so predictably we did not detect binding of HNF4α to the *SLCO2B1* 1e promoter. Results from the reporter assays and ChIP taken together, reveal that HNF4α binds to the functional DR1-1 site in the proximal region of the *SLCO2B1* 1e promoter to control expression of the truncated OATP2B1 variant.

Figure 4.5 A functional HNF4α-binding motif in the proximal region of the *SLCO2B1* 1e promoter. (A) Dual luciferase reporter assay in HepG2, Huh-7, Caco-2, and HeLa cells transfected with various constructs of the *SLCO2B1* 1e promoter (-2250 to +78, -1000 to +78, -500 to +78 and -200 to +78) or *SLCO2B1* 1b promoter (-2000 bp to +1). Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM (n=3-4). (B) Sequences of the proximal DR1-1 HNF4α-binding motif with a three base pair deletion (Del) or mutation of three key base pairs (Mut) based on the HNF4α position weight matrix (Bolotin et al., 2010). (C) Dual luciferase reporter assay in HeLa cells transfected with *SLCO2B1* 1e promoter constructs (WT, DR1-1 Del or DR1-1 Mut) and HNF4α or control pEF vector. Predicted HNF4α binding sites are shown in white along the 1e promoter constructs, the DR1-1 HNF4α-binding motif is shaded to indicate the binding motif disrupted by deletion or mutation. Results are presented as relative luciferase activity fold of pGL3-Basic control ± SEM (n=5), *$p<0.05$ versus wild type HNF4α binding site construct. (D) Western blot showing HNF4α expression in in HeLa, HepG2, Huh-7 and Caco-2 cells.

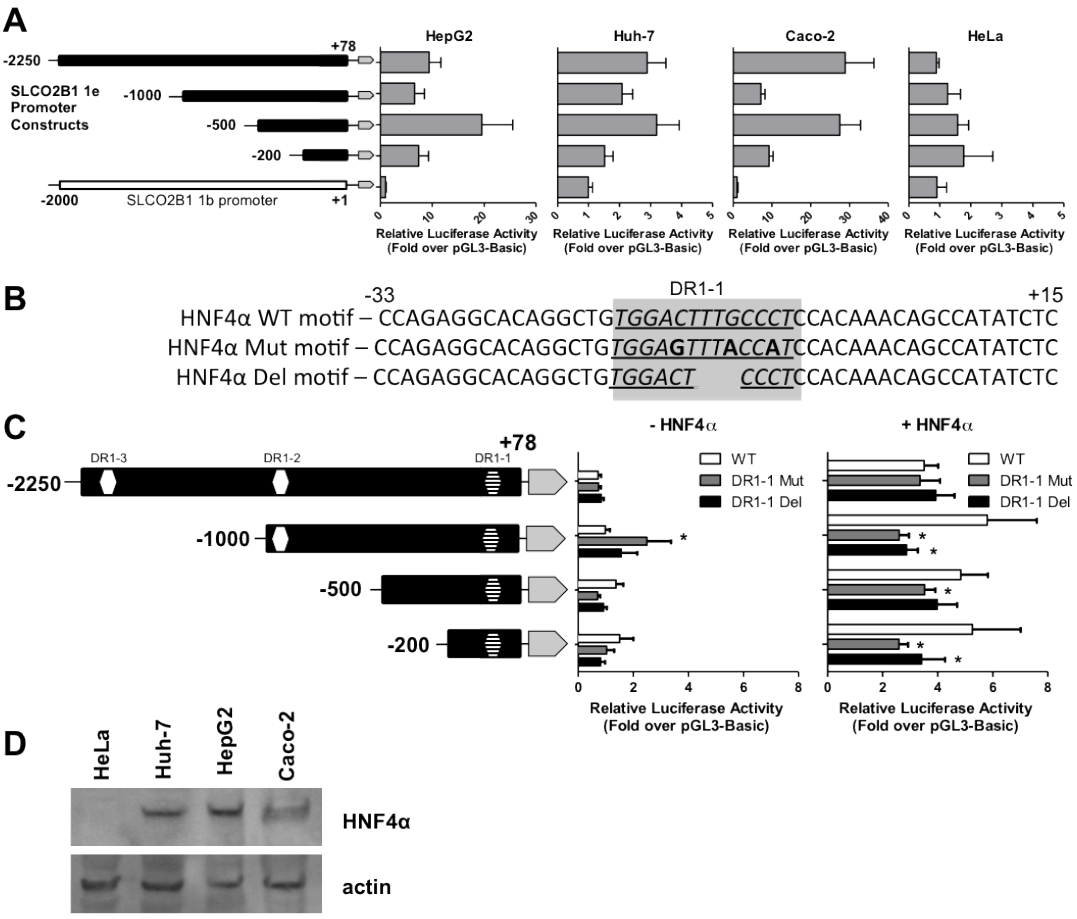

Figure 4.5 A functional HNF4α-binding motif in the proximal region of the *SLCO2B1* 1e promoter.

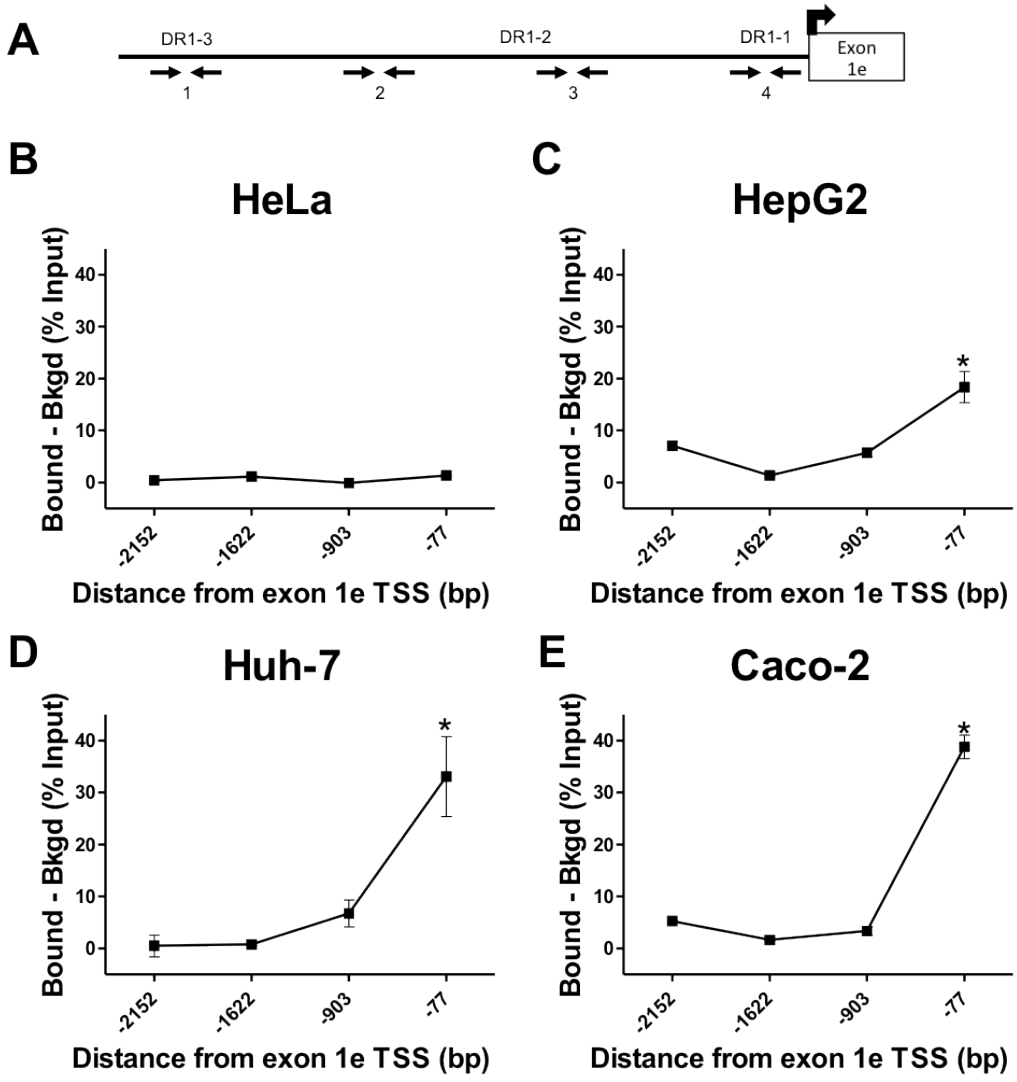

Figure 4.6 Chromatin Immunoprecipitation.

(A) Diagram of 2.3Kb of the *SLCO2B1* 1e promoter with the location of DR1 sites and primer pairs used for qPCR. ChIP using an anti-HNF4α antibody in HeLa (B), HepG2 (C), Huh-7 (D) and Caco-2 (E) cells. Quantification was done by qPCR on HNF4α immunoprecipitated DNA (bound fraction), IgG immunoprecipitated DNA (background fraction) and input DNA with four sets of primers located along the *SLCO2B1* 1e promoter. Results are presented as the difference between the recoveries in the bound and background fractions. *$p<0.05$ versus all other primer pairs

4.4 Discussion

In the present study, we characterized the transport function and transcriptional regulation of OATP2B1 1e variant. The *SLCO2B1* 1e variant encoding OATP2B1-Short was cloned from a human liver cDNA library. Heterologous expression of OATP2B1-Short (1e) in HeLa cells resulted in cell surface expression and functional transport activity. We found no pronounced differences in transport activity between the OATP2B1-FL and OATP2B1-Short protein for two typical OATP substrates: rosuvastatin and estrone 3-sulfate. However, we did see a significant increase in rosuvastatin uptake by OATP2B1-Short, but not for OATP2B1-FL at low pH. Reporter assays and chromatin immunoprecipitation revealed a functional HNF4α binding motif in the proximal region of the *SLCO2B1* 1e promoter.

The expression of the alternative OATP2B1 variants in intestine and kidney were reported previously (Pomari et al., 2009). We confirm that the OATP2B1 exon 1b transcription variant has broad expression pattern consistent with previous reports and regulation by a constitutive nuclear receptor Sp1 (Maeda et al., 2006; Pomari et al., 2009). Quantitative PCR revealed that expression of the OATP2B1 1b full length variant was highest in the brain, the tissue of first cloning (Tamai et al., 2000). We confirm the liver enriched expression of the OATP2B1 exon 1e variants using absolute quantitative PCR, as was previously suggested using semi-quantitative PCR (Pomari et al., 2009). Interestingly we report 100-fold variation in the hepatic mRNA expression of the OATP2B1 1e variant between individuals.

We demonstrate that the *SLCO2B1* 1e variant encodes a shortened protein capable of trafficking to the cell surface and has functional transport activity. While there was a non-significant trend towards higher transport efficiency for OATP2B1-Short in comparison to OATP2B1-FL during studies with concentration-dependent uptake, clear differences were observed in the transport efficiencies among the variants at low pH. Other studies have also documented the pH dependent transport of rosuvastatin, fluvastatin, pravastatin, atorvastatin, cerivastatin, and pitavastatin by OATP2B1 (Kobayashi et al., 2003; Varma et al., 2011). Previous studies have demonstrated pH dependent transport of

estrone 3-sulfate by OATP2B1; however, we did not detect a significant difference in the pH dependent transport of estrone 3-sulfate (Kis et al., 2010; Kobayashi et al., 2003; Nozawa et al., 2004a; Sai et al., 2006). It has been suggested that an inward proton gradient acts as the driving force for the OATP2B1 mediated intestinal absorption and cellular uptake of substrates (Nozawa et al., 2004a; Sai et al., 2006; Varma et al., 2011). The pH sensitivity of OATP2B1 has been linked to a conserved histidine in transmembrane domain three (Leuthold et al., 2009). Here, we propose a role for the amino acids in the predicted intracellular N-terminus in modulating substrate-specific, pH-dependent membrane transport of OATP2B1.

In this study, the liver specific nuclear receptor, HNF4α, was found to stimulate transcription of the *SLCO2B1* exon 1e promoter. Moreover, a functional HNF4α binding motif was found in the proximal region of the *SLCO2B1* 1e gene promoter very close to the transcription start site. Indeed, others have recently identified this HNF4α binding motif in the *SLCO2B1* exon 1e promoter using the ChIP-Seq method (Fang et al., 2012). HNF4α is expressed highly in metabolic tissues like the liver, kidney and intestine (Bookout et al., 2006). HNF4α has been shown to regulate key drug metabolism genes including Cytochromes P450 (*CYP*)*3A4, CYP3A5, CYP2D6, CYP2C9*, and CYP*2C19* as well as drug transporter genes such as *ABCC2*, *ABCB1* and *SLCO1B1* (Jover et al., 2001; Jover et al., 2009; Kamiyama et al., 2007; Kawashima et al., 2006; Tirona et al., 2003b). Like other transcriptional targets, we suspect that HNF4α-mediated regulation of OATP2B1 1e variant expression can be influenced by a variety of different factors including genetics, gender, environmental factors and diet (Hwang-Verslues and Sladek, 2010).

We have found 100-fold difference in OATP2B1 1e expression among individuals. It is interesting to speculate that interindividual differences in the tissue expression of OATP2B1 1e variant contributes to variable drug response. In principle, conditions that alter HNF4α expression and activity, such as diabetes and liver diseases, may impact on OATP2B1 1e expression. Factors enhancing HNF4α activity would increase expression of the OATP2B1 1e variant and could increase the hepatic and renal exposure OATP2B1

substrates. Increased HNF4α activity in the intestine would lead to upregulation of OATP2B1 1e and enhanced intestinal absorption of substrate drugs.

In this report, we assessed the transport function and regulation of the liver-enriched OATP2B1 1e transcription start site variant. The encoded OATP2B1-Short protein is a functional membrane transporter for estrone 3-sulfate and rosuvastatin. Analysis of the *SLCO2B1* 1e TSS variant promoter revealed a DR1 binding motif capable of binding HNF4α to control the liver specific expression of the short OATP2B1 1e variant. These findings indicate that differential regulation of OATP2B1 splice variant expression in tissues could contribute to variation in drug response.

