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Graduate Program in Pharmacology and Toxicology
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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In Vitro Functional Analysis Of Novel Single Nucleotide Polymorphisms In
OATP1B1 And Potential Clinical Relevance

(Thesis format: Monograph)

by

Zhiyuan Peter Yin

Graduate Program in Physiology and Pharmacology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
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London, Ontario, Canada

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Abstract

Statin-induced myopathy is a common adverse reaction of statin therapy. Patients with elevated plasma concentration of statins are thought to be at greater risk for myopathy. Statins are transported from the blood to hepatocytes via organic anion transporting polypeptide 1B1 (OATP1B1). Although single nucleotide polymorphisms (SNPs) in OATP1B1 have been associated with increased statin concentrations, we hypothesize that there may be other SNPs in OATP1B1 that can also contribute to reduced transport activity and increased plasma statin concentrations. OATP1B1 cDNA packaged in pEF6/V5-His TOPO was used as template, and 6 SNPs — c.298G>A, c.419C>T, c.463C>A (*4), c.1007C>G, c.1463G>C (*9), and c.1738C>T — were introduced separately and expressed in adenovirus. 3 SNPs abolished transport activity, 1 SNP decreased transport, 1 increased transport, and 1 did not affect transport activity. Our data support the hypothesis that there are additional loss of function SNPs in OATP1B1.

Keywords

Pharmacogenomics, drug transporters, OATP, statins, statin-induced myopathy, single nucleotide polymorphism, adverse drug reactions, personalized medicine

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List of Abbreviations

ABC	ATP-binding cassette
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BCRP	Breast cancer resistance protein
CCK-8	Cholecystokinin-8
Ct	Cycle threshold
CYP	Cytochrome P450
dbSNP	NCBI SNP database
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfide
E ₁ S	Estrone-3-sulfate
E ₂ 17βG	Estradiol-17-β-D-glucuronide
EDTA	Ethylenediaminetetraacetic acid
FXR	Farnesoid X receptor
HEK	Human embryonic kidney
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HNF	Hepatocyte nuclear factor
HRP	Horseradish peroxidase

KHB	Krebs Henseleit Bicarbonate
LB	Lysogeny broth
LDL	Low density lipoprotein
LXR	Liver receptor
MAF	Minor allele frequency
MDR	Multidrug resistance protein
MOI	Multiplicity of infection
MPP ⁺	1-Methyl-4-phenylpyridinium
MRP	Multidrug resistance-associated protein
MTT	Dimethylthiazolyl diphenyltetrazolium bromide
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
P-gp	P-glycoprotein
qPCR	Quantitative real-time PCR
RFLP	Restriction fragment length polymorphism
RIPA	Radioimmunoprecipitation assay
RPM	Revolutions per minute

SD	Standard deviation
SDS	Sodium dodecyl sulfate
SLC	Solute carrier
SNP	Single nucleotide polymorphism
TBS	Tris buffered saline
TEA	Tetraethylammonium
TKI	Tyrosine kinase inhibitor

Chapter 1

1 « Literature Review »

Human drug transporters are an important class of proteins that can affect pharmacokinetics and clinical outcomes. Additionally, single nucleotide polymorphisms (SNPs) in these transporters have emerged as a possible predictor of plasma drug levels, and can be used to maximize drug efficacy and minimize toxicity. This chapter seeks to introduce the classes of influx and efflux drug transporters, specifically the organic anion transporting polypeptides (OATPs) family, and outline some important SNPs that have been discovered in drug transporters. Finally, the chapter will discuss why this research may have significant clinical contributions.

1.1 Nomenclature of human drug transporters

Membrane transporters are membrane-bound proteins that facilitate the movement of compounds across a physiological barrier. Proper movement of compounds is essential for electrochemical and hormonal homeostasis. Small, non-polar molecules are able to pass through cell membranes by simple diffusion, but the movement of large polar molecules as well the movement of molecules against their concentration gradient require the aid of membrane transporters (1). Approximately 10% of all human genes are transporter-related, coding for either the transporter themselves or accessory proteins like subunits or regulatory proteins. Transporters are extremely diverse in their location and substrate specificity. Most carry important endogenous molecules, and some can transport exogenous compounds and drugs in addition to their endogenous ligands (2). These transporters with exogenous substrates are called drug transporters and can play major roles in a drug's absorption, distribution, and elimination. Therefore, full understanding of these transporters can greatly improve efficiency of drug discovery and development by being able to predict and control the pharmacokinetic profile of the drug and its target site of action (3).

Drug transporters are divided into two categories, the solute carrier (*SLC*) superfamily and the ATP-binding cassette (*ABC*) superfamily. The *ABC* superfamily consists of efflux

transporters that all have an ATP catalytic domain to generate energy to move compounds against their concentration gradient. Families within the *ABC* superfamily are given another letter (*ABCB*) based on sequence homology, and further division of individual members are given a final number (*ABCB1*) based on order of discovery (4). The first prominent drug transporter discovered was *ABCB1* in 1976, also known as multi-drug resistance protein 1 (MDR1), or P-glycoprotein (P-gp). It was located on the membrane of mutant colchicine-resistant Chinese hamster ovary cells, and was found to have direct correlation with degree of drug-resistance (5). The same protein was confirmed to exist in humans and have since become the most extensively studied transporter. In 1992, another transporter belonging to the *ABC* superfamily was discovered to convey drug resistance in cells that did not express P-gp. This transporter was later named multidrug resistance-associated protein 1 (MRP1), and given the gene name *ABCC1* (6). Throughout the 1990s and 2000s, advances in technology, methodology, and interest have catapulted the number of transporters discovered. Today there is a total of 48 known members in the *ABC* superfamily (4).

Contrary to the *ABC* superfamily, the *SLC* superfamily consists of uptake transporters that do not use energy from ATP to mediate transport. These transporters include passive transporters and ion exchangers/co-transporters. There are 52 members in the *SLC* superfamily named *SLC1* to *SLC52*, followed by the letter “A” as a space divider, then a numeral for the individual members of the family (*SLC10A1*). Exceptions to this nomenclature include *SLC35* which uses other letters in addition to “A” to represent different subfamilies (*SLC35A*, *SLC35B*), and the *SLC21* family, which has been renamed as *SLCO* to accommodate for easy organization and identification of the large number of members within the family (7). There is a total of 395 individual members within the *SLC* superfamily, which is far more expansive than the *ABC* superfamily (8). Over the last 2 decades, the discovery of expression cloning have allowed identification, and thus further research on *SLC* transporters (9). A comprehensive list of all *SLC* transporters and their clinical importance have all been reviewed by experts in the field (8). These *SLC* and *ABC* transporters are expressed in all major organs of the body, including the liver, kidneys, intestines, and blood-brain-barrier. Transporters that are critically important in pharmacokinetics are located mainly at the sites of drug elimination, the liver and kidneys

(2). One family of great importance is the *SLC21* family, renamed to *SLCO* in 2003 due to the rapid evolution of research on these transporters. This family encodes the protein family organic anion transporting polypeptides (OATP), which have been extensively studied because of their clinical and pharmacokinetic importance.

1.2 OATP

OATPs are a superfamily of membrane transporters that facilitate sodium-independent uptake of a broad number of endogenous and exogenous amphipathic molecules. Today, more than 300 members of the OATP family have been identified in several organisms including humans, rats, mice, monkeys, pigs, and dogs (2). However, no OATP homologues have been identified in bacteria or yeast. In humans, 11 members have been identified and categorized into 6 families (OATP1-OATP6) based on more than 40% sequence homology. Additional letters are added to further subdivide transporters that share more than 60% sequence homology (OATP1A). Finally, another number is added to represent the chronological order of discovery (OATP1A1) (2). The first OATP to be discovered was rat OATP1A1 in 1994 (10), and the first human OATP discovered was OATP1A2 in 1995 (11). OATPs all have 12 transmembrane domains with intracellular termini, but differ in their substrate specificity and tissue distribution (2). Below is an overview of all human members of the OATP family.

1.2.1 OATP1A2

OATP1A2 was cloned in 1995 and was the first human OATP discovered. It was found to have 67% identical sequence compared to the previously characterized rat OATP1A1. OATP1A2 was found to be expressed in the brain, lung, liver, kidney, intestine, and testes, with particular strong expression in the brain. This suggests an importance in mediating transport of drugs and hormones across the blood-brain barrier. Substrates for OATP1A2 include endogenous bile acids and steroid hormones as well as a broad spectrum of drugs (2, 11).

1.2.2 OATP1B1

OATP1B1 is a member of the OATP superfamily that is exclusively located on the basolateral membrane of hepatocytes. It was the second human OATP to be characterized and share 44% sequence identity with OATP1A2. It facilitates the hepatocellular uptake of a variety of endogenous molecules to be metabolized in the liver (12). Complete deficiency of OATP1B1 and a close family member OATP1B3 manifests as Rotor syndrome, an autosomal recessive disorder characterized by hyperbilirubinemia and jaundice during childhood, highlighting the significance of OATP1B1 in bilirubin transport. Patients with Rotor syndrome, however, live normal lives with no clinical complications (13). OATP1B1 is also the transporter for many xenobiotics, most importantly statins (2). The role of OATP1B1 as a drug transporter has been extensively studied, and the transporter's function has been associated with variability in patient sensitivity to these substrates. Changes in the function of the transporter can lead to unpredicted outcomes in drug therapy, and even cause severe adverse events (3). Due to this, OATP1B1 has been a focus in the field of personalized medicine to predict optimal dosing for drugs that are substrates.

1.2.3 OATP1B3

OATP1B3 was cloned in 2000 and shares 87% sequence identity with OATP1B1 and 61% with OATP1A2. Similar to OATP1B1, OATP1B3 is expressed highly, if not exclusively in the liver (14). Due to a high degree of similarity between OATP1B1 and OATP1B3, it is not surprising that they share many common substrates (2). However, there are also substrates that are exclusively transported by one and not the other. Cholecystokinin-8 (CCK-8) is a substrate that is highly transported by OATP1B3 but not OATP1B1. However, a study showed that by mutating 3 amino acid residues (A45G/L545S/T615I) in OATP1B1 to the corresponding amino acids in OATP1B3, transport of CCK-8 was attained in newly mutated OATP1B1. Conversely, mutating these 3 residues in OATP1B3 to the corresponding residues in OATP1B1 resulted in loss of CCK-8 transport (15). This suggests that there are specific amino acids that dictate recognition or transport of a particular substrate. The mutation of these 3 residues did not alter the transport of any other substrates, suggesting that different substrates utilize different sites as recognition targets (15).

1.2.4 OATP1C1

OATP1C1 was the last member of the OATP1 family discovered, and was found to share 48% sequence identity with OATP1A2, 1B1 and 1B3. OATP1C1 highly and selective transports thyroid hormones, specifically thyroxine, and is expressed bilocally in the brain and testes, suggesting an importance of this transporter in the uptake and distribution of iodothyronines in these organs (16). Compared to other members of the OATP1 family, OATP1C1 has a much narrower list of endogenous and exogenous substrates, indicating that it serves a specific function in the areas expressed rather than being a multipurpose transporter (16). Genomic organization of OATP1C1, as well as the 3 other members of the OATP1 family, are all clustered on chromosome 12p12. The sequence identity and genomic organization of the 4 OATP1 members suggest that they likely evolved by gene duplication from a common ancestor, and diverged to be expressed in different tissue to serve different functions (16).

1.2.5 OATP1A2

OATP2A1 was cloned in 1996 from human kidney cDNA library, and was found to be a high affinity prostaglandin transporter. Unlike members of the OATP1 family which are highly expressed in certain tissue, OATP2A1 is ubiquitously expressed in almost every tissue type (17). Recent studies in OATP2A1 revealed that it is a high affinity transporter for latanoprost, the most commonly prescribe prostaglandin for the treatment of glaucoma. Therefore, the function of OATP2A1 is important for the bioavailability of latanoprost in antiglaucoma treatment (18). In addition to prostaglandins, OATP2A1 shares a few common substrates with members of OATP1 family, but the true physiological significance of OATP2A1 in areas other than the eye remain unknown (2).

1.2.6 OATP2B1, OATP3A1, OATP4A1

OATP2B1, 3A1, and 4A1 were discovered simultaneously in 2000. OATP2B1 has broad tissue distribution with the highest expression in liver (19). OATP2B1, along with OATP1B1 and OATP1B3 are the 3 known OATPs that mediate hepatocellular uptake of substrates (1). Contrary to 1B1 and 1B3, OATP2B1 has limited substrate specificity, but includes drugs in the statin class that are also substrates for OATP1B1 (2). OATP2B1 was

shown to be expressed in muscle, and proposed to possibly mediate uptake of statins to the muscle to cause the common adverse effect of statin-induced myopathy (20). In the intestines, OATP2B1 was shown to mediate pH-dependent transport of pravastatin and fexofenadine, which is a feature not seen with other OATPs (21).

OATP3A1 and 4A1 have not seen as much research focus as OATP2B1 since their discovery. They are both ubiquitously expressed and share a similar list of substrates that include estrone-3-sulfate (E₁S) and prostaglandins (19). The physiological as well as clinical role of these transporters are currently unknown as research interest has been focused on other OATPs.

1.2.7 OATP4C1

OATP4C1 was characterized in 2004 and was found to be predominantly expressed in kidney. Only a few substrates for OATP4C1 have been identified and include digoxin, ouabain, and E₁S (22). Interestingly, inhibitors that inhibited E₁S uptake did not inhibit digoxin uptake, suggesting that E₁S and digoxin utilize different recognition sites for their transport (23). This is consistent with the conclusions made in the OATP1B1-OATP1B3 mutagenesis study previously discussed.

