INTERACTIONS OF ANTIRETROVIRAL PROTEASE INHIBITORS WITH HEPATIC TRANSPORT PROTEINS: MECHANISMS OF DRUG-INDUCED LIVER INJURY

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ABSTRACT

LATOYA M. GRIFFIN: Interactions of Antiretroviral Protease Inhibitors with Hepatic Transport Proteins: Mechanisms of Drug-induced Liver Injury (Under the direction of Dr. Kim L. R. Brouwer)

Lopinavir and ritonavir are protease inhibitors available as a coformulation for the management of HIV infection. However, liver enzyme elevations are associated with protease inhibitor use. Inhibition of bile acid transport leading to cellular accumulation of bile acids is one proposed mechanism of drug-induced liver injury (DILI). The global objective of this project was to investigate the influence of coadministered protease inhibitors on the hepatobiliary disposition of bile acids. Canalicular excretion of bile acid transport is facilitated by the bile salt export pump (BSEP). Impaired BSEP activity is a risk factor in the development of DILI. Drugs that decrease BSEP function are considered liver liabilities from a drug development perspective. Potent inhibitory activity of lopinavir and ritonavir *in vitro* has been demonstrated previously. However, the combined effect of lopinavir and ritonavir on the hepatobiliary disposition of bile acids has not been determined. Experiments were undertaken to determine the consequences of coadministered lopinavir and ritonavir on hepatocellular viability and bile acid transport. Lopinavir, alone and combined with ritonavir, demonstrated minimal toxicity but inhibited the biliary excretion of taurocholate and chenodeoxycholate in sandwich-cultured rat hepatocytes (SCRH). Studies in suspended rat hepatocytes revealed that neither lopinavir nor ritonavir altered the initial uptake

of either bile acid. Contrary to expectations, 24-hour exposure to lopinavir and ritonavir significantly *decreased* measured endogenous bile acid concentrations in SCRH. Lastly a genetic association study was carried out to explore the relationship between genetic variants in genes involved in bile acid transport or metabolism and risk of DILI. A comparison of data from patients in the Drug-induced Liver Injury Network to controls obtained from the British Birth Cohort revealed a significant association between the rs2919351 variant in OSTß and susceptibility to cholestatic and mixed liver injury. This work demonstrates that 10-minute lopinavir and ritonavir exposure, alone and combined, significantly impaired the biliary excretion of exogenously administered bile acids. However, 24-hour exposure to lopinavir and ritonavir evoked little toxicity *in vitro*. The lack of toxicity may be due to protective mechanisms in normal-functioning hepatocytes, such as a decrease in both the synthesis and cellular retention of endogenous bile acids.

I dedicate this work, with love and adoration, to the research project of a lifetime:

Jasmine Victoria Griffin.

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Above all, I give honor and thanks to God, for His perpetual favor and grace. May my accomplishments be a testimony to His power.

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ABC	ATP-Binding Cassette
AIDS	Acquired Immunodeficiency Syndrome
ATP	Adenosine Triphosphate
AUC	Area Under the Curve
BAC	BA–CoA synthase
BAT	BA-CoA:amino acid N‑acyltransferase
BCRP	Breast Cancer Resistant Protein
BEI	Biliary Excretion Index
BRIC	Benign Recurrent Intrahepatic Cholestasis
BSEP	Bile Salt Export Pump
CAR	Constitutive Androstane Receptor
CDCA	Chenodeoxycholic Acid
CGamF	Cholyl-Glycylamido-Fluorescein
CHO	Chinese Hamster Ovary
CYP	Cytochrome P450
DDI	Drug-drug Interaction
DILI	Drug-induced Liver Injury
DMSO	Dimethyl Sulfoxide
FXR	Farsenoid X Receptor
GCA	Glycocholic Acid
GCDCA	Glycochenodeoxycholic Acid

HAART	Highly Active Antiretroviral Therapy
HBMEC	Human Brain Microvascular Cell
HCV	Hepatitis C Virus
HEK	Human Embryonic Kidney
HIV	Human Immunodeficiency Virus
ICP	Intrahepatic Cholestasis of Pregancy
IL	Interleukin
LPV	Lopinavir
LPV/r	Lopinavir/Ritonavir
MDCK	Madin Darby Canine Kidney
MRP	Multidrug Resistance-associated Protein
NNRTI	Non-nucleotide Reverse Transcriptase Inhibitor
NRTI	Nucleotide Reverse Transcriptase Inhibitor
NTCP	Sodium-taurocholate Cotransporting Polypeptide
OATP	Organic Anion Transporting Polypeptide
OCT	Organic Cation Transporter
PFIC	Progressive Familial Intrahepatic Cholestasis
PBMC	Peripheral Blood Mononuclear Cell
P-gp	P-glycoprotein
PI	Protease Inhibitor
PXR	Pregnane X Receptor
RTV	Ritonavir
RXR	Retinoid X Receptor

SCRH	Sandwich-cultured Rat Hepatocyte
SLC	Solute Carrier
SNP	Single Nucleotide Polymorphism
TCA	Taurocholic Acid
TCDCA	Taurochenodeoxycholic Acid
TMCA	Tauromuricholic Acid
TNF	Tumor Necrosis Factor
UDCA	Ursodeoxycholate
UGT	Uridine diphosphate glucuronosyltransferase

CHAPTER 1

INTRODUCTION

Membrane transport proteins facilitate the absorption, distribution and elimination of numerous xenobiotics and endogenous compounds in humans. Consequently, transporters may be important determinants of the pharmacokinetic disposition and ultimately, the efficacy and safety of therapeutic agents. Many studies in knock-out rodent models and humans with loss-of-function genetic variants have demonstrated substantial changes in bioavailability, virologic resistance, and adverse drug reactions. These findings highlight the significance of drug-transporter interactions. Additionally, emerging studies in both humans and *in vitro* models (e.g. freshly isolated hepatocytes) support the hypothesis that impaired bile acid transport increases the risk of drug-induced liver injury (DILI).

Liver toxicity is a common adverse event associated with the use of antiretroviral protease inhibitors (PIs). Interestingly, the pharmacokinetic profile of PIs is highly variable, making it difficult to predict the risk of the development of hepatotoxicity in patients. Inhibition of the bile salt efflux pump (BSEP) leading to the cellular accumulation of bile acids has been shown for PIs. In an effort to better predict the risk of cholestasis, few clinical studies have attempted to characterize the

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relationship between antiretroviral therapy and plasma concentrations of bile acids however, the link remains unclear. Understanding the role of transport proteins in the overall disposition of drugs and/or bile acids is required to individualize drug therapy and improve health outcomes. The first section of this chapter highlights clinically significant interactions of transporters with PIs. The second and third sections discuss toxicity associated with PI therapy and regulation of bile acid synthesis and transport. The final portion of this introductory chapter provides a brief overview of *in vitro* tools currently employed to evaluate interactions between drugs, endogenous compounds, and transport proteins.

PART I. Influence of Drug Transport on Pharmacokinetics and Drug Interactions of HIV Protease Inhibitors

Saquinavir was the first PI introduced to the U.S. market in 1995 for the treatment of HIV/AIDS.[1] This class of life-saving antiretroviral agents has expanded to now include eight PIs that play an important role in the management of HIV infection.[2] Currently, the most frequently prescribed HIV PIs include lopinavir, atazanavir, darunavir and fosamprenavir, each of which is typically used in combination with one or more Nucleoside Reverse Transcriptase Inhibitor (NRTI) in Highly Active Antiretroviral Therapy (HAART) regimens.[3] In addition, more recent clinical data support the potential utility of HIV PI monotherapy in patients with prolonged viral suppression on HAART,[4] further illustrating the unique efficacy profile of these antiretroviral agents. The spectacular improvements in treatment success and life expectancy in patients with HIV infection can be attributed, in part,

to the long-term suppression of HIV replication by antiretroviral regimens with acceptable side-effect profiles.[5] HIV PIs currently are key components of first-line therapy in both treatment-naive and -experienced patients. A major challenge in antiretroviral pharmacotherapy is the potential for gradual development of viral resistance. The introduction of 2nd generation PIs such as darunavir, which require at least four concomitant mutations in the viral genome for resistance development, has provided clinicians with superior drugs to counter the development of resistance.[6]

Physicochemical properties of the HIV PIs are summarized in **Table 1.1**. In general, HIV PIs are peptidomimetic, large molecular weight, and often poorly water soluble compounds. Consistent with their physicochemical properties, HIV PIs tend to be highly protein bound and extensively metabolized by cytochrome P450 (CYP) 3A4 (**Table 1.8**), with relatively short terminal elimination half-lives in plasma. Long-term therapeutic success can be maintained only when minimum trough concentrations of the HIV PIs are achieved.[4] Rapid elimination from plasma requires multiple daily doses of HIV PIs to maintain therapeutic concentrations, which complicates patient adherence to therapy. Ritonavir is a remarkably potent mechanism-based inhibitor of CYP3A4. Concomitant administration of a subtherapeutic dose (100-200 mg) of ritonavir as a pharmacokinetic booster ("enhancer") together with HIV PIs increases exposure of lopinavir, atazanavir and darunavir several-fold.[7] The use of ritonavir as a "boosting" agent was a major advance in HIV PI-based therapy,[8,9] and has led to the development and marketing of once-daily dosage forms of HIV PIs, which has significantly increased patient

adherence. In addition, the clinical use of ritonavir-boosted HIV PIs has improved the side-effect and toxicity profile of HAART regimens.[10] For example, the addition of ritonavir to atazanavir-based dosing regimens resulted in decreased incidence of lipoatrophy as compared to unboosted treatments.[11]

Although the clinical strategy of using ritonavir as a boosting agent has enhanced the success of HIV PI-based antiretroviral regimens, it also has resulted in increased potential for drug-drug interactions (DDIs).[12] Drugs metabolized by CYP3A4 exhibit much longer elimination half-lives in ritonavir-treated patients as compared to other patients. Additional levels of complexity with respect to DDI potential are encountered in patients co-infected with *M. tuberculosis*, an infection that is increasing in prevalence in resource-limited countries.[13] Successful eradication of tuberculosis almost always requires administration of the very potent CYP3A4-inducer rifampicin, or the less potent but more expensive inducer rifabutin. These drugs reduce exposure to ritonavir-boosted HIV PIs.

Hepatic metabolism is an important step in the systemic elimination of HIV PIs. Importantly, drug transporters also play a key role in the oral bioavailability, hepatobiliary elimination and distribution of HIV PIs to target (lymphocytes) and peripheral (brain) tissues. The recent approval of PIs for the treatment of hepatitis C has increased the number of patients who are exposed to this class of drugs, and emphasizes the importance of understanding factors that influence their pharmacokinetics and DDI potential.

The Impact of Transporters on Protease Inhibitor Pharmacokinetics/ Pharmacodynamics. Pharmacological and toxicological effects of PIs are determined by drug absorption and distribution which are influenced by transporter-mediated processes. Thus, identifying transport proteins that interact with PIs and understanding the magnitude of their contribution to overall drug disposition is critical. Although PIs are known to inhibit active transport processes, data regarding the ability of PIs, themselves, to act as substrates for uptake proteins remains controversial. Significant temperature-dependent uptake of ritonavir, saquinavir and nelfinavir into suspended rat hepatocytes, indicative of active uptake processes, has been reported.[14] In addition to evidence provided by limited *in vitro* studies, the physicochemical properties of PIs (e.g., molecular size, protein binding, and lipophilicity) also should be considered. Localization and orientation of membrane transporters in a generalized cell is illustrated in **Figure 1.1**. Transporters have been well characterized in the liver, kidney and, to a lesser extent, the brain and intestine. Unfortunately, one challenge in the field is that the expression, localization and functional activity of transport proteins at target sites for viral transmission and sequestration, including the testicular system, female genital tract, lymphocytes and placenta are poorly characterized. However, this lapse in scientific knowledge is appreciated and studies in this area are ongoing. For an in depth discussion of the interactions between antiretroviral agents and transporters at these relevant organ systems see Kis *et al*. 2010.[15] The following discussion serves as an overview of solute carrier (SLC) and ATP-binding cassette (ABC) membrane transport proteins involved in the uptake and efflux of PIs known to date.

Impact of SLC Transporters on Protease Inhibitor Pharmacokinetics/Pharmacodynamics. Transporter-mediated uptake, largely governed by members of the SLC superfamily, may be rate limiting in the oral bioavailability and hepatobiliary clearance of drugs. The most prominent transporter interactions with PIs involve the organic anion transporting polypeptides (OATPs) and organic cation transporters (OCTs); the clinical relevance of these interactions has been well documented.

OATPs

OATPs, which are expressed in numerous organs and tissues including the intestine, liver, kidney, and placenta, mediate the sodium-independent bidirectional transport of diverse substrates including bile acids, bilirubin and xenobiotics.[16,17] OATPs interact with several PIs *in vitro*. OATP1A2, -1B1 and -1B3 expressed in *Xenopus laevis* oocytes mediate the uptake of lopinavir and saquinavir.[18-20] Darunavir transport via OATP1A2- and -1B1- also has been reported.[19,20] Lopinavir, atazanavir, darunavir, ritonavir, and saquinavir inhibit OATP1B1- and -1B3-mediated CGamF accumulation in chinese hamster ovary (CHO) cells. Inhibition of OATP2B1-mediated transport of estrone 3-sulfate by atazanavir, lopinavir, tipranavir, nelfinavir, indinavir, saquinavir, and ritonavir also has been shown in Caco-2 cells.[21,22] The clinical implications of these interactions are evident, for example, in the significant association between the OATP1B1 521T>C polymorphisms and elevated lopinavir plasma concentrations.[19] Additionally, a recent pharmacogenetics

study revealed that variability in lopinavir clearance was impacted by both genetic variants in OATP1B1 and ritonavir plasma concentrations.[23]

OCTs

OCTs, which are located predominantly in the kidneys and liver, are electrogenic uniporters that primarily transport small cations in a sodium-independent manner. Transport of uncharged and anionic compounds such as prostaglandins by OCTs has been described.[24] OCT1 and OCT3 are expressed at the sinusoidal membrane of liver tissue. OCT1 is expressed exclusively in the liver while OCT3 has a broader range of tissue distribution. Nelfinavir, ritonavir, indinavir, and saquinavir are reportedly potent inhibitors, but poor substrates, of OCT1- and OCT2-mediated transport.[25,26] Though the contribution of OCTs to PI transport remains unclear, several nucleoside NRTIs are translocated by OCTs and often are coadministered with PIs, increasing the risk of DDIs.

The Impact of ABC Transporters on Protease Inhibitor Pharmacokinetics/Pharmacodynamics. Members of the ABC transporter superfamily comprise one of the largest protein families with representatives in all living organisms. The structure and function of ABC transporters are relatively conserved across species. ABC transporters facilitate the transmembrane movement of substrates by utilizing the energy generated by ATP hydrolysis.[27] Mounting evidence suggests that ABC transport proteins confer drug resistance and alter PI pharmacokinetics/pharmacodynamics by decreasing bioavailability,

promoting sequestration at sanctuary sites, and decreasing accumulation in target organs and tissues.[28] This review focuses solely on ABC transporters clinically shown to impact the disposition of PIs.

P-gp

P-glycoprotein (P-gp; MDR1), which is expressed ubiquitously, protects cells from the accumulation of toxic drugs, metabolites, and endogenous compounds. P-gp exhibits broad substrate specificity, including PIs. Expression of P-gp in the intestine, brain and blood-testis barrier alters oral bioavailability and intracellular concentrations of PIs *in vivo*.[29-31] P-gp-mediated efflux of all currently marketed PIs has been demonstrated in several *in vitro* systems, including Caco-2 and MDCK-II cells.[32-36] Ritonavir, lopinavir, and nelfinavir also inhibit P-gp-dependent efflux of calcein-AM in MDCK-II cells.[37] In addition to inhibition of P-gp transport, saquinavir and darunavir induce P-gp mRNA expression and activity *in vitro*. Induction by darunavir increased cellular resistance, as measured by growth inhibition assays in LS-180 cell lines.[38]

BCRP

Breast cancer resistance protein (BCRP) is expressed in the liver, kidney, testis, GI tract and a many other tissues. BCRP is responsible for the extrusion of a broad range of both endogenous and exogenous compounds. Many PIs including lopinavir, nelfinavir, saquinavir, and ritonavir are effective inhibitors of BCRP-mediated transport, but appear to be poor substrates *in vitro*.[39-41] Although BCRP-

mediated transport of PIs has not been elucidated, BCRP activity is known to alter systemic and tissue concentrations of a antiretrovirals and in particular, PIs.[42] Consequently, the likelihood of DDIs between PIs and BCRP substrates remains a concern.

MRPs

To date, there are nine members of the multi-drug resistance-associated protein (MRP) transporter family. MRPs 1-5, all organic anion pumps, have been studied most extensively. MRP1 and MRP2 have similar substrate specificities; however, localization and tissue distribution differ. MRP1 is expressed widely and located in the basolateral membrane, while MRP2 is localized on the apical membrane and its expression is restricted primarily to the liver, kidney, and intestine. MRP3 is expressed on the basolateral membrane of the liver, kidney and gastrointestinal tract.[29,43] Common MRP1, MRP2, and MRP4 substrates include glutathione conjugates and anionic drugs. Bilirubin glucuronide is a substrate for both MRP2 and MRP3.[44,45] MRP2-mediated transport of saquinavir, ritonavir, indinavir, and lopinavir has been shown in stably transfected human MDCK-II cells.[32,46] Saquinavir, ritonavir, and atazanavir potently inhibit MRP2-mediated biliary efflux of CDF in human hepatocytes.[47] In a panel of ABC transporter over-expressing cell lines, atazanavir, lopinavir, and ritonavir inhibited MRP1 activity.[39] Furthermore, treatment with darunavir/ritonavir induced MRP1 protein expression in CD4 (+) T-cells from healthy human volunteers. MRP1-mediated efflux of carboxyfluorescein diacetate increased upon co-administration with efavirenz.[48] The contribution of MRPs to the transport of PIs remains unclear.

Drug-drug Interactions Involving Transporters and HIV Protease Inhibitors. Numerous *in vitro* and *in vivo* studies have demonstrated that most HIV PIs interact with both CYP3A4 and P-gp, either as a substrate, inhibitor or inducer.[8,49,50] Given the dominant roles of these proteins in drug disposition, most clinical DDI studies have focused on the contribution of CYP3A4 and/or P-gp.[8] In addition to the CYP3A4-mediated inhibition of PIs by ritonavir, a beneficial DDI that is utilized chemically in HAART regimens, synergistic effects have been observed with other combinations of HIV PIs. Dam and co-workers suggested that the synergistic inhibition of HIV-1 by a combination of saquinavir with lopinavir or atazanavir could be explained, at least in part, by enhanced inhibition of efflux mechanisms from target cells.[51] The complexity of HIV PI-based treatment regimens, often in combination with non-antiretroviral medication (e.g. anti-tuberculosis drugs), increases the potential for clinically significant DDIs (see www.hiv-druginteractions.org for a summary of risks and severity of antiretroviral DDIs). Unfortunately, details regarding the underlying mechanisms responsible for these DDIs are lacking, but clearly extend far beyond the involvement of CYP3A4 and P-gp. Clinically relevant changes in PI concentrations often may be the net result of multiple DDIs that have opposite effects (e.g. concomitant induction and inhibition); the outcome frequently depends on the exact dose and regimen (e.g. etravirine and darunavir/ritonavir in **Table 1.2**).[52] Another reason for the lack of mechanistic information is that the relative roles of drug metabolizing enzymes and transporters in drug disposition and DDIs remain poorly understood. **Tables 1.2** and **1.3** provide a summary of clinically relevant DDIs involving HIV PIs and drug

transporters; specific DDIs involving HIV PIs as 'perpetrator' drugs (**Table 1.2**), and those mediated by HIV PIs as 'victim' drugs (**Table 1.3**), are discussed below.

Mechanisms of HIV Protease Inhibitor DDIs: Drug Transporter Inhibition. HIV PIs are both substrates and potent inhibitors of some SLC and ABC transport proteins, and typically behave as perpetrators when considering DDIs elicited by transporter inhibition. In addition, when more than two HIV PIs are combined, different PIs can act as the perpetrator and victim. This is illustrated by the effect of atazanavir on the pharmacokinetics of saquinavir when coadministered with ritonavir.[53] Saturation and/or inhibition of efflux transporters modulating HIV PI accumulation may explain this interaction.

The most well documented DDIs with respect to transporter inhibition involve HIV PIs and the disposition of well-known P-gp substrates including digoxin, fexofenadine, and loperamide. For example, single or multiple dose regimens with indinavir/ritonavir increased fexofenadine plasma AUC up to 5- and 4.2-fold, respectively.[54] The most pronounced effects on digoxin exposure were reported after 300 mg bid ritonavir or 400/100 mg lopinavir/ritonavir in combination with intravenous or oral digoxin doses of 0.5 mg.[55] Loperamide exposure was increased more than 3-fold in the presence of 600 mg ritonavir.[56] Case reports of elevated tacrolimus or sirolimus concentrations when combined with ritonavir-boosted amprenavir or darunavir also support pronounced P-gp inhibition.[57,58] HIV PIs appear to exhibit much less pronounced (up to 37% increase) effects on the plasma exposure of the NRTI and P-gp substrate tenofovir following co-administration of the

disoproxil fumarate prodrug of tenofovir.[59] Minor increases in tenofovir plasma AUC values were observed, which were attributed to inhibition of P-gp mediated intestinal efflux of the prodrug.[59,60]

Compared to ABC transporter-based DDIs, much less is known about the potential role of HIV PIs in DDIs associated with uptake transporters. Limited data suggest that hepatic uptake transporters of the SLC family (specifically OATP1B1 and OATP1B3) are likely to play key roles in some DDIs involving HIV PIs. Shitara recently reviewed current clinical evidence demonstrating substantial alterations in the pharmacokinetics of OATP1B1 substrates (i.e. statins, repaglinide, and bosentan) in combination with the OATP1B1 inhibitor cyclosporin A. These data revealed increases in the AUC of atorvastatin of up to 9-fold.[61] Pronounced increases in the AUC of the lipid-lowering drugs atorvastatin and rosuvastatin have been reported with coadministration of boosted lopinavir and tipranavir; OATP1B1 inhibition has been suggested as a likely mechanism to explain this interaction.[62] Moderately decreased exposure to the NRTI elvucitabine when combined with a single 300 mg ritonavir dose may be attributed to ritonavir-mediated inhibition of intestinal uptake transporters.[63]

Finally, it is noteworthy that even though pronounced species differences exist,[47] several animal studies also support the role of transporters in mediating DDIs involving HIV PIs. For example, ritonavir enhanced darunavir absorption via P-gp inhibition in mouse *in situ* intestinal perfusions.[64]

Mechanisms of HIV Protease Inhibitor DDIs: Drug Transporter Induction.

Data obtained in various *in vitro* models have shown that HIV PIs show affinity for the pregnane X receptor (PXR), activation of which is clearly linked to regulation of drug metabolizing enzymes as well as drug transporter expression.[65,66] Induction of drug metabolizing enzymes by HIV PIs is a common mechanism underlying clinically relevant PI-associated DDIs.[67,68] Much less information is available with respect to the exact role of altered expression of drug transporters and changes in the pharmacokinetics of coadministered drugs relying on those drug transporters. Nevertheless, numerous examples in **Table 1.6** illustrate that most drug transporters are susceptible to the inducing effects of HIV PIs. Clinically relevant DDIs that may be attributed, at least in part, to HIV PI-mediated up-regulation of P-gp activity are included in **Table 1.2**. For example, there is a 2-3-fold decrease in loperamide exposure when combined with tipranavir/ritonavir (TPV/r).[69] The latter PI combination also significantly reduced exposure to the P-gp substrate digoxin, presumably through induction of P-gp, following concomitant doses of TPV/r.[70] It should be noted that the inducing effects of tipranavir predominate in contrast to ritonavir, which primarily inhibits P-gp when combined with loperamide or digoxin. The 25% reductions in fexofenadine C_{max} and half-life when combined with nelfinavir for 1 week, may be explained by induction of intestinal P-gp and/or hepatic OATPs.[71] The reduced exposure to delaviridine also could be due to induction of P-gp by amprenavir.[72] The NNRTI etravirine can be combined with several boosted PIs, including darunavir, lopinavir, and saquinavir;[73] however, when combined with TPV/r, the plasma exposure of etravirine is decreased by 76%. As etravirine is not a

P-gp, BCRP or MRP substrate,[74,75] induction of uptake transporters (e.g. OATPs) by tipranavir and/or ritonavir (in addition to induction of drug metabolizing enzymes) may contribute to this interaction.

Combined use of rifampicin, an anti-tuberculosis agent and potent inducer of drug metabolizing enzymes and transporters, with antiretroviral medication including HIV PIs is of high clinical relevance. As outlined in **Table 1.3**, reductions in HIV PI exposure when combined with rifampicin range from 75% to 89%, even in the presence of ritonavir as a boosting agent. When different LPV/r regimens combined with rifampicin were evaluated by La Porte *et al*., LPV/r combinations with higher ritonavir dose levels (i.e. LPV/r 400/400 > LPV/r 800/200) appeared to provide better compensation for the inducing effects of rifampicin.[76] This was especially reflected in the C_{min} concentrations achieved with the LPV/r 400/400 dose regimen, which tended to be comparable to the C_{min} concentrations achieved with the reference treatment of LPV/r 400/100 in the absence of rifampicin. Therefore, the use of rifabutin rather than rifampicin in the management of *M. tuberculosis* infection in HIV positive patients on antiretroviral therapy is highly recommended.