4.5 References

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5 TARGETED DISRUPTION OF MURINE ORGANIC ANION TRANSPORTING POLYPEPTIDE 2B1 (Oatp2b1/Slco2b1) AND THE IMPACT ON ROSUVASTATIN DISPOSITION[3]

[3] The material in this chapter is based on a manuscript in preparation: Knauer MJ, Takada H, Mansell SE, Kim RB, Tirona RG. Targeted disruption of murine organic anion transporting polypeptide 2b1 (Oatp2b1/Slco2b1) and the impact on rosuvastatin disposition. (2012)

5.1 Introduction

The Organic Anion Transporting Polypeptide (OATP) transporters belong to a large superfamily of membrane proteins that mediate the sodium-independent uptake of a variety of amphipathic compounds including hormones, bile acids, and drugs clinically used today. OATP transporters are found in many organs involved in drug disposition or response such as liver, intestine, brain and kidney. The superfamily of OATP transporters has currently identified 11 human OATPs and 15 mouse Oatps (Hagenbuch and Gui, 2008). There has been great interest in understanding the role of OATP transporters in drug disposition, particularly the members of the OATP1B family found highly expressed on the basolateral membrane of hepatocytes. It is now well appreciated that functionally relevant single nucleotide polymorphisms (SNPs) in OATP1B1, first identified by our group (Tirona et al., 2001), are associated with higher plasma concentrations of statins such as atorvastatin, rosuvastatin, simvastatin, pravastatin and pitavastatin (Chung et al., 2005; Ho et al., 2007; Pasanen et al., 2007; Pasanen et al., 2006). Importantly, a genome wide analysis revealed an association between a common reduced function variant OATP1B1 (*5, 521T>C, rs4149056) and statin myopathy in patients on high dose simvastatin (Link et al., 2008).

OATP2B1 (*SLCO2B1*) is another member of the OATP family, found much more widely expressed than OATP1B transporters in tissues that include liver, kidney, brain, intestine, colon, heart, lung, placenta, ovary, testis and skeletal muscle (Knauer et al., 2010; Kullak-Ublick et al., 2001; Tamai et al., 2000). Functionally, OATP2B1 has a much narrower substrate specificity compared to members of the OATP1B family (Hagenbuch and Gui, 2008). It also displays substrate specific, pH-dependent transport properties in human intestine epithelial cells (Kobayashi et al., 2003; Nozawa et al., 2004) indicating that OATP2B1 is involved in the intestinal absorption of statins (Varma et al., 2011). Moreover, OATP2B1 is thought to determine the extra-hepatic distribution of statins in the heart, skeletal muscle and platelets (Grube et al., 2006; Knauer et al., 2010; Niessen et al., 2009). While *SLCO2B1* genetic polymorphisms are associated with the pharmacokinetics of montelukast and fexofenadine, as well as androgen response in

prostate cancer, little is known regarding their effects on statin disposition (Imanaga et al., 2011; Mougey et al., 2009; Wright et al., 2011; Yang et al., 2011).

Knockout mouse models have been proven useful to study the roles of Oatps on drug disposition and glucose, cholesterol and bile acid homeostasis (Csanaky et al., 2011; Gong et al., 2011; Lu et al., 2008; Meyer zu Schwabedissen et al., 2011; van de Steeg et al., 2010; Zaher et al., 2008; Zhang et al., 2012a; Zhang et al., 2012b). The murine ortholog of human OATP1B1 and OATP1B3 is Oatp1b2. Oatp1b2 was the first Oatp to be studied in a knockout mouse model (Chen et al., 2008; Lu et al., 2008; Zaher et al., 2008). Oatp1b2$^{-/-}$ mice had significantly lower liver to plasma ratios for lovastatin, pravastatin, atorvastatin, and rosuvastatin, but not for simvastatin or cerivastatin (Chen et al., 2008; Degorter et al., 2012; Zaher et al., 2008), suggesting a variable impact in the hepatic uptake of different statins by Oatp1b2 (Chen et al., 2008). More recently an Oatp1a/1b$^{-/-}$ model was generated by targeted chromosomal deletion, eliminating expression of Oatp1a1, Oatp1a4, Oatp1a5, Oatp1a6 and Oatp1b2 (van de Steeg et al., 2010). The systemic exposure of pravastatin was greatly increased in Oatp1a/1b$^{-/-}$ mice, but only minor decreases were observed in liver exposure (Iusuf et al., 2012). They proposed that other transporters, like Oatp2b1, might compensate for the loss of Oatp1a/1b transporters in the hepatic uptake of pravastatin (Iusuf et al., 2012). Oatp2b1 is the murine ortholog of human OATP2B1 and it shares a ubiquitous expression pattern, but it is also found in the mouse liver at high levels (Cheng et al., 2005). To our knowledge, until now there have been no published reports on the generation and characterization of an Oatp2b1 knockout mouse.

Rosuvastatin is a hydrophilic statin with low membrane permeability that is thought to undergo very minimal metabolism; therefore, its disposition is highly dependent upon transporters (Kitamura et al., 2008; Shitara and Sugiyama, 2006). The liver selectivity of rosuvastatin is due to transport by hepatic uptake transporters, like OATP1B1, OATP1B3, OATP2B1 and the sodium-taurocholate co-transporting polypeptide (NTCP). In humans OATP1B1 and OATP1B3 are considered important transporters involved in the hepatic uptake of rosuvastatin (Ho et al., 2006). OATP2B1 is thought to be important for the absorption and extrahepatic distribution of rosuvastatin in humans; however, high

expression in the murine liver suggests it may play a significant role in the hepatic uptake as well.

Previously OATP2B1 has been implicated in the muscle uptake and myotoxicity of statins *in vitro* (Knauer et al., 2010; Sakamoto et al., 2008). However, there is a lack of *in vivo* studies examining statin transporters within skeletal muscle and their influence on myotoxic side effects of statins. We have proposed that the interplay between statin uptake versus efflux transporters modulates skeletal muscle statin exposure and toxicity. Inhibition of specific uptake transport mechanisms that control intracellular skeletal muscle concentrations of statins could lead to reduced accumulation of drug and decreased risk for toxicity. However, the *in vivo* relevance of OATP2B1 in statin intestinal absorption, hepatic clearance and muscle uptake has yet to be determined. In the present study, we describe the generation of a novel Oatp2b1 knockout mouse model with the aim of elucidating the *in vivo* role of Oatp2b1 in the pharmacokinetics and tissue distribution of rosuvastatin.

5.2 Methods

5.2.1 Materials

Rosuvastatin and rosuvastatin-d6 were obtained from Toronto Research Chemicals (North York, ON). [^{3}H] rosuvastatin (5 mCi/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

5.2.2 Targeting of Slco2b1

The Oatp2b1$^{tm1a/tm1a}$ mouse strain (Slco2b1$^{tm1a(KOMP)Wtsi}$) used for this research project was generated using a Knockout-First promoter driven approach by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org) (Fig 5.1) (Skarnes et al., 2011). The 'knockout-first' allele contains an IRES:*lacZ* trapping cassette and a floxed promoter driven cassette inserted into the intron between exons 3 and 4, disrupting *Slco2b1* gene function (Skarnes et al., 2011). NIH grants to Velocigene at Regeneron Inc (U01HG004085) and the CSD Consortium (U01HG004080) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244). For more information or to obtain KOMP products go to www.komp.org.

5.2.3 Gene Expression Analysis

Tissues were harvested from male C57BL/6 wild type and Oatp2b1$^{tm1a/tm1a}$ mice and snap frozen at -80°C until RNA isolation. Total RNA was extracted from tissues after homogenization using Trizol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems, Carlsbad, CA), according to manufacturer's

instruction, from 2.5 μg of RNA per reaction. The resulting cDNA was used for quantitative real-time PCR (qPCR).

Expression of Slco2b1 (Oatp2b1), Slco1a1 (Oatp1a1), Slco1a4 (Oatp1a4), Slco1a5 (Oatp1a5), Slco1c1 (Oatp1c1) were determined by TaqMan assays Mm00614448_m1, Mm01267415_m1, Mm01267407_m1, Mm01267394_m1 and Mm00451845_m1, respectively (Applied Biosystems). Expression of other transporters was done using SYBR green assays with primers listed in Table 5.1. All qPCR reactions were preformed with an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems). The amount of the transporter was normalized to 18S-rRNA and relative expression was determined using the $\Delta\Delta C_T$ method.

5.2.4 Transport Experiments

Human cervical adenocarcinoma (HeLa) (American Type Culture Collection, ATCC, Manassas, VA), cells were cultured in DMEM supplemented with 10% FBS, 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine (Invitrogen, Carlsbad, CA). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. HeLa cells were grown in 12-well plates (approximately 0.8×10^6 cells/well) and infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to absorb for 30 minutes. Cells were transfected with 1 μg of total transporter cDNA (mOatp2b1 or hOATP2B1) or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. Transport was evaluated using labeled substrate as previously reported (Cvetkovic et al., 1999). [^{3}H] rosuvastatin accumulation, after 10 mins, was determined by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

5.2.5 Pharmacokinetic Study Design

Animals were housed in a temperature-controlled environment with a 12h light/dark cycle and received a standard diet and water ad libitum. Male Oatp2b1$^{tm1a/tm1a}$ and wild-type C57BL/6 (Jackson Laboratory, Bar Harbour, MA) were between 8 and 16 weeks of age, weighing 20 – 30 g, were used for experiments. Animals were dosed with 10 mg/kg rosuvastatin by oral gavage in 0.5% hydroxypropylmethylcellulose or 1 mg/kg rosuvastatin by tail vein injection in saline with <2% DMSO. All blood samples were collect by serial sampling at 10, 20, 30 min and 1, 2, 4, and 8 hours after oral dosing and 5, 15, 30 min and 1, 2, 4, and 6 hours following intravenous dosing (Peng et al., 2009). 25 uL of blood was collected with a heparinized pipet after saphenous or tail vein puncture. Blood was transferred to a tube for centrifugation at 12,000 g for 5 min to obtain plasma (10 uL). At the final time point animals were euthanized by isoflurane overdose; blood was collected by cardiac puncture and liver, quadriceps and kidney were excised, rinsed in PBS, blotted and weighed. All plasma and tissue samples were stored at -80°C until analysis by LC-MS/MS. The study protocol was approved by the Animal Use Subcommittee of the University of Western Ontario, London, Canada.

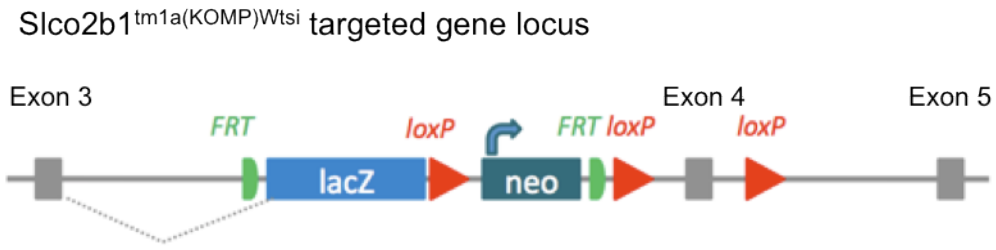

Figure 5.1 Schematic of the "knockout-first" conditional *Slco2b1* allele in Slco2b1$^{tm1a(KOMP)Wtsi}$ mice. The 'knockout-first allele' contains an IRES:*lacZ* trapping cassette and a floxed promoter driven cassette inserted into the intron between exons 3 and 4 disrupting *Slco2b1* gene function (Skarnes et al., 2011).

Table 5.1 Primer Sequences used for qPCR

Primer		Sequence
mOatp1b2 Fw	Sense	5′-TGGGCATTGGGAGTATTCTGA-3′
mOatp1b2 Rv	Antisense	5′-CCAGGTGTATGAGTTGGACCC-3′
mBcrp Fw	Sense	5′-AAATGGAGCACCTCAACCTG-3′
mBcrp Rv	Antisense	5′-CCCATCACAACGTCATCTTG-3′
mMrp2 Fw	Sense	5′-CTGAGTGCTTGGACCAGTGA-3′
mMrp2 Rv	Antisense	5′-CAAAGTCTGGGGGAGTGTGT-3′
mNtcp Fw	Sense	5′-GGCTTCCTGATGGGCTACATT-3′
mNtcp Rv	Antisense	5′-AGAGTTGGACGTTTTGGAATCC-3′
mOat3 Fw	Sense	5′-CTTCAGAAATGCAGCTCTTG-3′
mOat3 Rv	Antisense	5′-ACCTGTTTGCCTGAGGACTG-3′

5.2.6 LC-MS/MS Analysis

The concentration of rosuvastatin in plasma and tissues was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Agilent 1100 liquid chromatographic system and a TSQ-Vantage triple-quadrupole mass spectrometer (Thermo Scientific, Pittsburgh, PA) as previously reported (Degorter et al., 2012). Briefly plasma samples were precipitated with 45 uL of acetonitrile containing rosuvastatin–d6 as an internal standard and centrifuged for 20 min at 14000 rpm at 4°C. The supernatant was diluted 1:2 in 0.05% formic acid and analyzed by LC-MS/MS. Liver samples were homogenized 1:1 (w/v) in 0.05% formic acid while quadriceps and kidney samples were homogenized 1:2 (w/v) in 0.05% formic acid. Standard curves were prepared using blank tissue homogenates. Tissue homogenates were precipitated using 1:3 in acetonitrile containing rosuvastatin–d6 as an internal standard and centrifuged for 20 min at 14000 rpm at 4°C. The supernatant was diluted 1:2 with 0.05% formic acid and analyzed by LC-MS/MS like plasma. The samples were injected onto a Hypersil GOLD C18 column (50 × 3 mm, 5 μm particle size; Thermo Scientific) and separated using 0.05% formic acid and acetonitrile using a gradient ranging from 70:30 to 10:90 respectively. Detection of rosuvastatin was done using transitions m/z 482.1 → 258.2 for rosuvastatin and 488.0 → 264.3 for rosuvastatin-d6. The accuracy and precision of the assay were 5.8% and 8.9%, respectively.

5.2.7 Pharmacokinetic Calculations and Statistical Analysis

Tissue to plasma ratios were calculated by dividing the tissue level by the plasma concentration at the final sampling time point (6h IV and 8h PO). The area under the curve (AUC) from 0 to h ($AUC_{0\text{-}h}$) was calculated for each animal by the linear trapezoid rule. The area under the curve from 0 hours to infinity ($AUC_{0\text{-}\infty}$) was obtained as the sum of $AUC_{0\text{-}h}$ and the residual area, calculated by dividing the final concentration by the terminal rate constant (k_e). Half life (T½) was calculated as $Ln2/k_e$. The mean resonance time (MRT) was calculated as AUMC/AUC. CL_{plasma} was calculated using the equation $CL_{plasma} = dose/AUC_{0\text{-}\infty}$. The volume of distribution at steady state (V_{ss}) was

calculated as V_{ss} = AUMC × Dose/$AUC_{0-\infty}$[2]. Bioavailability (F) was calculated for each animal dosed by oral gavage. Results are presented as the mean ± SEM. A two-sided unpaired Student's t-test was used to evaluate the statically difference between Oatp2b1$^{tm1a/tm1a}$ and wild type mice in gene expression analysis and in pharmacokinetic studies. Statistical analysis was preformed using GraphPad Prism 5 (La Jolla, CA).