1.2.8 OATP5A1, OATP6A1, and OATPs in cancer

OATP5A1 and 6A1 are the most recent and poorly understood members of the OATP family. OATP5A1 was shown to be expressed predominantly in breast tissue (24), and OATP6A1 predominantly in testes (25). Endogenous substrates and physiological function of these transporters are also unknown. However, they have been discussed in their potential role as targets for cancer therapy, along with many other OATPs. In addition to the physiological expression of OATPs in various tissue, all 11 human OATPs have been shown to be expressed in various tumours (26). Many anti-tumour agents used today have also been shown to be substrates for various OATPs. This makes OATPs an attractive target for selective uptake of anti-tumour drugs into tumour cells. The expression of OATPs in tumour cells and the regulation of hormones by various OATPs, together with their involvement in hepatocellular uptake of several anti-tumour drugs, makes full understanding of all OATPs an essential task for outlining intricate pathways for

chemotherapeutic drug targeting (27). This has been an emerging field of research and could potentially result in significant progress in tumour targeting and treatment.

1.2.9 Factors affecting OATP regulation and function

Regulation of OATP expression have been shown to occur at the transcriptional and post-translational levels. Several nuclear receptors such as hepatocyte nuclear factor 4 α (HNF4 α), liver receptor α (LXR α), and farnesoid X receptor (FXR) have been shown to regulate the liver-specific OATP1B1 (28). Additionally, HNF1 α and FXR have both been shown to also regulate OATP1B3 (29). These nuclear receptors, when activated, bind to response elements located in the 5' untranslated region to regulate transcription. Ligands that activate these nuclear receptors also in turn enhance OATP expression and overall transport activity (2).

Post-translational regulation is also observed in OATPs. Glycosylation is a major post-translational modification in most membrane proteins and was proposed to regulate membrane targeting and protein stability. N-glycosylation sites on extracellular loops 2 and 5 are common features among OATPs (2). A study found that 3 asparagine residues at positions 134, 516, and 503 in OATP1B1 are glycosylated, and when simultaneously mutated to glutamine, glycosylation was prevented which resulted in significantly decreased OATP1B1 expression and transport activity (30).

The classes of drug transporters previously discussed have significant physiological contributions, but the true interest lies in their ability to transport and facilitate distribution of drugs. Due to the transporters' involvement in drug absorption, distribution, and elimination, interindividual variability in transporter function has been shown to be one of the causes for the variability observed in drug response (31). Transporters may be induced, inhibited, or have altered function due to genetic differences, and any of these changes can lead to unpredicted outcomes in drug therapy. The field of inherited differences in drug response, pharmacogenomics, has seen significant growth over the last decade since the publication of the human genome project. It is now widely accepted that drug metabolizing enzymes and drug transporters can possess genetic differences that lead to changes in protein function, and ultimately a difference in bioavailability and response to drugs (32).

1.3 Single nucleotide polymorphism

The most common form genetic variation that can affect protein function are SNPs. SNPs are defined as a change in one nucleotide in the gene that is observed in more than 1% of the population, and can be synonymous or non-synonymous. Any protein involved in the pathway of a drug is subject to polymorphisms, and thus may be a factor in determining altered drug response (31). Traditional approaches to drug therapy have been trial-and-error and one-size-fits-all. With the trial-and-error approach, an initial safe dose is given and titrated upwards until optimal treatment is obtained. This method is safe but requires time between initiation of treatment and reception of efficacious results. This length of time is crucial and may not be available with certain conditions or individuals. The one-size-fits-all approach treats the population as an average individual, and an average safe dose determined in large scale clinical trials are given to everyone in the population (33). However, interindividual variability is always observed with individuals that respond poorly or do not respond to the treatment. Due to growing understanding of the human genome and interest in genetic differences in drug metabolizing enzymes and transporters, personalized medicine has emerged as a new approach to drug therapy in hopes to pre-determine optimal choice of therapy and dose for each individual to minimize adverse reactions and maximize therapeutic efficacy (31).

1.4 SNPs in drug metabolizing enzymes and drug transporters

Initial studies in pharmacogenomics primarily focused on drug metabolizing enzyme cytochrome P450s (CYPs). There have been more than 50 CYP enzymes characterized, but a few of them, primarily CYP1A2, 3A4, 3A5, 2C9, and 2C19, metabolize more than 90% of all drugs on the market today. Thus it is not surprising that tremendous research has been placed on these enzymes and the genetic variations that exist in them (33). Many SNPs as well as other types of genetic variation such as copy number mutations were characterized, and the effects of these genetic differences on CYP enzyme function were determined by plasma drug or metabolite concentrations as well as phenotypic observations in drug response. For many of these enzymes, genetic variations have been determined to categorize individuals as poor, intermediate, extensive, or ultra-rapid metabolizers

depending on a SNP or a combination of SNPs. With this knowledge, drugs that are substrates for a specific CYP enzyme can be dosed according to an individual's CYP genotype to enhance clinical outcomes (33).

Due to complex interplay of proteins in drug absorption, distribution, metabolism, and elimination, knowledge of SNPs in CYP enzymes have been beneficial, but not absolute in guiding drug choice and dosage. Drug transporters are also significantly involved the pharmacokinetic pathway, and rapid evolution of drug transporter research in the 1990s and 2000s led to increased interest in genetic variations in these transporters (3). Both the *ABC* superfamily and *SLC* superfamily have numerous members that contain SNPs that affect transport function *in vitro* as well as drug concentrations *in vivo*. Most clinically significant transporters are located in the liver and the kidney, since these are major sites of drug metabolism and elimination, and transport of drug in and out of these organs have great impact on drug bioavailability and response (34). Figure 1 shows the major transporters expressed in the liver and kidneys with known SNPs that affect plasma drug concentrations and subsequently drug response.

1.4.1 P-gp

P-glycoprotein (P-gp; MDR1; gene symbol *ABCB1*) was the first drug transporter to be discovered, thus it is not surprising that over 1000 SNPs have been characterized in the gene (35). P-gp is an efflux transporter and contributes to the elimination of toxic compounds and xenobiotics and is located at several major organ systems including the intestines, liver, kidney, and the blood-brain barrier. P-gp also has an incredibly broad substrate specificity, further demonstrating the importance of P-gp in pharmacokinetics. The most frequently studied SNP is *ABCB1* 3435C>T (rs1045642), a synonymous SNP located in exon 26 with high frequencies observed across all ethnicities (36). Several studies have examined the functional consequences of this SNP, including its effects on mRNA and protein levels and transport of several substrates. Initial studies found this SNP to be associated with decreased intestinal expression leading to significantly higher plasma levels of digoxin (37). However, contradictory results have since been shown and the exact outcome of C3435T *in vitro* remains unknown (36). Since several classes of drugs are substrates for P-gp, attempts have been made to correlate SNPs with therapeutic outcomes.

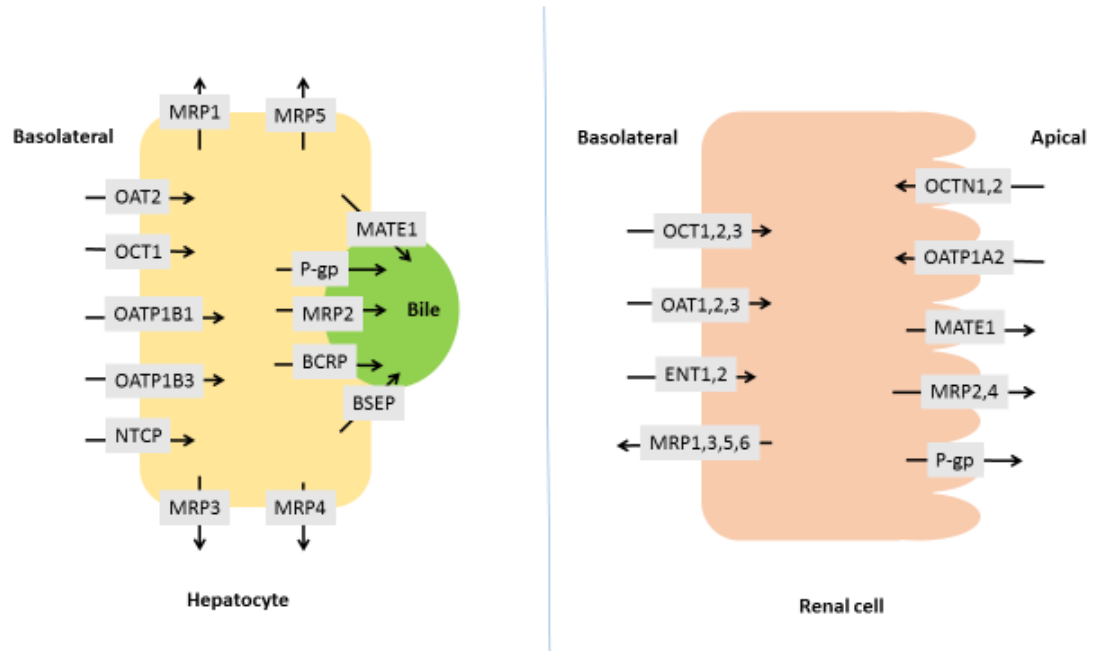


Figure 1. Human drug transporters with SNPs that may contribute to interindividual variability in pharmacokinetics.

A study looked at the treatment responsiveness of anticonvulsants in epilepsy and found the C3435T SNP to be associated with better drug response (38). This could possibly be due to lowered expression at the blood-brain barrier resulting in decreased drug excretion, but has not been confirmed by functional studies. Several studies later identified both similar and contradictory results, and meta-analyses summarized no significant association between any *ABCB1* SNPs and anticonvulsant treatment outcome (39).

Opioids are another major class of drugs that are substrates for P-gp. Multiple studies were in agreement with their results showing that patients who are homozygous variant for C3435T SNP required lower opioid dosage and had greater pain relief compared to wildtype individuals (40, 41).

The effect of *ABCB1* SNPs in anti-cancer therapy has also been a target of investigation. Studies involving patients with leukemia were again inconsistent with some studies showing a link between clinical outcomes and C3435T genotype and other studies showing no association. The studies that found an association were also contradictory, where some studies found 3435CC to have better outcomes and others found 3435TT to be beneficial (42, 43). The variability of results show that there is not a significant association between *ABCB1* SNPs and anti-leukemia treatment.

Although numerous studies have attempted to find significance in *ABCB1* SNPs, the overall picture is that there is no clear association between genotype and treatment outcome with P-gp substrate drugs. However P-gp continues to be of interest due to its broad substrate specificity and expression, and it has been suggested that SNPs may be more involved in mediating and altering drug-induction, inhibition, and drug-drug interaction at the transporter.

1.4.2 BCRP

Breast cancer resistance protein (BCRP; gene symbol *ABCG2*) is another prominent efflux transporter involved in chemoresistance. It was found to have broad expression including the intestines, bile canaliculi, mammary glands, and blood-brain barrier (44). BCRP expression is increased in the mammary glands during lactation, possibly facilitating the

secretion of vitamins and other nutrients into breast milk (45). Pharmacologically, BCRP also has a very broad range of drugs as substrates, most of which are anti-cancer drugs (35).

The most frequently studied SNPs in BCRP are 34G>A (rs2231137) and 421C>A (rs2231142), both with high minor allele frequencies (MAFs) of around 10% (35). These SNPs have had numerous *in vitro* and clinical studies done. One *in vitro* study investigating G34A and C421A found that they were both associated with decreased transport of indolocarbazoles mediated by decreased V_{max} , increased K_m , and decreased expression (46). Another study using E₁S as substrate found similar results with C421A, but no change in transport with G34A (47). Additional studies on these SNPs confirmed C421A as a loss-of-function polymorphism, but the effects of G34A are still inconclusive.

In vivo studies correlating BCRP genotype with plasma drug concentrations also focused on the two SNPs described. There have been a wide number of studies investigating anti-cancer drugs including camptothecins and tyrosine kinase inhibitors (TKIs). The camptothecins diflomotecan, topotecan, and irinotecan metabolite SN-38 are all known substrates for BCRP. Patients that are carriers for C421A SNP exhibited higher plasma concentrations of both diflomotecan and toptecan, but not irinotecan or SN-38 (48-50). This is possibly due to the more complex pharmacokinetic pathway that irinotecan undergoes. There were no association between camptothecin levels and G34A genotype. Studies involving tyrosine kinase inhibitors all found no association between G34A/C421A and AUC. However, two other SNPs, C-15622T and C1143T, emerged and were associated with increase severity of side effects following TKI therapy, suggesting prolonged system exposure to the drug (51).

Statins are another major class of drugs transported by BCRP. Higher plasma levels of rosuvastatin in carriers of C421A has been shown in multiple studies (52, 53), and it has also been observed that C421A associated with greater reduction in LDL-C following rosuvastatin therapy (54). Additionally, atorvastatin and fluvastatin plasma levels have also been correlated with C421A SNP, though not as significant as rosuvastatin (53). The increased plasma concentration and efficacy is likely due to decreased activity of the

transporter leading to increased absorption to the blood and liver. Other statins pitavastatin and pravastatin did not show any correlation with BCRP SNPs, and the reason for this lack of association is currently unknown and attributed somewhat to compensatory mechanisms (53, 55).