Transporter-mediated Processes Underlying Toxicity of HIV PIs. Both endogenous and exogenous (e.g. drugs) compounds are substrates for transporters. Interference of drugs with endogenous substrate transport may constitute a mechanism of drug-mediated toxicity. For example, interference of certain drugs (e.g. bosentan, troglitazone) with hepatic bile salt transport has been implicated as one mechanism in the development of drug-induced cholestasis.[77] Several HIV PIs

have been shown to interact with bile salt disposition in human and rat hepatocytes,[78] and this may explain, at least in part, the hepatotoxicity observed in some patients taking HIV PIs.[79] Rotger *et al*. quantified the effect of HIV PI-containing antiretroviral therapy on the incidence of hyperbilirubinemia in 96 HIV-infected patients. Atazanavir and indinavir (but not lopinavir, saquinavir, ritonavir, and nelfinavir) exhibited an increased incidence of elevated serum bilirubin concentrations.[80] Inhibition of the bilirubin conjugating enzyme UGT1A1 by these PIs has been proposed as a potential mechanism underlying this interaction. However, *in vitro* data generated by Campbell *et al.*[81] and Ye *et al*.[47] also support potent inhibition of OATP1B3, the bilirubin-transporter, by indinavir and atazanavir. As noted in **Table 1.7**, the altered lipid metabolism associated with HIV PI-based therapy may be caused by inhibition of transport of the endogenous substrate palmitate.[82]

Influence of HIV Infection, Co-infection and Antiretroviral Therapy on Transporters: Implications for Protease Inhibitor Pharmacokinetics/ Pharmacodynamics. The effect of HIV infection on transporter expression and activity is not well understood. Effects of diseases on P-gp mRNA expression and activity have been studied more extensively than other transport proteins. P-gp mRNA expression was decreased in leukocytes and PBMCs of SHIV infected macaques; changes in expression were more pronounced in animals receiving antiretroviral treatment that included indinavir. However, indinavir decreased P-gp expression, making it difficult to determine whether the disease state or indinavir

itself were responsible for the observed effects;[83] similar findings have been reported in humans. Lucia and colleagues reported that P-gp function in peripheral blood lymphocytes, as measured by rhodamine-123 efflux, was decreased in HIV-infected patients. Separate clinical studies in patients with HIV infection relative to healthy volunteers support these findings, although expression of MRP1 in PBMCs was not altered.[84] Increased MRP-mediated efflux also has been reported in patients with primary HIV infection that strongly correlates with disease progression.[85] In contrast, a time-dependent significant *increase* in P-gp expression in PBMCs from HIV+ individuals has been reported.[86]

The influence of hepatitis C co-infection on transporter function, and the potential implications for antiretroviral therapy, has been the subject of recent investigations due to the increasing prevalence of co-infection. MRP4 protein expression is induced in patients with cholestasis and animals with common bile duct ligation. These changes may facilitate compensatory MRP4-mediated basolateral efflux of endogenous compounds such as bile acids.[87-90] MRP2 mRNA levels also are significantly decreased in human HCV-infected liver tissue relative to non-infected tissue.[91] In addition, significant reductions in OCT1 and OATP1B1 mRNA which correlated with hepatitis C progression also have been reported in humans.[92]

MRP1 expression in total human lymphocytes is unaffected by atazanavir treatment, but increased in human brain microvascular endothelial cells (HBMECs). P-gp expression, however, was increased in both total lymphocytes and HBMECs.[93] In human PBMCs, efavirenz-mediated induction of MRP1 and MRP6 mRNA has

been reported. Tenofovir also was associated with a reduction in P-gp, MRP1, MRP5, and MRP6 mRNA expression in humans.[94]

Regulation of transporter expression by nuclear receptors such as PXR and CAR is now well-established. For example, induction of P-gp and MRP1 by ritonavir, and P-gp by saquinavir, both PXR agonists, has been reported.[95-97] Although a reduction in MRP1 protein expression in PBMCs of healthy volunteers following administration of darunavir/ritonavir was observed, the clinically relevant consequences of these changes remain unclear.[48]

Also, proinflammatory cytokines TNF-α, IL-1ß, and IL-6 are reportedly increased in HIV-infected patients and have been shown to modulate key transporters *in vitro.*[98-100] For example, all three aforementioned cytokines decreased MRP2 mRNA and protein expression in sandwich-cultured human hepatocytes. In the same study, IL-6 and IL-1ß BSEP mRNA expression was decreased while protein levels were increased.[99] NTCP, OCT1, OCT2, OATP1B1, -1B3, and -2B1 mRNA levels following 48-hour exposure to TNF-α or IL-6 were decreased. In addition, P-gp, MRP2, and BCRP mRNA were also reportedly decreased by IL-6. TNF-α also decreased BSEP mRNA and, conversely, increased BCRP and MRP3 protein expression levels.[100] Initiation of antiretroviral therapy is associated with a reduction in proinflammatory cytokine levels.[101] Cervia and colleagues report significantly decreased TNF-α and a nonsignificant trend towards reduced IL-6 in HIV-infected children initiating or changing antiretroviral therapeutic regimens.[98]

Evidence in the literature demonstrating a direct effect of HIV infection, co-infection and HAART therapy on transporter phenotype and function remains limited

and controversial for a number of reasons. The contribution of HIV infection, underlying symptoms, co-infection and antiretroviral therapy to pathophysiological changes are multifactorial and difficult to distinguish. In addition, appropriate models to investigate the intricate relationships are limited. The effect of HIV infection and co-infection on transporter function is the subject of ongoing investigations.

HIV PIs that interact with transport proteins are likely candidates for DDIs resulting in toxicity or the development of cellular resistance. Consequently, chemotherapeutic agents that exhibit minimal interactions with transport proteins such as P-gp are preferred.[102] Conversely, therapeutic agents that competitively inhibit transporters governing efflux may increase victim drug concentrations in relevant organs and tissues (e.g. lymphocytes), thereby enhancing efficacy and decreasing pill burden. For example, Pluronic P85, an amphiphilic block copolymer and P-gp inhibitor, increases saquinavir and nelfinavir accumulation in MDCKII-MDR1 cells.[103] Modulation of transport function is particularly promising given the difficulty of antiretrovirals to penetrate sites of viral sequestration, such as the brain, which expresses a number of efflux transporters known to interact with PIs, including P-gp, BCRP, and MRPs.[104-106] In addition to transporter interactions, HIV PIs may interact with cytochrome P450s, modify posttranscriptional regulation of nuclear receptors, and alter bile acid biosynthesis and metabolism. Gender, genetic polymorphisms and lifestyle choices such as smoking and alcohol consumption also must be taken into consideration when trying to predict the likelihood of drug-transporter interactions. Toxicity and efficacy associated with these interactions is undoubtedly multifactorial and remains difficult to predict. However clinicians,

scientists and regulatory agencies are becoming increasingly aware of the importance of understanding the dynamics of these relationships and are working together to ensure the emergence of safe and efficacious chemotherapeutic treatment options.

PART II. Hepatotoxicity Associated with Lopinavir and Ritonavir

LPV is only marketed in combination with RTV as a fixed-dose co-formulation under the tradename Kaletra®. LPV combined with RTV has become a front-line therapy in the treatment and management of HIV-1 infection. Despite the success of antiretroviral treatment, PI-associated hepatotoxicity, defined as > 5 times the upper limit of normal ALT or AST levels, may necessitate discontinuation of therapy and, consequently, virologic failure.[107] An accurate assessment of the incidence of PI-related hepatotoxicity in humans is difficult to establish due to limitations in study designs including patient inclusion/exclusion criteria, comorbidities, concomitant medications, and limited follow up. Although the results of studies combining data from multiple cohorts and databases continue to emerge, inconsistent definitions of liver toxicity and variability in patient populations often consisting of co-infected patients on numerous non-ARV drugs confound the interpretation of data.[108]

Unfortunately, mechanisms of liver toxicity related to PI exposure are poorly understood. The idiosyncratic nature of PI-induced hepatotoxicity makes prediction of adverse events in patients challenging. Growing evidence suggests that several factors increase the risk of hepatotoxicity in PI-treated patients including hepatitis B or hepatitis C co-infection, baseline liver function tests, length of drug therapy, and

gender (females exhibit a higher incidence than males). In an open, prospective, observational study conducted by Meraviglia and colleagues, 9.1% of patients treated with LPV/r developed liver enzyme elevations within the first 115 $\pm$ 85 days of initiating treatment. Of these patients, ~75% and 25% exhibited grades 2 and $\geq$ 3 toxicity, respectively.[109] In a one-year observational study conducted by Bongiovanni *et al*., hepatitis C co-infected patients treated with PI-containing HAART regimens exhibited a 7.4-fold greater risk of discontinuing LPV/r therapy due to drug-related adverse events, including liver toxicity. Additionally, high dose RTV has been identified as a risk factor for patients on ARV therapy.[110] RTV is now coadministered primarily at subtherapeutic doses in combination with other PIs to enhance their systemic concentrations. Thus, clinical reports of liver toxicity directly associated with high dose RTV has decreased over the years.

The pathogenesis of PI-associated liver injury remains unclear. One proposed mechanism is immune-mediated hypersensitivity in which the immune system's recognition of potential viral pathogens is restored following the successful initiation of HAART therapy, after which fulminant viral hepatitis ensues.[111] Harrill *et al.* reports an association between polymorphisms in the CD44 gene and high serum ALT levels after acetaminophen exposure in two separate patient cohorts. This gene encodes the CD44 antigen which is involved in an array of cellular functions including lymphocyte activation and tumor metastasis. Thus, differences in genes associated with the innate immune response may contribute to the variability in pharmacologic and toxicologic responses to drugs.

The liver is the principal site of metabolism for PIs. Another potential mechanism of PI-induced liver injury is that disturbances in metabolic pathways may cause an accumulation of parent compound and/or reactive metabolites, ultimately producing mitochondrial dysfunction. A recent review by Tuijos and colleagues highlights mitochondrial toxicity, typified by the deposition and accumulation of fat in hepatocytes, associated with a number of drugs including amiodarone and valproate.[112] The discovery that antiviral nucleoside analogs inhibit mitochondrial DNA polymerase gamma at physiological doses led to a black box warning regarding mitochondrial toxicity with the use of these compounds.[113]

A final potential mechanism of toxicity, which this project explores, is the inhibition of bile acid transport leading to the hepatocellular retention of bile acids (i.e., cholestasis). Perturbation of BSEP, the biliary efflux transporter, has been suggested to be a mechanism of DILI for a number of drugs reported to cause cholestasis such as troglitazone and bosentan.[77,114] Evidence supporting interference with bile salt transporters as a mechanism of DILI continues to emerge. Consequently, a number of *in vitro* methods and models, including sandwich-cultured hepatocytes, are now employed to screen for drug interactions with BSEP to predict and decrease the risk of DILI in humans.[115,116]

PART III. Bile Acid Synthesis, Hepatic Transport, and Molecular Regulation

A. *Bile Acid Synthesis.*

Bile acids, the main constituents of bile, are essential for the secretion of cholesterol from the liver. Additionally, bile acids play a major role in the molecular regulation of enzymes and transporters involved in the metabolism and distribution

of endogenous and exogenous compounds.[117] Bile acids are formed by the conversion of cholesterol in the liver via two pathways: the classical (or neutral) pathway and the alternative (or acidic) pathway. The classical pathway is common to all mammals, and accounts for ~75% of the total bile-acid pool. Cholesterol 7 α-hydroxylase (CYP7A1) is the first, rate-limiting enzyme in the classical pathway whereas the alternative pathway is initiated by sterol 27-hydroxylase (CYP27A1).[118] In the alternative pathway oxysterol intermediates are generated by 25-hydroxycholesterol 7-alpha-hydroxylase (CYP7B1). Sterol 12α-hydroxylase (CYP8B1) catalyzes the hydroxylation at position 12 of the steroid nucleus, forming the primary bile acid, cholic acid (CA) in both pathways. All 7α-hydroxylated sterols undergo a series of enzymatic steps ultimately ending in their conversion to primary bile acids.[119,120] A detailed depiction of bile acid synthesis is presented in **Figure 1.3**.

CA and chenodeoxycholic (CDCA) acid are the main primary bile acids common to most species. Primary bile acids are those formed in the liver via the synthetic pathways while secondary bile acids are formed by intestinal bacteria. CA and CDCA are differentiated by the number and position of hydroxyl groups.[121,122] Cholic acid has 3 hydroxyl groups while chenodeoxycholic acid (identified in the domestic goose, hence the prefix “cheno”) has only 2 hydroxyl groups (thus, the term “deoxy”). In humans, the most abundant pimary bile acids, in addition to CA and CDCA, are their respective secondary bile acids, deoxycholic acid (DCA) and lithocholic acid (LCA). In rats, CDCA is also converted to muricholic acid.[123] Most bile acids are conjugated predominantly to either glycine (in humans) or taurine (in

rats).[117] A list of serum bile acids and their concentrations in rats is provided in **Table 1.9**.

B. Hepatic Transport of Bile Acids

Following synthesis in the liver, bile acids are secreted into the bile and stored in the gallbladder. Gallbladder contractions transfer the stored bile into the small intestine. Once in the intestine, biliary bile acids move from the duodenum to the jejunum, and then into the ileum.[117] The liver maintains bile acid homeostasis via negative feedback regulatory mechanisms. Approximately 95% of bile acids are reabsorbed in the ileum, while the remaining bile acids undergo bacterial metabolism, forming secondary bile acids. The portal circulation carries primary and secondary bile acids back to the liver where they are taken up primarily by active transport processes, completing the enterohepatic recycling process. Enterohepatic recirculation (illustrated in **Figure 1.4**) enables efficient reusage of bile acids, and allows bile acids to act as regulators of their own synthesis and transport.[121,122]

The hepatocyte contains both basolateral (sinusoidal) and apical (canalicular) membrane domains. Bile acids are transported to and concentrated in the bile via active transport systems.[124] Bile acids move from the portal circulation into sinusoidal blood and through fenestrae, or pore-like openings, into the space of Disse. Basolateral uptake of bile acids into the hepatocyte is the initial step in the hepatic elimination of bile acids. Once disassociated from albumin, bile acids readily transverse the basolateral membrane via transport proteins, as detailed below.[125]

The canalicular membrane forms the border of the bile canaliculus and serves as the primary excretory route of bile acids. Biliary constituents are secreted into the bile against a steep concentration gradient; thus, canalicular excretion is the rate-limiting step in biliary elimination.[126] The following section highlights the localization and function of key bile acid transport proteins and discusses potential clinical implications of genetic defects.

Basolateral Transport Proteins

Sodium-taurocholate cotransporting polypeptide (NTCP) mediates the sodium-dependent uptake of conjugated bile acids from the portal blood. NTCP electrogenically transports sodium ions and bile acid molecules simultaneously with a stoichiometry of 2:1.[127] NTCP preferentially transports taurine- and glycine-conjugated bile acids relative to the unconjugated species. Also, NTCP displays a higher affinity for conjugates of dihydroxy bile acids (chenodeoxycholate and deoxycholate) than for conjugates of trihydroxy bile acids (cholate).[128] To date, no known genetic mutation in NTCP has been associated with liver disease. However, numerous studies have demonstrated that NTCP mRNA and/or protein expression is downregulated in cholestatic conditions such as progressive familial intrahepatic cholestasis (PFIC), biliary atresia, chronic hepatitis C, and late stage primary biliary cirrhosis.[129-132] Although genetic variants in NTCP have been identified, most variants display transport activity comparable to wildtype NTCP with the exception of

the c.668T>C variant, which exhibits minimal taurocholate and cholate transport and a greater affinity for rosuvastatin.[133,134]

Organic Anion Transporting Proteins (OATPs) comprise a family of multispecific organic anion transporters that are responsible for the sodium-independent uptake of bile acids as well as a broad range of organic anions and cations. OATP1A2 transports a number of endogenous and exogenous substrates including conjugated and unconjugated bile acids, bilirubin, dehydroepiandrosterone sulfate (DHEAS), estrogen conjugates, and the antihistamine fexofenadine; OATP1A2 appears to contribute only minimally to total bile acid uptake.[135] While there is overlap between OATP1B1 and -1A2 substrate specificity, OATP1B1 is reportedly the most important OATP transporter involved in sodium-independent bile acid uptake in humans. Interestingly, Xiang *et al*. reported significantly higher fasting plasma bile acid concentrations in individuals with OATP1B1 polymorphisms, supporting the premise that OATP1B1 plays a key role in overall bile acid uptake.[136] OATP1B3 also transports conjugated bile acids; however, the involvement of OATP1B3 in total bile acid transport remains unclear.

Rodent Oatp1a1 substrates are similar to OATP1A2, including conjugated and unconjugated bile acids. Oatp1a1 is the most important sodium-independent bile acid uptake transporter in rats.[135] Two additional transporters, Oatp1a4 and -1b2, have been demonstrated to play a lesser role in bile acid uptake, relative to Oatp1a1, in rat.[135]

Multidrug Resistance-associated Proteins (MRPs) are ATP-dependent efflux transporters. MRP3 and MRP4 are located on the basolateral membrane of hepatocytes. MRP3 predominantly transports glucoronidated substrates such as mono- and bisglucuronosyl bilirubin. Although rat Mrp3 has been shown to transport bile acids, including taurocholic acid and glycocholic acid with high affinity, human MRP3 only transports glycocholic acid with low affinity.[137-139] Conversely, MRP4 mediates the transport of monoanionic bile acids in a glutathione-dependent manner.[140,141] Thus, MRP4 may contribute to the basolateral efflux of glutathione and bile acids from the hepatocyte into blood or across the apical membrane of renal proximal tubules.

Under normal physiological conditions, translocation of bile acids across the basolateral membrane is predominantly influx, but under cholestatic conditions, basolateral efflux of bile acids via MRP3 and MRP4 is upregulated.[142] MRP4 reportedly transports sulfated bile acids and is significantly induced in the livers of farsenoid x receptor (FXR) null mice.[90] Additionally, Denk *et al*. demonstrated up-regulation of Mrp4 in the liver and down-regulation in the kidney of bile duct ligated rats.[87]

Although hepatic MRP3 expression in human livers under "normal" conditions is modest, induction of MRP3 has been reported in patients with primary biliary sclerosis and in individuals with Dubin-Johnson syndrome, a rare disorder characterized by prolonged conjugated hyperbilirubinemia.[143] Additionally, increased sulfated bile acids in the serum under cholestatic conditions, such as progressive familial intrahepatic cholestasis-2 and -3, support the premise that

MRP3 and MRP4 are induced as a hepatoprotective mechanism to prevent the intracellular accumulation of potentially toxic bile acids.[130]

Apical Transport Proteins

Bile Salt Export Pump (BSEP) is the predominant canalicular transport protein responsible for the translocation of monovalent bile acids into the bile.[124,126] Modest levels of Bsep mRNA expression in the kidney, brain, and intestine of rats has been reported. However, rodent Bsep is almost exclusively and homogenously expressed in the liver.[144-146] In humans, high levels of BSEP mRNA were reported in both the testis and the liver.[147] Low levels of Bsep also have been demonstrated in rat and human placenta during pregnancy.[148] Studies in Sf9 and HEK293 cellular systems reveal that cholate as well as the taurine and glycine conjugates of cholate and chenodeoxycholate are transported by rat Bsep with high affinity (km values between 2 and 22 μM). Similarly, human BSEP has been shown to transport taurocholate, glycocholate, and taurochenodeoxycholate.[128] In humans, BSEP mutations have been associated with type 2 PFIC.[149] Regulation of BSEP expression by bile acids via activation of FXR also has been reported.[150] FXR-mediated induction of BSEP is another hepatoprotective response to overcome cholestatic conditions.[151]

Multidrug Resistance-associated Protein 2 (MRP2), first identified in the apical membrane of human and rat hepatocytes, facilitates the canalicular excretion of various exogenous and endogenous compounds including sulfate conjugates,

glutathione conjugates, and some unconjugated drugs like ampicillin and irinotecan.[45,152] MRP2 also mediates the efflux of conjugated bilirubin and bile acids as a hepatic detoxification mechanism.[124] Several genetic polymorphisms in MRP2 also have been associated with Dubin-Johnson syndrome.[153] Stimulation of ATP-dependent vesicular bile acid transport by $E_2$17ßG has been shown in MRP2-expressing Sf9 vesicles.[154]

C. Molecular Regulation of Bile Acid Homeostasis

Bile acid synthesis, metabolism, and transport processes are tightly regulated by a number of feedforward and feedback mechanisms at both the gene transcription level and posttranscriptionally. Bile acids are natural detergents, and can elicit mitochondrial toxicity by increasing membrane permeability, oxygen free radicals, and lipid peroxidation.[155] Conversely, bile acids themselves act as signaling molecules in the regulation of enzymes and transport proteins involved in the metabolism and transport of bile acids, thereby preventing cellular accumulation and damage.[124]

Several nuclear hormone receptors and other transcriptional factors are key in the molecular regulation of bile acid formation and transport. Bile acids are activating ligands for FXR which, in concert with its heterodimer partner retinoid X receptor (RXR), is a transcription factor for several bile acid transporters, including BSEP.[124] Drugs like rifampin as well as endogenous compounds including lithocholate reportedly act as ligands for pregnane X receptor (PXR) in rodents and steroid X receptor (SXR) in humans to upregulate Oatp2 and Mrp2, respectively.

Upregulation of CYP7A1 by activation of the liver X receptor (LXR), culminating in increased bile acid synthesis, has been demonstrated by oxysterols.[124]

Wagner and colleagues demonstrated that agonists of the constitutive androstane receptor (CAR) and PXR stimulate alternate bile acid detoxification and elimination pathways in common bile duct-ligated mice. Alterations in these pathways included induction of efflux transporters Mrp2-4 and upregulation of sulfotransferase (SULT) 2A1, a key enzyme in bile acid sulfation, as well as uridine diphosphate (UDP)-glucuronosyltransferase 1A1, the enzyme responsible for bilirubin glucuronidation. These modifications resulted in decreased bile acids and bilirubin levels in plasma.[156,157]

Drugs and bile acids that alter hepatobiliary transporters have been employed as therapeutic drug targets. Ursodeoxycholic acid (UDCA), for example, induces the expression and function of multiple transporters and enzymes at numerous levels including Mrp2, Bsep, and CYP3A4. As such, evidence in the literature supporting the use of UDCA to promote adaptive reponses to combat cholestasis continues to emerge.[158,159]

The association between cholestasis, a common phenotype in a number of disease states, and altered expression and function of key bile acid enzymes and transporters remains unclear. The coordinated regulation of hepatic bile acid formation, metabolism, and transporter-mediated elimination is an intricate, multifactorial adaptive system designed to preserve the integrity of the liver. However, as data illustrating the effects of molecular changes on bile acid

homeostasis are unveiled, therapeutic options for the management of cholestasis will continue to increase.

Part IV. *In vitro* Model Systems to Investigate the Hepatobiliary Transport of Drugs and Endogenous Compounds

The concept that key drug-transporter interactions can influence the overall disposition of compounds is gaining recognition in the field. To date, *in vitro* models used to examine hepatic uptake and excretion are limited to transfected systems and cellular preparations from liver tissue. Transfected systems are useful to evaluate interactions between drugs and specific transport proteins; however, it is difficult to determine the relative contribution of each protein to the overall disposition of a given substrate or inhibitor. Additionally, the presence of endogenous transporters in transfected systems can make it difficult to accurately interpret experimental findings.

Freshly isolated hepatocytes in suspension are often employed to evaluate drug-transporter interactions. Limitations of this system, such as the rapid decline in cellular viability and an inability to distinguish between canalicular and basolateral efflux, confine the utility of this model to measuring short-term metabolism and characterizing initial hepatic uptake of substrates. Freshly plated hepatocytes in a gel entrapped design, i.e. sandwich-cultured hepatocytes, represent a diverse tool useful in evauating a number of physiological processes including hepatobiliary disposition of compounds, molecular regulation of transporters, and hepatotoxicity.[160] Hepatocytes cultured in this configuration exhibit liver specific *in*

vivo properties such as cellular polarity, intact bile canalicular networks, and the formation and secretion of numerous endogenous substances including albumin, fibrinogen, urea, and bile acids.[161] Additionally, studies demonstrating that calcium depletion disrupts tight junction networks of the bile canaliculi makes the model suitable for examining both the biliary excretion of compounds as well as the inhibitory potential of compounds on the biliary excretion of model substrates.[162] Sandwich-cultured hepatocytes are very useful to assess the CYP450 induction potential of compounds. Studies performed using typical inducers demonstrate that sandwich-cultured hepatocytes retain induction responses similar to those observed *in vivo*.[163]

In general, there are numerous powerful *in vitro* tools to investigate liver specific processes, each with advantages and disadvantages. The *in vivo* processes that govern drug disposition, efficacy, and toxicity are multifactorial. Consequently, while there is currently no comprehensive *in vitro* model to accurately predict *in vivo* interplay between drugs, transporters and metabolic enzymes, the combined application of a variety of *in vitro* model systems provides valuable insight to identifying safe, effective drug candidates.

Part V. Goals and Specific Aims

The global objective of this dissertation project was to develop a mechanistic understanding of how impaired bile acid transport proteins contribute to DILI. Particularly, the influence of combination antiretroviral PIs, LPV and RTV, on the hepatobiliary disposition of radiolabelled and endogenously formed bile acids was

investigated. Recent literature demonstrated that individually, LPV and RTV are potent inhibitors of BSEP. However, LPV is only available as a coformulation with RTV; their additive effect on BSEP remains unclear. Thus, the combined effect of LPV and RTV on bile acid transport is a fundamental question that represents the cornerstone of the present work. Several *in vitro* model systems, including freshly isolated suspended and sandwich-cultured hepatocytes were employed to conduct these investigations. A secondary goal of this dissertation was to determine whether genetic variants in genes involved in bile acid transport or synthesis predispose patients to DILI.

This dissertation addresses the following specific aims:

SPECIFIC AIM 1: Elucidate the effects of LPV and RTV, alone and combined, on hepatocellular toxicity and hepatobiliary bile acid transport.

Hypothesis: Coadministration of PIs increases the severity of inhibition of BA transport and, consequently, hepatocellular toxicity.

Experimental Approach:

- Following 24-hr exposure to LPV, RTV, and LPV/r, measure lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) medium and cellular content as indicators of cellular apoptosis and viability.
- Quantify the biliary excretion, biliary clearance, and cells + bile and cellular accumulation of model bile acids [^{3}H]TCA and [^{14}C]CDCA in sandwich-cultured rat hepatocytes.

- Quantify the sodium-dependent and sodium-independent initial uptake rates of [^{3}H]TCA and [^{14}C]CDCA in freshly isolated suspended rat hepatocytes in the presence and absence of LPV, RTV, and LPV/r.

SPECIFIC AIM 2: Investigate the influence of LPV and RTV, alone and combined, on the hepatocellular disposition of endogenous bile acids.

Hypothesis: Combination LPV and RTV exerts additive effects on bile acid transporters, causing cellular retention and accumulation of bile acids.

Experimental Approach:

- Quantify and compare the effects of LPV, RTV, and LPV/r on the cellular accumulation and biliary excretion of endogenously synthesized primary bile acids in cells, bile, and medium of day 4 sandwich-cultured rat hepatocytes.

SPECIFIC AIM 3: Determine whether genetic polymorphisms in key bile acid synthesis and transport genes are risk factors for DILI.

Hypothesis: Functional genetic variants in genes that play a role in bile acid transport or synthesis increase the risk of DILI in humans. Furthermore, variants in genes that act as compensatory mechanism(s) of BA excretion increase the risk of DILI.

Experimental Approach:

- Conduct a genetic association study using logistic regression analyses to determine whether the distribution of variants in bile acid metabolism and transport genes differ between patients from the Drug-induced Liver Injury Network (DILIN) and control subjects from the British Birth Cohort.

- Perform logistic regression analysis comparing distribution of genetic variants in DILIN patients who experienced toxicity due to BSEP inhibitors versus controls.

