Article Title: *Combination Lopinavir and Ritonavir Alter Exogenous and Endogenous Bile Acid Disposition in Sandwich-Cultured Rat Hepatocytes*

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## Nonstandard abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>α/β-TMCA</td>
<td>α/β-Taumuricholic Acid</td>
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<tr>
<td>BA</td>
<td>Bile Acid</td>
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<tr>
<td>BEI</td>
<td>Biliary Excretion Index</td>
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<tr>
<td>BLQ</td>
<td>Below Limit of Quantitation</td>
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<tr>
<td>BSEP</td>
<td>Bile Salt Export Pump</td>
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<tr>
<td>CDCA</td>
<td>Chenodeoxycholic Acid</td>
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<td>GCA</td>
<td>Glycocholic Acid</td>
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<td>GCDCDA</td>
<td>Glycochenodeoxycholic Acid</td>
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<td>LPV</td>
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<td>RTV</td>
<td>Ritonavir</td>
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<td>SCRH</td>
<td>Sandwich-cultured Rat Hepatocytes</td>
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<td>TCA</td>
<td>Taurocholic Acid</td>
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Abstract

Inhibition of the bile salt export pump (BSEP) can cause intracellular accumulation of bile acids and is a risk factor for drug-induced liver injury (DILI) in humans. Antiretroviral protease inhibitors lopinavir (LPV) and ritonavir (RTV) are reported BSEP inhibitors. However, the consequences of LPV and RTV, alone and combined (LPV/r) on hepatocyte viability, bile acid transport, and endogenous bile acid disposition in rat hepatocytes have not been examined. The effect of LPV, RTV, and LPV/r on cellular viability and the disposition of $[^3]$H]taurocholic acid ($[^3]$H[TCA]) and $[^14]$C]chenodeoxycholic acid ($[^14]$C[CDCA]) was determined in sandwich-cultured rat hepatocytes (SCRH) and suspended rat hepatocytes. LDH and ATP assays revealed a concentration-dependent effect of LPV and RTV on cellular viability. LPV (5 µM), alone and combined with 5 µM RTV, significantly decreased $[^3]$H[TCA] accumulation in cells+bile of SCRH compared to control. LPV/r significantly increased $[^3]$H[TCA] cellular accumulation (7.7±0.1 pmol/mg protein) compared to vehicle and 5 µM LPV alone (5.1±0.7 and 5.0±0.5 pmol/mg protein). $[^3]$H[TCA] biliary clearance was reduced significantly by LPV and RTV, and further reduced by LPV/r. LPV and RTV did not affect initial uptake rates of $[^3]$H[TCA] and $[^14]$C[CDCA] in suspended rat hepatocytes. LPV (50 µM), RTV (5 µM), and LPV/r (5 and 50 µM/ 5 µM) significantly decreased accumulation of total measured endogenous bile acids (TCA, taurochenodeoxycholic acid, and α/β-tauromuricholic acid) in SCRH. Quantification of endogenous bile acids in SCRH may reveal important adaptive responses associated with exposure to known Bsep inhibitors.
INTRODUCTION

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 (Sulkowski et al., 2000; Bongiovanni et al., 2005). 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 (Sulkowski, 2004). 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 (Sulkowski, 2003). In particular, ritonavir (RTV)-containing regimens reportedly increased the risk of hepatotoxicity by 8.6-fold (Sulkowski et al., 2000). RTV is now administered at subtherapeutic (and subtoxic) doses to enhance systemic concentrations of co-administered 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 exhibited higher LPV/r plasma concentrations compared to patients with normal functioning livers (Abbott Laboratories, package insert).