5.3 Results

5.3.1 Characterization of Oatp2b1$^{tm1a/tm1a}$ Mice

We have observed the Oatp2b1$^{tm1a/tm1a}$ mice to an age of 18 months in our laboratory. Oatp2b1$^{tm1a/tm1a}$ mice were viable and fertile and appear to have a normal life span. No differences were noted in the weight or growth rate of Oatp2b1$^{tm1a/tm1a}$ mice compared to wild type animals. Macro and microscopic histological and pathological analysis has not revealed any obvious aberrations in tissues of Oatp2b1$^{tm1a/tm1a}$ mice.

The Oatp2b1$^{tm1a/tm1a}$ mice have a LacZ trapping cassette located between exon 3 and 4 to disrupt splicing of Oatp2b1 resulting in gene knockout. For confirmation of the Oatp2b1 knockout by RT-PCR analysis in liver, kidney, ileum, heart, and quadriceps, TaqMan primers and probes were located after the LacZ trapping cassette in exon 9 and 10. We demonstrated a significant decrease (>98.2%) in Oatp2b1 mRNA expression in Oatp2b1$^{tm1a/tm1a}$ mice compared with wild type animals (Fig. 5.2). Confirmation of Oatp2b1 knockout at the protein level is ongoing in the laboratory.

5.3.2 Expression Analysis of Other Transporter Proteins in Tissues of The Oatp2b1$^{tm1a/tm1a}$ Mice

Real time PCR analysis was preformed to determine if compensatory changes occurred in the expression levels of various uptake and efflux transporters in liver, kidney, ileum, heart, and quadriceps of male wild type and Oatp2b1$^{tm1a/tm1a}$ mice. The expression of 4 other Oatps, Oatp1a1, Oatp1a4, Oatp1b2 and Oatp1c1 were not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$ mice (Fig. 5.3). Oatp1a5 has been reported to be expressed in the intestine (Cheng et al., 2005); however, we were unable to detect expression of Oatp1a5 using a TaqMan assay. We found that expression of Oatp1a transporters tended to be reduced in Oatp2b1$^{tm1a/tm1a}$ mice, but this did not reach significance.

Expression of the rosuvastatin uptake and efflux transporters sodium-taurocholate co-transporting polypeptide (Ntcp), organic anion transporter 3 (Oat3), breast cancer resistance protein (Bcrp) and multidrug resistance-associated protein 2 (Mrp2) was examined in liver and kidney. Ntcp expression in the liver was not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$ mice (Fig. 5.4A). Expression of Oat3 in the kidney was upregulated in Oatp2b1$^{tm1a/tm1a}$ mice but it did not reach significance (Fig. 5.4C). Bcrp expression in liver and kidney was also not significantly different between groups (Fig. 5.4B). However, expression of Mrp2 was significantly upregulated in the liver but not the kidney of Oatp2b1$^{tm1a/tm1a}$ when compared to wild type animals (Fig. 5.4D).

5.3.3 Rosuvastatin is a Substrate of mOatp2b1

Mouse Oatp2b1 is highly expressed in liver, intestine and skeletal muscle like OATP2B1, but it was not known whether mOatp2b1 also transported statins. Unpublished work from the laboratory has demonstrated in HeLa cells transfected with mOatp1b2 or hOATP2B1 that mOatp2b1 is capable of transporting rosuvastatin. Rosuvastatin uptake in cells expressing mOatp1b2 or hOATP2B1 was greater (113 and 165% higher, respectively) than cells transfected with vector alone (Fig. 5.5). This finding demonstrates that mouse Oatp2b1, like human OATP2B1, is capable of transporting rosuvastatin.

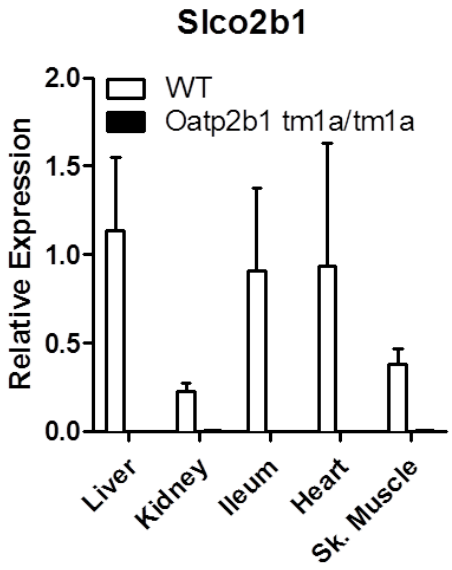

Figure 5.2 Expression of *Slco2b1* mRNA in liver, kidney, ileum, heart and skeletal muscle (quadriceps) of wild type and Oatp2b1$^{tm1a/tm1a}$ mice.

mRNA expression as assessed by real-time RT-PCR. Data are presented as mean ± SEM of 3 male mice per group.

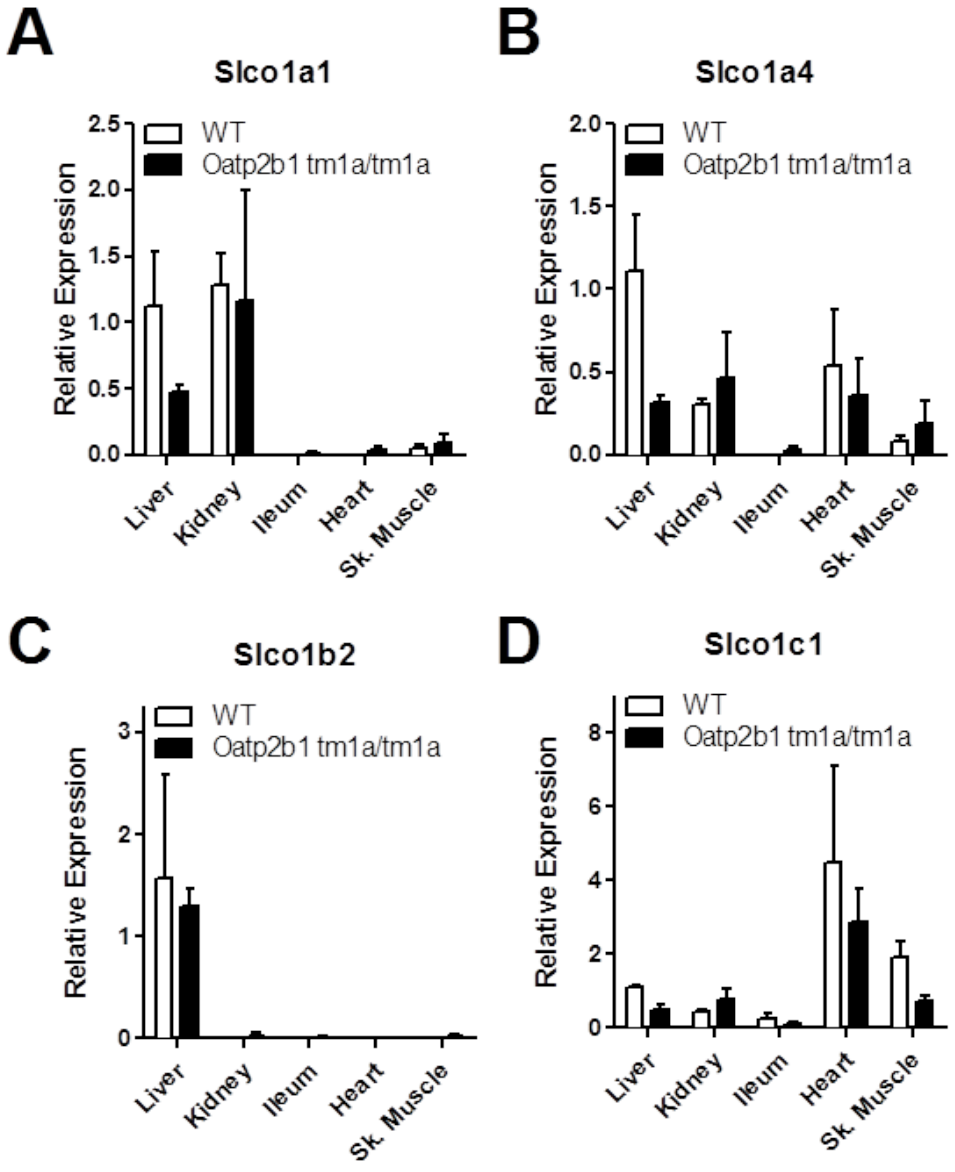

Figure 5.3 Expression of *Slco1a1* (A), *Slco1a4* (B), *Slco1b2* (C), and *Slco1c1* (D), in liver, kidney, ileum, heart and skeletal muscle (quadriceps) of wild type and Oatp2b1$^{tm1a/tm1a}$ mice.

mRNA expression was assessed by real-time RT-PCR. Data are presented as mean ± SEM of 3 male mice per group.

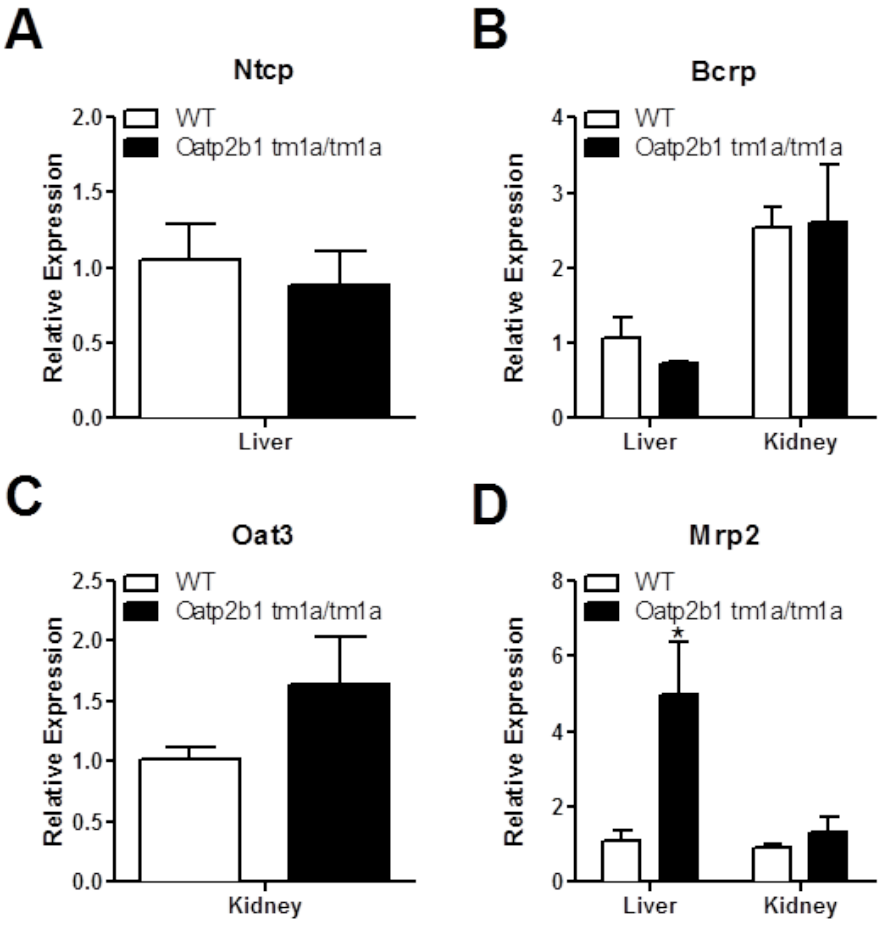

Figure 5.4 Expression of uptake and efflux transporters in liver and kidney of wild type and Oatp2b1$^{tm1a/tm1a}$ mice.

mRNA expression of (A) sodium-taurocholate co-transporting polypeptide (Ntcp), (B) organic anion transporter 3 (Oat3), (C) breast cancer resistance protein (Bcrp) and (D) multidrug resistance-associated protein 2 (Mrp2) was assessed by real-time PCR. Data are presented as mean ± SEM of 3 male mice per group. * $p < 0.05$ significantly different from wild type mice.

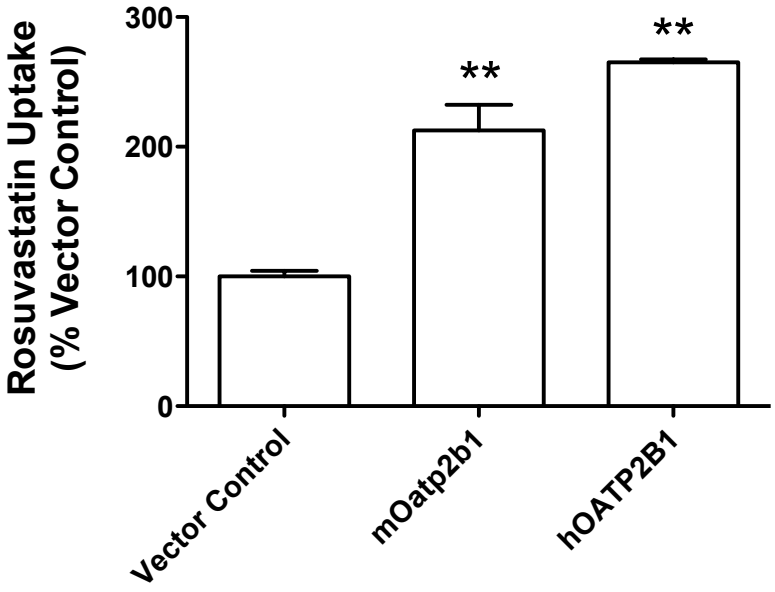

Figure 5.5 Intracellular accumulation of [^{3}H] rosuvastatin in HeLa cells transiently transfected with mOatp2b1 and hOATP2B1.

Results are presented as percent of vector control ± SEM. **p<0.01 compared to vector control.