BCRP, like P-gp, has very broad tissue distribution and substrate specificity. The SNP C421A seemed to be the most significant SNP in terms of pharmacokinetics camptothecins and statins, but the effects of G34A were not as conclusive. The overall impact of BCRP SNPs on pharmacokinetics appeared to be minor and does not justify pharmacogenomics-guided dosing and selection. However, only a few SNPs have been studied in clinical settings, so there is definitely a large area of unknown in BCRP pharmacogenomics that have yet to be explored.

1.4.3 MRPs

Multidrug resistance-associated proteins (MRPs; gene family *ABCC*) are another class of highly polymorphic efflux drug transporters primarily located in the liver, kidneys, and intestines (35). MRP2 and 8 (*ABCC2*, *ABCC11*) have been extensively studied for their polymorphisms and clinical effects. Interestingly, MRP1 was the second transporter discovered after P-gp, but studies regarding polymorphisms in this transporter have not been as comprehensive. *In vitro* functional studies of MRP1 SNPs found 1299G>T (rs60782127) to be associated with decreased transport of estradiol-17- β -D-glucuronide (E₂17 β G), but was also associated with increased doxorubicin resistance, demonstrating substrate specific effects. The SNP 2965G>A (rs35529209) also showed significant reduction in E₂17 β G transport (56). Clinical studies correlating MRP1 SNPs to drug treatment outcomes have also been unconvincing, and the SNPs discovered do not coincide with any of the SNPs with significant *in vitro* effects (57).

MRP2 arose as a protein of interest due to the discovery that genetic variants in MRP2 resulting in a loss-of-function protein results in the hereditary disorder Dubin-Johnson syndrome, characterized by conjugated hyperbilirubinemia (58). MRP2 secretes conjugated bilirubin from liver into bile, and the loss of function mutant is compensated by secretion of conjugated bilirubin into blood by MRP3, resulting in hyperbilirubinemia

(59). Clinically, one MRP2 SNP, -24C>T, had high frequencies and was associated with decreased clearance of substrate drugs mycophenolic acid and methotrexate (60, 61).

MRP8 SNPs have also been studied in detail. This was because the SNP 538G>A (rs17822931) was found to determine wet/dry earwax type (62). It was the first time that a single allele was discovered to correspond to a specific physical trait. In addition to dictating earwax type, G538A SNP has also been associated with axillary osmidrosis. Carriers of the G allele were found to have larger apocrine glands, thus secreting more preodoriferous compounds (63). Clinically, this SNP has been investigated in relation to breast cancer risk and anti-cancer drug resistance. However, no significant associations have been discovered with any of the drug investigated, and it remains to be seen if G538A allele is pharmacologically significant (64).

1.4.4 OCTs

Organic cation transporters (OCTs; gene family *SLC22*) are uptake transporter expressed in many tissues in the body. There are 3 members in the family, OCT1, 2, and 3 (*SLC22A1*, 2, 3). OCT1 is primarily expressed in liver while OCT2 is primarily expressed in the kidneys. OCT3 seemed to show a more ubiquitous expression through various tissues (35). A broad range of substrates have been found for OCTs, and SNPs in OCTs have been investigated for clinical effects. Most studies OCT SNPs have focused on the drug metformin, which has been shown as a substrate for all 3 members, but detailed clinical studies have only been completed for OCT1 and 2.

There has been many SNPs identified in OCT1, and many have demonstrated reduced *in vitro* uptake of prototypical OCT substrates MPP⁺ and TEA as well as metformin (65). However, the majority of the polymorphisms discovered were quite rare and the effects were only observed by single studies. The SNPs with higher frequencies did not affect MPP⁺ or metformin transport. Divergent results have been found in studies correlating SNPs with metformin plasma concentrations and clinical outcome (66, 67). Combinations of SNPs seemed to have amplified effect on metformin renal clearance and plasma glucose levels, and has yet been verified (68). Despite the inconsistent results, OCT1 genotype may still play an important role in understanding the variability in metformin therapy.

Significantly less SNPs have been identified for OCT2. One SNP, 808G>T (rs316019), has fairly high frequencies across all ethnic groups and has been investigated in many studies to be a reduced function polymorphism. Patients that carry the G808T variant allele exhibited higher plasma concentrations of metformin due to decreased renal clearance (69).

Although rare, SNPs in OCT1 and OCT2 definitely affect transport *in vitro*. Whether their impact on metformin pharmacokinetics is significant enough to warrant genotyping for patients has yet to be seen. Further research needs to be done to fully understand the contribution of OCTs in OCT substrate pharmacokinetics.

1.4.5 OATs

Organic anion transporters (OATs) also belong to the gene family *SLC22*, and the 3 members OAT1/2/3 are given the gene names *SLC22A6/7/8*. OATs are primarily expressed in kidneys and are transporters for a number of drugs (35). Polymorphisms in OATs have not been extensively studied, and there has been very minimal genetic variation seen in OAT2 (70). Rare SNPs have been identified in OAT1 and OAT3, and studies have attempted to correlate OAT genotype with diuretics and antiviral drugs. The results however show no correlation, possibly due to low patient population or the rescuing of a deficient polymorphism by other members of the family (35). The role of genetics in OATs remain unclear and more studies are necessary for a better understanding of the effects of OAT genotypes.

1.4.6 OATPs

OATP1A2, OATP2B1, OATP1B3, and OATP1B1 are 4 members of the OATP family that have been studied for genetic polymorphisms. Studies involving OATP1A2, 2B1, and 1B3 have been fairly inconsistent, and polymorphisms in these 3 transporters seemed to play a small role in substrate drug pharmacokinetics (35). Studies in OATP1A2 have attempted to correlate known SNPs with lopinavir and imatinib, but found no association (71, 72). OATP1B3 522G>C SNP seemed to facilitate substrate dependent changes in transport activity, while other identified SNPs in the same study did not show functional effects (73). *In vitro* studies in OATP2B1 found the *3 variant to be a significant loss-of-function

polymorphism when transporting E₁S (74), and the SNP 1457C>T was associated with decreased fexofenadine transport (75).

By far the most clinically significant and widely studied transporter is OATP1B1, a liver-specific member of the OATP family. SNPs in OATP1B1 have been shown to functionally affect transporter activity *in vitro* and *in vivo*, and significantly affect plasma concentrations of substrate drugs, which include several widely prescribed medications. This allowed the acknowledgement of the potential benefits of genotype-guided dosing and the advancement of personalized medicine. The effects of OATP1B1 polymorphisms are described in detail in the section below.

1.4.7 OATP1B1

OATP1B1, described previously, is a 12 transmembrane uptake transporter located on the basolateral membrane of human hepatocytes. Figure 2 shows the predicted topology of OATP1B1 and some of the important SNPs discovered. Functional SNPs in OATP1B1 were first characterized in 2001. 16 variants of OATP1B1 were identified, and 7 of these variants were found to have altered transport of *in vitro* probes E₁S and E₂17βG compared to wildtype. The changes in transport were mediated by a combination of decreased affinity between substrate and transporter, and decreased maximum transport velocity which could be explained by a decrease in total cell surface expression of the transporter (76). Among the SNPs identified were *SLCO1B1**1*b* (c.388A>G; rs2308283), *5 (c.521T>C; rs4149056), and *15 (*1*b* + *5), which have all since been extensively studied *in vitro* and *in vivo*. *SLCO1B1**1*b* is listed to have a MAF of 0.4050, and *SLCO1B1**5 has a MAF of 0.1230 according to NCBI's SNP database (dbSNP). Due to high frequencies in the population, effects caused by these SNPs would affect a large number of patients. *SLCO1B1**1*b* was found not to affect the transport of E₁S, E₂17βG, and rosuvastatin *in vitro* (76), and *in vivo* studies show no association with plasma concentrations of pravastatin and rosuvastatin (77). Other studies of *SLCO1B1**1*b* however showed that this SNP increased OATP1B1 function and was associated with increased transport of E₁S and E₂17βG and decreased plasma levels of pravastatin (78, 79). The disparity between studies suggest that *SLCO1B1**1*b* likely does not have a major impact on transport function. *SLCO1B1**5 and *15 were both found to decrease the transport of most statins and other

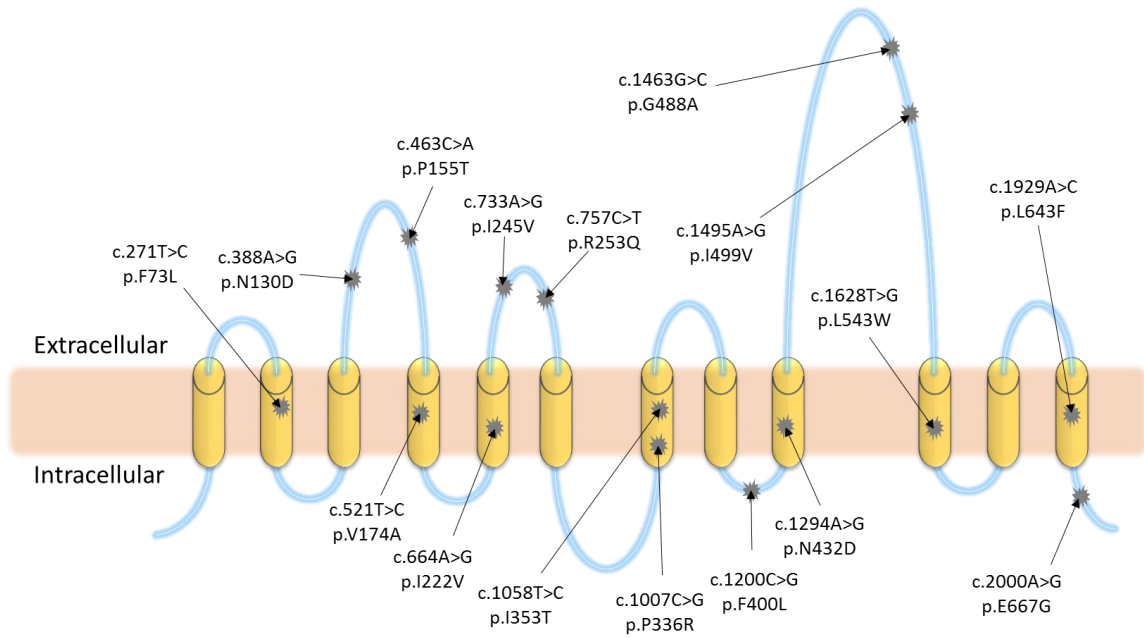


Figure 2. Predicted 12-transmembrane topology of OATP1B1 and some significant SNPs that have been discovered.

substrates *in vitro* (76, 80). *In vivo* studies found an increase in AUC and C_{\max} of these substrates (81-83). A genome-wide association study on more than 300 000 markers found *SLCO1B1**5 to be the only SNP linked with simvastatin-induced muscle toxicity (84). Due to these studies, *SLCO1B1**5 and other haplotypes containing this SNP can significantly affect plasma statin levels, and dosing of statins may be adjusted if the patient was found to be a carrier for this SNP (52).

Chapter 2

2 « Objectives and Aims »

2.1 Rationale

The primary class of drugs that are substrates for OATP1B1 are statins, which are drugs used to reduce cholesterol and LDL to decrease risk for cardiovascular disease (34). Its therapeutic effects are profound, but adverse reactions are seen in some patients. Cholesterol synthesis primarily takes place in the liver and begins with the combination of 3 acetyl-CoA molecules to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). HMG-CoA is then reduced by HMG-CoA reductase into mevalonate, which is the rate-limiting step of the cholesterol synthesis pathway. Mevalonate then undergoes a long series of reactions to form cholesterol. Statins lower cholesterol by inhibiting HMG-CoA reductase, preventing the formation of mevalonate and ultimately cholesterol (85). Since the majority of cholesterol is synthesized in the liver, successful uptake of statins into hepatocytes are important not only for it to exert its effect in the liver, but also to reduce toxicities associated with prolonged systemic exposure (32). The most common side effect of statin therapy is myalgia and myopathy, and patients experiencing these side effects will often recover once statin therapy is terminated. In a very small number of patients, the effect can develop into a fatal condition rhabdomyolysis, the total breakdown of muscle tissue. The cause of this side effect is unknown, and different mechanisms have been proposed and have yet been absolutely verified (86). Risk factors for myopathy include advanced age, low BMI, female gender, Asian origin, and the existence of systemic disease involving renal or liver complications (87). A common characteristic among patients experiencing statin-induced muscle toxicity is elevated concentrations of statins in blood (88). Since *SLCO1B1**5 allele has been shown to be associated with increased plasma concentrations of almost all statins, patients that are carriers for variant alleles are more susceptible to statin-induced muscle toxicity and should be monitored carefully if on statin therapy (31). Although studies unanimously agree that *SLCO1B1**5 allele contributes to significant loss of function of the transporter and elevated plasma concentrations of statins, attempts at showing correlation between *5 genotype and clinical outcome of statin therapy have been

inconsistent. Due to a tremendous amount of SNPs that have been discovered in OATP1B1, the possibility exists that other SNPs may also affect the function of OATP1B1.