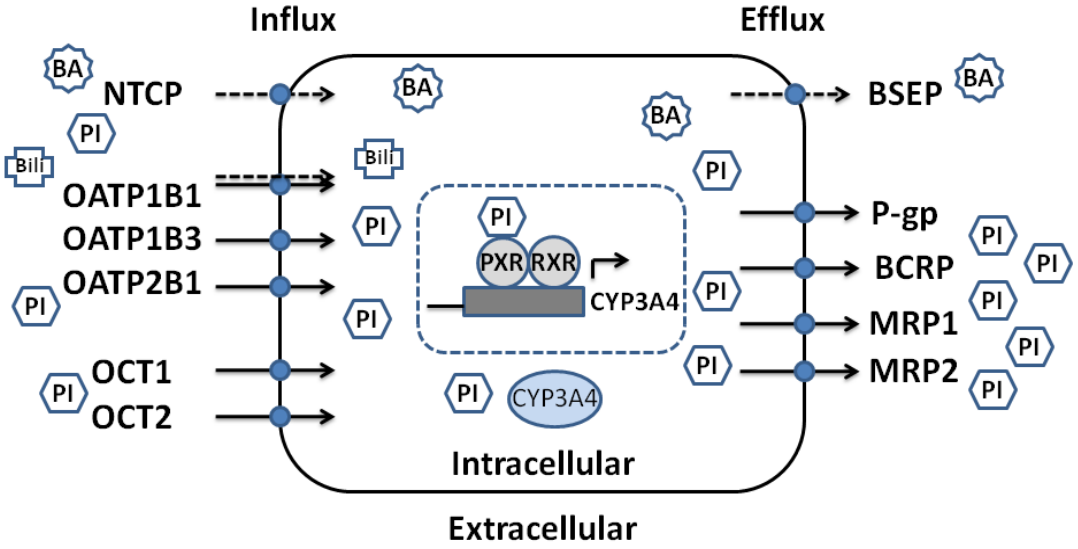

Figure 1.1 Schematic depicting the localization of SLC and ABC transport proteins involved in the translocation of protease inhibitors (PI) at sites of absorption (intestine), excretion (liver and kidney), and at target sites (central nervous system, lymphatic system, placenta, blood–testis barrier, and female genital tract). In general, PIs are transported (denoted by solid lines) into cells by proteins of the SLC family (e.g., OATPs and OCTs) and transported out of cells by proteins of the ABC family (e.g., P-gp, BCRP, and MRPs). The hepatic uptake and excretion of bile acids (BA), which are mediated by NTCP and BSEP, respectively, are inhibited (denoted by dashed lines) by PIs. The hepatic transport of bilirubin (bili), which is mediated by OATP1B1, is inhibited (denoted by dashed line) by PIs. PIs bind to pregnane X receptor (PXR), an orphan nuclear receptor, which forms a heterodimer with the retinoid X receptor (RXR) and mediates the induction of cytochrome P450 3A4 (CYP3A4).

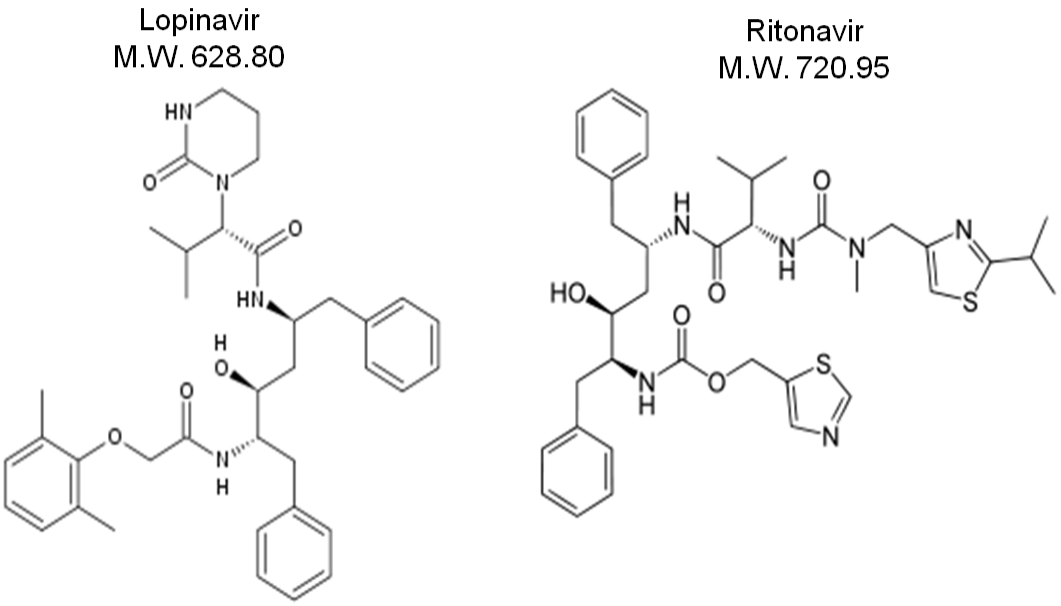

Figure 1.2 Chemical Structures of Selected Protease Inhibitors

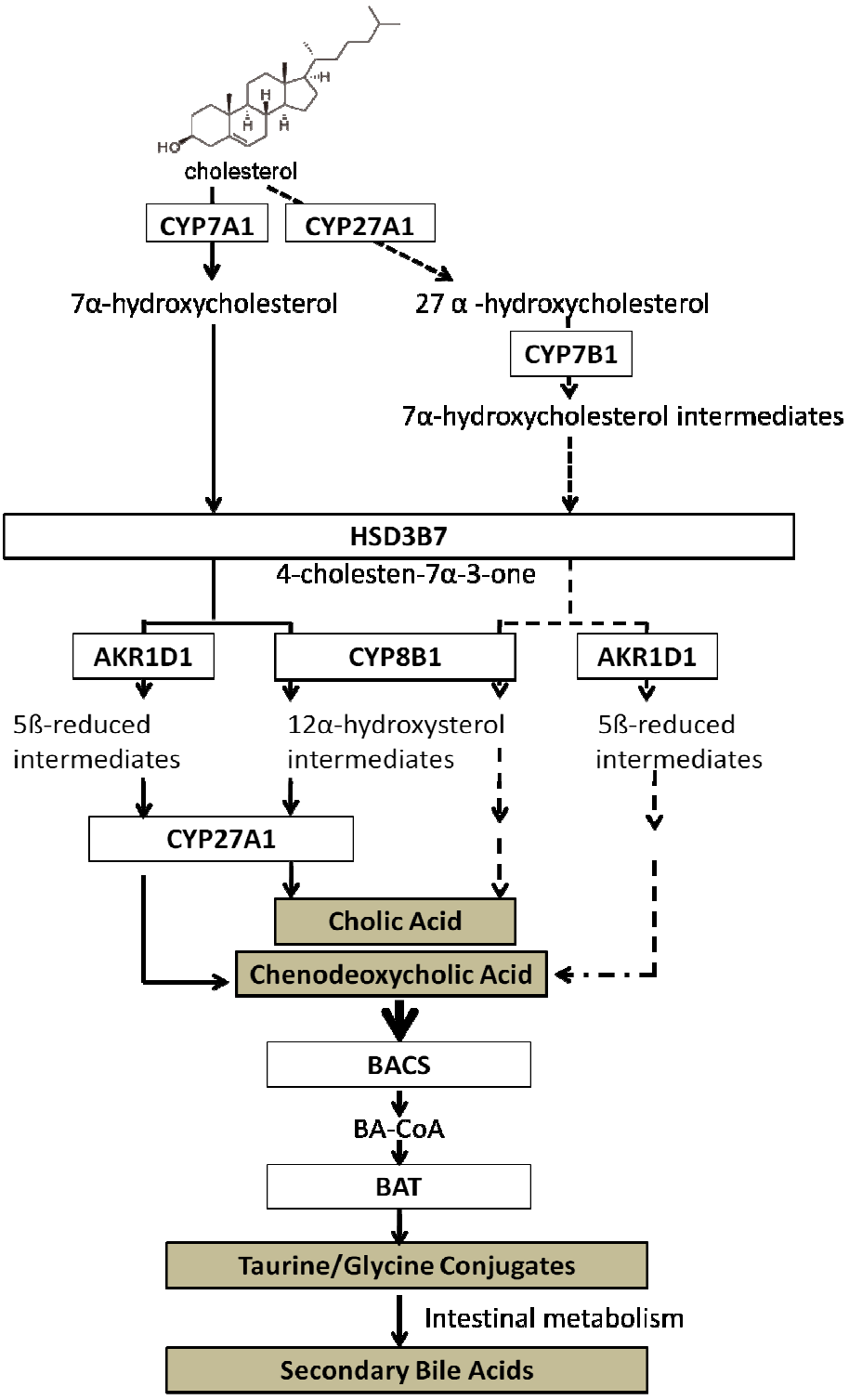

Figure 1.3 Classical (solid arrows) and Alternative (dashed arrows) Pathways of Bile Acid Synthesis.

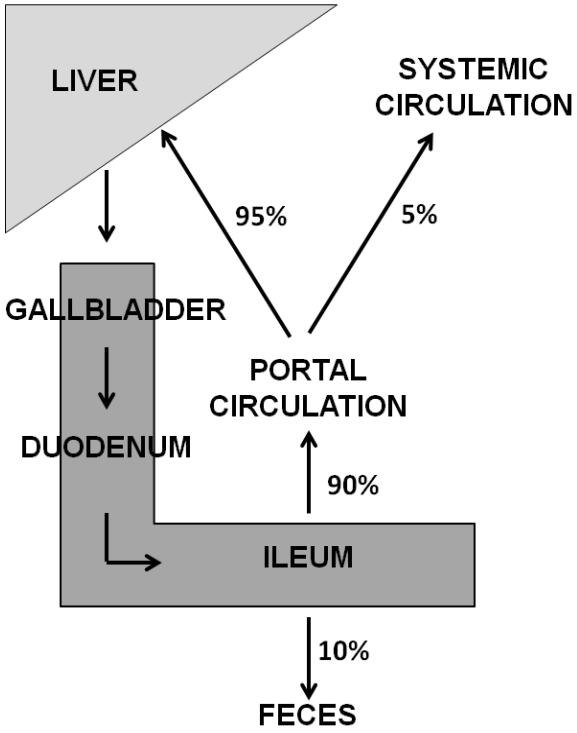

Figure 1.4 Enterohepatic Circulation of Bile Acids

Table 1.1 Physicochemical properties and *in vitro* cellular accumulation ratios of HIV protease inhibitors

	Amprenavir (APV) [Fosamprenavir]	Atazanavir (ATZ)	Darunavir (DRV)	Indinavir (IDV)	Lopinavir (LPV)	Nelfinavir (NLV)	Ritonavir (RTV)	Saquinavir (SQV)	Tipranavir (TPV)
pKa	1.9 [a] [6.28]	4.3	14.2	6.2; 5.9, 3.7	1.6	6.0, 11.1	2.8	1.1, 7.1; 7.0; 5.5 [b]	7.8
Lipophilicity (Log P o/w) Log D (pH)	1.7; 3.3 or 4.2[b] [0.84]	4.25; 4.5 [a]	1.8 [a]	0.9; 2.9 [a]	1.7	2.9; 6 [a] 4.0 (pH 7.4); 4.1 (pH 6.0)	1.2; 5.2 3.9 [a]	1.9; 4.1 (mesylate); 3.8	6.9 [a]
Solubility (µg/ml)	Mesylate: Aq: 190 pH 7.4: 60 pH 6.8: 190 [Calcium: Aq: 700][d]	Aq: 4-5 mg/mL[a]	Ethanolate: Aq: 150 [a]	Aq: 15 [a] Sulfate: Aq: > 100 mg/ml pH 7.4: 70 pH 4.8: 300 pH 3.5: 60 mg/ml	Aq: very low	Mesylate: Aq: 4500 pH 7.4: very low pH 3.5: 500 pH 2.6: 4500	Aq: 1 pH 7.4: 5.3 pH 4: 6.9	Mesylate: Aq: 2220 pH 7.4: 36 pH 6.5: 73	Aq: insoluble
Intracellular Accumulation ratio's (*in vivo* in blood)[c]	3.2	1.2		0.29	1.55; 0.7-2.1	5.3	1.25; 1.7; 0.8-4.2	3.64; 4.9; 1.5-6.7	
References	164-166	22,53,167		164-166,168,169	164,170	164-166,170,171	53,164-166,170	1,53,164-166,170	

[a]Data obtained from Drugbank (http://www.drugbank.ca/drugs)
[b]Conflicting results reported
[c]When coadministered with ritonavir (except nelfinavir); ritonavir: coadministered with saquinavir
[d]Fosamprenavir

Table 1.2 Summary of clinically relevant drug-drug interactions involving HIV protease inhibitors with evidence for a role of drug transporters in mediating the interactions: protease inhibitor as perpetrator drug.

Transport Protein	Victim Drug	Protease Inhibitor	Clinical Exposure Changes	*In Vitro* Studies
MDR1	Tenofovir (TFV) disoproxil fumarate (TDF)	ATV/r, DRV/r, LPV/r, SQV/r	Plasma TFV AUC 1.1-1.4 × ↑ in combination with ATV/r, DRV/r, LPV/r, SQV/r.[59,172]	• Efflux Ratio (ER) of TDF across MDCK-MDR1 is 34 (control); ER is significantly reduced to 4.3 (NFV), 4.4 (LPV, RTV); 16 (ATV); 22 (SQV); 24 (APV);[59] • Limited interaction of HIV PI with transporters involved in TFV disposition in the kidney (hOAT1/3, MRP4).[173]
	Fexofenadine 120 mg	LPV/r RTV	AUC 2.2 × ↑ (single RTV 100) AUC 4.0 × ↑ (single LPV/r 400/100) AUC 2.9 × ↑ (steady-state LPV/r 400/100)[174]	• RTV IC_{50} = 5.4 µM for P-gp-mediated fexofenadine transport across Caco-2[175] • LPV and RTV are P-gp inhibitors and inducers • RTV causes net induction (rather than inhibition) of P-gp *in vivo* in rats – based on CsA oral BA [176]
	Fexofenadine 60 mg	RTV 200 tid - 400 bid	AUC 2.8 × ↑ (acute RTV) AUC 1.4 × ↑ (steady-state RTV)[177]	
	Fexofenadine 60 mg	IDV/r 800/100 bid	AUC 5.0 × ↑ (single dose IDV/r) AUC 4.2 × ↑ (steady-state IDV/r) [54]	Largest change for IDV may be explained by P-gp being an inhibitor but not an inducer

Transport Protein	Victim Drug	Protease Inhibitor	Clinical Exposure Changes	*In Vitro* Studies
MDR1	Digoxin 0.4 mg oral	RTV200; 14d	Plasma AUC 1.2 × ↑ [178]	• RTV blocks P-gp activity (Table 1.4) • RTV (>5µM) enhances digoxin (0.1µM) accumulation in RBE4 cells[179]
	Digoxin 0.5 mg iv	RTV300 bid	Plasma AUC 1.9 × ↑, V_d 1.8 × ↑, Cl_{renal} 1.5 × ↓, $Cl_{non\text{-}renal}$ 2 × ↓[180]	
	Digoxin 0.4mg qd	DRV600/r100 bid	Plasma AUC 1.4 × ↑ [181]	• DRV and RTV block P-gp (Table 1.4)
	Digoxin 0.5 mg (oral)	SQV1,000/r100 bid	Plasma AUC 1.5 × ↑[182]	• SQV (>10µM) and RTV (>5µM) enhance digoxin (0.1 µM) accumulation in RBE4 cells[179]
	Digoxin 0.25 mg (oral)	TPV/r	Plasma AUC 1.9 × ↑ after first dose Plasma AUC unchanged and Cmax 1.5 × ↓ at steady-state[70]	See Tables1.4 and 1.6
	Digoxin 0.5 mg (oral)	LPV400/r100 bid (14 d)	Plasma AUC 1.8 × ↑[55]	
	Loperamide	TPV750(/r200)	Plasma AUC 2-3 × ↓: in vivo intestinal P-gp induction, also in presence of RTV as inhibitor [69]	

Transport Protein	Victim Drug	Protease Inhibitor	Clinical Exposure Changes	*In Vitro* Studies
MDR1		RTV200	Plasma AUC 2.2 × ↑: in vivo intestinal P-gp inhibition;[69] no effect on brain PD (loperamide)	See Table 1.4
		RTV600	Plasma AUC 3.2 × ↑: in vivo intestinal P-gp inhibition[56]	
	Delaveridine	APV600 bid	possibly partly due to intestinal P-gp induction[72]	See Table 1.6
	Tacrolimus Sirolimus	APV/r	Case report in HIV-infected patient indicates increased tacrolimus/sirolimus half-life and trough levels, attributed to CYP and/or P-gp inhibition by APV/r[57]	See Table 1.4
	Tacrolimus	DRV/r	Case report: HIV-infected kidney-transplant patient required a tacrolimus dose equal to 3.5% of usual dose.[58]	
	Sildenafil	DRV/r 400/100 bid	Plasma AUC 4 × ↑: possibly due to P-gp inhibition (or OATP inhibition).[183]	
	Ketoconazole 200 bid	DRV/r 400/100 bid	Plasma AUC 3.1 × ↑: possibly due to P-gp inhibition (or OATP inhibition)[184]	

Transport Protein	Victim Drug	Protease Inhibitor	Clinical Exposure Changes	*In Vitro* Studies
MDR1	Ketoconazole 200 qd	RTV	Plasma AUC 3.4 × ↑: possibly due to P-gp inhibition (or OATP inhibition) [185]	See Table 1.4
	Ketoconazole 200 single dose	LPV/r 400/100 bid	Plasma AUC 3.0 × ↑: possibly due to P-gp inhibition (or OATP inhibition)[186]	
	Ketoconazole 200 qd	FPV/r 700/100 bid	Plasma AUC 2.7 × ↑: possibly due to P-gp inhibition (or OATP inhibition) [187]	
OATP/ BCRP	Atorvastatin	LPV/r TPV/r SQV/r (400/400 bid) DRV/r (300/100 bid)	AUC 5.9 × ↑(LPV/r) AUC 9.4 × ↑(TPV/r)[62] AUC 3.4 ×↑(SQV/r) [188]AUC 4.0 × ↑ (DRV/r) [62,189]	• Atorvastatin is an OATP1B1 and BCRP substrate [190,191] • HIV PI are OATP and BCRP inhibitors [41,192,193]
OATP1B1/ BCRP	Rosuvastatin	TPV/r LPV/r ATV/r	AUC 1.4× ↑ (TPV/r)[62] AUC 2.1× ↑ (LPV/r); $t_{1/2}$ not affected [172] AUC 3.1× ↑ (APV/r) [194]	• Rosuvastatin is an OATP1B1 and BCRP substrate [190,191] • LPV, TPV, ATV and RTV are OATP and BCRP inhibitors [41,192,193]
OATP1B1 MRP2	Pravastatin 40 mg qd	DRV/r 600/100bid	Plasma AUC 1.8 × ↑ [181]	See Table 1.4[195]

Transport Protein	Victim Drug	Protease Inhibitor	Clinical Exposure Changes	*In Vitro* Studies
OATP/ MDR1	Fexofenadine	NLF 1250 bid (1wk)	Fexofenadine C_{max} 1.3 × ↓, $t_{1/2}$ 1.3 × ↓; possibly due to intestinal P-gp and hepatic OATP induction[71]	See Table 1.6
OATP2B1	Elvucitabine 20 mg	RTV 300 (single dose)	Elvucitabine AUC 1.3 × ↓ and C_{max} 1.7 × ↓; possibly due to inhibition of intestinal influx transporters [63]	See Table 1.4 for effect of RTV on OATP activity
Uptake transporters	Etravirine	DRV/r (600/100 bid)	100 bid: plasma AUC 1.6 × ↓ 200 bid: plasma AUC 1.8 × ↑[52,196]	Etravirine is not a substrate for P-gp, BCRP or MRP1-3.[75] The role of uptake transporters has not been investigated.
	Etravirine	TPV/r (500/200 bid)	Plasma AUC 4.2 × ↓ [73]	See Table 1.6

Table 1.3 Summary of clinically relevant drug-drug interactions involving HIV protease inhibitors with evidence for a role of drug transporters in mediating the interactions: protease nhibitor as victim drug.

Transport Protein	**Perpetrator Drug (Inhibitor/Inducer)**	**Victim Protease Inhibitor**	**Clinical Exposure changes**	**In Vitro Studies**
MDR1 (ABCB1, P-gp)	Rifampicin 600 mg qd	Most HIV PI	Plasma AUC 5.6 × ↓ (APV) Plasma AUC significantly ↓ (ATV) Plasma AUC 9.1 × ↓ (IDV) Plasma AUC 5.6 × ↓ (NFV) Plasma AUC 2.9 × ↓ (RTV) Plasma AUC 6.3 × ↓ (SQV) (possibly partly attributable to intestinal P-gp induction)[197]	HIV PI are (poor) P-gp substrates, but exact role of intestinal efflux transporters in their absorption unclear.[31,39,198,199]
	Rifampicin 600 mg qd	LPV/r 800/200 mg qd 400/400 mg qd	Plasma AUC 4 × ↓ (LPV/r)[76]	LPV is an ABCB1 (but not ABCC2) substrate[36]
	Rifampicin 600 mg qd	ATV/r 300/100 mg qd	Plasma AUC 6.7 × ↓ (ATV) and 2.9 × ↓ (RTV) (possibly partly attributable to intestinal P-gp induction; evaluated in three patients only) [200]	ATV and RTV are (poor) P-gp substrates.[39]
MDR1 (ABCB1, P-gp)	Rifabutin	APV IDV NFV SQV	Inductive effects on HIV PI PK is less pronounced (1.2-1.7×↓) than for rifampicin.[197,201]	cfr. above
MDR1 (ABCB1, P-gp)	SJW, Ginkgo and other herbal medicines	Several HIV PI	Reduced exposure, potentially leading to therapy failure (exact contribution of efflux transporters versus drug metabolizing enzymes not clear)[202]	see Table 1.5 illustrating that HIV PI are substrates for efflux transporters

Transport Protein	Perpetrator Drug (Inhibitor/Inducer)	Victim Protease Inhibitor	Clinical Exposure changes	In Vitro Studies
MDR1 (ABCB1, P-gp)	Ketoconazole 200 bid	DRV/r 400/100 bid	Plasma DRV AUC 1.4 × ↑[203]	DRV is a P-gp substrate, even though RTV co-administration limits the role of P-gp. [64,204]
MDR1 (ABCB1, P-gp)	Ketoconazole 200-400 qd	SQV/r 400/400 bid	Plasma SQV AUC 1.4 × ↑[205]	Ketoconazole inhibits P-gp-mediated SQV transport across Caco-2 monolayers.[206]
MDR1 (ABCB1, P-gp)	Etravirine	FPV700/r100 bid IDV800 tid TPV500/r200 bid ATV400 qd SQV1200 single	Plasma APV AUC 1.7 × ↑ Plasma IDV AUC 1.5 × ↓ Plasma TPV AUC 1.2 × ↑ Plasma ATV AUC 1.2 × ↓ Plasma SQV AUC 1.5 × ↓[73]	Etravirine has been shown to be a potent BCRP inhibitor and inducer *in vitro,* however PI are not BCRP substrates;[40] etravirine shows no significant P-gp inhibition, but modest induction of P-gp and MRP3 [75]
OATP1B (SLCO1B)	Rifabutin 150 mg qod	DRV/r 600/100 mg bid	DRV plasma AUC 1.6 × ↑ RTV plasma AUC 1.7 × ↑ Mechanism unknown but possibility of SLC inhibition has been suggested.[184]	No data available on effect of rifabutin on OATP activity

Table 1.4 HIV protease inhibitors as inhibitors of ABC and SLC transporters.

ABC					SLC			
Protease Inhibitor	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**
Amprenavir	**P-gp**	23.1*	BBMEC (rhodamine 123)	207	**OATP1B1**	14.4*, 12.8**	CHO (CGamF)	21
	BCRP	181*	MDCKII (Pheophorbide A)	41	**OATP1B3**	19.1*, 13.1**	CHO (CGamF)	21
Atazanavir	**P-gp**	67.8*	MDCKII (Calcein-AM)	37	**OATP1B1**	1.7*, 1,5**	CHO (CGamF)	21
	BCRP	69.1*	MDCKII (Pheophorbide A)	41	**OATP1B3**	3*, 3**	CHO (CGamF)	21
					OATP2B1	2.2*	Caco-2 (E3S)	22
						3.6*	MDCKII (E3S)	22
Darunavir	**P-gp**	33*	Not reported	208	**OATP1B1**	3.5*, 3.1**	CHO (CGamF)	21
		>100*	MDCKII (Calcein-AM)	37	**OATP1B3**	4.8*, 3.3**	CHO (CGamF)	21
					OATP2B1	29*	Caco-2 (E3S)	22
						26*	MDCKII (E3S)	22
Indinavir	**P-gp**	54.6*	BBMEC (rhodamine 123)	207	**OATP1B1**	12.2*, 10.8**	CHO (CGamF)	21
		>100*	MDCKII (Calcein-AM)	37		5.84*	HeLa	209
					OATP1B3	12.3*, 8.5**	CHO (CGamF)	21
					OATP2B1	3.9*, 3**	Caco-2 (E3S)	21
					OCT1	37.6*	HEK293 (MPP)	25
						62*	HeLa	26

ABC					SLC			
Protease Inhibitor	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**
Lopinavir	**P-gp**	10.3*	MDCKII (calcein-AM)	37	**OATP1B1**	0.5*,0.5**	CHO (CGamF)	21
	BCRP	7.66*	MDCKII (Pheophorbide A)	41	**OATP1B3**	2*, 1.4**	CHO (CGamF)	21
					OATP2B1	1.7*	Caco-2 (E3S)	22
						0.72*	MDCKII (E3S)	22
Nelfinavir	**P-gp**	1.7*	BBMEC (rhodamine 123)	207	**OATP1B1**	0.93*	HeLa (E217ßG)	209
		19.9*	MDCKII (calcein-AM)	37	**OATP2B1**	2.2*	Caco-2 (E3S)	22
	BCRP	13.5*	MDCKII (Pheophorbide A)	41	**OCT1**	0.9*	MDCKII (E3S)	22
						22*	HeLa	26
		12.5*	HEK293 (mitoxantrone)	40	**OCT2**	7*	HEK293 (MPP)	25
						13*	HEK293 (MPP)	25
Ritonavir	**P-gp**	3.8*	Caco-2 (digoxin)	106	**OATP1B1**	0.71*	HeLa (E217ßG)	209
		5*	Caco-2 (digoxin)	210		0.78**	HEK293 (pitavastatin)	213
		6.7*	Caco-2 (rhodamine 123)	211		1.6*, 1.4**	CHO (CGamF)	21
		26.4*	BBMEC (rhodamine 123)	207	**OATP1B3**	3.6*, 2.5**	CHO (CGamF)	21
		28.2*	MDCKII (digoxin)	212	**OATP2B1**	6.3*, 4.8**	Caco-2 (E3S)	21
		39.6*	MDCKII (calcein-AM)	37		0.93*	Caco-2 (E3S)	22
	BCRP	19.5*	HEK293 (mitoxantrone)	40		2.2*	MDCKII (E3S)	22

ABC					SLC			
Protease Inhibitor	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**	**Transporter**	**IC50* or Ki** (μM)**	**System (Substrate)**	**Ref**
					OATP1A2	<10*	HeLa (fexaofenadine)	214
						5.2*	HeLa	26
					OCT1	14*	HEK (MPP)	25
						25*	HEK (MPP)	25
					OCT2	13.9*	HeLa	215
					MATE1	15.4*	HeLa (metformin)	215
Saquinavir	**P-gp**	1.4*	BBMEC (rhodamine 123)	207	**OATP1B1**	1.23*	HeLa (E217ßG)	209
	BCRP	27.4*	MDCKII (Pheophorbide A)	41		2.1*, 1.8**	CHO (CGamF)	21
		19.5*	HEK293 (mitoxantrone)	40		1.59**	HEK293 (pitavastatin)	213
					OATP1B3	4.1*, 2.8**	CHO (CGamF)	21
					OATP1A2	<10	HeLa (Fexofenadine)	214
					OATP2B1	5.3*, 4**	Caco-2 (E3S)	21
						3.5*	Caco-2 (E3S)	22
					OCT1	4.6*	MDCKII (E3S)	22
						8.3*	HeLa [216]	26
						37*	HEK293 (MPP)	25
Tipranavir					**OATP2B1**	0.77*	Caco-2 (E3S)	22
						0.88*	MDCKII (E3S)	22