5.3.4 Rosuvastatin Pharmacokinetics In Oatp2b1$^{tm1a/tm1a}$ And Wild Type Mice After Oral Administration

Like all statins, rosuvastatin is orally administered and little is known about transporters involved with the *in vivo* intestinal absorption of rosuvastatin. However, in humans, absorption of rosuvastatin is thought to be partially facilitated by OATP1A2 and OATP2B1 (Shitara and Sugiyama, 2006). In mice, Oatp2b1 is highly expressed throughout the gastrointestinal tract and may facilitate the absorption of rosuvastatin (Cheng et al., 2005). Therefore, we investigated the role of Oatp2b1 in the oral absorption of rosuvastatin in wild type and Oatp2b1$^{tm1a/tm1a}$ mice. We measured rosuvastatin plasma levels after the administration of 10 mg/kg of rosuvastatin by oral gavage. Similar to previous reports, we found that the oral absorption of rosuvastatin to be very rapid (Peng et al., 2009). The highest plasma concentrations were observed at the first time point, assessed at 10 min. The plasma rosuvastatin concentrations were not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$ mice (Fig. 5.6). Analysis of the pharmacokinetic parameters revealed no significant difference in the $AUC_{0-\infty}$, C_{max}, T_{max} or half life (T½) between wild type and Oatp2b1$^{tm1a/tm1a}$ mice (Table 5.2). The systemic bioavailability of rosuvastatin was also not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$, 9.2 % vs 10.9% respectively. Theses results indicate that Oatp2b1 is not essential for the oral absorption of rosuvastatin and that Oatp2b1 has no significant effect on the bioavailability or systemic exposure of rosuvastatin when examined in this mouse model.

5.3.5 Rosuvastatin Pharmacokinetics in Oatp2b1$^{tm1a/tm1a}$ and Wild Type Mice After Intravenous Administration

Rosuvastatin pharmacokinetics was also examined after intravenous dosing (1mg/kg). The results were similar to oral administration experiment; plasma rosuvastatin concentrations were not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$ mice (Fig. 5.7). Pharmacokinetic analysis revealed no differences in the $AUC_{0-\infty}$, C_0, clearance or half life (T½) between wild type or Oatp2b1$^{tm1a/tm1a}$ mice (Table 5.3). The

volume of distribution was elevated in the wild type *vs*. Oatp2b1$^{tm1a/tm1a}$ mice, 207 mL vs 128 mL respectively, but the difference did not reach statistical significance (p value = 0.42). The volume of distribution of rosuvastatin is greater than the reported total body water volume of 21.75 mL for a 30 g mouse (Davies and Morris, 1993). This suggests that rosuvastatin has significant accumulation in tissues. Rosuvastatin tissue distribution studies conducted in rats demonstrate that the liver is the primary site for rosuvastatin accumulation (Nezasa et al., 2002).

The plasma clearance of rosuvastatin was not significantly different between wild type and Oatp2b1$^{tm1a/tm1a}$ mice, 277 mL/h versus 242 mL/h respectively. Rosuvastatin is reported to be primarily excreted in the bile unchanged (Nezasa et al., 2002), however the values for rosuvastatin clearance are significantly greater than plasma flow to the liver of 59 mL/h (Davies and Morris, 1993). These results indicate significant non-hepatic clearance of rosuvastatin unaffected by Oatp2b1.

Taken together, the results from both oral and intravenous experiments indicate that Oatp2b1 does not significantly affect the systemic exposure of rosuvastatin.

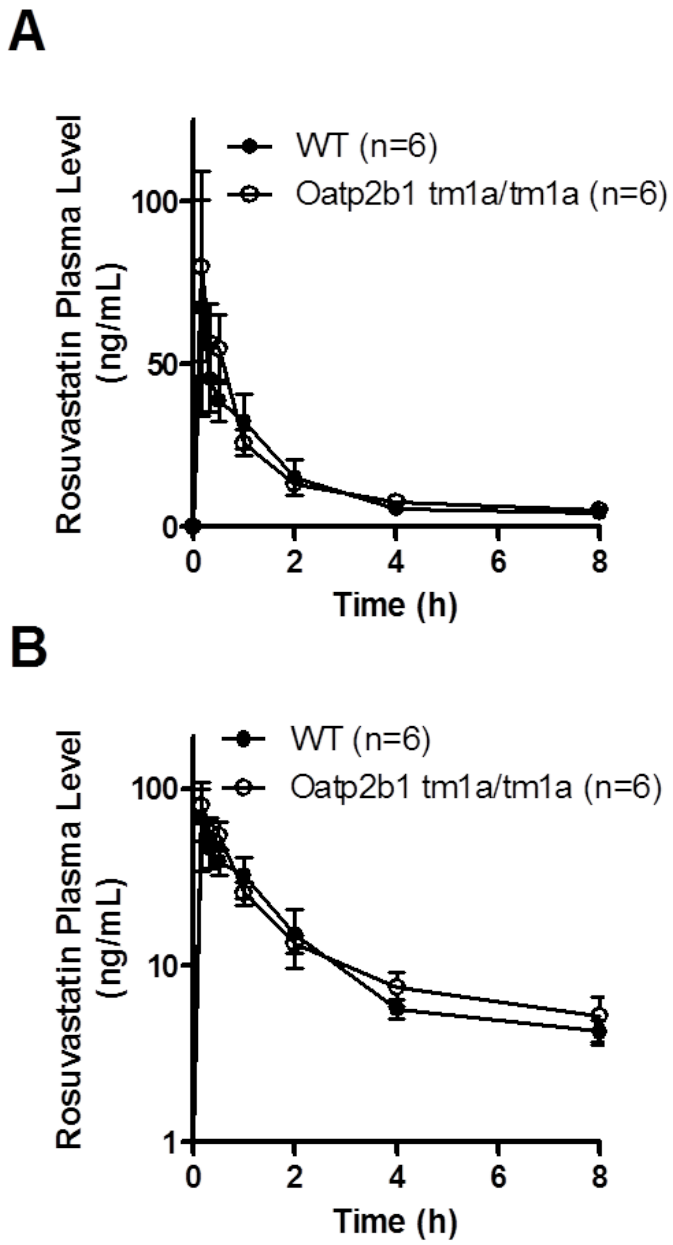

Figure 5.6 Plasma Concentration-time curves of rosuvastatin in wild type and Oatp2b1$^{tm1a/tm1a}$ mice after administration of rosuvastatin by oral gavage (10mg/kg) (A).

Rosuvastatin concentrations shown on a semi-log plot (B). Blood samples were taken at various time points by serial sampling. Data are presented as the mean ± SEM of 6 male mice per group.

Table 5.2 Non-compartmental analysis of pharmacokinetic data in wild type and Oatp2b1 knockout mice after oral administration of rosuvastatin 10 mg/kg. Data are presented as the mean ± SEM of 6 male mice per group.

Parameter	**Wild Type (n=6)**	**Oatp2b1$^{tm1a/tm1a}$ (n=6)**	**p-value**
Dose (mg/kg)	10	10	
$AUC_{0-\infty}$ (ng*h/mL)	127.62 ± 23.23	154.33 ± 26.14	0.57
C_{max} (ng/mL)	78.34 ± 31.39	89.42 ± 28.10	
T_{max} (h)	0.39 ± 0.33	0.31 ± 0.16	
MRT (h)	4.31 ± 0.46	5.38 ± 1.19	0.46
T ½ (h)	3.76 ± 0.63	4.48 ± 0.91	0.58
F (%)	9.19% ± 1.67%	10.92% ± 1.85%	0.50

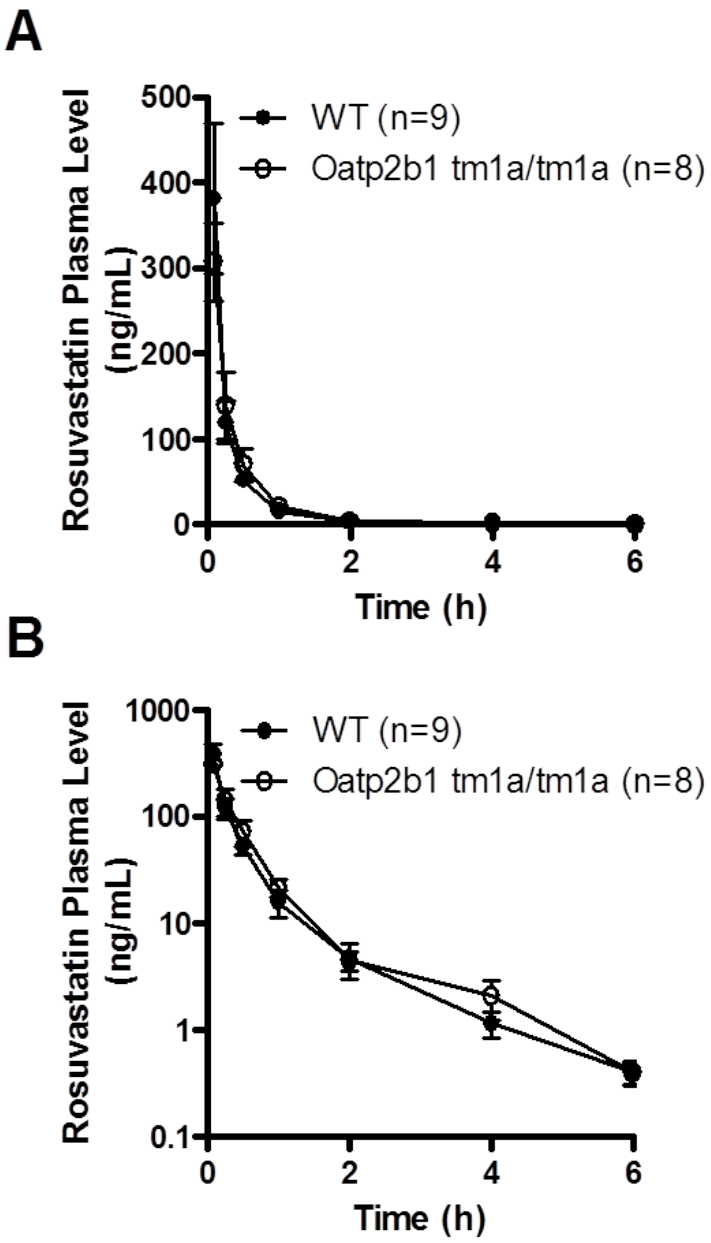

Figure 5.7 Plasma Concentration-time curves of rosuvastatin in wild type and Oatp2b1$^{tm1a/tm1a}$ mice after a single intravenous dose of rosuvastatin (1mg/kg) (A).

Rosuvastatin concentrations shown on a semi-log plot (B). Blood samples were taken at various time points by serial sampling. Data are presented as the mean ± SEM of 8-9 male mice per group.

Table 5.3 Non-compartmental analysis of pharmacokinetic data in wild type and Oatp2b1 knockout mice after intravenous administration of rosuvastatin 1 mg/kg. Data are presented as the mean ± SEM of 8-9 male mice per group.

Parameter	**Wild Type (n=9)**	**Oatp2b1$^{tm1a/tm1a}$ (n=8)**	**p-value**
Dose (mg/kg)	1	1	
$AUC_{0-\infty}$ (ng*h/mL)	138.91 ± 31.56	141.32 ± 24.38	0.95
C_0 (ng/mL)	577.74 ± 181.66	484.17 ± 59.55	0.65
MRT (h)	0.59 ± 0.13	0.52 ± 0.05	0.62
T ½ (h)	1.41 ± 0.31	1.17 ± 0.13	0.50
CL_{plasma} (mL/h)	277.21 ± 62.90	242.33 ± 44.30	0.66
V_{ss} (mL)	207.90 ± 87.89	128.01 ± 31.44	0.42

5.3.6 Tissue Distribution of Rosuvastatin in Oatp2b1$^{tm1a/tm1a}$ Mice

Oatp2b1 is thought to facilitate the extrahepatic tissue distribution of rosuvastatin. We have reported previously that OATP2B1 contributes to the distribution of rosuvastatin into skeletal muscle and sensitization to myotoxicity (Knauer et al., 2010). We investigated the tissue distribution of rosuvastatin into liver, kidney and skeletal muscle (quadriceps) after both oral and intravenous dosing. Previous studies in rats demonstrate that rosuvastatin primarily accumulates in the liver while the muscle to plasma ratio is less than unity (Nezasa et al., 2002; Sidaway et al., 2009). The tissue distribution of rosuvastatin 8 hours after oral dosing is reported in Figure 5.8 for wild type and Oatp2b1$^{tm1a/tm1a}$ mice. The mean rosuvastatin liver to plasma ratio was 143 in wild type and 109 in Oatp2b1$^{tm1a/tm1a}$ mice. Unlike studies in rats where the muscle to plasma ratio was less than one, the mean rosuvastatin muscle to plasma ratio was 7 in wild type and 2 in Oatp2b1$^{tm1a/tm1a}$ mice. However, we found no difference between the rosuvastatin tissue concentration, tissue to plasma ratio in liver or quadriceps between wild type and Oatp2b1$^{tm1a/tm1a}$ mice.

The rosuvastatin tissue distribution after 6 hours after intravenous dosing is reported in Figure 5.9. The mean rosuvastatin kidney to plasma ratio was 2.3 in wild type and 8.6 in Oatp2b1$^{tm1a/tm1a}$ mice. The muscle to plasma ratio was 17 in wild type and 15 in Oatp2b1$^{tm1a/tm1a}$ mice after intravenous dosing. We found no difference between the rosuvastatin tissue concentration, tissue to plasma ratio in kidney or quadriceps between wild type and Oatp2b1$^{tm1a/tm1a}$ mice. However, Oatp2b1$^{tm1a/tm1a}$ mice had a higher rosuvastatin liver concentration compared with wild type mice. This resulted in a significant elevation in the liver to plasma ratio for Oatp2b1$^{tm1a/tm1a}$ mice versus wild type animals, 195 versus 79 respectively. These results indicate that Oatp2b1 does not contribute significantly to the distribution of rosuvastatin to skeletal muscle or kidney in mice.

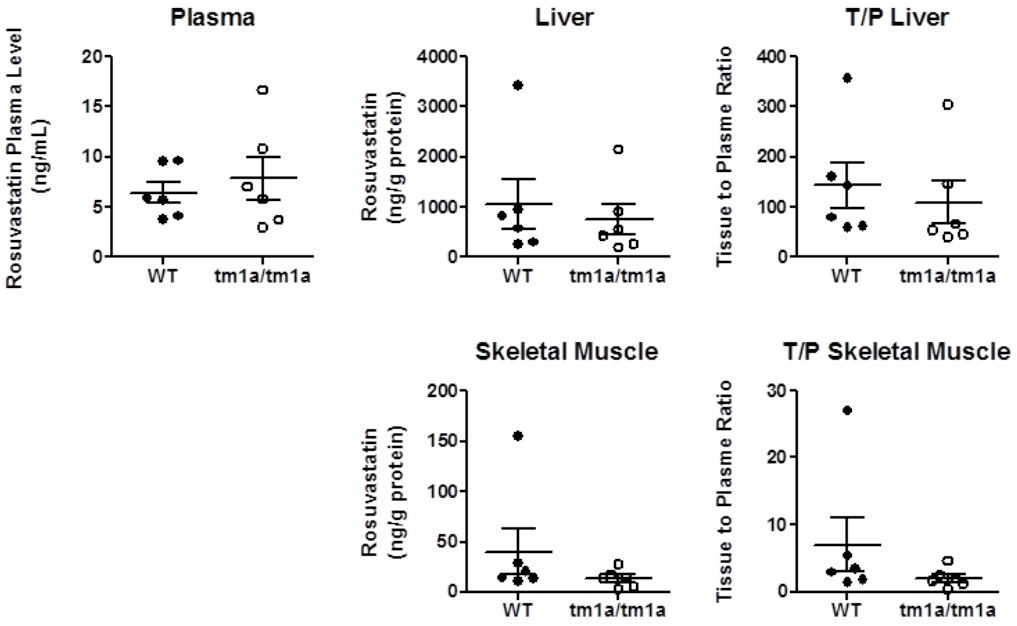

Figure 5.8 Concentrations in plasma, liver, and skeletal muscle (quadriceps), and liver to plasma and skeletal muscle (quadriceps) to plasma concentration ratios of rosuvastatin in Oatp2b1$^{tm1a/tm1a}$ mice compared to wild-type controls, 8 hours after a 10 mg/kg oral dose.