Six non-synonymous SNPs in OATP1B1 were chosen to be analyzed *in vitro*. Due to lack of murine model of OATP1B1, *in vitro* functional studies are the best predictors of how SNPs can affect the transporter *in vivo* (3). These 6 SNPs were found on dbSNP which collects new and seldom-studied SNPs from a wide range of sources. Three criteria were developed to select for SNPs that were most likely to have significant functional consequences. The first criterion was MAF. A higher MAF indicates a greater number of carriers in the population and greater potential for clinical significance if the SNP was found to be functional. The next criterion was the position of the SNP in the transporter. It has been demonstrated in previous studies that SNPs in the transmembrane and extracellular regions of the transporter were more likely to lead to functional consequences. Transmembrane domains 8 and 9, as well as extracellular loops 2 and 5 have all been suggested as important regions for substrate binding and transport (89, 90). Therefore only SNPs in the transmembrane and extracellular domains were considered. Lastly, the resultant amino acid change caused by the SNP was taken into account. If the amino acids before and after the polymorphism had drastically different side chains, it would be more likely to affect the overall folding of the protein and more likely to affect substrate binding, transport, and other interactions. With these criteria, the SNPs c.298G>A (rs144508550, Gly100→Ser), c.419C>T (rs147450830, Ser140→Phe), c. 463C>A (*4, rs11045819, Pro155→Thr), c.1007C>G (rs72559747, Pro336→Arg), c.1463G>C (*9, rs59502379, Gly488→Ala), and c.1738C>T (rs71581941, Arg580→Stop) were chosen to be functionally characterized (Table 1). Of these 6 SNPs, *4, *9, and C1007G have been studied previously. *SLCO1B1**4 was found to not significantly affect the uptake of E₁S and E₂17G, while *SLCO1B1**9 was found to nearly abolish OATP1B1 uptake of rosuvastatin (76). *SLCO1B1**4 has also been studied *in vivo* and was found to be correlated with lower atorvastatin AUC (91). C1007G was also found to not have any significant effects on OATP1B1 transport (92). The other 3 SNPs G298A, C419T, and C1738T have never been studied before and will be characterized for the first time.

2.2 Objectives

This study seeks to confirm previous *in vitro* data on *SLCO1B1**4, *9, and C1007G, as well as functionally characterize the new SNPs G298A, C419T, and C1738T using substrates E₁S, E₂17βG, and rosuvastatin. Subsequently, these SNPs will be studied *in vivo* by genotyping patients on rosuvastatin and determining any associations between plasma statin concentrations and genotype. This would contribute to the database of known functional OATP1B1 polymorphisms, as well as determine new pharmacogenomics predictors of plasma statin levels. With this information, patients starting rosuvastatin could be genotyped for these functional SNPs and be dosed accordingly to minimize the chance of adverse reactions.

2.3 Hypothesis

We predict that OATP1B1 containing the SNPs to be investigated will have decreased total uptake of all 3 substrates compared to wildtype facilitated by lower V_{\max} , higher K_m , and lower cell-surface expression. Due to impaired function of the transporter, patients carrying these SNPs will be associated with higher plasma concentrations of rosuvastatin due to reduced hepatic uptake of statins compared to wildtype individuals.

Table 1. SNPs chosen to be functionally analyzed in this study.

dbSNP rs #	Nucleotide	Amino Acid	Domain	MAF
rs144508550	G298A	G100S	3 rd TM	N/A
rs147450830	C419T	S140F	2 nd EX	N/A
rs11045819	C463A	P155T	2 nd EX	0.078
rs72559747	C1007G	P336R	7 th TM	0.001
rs59502379	G1463C	G488A	5 th EX	0.01
rs71581941	C1738T	R580Stop	11 th TM	0.005

Chapter 3

3 « Methods »

3.1 Materials

Radiolabelled [H^3]-E₁S (45 Ci/mmol, >97% radiochemical purity) and [H^3]-E₂17βG (34.3 Ci/mmol, >97% radiochemical purity) were purchased from Perkin Elmer, and radiolabelled [H^3]-rosuvastatin (74 Ci/mmol, 97.1% radiochemical purity) was purchased from GE Healthcare. Unlabelled E₁S sodium salt and E₂17βG sodium salt were purchased from Sigma-Aldrich, and unlabelled rosuvastatin calcium was purchased from Toronto Research Chemicals. *SLCO1B1*1a* cDNA packaged in pEF-V5-His-Topo expression vector was provided by Dr. Kim. Custom rabbit OATP1B1 polyclonal antibody were developed by Research Genetics (Huntsville, AL), and was raised against C-terminal peptide of OATP1B1 (ESLNKNKHFVPSAGADSETHC). Actin antibody HRP conjugate was purchased from Santa Cruz Biotechnology, rabbit polyclonal calnexin antibody was purchased from Cedarlane Labs, and mouse monoclonal Na⁺/K⁺-ATPase antibody was purchased from Axxora. Rosuvastatin patient plasma levels were measured by Dr. Marianne DeGorter, and purified patient DNA were provided by Dr. Kim at 10 ng/μL. All dry ingredients used in buffers were purchased from Sigma-Aldrich of the highest quality.

3.2 Cell culture

Cell types used were HeLa, HEK293, and HEK293A. Media used for all cell culture was Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing fetal bovine serum, L-glutamine, and penicillin/streptavidin. Cells were incubated at 37 °C and 5% CO₂. Viral cell culture were incubated in a separate designated viral incubator in the same conditions. Media was replaced every 2 days, and cells were trypsinized and replated before reaching confluency. When plating cells for experiments, cells were trypsinized and pelleted by centrifugation at 1000 rpm for 3 min. Media and trypsin were removed and the pellet was resuspended in fresh media. Cell number was counted on a haemocytometer and the appropriate number of cells was plated for experiments.

3.3 Introducing SNPs in *SLCO1B1*

SNPs were introduced to *SLCO1B1*1a* plasmid expressed in pEF-V5-His-Topo using QuickChange Site-directed Mutagenesis as per manufacturer's protocol. Mutated plasmids were transformed to XL10-Gold Ultracompetent Cells, plated on LB-Ampicillin agar plates and incubated overnight at 37 °C. The following day, plasmids were extracted from 6 separate colonies using QIAprep® Spin Miniprep Kit. Successful mutagenesis for variants G298A, C419T, and C463A (*4) were confirmed by sequencing with T7 promoter. Successful mutagenesis for variants C1007G, G1463C (*9), and C1738T were confirmed by sequencing with custom primers. In addition to generating *SLCO1B1* variants, site-directed mutagenesis was used again to remove a Pac-I digestion site in *SLCO1B1*. Pac-I digestion is an essential step for further propagation of adenovirus in cells, so Pac-I sites within *SLCO1B1* must be removed. The SNP C419T is located close to the Pac-I site, so a separate mutagenesis primer was required to mutate the Pac-I site as well as maintain the C419T SNP. All mutagenesis and sequencing primers used are listed in Tables 2 and 3.

3.4 Expression of OATP1B1 variants as adenovirus

OATP1B1 wildtype and variants were expressed using ViraPower Adenoviral Expression System (Invitrogen). *SLCO1B1*1a* and variant DNA were isolated from pEF vector by digestion with KpnI and NotI (New England Biolabs), followed by separation by gel electrophoresis and purification of the ~2000 bp band. pENTR1A dual selection vector was also digested with KpnI and NotI and separated by gel electrophoresis and a different ~2000 bp band was isolated from the gel. *SLCO1B1* entry clone was generated by ligating isolated *SLCO1B1* DNA to digested pENTR1A vector using T4 DNA ligase with *OATP1B1* DNA to pENTR1A DNA ratio of 10:1. Ligated product were transformed to One Shot® TOP10 Chemically Competent *E. coli* and plated on LB-Kanamycin agar plates. 6 colonies were selected following overnight incubation at 37 °C, and plasmids were purified again with QIAgen miniprep kit. Correct insertion of *SLCO1B1* into pENTR1A dual selection vector was verified by digestion with BamHI restriction enzyme. Plasmids with a 313 bps digestion product were selected for further propagation in adenoviral plasmid. Selected *SLCO1B1* entry clones were then recombined with pAd/CMV/V5-DEST vector as per manufacturer's protocol, transformed to One Shot® TOP10

Table 2. Mutagenesis primers.

Primer	Sequence	Length	T_m (°C)
G298A	5'-ATAGACCAAAGTTAATTGGAAT CAGTTGTTTCATTATGGGAATTGGA-3'	47	67.3
C419T	5'-CATCAGAAAATTCAACATCGA CCTTATTCACTTGTTTAATTAATC-3'	45	65.6
C463A	5'-ATCACTCAATAGAGCATCAACT GAGATAGTGGGAAAAGG-3'	39	68.9
C1007G	5'-TTTTAAAAGCATCCTTACTAATC GCCTGTATGTTATGTTTGTGCTTT-3'	47	67.3
G1463C	5'-CTCACCTGTCTAGCA GCTTGCAAATCTTCAAGTG-3'	35	70.5
C1738T	5'-GGGTTTCCACTCAATGGTT ATATGAGCACTAGGAG-3'	35	69.4
PacI	5'-CATCGACCTTATC AATTTTATCACTC-3'	26	66.6
C419T-PacI	5'-AAAATTCAACAT CATCACTCAATAGA-3'	26	66.8

Table 3. Sequencing primers.

Primer	Sequence	Length	T_m (°C)
Forward Primer 1	5'-GGAAGCTTTGAAATTGGAAA-3'	20	54.2
Forward Primer 2	5'-ATTCAACATCGACCTTATCC-3'	20	56.3
Forward Primer 3	5'-AAGTCAATGGGAACCAGTCT-3'	20	58.4
Reverse Primer 1	5'-CCAGATTCCTTTAAACAACC-3'	20	56.3
Reverse Primer 2	5'-TAAGGATGCTTTTAAAAGAC-3'	20	52.2

Chemically Competent *E. coli* and plated on LB-Ampicillin plates to generate adenoviral expression clones. GenElute™ HP Plasmid Maxiprep Kit was used to isolate plasmids from 3 separate clones and successful recombination was verified by digest with SpeI forming a 735 bp product. After confirmation of recombination with SpeI, 10 µg of adenoviral plasmid DNA were digested with PacI and purified with QIAquick® PCR Purification Kit. 1 µg of PacI digested adenoviral plasmid DNA were then transfected to HEK293A cells using Lipofectamine 2000 following manufacturer's instructions to produce crude adenoviral stock containing OATP1B1 wildtype and variant genes.

3.5 Purification of adenovirus from crude stock

Crude adenovirus stock generated from ViraPower Adenoviral Expression System protocol was purified to increase virus concentration and reduce toxic cellular by-products. HEK293A cells were grown to confluency on 150 mm tissue culture plates, then transduced with 100 µL of crude adenovirus for 2-3 days until cells are noticeably filled with virus. Cells were washed off and resuspended in 10 mM Tris at pH 9, then homogenized in a dounce homogenizer. The solution was then spun at 1500 g for 5 min, and 1.5 mL of the supernatant was layered on top of 3 mL of 10 mM Tris containing 36% sucrose in an ultracentrifuge tube and spun at 100 000 g for 1 hour. Supernatant was then removed, and the pellet was resuspended in 1 mL of 10 mM Tris as purified adenovirus. Purified adenovirus concentration was determined with Adeno-X Rapid Titer kit following manufacturer's protocol.

3.6 Transduction of adenovirus in HeLa cells

HeLa cells were chosen to be transduced with *SLCO1B1* adenovirus because they are a stable adherent mammalian cell line that does not express OATP1B1. On day 1, appropriate number of HeLa cells were plated and incubated overnight at 37 °C and 5% CO₂ until cells attached. On day 2, the media was removed and replaced with media containing adenovirus at MOI of 1000. Cells were incubated with viral media overnight. The next day, viral media was removed and replaced with fresh media and incubated overnight again. On day 4, cells were stably overexpressing the transporter and ready for experiments. Table 4 lists the number of cells and volume of media used for different types of plates.

Table 4. Amount of cells plated for different sized plates.

Plate	# of cells to plate	Volume of media (mL)
6-well	500 000	2
12-well	200 000	1
24-well	100 000	0.5
96-well	10 000	0.2

3.7 MTT assay to determine cell viability after adenoviral transduction

HeLa cells were plated in 96-well plate and transduced with virus according to the protocol above. On day 4 of transduction, 20 μ L of Thiazolyl Blue Tetrazolium Bromide (MTT) at 5 mg/mL dissolved in PBS was added to each well. The plate was shaken for 5 min at 150 rpm and incubated at 37 °C and 5% CO₂ for 4 hours to allow MTT to be metabolized to formazan. Residual media was removed and the formazan formed was resuspended with 200 μ L of DMSO and shook for 5 min. Optical density of each well was read at 560 nm and subtracted background readings at 670 nm. Cells transduced with adenovirus were compared to non-viral controls and expressed as a percentage. Each variant was performed in triplicate and repeated on 2 separate days.

3.8 Functional studies of OATP1B1 variants

HeLa cells were plated and transduced with adenovirus following the protocol previously described. On day 4, cells were washed with Krebs Henseleit Bicarbonate (KHB) buffer (1.2 mM MgSO₄, 0.96 mM KH₂PO₄, 4.83 mM KCl, 118 mM NaCl, 1.53 mM CaCl₂, 23.8 mM NaHCO₃, 12.5 mM HEPES, 5 mM glucose, pH 7.4), and allowed to equilibrate to 37 °C in the incubator. Transport was initiated by replacing KHB buffer with KHB buffer containing radiolabelled and un-labelled substrates at various concentrations and incubated for various periods of time depending on substrate and experiment. Un-labelled substrate was used to acquire the desired drug concentration and minimal radiolabelled substrate was used to achieve detection. After incubation with substrates, cells were washed 3 times with ice-cold PBS and harvested in 500 μ L of 1% SDS. Radioactivity was measured by Tri-Carb 2900TR Liquid Scintillation Analyzer (PerkinElmer) and protein content was measured with BCA Protein Assay Kit (Thermo).