Table 1.5 HIV protease inhibitors as substrates of ABC and SLC transporters

	ABC				SLC			
Protease Inhibitor	**Transporter**	**K_m* (μM) or ER (PI dose)****	**In vitro System**	**Ref**	**Transporter**	**K_m* (μM) or ER (PI dose)****	**In vitro System**	**Ref**
Amprenavir	P-gp	47* 24.2 (10 μM)**	High Five membranes MDCKII-MDR1	207 217				
Indinavir	P-gp	0.47* 2.1*	High Five membranes High Five membranes	218 207				
Nelfinavir	P-gp	3.6*	High Five membranes	207				
Ritonavir	P-gp	0.8*	LLC-PK1	219				
Saquinavir	P-gp	1.4* 14.5* 15.4*	High Five membranes LLC-PK1 Caco-2	207 219 220	OATP1A2	36.4*	Oocytes	18
Tipranavir	P-gp	5.9 (8.1 μM)**	Caco-2	69				

Table 1.6 *In vitro* induction data with HIV protease inhibitors

Transporter	HIV PI	Model System (marker): Effect (conc)	Reference
MDR1	APV	T84 (0.1µM digoxin ER): ER doubled (10µM; 72h) T84 (mRNA): 5× ↑ (10µM; 72h) LS180 (mRNA): 17× ↑ (10µM; 96h)	96,221
	ATV	LS180V (protein): 2.5× ↑ (30 µM, 3 d) LS180V (Rh123 uptake): 55%↓ (30 µM, 3 d) hCMEC/D3 cells (protein): 2.5× ↑ (10 µM; 3d) LS180 (mRNA): 5× ↑ (10µM; 96h)	96,222,223
	DRV	LS180 (mRNA): 3.8× ↑ (10µM; 1wk)	34
	IDV	No significant P-gp induction	96,222,223
	LPV	LS-180V (protein/mRNA): 3× ↑ (30 µM 72h) LS-180V (Rh123 uptake): 50%↓ (30 µM 72h) LS180 (mRNA): 12× ↑ (10µM; 96h)	96,222-224
	NFV	Cultured Hepatocytes (mRNA): 4-6× ↑ (10-25 µM) Cytotrophoblast culture (Rh123 uptake): 23% ↓ (3 µg/ml; 24h) LS180 (mRNA): EC50 = 1.2 µM (96h); LS180 (mRNA): 7× ↑ (10µM; 96h)	67,96,225
	RTV	Human hepatocytes (mRNA): 9-10× ↑ (10-25 µM) LS-180V cells (protein): 6× ↑ (1-100 µM; 3d) LS-180V (Rh123 uptake): 50%↓ (>10 µM; 3d) hCMEC/D3 cells (protein): 2× ↑ (10 µM; 3d) LS180 (mRNA): EC50 = 1.7 µM (96h); LS180 (mRNA): 12× ↑ (10µM; 96h)	67,96,97,223,226

Transporter	HIV PI	Model System (marker): Effect (conc)	Reference
	SQV	LS180 (mRNA): 5.7× ↑ (10μM; 1wk) Cytotrophoblast culture (protein): 2× ↑ (1 μg/ml; 24h) Cytotrophoblast culture (Rh123 uptake): 18% ↓ (1 μg/ml; 24h) LS180 (mRNA): 5× ↑ (10μM; 96h)	34,96,225
	TPV	LS180 (mRNA): 10× ↑ (10μM; 96h)	96
MRP1	RTV	LS-180V cells (protein): 3× ↑ (1-100 μM; 3d) LS-180V (CBF uptake): 30% ↓ (30 μM; 3d)	97
	SQV	LS180 (mRNA): 2.3× ↑ (10μM; 1wk)	34
MRP2	NFV	Human hepatocytes (mRNA): 2-4× ↑ (10-25 μM)	67
	RTV	Human hepatocytes (mRNA): 5-6× ↑ (10-25 μM)	67
	SQV	LS180 (mRNA): 4.5× ↑ (10μM; 1wk)	34
MRP3	SQV	LS180 (mRNA): 2× ↑ (10μM; 1wk)	34
MRP4	SQV	LS180 (mRNA): 1.8× ↑ (10μM; 1wk)	34
MRP5	SQV	LS180 (mRNA): 3.8× ↑ (10μM; 1wk)	34
BCRP	NFV	Human hepatocytes (mRNA): < 2× ↑ (10-25 μM)	67
	RTV	Human hepatocytes (mRNA): 2-3× ↑ (10-25 μM)	67
	SQV	LS180 (mRNA): 4.1× ↑ (10μM; 1wk)	34
OATP1B1	NFV	Hepatocytes (mRNA): 2-3× ↑ (10-25 μM)	67
	RTV	Hepatocytes (mRNA): 2× ↑ (10-25 μM)	67
	SQV	LS180 (mRNA): 4.6× ↑ (10μM; 1wk)	34
OATP1B3	NFV	Human hepatocytes (mRNA): 2-5× ↑ (10-25 μM)	67
	RTV	Human hepatocytes (mRNA): 3-4× ↑ (10-25 μM)	67
OATP2B1	DRV	LS180 (mRNA): 1.9× ↑ (10μM; 1wk)	34
	SQV	LS180 (mRNA): 1.8× ↑ (10μM; 1wk)	34

Table 1.7 Clinically Relevant Examples of Transporter-mediated Interactions between HIV Protease Inhibitors and Endogenous Compounds.

Endogenous Compound	Transport Protein	Protease Inhibitor	In Vitro Studies	Clinical Relevance
Bile salts	NTCP BSEP	RTV, SQV	Inhibition of bile acid transport [78]	Increased serum bile acids; Increased hepatocyte bile acids; Increased risk for hepatotoxicity
Palmitate	CD36 and CPT1 fatty acid transporters	LPV/r and DRV/r (not ATV/r)	Inhibition of palmitate uptake in cultured skeletal muscle cells (myotubes)[82]	Dyslipidaemia, insulin resistance
Bilirubin	OATP1B1	ATV, IDV	Potent inhibition of OATP1B activity by HIV protease inhibitors causing increased incidence of hyperbilirubinemia and jaundice[47,81]	Increased serum bilirubin levels associated with the use of specific protease inhibitors.[80]

Table1.8 Key Pharmacokinetic Parameters of the HIV Protease Inhibitors.

Compound	Standard Dosing Level	Metabolism and Elimination	$t1/2_{ß}$ (h)	Plasma Protein Binding (%)	Ritonavir Boosting effect	Oral Bioavailability (%); [unboosted]	DME interactions (based on boosted use in the clinic)	Refs
Amprenavir (from fosamprenavir)	700 mg b.i.d. /r 100 mg b.i.d.	hepatic 3A4, 2D6 urine:14%; feces:75 % ; (unchanged:1% urine; ND in feces)	7-12	90	C_{max} 1.5 × ↑ AUC > 2 × ↑ C_{trough} 4 × ↑	30-70	CYP3A	187,227
Atazanavir	300 mg q.d. /r 100 mg q.d.	hepatic 3A4; Non-linear (300-600 mg), 79% bile/13% urine; UD % dose in bile 20%, in urine 7%	6 (in HIV patients; 2 × ↓ in healthy volunteers)	86	C_{trough} 5 × ↑ AUC 3 × ↑	68; pH dependent, AUC 1.7 × ↑ with food	CYP3A, UGT1A1	227,228
Darunavir	600-800 mg b.i.d. /r 100 mg b.i.d.	Hepatic CYP3A4 Feces: 79.5%; urine: 13.9% Unchanged (unchanged: 41.2% in feces; 7.7 % in urine)	15	95	AUC 10 × ↑	82 [37]	Inh.: CYP3A4, CYP2D6 Ind.: CYP2C9 CYP2C19	181,227,229

Compound	Standard Dosing Level	Metabolism and Elimination	t1/2$_{ß}$ (h)	Plasma Protein Binding (%)	Ritonavir Boosting effect	Oral Bioavailability (%); [unboosted]	DME interactions (based on boosted use in the clinic)	Refs
Indinavir	800 mg b.i.d. /r 100 mg b.i.d.	hepatic CYP3A4; 19% and 83% recovered in urine and feces, respectively; Of this unchanged drugs accounted for 19.1% and 9.4% and in the urine and feces, respectively	2	61	AUC 2 × ⬆ C_{min} > 4 × ⬆	60-65	CYP3A4 Weak 2D6 inhibitor	227,230
Lopinavir	400 mg b.i.d. /r 100 mg b.i.d.	hepatic CYP3A4 10.4 % and 82% in urine and feces, respectively. Of this, 2.2 and 19.8% appeared unchanged in the urine and feces respectively	5-6	99	AUC 1.5 × ⬆ C_{min} 2 × ⬆ C_{SS} 15-20 × ⬆	Not established (increased AUC and Cmax under fed conditions however)	CYP3A4	186,227

Compound	Standard Dosing Level	Metabolism and Elimination	t1/2ß (h)	Plasma Protein Binding (%)	Ritonavir Boosting effect	Oral Bioavailability (%); [unboosted]	DME interactions (based on boosted use in the clinic)	Refs
Nelfinavir	635 mg b.i.d.	hepatic CYP3A4, 2C19, 2D6, 2C9 2% and 87% recovered in urine and feces, respectively. Of this, 22% and 1 % was unchanged in the urine and feces, respectively	1.8-3.4	99	AUC 2.5 × ↑ C_{max} 1.4 × ↑	> 78	CYP3A CYP2C19	227
Ritonavir	PI + 100 mg b.i.d.	hepatic CYP3A4	3-5	99		66-75		227
Saquinavir	1 g b.i.d. /r 100 mg b.i.d.	hepatic CYP3A4	13	98		< 20 (Soft Gelatin Capsule)		227

Table 1.9 Serum bile acid concentrations in the rat

	Concentration (μM)	
Bile Acid Species	**Wang *et al.*[231]**	**Bai *et al.*[232]**
GCA	0.1 ± 0	0.12 ± 0.07
GCDCA	0.2 ± 0.1	0.04 ± 0.0
GDCA	0.3 ± 0.2	0.12 ± 0.07
CA	3.2 ± 0.8	1.81 ± 1.34
UDCA	0.6± 0.2	0.04 ± 0
GLCA	0.04 ± 0	0.04 ± 0.0
CDCA	0.8 ± 0.4	0.16 ± 0.16
DCA	0.6 ± 0.1	0.04 ± 0.0
TCA	0.3 ± 0.1	1.09 ± 0.15
TUDCA	0.1 ± 0	0.04 ± 0
TCDCA	0.2 ± 0.1	0.04 ± 0
TDCA	0.2 ± 0.1	0.04 ± 0
Total Bile Acids	**7.3 ± 1.4**	**3.28 ± 1.66**

CHAPTER 2

COMBINATION ANTIRETROVIRAL PROTEASTE INHIBITORS ALTER EXOGENOUS AND ENDOGENOUS BILE ACID DISPOSITION IN SANDWICH-CULTURED RAT HEPATOCYTES

Antiretroviral protease inhibitors (PIs) continue to be a mainstay in the treatment of HIV infection. Despite their success, PIs have been associated with drug-induced liver injury (DILI) which is one of the most common adverse events leading to the discontinuation of PI-inclusive antiretroviral therapy.[233,234] Liver injury occurred in 1% to 9.5% of PI-treated patients in randomized clinical trials conducted prior to US Food and Drug Administration approval.[235] Retrospective and prospective cohort studies report an overall incidence rate of hepatotoxicity associated with PI-inclusive drug therapy between 5% and 23%. However, the PI dose and the definition of hepatotoxicity varied across studies.[110] In particular, ritonavir (RTV)-containing regimens reportedly increased the risk of hepatotoxicity by 8.6-fold.[234] RTV is now administered at subtherapeutic (and subtoxic) doses to enhance systemic concentrations of coadministered PIs. One commonly prescribed PI combination is lopinavir and ritonavir (LPV/r). Reportedly, patients on highly active antiretroviral therapy (HAART) containing LPV/r who experienced liver failure had higher LPV/r plasma concentrations compared to patients with normal functioning livers.[186] One proposed mechanism for DILI is that drugs and/or their metabolites impair the function of transport proteins responsible for the efflux of bile acids from

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the hepatocyte.[115,116,236] Bile acids can cause cellular necrosis and apoptosis as a result of mitochondrial damage and disruption of cell membranes due to the detergent-like effects of these molecules.[237] Interference with the efflux of bile acids from hepatocytes could cause intracellular accumulation of bile acids, leading to toxicity.

The major transport protein responsible for biliary excretion of bile acids from the hepatocyte is the bile salt export pump (BSEP). Recent studies have shown that many drugs implicated in DILI inhibit BSEP.[238] PIs including LPV and RTV also have been shown to inhibit bile acid transport via BSEP,[78,239] supporting the idea that intracellular accumulation of bile acids may be a mechanism for DILI observed in patients treated with this combination.[238,239] If this is correct, we reasoned that the combination of LPV and RTV used in the clinic may have an additive or even synergistic effect on BSEP inhibition, resulting in an increased risk of DILI.

To our knowledge, the effect of PI combinations on hepatocyte viability and bile acid uptake and/or efflux, has not been studied previously. Therefore, we examined the effects of LPV, alone and combined with RTV, on hepatocyte viability, bile acid transport, and endogenous bile acid disposition in rat hepatocytes. We hypothesized that each PI would cause hepatocellular accumulation of bile acids and toxicity, and that co-administration of RTV and LPV would have at least an additive effect on bile acid accumulation and toxicity.

MATERIALS AND METHODS

Chemicals. [^{3}H]Taurocholic acid (TCA, 5 Ci/mmol; purity > 97%) was purchased from Perkin Elmer (Waltham, MA). [^{14}C]Chenodeoxycholic acid (CDCA; 50 mCi/mmol; purity > 97%) and [^{14}C]inulin (2.8 mCi/g, purity > 97%) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). RTV was obtained initially from the National Institutes of Health AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health. In addition, RTV, LPV and d_4 TCA were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). The d_8 TCA was purchased from Martrex, Inc. (Minnetonka, MN). All other deuterated bile acids were purchased from CDN Isotopes, Inc. (Pointe-Claire, Quebec, Canada). The bile acids α- and ß-tauromuricholic acid (α/ß-TMCA) were purchased from Steraloids, Inc. (Newport, RI). TCA, lactate dehydrogenase (LDH), adenosine triphosphate (ATP), Triton X-100, Hanks' balanced salt solution (HBSS) premix, HBSS modified (with no calcium chloride, magnesium sulfate, phenol red and sodium bicarbonate) premix, dexamethasone, and collagenase (type IV) were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide (DMSO) was obtained from Fisher Scientific (Fairlawn, NJ). GIBCO brand fetal bovine serum, recombinant human insulin, and Dulbecco's modified Eagle's medium (DMEM) were purchased from Invitrogen (Carlsbad, CA). Insulin, transferrin, and selenium (ITS) Universal Culture Supplement Premix and Matrigel™ Basement Membrane Matrix were obtained from BD Biosciences (Palo Alto, CA). The CellTiter-Glo® Luminescent Cell Viability Assay was purchased from Promega (Madison, WI). LDH Cytotoxicity

Detection Kit was purchased from Roche Applied Sciences (Indianapolis, IN). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Hepatocyte Isolation and Culture in a Sandwich Configuration. Hepatocytes were isolated from male Wistar rats (270–300 g) obtained from Charles River Laboratories, Inc. (Raleigh, NC) using a two-step collagenase perfusion method previously described.[163] Animals had free access to water and food before surgery and were allowed to acclimate for at least five days. All animal procedures complied with the guidelines of the Institutional Animal Care and Use Committee (University of North Carolina, Chapel Hill, NC).

Hepatocytes were seeded at 1.75 x 10^6 cells/well on 6-well, or 0.35 x 10^6 cells/well on 24-well, BioCoat™ collagen plates in DMEM containing 5% fetal bovine serum, 10 µM insulin, 1 µM dexamethasone, 2 mM L-glutamine, 1% MEM non-essential amino acids, 100 units penicillin G sodium and 100 µg streptomycin sulfate. Cells were incubated for 2 h at 37ºC in a humidified incubator (95% O_2, 5% CO_2) and allowed to attach to the collagen substratum, after which time the medium was aspirated to remove unattached cells, and replaced with fresh medium. Approximately 24 hours later cells were overlaid with BD Matrigel™ at a concentration of 0.25 mg/ml in ice-cold feeding medium (DMEM with 1% ITS, 0.1 µM dexamethasone, 2 mM L-glutamine, 1% MEM non-essential amino acids, 100 units penicillin G sodium and 100 µg/ml streptomycin sulfate). The culture medium was changed daily thereafter. Rat hepatocytes were cultured for at least 3 days to allow for the formation of bile canalicular networks.

Cytotoxicity and Cell Viability Assays. Following 24-hour exposure to PIs, intracellular ATP levels were measured using the CellTiter-Glo® Luminescent Cell Viability Assay. All reagents were allowed to equilibrate to room temperature prior to use. The CellTiter-Glo® Reagent was prepared by adding lyophilized CellTiter-Glo® substrate to CellTiter-Glo® buffer and mixing by vortex. Hepatocytes cultured in 24-well plates were allowed to equilibrate for at least 30 min to reach room temperature before the assay was performed. Medium was aspirated from each well twice, and replaced with equal volumes of fresh feeding medium and CellTiter-Glo® reagent. Plates were placed on an orbital shaker for 2 min to induce cell lysis, and then incubated at room temperature for 10 min to allow the luminescent signal to stabilize.

LDH leakage into sandwich-cultured rat hepatocyte (SCRH) medium was determined using the LDH Cytotoxicity Detection Kit. Briefly, day 3 SCRH in 24-well plates were exposed to PIs for 24 hours, after which cell-free supernatant was collected and aliquots were placed in individual wells of a 96-well plate. The substrate mixture was added to the culture supernatant and incubated for 30 min. During this time, LDH released from hepatocytes into the supernatant reduced the tetrazolium salt 2-(4-iodophenyl)-3-(4- nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) to formazan by a coupled enzymatic reaction. Following incubation, formazen formation was measured directly in the 96-well opaque-walled microplate by an ELISA absorbance plate reader. To directly compare assays, LDH data were converted to viability, and expressed as a percentage of control by subtracting the

degree of toxicity (%) from 100%. Maximum cell death was represented by the values measured following complete cell lysis by 0.5% Triton X-100.

Bile Acid ([^{3}H]TCA and [^{14}C]CDCA) Accumulation Studies in Sandwich-Cultured Rat Hepatocytes. The model bile acid, TCA, and the unconjugated organic acid, CDCA, were used for transport studies. Day 4 SCRH seeded in 24-well plates were washed 3 times (20 sec per wash) and co-incubated for 10 min with Ca^{2+}-containing (standard; cells + bile) or Ca^{2+}-free (cells) HBSS buffer to maintain or disrupt tight junctions, respectively. Next hepatocytes were co-incubated for 10 min with TCA (1 µM cold TCA plus trace [^{3}H]TCA) or [^{14}C]CDCA (1 µM cold CDCA plus 4 µM [^{14}C]CDCA) in the presence or absence of individual or combined PIs in standard HBSS at 37ºC. Cells were then aspirated twice and uptake was terminated by rinsing wells with 2.0 ml of ice-cold standard HBSS. Following rinsing, cells were lysed with 0.1% Triton X-100 in phosphate-buffered saline, and placed on an orbital shaker for 20 min. Aliquots of sample (500 µL) and dosing solution (100 µL) were collected for quantification of radioactivity by liquid scintillation counting. Another 500 µl aliquot of sample was reserved for protein quantification using the Pierce BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL). To correct for nonspecific binding to the collagen substratum, [^{3}H]TCA and [^{14}C]CDCA accumulation in BioCoat™ plates without cells was subtracted from raw values.

[^{3}H]TCA and [^{14}C]CDCA Initial Uptake in Suspended Rat Hepatocytes. The initial uptake of TCA (1 µM cold TCA plus trace [^{3}H]TCA; 60 nCi/ml) and CDCA (0.5 µM cold CDCA plus 0.5 µM [^{14}C]CDCA; 25 nCi/ml) in suspended rat hepatocytes was measured in the presence of vehicle (DMSO), LPV (10 µM) or RTV

(5 μM), alone and combined, using methods previously described.[240] Uptake studies were performed in Na^+-containing buffer to measure total uptake (Na^+-dependent and Na^+-independent), and Na^+-free, choline-containing buffer (Na^+-independent uptake only). Na^+-dependent uptake was calculated by subtracting the Na^+-independent uptake from the total uptake). Briefly, cells were washed 2 times in ice-cold buffer containing sodium chloride or choline chloride (137 mM NaCl or choline chloride, 0.8 mM $MgSO_4$, 10 mM HEPES, 1.2 mM $CaSO_4$, 0.86 mM K_2HPO_4, 0.14 mM KH_2PO_4, and 5 mM glucose, pH 7.4). Cells were resuspended at 1.0 x 10^6 cells/ml in the same buffer, kept on ice, and used immediately in experiments. Hepatocyte suspensions (4 ml; n = 3 livers, in triplicate) were preincubated in bottom inverted Erlenmeyer flasks at 37℃ for 5 min; 0.1% DMS O or PIs were added 30 sec before, followed by [^{3}H]TCA (1 μM unlabeled TCA plus trace [^{3}H]TCA, 60 nCi/ml). At 15, 30, and 45 sec, 200 μL samples of the cell suspension were collected and placed in a 0.4 ml polyethylene tube containing a top layer of silicone oil:mineral oil (82:18 [v/v], 100 μL) and a bottom layer of 3M KOH (50 μL), and immediately centrifuged. Radioactivity in the cell pellet and in the supernatant was measured by liquid scintillation counting. Adherent fluid volume was determined by incubating cells with [^{14}C] inulin (60 nCi/ml) as reported by Baur *et al*.[241] Uptake was normalized to protein concentrations for individual hepatocyte suspensions as determined by the BCA protein assay reagent kit. Cellular viability of the suspended hepatocytes (> 90%) was determined by trypan blue exclusion at the beginning and end of each experiment.

Accumulation of Endogenous Bile Acids in Cells + Bile, Cells, and Culture Medium of Sandwich-Cultured Rat Hepatocytes. Following 24 hour exposure to vehicle or PIs, 1 mL aliquots of medium were collected from day 4 SCRH in 6-well format and stored at -80˚ C until analysis. The remaining culture medium was aspirated from all wells, and triplicate wells were rinsed with 1.5 ml/well of warmed HBSS containing calcium (cells + bile) or HBSS without calcium (cells alone). Following rinses, wells were aspirated twice and another 1.5 ml of HBSS with or without calcium was added to the wells and cells were incubated at 37˚C for 4 min. After incubation, the HBSS buffer was aspirated from all wells. Plates were sealed and stored at -80˚ C until analysis.

LC-MS/MS Analysis. Culture medium and cell lysate samples were prepared for LC-MS/MS analysis as described previously [242]. Briefly, six endogenous conjugated bile acid species [taurocholic acid (TCA), glycoholic acid (GCA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), and α/ß-tauromuricholic acid (α/ß TMCA)] were detected simultaneously; 10 µL of sample or calibration standards were injected onto a Shimadzu binary high-performance liquid chromatography system (Columbia, MD). Chromatographic conditions used were as follows: 60% 0.5 mM ammonium acetate:40% MeOH (solvent A) and 20% 0.5 mM ammonium acetate:80% MeOH (solvent B) at a flow rate of 50 µL/min. The initial mobile phase was 70% solvent A:30% solvent B. The gradient was increased rapidly to 100% of solvent B for 2-15 min, and then returned to initial conditions (solvent A) for 1 min. The autosampler was maintained at 4℃ and rinsed with 1500 µl of 50:50 (v/v) 50% methanol:50% water following aspiration.

Methanol (100%) was added at 10 µl/min as a post-column solvent. Tandem mass spectrometry used to quantify analytes was performed using a Thermo Electron TSQ Quantum Discovery MAX (Thermo Fisher Scientific) with an Ion Max ESI source in negative ion electrospray ionization mode using selected reaction monitoring. The concentration ranges of the standard curves for rat cell lysate and medium of each bile acid were 0.5-100 pmol/well and 0.5-50 pmol/100 µl of medium, respectively. For a detailed list of the transitions monitored at unit resolution, see Marion *et al*., 2011.

When rat lysate and medium samples were analyzed initially, LC-MS/MS raw data were collected on α- and β-TMCA, but not processed. Both α- and β-TMCA have the same MS precursor and product negative ions as TCA, thus, their MS data were collected in the same analytical run as TCA. Once standards for α- and β-TMCA became available, they were utilized to confirm the identity of the LC-MS/MS response in the TCA channel thought to be α/β-TMCA. Because of the chromatographic separation utilized here, TCA was well resolved from α- and β-TMCA; however, α- and β-TMCA, which are stereoisomers, were measured collectively (designated α/β-TMCA). Utilizing recently generated standard curves for β-TMCA from rat lysate (10 – 2000 pmol/well) and media (1.0 – 500 pmol/100µL), the original raw data collected for α/β-TMCA, along with the data for the other bile acids, was processed. The new α/β-TMCA standard curves were not generated with a stable isotope equivalent but were corrected for endogenous α/β -MCA background. Similarly, the raw data for the glycine conjugates of α- and β-muricholic

acid were collected but not processed in the original analytical run. Unfortunately, standards for these glycine conjugates are currently not available.

Data Analysis. Cells + bile and cellular concentrations of bile acids were calculated based on estimates of hepatocyte intracellular volume (6.83 µl/well) and number of cells/well.[243] Medium concentrations were calculated based on a volume of 1.5 ml/well. For bile acid accumulation studies, the *in vitro* biliary excretion index (BEI; %), defined as the percentage of accumulated substrate residing within the bile canaliculi, was calculated using B-CLEAR® technology (Qualyst, Inc. Durham, NC) according to the following equation: BEI = [($Accumulation_{standard\ buffer}$ - $Accumulation_{Calcium\text{-}free\ buffer}$)/($Accumulation_{standard\ buffer}$)] X 100%.[162] The *in vitro* biliary clearance (Cl_{bile}) was calculated based on the following equation: Cl_{bile}= ($Accumulation_{standard\ buffer}$ – $Accumulation_{Calcium\text{-}free\ buffer}$) / ($AUC_{medium}$), where AUC represents the area under the substrate concentration-time profile in the incubation buffer. Statistical analyses (one-way ANOVA and Bonferroni's multiple comparison post test) were performed using GraphPadPrism 3.0. In all cases, $p < 0.05$ was considered statistically significant.

RESULTS

Assessment of Cellular Viability in Sandwich-cultured Rat Hepatocytes. Prior studies have shown that it takes 3 days for rat hepatocytes to regain polarity in sandwich culture.[161,244,245] Because polarity is desirable to assess bile acid transport, we examined the effects of 24-hr RTV and LPV treatment, alone and combined, on

cellular viability and bile acid disposition between culture day 3 and day 4. LDH release and cellular adenosine triphospate (ATP) content were measured after individual and combination treatment with LPV and RTV. Alone, LPV and RTV demonstrated dose-dependent effects on cellular viability; the observed differences between the two treatments were not significant (**Figure 2.1**). Toxicity was not detected, or was minimal, at concentrations < 50 µM for each PI. Since toxicity may affect metabolic and transport processes involved in bile acid disposition in the SCRH model, PI concentrations $\leq$ 50 µM were used in subsequent studies. Cellular viability following exposure to the combination of LPV (5-50 µM) and RTV (5 µM) was comparable to LPV alone (**Table 2.1**) and the trend towards increased toxicity at 50 µM LPV was not statistically significant.