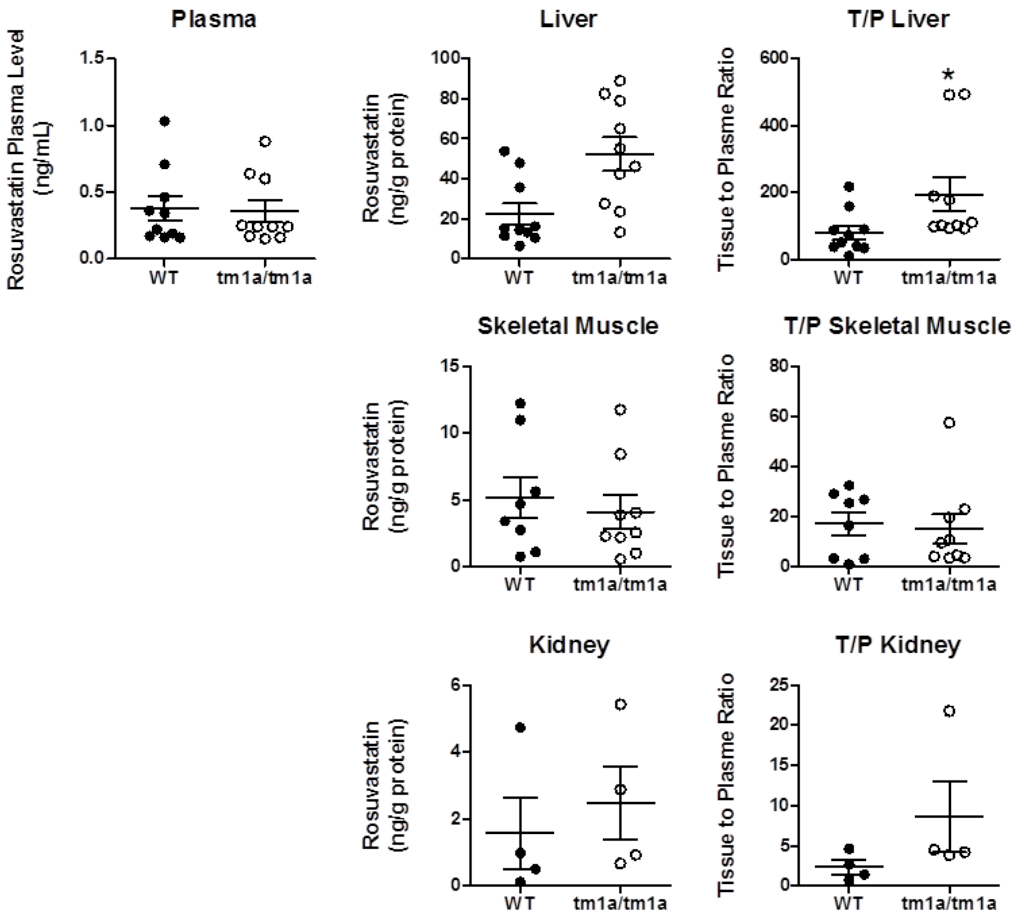

Figure 5.9 Concentration in plasma, liver, skeletal muscle (quadriceps), and kidney, and liver to plasma, skeletal muscle (quadriceps) to plasma, and kidney to plasma concentration ratios of rosuvastatin in Oatp2b1$^{tm1a/tm1a}$ mice compared to wild-type controls, 6 hours after a 1 mg/kg intravenous dose.

5.4 Discussion

Here we report for the first time the generation and characterization of a novel mouse model lacking Oatp2b1. Oatp2b1$^{tm1a/tm1a}$ mice were viable, fertile and did not display any clear pathological abnormalities. The present study demonstrates that Oatp2b1 is not significantly involved in the intestinal absorption of rosuvastatin after oral administration. We show that Oatp2b1 does not affect the systemic exposure, clearance or volume of distribution of rosuvastatin after oral and intravenous dosing. The extrahepatic distribution of rosuvastatin in Oatp2b1$^{tm1a/tm1a}$ mice is unchanged compared to wild type animals. However, we did see an increase in the rosuvastatin liver to plasma ratio after intravenous dosing.

OATP1A2 and OATP2B1 transporters in the intestine have been proposed to mediate the intestinal absorption of statins in humans. We show that Oatp2b1 is not significantly involved in the intestinal absorption of rosuvastatin in our knockout mouse model. Interestingly, the intestinal absorption of rosuvastatin in pigs has also been shown to be unaffected by co-administration of gemfibrozil, an OATP2B1 inhibitor (Bergman et al., 2009). Rosuvastatin is quite polar, LogD -0.25 to -0.5 (Table 1.1), and drug transporters are likely involved in its absorption. The murine Oatp1a family of transporters are highly expressed in the intestine, like human OATP1A2, and maybe also be involved in rosuvastatin absorption. However, studies using Oatp1a/1b knockout mice demonstrate that intestinal absorption of pravastatin, another hydrophilic statin, is not dependent on Oatp1a/1b transporters (Iusuf et al., 2012). It is possible that Oatp2b1 and Oatp1a transporters compensate for the loss of the other transporter or that another transporter is responsible for rosuvastatin absorption in mice. However, we did not observe significant changes to the expression of Oatps in the ileum of the Oatp2b1$^{tm1a/tm1a}$ mice. We used whole ileum to extract total RNA not just enterocytes, so it is possible that Oatp expression is altered in other sections of the small intestine or that the expression is altered selectively in enterocytes. Additional studies are required to determine the transporter(s) responsible for the intestinal absorption of rosuvastatin.

The systemic exposure of rosuvastatin was unchanged after oral or intravenous dosing in Oatp2b1$^{tm1a/tm1a}$ mice. This suggests that Oatp2b1 does not have a significant contribution to the clearance or volume of distribution of rosuvastatin. This could be expected because, at least in humans, rosuvastatin hepatic uptake is probably mediated by multiple transporters including OATP1B1, OATP1B3, OATP2B1 and NTCP (Ho et al., 2006). Data from Oatp1b2$^{-/-}$ mice suggests that the effect of Oatp1b2 increases plasma levels by about 1.5 fold (Degorter et al., 2012). It has been suggested that other hepatic uptake processes can compensate for loss of the hepatic Oatp1b2 like Oatp2b1.

Although the volume of distribution of rosuvastatin is slightly smaller in Oatp2b1$^{tm1a/tm1a}$ mice, the extrahepatic distribution of rosuvastatin into muscle and kidney appears unchanged compared to wild type animals. However, we did not find any significant differences in the rosuvastatin muscle concentration or muscle to plasma ratio in the quadriceps of Oatp2b1$^{tm1a/tm1a}$ mice compared to wild type animals. A repeated dosing study would allow for the study of rosuvastatin after drug levels have reached steady state. Oatp2b1 knockout may lead to changes in the steady state rosuvastatin muscle levels, which are not apparent in this single dose study. As mentioned before the muscle to plasma ratio was greater than one in wild type and Oatp2b1$^{tm1a/tm1a}$ mice compared to reports of less than one in rats and guinea pigs (Madsen et al., 2008; Sidaway et al., 2009). Unpublished data from studies we have done in Mrp1$^{-/-}$ mice suggest a muscle to plasma ratio less then one for [^{3}H]rosuvastatin (Appendix D). An explanation for this difference could be the differences in the rosuvastatin dosage or time of sampling. Muscle may be a slowly equilibrating tissue in the distribution of statins and time and dose may factor into the muscle to plasma ratio.

Assuming there is no difference in other transporters relevant to the hepatic disposition of a drug between the Oatp2b1$^{tm1a/tm1a}$ and wild type mice, when Oatp2b1-mediated uptake does not represent a rate-limiting step, the liver to plasma ratio would be equal between Oatp2b1$^{tm1a/tm1a}$ and wild type mice. In contrast, when Oatp2b1 uptake plays a critical role in the hepatic disposition of a drug, liver to plasma ratios in wild type mice would be significantly higher than that of Oatp2b1$^{tm1a/tm1a}$ mice. Our data showed that Oatp2b1 did not play a significant part in the uptake of rosuvastatin in the liver. Interestingly, we saw

the opposite to what was expected and there was an increase in the liver to plasma ratio for rosuvastatin after intravenous dosing. This result does not appear to be related to changes in the expression of other transporters. Although we see that Mrp2 is upregulated in the liver, we would expect this change to increase elimination into the bile and decrease liver to plasma ratios. Importantly, we demonstrate there does not appear to be any major compensatory upregulation of other Oatps in the liver or other tissues. The underlying mechanism for the increased liver to plasma ratio in Oatp2b1$^{tm1a/tm1a}$ mice is not known and requires further study. Interestingly, a similar increase in the liver to plasma ratio was shown for pravastatin in Oatp1b2$^{-/-}$ mice, with no explanation given (Chen et al., 2008). The hepatic extraction of rosuvastatin is considered very high, 0.63 in humans (Martin et al., 2003) and 0.89 in pigs (Bergman et al., 2009), so the increase in the rosuvastatin liver to plasma ratio in Oatp2b1$^{tm1a/tm1a}$ mice could be attributed to a decrease in hepatic elimination versus an increase in uptake. However, we do not have evidence to support this hypothesis. We see a trend toward reduced volume of distribution in Oatp2b1$^{tm1a/tm1a}$ mice, and lower muscle to plasma levels. Skeletal muscle has the largest organ volume in the mouse with total volume of muscle almost 8 fold higher than the liver (1.3 *vs.* 10 mL) (Davies and Morris, 1993). Even a small reduction in the rosuvastatin distribution into one muscle group would translate into a much larger reduction in the total muscle distribution. In turn this would lead to increased plasma levels and AUC, which are slightly higher in the Oatp2b1$^{tm1a/tm1a}$ mice.

The liver accounts for the majority of the volume of distribution of statins. In the Oatp2b1$^{tm1a/tm1a}$ mice we see a small reduction in the V_{ss} but a significant increase in liver levels. Theoretically this means that rosuvastatin has much less extrahepatic distribution in Oatp2b1$^{tm1a/tm1a}$ mice then wild type mice, resulting in a large reduction in the tissue distribution of a few tissues or a small reduction in distribution to many tissues. Unfortunately, we only examined the tissue distribution in liver, kidney and quadriceps, so it is possible that some tissues have large decreases in the rosuvastatin distribution or that there is a small reduction in the rosuvastatin tissue distribution in many tissues.

This study has many limitations, one of which is the lack of data to confirm Oatp2b1 knockout at the protein expression level. Studies are ongoing in our laboratory to test

newly made custom Oatp2b1 antibodies and LC-MS/MS to quantify protein expression in mice. There are other transporters involved in rosuvastatin absorption, elimination and distribution. We have suggested that Mrp1, Mrp4 and Mrp5 may be involved in the muscle exposure of rosuvastatin (Knauer et al., 2010) but we have not examined expression of efflux transporters in the muscle of Oatp2b1$^{tm1a/tm1a}$ mice. Bcrp, P-gp and Mrp2 may also play a role in the intestinal bioavailability of rosuvastatin; therefore, expression of efflux transporters needs to be assessed in the intestinal enterocytes of Oatp2b1$^{tm1a/tm1a}$ mice.

In conclusion, we report the generation of a novel knockout mouse model of Oatp2b1, as a tool to study the *in vivo* role of Oatp2b1 in drug disposition. We show that knockout of Oatp2b1 does not have a significant effect on the absorption, systemic exposure, or extrahepatic tissue distribution of rosuvastatin. A role for Oatp2b1 in the skeletal muscle toxicity of statins remains to be determined.

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6 DISCUSSION AND CONCLUSIONS

6.1 Summary and Discussion

6.1.1 Chapter Three

The aim of this chapter was to determine the spectrum, localization and function of statin transporters expressed in human skeletal muscle. We hypothesized that the functional expression of statin uptake and efflux transporters control the intracellular statin concentration and modulate the response to skeletal muscle exposure. We describe in Chapter Three that the uptake transporter OATP2B1 and 3 novel statin efflux transporters, MRP1, MRP4 and MRP5, are found in skeletal muscle and are able to control the *in vitro* intracellular accumulation of atorvastatin and rosuvastatin. We demonstrate that OATP2B1 sensitizes muscle to toxicity and MRP1 attenuates the toxicity of atorvastatin and rosuvastatin using an *in vitro* skeletal muscle model. This chapter confirms the importance of intracellular muscle exposure in the development of myotoxicity and describes how novel statin transporters present within skeletal muscle can affect the intracellular accumulation of statins and their toxic adverse effects. The results of this Chapter support the overall hypothesis of this thesis.