For time-dependent uptake studies, cells transduced with Lac Z and OATP1B1*1a adenovirus were incubated with E₁S, E₂17 β G, or rosuvastatin at 100 nM for 3, 5, 10, 15, 20, and 30 min. Phase of linear active transport was determined to occur at 3 min.

For total uptake studies, cells transduced with OATP1B1*1a and variants were incubated with substrates at 100 nM for 3 min.

For concentration-dependent uptake studies, cells expressing OATP1B1*1a and variants were incubated with substrates at varying concentrations for 3 min. E₁S concentrations used were 0.1, 0.5, 1, 2, and 5 μ M; E₂17 β G concentrations used were 0.1, 1, 5, 10, and 20 μ M; rosuvastatin concentrations used were also 0.1, 1, 5, 10, and 20 μ M. Concentrations beyond the maximum concentrations used were determined to be toxic. All transport experiments were done in triplicates on at least 3 different days.

3.9 Immunoblots to determine total cellular OATP1B1 expression

HeLa cells were grown and transduced with Lac Z and OATP1B1 wildtype and mutant adenovirus as described above. For total cell lysate immunoblots, cells were scraped off with PBS, spun at 500 g for 3 min, and resuspended in lysis buffer (5 mM Tris-HCl, 1% Triton-X-100, 150 mM NaCl, 5 mM EDTA, protease inhibitor (Sigma)). Protein concentrations were determined with BCA Protein Assay Kit (Table 5) and 20 μ g of protein from each OATP1B1 variant was analyzed. All reagents and equipment used in immunoblots were purchased from Novex by Life Technologies. Samples were then mixed with NuPAGE® LDS Sample Buffer and reducing agent 2-mercaptoethanol and incubated at 70 °C for 10 min to linearize protein. Gel electrophoresis was run on NuPAGE® 4-12% Bis-Tris Protein Gels in X-Cell SureLock™ Mini-Cell Apparatus with NuPAGE® MES SDS Running Buffer. Electrophoresis was done at constant voltage of 200 V until protein has run through the gel. After gel electrophoresis, proteins were blotted onto nitrocellulose membranes with 0.45 μ m pores. Blotting was performed with X-Cell II™ Blot Module and the gels and nitrocellulose membranes were incubated in NuPAGE® Transfer Buffer. Membranes were blotted at constant voltage of 30 V for 1.5 hrs. After blotting, membranes were washed with tris buffered saline (TBS) with TWEEN-20 (Sigma) and blocked with Western Blocking Reagent (Roche) for 1 hour at room temperature. After blocking, membranes were washed again and incubated with primary OATP1B1 antibody at 1:10000 dilution at 4 °C overnight. Membranes were then subsequently incubated with secondary antibody (anti-rabbit HRP conjugate) at 1:20000 dilution at room temperature for 1 hr. After secondary antibody incubation, membranes were washed again and soaked with Amersham ECL Western Blotting Detection Reagents (GE) and exposed on image station

Table 5. Protein concentrations of Lac Z and OATP1B1 variants determined by BCA Protein Assay Kit

OATP1B1 Variant	Protein concentration ($\mu\text{g}/\mu\text{L}$)
Lac Z	0.97
*1a	0.95
*1b	1.09
*5	1.02
*15	0.88
G298A	0.77
C419T	0.93
C463A	0.99
C1007G	0.92
G1463C	0.99
C1738T	0.87

to visualize. Membranes were then stripped with Restore Western Blot Stripping Buffer (Thermo). Membranes were blocked again, incubated with actin HRP conjugate antibody at 1:500 dilution, and visualized on image station.

3.10 RNA extraction, reverse-transcription, and quantitative real-time PCR to analyze C1738T variant mRNA expression

The OATP1B1 variant C1738T causes a premature stop codon that truncates the protein before the terminal 21-mer sequence that the OATP1B1 antibody binds to, so no protein levels were detected by immunoblot analysis. Therefore, quantitative real-time PCR was used to quantify C1738T mRNA levels.

HeLa cells were plated in 6-well plates and transduced with OATP1B1*1a and C1738T adenovirus. When ready, RNA was extracted using TRIzol® Reagent (Applied Biosystems). 1 mL of TRIzol® Reagent was added to each well and incubated for 5 min at room temperature. The contents of each well were harvested in Eppendorf tubes and 200 µL of chloroform was added to each tube and shaken for 15 sec. Tubes were incubated for 3 min at room temperature and centrifuged at 12 000 g and 4 °C for 15 min. The clear aqueous layer was transferred to a new tube and 500 µL of isopropanol was added. Tubes were incubated at room temperature for 10 min and centrifuged again at 12 000 g and 4 °C for 10 min. Supernatant was removed and the pellet was washed with 1 mL of 75% ethanol solution. Tubes were centrifuged again at the same speed and temperature for 5 min, and the supernatant was removed. 50 µL of RNAase-free water was used to resuspend RNA, and RNA concentration was measured by NanoVue Plus Spectrophotometer (GE).

After RNA extraction, reverse-transcription was done using TaqMan® Reverse Transcription Reagents (Invitrogen) to obtain cDNA. 1500 ng of OATP1B1*1a and C1738T RNA were mixed with reverse-transcription reagents and placed in thermocycler. cDNA was synthesized by incubating reagents for 10 min at 25 °C, followed by 30 min at 48 °C, and finally 5 min at 95 °C.

After RNA extraction and reverse-transcription to cDNA, qPCR was performed to quantify cDNA levels of OATP1B1 *1a and C1738T. All qPCR reagents were purchased from

Applied Biosystems®. Primers and PCR conditions are described in Table 6. Housekeeping gene used was human 18S rRNA. OATP1B1*1a and C1738T gene expression was detected using Fast SYBR® Green Master Mix, and 18S rRNA expression was detected using Taqman® Fast Advanced Master Mix. $\Delta\Delta$ Cycle threshold (Ct) was calculated by subtracting 18S Ct from OATP1B1 Ct, and then subtracting reference gene (*1a) Δ Ct from experimental (C1738T) Δ Ct. Relative gene expression is then calculated by the formula $2^{(-\Delta\Delta Ct)}$.

3.11 Cell surface biotinylation

Cell surface biotinylation was done to isolate cell-membrane proteins. HeLa cells were grown and transduced with Lac Z and OATP1B1 wildtype and mutant adenovirus. After transduction, cells were washed with PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂ (PBS/Mg/Ca), then incubated with biotinylating agent Sulfo-NHS-SS-biotin (1.5 mg/mL, Pierce) in PBS/Mg/Ca for 1 hour at 4 °C. Following incubation with biotin, cells were washed with PBS/Mg/Ca containing 100 mM glycine at pH 3 and incubated for 20 min. Cells were washed again with PBS/Mg/Ca and harvested in 700 uL of radioimmunoprecipitation assay (RIPA) buffer (10 mM Tris-base, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X-100, protease inhibitor (Sigma), pH 7.4). Samples were then centrifuged, and 600 uL of RIPA supernatant was mixed with 140 uL of Immunopure Streptavidin beads (Stratagene) for 1 hour at room temperature. Samples were centrifuged again, washed with RIPA, and the final pellet was mixed with 50 uL 2X Laemmli buffer (0.125 M Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.01% bromophenol blue) for 30 min at room temperature. After final centrifugation, the supernatant was used for western blot analysis. Protein samples were separated by gel electrophoresis, transferred to nitrocellulose membrane and probed with OATP1B1 antibody. Blots were stripped and probed with calnexin antibody (1:1000) as intracellular control, and probed again with Na⁺/K⁺-ATPase antibody (1:500) as cell-surface control.

3.12 Rosuvastatin patient selection

Rosuvastatin cohort from a previous study by our group was obtained (52). Adult outpatients at London Health Sciences Center (London, ON) taking daily dose of

rosuvastatin were enrolled in the study. Patients were excluded if the last dose taken was more than 24 hrs prior to blood draw, and all patients were on the same dose of rosuvastatin for ≥ 6 weeks.

3.13 Determining OATP1B1*4 genotype

Restriction fragment length polymorphism (RFLP) was used to genotype statin patients for OATP1B1*4. Patient DNA samples (10 ng/uL) were amplified by PCR and digested with

Table 6. Real-time PCR primers and conditions.

Primer	Sequence	Length	Tm (°C)
OATP1B1 FW	TGAACACCGTTGGAATTGC	19	65.0
OATP1B1 RV	TCTCTATGAGATGTCACTGGAT	22	58.3
PCR Conditions			
Denaturation (°C)	95		
Denaturation time (s)	15		
Anneal/Extend (°C)	60		
Extension time (s)	60		
Cycles	40		

Hph-I. Primers for PCR are listed in Table 7. PCR was initialized for 5 min at 94 °C, followed by 1 min at 94 °C, 1 min at 54 °C, and 1 min at 72 °C repeated for 35 cycles, and concluded with 7 min at 72 °C. Total amplicon size after PCR is 532 bps. 4 units of Hph-I was used to digest 3 µg of PCR product for 2 hrs and analyzed by gel electrophoresis. Digested samples with 2 bands at 209 and 323 bps indicate wildtype genotype, undigested samples with 1 band at 532 bps indicate homozygous mutant genotype, and semi-digested products with 3 bands at 532, 209, and 323 bps indicate heterozygous genotype.

3.14 Statistical analysis

Comparison of means for transport experiments were done by Student's t test, one-way ANOVA, and Tukey multiple comparisons test. Multiple linear regression was done to correlate log-transformed rosuvastatin plasma concentration with variables age, sex, ethnicity, body mass index (BMI), dose, time since last does, and 3 OATP1B1 SNPs *1b, *4, and *5. Additional haplotype analysis was also performed and the percentage of genetic contribution to the observed variability was calculated by totalling the sum of squares for each variable and calculating the proportion of the genetic component.

Table 7. RFLP PCR primers and conditions.

Primer	Sequence	Length	T_m (°C)
OATP1B1*4 FW	GATAACCCACTTAGCCTGGGG	21	64.5
OATP1B1*4 RV	ACCTGTGTTGTTAATGGGCGA	21	60.6
PCR Conditions			
Denaturation (°C)	94		
Annealing (°C)	54		
Elongation (°C)	72		
Cycles	35		

Chapter 4

4 « Results »

4.1 MTT assay shows cells were viable at time of transport

All viral variants shows $\geq 80\%$ viability compared to no virus control and were all not significantly different from *1a variant (Fig. 3). MTT assays were performed in triplicates on 2 separate experimental days.

4.2 Time-dependent uptake shows active transport within the first 3 minutes

HeLa cells were transduced with Lac Z and OATP1B1*1a (wildtype) adenovirus and incubated with substrates E₁S, E₂17 β G, and rosuvastatin for 3, 5, 10, 20, and 30 minutes. Significant transport was observed in HeLa cells transduced with OATP1B1*1a while minimal transport was observed in HeLa cells transduced with Lac Z. Unsaturated active transport was observed in the first 3 minutes of uptake for all substrates, which will be the time point used for all subsequent uptake experiments (Fig. 4).

4.3 Total uptake for 3 minutes with all substrates reveals significant differences in uptake between OATP1B1 wildtype and variants

Single 3 minute uptake of E₁S showed significantly decreased uptake in OATP1B1*1b, *5, *15, and C419T, and almost no uptake in G298A, *9, and C1738T. Uptake in *4 and C1007G were not significantly different from *1a, although *4 showed slightly decreased uptake while C1007G showed slightly increased uptake (Fig. 5).

Experiments with E₂17 β G showed similar results with significantly decreased uptake in *1b, *5, *15, and C419T, and no uptake in G298A, *9, and C1738T. *4 again showed no significant difference compared to *1a, but C1007G had significantly greater uptake than *1a (Fig. 5).

Rosuvastatin single uptake experiments showed significantly decreased uptake with all SNPs except for C1007G, which again showed slight increase in uptake but not statistically

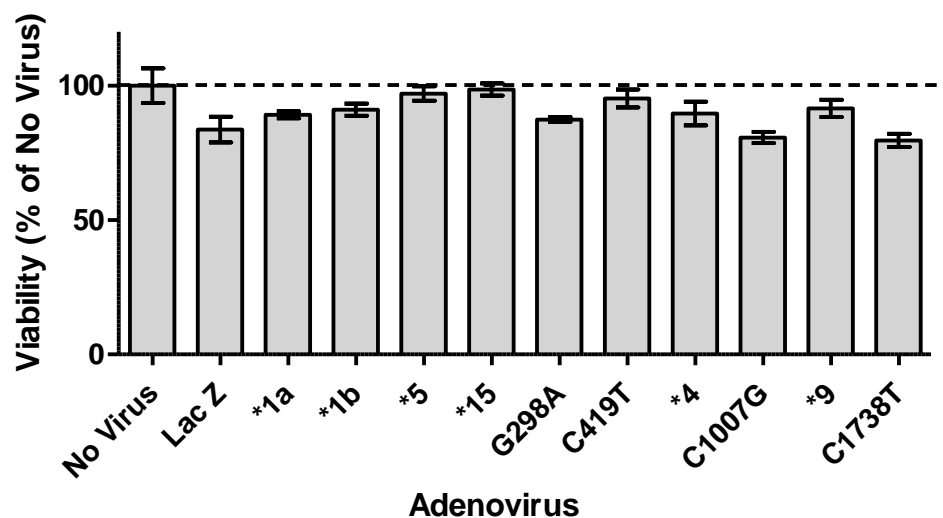


Figure 3. MTT assay of HeLa cells transduced with various adenovirus. Values are expressed as percentage of no virus control. No variants were significantly different from control ($n=2$).