[^{3}H]TCA and [^{14}C]CDCA Accumulation in Sandwich-cultured Rat Hepatocytes. Accumulation of [^{3}H]TCA (1 µM) or [^{14}C]CDCA (5 µM) from the culture medium into cells + bile vs cells alone was measured following 10-min co-incubation with vehicle (0.1% DMSO), RTV (5 µM), LPV (5-50 µM), or combined LPV and RTV (LPV/r). As shown in Figure 2, the mean accumulation of [^{3}H]TCA in cells + bile was reduced by both LPV and RTV when administered alone, and the reduction was significant for LPV. A significant reduction relative to vehicle treatment also was observed for the combination treatment LPV/r (from 16.0 $\pm$ 2.2 vehicle alone to 7.6 $\pm$ 1.2 pmol/mg protein). It appeared that co-administration of LPV with RTV resulted in additional reduction in cells + bile concentration of [^{3}H]TCA compared to LPV treatment alone, but this decrease was not significant. The hepatocyte (cell) concentration of [^{3}H]TCA was not significantly increased by RTV or LPV alone

(**Figure 2.2**). However, when RTV was combined with LPV, the hepatocyte concentration of [^{3}H]TCA (7.7 $\pm$ 0.1 pmol/mg protein) was significantly increased relative to the cellular concentrations observed with either vehicle or 5 µM LPV alone (5.1 $\pm$ 0.7 and 5.0 $\pm$ 0.5 pmol/mg protein, respectively). When the same experiment was repeated with [^{14}C]CDCA, the treatments did not significantly alter the accumulation of [^{14}C]CDCA species in cells + bile or cells alone (**Figure 2.3**).

Biliary excretion of [^{3}H]TCA and [^{14}C]CDCA in Sandwich-cultured Rat Hepatocytes.

The calculated Biliary Excretion Index (BEI; %) for [^{3}H]TCA was reduced by both LPV and RTV alone, and further reduced by the combination treatment (**Table 2.2**). The calculated biliary clearance values ($Cl_{bile)}$ followed a similar pattern, but the reductions caused by RTV and LPV were statistically significant relative to vehicle treatment. Moreover, the reduction in Cl_{bile} observed with the combination of LPV and RTV was significantly greater than that observed with LPV alone, suggesting an additive effect on impaired biliary clearance. Concentrations exceeding 10 µM of LPV virtually ablated the BEI of [^{3}H]TCA, regardless of co-administration with RTV (data not shown).

[^{14}C]CDCA cellular concentrations in vehicle treated hepatocytes were 120-fold greater compared to [^{3}H]TCA, and the BEI of [^{14}C]CDCA species was 3-fold lower than for [^{3}H]TCA. Thus, changes in biliary clearance may not affect the cellular accumulation of [^{14}C]CDCA to the same extent as that of [^{3}H]TCA. LPV, alone or in combination with RTV reduced the BEI and essentially abolished the Cl_{bile} of [^{14}C]CDCA species (**Table 2.2**).

[^{3}H]TCA and [^{14}C]CDCA Initial Uptake in Suspended Rat Hepatocytes. To determine whether inhibition of bile acid uptake contributed to the reduction in Cl_{bile} caused by the PIs, [^{3}H]TCA and [^{14}C]CDCA influx into hepatocytes was measured during the linear uptake time interval (15 to 45 sec) in suspended rat hepatocytes [115,246]. Initial uptake rates of [^{3}H]TCA in Na^{+}-containing and Na^{+}-free buffer were 1.53 $\pm$ 0.11 and 0.15 $\pm$ 0.07 pmol/sec/mg protein, respectively (n=3; **Figure 2.4**). LPV (10 µM), RTV (5 µM), and LPV/r had no effect on the initial uptake rates of [^{3}H]TCA in Na^{+}-containing, or Na^{+}-free buffer compared to vehicle control. Similarly, LPV, RTV, and LPV/r had no effect on the initial uptake rates of [^{14}C]CDCA in Na^{+}-containing and Na^{+}-free buffer of vehicle control hepatocytes (9.92 $\pm$ 3.02 and 6.73 $\pm$ 2.19 pmol/sec/mg protein, respectively; n=3; **Figure 2.5**).

Accumulation of Endogenous Bile Acids in Cells + Bile, Cells, and Medium of Sandwich-cultured Rat Hepatocytes. TCA, GCA, TCDCA, GCDCA, and α/β-TMCA were measured in cells + bile, cells, and medium of SCRH. Taurine-conjugated bile acids accounted for the majority (approximately 99%) of bile acid species detected in vehicle-treated SCRH, consistent with data from *in vitro* rat studies published previously.[247] Concentrations (µM) of each bile acid species in cells + bile, cells, and medium of vehicle-treated SCRH are listed in **Table 2.3**. The α- and β-TMCA species comprised the majority of the total measured bile acid pool and appeared predominantly in the cells + bile and cells of SCRH. The BEI value of endogenous TCA (49%) was in the same range as the BEI calculated following addition of 1 µM [^{3}H]TCA (68%; **Table 2**). It is not possible to assess biliary

clearance of endogenously synthesized bile acids based on the current study design.

Total endogenous bile acid (sum of TCA, GCA, TCDCA, GCDCA and α/ß-TMCA) accumulation in medium, cells, and bile of SCRH also was determined following 24-hr incubation with vehicle, LPV (5 or 50 µM), and RTV (5 µM), alone or combined. Surprisingly, all treatments, except 5 µM LPV, significantly decreased total bile acid accumulation compared to vehicle control by (**Figure 2.6**). Interestingly, this marked reduction in total measured bile acids occurred despite the observation that LPV yielded minimal apparent toxicity to SCRH at these concentrations (**Figure 2.2**). The addition of 5 µM RTV to 50 µM LPV did not further decrease endogenous bile acid accumulation relative to 50 µM LPV alone (**Figures 2.6-2.9**). Conversely, the addition of 5 µM RTV to low dose LPV (5 µM), significantly decreased both total bile acid accumulation (**Figure 2.6**) as well as TCDCA accumulation in cells + bile (**Figure 2.8**). Similar trends were observed for the two principal bile acids measured, TCA and α/ß-TMCA (**Figures 2.7 and 2.9**).

LPV (50 µM) reduced the amount of TCA in medium, cells + bile, and cells alone (**Figure 2.7**); the reductions were roughly proportional in each of these three compartments. Similarly, TCDCA accumulation in cells + bile and cells alone was significantly decreased by 50 µM LPV; the addition of RTV did not appear to alter the effect of LPV alone (**Figure 2.8**). Notably, the BEI of TCDCA was markedly decreased by RTV, alone or in combination with LPV (values at the top of **Figure 2.8**). No significant differences in the accumulation of TCDCA in the medium were noted.

GCA accumulation in cells + bile was significantly decreased from control by 5 µM LPV combined with 5 µM RTV (1.53 ± 0.42 vs. 0.14 ± 0.14 pmol/mg protein), and nearly abolished by exposure to high dose LPV, in the absence and presence of RTV. GCDCA was essentially undetectable in cells + bile and cells of SCRH treated with 5 µM LPV combined with RTV, or with high dose LPV (50 µM), alone or combined with 5 µM RTV. Medium GCA and GCDCA were not statistically different following PI exposure relative to vehicle control values.

DISCUSSION

Inhibition of BSEP-mediated biliary excretion of bile acids is a proposed mechanism of DILI. Several PIs, including LPV and RTV, are inhibitors of BSEP *in vitro* and are associated with hepatotoxicity. Moreover, HIV treatment regimens frequently combine RTV with other PIs to improve oral availability (boosting effect), and these regimens may have increased potential for liver toxicity. The present work further characterizes the complex interactions between hepatocytes, PIs, and endogenous bile acids. We hypothesized that addition of RTV to LPV would result in increased intracellular accumulation of bile acids and increased toxicity in SCRH.

Hepatocytes cultured in a sandwich configuration regain morphological properties similar to those observed *in vivo*, including the development of tight junctions, canalicular networks, and polarized transport.[160] Additionally, SCRH exhibit toxicity when BSEP is inhibited.[115,248,249] Thus, the SCRH model was selected as the most suitable system to evaluate the effect of the PIs, LPV and RTV, on cytotoxicity, bile acid transport and endogenous bile acid disposition.

Contrary to our hypothesis, the combination of RTV and LPV did not produce a detectable increase in toxicity relative to LPV alone (**Table 2.1**). Nonetheless, exposure of SCRH to LPV coadministered with RTV further increased the cellular accumulation of TCA compared to LPV alone (**Figure 2.2**). It is important to note that our transport inhibition studies were conducted after 10 min of PI exposure, whereas toxicity was assessed after 24 hour PI exposure. The lack of toxicity observed at 24 hr may indicate that normal functioning hepatocytes are capable of responding to cellular injury via hepatoprotective mechanisms that maintain hepatocyte health despite accumulation of bile acids. Alternatively, feedback mechanisms could downregulate bile acid synthesis and/or upregulate bile acid efflux resulting in only a transient increase in intracellular bile acid concentrations.

As expected from previous reports,[78] RTV inhibited [^{3}H]TCA Cl_{bile} and BEI. Exposure to LPV inhibited the Cl_{bile} of [^{3}H]TCA, and addition of RTV resulted in further inhibition. It should be noted that the marked additional reduction in [^{3}H]TCA Cl_{bile} and BEI resulting from addition of RTV to LPV is consistent with additive effects of each drug and not a synergistic interaction. Doubling the concentration of LPV (to 10 µM) essentially ablated both Cl_{bile} and BEI for [^{3}H]TCA. This effect was similar to that observed when LPV (5 µM) was coadministered with RTV (5 µM).

In contrast to the result with [^{3}H]TCA, we were unable to detect any effect of LPV alone or in combination with RTV on the cellular content of [^{14}C]CDCA species. This may suggest that the effects of PIs on bile acid transport are specific for certain bile acids. Nonetheless, the effects of LPV and RTV on the calculated BEI and biliary clearance of [^{14}C]CDCA species were similar to those observed with [^{3}H]TCA.

Because the marked effects of the PIs on biliary excretion of [^{3}H]TCA and [^{14}C]CDCA species generally were not associated with similar increases in hepatocyte content of bile acids, it was possible that the PIs were differentially inhibiting basolateral uptake of bile acids. Modulating the Na^{+}-content of the buffer provides an accurate estimate of the contribution of the Na^{+}-dependent transporter, Ntcp, and the sodium-independent organic anion transporting polypeptides (Oatps), to total uptake. Basolateral uptake of TCA is governed primarily by Ntcp, and to a lesser extent by Oatps.[237] Conversely, CDCA uptake is reportedly driven predominantly by Oatps, while Ntcp contributes to a lesser degree.[242] Consistent with previous work, ~90% of the initial uptake rate of TCA into hepatocytes pre-incubated with vehicle (0.1% DMSO) was Ntcp-mediated, while the remaining ~10% was driven by sodium-independent transporter-mediated processes (presumably Oatps). Conversely, ~69% of transporter-mediated [^{14}C]CDCA uptake occurred in Na^{+}-free buffer, consistent with the literature findings that Oatp transporters are primarily responsible for initial CDCA uptake.[242,250] LPV and RTV, alone and combined, had no significant effect on the initial uptake of [^{3}H]TCA or [^{14}C]CDCA under Na^{+}-containing and Na^{+}-free conditions. Based on these findings, we concluded that disruption of canalicular efflux is the primary mechanism responsible for the PI-mediated decrease in the biliary clearance of [^{3}H]TCA and [^{14}C]CDCA.

Reported in this manuscript, for the first time, are the effects of PIs on the disposition of bile acids synthesized by SCRH. While the bile acid pool is comprised of numerous bile acid species, the present study focused on the quantification of taurine- and glycine-conjugated cholic acid and chenodeoxycholic acid due to their

potential cytotoxic effects.[119,251,252] In addition, the aforementioned bile acids are common constituents of both human and rodent bile. The rodent-specific α/ß-TMCA species also were quantified since they make up the majority of the bile acid pool in the rat. Secondary bile acids, i.e. those produced via intestinal metabolism, are not synthesized in the SCRH system; thus, these bile acid species were not quantified.[120] BEI values for endogenous TCA were comparable to those estimated following addition of radiolabelled TCA. However, very different results were obtained when we investigated the effects of the PIs on intracellular concentrations of endogenously synthesized TCA. Contrary to our results with exogenous [^{3}H]TCA administration and short-term PI exposure, RTV and LPV treatment (5 and 50 µm; 24 hr) resulted in a significant reduction in hepatocyte concentrations of endogenous TCA and α/ß-TMCA. Addition of RTV to high dose LPV (50 µM) appeared to have little additive effect. However, addition of RTV to low dose LPV (µM) significantly reduced the accumulation of endogenously synthesized total bile acids and TCDCA in SCRH (**Figures 2.6 and 2.8**). This observation may indicate that RTV inhibits LPV metabolism leading to increased cellular LPV concentrations, which may result in altered bile acid synthesis. These studies suggest that LPV and RTV may alter the synthesis and biliary excretion of individual bile acids differentially.

Fresh medium was applied to the SCRH every 24 hours. Thus, the remarkable decrease in total measured bile acid content may be due to reduced bile acid synthesis. Consistent with this conclusion, RTV (15-100 µM) exposure for 24 hr has been reported to disrupt cholesterol homeostasis and perturb bile acid synthesis in a concentration-dependent manner by decreasing the activity of cholesterol 7α

hydroxylase, the rate-limiting enzyme responsible for the catabolism of cholesterol to bile acids.[253] Based on these findings, the observed decrease in total measured bile acids following PI exposure in SCRH probably involves regulatory feedback mechanisms that promptly reduce synthesis of bile acids as a protective mechanism. An important conclusion drawn from our studies is that it may be necessary to quantify hepatocellular concentrations of *endogenous* bile acids when establishing a relationship between drug-mediated inhibition of hepatic transporters and hepatotoxicity.

An important question is how the effects of LPV and RTV on bile acid excretion from hepatocytes may relate to the hepatotoxicity observed in the clinic with these drugs. At steady-state, LPV and RTV are 98-99% bound to plasma proteins, albumin, and AAG. The average unbound fraction of LPV was 0.73% and ranged from 0.14-1.68%.[254] Total and unbound LPV concentrations in HIV-infected patients ranged from 677 to 23,767 ng/ml (~1-38 µM) and 4.2 to 209.2 ng/ml (0.007-0.33 µM), respectively. PI concentrations selected for these studies exceeded reported unbound plasma concentrations by 10-fold or more. However, pilot data indicated that intracellular LPV concentrations in SCRH were up to 20-fold greater than medium concentrations after co-administration with RTV (data not shown).

In summary, we found that short term exposure of hepatocytes to LPV and RTV resulted in reduced biliary excretion and, consequently, intracellular accumulation of TCA. However, following 24 hr exposure to LPV and RTV, we were unable to demonstrate even additive toxicity, and we observed a marked reduction in hepatocyte accumulation of endogenous bile acids (sum total of TCA, GCA,

TCDCA, GCDCA and α/ß-TMCA), primarily attributed to decreased α/ß-TMCA. These observations do not necessarily refute a role for bile acid transport inhibition in the DILI observed in patients treated with PIs. This is because most patients treated with PIs do not develop hepatotoxicity. We speculate that initial PI-mediated increases in cellular bile acid concentrations initiate a cascade of events that enables the hepatocytes to remain healthy in most patients. This adaptive response includes mechanisms that result in a marked decrease in hepatocyte content of bile acids, most likely involving reduced synthesis. We further speculate that this adaptive response may not occur in all patients treated with these drugs. If such deficiencies have a genetic basis, their identification could lead to a personalized medicine approach to avoid DILI in PI-containing regimens.

Table 2.1 Effect of 24-hour lopinavir exposure, in the presence or absence of ritonavir, on sandwich-cultured rat hepatocyte viability.

	LPV		**LPV/r**	
	Viability (% Control)			
Dose (μM)	**LDH assay**	**ATP assay**	**LDH assay**	**ATP assay**
5	99 ± 1	102 ± 15	99 ± 1	81 ± 7
10	100 ± 1	105 ± 7	99 ± 1	80 ± 7
25	99 ± 1	101 ± 1	98 + 1	79 ± 4
50	98 ± 2	81 ± 22	88 ± 8	68 ± 25

Notes. Day 3 sandwich-cultured rat hepatocytes were treated for 24 hr with lopinavir (LPV) in the absence or presence of ritonavir (LPV/r); mean ± SEM (n=3 livers in triplicate).

Table 2.2 Effect of lopinavir and ritonavir on the biliary excretion index and *in vitro* biliary clearance of [^{3}H]taurocholic acid and [^{14}C]chenodeoxycholic acid in sandwich-cultured rat hepatocytes.

	BEI (%)		**Cl_{bile} (ml/min/kg)**	
	[^{3}H]TCA	**[^{14}C]CDCA**	**[^{3}H]TCA**	**[^{14}C]CDCA**
Vehicle	68 ± 3	27 ± 2	8.7 ± 1.3	37.2 ± 8.1
5 µM RTV	21 ± 15	3 ± 3	2.5 ± 2.1[a]	0
5 µM LPV	49 ± 11	4 ± 4	4.4 ± 1.7[a]	0
5 µM LPV/r	9 ± 5	1 ± 1	0.61 ± 0.35[b]	0

Notes. Data from Figures 2 and 3 were used to calculate the biliary excretion index (BEI) and *in vitro* biliary clearance (Cl_{bile}), as described in the methods, in the absence or presence of RTV (LPV/r); mean ± SEM (n=3 livers in triplicate, analysis of variance followed by a Bonferroni post test; [a], versus vehicle control; [b] versus 5 µM LPV alone, $p < 0.05$).

Table 2.3 Bile acid concentrations (μM) in cells + bile, cells, and medium, and biliary excretion index values for each bile acid species, in day 4 sandwich-cultured rat hepatocytes.

Species	Cells + bile	Cells	Medium	BEI (%)
TCA	5.14 ± 1.71	2.61 ± 1.78	0.651 ± 0.127	49
GCA	0.20 ± 0.06	0.13 ± 0.08	0.07± 0.03	35
TCDCA	1.07 ± 0.20	0.63 ± 0.20	0.017 ± 0.003	41
GCDCA	0.12 ± 0.08	0.07 ± 0.04	0.004 ± 0.003	42
α/ß TMCA	168 ± 65	133 ± 72	1.59 ± 0.37	20
Total	174 ± 67	137 ± 74	2.34 ± 0.412	

Notes. Data represent mean ± SEM (n=3 livers in triplicate). Calculations assume a hepatocyte volume of 6.83 μl/well. The biliary excretion index (BEI) was calculated as described in the methods.

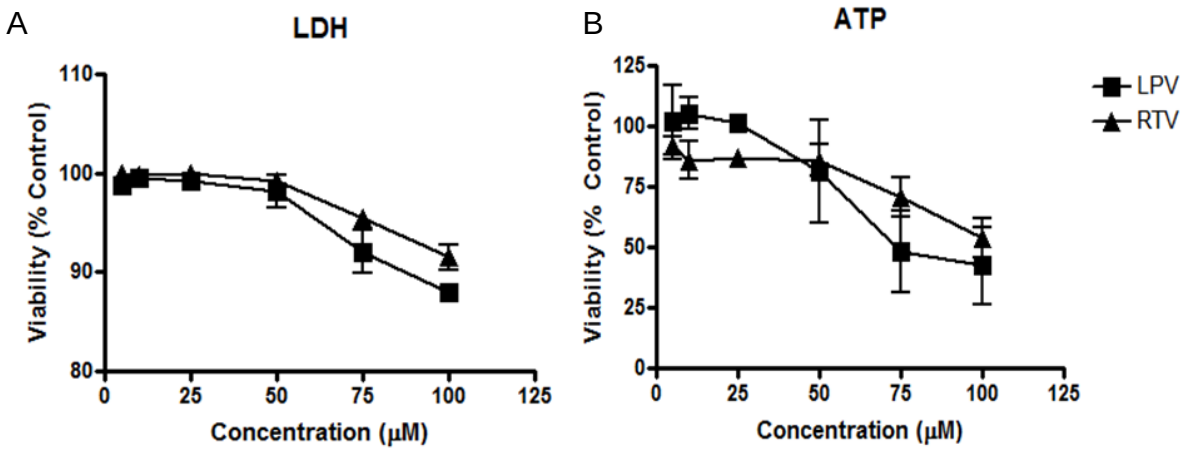

Figure 2.1 Effect of 24-hr exposure to LPV or RTV on hepatocyte viability in sandwich-cultured rat hepatocytes (SCRH). Day 3 SCRH were treated with LPV (squares; 5-100 μM) or RTV (triangles; 5-100 μM) for 24 hours. Following incubation, LDH release (A) and cellular ATP (B) levels were measured. Data are presented as mean ± SEM (*n* =3).

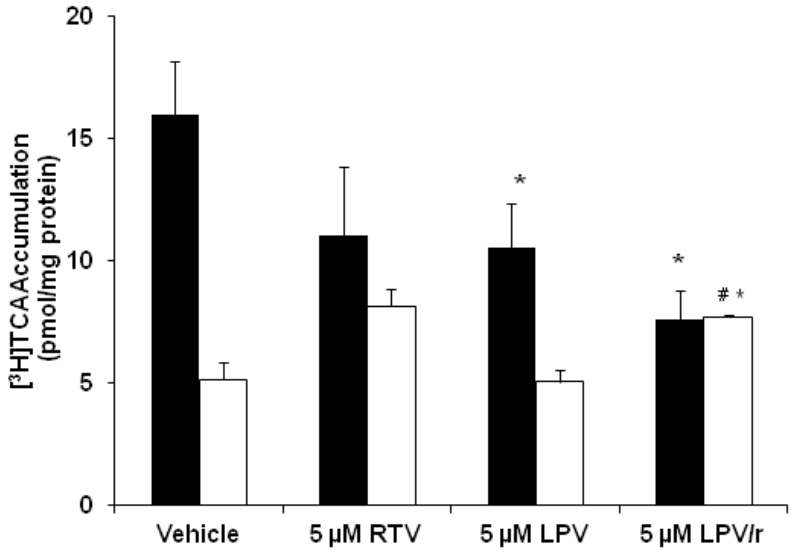

Figure 2.2 Effect of LPV and RTV, alone and combined, on taurocholic acid (TCA) accumulation in SCRH. [^{3}H]TCA BEI and accumulation in cells + bile (black bars) and cells (white bars), in day 4 SCRH were determined following a 10-min co-incubation with ritonavir (RTV, 5 µM) and lopinavir (LPV; 5 µM) alone or combined (LPV/r) (mean $\pm$ SEM; *n* = 3 livers in triplicate; analysis of variance followed by a Bonferroni post test , * versus cells + bile vehicle control, # vs. 5 µM LPV alone; $p < 0.05$).

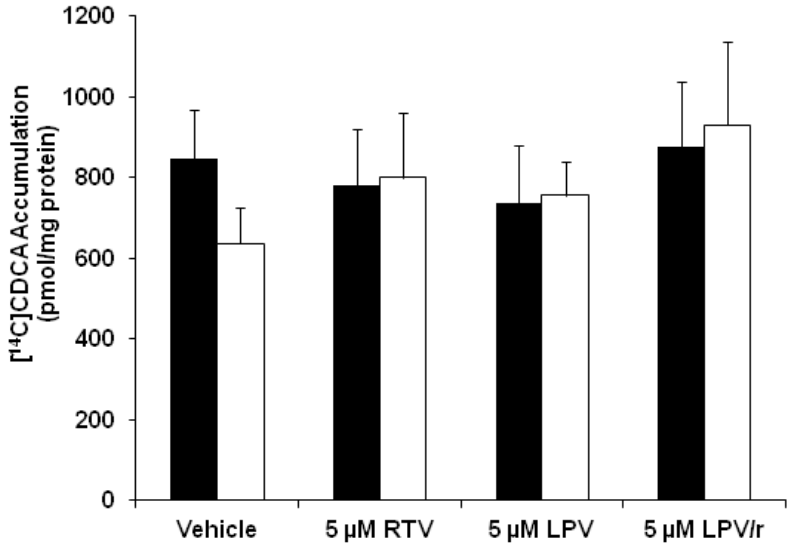

Figure 2.3 Effect of LPV and RTV, alone and combined, on chenodeoxycholic acid (CDCA) accumulation in SCRH. [^{14}C]CDCA BEI and accumulation in cells + bile (black bars) and cells (white bars), in day 4 SCRH were determined following a 10-min co-incubation with ritonavir (RTV; 5 µM) and lopinavir (LPV; 5 µM) alone or combined (LPV/r) (mean ± SEM; *n* = 3 livers in triplicate).

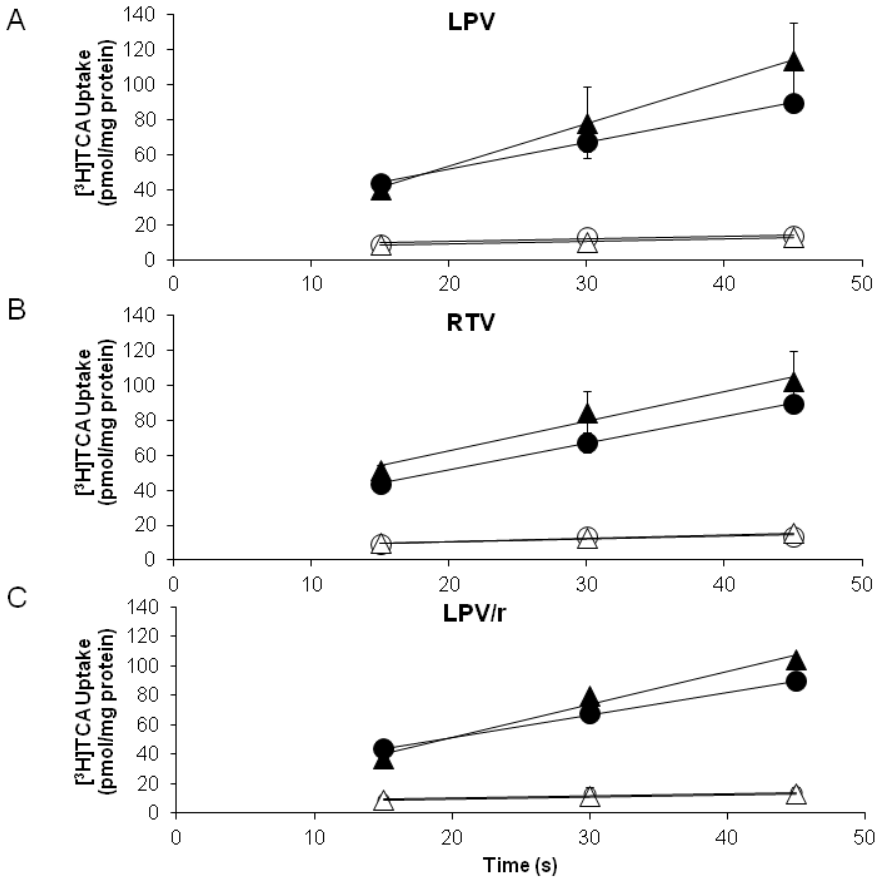

Figure 2.4 Effect of LPV and RTV, alone and combined, on the Na^+-dependent and Na^+-independent uptake of [^{3}H]TCA into freshly isolated suspended rat hepatocytes. [^{3}H]TCA accumulation in freshly isolated rat hepatocytes was determined following pre-incubation with LPV (10 µM; A) or RTV (5 µM; B), alone and in combination (C), in the absence or presence of sodium. Closed and open circles represent vehicle treated cells in Na^+-containing or Na^+-free buffer, respectively. Closed and open triangles represent treated cells in Na^+-containing or Na^+-free buffer, respectively. Uptake into cells is reported as pmol/ mg protein (mean $\pm$ SEM; *n*=3 livers in triplicate).