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 the hepatocyte (McRae et al., 2006; Marion et al., 2007; Wolf et al., 2010). 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 (Pauli-
Magnus et al., 2005). 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 (Morgan et al., 2010). PIs including LPV and RTV also have been shown to inhibit bile acid transport via BSEP (McRae et al., 2006; Dawson et al., 2012), supporting the idea that intracellular accumulation of bile acids may be a mechanism for DILI observed in patients treated with this combination (Morgan et al., 2010; Dawson et al., 2012). 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. [3H]Taurocholic acid (TCA, 5 Ci/mmol; purity > 97%) was purchased from Perkin Elmer (Waltham, MA). [14C]Chenodeoxycholic acid (CDCA; 50 mCi/mmol; purity > 97%) and [14C]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 d₄ TCA were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). The d₈ 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 β-tauromuricholic acid (α/β-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 (LeCluyse et al., 1996). 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 37°C in a humidified incubator (95% O₂, 5% CO₂) 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 hr 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-hr 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 hr, 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-nitrophenoxy)-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 at 492 nm. 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 ([³H]TCA and [¹⁴C]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 Ca²⁺-containing (standard; cells + bile) or Ca²⁺-free (cells) HBSS buffer containing EGTA to maintain or disrupt tight junctions, respectively. Next, hepatocytes were co-incubated for
10 min with TCA (1 µM cold TCA plus 0.07 µM [3H]TCA) or [14C]CDCA (1 µM cold CDCA plus 4 µM [14C]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, [3H]TCA and [14C]CDCA accumulation in BioCoat™ plates without cells was subtracted from raw values.

**[3H]TCA and [14C]CDCA Initial Uptake in Suspended Rat Hepatocytes.** The initial uptake of TCA (1 µM cold TCA plus [3H]TCA; 60 nCi/ml) and CDCA (0.5 µM cold CDCA plus 0.5 µM [14C]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 (Leslie et al., 2007). 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 MgSO₄, 10 mM HEPES, 1.2 mM CaSO₄, 0.86 mM K₂HPO₄, 0.14 mM KH₂PO₄, and 5 mM glucose, pH 7.4). Cells were resuspended at 1.0 x 10⁶ 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°C for 5 min; 0.1% DMSO or PIs were added 30 sec before the addition of [³H]TCA (1 μM unlabeled TCA plus [³H]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 [¹⁴C] inulin (60 nCi/ml) as reported by Baur and colleagues (Baur et al., 1975). 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-hr 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 (Marion et al., 2011). Briefly, six endogenous conjugated bile acid species [taurocholic acid (TCA), glycocholic acid (GCA), taurochenodeoxycholic acid (TCDDCA), glycochenodeoxycholic acid (GCDCA), and α/β-tauromuricholic acid (α/β-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°C 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/100 μL), 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-TMCA 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 currently are not commercially 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 (Lee and Brouwer, 2010). 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-free buffer})/(Accumulation_{standard buffer})] × 100% (Liu *et al*., 1999b). The *in vitro* biliary clearance (Cl_{bile}) was calculated based on the following equation: Cl_{bile} = (Accumulation_{standard buffer} - Accumulation_{Calcium-free buffer}) / (AUC_{medium}), where AUC_{medium}
represents the product of the incubation time (10 min) and the initial concentration in the incubation medium. 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 (Dunn et al., 1991; Liu et al., 1998; Liu et al., 1999a). Because polarity is necessary to assess vectorial transport of bile acids, 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 triphosphate (ATP) content were measured after individual and combination treatment with LPV and RTV. Alone, LPV and RTV demonstrated concentration-dependent effects on cellular viability; the observed differences between the two treatments were not significant (Figure 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 < 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 1) and the trend towards increased toxicity at 50 µM LPV was not statistically significant.

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 5 µM RTV (LPV/r). As shown in Figure 2, the mean accumulation of [\textsuperscript{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 in cells + bile relative to vehicle treatment also was observed for the combination treatment LPV/r (from 16.0 ± 2.2 to 7.6 ± 1.2 pmol/mg protein). It appeared that co-administration of LPV with RTV resulted in additional reduction in the concentration of [\textsuperscript{3}H]TCA in cells + bile compared to LPV treatment alone, but this decrease was not significant. The hepatocyte (cell) concentration of [\textsuperscript{3}H]TCA was not significantly increased by RTV or LPV alone (Figure 2). However, when RTV was combined with LPV, the hepatocyte concentration of [\textsuperscript{3}H]TCA (7.7 ± 0.1 pmol/mg protein) was significantly increased relative to the cellular concentrations observed with either vehicle or 5 µM LPV alone (5.1 ± 0.7 and 5.0 ± 0.5 pmol/mg protein, respectively). When the same experiment was repeated with [\textsuperscript{14}C]CDCA, the treatments did not significantly alter the accumulation of [\textsuperscript{14}C]CDCA species in cells + bile or cells alone (Figure 3).