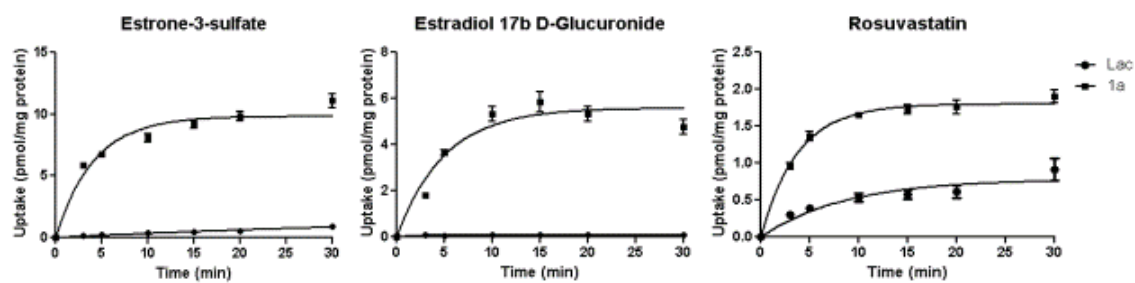


Figure 4. Time-dependent uptake of all three substrates by wildtype OATP1B1 (*1a) and Lac Z ($n=3$).

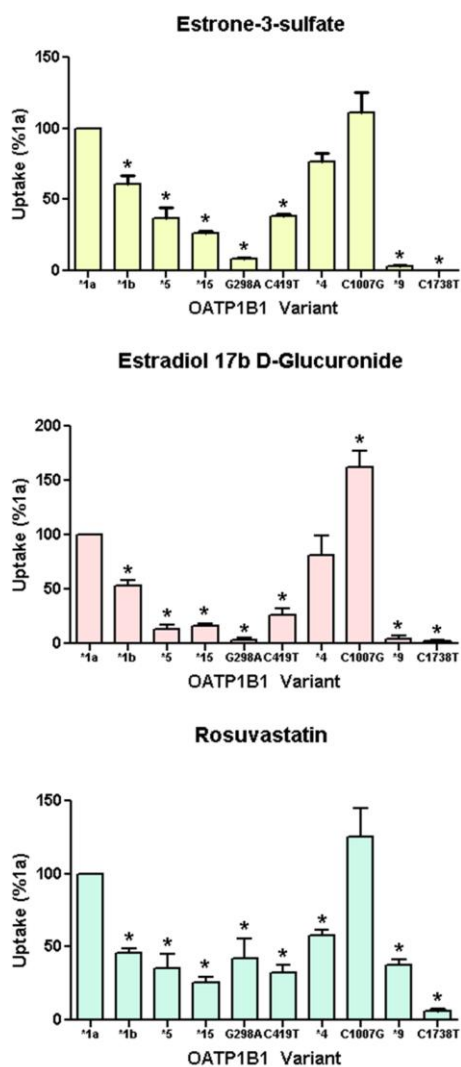


Figure 5. Total uptake in the first 3 min of all substrates at 100 nM by HeLa cells transduced with OATP1B1*1a and variants. Values were corrected for Lac Z and expressed as percentage of *1a ($n=3$). * denotes that value is significantly different from *1a; $p<0.001$.

significant. Only C1738T SNP seemed to completely abolish transport function as uptake was observed in the other two SNPs that previously showed no uptake (Fig. 5).

4.4 Western blot analysis of total cellular OATP1B1 expression

Two distinct bands were observed in western blots for OATP1B1, one at ~80 kDa and one at ~60 kDa. OATP1B1*1b, *5, and *15 SNPs all showed a decrease expression of total OATP1B1. G298A, C419T, and *9 showed decreases in the ~80 kDa band, but more expression of the ~60 kDa band. OATP1B1 *4 showed slight decrease in ~80 kDa band and a slight increase in ~60 kDa band. C1007G showed increased expression of both bands of OATP1B1. OATP1B1 was not detected in C1738T due to inability of antibody to bind to the transporter. Actin control shows uniform protein loading (Fig. 6).

4.5 Quantitative real-time PCR analysis shows increased production of C1738T mRNA compared to *1a

Due to C1738T variant causing a premature stop codon that prevents binding of OATP1B1 antibody to the terminal sequence, protein levels could not be detected by western blot. qPCR was used and found that C1738T had a 1.8-fold increase in mRNA levels compared to *1a (Fig. 7).

4.6 Concentration-dependent uptake

Uptake experiments with E₁S were performed with 0.1, 0.5, 1, 2, and 5 μM of substrate incubated for 3 minutes. No uptake was observed for variants G298A, *9, and C1738T. V_{max} was decreased for C419T and *4 and increased for C1007G, but all were not significant from *1a. K_m remained unchanged for these three SNPs (Fig 8). Intrinsic clearance for was significantly decreased in C419T compared to *1a, slightly decreased in *4 and remained the same in C1007G (Table 8).

Uptake experiments with E₂17G were performed with 0.1, 1, 5, 10, and 20 μM of substrate incubated for 3 minutes. No uptake was observed again for variants G298A, *9, and

C1738T. V_{\max} was again slightly lowered in C419T and *4, and slightly increased in C1007G, but not statistically significant. K_m in C419T and C1007G remained the same as

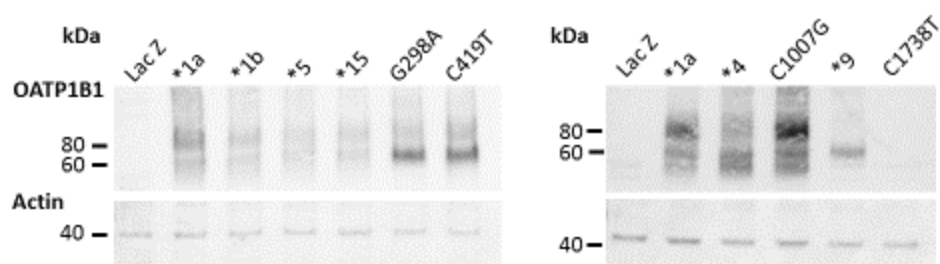


Figure 6. Immunoblots of whole cell lysates obtained from HeLa cells transfected with Lac Z, OATP1B1*1a and variants. Blots were probed with OATP1B1 antibody (top panels), stripped, and probed again with actin antibody (bottom panels).

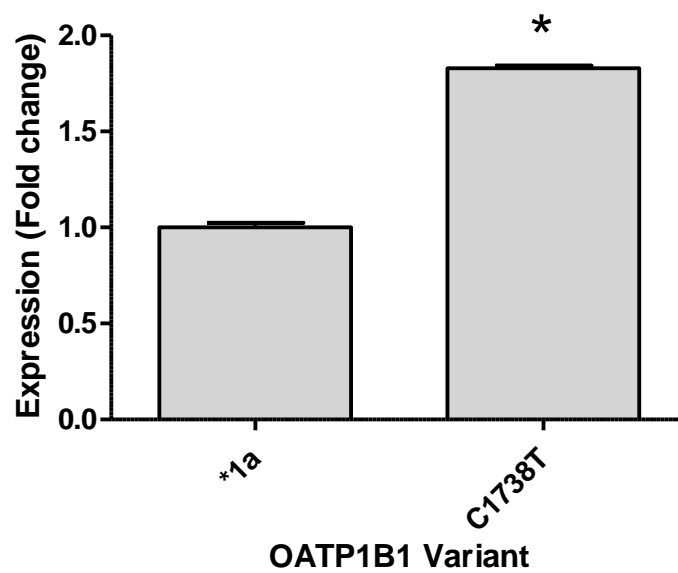


Figure 7. mRNA expression of *SLCO1B1**1a and *C1738T* determined by qPCR. *C1738T* mRNA was 1.8-fold higher than *1a ($p < 0.0001$).

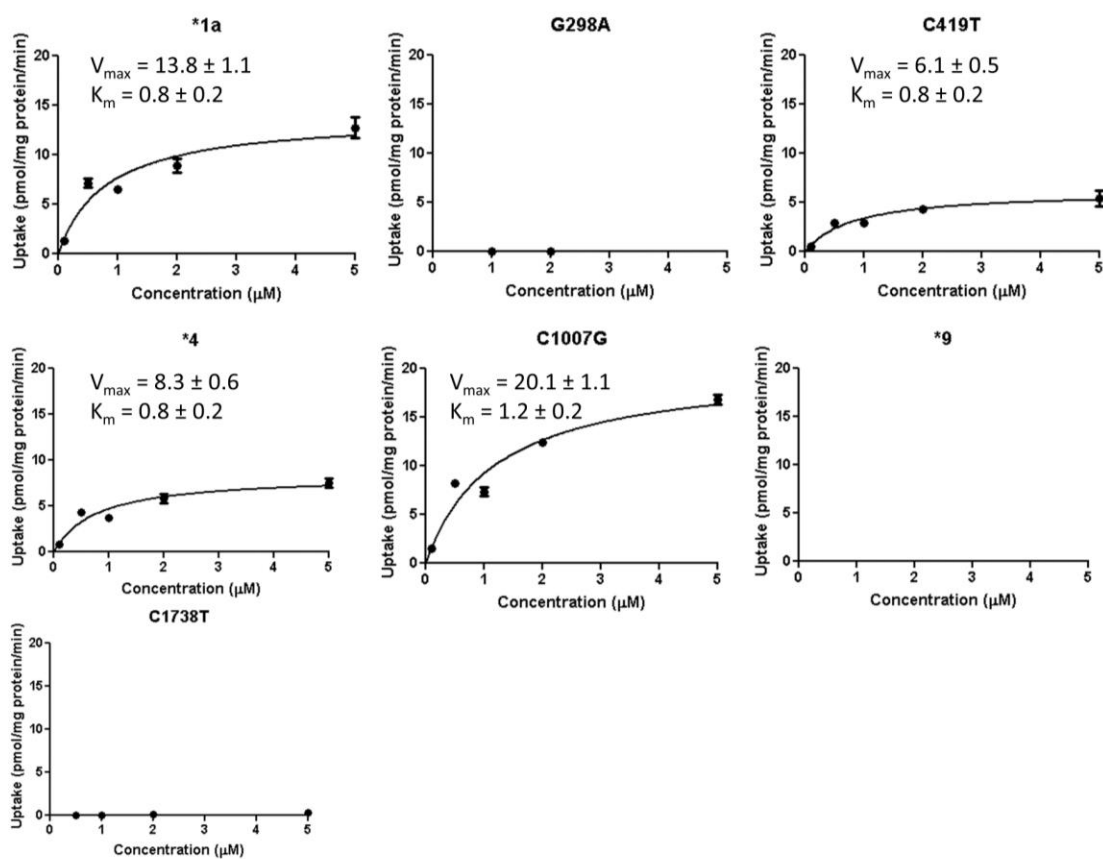


Figure 8. Concentration-dependent uptake of E1S by OATP1B1 wildtype and variants. Data are expressed as mean \pm S.E. ($n = 3 - 5$). Kinetic parameters were obtained by non-linear curve fitting.

Table 8. Intrinsic clearance of E1S uptake by OATP1B1 wildtype and variants.

OATP1B1	V_{\max}/K_m
*1a	17.9 ± 2.7
C419T	$8.4 \pm 2.2^*$
*4	10.9 ± 1.0
C1007G	17.3 ± 1.5

* denotes that value is significant different from *1a; $p < 0.05$.

*1a. *4 had an increase in K_m , but still not statistically significant due to variation (Fig. 9). Intrinsic clearance was decreased in C419T, remained the same in *4, and increased in C1007G, but all not statistically significant (Table 9).

Uptake experiments with rosuvastatin were also performed with 0.1, 1, 5, 10, and 20 μM of substrate incubated for 3 minutes. G298A, *9, and C1738T showed no uptake again. V_{max} was unchanged in *4 and increased significantly in C1007G compared to *1a, and K_m was approximately the same for both *4 and C1007G compared to *1a. (Fig. 10). Curve fitting could not be done for variant C419T so kinetic parameters could not be calculated. Intrinsic clearance was about the same in *4, and increased significantly in C1007G compared to *1a (Table 10).

4.7 Cell surface biotinylation reveals changes in cell surface OATP1B1 expression

Cell-surface protein isolation revealed profound changes in OATP1B1 expression between variants. G298A, and *9 variants had almost no expression of OATP1B1. C419T had a slightly decreased expression; *4 had similar expression compared to *1a and C1007G had an increase in expression. Calnexin, an intracellular protein, and Na^+/K^+ -ATPase, a membrane exclusive protein were probed to confirm cell-surface fraction (Fig. 11).

4.8 OATP1B1*4 genotyping

126 patients taking rosuvastatin were genotyped for their OATP1B1 *4 polymorphism. 85 were determined to be wildtype, 35 were heterozygous, and 6 were homozygous mutant. Figure 12 shows restriction fragment length polymorphism analysis of undigest, wildtype, heterozygous, and homozygous mutant samples. Patient characteristics are described in Table 11.