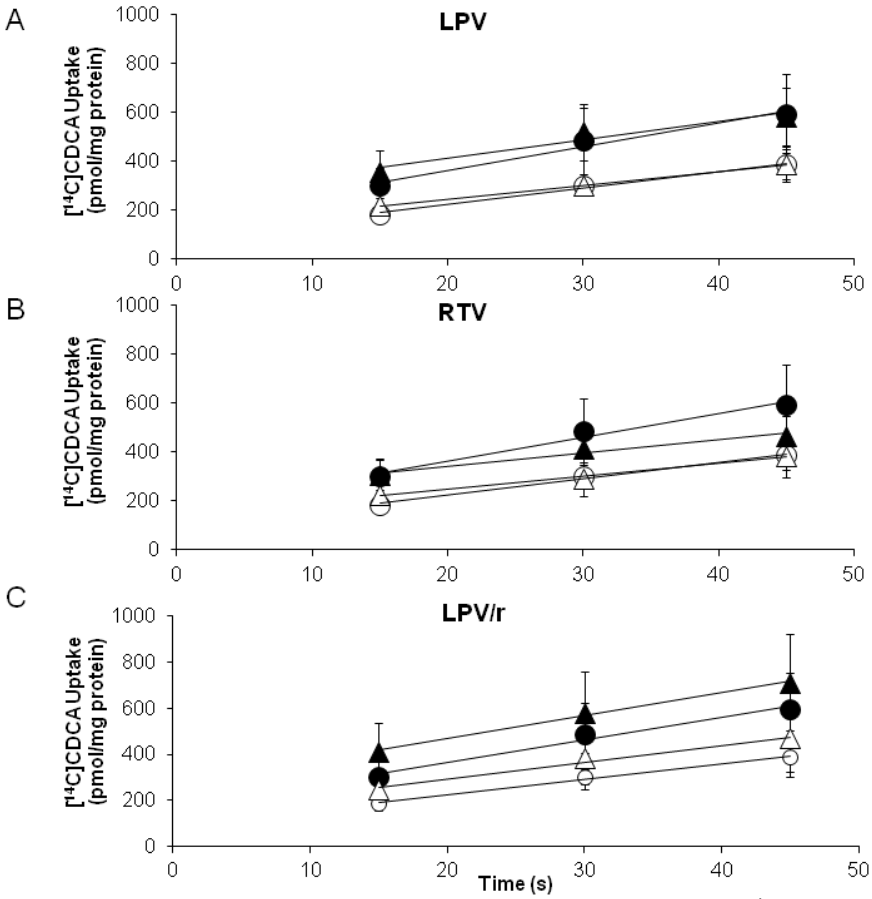

Figure 2.5 Effect of LPV and RTV, alone and combined, on the Na^+-dependent and Na^+-independent uptake of [^{14}C]CDCA into freshly isolated suspended rat hepatocytes. [^{14}C]CDCA accumulation in freshly isolated rat hepatocytes was determined following pre-incubation with LPV (10 µM; A) or RTV (5 µM; B) alone and in combination, in the absence or presence of sodium (C). Closed and open circles represent vehicle treated cells in Na^+-containing or Na^+-free buffer, respectively. Closed and open triangles represent treated cells in Na^+-containing or Na^+-free buffer, respectively. Uptake into cells is reported as pmol/ mg protein (mean ± SEM; *n*=3 livers in triplicate).

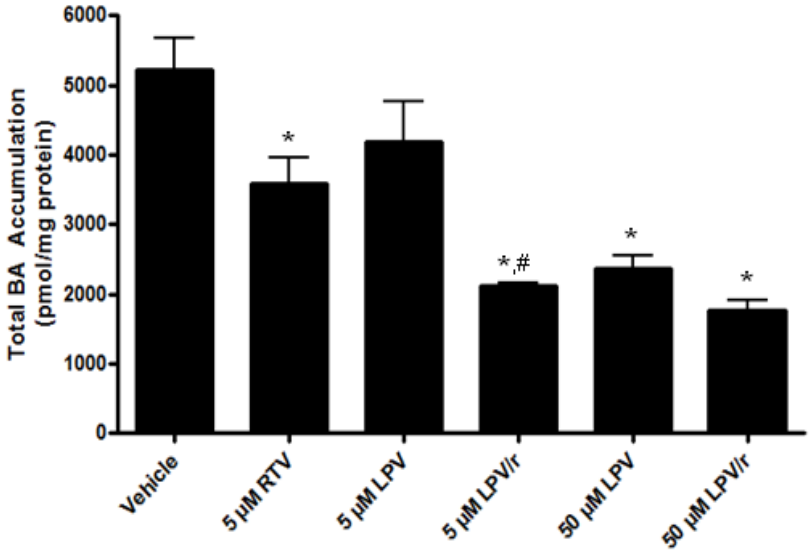

Figure 2.6 Accumulation of total measured bile acids (sum of TCA, GCA, TCDCA, GCDCA, and α/ß-TMCA) in SCRH (cells, bile, and medium) following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined (mean ± SEM; *n*=4 livers in triplicate; analysis of variance followed by a Bonferroni post test, *, versus vehicle control, $p < 0.05$).

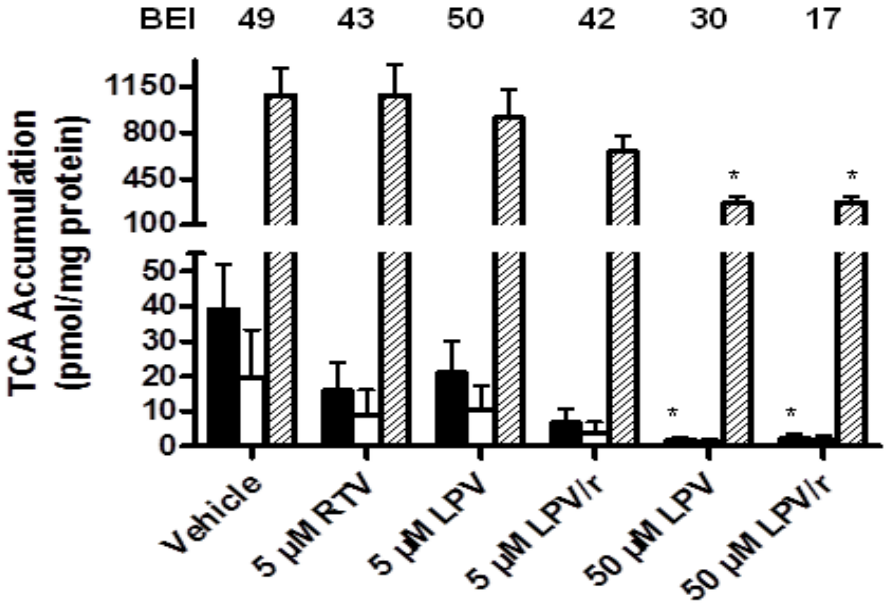

Figure 2.7 Accumulation of TCA in cells + bile (solid bars), cells (open bars), and medium (hatched bars) and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined (mean ± SEM; *n*=4 livers in triplicate; analysis of variance followed by a Bonferroni post test, *, versus vehicle control, $p < 0.05$).

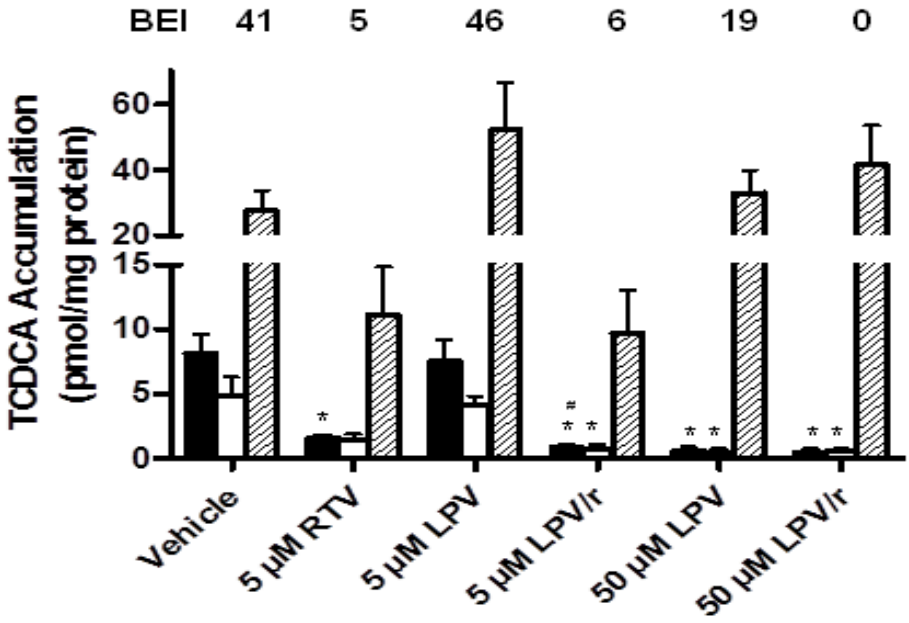

Figure 2.8 Accumulation of TCDCA in cells + bile (solid bars), cells (open bars), and medium (hatched bars) and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined (mean ± SEM; *n*=4 livers in triplicate; analysis of variance followed by a Bonferroni post test, *, versus vehicle control; #, versus 5 µM LPV, $p < 0.05$)

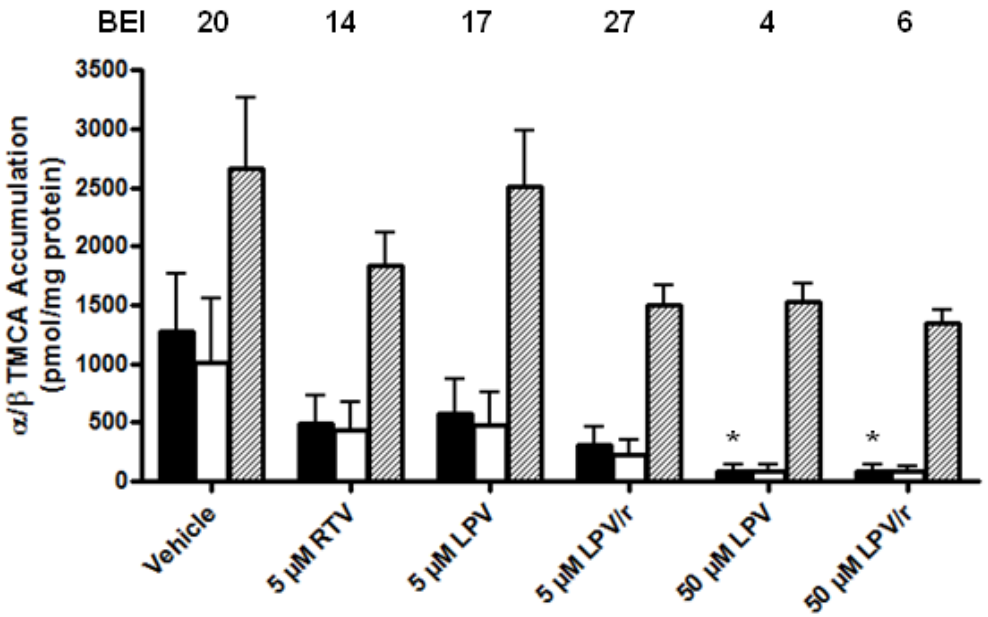

Figure 2.9 Accumulation of α/ß-TMCA in cells + bile (solid bars), cells (open bars), and medium (hatched bars) and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined (mean ± SEM; *n*=4 livers in triplicate; analysis of variance followed by a Bonferroni post test, *, versus vehicle control; #, versus 5 µM LPV, $p < 0.05$)

CHAPTER 3

GENETIC VARIATION IN BILE ACID TRANSPORT AND SYNTHESIS GENES: A POTENTIAL RISK FACTOR FOR DRUG-INDUCED LIVER INJURY

Drug-induced liver injury (DILI) is the leading cause of acute liver failure in the United States and is the most common adverse event leading to the withdrawal of drugs from the market.[255-257] DILI is rare, which makes predicting hepatotoxic events associated with drug therapy challenging. Impaired bile acid transport leading to the accumulation of bile acids known to cause mitochondrial damage and decreased membrane integrity is a proposed mechanism of DILI.[77,114,258] The bile salt export pump (BSEP) is the primary transport protein responsible for the canalicular excretion of bile acids.[126,128,259,260] Increasing evidence in the literature has established a correlation between inhibition of BSEP and cholestasis.[124,159,258] However, numerous studies demonstrate drug- and/or cholestasis-induced upregulation of alternate bile acid elimination pathways, and changes in the expression and activity of enzymes involved in bile acid synthesis. These changes in feedback regulatory mechanisms may offer hepatoprotection against the cellular accumulation of bile acids.[87,88,130,135,253] For example, a seven-fold increase in the protein expression of the multidrug resistance-associated protein (MRP) 4, which facilitates basolateral efflux of an array of compounds including bile acids, has been reported in cholestasis-induced bile duct ligated rats.[87]

Drugs can also indirectly affect bile acid synthesis and transport. Ritonavir, a potent inhibitor of BSEP, has been shown to significantly decrease cytochrome P450 (CYP)7A1 mRNA and protein expression levels in primary rat hepatocytes. CYP7A1 is the rate-limiting enzyme responsible for the conversion of cholesterol to bile acids. In the same study, bile acid synthesis also was decreased following exposure to ritonavir.[253]

Bile acids are taken up from the systemic circulation into the hepatocyte largely by the sodium-dependent co-transporting polypeptide (NTCP). NTCP is a member of the solute carrier (SLC) family, and is primarily responsible for the uptake of monovalent taurine- and glycine-conjugated bile acids. Sulfated compounds, thyroid hormones and a few drugs are also substrates for NTCP.[128] Organic anion transporting polypeptides (OATPs) mediate the sodium-independent basolateral uptake of bile acids. Two liver-specific isoforms, OATP1B1 and -1B3 contribute to the influx of bile acids and endogenous compounds such as bilirubin. While transporter affinity varies between bile acids species, the sodium-dependent uptake of bile acids is quantitatively more important in humans than the sodium-independent uptake processes.[128,242]

Canalicular efflux, the rate-limiting step in hepatocellular transport of bile acids, is driven predominantly by BSEP and thus, this protein is the focus of the present study.[128] The importance of BSEP in bile acid homeostasis has been demonstrated repeatedly in the literature. Decreased mRNA and protein levels of BSEP in liver slices incubated with lipopolysaccarides from patients with inflammatory liver disease have been reported by Elfereink and colleagues.[232]

Administration of ursodeoxycholic acid, used to treat cholestasis, is associated with upregulation of BSEP in patients with gallstones.[261] Genetic mutations in BSEP resulting in cholestatic diseases in humans also have been reported. One of the most severe diseases associated with a polymorphism in BSEP is progressive familial intrahepatic cholestasis type 2 (PFIC2). Clinical presentation often begins during childhood and usually progresses to severe cholestasis warranting liver transplantation.[130,262,263]

Other transporters located on the canalicular membrane play a minimal role in the efflux of some bile acids. MRP2 excretes sulfated and glucuronidated bile acids as well as bilirubin into the bile, while p-glycoprotein (P-gp) transports taurine- and glycine-conjugated bile acids.[120,137,264] However, these proteins are primarily responsible for the canalicular efflux of a diverse range of drugs, including compounds that interact with BSEP.

Basolateral efflux transporters MRP3 and MRP4 are expressed at low levels in healthy hepatocytes. While these proteins generally contribute to the basolateral efflux of numerous, structurally diverse drugs, MRP3- and MRP4-mediated bile acid transport has been shown.[137,140,141] Furthermore, MRP3 and MRP4 may be upregulated during cholestasis as a hepatoprotective mechanism. Increased renal excretion of bile acids in patients with chronic cholestasis corroborates this observation.[139,265,266] The organic solute transporter (OST) α, combined with OSTß, transports bile acids in a sodium-independent fashion. OSTα is modestly expressed in the human liver while OSTß liver expression is virtually undetectable. While the independent function of each subunit has yet to be determined, it is clear that co-

expression and assembly is required for trafficking of this protein to the plasma membrane. The localization of proteins involved in hepatic bile acid transport is depicted in **Figure 3.1.**

The mechanisms underlying DILI are complex and most likely involve a number of factors including, age, gender, duration of drug exposure, concomitant medications, and co-morbidities. Several studies also suggest that genetic variants in specific transport proteins may alter the disposition of drugs and endogenous bile acids, thereby predisposing some individuals to drug-induced hepatotoxicity.[130,149,153,267-269] In addition, genes involved in bile acid synthesis can indirectly influence bile acid transport. Thus, deleterious genetic mutations in such genes may indirectly contribute to the risk of DILI. Based on this rationale, we tested the hypothesis that single nucleotide polymorphisms (SNPs) in genes that play a role in bile acid transport and synthesis are predictive risk factors for DILI. Furthermore, multiple variants in genes that serve as alternate routes of bile acid excretion may have an additive effect on the risk of DILI.

METHODS

Study Subjects

Polymorphisms in candidate genes selected based on literature review were used to conduct a SNP association analysis to elucidate the role of genetic variants in DILI. After patients provided informed consent, DNA samples were obtained and prepared as reported previously.[270,271] Cases (n=401) of European ancestry enrolled in the Drug-induced Liver Injury Network (DILIN)[270] between August 2004 and April

2009 were included in the study. Cases were genotyped at the Duke Center for Human Genome Variation using the Illumina Human1M-Duo BeadChip. Genotype data from 2,346 controls from the 1958 British Birth Cohort supplied by the Wellcome Trust Case-Control Consortium 2 (www.wtccc.org.uk) were used for comparison. Cases were categorized as hepatocellular, cholestatic or mixed using the R value as described by Danan *et al*.[272] Severity of hepatic injury (ranging from mild to fatal) and causality scores also were determined.[270] Characteristics of the DILIN patients included in the present study are listed in **Table 3.1**

Selection and Analysis of Genetic Variants.

In the present study, variants in genes implicated in bile acid metabolism and hepatobiliary transport were selected for analysis. The genetic variants were chosen from a subset of drug absorption, distribution, metabolism and elimination (ADME) genes for which genotyping data were previously generated in a genome-wide association analysis.[270,271] The majority of the selected variants were located in exomic regions. Two variants were located at the 5' or 3' untranslated region, and seven variants were located in intronic regions. Although some SNPs were selected based on reported functional consequences, to date, evidence demonstrating functional roles of genetic variants, particularly for drug transporters, is limited. **Table 3.2** lists the SNPs and genes selected, their physiological function, associated phenotypes (where clinically reported), and genomic location.

To evaluate potential associations of individual variants with DILI, genotypes were analyzed as wild-type versus variant carriers, where variant carriers were

either heterozygotes or homozygotes. Logistic regression analyses were performed to evaluate the relationship between individual polymorphisms and DILI in wild-type and variant carriers. Studies examining the influence of multiple MRP3 and MRP4 variants on DILI cases involving drugs known to inhibit BSEP[238] also were analyzed using logistic regression. All tests were carried out using the top 10 principal components emerging from the EIGENSTRAT analyses[273] as covariates in the model. Drugs reported to inhibit BSEP that were suspected of causing liver toxicity in the DILIN cases are listed in **Table 3.3**. The outcome was dichotomized based on the absence or presence of DILI, and the number of variants present in MRP3 and/or MRP4 was counted. The specified threshold for significance after multiple test correction was $p < 0.001$. All hypotheses tested were determined *a priori*.

RESULTS

A total of 30 out of 36 selected variants were analyzed by logistic regression. Four variants were omitted from the analysis because they were present only in a small number of controls, which caused collinearity problems in the regression; these variants were found in MRP4 (rs11568668), OSTα (rs9849888) and OSTß (rs2919347 and rs4961295). Additionally, CYP7A1 (rs8192875) and SREBF2 (rs2229440) variants were only found in one individual. Consequently, there was not sufficient data to perform logistic regression analysis for these variants. Odds ratios, p-values and 95% confidence intervals for each comparison are listed in **Table 3.4**. Quantile-quantile plots were constructed for each analysis to evaluate the distribution of each variant (**Figures 3.2, 3.3 and 3.4**). Generally, the distribution of

p-values resulting from the set of tests performed were no different from those expected under the null hypothesis. None of the individual variants assessed were significantly associated with an increased risk of DILI when controls were compared to all DILI cases, or when controls were compared to cholestatic cases. However the association of the OSTß variant, rs2919351, was notable when controls were compared to all DILI cases and cholestatic DILI cases (odds ratios of 3.6 and 6.1, respectively). The rs2919351 variant yielded a significantly increased odds ratio of 10.1 ($p<0.0015$) when controls were compared to cholestatic and mixed DILI cases. This odds ratio was greater for mixed cases alone (17.6, $p=3 \times 10^{-4}$). It is important to note that this variant clearly deviates from the expected distribution for the cholestatic and mixed model (**Figure 3.4**). We next examined whether the association would strengthen with increased confidence in the diagnosis of DILI. Cases without causality scores and those with scores of "unlikely" and "possible" were omitted from the analysis. We found that the odds ratio and strength of association were modestly decreased when the analysis was restricted to confirmed probable cases (**Table 3.5**).

Because MRP3 and MRP4 may compensate for loss of BSEP activity in the setting of BSEP inhibition, we hypothesized that individuals carrying variants in MRP3 and/or MRP4 may be at increased risk of DILI due to BSEP inhibiting drugs, and that the risk may be additive with increasing burden of MRP3/4 variants. Of the 401 cases, approximately 12% of the indicated drugs have been reported to inhibit BSEP (**Table 3.3**). Logistic regression analysis of 49 DILI cases due to known

BSEP inhibitors and 2,346 controls revealed no significant differences in risk of DILI, irrespective of DILI category.

DISCUSSION

Our hypothesis was that variations with functional consequences in genes involved in bile acid transport and synthesis may influence the risk of drug-induced hepatotoxicity. The present study investigated the impact of genetic variants on the risk of DILI. Additionally, the hypothesis that multiple variants in transporter genes that act as compensatory elimination routes (i.e. MRP3 and MRP4) have an additive effect on the risk of DILI was examined. We found that the OSTß variant rs2919351 was associated with cholestatic and mixed DILI, and that this association approached significance even after correction for multiple comparisons. Moreover, this association achieved significance when only mixed DILI cases were examined. This observation suggests that this variant in OSTß may increase patient susceptibility to hepatotoxic events following drug exposure. Secondary analysis in which cases with less evidence of causality were omitted showed no differences in the variant contribution to DILI. The observation that the association was strongest with mixed rather than cholestatic DILI could be explained by the theory that DILI is a progressive adverse event in which hepatocellular death is preceded by cholestasis. Thus, “mixed” DILI may, in fact, be the result of cholestasis and ultimately progression to hepatocellular liver injury.

Since the phenotypic outcome of interest (DILI) is a rare event, population controls (rather than drug-treated controls) were chosen for comparison to DILI

patients. The present study was a retrospective, hypothesis-driven investigation that was exploratory in nature, and as such, there are obvious limitations. Firstly, only subjects of European descent were included in the analysis. Although this restriction creates a population that is not representative of the general North American population, it does eliminate the risk of spurious findings due to population stratification. Also, variants selected for interrogation were restricted to those genotyped or tagged on the Illumina 1Mduo BeadChip, which generally only contains polymorphisms with allelic frequencies of at least 5%. As a result, we were unable to characterize the influence of more rare genetic variants on DILI. It is often assumed that variants that are apt to markedly affect transporter and enzyme function are likely to be deleterious and subject to purifying selection, and are therefore expected to be rare in the population. This concept has been demonstrated for transporter genes in particular.[274,275] Studies clearly demonstrating a functional consequence of genetic variants on genes involved in drug and/or bile acid disposition, particularly those in transporter genes, are limited. Thus, it is unclear whether some of the SNPs selected in the present study have a notable influence on the hepatic disposition of bile acids in humans. A final limitation is that information regarding which drugs inhibit BSEP in humans is minimal. Cases involving BSEP inhibitors were selected based on evidence in the literature. Consequently, compounds that inhibit BSEP but lack data supporting this interaction were not included in the BSEP-focused association analysis.

In conclusion, a variant in the basolateral bile acid efflux transporter, OSTß significantly increased the risk of cholestatic and mixed DILI. If confirmed in

additional cohorts, this finding supports our hypothesis that genetic variants in bile acid transporters and metabolic enzymes might contribute to the disposition of endogenous bile acids, thereby increasing the risk of DILI. Further studies are warranted to understand the potential role of rare variants, characterize the functional consequences of individual variants, and examine the contribution of putatively functional variants to drug-induced hepatotoxicity.

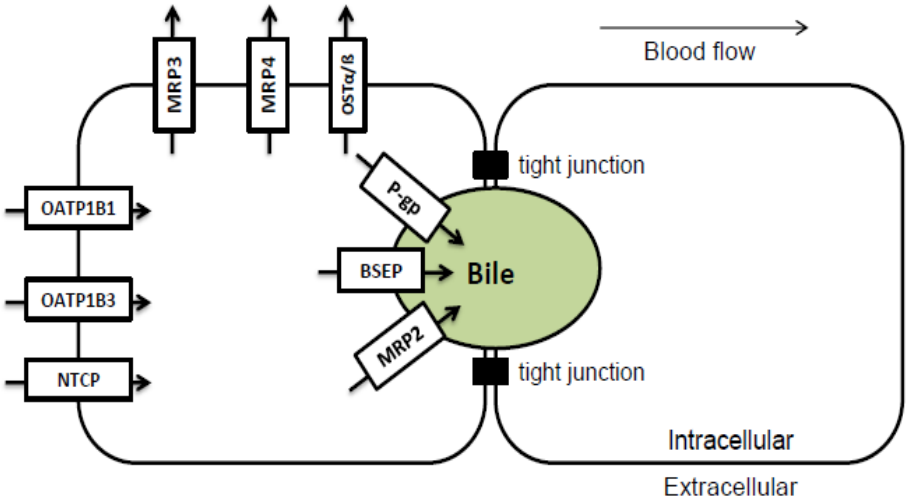

Figure 3.1 Illustration of hepatic transport proteins involved in bile acid transport in humans. Basolateral uptake of bile acids is governed primarily by NTCP, and to a lesser extent by OATPs. Canalicular efflux of bile acids is facilitated by BSEP, which represents the rate-limiting step in the hepatocellular disposition of bile acids. MRP3 and MRP4 are basolateral drug transporters that are capable of effluxing bile acids under cholestatic conditions. The OSTα/OSTß heterodimer, while predominantly expressed in the intestine, contributes to the basolateral efflux of bile acids from hepatocytes. P-gp and MRP2 are responsible for the canalicular efflux of an array of drugs and endogenous compounds (e.g., bilirubin). However, modest canalicular efflux of taurine- and glycine-conjugated (P-gp) as well as sulfated (MRP2) bile acids has been demonstrated.