**Biliary excretion of [\textsuperscript{3}H]TCA and [\textsuperscript{14}C]CDCA in Sandwich-cultured Rat Hepatocytes.** The calculated Biliary Excretion Index (BEI; %) for [\textsuperscript{3}H]TCA was reduced by both LPV and RTV alone, and further reduced by the combination treatment (Table 2). The calculated biliary clearance values (Cl\textsubscript{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\textsubscript{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 [³H]TCA, regardless of co-administration with RTV (data not shown).

[¹⁴C]CDCA cellular concentrations in vehicle treated hepatocytes were 120-fold greater compared to [³H]TCA, and the BEI of [¹⁴C]CDCA species was two- to three-fold lower than for [³H]TCA. Thus, a decrease in biliary excretion may not affect the cellular accumulation of [¹⁴C]CDCA to the same extent as that of [³H]TCA. LPV, alone or combined with RTV reduced the BEI and Cl_bile of [¹⁴C]CDCA species to values below the limit of quantitation (Table 2).

³H]TCA and [¹⁴C]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, [³H]TCA and [¹⁴C]CDCA influx into hepatocytes was measured during the linear uptake time interval (15 to 45 sec) in suspended rat hepatocytes (Iga and Klaassen, 1982; Marion et al., 2007). Initial uptake rates of [³H]TCA in Na+-containing and Na+-free buffer were 1.53 ± 0.11 and 0.15 ± 0.07 pmol/sec/mg protein, respectively (n=3; Figure 4). LPV (10 µM), RTV (5 µM), and LPV/r had no effect on the initial uptake rates of [³H]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 [¹⁴C]CDCA in Na+-containing and Na+-free buffer of vehicle control hepatocytes (9.92 ± 3.02 and 6.73 ± 2.19 pmol/sec/mg protein, respectively; n=3; Figure 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 (Barth et al., 2006). Concentrations (µM) of each bile acid species in cells + bile, cells, and medium of vehicle-treated SCRH are listed in Table 3. The α- and β-TMCA species comprised the majority of the total measured bile acid pool and appeared predominantly in the cells of SCRH. The BEI value of endogenous TCA (49%) was in the same range as the BEI calculated following addition of 1 µM [3H]TCA (68%; Table 2). It is not possible to calculate the 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/β-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. This marked reduction in total measured bile acids is consistent with the observation that 24-hr LPV exposure yielded minimal apparent toxicity to SCRH at these concentrations (Figure 2). The addition of 5 µM RTV to low dose LPV (5 µM) significantly decreased total bile acid accumulation relative to both vehicle and 5 µM LPV alone (Figure 6). 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 6-9).

The addition of 5 µM RTV to low dose LPV (5 µM) significantly decreased TCDCA accumulation in cells + bile and cells alone relative to both vehicle and 5 µM LPV alone (Figure 8). In contrast, TCA and α/β-TMCA accumulation in cells + bile and cells alone were not significantly influenced by LPV (5 µM), alone or combined with RTV.
(Figures 7 and 9, respectively). LPV (50 µM) significantly reduced TCA accumulation in medium and cells + bile (Figure 7), TCDCA accumulation in cells + bile and cells alone (Figure 8), and α/β-TMCA accumulation in cells + bile (Figure 9), relative to vehicle. Interestingly, TCDCA accumulation in cells + bile and cells alone was significantly decreased by 5 µM RTV (Figure 8). Notably, the BEI of TCDCA was markedly decreased by RTV, alone or in combination with LPV (values at the top of Figure 8).

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. LPV and RTV inhibit BSEP in vitro and are associated with hepatotoxicity. HIV treatment regimens frequently combine RTV with other PIs to improve oral availability, but are associated with increased liver toxicity. The present work characterizes the interactions between hepatocytes, PIs, and bile acids. We hypothesized that addition of RTV to LPV would result in increased toxicity and intracellular accumulation of bile acids in SCRH.
SCRH regain *in vivo*-like morphological properties, including the development of tight junctions, polarized transport, and functional canalicular networks; metabolic capacity (e.g. bile acid synthesis and secretion) and functional regulatory machinery are well-documented in SCRH (Swift *et al*., 2010). Periodic contractions of the networks, as described for isolated hepatocyte couplets/hepatocyte groups (Oshio and Phillips, 1981; Phillips *et al*., 1982), return canalicular contents to the medium (LeCluyse *et al*., 1994). Mathematical modeling of SCRH data consistently requires a rate constant for substrate flux from the canalicular compartment (Liu *et al*., 1999b; Hoffmaster *et al*., 2005; Lee *et al*., 2010). Thus, when cultured under appropriate conditions, SCRH do not exhibit biliary stasis, intracellular bile acids are within the range of previously reported values (Marion *et al*., 2012), and SCRH exhibit toxicity when BSEP is inhibited (Kemp and Brouwer, 2004; Marion *et al*., 2007; Ogimura *et al*., 2011). Reduced glutathione (GSH) content in SCRH is normal, and markedly decreased by exposure to toxicants that deplete GSH *in vivo* (Kiang *et al*., 2011). Therefore, we selected the SCRH model for the present studies. Indisputably, species differences exist between rodents and humans regarding drug-mediated toxicity and drug-transporter interactions. For example, potent inhibition of the bile salt analogue cholyl-glycylamido-fluorescein by RTV in rat but not human hepatocytes has been reported (Ye *et al*., 2010). However, rodents remain the major preclinical *in vivo* screen to assess potential hepatotoxicity of new drug candidates.