6.1.2 Chapter Four

The aim of Chapter Four was to examine the regulation and function of OATP2B1 transcription start site variants. OATP2B1 is ubiquitously expressed in various tissues in the body including; small intestine, liver, brain, kidney and skeletal muscle (Knauer et al., 2010; Kullak-Ublick et al., 2001; Tamai et al., 2000). Many statins are OATP2B1 substrates and OATP2B1 is believed to be involved in the bioavailability and distribution of statins into skeletal muscle as suggested in Chapter Three. Recently, it was shown that differential promoter usage in tissues results in the expression of five OATP2B1 transcriptional start site variants that encode either a full length or shortened protein lacking 22 amino acids (Pomari et al., 2009). There was an absence of information regarding the relative expression of the OATP2B1 transporter variants in key tissues responsible for statin absorption, elimination or muscle distribution. We hypothesized that OATP2B1 full length and short variant proteins would have differential expression

or function. We found differing expression patterns for the OATP2B1 variants in different tissues. The OATP2B1 short variant was highly expressed in liver and its hepatic expression was found to be regulated by HNF4α. Functional analysis of the OATP2B1 variants with estrone sulfate and rosuvastatin revealed similar results to the well-characterized full length variant. However, we observed that the OATP2B1 short variant was significantly better at transporting rosuvastatin at low pH compared to the full length form. Interestingly, we saw over a 100-fold variation in the hepatic expression of the OATP2B1 short variant regulated by HNF4α. HNF4α-mediated gene regulation can be influenced by a variety of different factors including genetics, gender, environmental factors and diet (Hwang-Verslues and Sladek, 2010). Alterations in the expression of OATP2B1 variants, such as the short variant in liver, could lead to changes in substrate pharmacokinetics. In the case of statins, changes to OATP2B1 short variant expression could affect systemic exposure through alterations in statin absorption or hepatic uptake. As discussed above alterations in systemic exposure can lead to changes in myopathy risk. These findings indicate that variation in HNF4α-mediated regulation of OATP2B1 short variant expression in liver could contribute to variable statin response.

6.1.3 Chapter Five

The aim of Chapter Five was to investigate the *in vivo* contribution of Oatp2b1 to the pharmacokinetics and tissue distribution of rosuvastatin using a novel Oatp2b1 knockout mouse model. We hypothesized that Oatp2b1 is involved in the absorption, hepatic uptake and muscle distribution of rosuvastatin. However, we observed that Oatp2b1 does not have a significant effect on the systemic exposure, clearance or volume of distribution of rosuvastatin. Rosuvastatin absorption and extrahepatic distribution in Oatp2b1 KO mice was unchanged compared to wild type animals. These results do not support the hypothesis that Oatp2b1 is significantly involved in rosuvastatin pharmacokinetics or risk for myopathy.

6.2 Therapeutic Implications

It is envisioned that the knowledge gained from this project may provide the basis for new preventative strategies to lower the risks of statin associated muscle injury and develop methods to treat those patients affected with this problematic drug side effect. It is accepted that statin myotoxicity is dose dependent and systemic exposure affects the risk for myopathy. However, we have shown that muscle exposure can also affect the risk of myopathy. Drug interactions at the level of metabolism have long been thought of as mechanisms for increasing the systemic exposure of drugs. The cerivastatin experience highlighted that drug interactions are also possible at the level of transporters to alter systemic exposure. We have demonstrated that drug interactions can also occur at the level of the muscle involving transporters and could prove important for therapeutics.

Pharmacogenetics and genetics-based dosing for statins is becoming more important given the evidence that polymorphisms in BCRP and OATP1B1 alter statin efficacy (Bailey et al., 2010; Chasman et al., 2012). The SEARCH study was the first to demonstrate a genetic predictor of simvastatin-induced myopathy (Link et al., 2008). Dosing algorithms have already been proposed based on the *SLCO1B1**5 polymorphism to lower the risk of statin induced myopathy (Niemi, 2010). This work demonstrates a role for transporters in skeletal muscle as potential determinates of toxicity. This may lead to discoveries relating genetic polymorphisms in muscle transporters and myopathy.

6.3 Future Directions

One of the most important questions that needs to be addressed is whether muscle concentrations are different between individuals with and without statin myopathies. This will not be an easy question to answer in humans but it may prove important for understanding the mechanism of statin induced myopathy. Recently, a non-invasive PET imaging approach has been used to study the hepatic distribution of pravastatin in rats (Ijuin et al., 2012). In the future, this approach could be applied to humans to non-

invasively measure the statin concentrations in skeletal muscle in patients with and without statin myopathy.

Interindividual differences in intestinal and hepatic drug transporters have been shown to be important for efficacy and toxicity. Assuming that muscle statin levels are different between affected and non-affected individuals we propose that variation in muscle transporters and the balance of uptake and efflux processes will be important for muscle exposure and risk. However, there is an absence of information on the expression of muscle transporters in patients. More studies investigating the genetic variation, transporter expression and function of muscle transporters from muscle biopsies are required.

We reported in Chapter Three that OATP2B1 is responsible for the uptake of statins into muscle and can potentiate the myotoxic effects of theses drugs. However, It is not known if there are interindividual differences in OATP2B1 expression within skeletal muscle or if expression can be regulated in muscle. Alterations in HNF4α activity due to genetics or environmental factors could lead to changes in expression and activity of drug metabolizing enzymes and transporters, including OATP2B1, responsible for statin disposition. OATP2B1 has a role in determining the systemic exposure and muscle exposure of statins; however, it is not known if changes in HNF4α regulation translate into differences in risk for myopathy.

As mentioned before, generation of a novel Oatp2b1 KO mouse could be potentially used for many different research areas. We must confirm Oatp2b1 knockout at the protein level using mass spectrometry or Western blotting with custom antibodies.

Our preliminary work in the Oatp2b1$^{tm1a/tm1a}$ mouse demonstrates the need for more experiments on the involvement of Oatps in the intestinal absorption of statins measuring the portal vein statin concentrations (Matsuda et al., 2012) or using everted gut sac experiments (Alam et al., 2012). Future studies will also include long term multiple dosing pharmacokinetic experiments to elucidate the role of Oatp2b1 in steady state statin levels. Although, most statins are substrates of OATP2B1 we cannot rule out pharmacokinetic differences between substrates or other statins. Other substrates and

statins need to be assessed with pharmacokinetic studies in the Oatp2b1 KO mouse to understand potential substrate specific differences in pharmacokinetics.

Research suggests that multiple transporters are involved in the hepatobilillary transport of statins and that redundancies exist in uptake and efflux transport processes. We have demonstrated that single transporter knockout mice of mOatp2b1 and mMrp1 (Chapter 5 and Appendix D) do not have significant alterations in the rosuvastatin muscle to plasma ratio. We have suggested that compensation by other transporters may occur in knockout animals. Future experiments preformed in mouse models with multiple transporter knockouts or tissue specific transporter knockouts may prove valuable to assess the role of transporters in specific tissues. One unique feature about the Oatp2b1$^{tm1a/tm1a}$ mouse strain is that the "knockout mouse first" targeting strategy used can be used to generate tissue specific knockout animals.

6.4 Conclusions

The role of drug transport proteins in drug absorption and distribution is becoming increasingly evident. We now recognize that interindividual variability in drug transport activity can lead to variation in pharmacokinetics, drug response and toxicity. The focus of this thesis was the involvement of drug transporters in statin induced myopathy, with focus on those expressed in skeletal muscle. Statins are substrates of numerous drug transporters and variation in these transporters has been shown to alter statin efficacy and system exposure. We proposed that drug transporters also control skeletal muscle exposure and modulate the response to statin myopathy.

The first section of the thesis focused on the identification of novel statin transporters in skeletal muscle and their involvement in controlling muscle exposure and toxicity. The second part of this thesis examined the regulation of OATP2B1 variants and the interindividual differences in expression of OATP2B1 variant proteins. Finally, we employed an *in vivo* mouse model of Oatp2b1 KO to assess rosuvastatin pharmacokinetics and tissue distribution. Taken together these studies provide insight into the *in vitro* and *in vivo* role of drug transporters in statin skeletal muscle exposure and myotoxic response.

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Appendices

Appendix A: Ethics Approval

Western

April 14, 2008

This is the Original Approval for this protocol
A Full Protocol submission will be required in 2012

Dear Dr. Tirona:

Your Animal Use Protocol form entitled:
The Role of Skeletal Muscle Drug Transporters in Statin-Induced Myopathy
Funding Agency CANADIAN INSTITUTES OF HEALTH RESEARCH
Role of Skeletal Muscle Transporters in Statin-Induced Myopathy - Grant #177829

has been approved by the University Council on Animal Care. This approval is valid from **April 14, 2008 to April 30, 2009.** The protocol number for this project is **2008-037-04**.

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3. If no number appears please contact this office when grant approval is received.
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ANIMALS APPROVED FOR 1 YR.

Species	Strain	Other Detail	Pain Level	Animal # Total for 1 Year
Mouse	FVB	30 gm Male	B	60
Mouse	FVB abcc1-/-	30 gm Male	B	60
Mouse	FVB abcg2-/-	30 gm Male	B	60

STANDARD OPERATING PROCEDURES
Procedures in this protocol should be carried out according to the following SOPs. Please contact the Animal Use Subcommittee office (661-2111 ext. 86770) in case of difficulties or if you require copies.
SOP's are also available at http://www.uwo.ca/animal/acvs
310 Holding Period Post-Admission
320 Euthanasia
321 Criteria for Early Euthanasia/Rodents

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.
1. Please review the safety section as new information has been added by the safety officials.

c.c. Approved Protocol - R. Tirona, W. Lagerwerf
Approval Letter - W. Lagerwerf

From: eSiriusWebServer **Sent:** Wednesday, June 20, 2012 1:06 PM **To:**

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AUP Number: 2012-006 **PI Name:** Tirona, Rommel **AUP Title:** The Role Of Skeletal Muscle Drug Transporters In Statin-induced Myopathy **Approval Date:** 06/20/2012

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "The Role Of Skeletal Muscle Drug Transporters In Statin-induced Myopathy " has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2012-006::1

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Submitted by: Copeman, Laura on behalf of the Animal Use Subcommittee University Council on Animal Care

Appendix B: Copyright Approval

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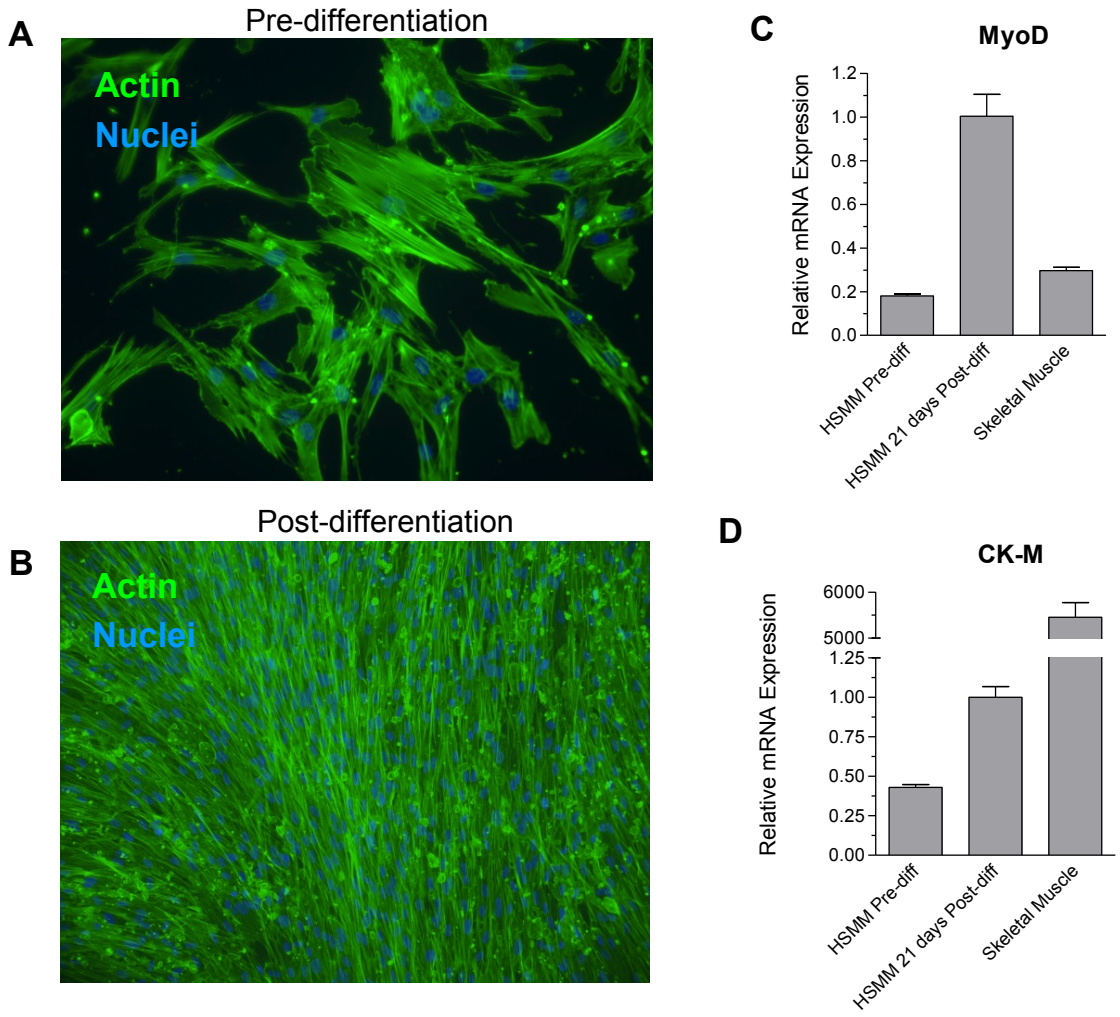
Appendix C: Supplementary Information I



Supplementary Figure C1. Characterization of primary cultured human skeletal myoblasts (HSMM). Morphology of HSMM cells pre-differentiation (A) and post-differentiation (B). HSMM cells were stained with phalloidin Alexa 488 (Invitrogen) to visualize actin and DAPI for nuclei. Expression of the skeletal muscle-selective genes MyoD (C) and creatine kinase isoform M (CK-M) (D) in pre-differentiated and post-differentiated cultured HSMM cells, as well as, intact human skeletal muscle was determined by quantitative PCR.

Supplementary Table C1. Real-time PCR primers used for gene expression quantitation.

Gene	Forward Primer	Reverse Primer
18S	5'-GTAACCCGTTGAACCCATT-3'	5'-CCATCCAATCGGTAGTAGCG-3'
OATP2B1	5'-CTTCATCTCGGAGCCATACC-3'	5'-AGATGCTGGGTTTCTGTGTAG-3'
MRP1	5'-CGGAAACCATCCACGACCCTAATC-3'	5'-ACCTCCTCATTCGCATCCACCTTG-3'
MRP2	5'-ATGCTTCCTGGGGATAAT-3'	5'-TCAAAGGCACGGATAACT-3'
MRP4	5'-AAGTGAACAACCTCCAGTTCCAG-3'	5'-GGCTCTCCAGAGCACCATCT-3'
MRP5	5'-CCCAGGCAACAGAGTCTAACC-3'	5'-CGGTAATTCAATGCCCAAGTC-3'
BCRP	5'-TGGCTGTCATGGCTTCAGTA-3'	5'- GCCACGTGATTCTTCCACAA -3'
CK-M	5'-CTAACTACGTGCTCAGCAGC-3'	5'-GGGTAGTACTTCCCTTTGAAC-3'
MyoD	5'-AGTAAATGAGGCCTTTGAGACACTC-3'	5'-TCGATATAGCGGATGGCGTT-3'

Appendix D: Supplementary Information II - Impact of Drug Transport on the Tissue Distribution of Rosuvastatin: Studies in Multidrug Resistance Associated Protein 1 Knockout Mice[4]

[4] This section is based on unpublished work: Knauer MJ, Mansell SE and Tirona RG. Impact of Drug Transport on the Tissue Distribution of Rosuvastatin: Studies in Multidrug Resistance Associated Protein 1 Knockout Mice. 2012.