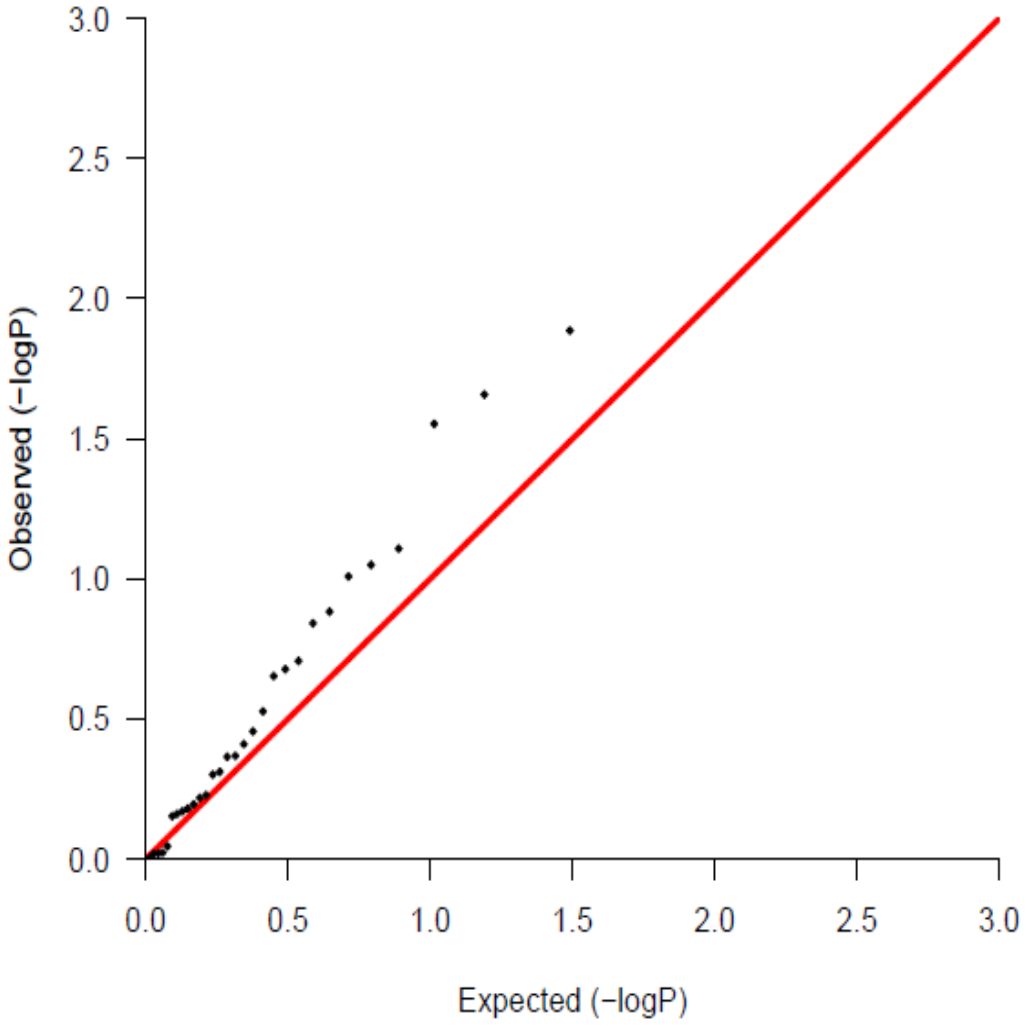

Figure 3.2 Normal probability plot of all DILI cases.

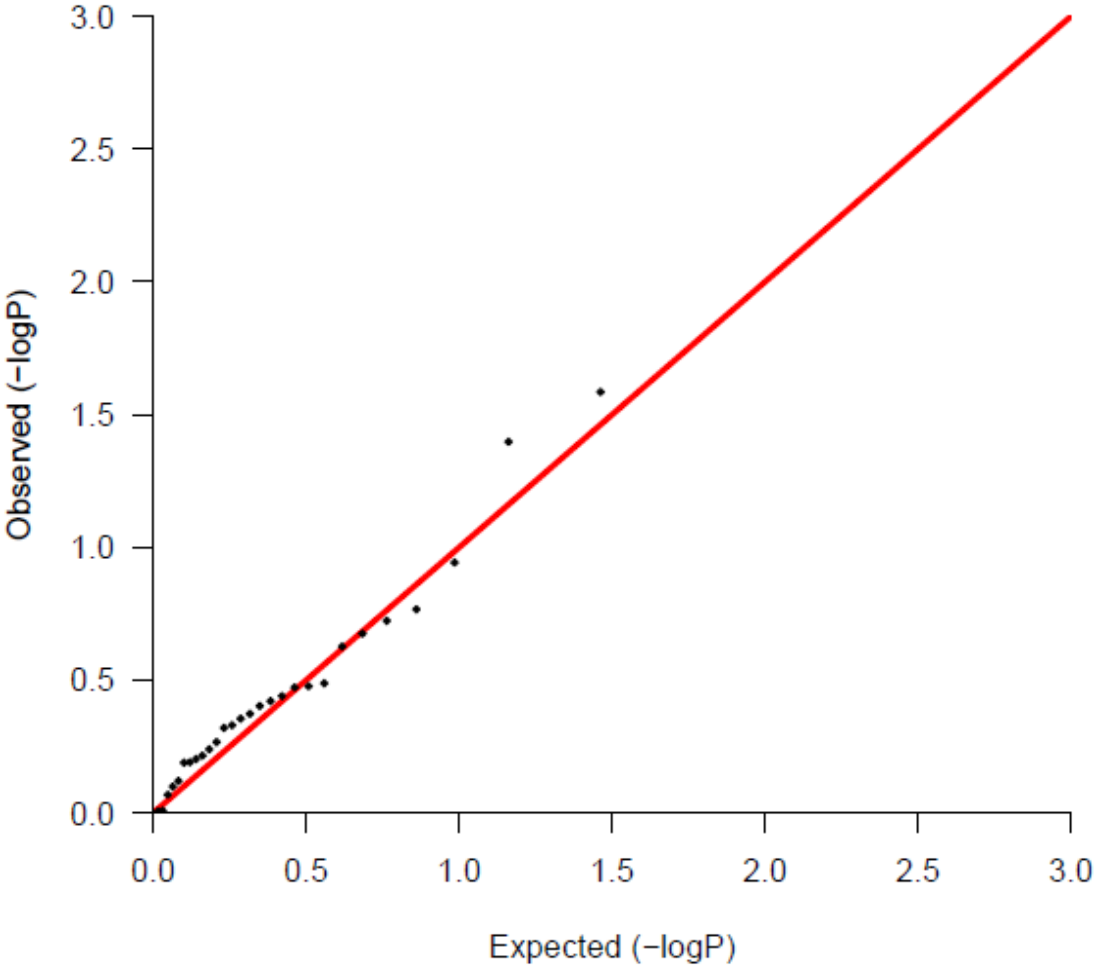

Figure 3.3 Normal probability plot of cholestatic DILI cases.

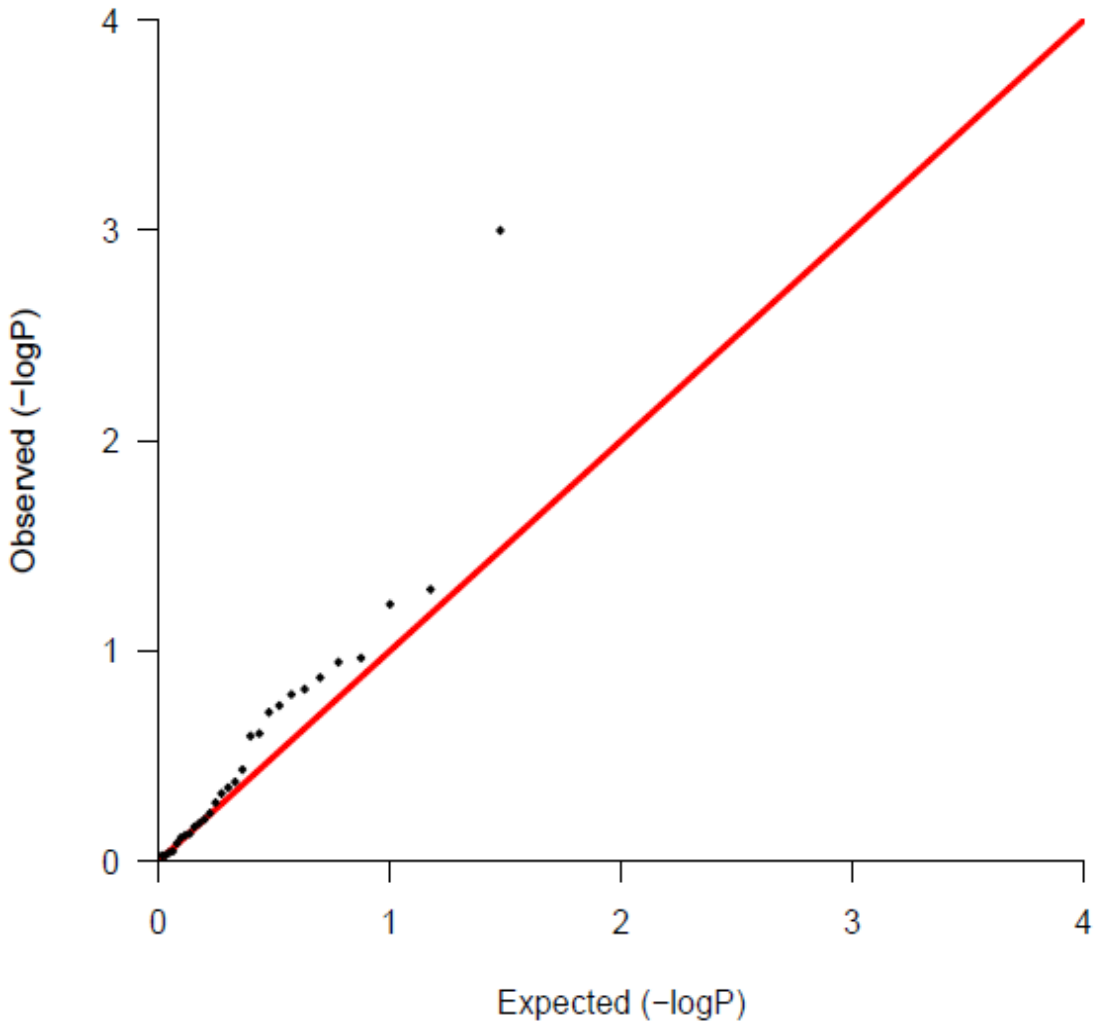

Figure 3.4 Normal probability plot of cholestatic and mixed DILI cases.

Table 3.1 DILIN subject characteristics

Variable	DILIN patients, N (%)
Gender	
Male	159 (40)
Female	241 (60)
DILI Category	
Cholestatic	82 (21)
Hepatocellular	158 (40)
Mixed	80 (20)
N.D.	60 (15)
Severity	
Mild	105 (26)
Moderate	80 (20)
Moderate-hospitalized	47 (12)
Severe	20 (5)
Fatal	124 (31)
N.D.	24 (6)

N.D.: not determined

Table 3.2 Genes and SNPs interrogated

Gene	Function	dbSNP and location	Associated Phenotype/ Functional Evidence
BSEP	Canalicular efflux of bile acids.	rs2287622[a]	↑risk of ICP[269], CC, DC, BRIC[267]
		rs497692[b]	PBC; Severe exon skipping[267]
		rs4148777[b]	N.R.
MRP2	Canalicular efflux of organic anions, including drugs and some endogenous compounds.	rs2273697[a]	↓ affinity for LTC_4, $E_2$3G, and $E_2$17G in Sf9 transfected cells ↓ carbamazepine transport[276,277]
		rs8187707[b]	N.R.
		rs8187710[a]	↑LPV accumulation in PBMCs of HIV-infected patients[276]
MRP3	Basolateral efflux of organic anions, including drugs and endogenous compounds such as bile acid conjugates (under cholestatic conditions).	rs4794175[c]	N.R.
		rs11568605[a]	N.R.
		rs4148416[b]	N.R.
		rs11568591[a]	N.R.
		rs2277624[b]	N.R.
		rs11568589[b]	N.R.
		rs1051640[b]	N.R.
MRP4	Basolateral efflux of drugs and endogenous compounds, including bile acids (under cholestatic conditions).	rs3742106[d]	N.R.
		rs3765534[a]	↓ surface membrane protein expression[278]; ↑ sensitivity to 6-mercaptopurine toxicity[278]
		rs11568668[a]	↑ intracellular levels of azidothymidine and PMEA[279]
		rs11568658[a]	↑ intracellular levels of azidothymidine and PMEA[279]
P-gp	Canalicular efflux of drugs and some endogenous compounds (e.g. bilirubin).	rs2235035[c]	↑ susceptibility to colorectal cancer[280]
		rs1128503[b]	↑ exposure and ↓ clearance of doxorubicin[281]; ↑ systemic tipifarnib exposure in cancer patients[282]
		rs1202168[c]	Altered menopausal hormone replacement-associated colorectal cancer risk[283]

Gene	Function	dbSNP and location	Associated Phenotype/ Functional Evidence
P-gp	Canalicular efflux of drugs and some endogenous compounds (e.g. bilirubin).	rs3789243[c]	↑ drug resistance in epilepsy patients[284]
		rs3213619[e]	↓mRNA expression in colorectal cancer cells[285]; ↓tacrolimus systemic concentrations[286]
OSTα	Basolateral efflux of bile acids.	rs11719281[b]	N.R.
		rs1522394[b]	N.R.
		rs939885[a]	N.R.
		rs17852687[b]	N.R.
		rs9849888[a]	N.R.
OSTß	Basolateral efflux of bile acids.	rs2414870[c]	N.R.
		rs2919347[c]	N.R.
		rs34961295[c]	N.R.
		rs2919351[c]	N.R.
CYP7A1	Rate-limiting enzyme in the classical pathway of bile acid synthesis.	rs8192875[a]	N.R.
CYP39A1	Enzyme involved in the conversion of cholesterol to bile acids.	rs2277119[a]	N.R.
HSD3B7	Enzyme involved in the conversion of cholesterol to bile acids.	rs9938550[a]	PFIC type 4[287]
		rs34212827[a]	N.R.
SREBF2	Transcription factor that regulates cholesterol homeostasis.	rs2229440[a]	N.R.

N.R.: not reported; CC: contraception-induced cholestasis; DC: drug-induced cholestasis; BRIC: benign recurrent extrahepatic cholestasis; PBMC: peripheral blood mononuclear cells; ICP: intrahepatic cholestasis of pregnancy; PFIC: progressive familial intrahepatic cholestasis; LTC_4: cysteinyl leukotriene; $E_2$3G:Estradiol-3-glucuronide; $E_2$17G: Estradiol-17-beta-

glucuronide; PMEA: para-methoxyethylam1phetamine; a: coding non-synonymous missense mutation; b: synonymous mutation; c:intron; d: 3 prime untranslated region; e: 5 prime untranslated region.

Table 3.3 List of BSEP Inhibitors Implicated in DILI cases

Implicated Drug	# of Cases
Amiodarone	3
Amitriptyline Hydrochloride	1
Ciprofloxacin	5
Cylophosphamide	2
Erythromycin	1
Estradiol	1
Fluconazole	2
Fluoxetine	2
Glipizide	1
Isoniazid	16
Metformin	2
Methotrexate	2
Metoprolol	1
Nevirapine	2
Ranitidine	2
Simvastatin	4
Tizanidine	1
Verapamil	1

Table 3.4 Logistic Regression Analysis of Controls versus DILIN Cases

		All Cases (n=401)				Cholestatic (n=82)				Cholestatic/mixed (n=162)			
		Odds Ratio	p value	95% Confidence Interval		Odds Ratio	p value	95% Confidence Interval		Odds Ratio	p value	95% Confidence Interval	
BSEP	**rs497692**	1.1	0.210	0.940	1.325	1.2	0.337	0.840	1.664	1.2	0.161	0.930	1.546
	rs2287622	0.9	0.431	0.783	1.110	0.9	0.396	0.601	1.223	0.9	0.591	0.721	1.205
	rs4148777	1.4	0.098	0.940	2.074	1.4	0.325	0.698	2.963	1.2	0.632	0.634	2.119
MRP2	**rs2273697**	0.8	0.013	0.599	0.942	0.8	0.379	0.535	1.269	0.8	0.182	0.579	1.109
	rs8187707	1.2	0.388	0.825	1.641	1.2	0.608	0.609	2.337	1.1	0.754	0.648	1.820
	rs8187710	1.2	0.428	0.814	1.625	1.2	0.575	0.618	2.377	1.0	0.897	0.610	1.756
MRP3	**rs4794175**	1.1	0.488	0.817	1.526	1.2	0.644	0.619	2.171	1.4	0.152	0.890	2.118
	rs11568605	0.1	0.028	0.011	0.773	N.A.				0.2	0.196	0.017	2.310
	rs4148416	1.3	0.131	0.923	1.857	1.3	0.441	0.660	2.601	1.3	0.254	0.809	2.236
	rs11568591	0.9	0.499	0.622	1.260	1.3	0.363	0.726	2.397	1.0	0.911	0.593	1.595
	rs2277624	1.0	0.991	0.814	1.232	0.9	0.795	0.623	1.436	1.0	0.772	0.701	1.302
	rs11568589	1.0	0.947	0.408	2.609	N.A.				N.A.			
	rs1051640	1.0	0.703	0.763	1.200	0.8	0.333	0.491	1.272	0.9	0.659	0.664	1.296
MRP4	**rs3742106**	1.0	0.591	0.880	1.252	0.9	0.626	0.644	1.304	1.0	0.740	0.738	1.241
	rs3765534	0.8	0.674	0.328	2.057	1.2	0.854	0.259	5.111	0.5	0.420	0.120	2.419
	rs11568658	1.8	0.022	1.091	2.997	2.5	0.026	1.117	5.777	1.7	0.134	0.843	3.604
P-gp	**rs2235035**	1.0	0.957	0.830	1.193	0.8	0.189	0.560	1.122	0.9	0.476	0.697	1.183
	rs1128503	1.1	0.297	0.923	1.299	1.1	0.541	0.793	1.557	1.3	0.060	0.990	1.644
	rs1202168	1.1	0.222	0.938	1.319	1.1	0.423	0.819	1.609	1.3	0.051	0.999	1.658
	rs3789243	1.0	0.689	0.818	1.142	0.9	0.467	0.634	1.232	0.8	0.113	0.640	1.048
	rs3213619	1.3	0.196	0.869	1.987	0.4	0.211	0.128	1.576	1.3	0.447	0.688	2.334
OSTα	**rs11719281**	1.1	0.659	0.822	1.363	1.0	0.986	0.606	1.635	1.0	0.938	0.700	1.471
	rs1522394	1.1	0.350	0.903	1.334	1.1	0.758	0.725	1.556	1.0	0.822	0.775	1.379
	rs939885	1.0	0.899	0.835	1.171	1.0	0.979	0.720	1.402	1.1	0.367	0.874	1.439
	rs17852687	1.2	0.089	0.978	1.374	1.3	0.171	0.905	1.759	1.1	0.528	0.845	1.389

		All Cases (n=401)				Cholestatic (n=82)				Cholestatic/mixed (n=162)			
		Odds Ratio	p value	95% Confidence Interval		Odds Ratio	p value	95% Confidence Interval		Odds Ratio	p value	95% Confidence Interval	
OSTß	**rs2414870**	1.0	0.953	0.787	1.289	0.9	0.646	0.534	1.476	0.9	0.688	0.637	1.346
	rs2919351	3.6	0.078	0.868	14.824	6.1	0.114	0.649	57.703	10.1	**0.001**	2.519	40.325
CYP39A1	**rs2277119**	0.9	0.144	0.697	1.054	0.8	0.236	0.510	1.180	0.8	0.108	0.565	1.058
HSD3B7	**rs9938550**	1.0	0.637	0.803	1.144	0.9	0.478	0.616	1.254	1.0	0.939	0.781	1.306
	rs34212827	1.4	0.604	0.427	4.316	4.3	0.040	1.070	17.309	2.3	0.247	0.566	9.177

N.A.: Not Applicable

Table 3.5 Secondary Analysis of rs2919351: Influence of DILI Category and Causality

	Odds Ratio	p value	95% Confidence Interval	
Control vs. mixed DILI[a]	17.6	0.000344	3.67	84.79
Control vs. cholestatic + mixed DILI	10.1	0.001075	2.52	40.33
Control vs. cholestatic + mixed DILI[b]	9.7	0.003000	2.19	42.64

[a]statistically significant
[b]cases restricted to those with causality scores $\geq$ probable

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

Drug-induced liver injury (DILI) is a rare but severe adverse event, often resulting in the withdrawal of otherwise effective drugs from the market.[255,257] DILI accounts for at least 13% of US acute liver failure cases[256] and the incidence of DILI reportedly ranges from one in 10,000 to one in 100,000 patients on medications.[288] Unfortunately, the number of DILI-inducing drugs that have known mechanisms of toxicity and/or exhibit dose-dependent toxicity is marginal. Furthermore, not all drugs that elicit abnormal liver function tests cause patients to develop persistent hepatotoxicity. Several examples in the literature provide evidence of adaptation, in which initial elevations in liver enzymes following initiation of drug therapy return to normal after continued drug exposure.[289,290] The infrequency and irregularity of DILI further complicates our understanding of its pathophysiology, making it difficult to predict the risk of DILI in humans. Though mechanisms of DILI remain unclear, several hypotheses have been proposed, including the production of reactive metabolites, auto-immune responses, or cellular oxidative stress. All of the aforementioned mechanisms are known to alter membrane permeability, resulting in perturbation of ATP synthesis.[291]

Inhibition of bile acid transport culminating in hepatocellular retention and accumulation of bile acids (i.e. cholestasis) also has been proposed as a mechanism

of drug-induced liver injury.[238,239,292] Mounting evidence in the literature demonstrates inhibition of the bile salt export pump (BSEP), the main canalicular efflux protein responsible for bile acid excretion, by a number of drugs is associated with cholestasis. Currently, potent inhibition of BSEP is considered a potential risk, often leading to the termination of a compound during the drug development process. One aim of this dissertation research was to utilize the sandwich-cultured rat hepatocyte (SCRH) model and freshly isolated suspended rat hepatocytes to determine the inhibitory effect of combination protease inhibitors on the hepatic transport of bile acids as a mechanism of toxicity (**Chapter 2**). A second aim was to perform an association study using a candidate-gene approach to elucidate the contribution of genetic variants in key bile acid transport and metabolism genes to DILI (**Chapter 3**).

Cellular Viability in Sandwich-Cultured Hepatocytes: Effects of Culture Day and Protease Inhibitors.

In Aim 1, cellular viability assays were conducted to investigate the effect of days in culture on drug-mediated cytotoxicity in SCRH (**Appendix A**), and to determine subtoxic protease inhibitor (PI) concentrations for use in subsequent studies (**Chapter 2**). Lactate dehydrogenase (LDH) and adenosine triphosphate (ATP) assays were selected based on work published by Kemp *et al.* demonstrating that the LDH assay is more sensitive than other conventional assays utilized in SCRH (MTT, alamar blue, and propidium iodide staining).[248] Additionally, the nondestructive nature of the LDH assay allowed multiple studies to be performed on

a single sample set. Quantification of cellular ATP content is also a standard approach to determine toxicity and was used to corroborate findings from the LDH assay.

Based on the LDH assay, 24 hour exposure to 100 μM ritonavir (RTV) and lopinavir (LPV) yielded significantly lower toxicity on culture day 3 compared to culture day 1 (**Table A.1**). Next, dose-response studies performed on culture day 3 demonstrated that RTV and LPV were not toxic at concentrations less than or equal to 50 μM; toxicity in these studies was assessed by both LDH and ATP assays. Also, both assays revealed that LPV (5-50 μM) combined with RTV (5 μM) did not significantly decrease cellular viability compared to LPV alone. As a class, PIs are associated with a number of adverse reactions including the production of reactive oxygen species, elevated liver function tests, hyperbilirubinemia, jaundice and dyslipidemia.[293] Based on evidence in the literature, we hypothesized that PIs used in combination might exhibit additive hepatotoxic effects compared to single agents. However, findings of toxicity studies carried out in SCRH did not support this hypothesis.

In retrospect, determination of protein expression levels of genes implicated in the metabolism of LPV, RTV, and bile acids following 24 hour exposure over days in culture would have been helpful to interpret the results. While studies characterizing the effects of various culture conditions (e.g. supplemental medium content) have been performed to optimize the model, the influence of sustained PI exposure on transporters and drug-metabolizing enzymes in sandwich-cultured hepatocytes remains unclear. Understanding the impact of LPV and RTV on

metabolic enzymes and transport proteins in SCRH would help determine whether there was a shift in the formation of potentially toxic drug or bile acid intermediates and/or metabolites.

Also, a time-course study evaluating the toxicity of LPV and RTV over 24 hours, and perhaps beyond, would have proven useful. Because toxicity was evaluated at a single 24-hour time point, we are unable to comment on the effects of chronic drug exposure on cellular viability. It remains unclear how 24-hr incubation *in vitro* corresponds to *in vivo* exposure. Thus, it is difficult to extrapolate the present results to clinical circumstances. Nevertheless, the findings of experiments outlined in this dissertation work demonstrated that SCRH did not succumb to toxicity using PI doses that were within and above the clinically relevant plasma concentrations reported in humans. Recent published data demonstrated bile-acid dependent hepatotoxicity of BSEP inhibitors in sandwich-cultured rat hepatocytes.[249] Subsequent studies evaluating the effect of coadministered BSEP inhibitors and bile acids on DILI and hepatobiliary bile acid transport are necessary to further clarify the mechanisms by which bile acids induce DILI.

Individual and Coadministered Protease Inhibitors Impaired Canalicular Excretion of Bile Acids but did not Affect Initial Uptake.

The purpose of Aim 2 was to investigate the impact of RTV and LPV, alone and combined, on the hepatobiliary disposition of the bile acids taurocholate (TCA) and chenodeoxycholate (CDCA). Previous work by McRae and colleagues showed that RTV inhibited Bsep-mediated [^{3}H]TCA biliary excretion in SCRH, and to a lesser

extent, NTCP-mediated uptake.[78] While a small number of studies examining the effect of LPV on the initial uptake of bile acid structural analogues have been performed, the influence of LPV on [^{3}H]TCA and [^{14}C]CDCA uptake and biliary efflux had not been elucidated. Furthermore, the impact of coadministered PIs (as used clinically) on bile acid transport had not been investigated.

Studies measuring the effects of LPV and RTV, alone and combined, on [^{3}H]TCA and [^{14}C]CDCA accumulation in cells + bile and cells of SCRH were performed on culture day 4. Modulation of calcium content in the medium disrupts tight junctions causing release of the content of bile canalicular networks permitting the accurate determination of cellular substrate concentrations.[160,162,245] Co-incubation (10 min) with LPV, alone and combined with RTV (LPV/r), significantly decreased the accumulation of [^{3}H]TCA in cells + bile. LPV/r significantly increased the hepatocellular concentration of [^{3}H]TCA. While [^{14}C]CDCA accumulation in cells + bile and cells was not significantly altered by LPV and RTV, the biliary clearance of [^{14}C]CDCA was ablated by LPV and RTV, alone and combined.

Initial uptake studies using suspended rat hepatocytes were performed to characterize the effect of LPV and RTV on [^{3}H]TCA and [^{14}C]CDCA uptake. Freshly isolated suspended hepatocytes are ideal for measuring the initial uptake of compounds, however, the utility of suspended hepatocytes is limited due to the rapid decrease in cellular viability. Manipulation of sodium content in the incubation buffer allows determination of the contribution of Na^{+}-dependent (Ntcp-driven) and Na^{+}-independent (Oatp-mediated) transport processes to total uptake.[240] Interestingly, these studies revealed that the initial uptake rates of [^{3}H]TCA and [^{14}C]CDCA were

not affected by LPV and/or RTV at clinically relevant concentrations, suggesting that the observed decrease in the biliary excretion of [^{3}H]TCA and [^{14}C]CDCA following PI exposure was not due to decreased bile acid uptake.