4.9 Haplotype analysis of rosuvastatin patient population

Statistical analysis program R was used to predict haplotypes based on genotype. 3 SNPs: *1b, *4, and *5 were considered in the analysis to generate a total of 7 different haplotypes and their observed frequencies. The combination of *1b and *4 SNPs has previously been defined as *14, and the combination of *1b and *5 has been defined as *15. However, this

is the first time that the 3 SNPs *1b, *4, and *5 have been identified on one haplotype, and will be defined as *37 (Table 12).

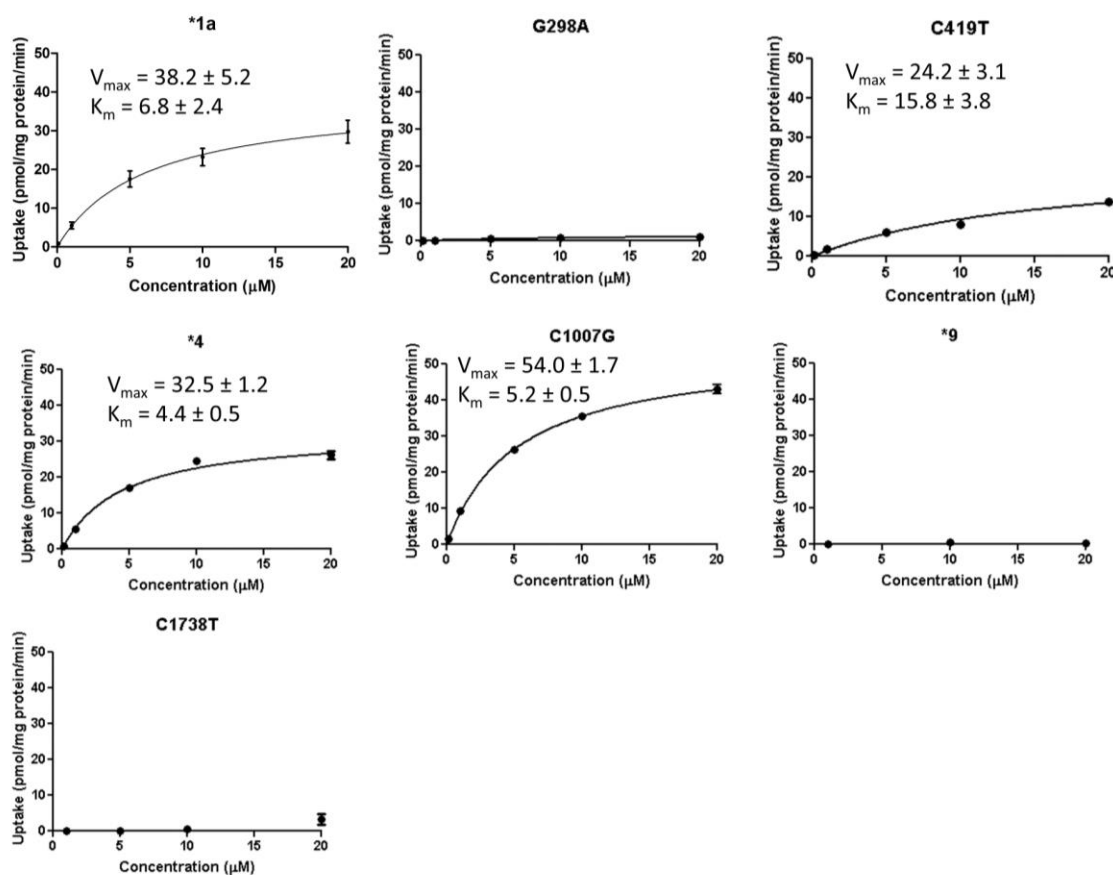


Figure 9. Concentration-dependent uptake of E₂₁₇βG by OATP1B1 wildtype and variants. Data are expressed as mean ± S.E. ($n = 3 - 5$). Kinetic parameters were obtained by non-linear curve fitting.

Table 9. Intrinsic clearance of E₂₁₇βG uptake by OATP1B1 wildtype and variants.

OATP1B1	V_{max}/K_m
*1a	6.6 ± 1.9
C419T	1.8 ± 0.4
*4	7.3 ± 0.5
C1007G	10.5 ± 1.7

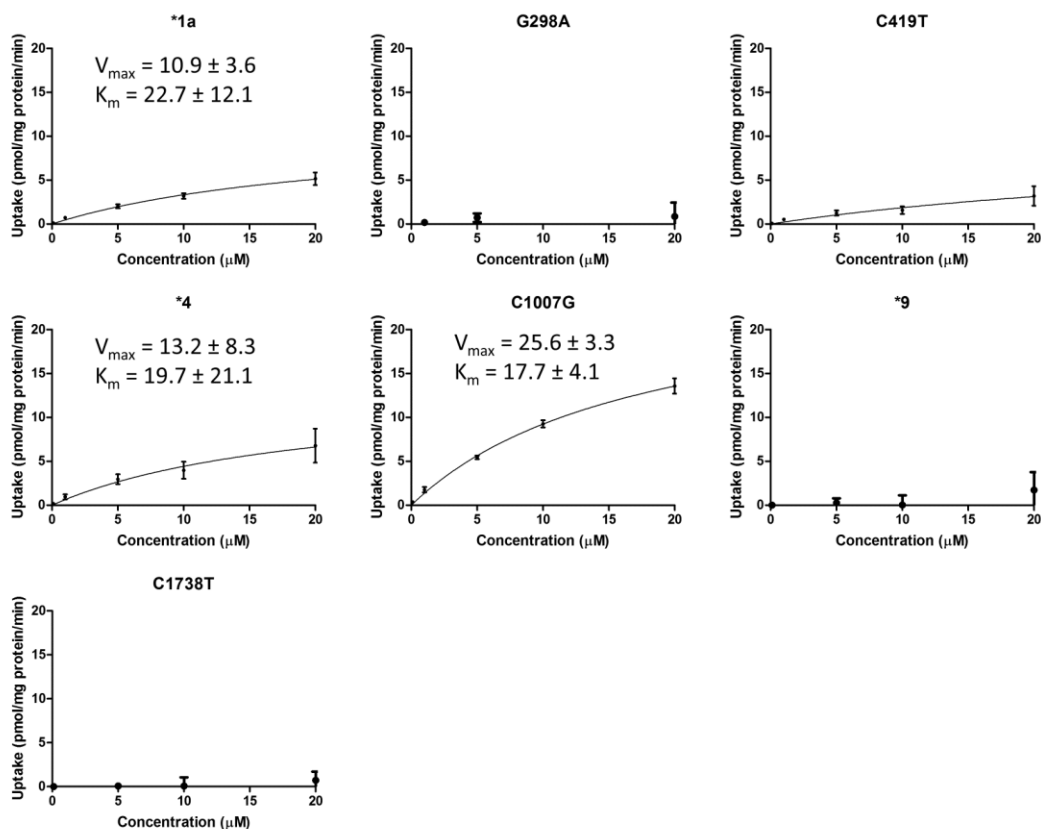


Figure 10. Concentration-dependent uptake of rosuvastatin by OATP1B1 wildtype and variants. Data are expressed as mean \pm S.E. ($n = 3 - 5$). Kinetic parameters were obtained by non-linear curve fitting.

Table 10. Intrinsic clearance of rosuvastatin uptake by OATP1B1 wildtype and variants.

OATP1B1	V_{max}/K_m
*1a	0.5 ± 0.1
*4	0.7 ± 0.2
C1007G	$1.5 \pm 0.2^*$

* denotes that value is significant different from *1a; $p < 0.05$.

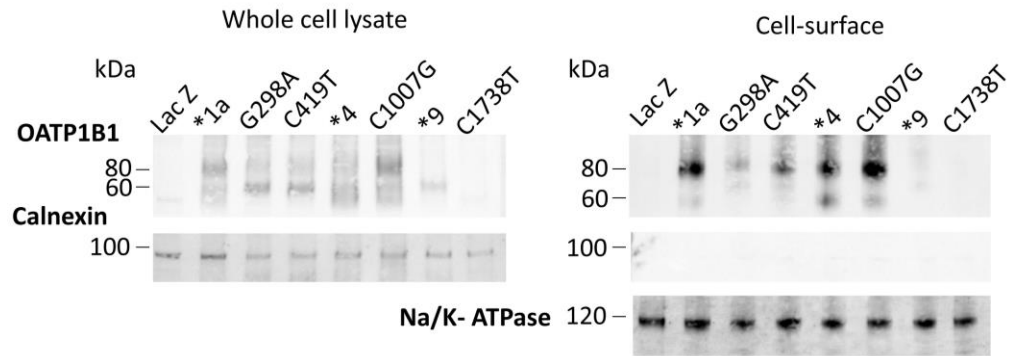


Figure 11. Immunoblot of whole cell lysates and cell-surface fractions obtained from HeLa cells transduced with OATP1B1 wildtype and variants. Blots were probed with OATP1B1 antibody (top panels), stripped and probed again with actin calnexin antibody (middle panels), then stripped again and probed with Na⁺/K⁺-ATPase antibody (bottom panel).

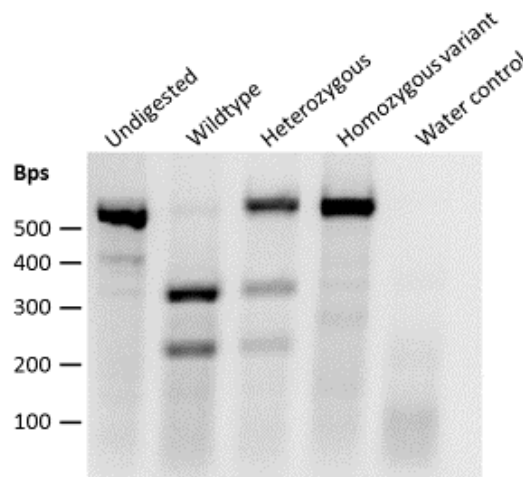


Figure 12. RFLP of wildtype, heterozygous, and homozygous variant *SLCO1B14 genotypes.**

Table 11. Rosuvastatin patient characteristics.

Number of patients	126
Sex (male)	88
Age (Years)	57.1 (19-80)
Ethnicity (Caucasian)	105
BMI (kg/m ²)	30.4 ± 7.2
Dose (mg/kg)	0.23 ± 0.14
5 mg	19
10 mg	37
15 mg	1
20 mg	35
30 mg	2
40 mg	32
Time since last dose (hours)	13.7 ± 3.3
Minor allele frequency	
SLCO1B1*1b	109/252 (43.2%)
SLCO1B1*4	47/252 (18.7%)
SLCO1B1*5	47/252 (18.7%)

Data are presented as mean (range) or mean ± SD.

Table 12. Observed haplotypes and frequencies in rosuvastatin patient population.

*1b	*4	*5	Haplotype	Frequency
			*1a	0.525
X			*1b	0.117
	X		*4	0.005
		X	*5	0.037
X	X		*14	0.166
X		X	*15	0.134
X	X	X	*37	0.016

X represents presence of polymorphic allele.

4.10 Effect of haplotypes on plasma rosuvastatin concentration

Multiple linear regression analysis of rosuvastatin patient population found that OATP1B1 *5 and *4 had significant effects on plasma rosuvastatin levels with p values of 0.0004 and 0.034 respectively, while *1b did not have significance with p value of 0.891 after adjusting for age, sex, ethnicity, BMI, dose, and time since last dose. However, since the SNPs *4 and *5 often occurs with *1b as *14 and *15, analysis was performed correlating haplotypes with rosuvastatin concentrations. 3 haplotypes were found to be statistically significant after adjusting for the same variables as above. *37 positively correlated with rosuvastatin concentration with $p = 0.02096$. *14 negatively correlated with rosuvastatin levels with $p = 0.00317$, and *15 showed positive correlation with $p = 0.00590$. A full list of haplotypes and their effect are listed in Table 13. The adjusted R^2 value for this model was 0.61. Dose and time since last dose unsurprisingly were major determinants of the variability observed, and the genetic component of the model contributed to 14% of the total variability with $p = 0.00026$ (Table 14).

Table 13. Effect and significance of haplotypes on log-transformed plasma rosuvastatin concentration.

Haplotype	Effect	Significance (p value)
*37	0.25	0.021
*14	-0.10	0.0032
*15	0.098	0.0059
*1b	0.0086	0.815
*4	-0.19	0.291
*5	0.10	0.151

Table 14. Contribution of variables considered in ANOVA model to variation in plasma rosuvastatin concentration.

Variable	Sum of Squares
Haplotype	0.8903
Age	0.0094
Sex	0.0061
Ethnicity	0.0147
BMI	0.0048
Dose	4.9021
Time since last dose	0.6939

Chapter 5

5 « Discussion »

Six non-synonymous SNPs in OATP1B1 were analyzed by *in vitro* techniques and their functional consequences were characterized. Loss-of-function, gain-of-function, and null-effect SNPs were all identified. One SNP with a high MAF was genotyped in rosuvastatin patients, and a significant correlation was found between genotype and plasma rosuvastatin levels. These findings further demonstrate the importance of understanding OATP1B1 SNPs in statin therapy and the prediction of statin-induced myopathy.

5.1 *In vitro* data show consistent results among substrates

In vitro functional analyses were performed with 3 different substrates E₁S, E₂17G, and rosuvastatin, and the effect of each SNP was consistent for each substrate. Substrate-dependent differences have often been shown by previous studies involving OATP1B1 SNPs and these substrates, but were not seen here (31). Transport for all substrates were totally abolished in G298A, *9, and C1738T variants, decreased in C419T, increased in C1007G, and unaffected in *4 (Table 15). The absence of substrate-dependent differences suggest that these SNPs are not located at sites of substrate recognition, and that the changes induced by these SNPs are purely due to changes in protein structure or interaction between the transporter and other proteins that changes the expression of the transporter.