Introduction

Statins, or 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are highly effective drugs for the treatment of hypercholesterolemia. Statins lower blood cholesterol by inhibiting the hepatic synthesis of mevalonate, the rate-limiting step in cholesterol biosynthesis, leading to increased low-density lipoprotein receptor expression, and reductions in LDL-C (Endo, 1992; Jacobson, 2008). Statins have a favourable risk-benefit ratio and are generally well tolerated (Pasternak, 2002). However, statins are known to cause skeletal muscle side-effects, ranging from mild myalgia to fatal rhabdomyolysis (Thompson et al., 2006). Observational reports suggest that myalgia, defined as muscle aches or weakness in the absence of blood creatine kinase (CK) elevation, occurs in 5-15% of statin-treated patients (Bruckert et al., 2005; Buettner et al., 2008; Evans and Rees, 2002; Jacobson, 2008; Thompson et al., 2003). It is well documented that myotoxicity is statin dose-dependent, but little is known regarding the precise mechanism of toxicity (Ballantyne et al., 2003; Huerta-Alardin et al., 2005).

Recently, we demonstrated a role for human Organic Anion Transporting Polypeptide (OATP) 2B1 and Multidrug Resistance Associated Proteins (MRP) 1, MRP4 and MRP5 in the uptake and efflux transport as well as toxicity of statins in skeletal muscle cells *in vitro* (Knauer et al., 2010). However, there is a lack of *in vivo* studies examining statin transporters within skeletal muscle and their influence on myotoxic side effects of statins. We have proposed that the interplay between statin uptake versus efflux transporters modulates skeletal muscle statin exposure and toxicity. Inhibition of specific efflux transport mechanisms controlling intracellular skeletal muscle concentrations of statins could lead to local accumulation of drug and increased risk for toxicity. Studies in the guinea pig and rat suggest that statins do not accumulate considerably into skeletal muscle as evidenced by a low ratio of muscle to plasma drug levels of < 1 (Madsen et al., 2008; Nezasa et al., 2002; Sidaway et al., 2009). This indicates that efflux mechanisms present in skeletal muscle may be acting to prevent drug accumulation. Another study in rats showed that co-administration of an Mrp1 inhibitor, probenecid, promoted rosuvastatin myotoxicity (Dorajoo et al., 2008). However, this study did not

measure plasma or skeletal muscle rosuvastatin concentrations and failed to demonstrate rosuvastatin transport by rMrp1. Hence, it is unclear if the rosuvastatin skeletal muscle toxicity was due to changes in the systemic exposure or myofibre levels of rosuvastatin. In this report, we have investigated the *in vivo* role the efflux transporters in regulating the tissue distribution of rosuvastatin in a mouse model of Mrp1 deficiency. Rosuvastatin was chosen for this study because it is among the most prescribed statins in Canada, it is said to undergo minimal metabolism, which could complicate pharmacokinetic analysis, and it is highly dependent on transporters for systemic clearance (Kitamura et al., 2008).

In this study, we demonstrate that rosuvastatin is indeed a transport substrate for mouse Mrp1. The distribution of rosuvastatin in $Mrp1^{-/-}$ mice was similar to wild-type animals. However, $Mrp1^{-/-}$ animals had a significantly higher liver to plasma ratio of rosuvastatin compared to wild-type animals. Importantly, we observed a lack of difference in skeletal muscle rosuvastatin distribution between $Mrp1^{-/-}$ and wild-type mice. Gene expression analysis demonstrated elevated levels of Mrp2, Mrp4 and Mrp5 in KO mouse tissues, which normally express high levels of Mrp1. Tissue distribution of rosuvastatin was not different in tissues of $Mrp1^{-/-}$ animals despite that rosuvastatin is an Mrp1 substrate. These findings highlight the interplay between multiple redundant transporters in the tissue pharmacokinetics of rosuvastatin.

Methods

Reagents

[^{3}H] rosuvastatin (5 mCi/mmol, 99% radiochemical purity) was purchased from American Radiolabeled Chemicals (St. Louis, MO). [^{3}H] rosuvastatin (79 Ci/mmol, 97.1% radiochemical purity) and unlabeled rosuvastatin were also kindly provided by Dr. Yi Wang (AstraZeneca, Wilmington, DE). Unlabeled rosuvastatin was obtained from Toronto Research Chemicals (North York, ON). All other chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

Cloning of Mouse Mrp1

Expression plasmids for rOatp1b2 and MRP1 are described elsewhere (Knauer et al., 2010; Tirona et al., 2003). Mouse Mrp1 (mMrp1) cDNA was obtained by PCR, using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN), from a cDNA library of mouse skeletal muscle. mMrp1 cDNA was amplified in two parts from skeletal muscle with the following primers: 5'-AGCATGGCGCTGCGCAGCTTCTGCAGCG-3' and 5'-GGTTCACACCCTTCTCACCGATCTCTGTGCGGTC -3'; 5'-GCTCCGTGGCCTACGTGCCCCAGCAGG-3' and 5'-CATTGGATATGCCAGAGACCAGCTCACACCAAGCC -3'. Full-length mMrp1 was assembled from the two parts by ligation at the *Spf* I site. mMrp1 was sequence verified and cloned into pcDNA3.1/V5-His-TOPO vector (Invitrogen).

Transport Experiments

HeLa cells (human cervical cancer cell line) were grown in 12-well plates (approximately 0.8×10^6 cells/well) and infected with vaccinia (vtf-7) at a multiplicity of infection of 10 plaque-forming units/cell in serum-free Opti-MEM I medium (Invitrogen) and allowed to

absorb for 30 minutes. Cells were transfected with 1 μg of total transporter cDNA, in a 1:4 ratio of uptake to efflux, or parental plasmid lacking insert as control using Lipofectin (Invitrogen) and incubated at 37°C for 16h. Transport was evaluated using labeled substrate as previously reported (Cvetkovic et al., 1999). Drug accumulation was determined at selected time intervals by washing cells three times with ice-cold PBS followed by lysis with 1% sodium dodecylsulfate. Retained cellular radioactivity was quantified by liquid scintillation spectrometry.

Animals

Animals were housed in a temperature-controlled environment with a 12h light/dark cycle and received a standard diet and water ad libitum. Male Mrp1$^{-/-}$ (FVB.129P2-*Abcc1a*^{tm1Bor} N12) (Taconic, Hudson, NY) and wild-type FVB mice were between 10 and 16 weeks of age were used for experiments. Genotype was confirmed PCR using the following primers: 5'-TGCTGGCTGAGATGGACAAG-3' and 5'-CGGTCTAGCAGCTCCTGATA-3' (Schuetz et al., 2001) and protein expression by Western blot using MRPr1 antibody (Alexis Biochemicals) (Knauer et al., 2010). The study protocol was approved by the Animal Use Subcommittee of the University of Western Ontario, London, Canada.

Rosuvastatin Tissue Distribution

Male wild type FVB and Mrp1$^{-/-}$ mice were dosed with 1 mg/kg rosuvastatin with 2 μCi [^{3}H] rosuvastatin by tail vein injection in in saline with <2% DMSO. After 6 hrs animals were euthanized with isoflurane; blood was collected by cardiac puncture and various tissues were excised, rinsed in PBS, blotted and weighed. Blood was transferred to a tube for centrifugation at 12,000 g for 5 min to obtain plasma. Tissues were homogenized in water with 4% BSA in a ratio of 1:10 (w:v). Rosuvastatin concentration in plasma and tissue was quantified by liquid scintillation spectrometry. All plasma and tissue samples were stored at -20°C until analysis.

Gene Expression Analysis

The mRNA expression of Oatp2b1, Mrp2, Mrp4, and Mrp5 were measured by SYBR green quantitative real-time PCR with an ABI Prism 7700 sequence detection system (Applied Biosystems). Total RNA was extracted from various tissues using Trizol reagent (Invitrogen) and RNA quality and quantity was determined using Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA). cDNA was synthesized using Multiscribe reverse transcriptase (Applied Biosystems) according to manufacturer's instruction and 30 ng of cDNA was used in each qPCR reaction. The sequences of primers used for quantitative PCR are listed in Table D1. The amount of the transporter was normalized to 18S-rRNA and relative expression was determined using the $\Delta\Delta C_T$ method.

Statistical Analysis

Statistical differences between groups were determined by unpaired Student's T-test or 1-way ANOVA or 2-way ANOVA using Bonferroni's multiple comparison test, as appropriate (GraphPad Software Inc, San Diego, CA). A *P* value of <0.05 was considered statistically significant.

Table D1. Primer Sequences for qPCR

Primer		Sequence
mOatp2b1 Fw	Sense	5′-CTTCATCTCAGAACCATACC-3′
mOatp2b1 Rv	Antisense	5′-ACTGGAACAGCTGCCATTG-3′
mMrp2 Fw	Sense	5′-GTCATCACTATCGCACACAG-3′
mMrp2 Rv	Antisense	5′-TTCTACAGGGTGGTTGAGAC-3′
mMrp4 Fw	Sense	5′-GGTTGGAATTGTGGGCAGAA-3′
mMrp4 Rv	Antisense	5′-TCGTCCGTGTGCTCATTGAA -3′
mMrp5 Fw	Sense	5′-GTCAAACTCCACCACCTGTC-3′
mMrp5 Rv	Antisense	5′-AGAGAGAACGCACATGAAGG-3′

Results

Rosuvastatin is a Substrate of mMrp1

Mouse Mrp1 is highly expressed in skeletal muscle like human MRP1, but it was not known whether mMRP1 transported statins. Hence, HeLa cells were double transfected with the uptake transporter, rOatp1b2, and human MRP1 or mouse Mrp1. In this system, modulation of cellular retention of rosuvastatin served as an indicator for efflux transport activity. Rosuvastatin retention in cells expressing rOatp1b2 together with MRP1, Mrp1 was lower (78 and 73% lower, respectively) than cells expressing rOatp1b2, alone (Fig. D1). This finding demonstrates that mouse Mrp1, like human MRP1, is capable of transporting rosuvastatin.

Rosuvastatin Tissue Distribution in Mrp1$^{-/-}$ Mice

MRP1 is thought to facilitate the *in vivo* export of rosuvastatin from human skeletal muscle. We have reported previously that MRP1 *in vitro* is responsible for the efflux of rosuvastatin from skeletal muscle and sensitization to myotoxicity (Knauer et al., 2010). Here, we investigated the tissue distribution of rosuvastatin into liver, kidney, small intestine, spleen, heart, brain, lung, testis, and skeletal muscle (quadriceps and gastrocnemius) after intravenous dosing in wild type and Mrp1$^{-/-}$ mice. Deletion of Mrp1 was confirmed by western blot and PCR analysis of genomic DNA (Fig. D2). The rosuvastatin tissue concentration and tissue to plasma ratio is reported in Table D2 for wild type and Mrp1$^{-/-}$ mice. Mrp1$^{-/-}$ mice had significantly elevated plasma levels of rosuvastatin compared to wild type mice. Also the rosuvastatin tissue concentration in Mrp1$^{-/-}$ mice was significantly higher in liver, testis and lungs. The tissue to plasma ratio of rosuvastatin in kidney, spleen, brain, heart, lung, testis and small intestine was similar in Mrp1$^{-/-}$ mice in comparison to wild-type animals. However, Mrp1$^{-/-}$ animals had a significantly higher liver to plasma ratio of rosuvastatin compared to wild-type animals. Importantly, we observed no difference in skeletal muscle concentration or tissue to plasma ratio for rosuvastatin between Mrp1$^{-/-}$ and wild-type mice. Interestingly, like skeletal muscle, tissues with high expression of Mrp1 such as brain, testis, and lung

(Leslie et al., 2005), also do not show a difference in the tissue to plasma drug concentration ratio between wild type and Mrp1$^{-/-}$ animals. These results indicate that Mrp1 does not appear to be significantly involved in the efflux of rosuvastatin from skeletal muscle or other tissues with high Mrp1 expression. However, the increase in rosuvastatin plasma levels in Mrp1$^{-/-}$ mice suggests that Mrp1 contributes to plasma clearance or volume of distribution of rosuvastatin.

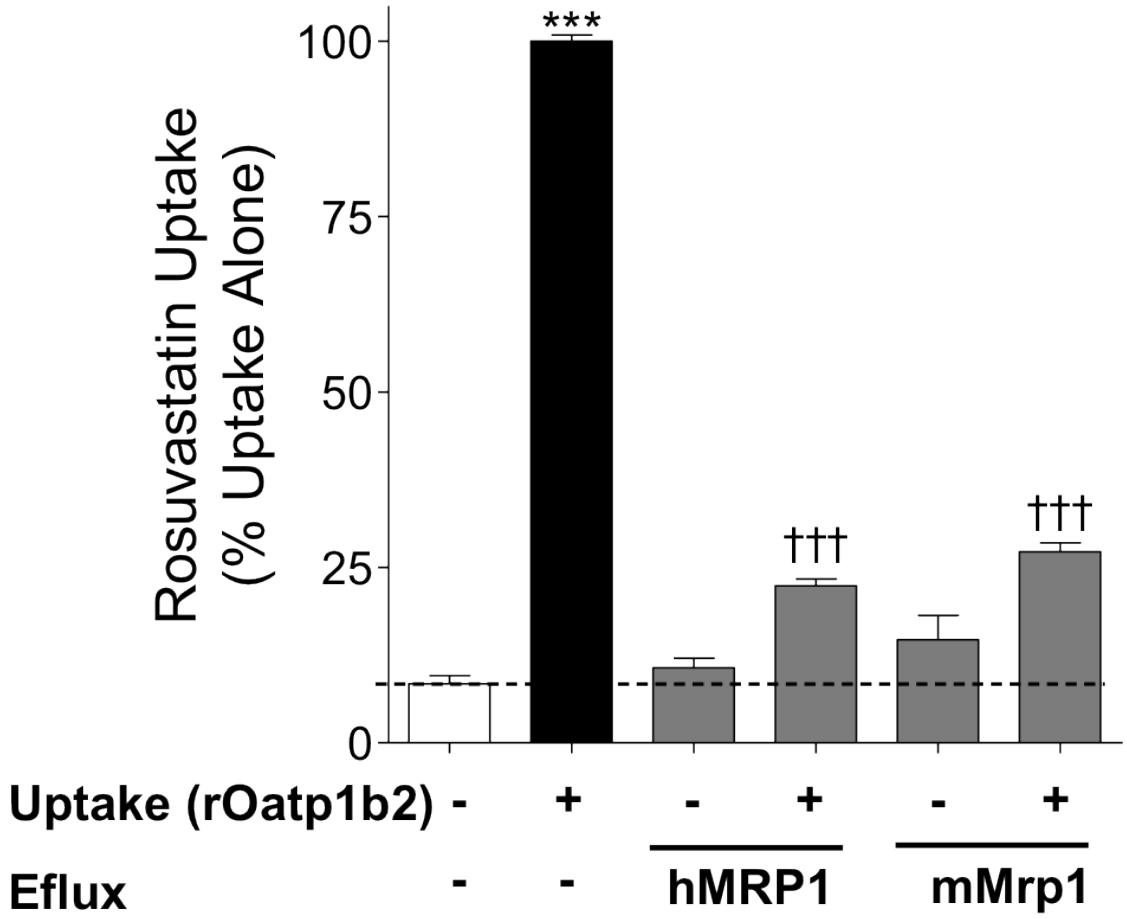


Figure D1. Intracellular accumulation of rosuvastatin in HeLa cells transiently transfected with rOatp1b2 and human MRP1 (hMRP1) or mouse (mMrp1). Results are presented as percent of rOatp1b2 mediated uptake ± SEM (n=3). ***p<0.001 compared to vector control and efflux transporter alone. †††p<0.001 compared with rOatp1b2 mediated uptake.