Protease Inhibitors Decreased Total Endogenous Bile Acid Concentrations in Sandwich-cultured Rat Hepatocytes.

The objective of Aim 3 was to evaluate the effect of LPV and RTV, alone and combined, on the accumulation of endogenous bile acids in cells + bile, cells, and medium of SCRH. Hepatocytes were treated with LPV and RTV for 24 hours, beginning on day 3. Next, concentrations of endogenous bile acids [TCA, taurochenodeoxycholic acid (TCDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), α- and ß-tauromuricholic acid (TMCA)] were measured on day 4 by high performance liquid chromatography and mass spectrometry.

The 10-minute accumulation studies in day 4 SCRH showed potent inhibition of [^{3}H]TCA and [^{14}C]CDCA biliary excretion due to LPV and RTV exposure. Therefore, culturing hepatocytes with LPV and RTV for 24 hours was expected to significantly inhibit the biliary clearance of endogenous bile acids, thereby increasing intracellular accumulation of bile acids. Surprisingly, LPV and RTV treatment significantly *decreased* total bile acid accumulation (the sum of all measured bile acids) in cells + bile, cells, and medium of SCRH compared to vehicle control. One explanation for these findings is that PI treatment decreased bile acid synthesis. Although Zhou and colleagues reported that RTV inhibited CYP7A1, the rate-limiting

enzyme in the conversion of cholesterol to bile acids, in a concentration-dependent manner, the effect of LPV on bile acid biosynthesis has not been examined. Alternatively, the observed decrease in total bile acid accumulation may be attributed to increased metabolism of bile acids following PI exposure. Subsequent studies are necessary to determine the impact of PIs on the formation and catabolism of endogenous bile acids in SCRH. It is also plausible that compensatory efflux via the basolateral transporters (*e.g.,* MRP3 and/or MRP4) played a role in decreased cellular concentrations of bile acids. While it is difficult to design and implement basolateral efflux studies in sandwich-cultured hepatocytes, future experiments should be conducted to measure the contribution of basolateral efflux transporters following PI exposure. Whole-animal experiments using wild-type and Mrp2-deficient rats, in which both renal and hepatic mRNA and protein expression of basolateral efflux transporters (*e.g.* Mrp3 and Mrp4) are evaluated following short-term and chronic administration of LPV and RTV, would be novel and relevant. Also, the correlation between drug and bile acid concentrations in the plasma and concentrations at target organs remain unclear. Concentrations of PIs, cholesterol, bilirubin, and bile acids also could be quantified from serum as well as renal and hepatic tissues harvested from these animals. These studies would help clarify the relationship between the concentrations of drug and endogenous compounds in the serum versus target organs and ultimately aid in better prediction of pharmacological outcomes.

Role of Genetic Variants in Drug-induced Liver Injury

Increasing evidence in the literature suggests that single nucleotide polymorphisms (SNPs) that alter the function of key enzyme and transporter genes influence the pharmacokinetic profile of substrates, including drugs and endogenous compounds. Thus, the goal of Aim 4 was to determine the contribution of genetic variants in relevant bile acid metabolism and transport genes to the risk of DILI by performing an association analysis using a candidate-gene approach. The investigated genes included two canalicular bile acid export transporters: bile salt export pump (BSEP) and multidrug resistance associated protein 2 (MRP2). Using cases obtained from the Drug-induced Liver Injury Network (DILIN) and controls from the 1958 British Birth Cohort, patients who had experienced DILI were compared to controls to determine whether there was an increased risk of DILI in persons with SNPs in the genes of interest.

Importantly, the rs2919351 variant of OSTß yielded a significantly increased odds ratio of 10.1 ($p<0.0015$) when controls were compared to cholestatic and mixed DILI cases. The odds ratio was notably greater for mixed cases alone (17.6, $p=3 \times 10^{-4}$). This novel and exciting finding suggests that a genetic variant in the basolateral efflux transporter OSTß may contribute to increased risk of DILI in humans. Replication in a larger cohort is necessary to confirm this association.

Future Directions

Throughout the course of this dissertation research, my major advisor often optimistically reminded me that “good science” generates more questions than it

answers. The studies presented herein yielded exciting and, at times, unexpected, observations. Thus, there are a number of studies that would be logical next-steps in the future directions of this research program. While this work addressed the impact of coadministered PIs on bile acid transport, management of HIV infection often involves multiple drugs from several classes with distinct mechanisms of action. Subsequent studies evaluating the influence of coadministered antiretroviral agents, using a cassette-dosing approach, on hepatotoxicity and bile acid disposition is warranted. Data generated from these studies would provide insight regarding the drug-bile acid interactions between antiretrovirals from numerous drug classes. This knowledge is key to understanding the potential risks associated with highly active antiretroviral therapy at both the drug transport and metabolism level.

Also, LPV and RTV are rapidly and extensively metabolized by CYP450 enzymes; at least one RTV metabolite is pharmacologically active. Therefore, it would be useful to characterize the extent of LPV and RTV metabolism in rat and human hepatocytes relative to *in vivo.* These data would provide substantial information about the applicability and precision of *in vitro* model systems in the prediction of drug disposition in vivo, because it is costly and time prohibitive to perform extensive pharmacokinetic studies of this nature in humans. To date, the ability of drug-transporter interactions to influence the pharmacokinetic disposition, and thus, efficacy, and safety profiles of drugs has only recently gained recognition. These studies would allow scientists to more accurately predict the physiological consequences of drug-transporter interactions, if any, observed *in vitro*.

Although some preliminary data examining the effect of antiretrovirals on systemic bile acid concentrations in HIV-infected patients has been published, findings were inconclusive due to small sample sizes and high interindividual variability.[79] Consequently, despite the association of PIs with disturbances in lipid homeostasis, the association between circulating bile acids and antiretroviral use remains unclear. Future studies investigating the consequences of PIs on bile acid concentrations in plasma, urine, and bile from HIV-infected patients compared to healthy control subjects should be performed. These data could be used to develop a pharmacokinetic model capable of predicting perturbations in bile acid disposition. This information would enable scientists to determine the risk of toxicity associated with bile acid disposition in humans. Importantly, these studies could reveal the utility of serum bile acids as a biomarker for DILI, and might help elucidate the contribution of HIV-infection itself to alterations in bile acid disposition.

One limitation of the present work is that only six major bile acids were measured by HPLC-MS/MS. Bile acid metabolism is complex and tightly controlled by several regulatory feedback mechanisms.[261,294] Additional studies quantifying other bile acids, including more toxic bile acid species such as LCA, DCA, as well as sulfate and glucuronide conjugates of the major bile acids in both hepatocytes and in plasma after PI exposure should also be carried out. These findings would be necessary to detect drug-mediated shifts in the composition of the bile acid pool. Such changes may signal either a hepatoprotective response or drug-induced changes in key proteins that regulate bile acid synthesis and/or excretion from the hepatocyte. These comprehensive studies should be conducted in both human and

rodent models to delineate species differences because bile acid composition and the inhibitory potential of drugs on bile acid metabolism and transport reportedly differ across species. Identifying preclinical signals of hepatotoxicity would be an important contribution to the development of safer drugs.

A novel and exciting finding of the current work is the observed decrease in bile acid concentrations following 24 hour PI co-incubation with SCRH (**Chapter 2**). Follow-up studies measuring bile acid concentrations over 24 hours, as opposed to a single end-point, would provide key information regarding the extent and time course of decreased bile acid formation or increased bile acid metabolism. Measurement of bile acid precursors, such as cholesterol and its intermediate metabolites, and bile acid metabolites would help determine where perturbations occur in the bile acid synthesis or catabolism cascade. Also, quantifying protein and/or mRNA expression of key enzymes involved in the classical and alternate pathways of cholesterol metabolism, such as cholesterol 7-α hydroxylase (CYP7A1) and sterol 27-hydroxylase (CYP27A1) could help to determine whether induction or inhibition of metabolic enzymes contribute to the changes in bile acid concentrations.

The sandwich-cultured hepatocyte model is a relatively new tool to assess hepatic uptake and excretory processes of drugs and endogenous compounds. Consequently, additional research is needed to characterize bile acid disposition and elucidate new applications of this *in vitro* system. For example, cholesterol and bile acid content over days in culture, and the effects of daily medium changes on these levels, have yet to be determined in sandwich-cultured hepatocytes. Thus, interpretation of studies in SCRH exploring drug effects on endogenous bile acids is

limited. Future work should include comprehensive studies describing the inherent characteristics of the sandwich-cultured hepatocyte system with respect to bile acid disposition, and how this *in vitro* model compares to the *in vivo* situation.

As discussed in Chapter 3, the genetic study detected a significant association between a single variant in OSTß and an increased risk of DILI. Subsequent studies clearly identifying functional consequences of genetic variants are required to accurately identify and assess the influence of variants on drug-induced hepatotoxicity. Evidence correlating genetic polymorphisms with functional consequences is marginal. This limitation confounds our understanding of interindividual variability in drug pharmacokinetics, and, consequently, efficacy and toxicity. To date, resources that provide useful, accurate information regarding drug-transporter interactions due to genetic variants are limited. Initiatives to address this scientific need will develop as the field continues to evolve.

In conclusion, the present work has enhanced our understanding of the interactions between hepatic transport proteins and coadministered antiretroviral drugs, specifically LPV and RTV. Additionally, this project illustrated the consequences of those interactions on bile acid disposition in an *in vitro* model, and highlighted the importance of employing a system capable of retaining *in vivo* like properties. One key finding is that despite the short-term, potent inhibition of Bsep-mediated bile acid transport, PIs such as LPV and RTV *decrease* bile acid accumulation when incubated for 24 hours with normal rat hepatocytes cultured in a sandwich configuration for 4 days. This observation may explain the lack of toxicity that was observed in SCRH when incubated with clinically relevant concentrations of

PIs. Lastly, the genetic study provided a framework for future hypothesis-driven association studies, particularly focused on transport proteins that play a role in the disposition of bile acids.

APPENDIX

RAW DATA SUMMARY

Figure 2.1 Sandwich-cultured rat hepatocyte viability (mean and SEM; % control) following 24-hr exposure to LPV or RTV. Day 3 SCRH were treated with LPV or RTV (5-100 µM) for 24 hours. Following incubation, LDH release and cellular ATP (B) levels were measured.

Viability (%)										
LDH										
	Lopinavir					Ritonavir				
Dose (µM)	N1	N2	N3	mean	SEM	N1	N2	N3	mean	SEM
5	98.9	98.1	99.5	98.8	0.4	99.8	100.9	99.6	100.1	0.4
10	100.0	100.0	98.6	99.5	0.5	100.0	100.0	99.6	99.9	0.1
25	100.0	99.4	98.1	99.2	0.6	100.0	100.0	99.7	99.9	0.1
50	100.0	99.6	94.8	98.1	1.7	100.0	99.8	98.0	99.3	0.6
75	93.8	94.8	87.7	92.1	2.2	96.1	93.9	96.5	95.5	0.8
100	89.4	87.3	87.3	88.0	0.7	89.6	93.9	91.1	91.5	1.3
ATP										
	Lopinavir					Ritonavir				
Dose (µM)	N1	N2	N3	mean	SEM	N1	N2	N3	mean	SEM
5	131.8	84.2	89.2	101.8	15.1	98.2	87.9	90.4	92.1	3.1
10	117.5	103.6	94.6	105.2	6.6	98.7	71.7	87.4	85.9	7.8
25	101.7	100.1	102.7	101.5	0.8	91.8	87.6	81.2	86.9	3.1
50	81.9	118.3	43.8	81.3	21.5	98.8	75.6	83.8	86.0	6.8
75	71.1	58.7	15.4	48.4	16.9	87.2	61.6	63.5	70.8	8.2
100	59.1	57.2	10.7	42.3	15.9	67.8	53.9	39.3	53.6	8.2

Figure 2.2 Accumulation (mean and SEM; pmol/mg protein), BEI (%) and Cl_{bile} (ml/min/mg protein) of ^{3}H[TCA] in cells + bile and cells of day 4 SCRH following a 10-min co-incubation with vehicle control (0.1 % DMSO), RTV (5 µM), and LPV (5 µM), alone or combined (LPV/r).

	cells + bile					cells				
	N1	**N2**	**N3**	**Mean**	**SEM**	**N1**	**N2**	**N3**	**mean**	**SEM**
Vehicle	15.9	19.8	12.3	16.00	2.17	6.0	5.7	3.7	5.13	0.72
RTV	8.8	16.6	7.8	11.07	2.78	9.3	8.3	6.8	8.13	0.73
LPV	8.3	14.1	9.3	10.57	1.79	6.0	4.7	4.4	5.03	0.49
LPV/r	8.3	9.2	5.3	7.60	1.18	7.5	7.7	7.9	7.70	0.12

	BEI (%)					Cl_{bile} (ml/min/kg)				
	N1	**N2**	**N3**	**Mean**	**SEM**	**N1**	**N2**	**N3**	**mean**	**SEM**
Vehicle	62.30	71.21	69.92	67.80	2.78	7.92	11.28	6.88	8.70	1.33
RTV	0.00	50.00	12.82	20.93	15.00	0.00	6.64	0.80	2.47	2.08
LPV	27.70	66.67	52.69	49.03	11.41	1.84	7.52	3.92	4.40	1.66
LPV/r	9.60	16.30	0.00	8.63	4.73	0.64	1.20	0.00	0.60	0.35

Figure 2.3 Accumulation (mean and SEM; pmol/mg protein), BEI (%), and Cl_{bile} (ml/mink/kg) of [^{14}C]CDCA in cells + bile and cells of day 4 SCRH following a 10-min co-incubation with vehicle control (0.1% DMSO), RTV (5 µM), and LPV (5 µM), alone or combined (LPV/r).

	cells + bile					cells				
	N1	**N2**	**N3**	**mean**	**SEM**	**N1**	**N2**	**N3**	**Mean**	**SEM**
Vehicle	1077.4	793.0	672.2	847.5	120.1	813.3	580.6	515.4	636.4	90.4
RTV	1026.6	768.7	546.5	780.6	138.7	1115.3	693.3	592.4	800.3	160.2
LPV	930.9	823.2	456.1	736.7	143.7	812.6	862.1	593.1	755.9	82.7
LPV/r	1079.4	989.3	558.9	875.9	160.6	1252.7	995.3	542.3	930.1	207.6

	BEI (%)					Cl_{bile} (ml/min/kg)				
	N1	**N2**	**N3**	**mean**	**SEM**	**N1**	**N2**	**N3**	**Mean**	**SEM**
Vehicle	30.6	26.8	23.3	26.9	2.1	26.4	21.2	15.7	37.2	8.1
RTV	0.0	9.8	0.0	3.3	3.3	0.0	7.5	0.0	0.0	0.0
LPV	12.7	0.0	0.0	4.2	4.2	11.8	0.0	0.0	0.0	0.0
LPV/r	0.0	0.0	3.0	1.0	1.0	0.0	0.0	1.7	0.0	0.0

Figure 2.4. Na^+-dependent and Na^+-independent uptake of [^{3}H]TCA into freshly isolated suspended rat hepatocytes. [^{3}H]TCA accumulation in freshly isolated rat hepatocytes was determined following pre-incubation with vehicle control (0.1% DMSO), LPV (10 µM; A) or RTV (5 µM; B), alone and in combination (C), in the presence or absence of sodium.

		Na+-containing buffer					Na+-free buffer				
	Time (s)	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
Control	15	48.24	40.04	42.74	43.67	2.41	12.6	8.56	6.03	9.06	1.91
	30	78.36	66.15	58.39	67.63	5.81	22.37	11.7	5.86	13.31	4.83
	45	92.39	80.86	95.13	89.46	4.37	21.35	11.26	8.44	13.68	3.92
	rate (pmol/s/mg p)	1.472	1.361	1.746	1.53	0.04	0.29	0.09	0.08	0.15	0.07
		Na+-containing buffer					Na+-free buffer				
	Time (s)	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
LPV	15	52.54	38.54	30.83	40.64	6.35	11.78	8.63	6.4	8.94	1.56
	30	78.16	43.46	114.3	78.64	20.45	8.15	12.15	9.89	10.06	1.16
	45	142.45	72.33	127.58	114.12	21.33	16.14	13.26	9.67	13.02	1.87
	rate (pmol/s/mg p)	2.997	1.126	3.225	2.45	0.05	0.15	0.15	0.11	0.14	0.04
		Na+-containing buffer					Na+-free buffer				
	Time (s)	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
RTV	15	56.01	45.87	52.54	51.47	2.98	12	9.11	8.25	9.79	1.13
	30	90.37	61.94	101.98	84.76	11.89	15.69	10.77	11.59	12.68	1.52
	45	92.66	79.03	135.95	102.55	17.16	18.17	14.43	14.02	15.54	1.32
	rate (pmol/s/mg p)	1.222	1.105	2.78	1.70	0.30	0.21	0.18	0.19	0.19	0.00
		Na+-containing buffer					Na+-free buffer				
	Time (s)	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
LPV/r	15	46.82	44.13	21.23	37.39	8.12	13.22	7.11	5.74	8.69	2.30
	30	79.69	84.95	74.58	79.74	2.99	16.06	10.83	8.78	11.89	2.17
	45	97.19	96.23	119.35	104.26	7.55	19.32	9.55	9.78	12.88	3.22
	rate (pmol/s/mg p)	1.679	1.737	3.271	2.23	0.34	0.2	0.08	0.13	0.14	0.04

Figure 2.5. Na^+-dependent and Na^+-independent uptake of [^{14}C]CDCA into freshly isolated suspended rat hepatocytes. [^{14}C]CDCA accumulation in freshly isolated rat hepatocytes was determined following pre-incubation with vehicle control (0.1% DMSO), LPV (10 µM; A) or RTV (5 µM; B), alone and in combination (C), in the presence or absence of sodium.

		Na+-containing buffer					Na+-free buffer				
Control	**Time (s)**	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
	15	374.1	377.5	154.4	302.0	73.8	137.9	248.8	171.3	186.0	32.9
	30	681.7	553.0	223.7	486.2	136.4	315.5	353.9	242.8	304.1	32.6
	45	821.9	679.4	288.6	596.6	159.4	458.2	440.7	264.6	387.9	61.8
	rate (pmol/s/mg p)	14.9	10.1	4.5	9.8	3.0	10.7	6.4	3.1	6.7	2.2
		Na+-containing buffer					Na+-free buffer				
LPV	**Time (s)**	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
	15	471.1	419.0	186.7	359.0	87.4	284.2	195.2	175.1	218.2	33.5
	30	692.6	566.3	302.9	520.6	114.8	372.8	275.6	257.9	302.1	35.7
	45	755.7	642.9	358.4	585.7	118.2	535.0	320.1	310.7	388.6	73.2
	rate (pmol/s/mg p)	9.5	7.5	5.7	7.6	1.1	8.4	4.2	4.5	5.7	1.3
		Na+-containing buffer					Na+-free buffer				
RTV	**Time (s)**	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
	15	387.4	342.2	189.2	306.3	60.0	332.9	190.4	158.2	227.2	53.7
	30	527.6	464.5	258.1	416.7	81.4	421.5	255.3	191.6	289.5	68.5
	45	597.3	488.6	315.8	467.2	82.0	568.4	298.8	291.5	386.2	91.1
	rate (pmol/s/mg p)	7.0	4.9	4.2	5.4	0.8	7.9	3.6	4.4	5.3	1.3
		Na+-containing buffer					Na+-free buffer				
LPV/r	**Time (s)**	N1	N2	N3	Mean	SEM	N1	N2	N3	mean	SEM
	15	627.9	407.5	199.2	411.5	123.8	404.7	204.4	142.1	250.4	79.2
	30	908.5	535.4	302.6	582.2	176.5	640.4	303.2	201.3	381.7	132.7
	45	1094.5	680.3	364.2	713.0	211.5	788.6	383.9	234.2	468.9	165.6
	rate (pmol/s/mg p)	15.6	9.1	5.5	10.0	2.9	12.8	6.0	3.1	7.3	2.9

Figure 2.6 Accumulation (pmol/mg protein) of total bile acids (TCA + GCA + TCDCA + GCDCA + α/ß-TMCA) in SCRH (cells, bile, and medium) following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined.

	N1	N2	N3	N4	mean	SEM
Vehicle Control	5060	4169	5202	6432	5216	466
5 µM RTV	3921	2642	3403	4362	3582	369
5 µM LPV	4866	2605	4109	5162	4185	572
5 µM LPV + 5 µM RTV	2175	2033	2125	2137	2118	30
50 µM LPV	2666	1829	2477	2500	2368	185
50 µM LPV + 5 µM RTV	2015	1409	1979	1669	1768	143

Figure 2.7 Accumulation (pmol/mg protein) of endogenous TCA in cells + bile, cells, and medium and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined

		N1	N2	N3	N4	mean	SEM
cells + bile	**Vehicle Control**	38.17	12.87	74.45	30.48	38.99	12.95
	5 µM RTV	12.80	4.91	39.07	6.61	15.85	7.93
	5 µM LPV	21.70	3.85	45.24	13.39	21.04	8.85
	50 µM LPV	3.65	2.32	18.83	2.63	6.86	4.00
	5 µM LPV+ 5 µM RTV	2.88	0.60	3.55	0.00	1.76	0.86
	50 µM LPV + 5 µM RTV	1.06	0.80	6.72	0.92	2.37	1.45
cells		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	6.19	3.39	60.19	9.50	19.82	13.51
	5 µM RTV	1.89	1.41	30.21	2.76	9.07	7.05
	5 µM LPV	3.49	1.69	31.13	5.65	10.49	6.93
	50 µM LPV	0.84	0.60	13.07	1.38	3.97	3.04
	5 µM LPV + 5 µM RTV	0.60	0.20	3.83	0.30	1.23	0.87
	50 µM LPV + 5 µM RTV	0.83	0.43	5.80	0.79	1.96	1.28
medium		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	1039.90	512.79	1305.87	1482.57	1085.28	211.40
	5 µM RTV	1309.64	426.00	1062.36	1538.00	1084.00	239.87
	5 µM LPV	1049.84	269.00	1108.77	1225.00	913.15	217.78
	50 µM LPV	601.02	399.00	661.87	961.00	655.72	116.24
	5 µM LPV + 5 µM RTV	365.12	124.00	310.55	252.00	262.92	51.75
	50 µM LPV + 5 µM RTV	300.00	140.00	316.68	313.00	267.42	42.62

Figure 2.8 Accumulation (pmol/mg protein) of endogenous TCDCA in cells + bile, cells, and medium and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined

		N1	N2	N3	N4	mean	SEM
cells + bile	**Vehicle Control**	8.93	4.02	8.09	11.3	8.09	1.52
	5 µM RTV	1.33	1.20	2.34	1.39	1.57	0.26
	5 µM LPV	10.00	3.59	5.75	10.7	7.50	1.70
	50 µM LPV	0.96	0.00	1.34	1.05	0.84	0.29
	5 µM LPV + 5 µM RTV	1.14	0.00	1.10	0.00	0.56	0.32
	50 µM LPV + 5 µM RTV	0.00	0.95	0.93	0.00	0.47	0.27
cells		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	3.12	2.89	9.30	3.81	4.78	1.52
	5 µM RTV	0.97	1.32	2.68	0.98	1.49	0.41
	5 µM LPV	3.49	2.89	5.94	4.31	4.16	0.66
	50 µM LPV	0.00	0.90	1.32	0.93	0.79	0.28
	5 µM LPV + 5 µM RTV	0.00	0.61	1.21	0.00	0.46	0.29
	50 µM LPV + 5 µM RTV	0.30	0.61	1.23	0.00	0.54	0.26
medium		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	38.96	30.79	10.02	30.26	27.50	6.16
	5 µM RTV	12.50	19.69	1.44	10.82	11.11	3.75
	5 µM LPV	82.38	43.54	15.81	66.51	52.06	14.48
	50 µM LPV	13.08	14.94	0.00	10.86	9.72	3.35
	5 µM LPV + 5 µM RTV	45.40	28.27	16.15	42.18	33.0	6.74
	50 µM LPV + 5 µM RTV	33.32	44.48	14.68	73.04	41.38	12.21

Figure 2.9 Accumulation (pmol/mg protein) of endogenous α/ß-TMCA in cells + bile, cells, and medium and BEI values in SCRH following 24-h treatment with vehicle (0.1% DMSO), RTV (5 µM), and LPV (5 or 50 µM), alone or combined

		N1	N2	N3	N4	mean	SEM
cells + bile	**Vehicle Control**	899	701	2756	740	1274	496
	5 µM RTV	348	307	1166	172	498	226
	5 µM LPV	404	246	1454	203	577	296
	50 µM LPV	194	153	782	100	307	159
	5 µM LPV + 5 µM RTV	51	50	234	30	91	48
	50 µM LPV + 5 µM RTV	52	27	248	27	89	53
cells		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	478	503	2656	416	1013	548
	5 µM RTV	190	251	1160	122	431	245
	5 µM LPV	219	239	1318	142	480	280
	50 µM LPV	107	115	598	74	224	125
	5 µM LPV + 5 µM RTV	38	50	239	23	88	51
	50 µM LPV + 5 µM RTV	42	30	235	25	83	51
medium		**N1**	**N2**	**N3**	**N4**	**mean**	**SEM**
	Vehicle Control	2999	2827	938	3859	2656	615
	5 µM RTV	2173	1818	1074	2294	1840	275
	5 µM LPV	3257	2003	1409	3372	2510	480
	50 µM LPV	1560	1723	996	1701	1495	170
	5 µM LPV + 5 µM RTV	1975	1305	1526	1302	1527	158
	50 µM LPV + 5 µM RTV	1629	1185	1378	1215	1352	102

Table A.1 Toxicity (%) after 24 hour drug exposure. LDH release was measured in sandwich-cultured rat hepatocytes after 24 hour incubation with 100 µM LPV, RTV, triclosan (TCS), or vehicle (0.1 DMSO) beginning on day 1, 2, or 3 in culture.

	N	Day	Toxicity (%)	AVG	SEM
RTV	1	day 1	37.010	60.16	12.21068
	2	day 1	78.470		
	3	day 1	65.000		
	1	day 2	14.720	20.24	9.149541
	2	day 2	7.891		
	3	day 2	38.110		
	1	day 3	1.500	6.39	2.477707
	2	day 3	9.530		
	3	day 3	8.140		
LPV	1	day 1	98.670	96.69	2.672719
	2	day 1	100.000		
	3	day 1	91.400		
	1	day 2	56.180	49.75	13.1825
	2	day 2	24.400		
	3	day 2	68.680		
	1	day 3	36.540	38.55	16.63488
	2	day 3	10.790		
	3	day 3	68.310		
TCS	1	day 1	95.900	97.30	1.350308
	2	day 1	100.000		
	3	day 1	96.000		
	1	day 2	90.210	88.66	1.348833
	2	day 2	89.790		
	3	day 2	85.970		
	1	day 3	83.660	72.48	9.886085
	2	day 3	52.770		
	3	day 3	81.020		
Vehicle	1	day 1	2.708	0.685	1.029
	2	day 1	0.000		
	3	day 1	-0.652		
	1	day 2	0.504	0.40	0.21065
	2	day 2	0.000		
	3	day 2	0.709		
	1	day 3	0.106	-0.026	0.258237
	2	day 3	0.341		
	3	day 3	-0.524		

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