5.2 No significant differences in intrinsic clearance was observed in OATP1B1 variants that exhibited transport

Concentration-dependent uptake studies were performed with all 6 OATP1B1 variants, and the results were analyzed by non-linear curve fitting to calculate V_{\max} and K_m . Subsequently, intrinsic clearances (V_{\max}/K_m) were calculated and compared against wildtype. G298A, *9, and C1738T variants did not exhibit any transport activity with any substrate, so Michaelis-Menten kinetic parameters could not be calculated. This is consistent with total uptake results as these variants also showed minimal total uptake.

For E₁S, V_{\max} and K_m were calculated for wildtype and variants C419T, *4, and C1007G. No significant differences were observed for V_{\max} and K_m of any of these variants compared

Table 15. Summary of *in vitro* findings for all substrates and OATP1B1 variants.

	G298A	C419T	*4	C1007G	*9	C1738T
E ₁ S	No transport	Reduced transport	No Change	Increased transport	No transport	No transport
E ₂ 17βG	No transport	Reduced transport	No Change	Increased transport	No transport	No transport
Rosuvastatin	No transport	Reduced transport	No Change	Increased transport	No transport	No transport

to wildtype (Fig. 8). Looking at intrinsic clearance, only C419T had a significantly lower intrinsic clearance, which is consistent again with total uptake data (Table 8). *4 had slightly lower intrinsic clearance that was not statistically significant while C1007G had similar intrinsic clearance compared to wildtype. Both of these observations were also consistent with total uptake of E₁S (Fig. 5).

For E₂17G uptake, V_{\max} and K_m were again calculated for wildtype and variants C419T, *4, and C1007G. V_{\max} , K_m , and intrinsic clearance for the variants were all not significantly different compared to wildtype, but show a similar trend as uptake with E₁S. C419T had slightly lower intrinsic clearance, *4 had intrinsic clearance similar to wildtype, and C1007G had slightly higher intrinsic clearance (Fig. 9, Table 9). This again is consistent with total uptake results with E₂17G which showed significantly lower uptake by C419T, no change in uptake with *4, and significantly higher uptake with C1007G (Fig. 5).

For rosuvastatin uptake, V_{\max} and K_m could only be calculated for wildtype and variants *4 and C1007G. Uptake was observed for C419T, but curve fitting did not reach an asymptote and therefore kinetic parameters could not be determined (Fig. 10). Increasing drug concentration beyond 20 μM in an attempt to extend the curve proved to be toxic to cells and could not be done. There were again no significant differences in V_{\max} and K_m of variants compared to wildtype (Fig. 10). Intrinsic clearance of *4 was again similar to wildtype, and intrinsic clearance of C1007G was significantly greater than wildtype (Table 10). Although intrinsic clearance of C419T could not be calculated, it can be seen on the graph that it seems to have lower V_{\max} compared to wildtype (Fig. 19). These results are again in agreement with total uptake results as well as the intrinsic clearances of these variants with the other two substrates (Fig. 5).

Although the intrinsic clearances were not statistically significant for most of the variants, it is apparent that for the variants exhibiting transport, the transport activity from lowest to highest is ordered: C419T, *4, and C1007G, with wildtype transport activity around the same as *4.

5.3 Changes in total transport is mediated by changes in transporter expression and not intrinsic transporter kinetics

Immunoblots for whole cell lysates of HeLa cells overexpressing OATP1B1 show 2 bands of OATP1B1 at ~80 kDa and ~60 kDa (Fig 6). The ~80 kDa OATP1B1 is a glycosylated version of the protein, and has been proposed to be the stable version expressed at the cell membrane. The ~60 kDa protein is unglycosylated, and as a result is unstable and likely degraded before implanted in the membrane (30, 80). Therefore it would be reasonable to assume that the expression of ~80 kDa protein would correlate with transport activity, which is observed here. Variants G298A, C419T, and *9 all have strong expression of the ~60 kDa band and very weak or no expression of the ~80 kDa band, and these variants were determined to have weak or no transport activity. *4 variant has slightly weaker expression of ~80 kDa band, and therefore has no changes in total transport with E₁S and E₂17G and slightly lower transporter with rosuvastatin. C1007G variant has much stronger expression of ~80 kDa band, and is again correlated with higher total transport of all substrates.

Concentration-dependent uptake studies revealed few significant differences in intrinsic clearance with most variants. Intrinsic clearance was significantly lower for C419T when transporting E₁S, and significantly higher for C1007G when transporting rosuvastatin. Overall, the lack of significance show that intrinsic properties of the transporter were not altered. Therefore the SNPs investigated in this study do not significantly change transporter structure, but rather protein-protein interaction and transporter expression.

5.4 Cell membrane fraction exclusive expresses ~80 kDa OATP1B1 and correlates strongly with transport activity

Cell-surface biotinylation was used to isolate membrane bound proteins, and the isolated fraction was probed with OATP1B1 antibody. Unlike whole cell lysates where 2 bands were identified at ~80 kDa and ~60 kDa, only 1 band at ~80 kDa was observed (Fig. 11). This is consistent with the idea that ~80 kDa OATP1B1 is stably expressed at the cell membrane and the ~60 kDa version is not (80). Furthermore, the strength of the ~80 kDa

band correlates very well with total uptake, similar to but more profound than the correlation seen with whole cell lysates. *4 variant has cell membrane OATP1B1 expression comparable to that of wildtype, and therefore also have similar transport activity. C419T has decreased expression and decreased transport compared to wildtype. C1007G has increased expression with higher transport compared to wildtype. G298A, *9, and C1738T variants have minimal or no expression of OATP1B1, and predictably have no transport activity. This data shows again that transport activity is more strongly dictated by cell surface expression level of ~80 kDa OATP1B1, and less so by intrinsic activity of the transporter.

5.5 OATP1B1*4 SNP is more prominent in Caucasian population

NCBI database reports the OATP1B1 *4 variant to have a MAF of 0.0785. Detailed look at NCBI data revealed Americans with European descent to have the highest frequency of heterozygotes compared to all other ethnicities. In our rosuvastatin patient population, 126 patients were genotyped for *4 SNP and a MAF of 0.18 was observed, more than double the MAF reported by NCBI. Out of 126 patients, 105 identified to be Caucasian and 21 were non-Caucasian (Table 11). MAF within the Caucasian population was slightly higher at 0.20 and out of the 21 non-Caucasians, only 3 were observed to be heterozygous and the rest were homozygous wildtype. This shows that the variant allele of OATP1B1 *4 may have higher frequency in Caucasians than other ethnicities.

5.6 OATP1B1 genotype significant contributes to variability in plasma rosuvastatin concentrations

Variables age, sex, ethnicity, BMI, dose, time since last dose, and 7 different observed haplotypes were analyzed in a regression model against log-transformed rosuvastatin plasma concentrations. It was determined that the genotype, dose, and time since last dose were significant contributors to variability in plasma rosuvastatin concentrations, with the genotype contributing 14% (Table 14). If dose and time since last dose are excluded from the model, genotype contributes to 97% of the observed variability, which means that apart

from dosage, almost all the variability in plasma rosuvastatin concentrations stem from the SNPs *1b, *4, and *5.

5.7 OATP1B1*4 almost always occurs with *1b as *14 and correlates with lower plasma rosuvastatin concentrations

OATP1B1 *4 and *14 were characterized in 2001 but have not been studied in detail since. After genotyping rosuvastatin patients for OATP1B1 *4, it was observed that carriers for *4 were almost always also carriers for *1b. Using haplotype analysis, it was revealed that the *4 genotype alone only has a frequency of 0.5% while the *14 genotype has a frequency of 17% (Table 12).

OATP1B1 *14 is significantly correlated with lower plasma rosuvastatin levels with $p = 0.003$. Patients with the *14 haplotype would be predicted to have lower chance of rosuvastatin-induced myopathy due to lower plasma levels (Table 13). A recent study found that *14 correlated with lower atorvastatin AUC (91). This is consistent with our findings and further show that *14 may be a protective genotype for statin therapy.

Previous findings of OATP1B1 *1b have been controversial in correlating genotype with drug concentrations (31). This could be due to lack of consideration of *4. Since *14 is fairly common in the population, it is likely that many individuals genotyped as *1b is actually *14. While *14 significantly affects rosuvastatin concentrations and *1b does not, it would explain to a degree the variability seen in studies examining the *1b genotype.

5.8 *In vitro* data with *4 does not correlate with *in vivo* findings

Total uptake and kinetic data with *4 polymorphism showed that it has a very moderately decreased to no change in uptake compared to wildtype. Interestingly, total uptake with rosuvastatin actually showed a significantly decreased uptake (Fig. 5). This would mean that patients that are carriers for *4 should have significantly higher rosuvastatin concentrations due to impaired hepatic uptake. However, this is not the case as seen above. Although *in vitro* studies with *14 were not conducted, previous studies showed that *14

does not show significantly different uptake compared to *1a (76). Since functional studies showed that changes in cell-surface expression was the primary driver of changes in transport, it is likely that there are regulatory mechanisms affected by *14 that increased the expression of OATP1B1 at the basolateral membrane of hepatocytes. There is a great amount of variation in OATP1B1 expression in the liver that cannot be explained by genetics, and the mechanisms of this variation remain unknown (93). With further research into the regulatory mechanisms of OATP1B1 expression, we may be able to identify the reason that *14 is correlated with lower plasma rosuvastatin levels.

5.9 Carriers for C1007G may have decreased chance of statin-induced myopathy

Although data with *4 did not suggest correlation between *in vitro* and *in vivo* results, previous studies with *5 and *15 did show strong correlation with decreased uptake *in vitro* and increased statin concentrations *in vivo* (76, 80). Therefore it is possible that carriers for the gain-of-function polymorphism C1007G would have a more active OATP1B1, and thus have better hepatic uptake of statins and lower risk for myopathy. Since this polymorphism is rare, it was not genotyped in our population. It would be interesting to see in a larger population if this SNP significantly affects plasma statin levels.

5.10 Conclusions

The results partly support the hypothesis that OATP1B1 variants will have decreased transport of the substrates E₁S, E₂17βG, and rosuvastatin compared to wildtype. Variants G298A, C419T, *9, and C1738T all exhibited decreased uptake, while *4 showed no significant difference in uptake and C1007G showed an increase in uptake. The *4 findings are consistent with previous *in vitro* data, but it was surprising to find C1007G to be a gain-of-function polymorphism. The mechanism for the gain-of-function is unknown and would require deeper insight into the tertiary structure of the transporter. The changes in uptake observed were all facilitated by changes in cell-surface expression, and not by changes in V_{\max} or K_m .

Only *SLCO1B1*4* was studied *in vivo* due to its high MAF. Contrary to the hypothesis, carriers for this allele had lower plasma rosuvastatin concentrations despite it having no effect on the uptake of rosuvastatin *in vitro*. This shows that a SNP's *in vitro* effect can be opposite of its *in vivo* effect, and that polymorphisms in the transporter can affect other biological mechanisms apart from intrinsic transporter properties to generate an overall *in vivo* effect.

This study was successful in determining new pharmacogenomic predictors for plasma statin levels. Patients carrying the *SLCO1B1*14* genotype are predicted to have lower plasma rosuvastatin concentrations and have lower chance of experiencing statin-induced myopathy, so their rosuvastatin dosing can be more aggressive to achieve desired LDL-C levels.

5.11 Limitations

One of the significant limitations of the adenoviral expression system is that there is no control of the amount of transporter expressed other than transducing cells with the same MOI of adenovirus. The viral production machinery in cells may vary and each cell would be expressing a different amount of transporter. Also, a different adenovirus is generated for each SNP, so it is assumed that the different viruses have the same capacity for infection. This however may not be the case and may be the cause for difference in OATP1B1 expression rather than the SNPs.

Protein expression for C1738T could not be analyzed by western blot due to the absence of C-terminus sequence that the antibody binds to. Although mRNA levels were measured, it is not a perfect predictor of what occurs at the protein level. It is likely that the protein is degraded prematurely and is not implanted at the membrane, but an antibody that binds to another sequence along the protein would confirm expression of the protein. However OATP1B1 antibodies are not common and the one used in this study is the only one available and functional.

5.12 Future directions

OATP1B1 SNPs and pharmacogenomics in general have advanced a long way since the Human Genome Project. There is now undeniable evidence that personalized medicine can improve clinical outcomes (33). Pharmacogenomics began with the identification of SNPs and other small genetic variations, but it is now economically feasible to sequence entire genes to search for novel SNPs and other types of genetic differences that can contribute to variation in drug response. Patients experience severe cases of statin-induced myopathy can have hundreds of candidate genes sequenced that may contribute to statin pharmacokinetics, and genetic differences can be noted and compared with other individuals. By sequencing entire genes, rare or novel mutations would be identified. Since the approach starts with a phenotype and works backwards to identify a genotype, the genetic variations observed are more likely to have clinical significance. The genetic differences found by this method can then be taken back to the lab and functionally analyzed in *in vitro* or *in vivo* models with the knowledge of the corresponding clinical phenotype. With sequencing of entire genes, there would surely be a rapid growth in the number of clinically significant mutations, and would greatly enhance the impact of personalized medicine.

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