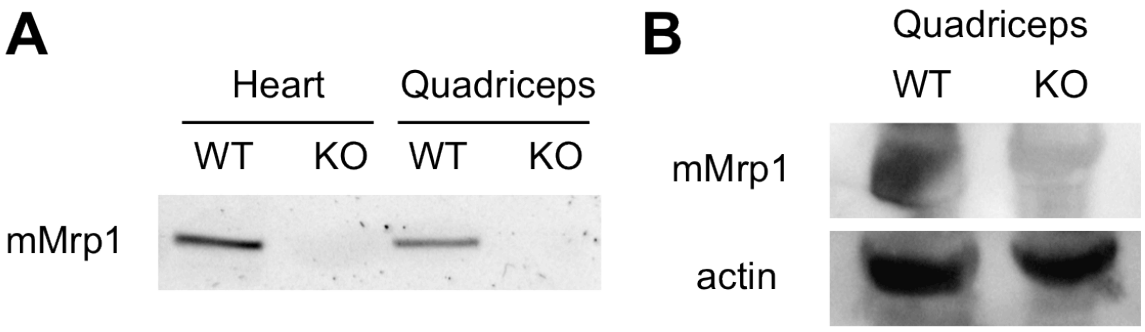


Figure D2. Confirmation of mMrp1 Knockout (KO) in mice by (A) PCR based genotyping and (B) Western blot analysis.

Table D2. Tissue Distribution of Rosuvastatin in Mrp1$^{-/-}$ Mice

	Rosuvastatin Concentration (ng/g tissue)			Rosuvastatin Tissue/Plasma Ratio		
	Wild Type (n=6)	Mrp1-/- (n=6)		Wild Type (n=6)	Mrp1-/- (n=6)	
Plasma (ng/mL)	1101.28 ± 62.99	1232.45 ± 104.21	*			
Liver	12919.80 ± 2154.49	18725.21 ± 2513.30	*	11.81 ± 2.37	15.35 ± 2.95	*
Kidney	1095.73 ± 205.59	1311.92 ± 125.30		1.00 ± 0.22	1.07 ± 0.17	
Spleen	609.49 ± 61.65	749.14 ± 172.31		0.56 ± 0.08	0.61 ± 0.17	
Testis	657.56 ± 83.56	804.48 ± 88.26	*	0.60 ± 0.05	0.66 ± 0.11	
Small Int.	4939.85 ± 2140.91	4313.05 ± 1680.40		4.57 ± 2.30	3.60 ± 1.65	
Brain	598.06 ± 60.04	623.88 ± 71.42		0.55 ± 0.07	0.51 ± 0.08	
Heart	881.39 ± 132.18	1140.09 ± 313.78		0.81 ± 0.15	0.94 ± 0.30	
Lungs	856.51 ± 89.50	995.68 ± 97.84	*	0.78 ± 0.10	0.81 ± 0.11	
Quadriceps	616.36 ± 77.72	840.35 ± 411.49		0.56 ± 0.10	0.70 ± 0.38	
Gastrocnemius	578.65 ± 52.99	678.76 ± 118.43		0.53 ± 0.05	0.56 ± 0.11	

*$p < 0.05$ compared to wild type mice.

Expression of Other Transporters in Mrp1$^{-/-}$ Mice

With the exception of liver, we did not find differences in the tissue to plasma concentration ratio for rosuvastatin. Hence, we examined the expression of other transporters in tissues that may compensate for deletion of Mrp1. We chose to examine the relative expression of uptake transporter Oatp2b1 and three other efflux transporters Mrp2, Mrp4 and Mrp5. Expression of Oatp2b1 and Mrp2 were lower in the liver and small intestine of Mrp1$^{-/-}$ mice compared to wild type mice (Fig. D3). No significant changes were seen in the expression of Oatp2b1 or Mrp2 in other tissues. Expression of Mrp4 and Mrp5 was higher in the skeletal muscle (Mrp4 – quadriceps; Mrp5 – gastrocnemius) and lung of Mrp1$^{-/-}$ mice compared to wild type mice (Fig. D3). Expression of Mrp4 and Mrp5 were slightly higher in brain and testis of Mrp1$^{-/-}$ mice, but this difference did not reach significance. No significant changes were seen in the expression of Mrp4 or Mrp5 in the other tissues examined.

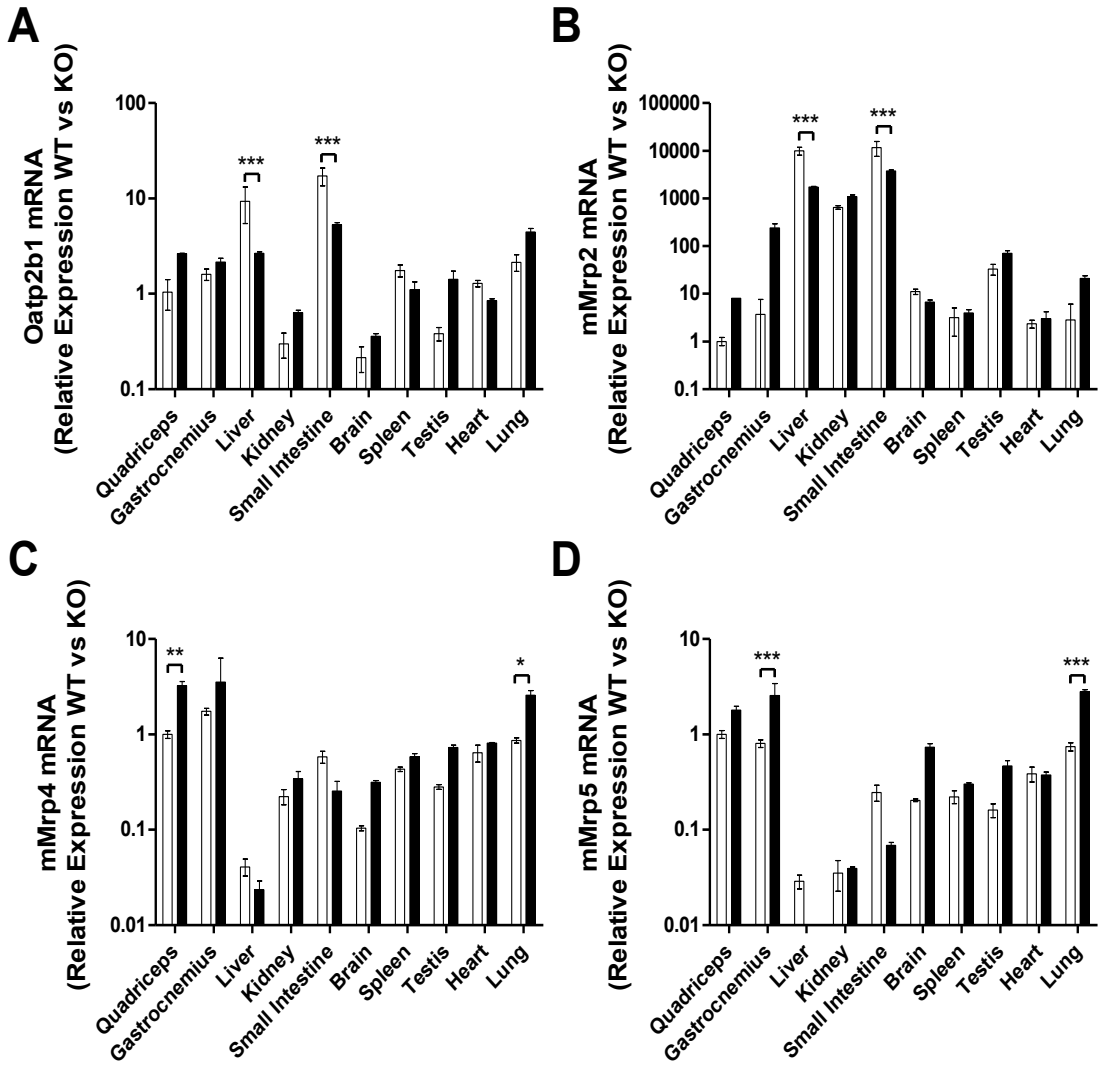


Figure D3. Relative expression of statin uptake and efflux transporters in Wild-type (white bars) and mMrp1 KO (black bars) mice. Results are normalized to WT Quadriceps expression (set to 1). *p<0.05, **p<0.01, ***p<0.001

Discussion

Here we report that rosuvastatin is a substrate of mouse Mrp1 similar to our previous findings with human MRP1. The tissue distribution of rosuvastatin in kidney, spleen, brain, heart, and small intestine was similar in $Mrp1^{-/-}$ mice in comparison to wild-type animals. The tissue rosuvastatin levels were elevated in the liver, testis and lungs of $Mrp1^{-/-}$ mice. However, $Mrp1^{-/-}$ animals had a significantly higher rosuvastatin plasma concentration and only liver to plasma concentration ratio of rosuvastatin was significantly higher. Importantly, we observed a lack of difference in skeletal muscle rosuvastatin distribution between $Mrp1^{-/-}$ and wild-type mice. Gene expression analysis demonstrated elevated levels of Mrp2, Mrp4 or Mrp5 in KO mouse tissues that normally express high levels of Mrp1.

Despite being a Mrp1 substrate, the tissue distribution of rosuvastatin was not different in tissues of $Mrp1^{-/-}$ animals. However, we did see an increase in the rosuvastatin plasma levels in $Mrp1^{-/-}$ animals. This suggests that Mrp1 may have an effect on the clearance of rosuvastatin. Our result suggests that deletion of Mrp1 would increase the systemic exposure of rosuvastatin and potentially the toxic side effect. Dorajoo and colleagues showed that inhibition of Mrp1 by probenecid in rats lead to muscle toxicity (Dorajoo et al., 2008). Unfortunately they did not report measuring the plasma or muscle concentrations of rosuvastatin. So it remains unclear if Mrp1 inhibition results in an increase in systemic or local muscle exposure. More studies are needed to examine the potential effect of Mrp1 has on rosuvastatin clearance.

The Mrp1 is expressed at low levels in the liver; however, the rosuvastatin liver to plasma ratio is elevated in the $Mrp1^{-/-}$ mice. This could be attributed to reduced biliary excretion by Mrp2, whose expression is lower in $Mrp1^{-/-}$ mice, or other bile canalicular efflux transporters like breast cancer resistance protein (Bcrp) and P-glycoprotein (P-gp) (Kitamura et al., 2008). Another explanation for increased liver to plasma ratio would be an increased hepatic uptake of rosuvastatin by Oatp1a1, Oatp1a5, Oatp1b2, Oatp2b1 or sodium-taurocholate co-transporting polypeptide (Ntcp). We did not examine the expression of Oatp1a/1b or Ntcp, but we observed a downregulation in Oatp2b1

expression in Mrp1$^{-/-}$ liver. This downregulation of Oatp2b1 is not consistent with increased hepatic uptake. Clearly, more analysis is required to better understand the mechanisms involved in the increased hepatic accumulation of rosuvastatin in Mrp1$^{-/-}$ mice.

Mrp1 is highly expressed in brain, testis, lung, and skeletal muscle, but the rosuvastatin tissue distribution as monitored by tissue/plasma concentration ratio, was not affected by Mrp1 gene deletion. Gene expression analysis of other Mrps revealed that Mrp2, Mrp4 and/or Mrp5 are upregulated in these tissues. This suggests that there is functional compensation from other statin transporters in regulating statin accumulation in these tissues.

There are a number of limitations to this study that impact on the interpretation of the results. First, we used scintillation spectroscopy to measure statin levels in plasma and tissues under the assumption that rosuvastatin is largely unmetabolized. Recent studies in our laboratory and elsewhere have demonstrated a significant proportion of radiolabel cannot be attributed to rosuvastatin itself and that metabolism is an unappreciated mechanism of elimination in mouse and perhaps humans (Kitamura et al., 2008; Nezasa et al., 2002). Specific analytical methods as that described in Chapter 5 are required to better understand the role of drug transporters in the tissue distribution of rosuvastatin. Second, we measured plasma and tissue rosuvastatin levels at a single time point (6 hours). This sparse sampling experimental design precludes the ability to formally estimate key pharmacokinetic parameters such as volume of distribution and clearance. Lastly, the expression of the entire complement of statin transporters was not examined in mouse tissues further preventing a broader understanding of the influence of other statin transporters in the tissue distribution and pharmacokinetics of rosuvastatin in the Mrp1$^{-/-}$ knockout model.

In conclusion, knockout of Mrp1 in mice does not appear to affect the tissue distribution of rosuvastatin into skeletal muscle. Upregulation of Mrp4 and Mrp5 in muscle may compensate for the loss of Mrp1 in regulating rosuvastatin tissue distribution. A role for Mrp1 in the skeletal muscle toxicity of statins remains to be determined.

References

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University of Western Ontario
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Government of Ontario
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Honours and Awards:

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Publications:

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