Contrary to our hypothesis, LPV/r did not significantly increase toxicity relative to LPV alone (*Table 1*). Nonetheless, short term (10-min) exposure of SCRH to LPV/r further increased TCA cellular accumulation compared to LPV alone (*Figure 2*). It is
important to note that our transport inhibition studies were conducted after 10-min PI exposure, whereas toxicity was assessed after 24-hr PI exposure. The lack of toxicity observed at 24 hr may indicate that normal-functioning hepatocytes respond to cellular injury via hepatoprotective mechanisms. Alternatively, feedback mechanisms may downregulate bile acid synthesis and/or upregulate bile acid efflux causing only transient increases in intracellular bile acids.

As expected from previous reports (McRae et al., 2006), RTV inhibited [3H]TCA Clbile and BEI. LPV also inhibited the Clbile of [3H]TCA; addition of RTV resulted in further inhibition. It should be noted that the additional reduction in [3H]TCA Clbile and BEI resulting from addition of RTV to LPV is consistent with additive effects of each drug and not a synergistic interaction. Doubling the LPV concentration (to 10 µM) ablated Clbile and BEI for [3H]TCA. Similar effects were observed when LPV (5 µM) was co-administered with RTV (5 µM).

The effects of LPV and RTV on the BEI and Clbile of [14C]CDCA species were similar to those observed with [3H]TCA; values were reduced below the limit of quantitation. In contrast to the result with [3H]TCA, we were unable to detect any effect of LPV alone or LPV/r on the cellular content of [14C]CDCA species because cellular accumulation was already extensive for these bile acid species. Thus, cellular concentrations of [14C]CDCA species are less sensitive to a modest decrease in canalicular efflux.

Because the marked effects of the PIs on biliary excretion of [3H]TCA and [14C]CDCA species were not associated with similar increases in hepatocyte content of bile acids, we speculated that the PIs differentially inhibited 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 (Pauli-Magnus et al., 2005). Conversely, CDCA uptake is driven predominantly by Oatps, while Ntcp contributes to a lesser degree (Marion et al., 2011). Consistent with previous work, ~90% of the initial uptake rate of TCA into hepatocytes pre-incubated with vehicle (0.1% DMSO) was Ntcp-mediated; ~10% was driven by sodium-independent transporter-mediated processes (presumably Oatps). Conversely, ~69% of transporter-mediated \( ^{14}\text{C} \)CDCA uptake occurred in Na\(^+\)-free buffer, consistent with published reports that Oatp transporters are primarily responsible for initial CDCA uptake (Kemp et al., 2005; Marion et al., 2011). LPV and RTV, alone and combined, did not affect the initial uptake of \( ^{3}\text{H} \)TCA or \( ^{14}\text{C} \)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 Cl\text{ bile} of \( ^{3}\text{H} \)TCA and \( ^{14}\text{C} \)CDCA species.

This manuscript reports, for the first time, the effects of PIs on the disposition of bile acids synthesized by SCRH. While the bile acid pool constitutes numerous bile acid species, this study focused on quantification of taurine- and glycine-conjugated cholic acid and chenodeoxycholic acid due to their potential cytotoxicity (Danielsson, 1973b; Danielsson, 1973a; Ellis et al., 1998). Additionally, the aforementioned bile acids are common to both human and rodent bile. The rodent-specific \( \alpha/\beta \)-TMCA species represent the majority of the rat bile acid pool. Secondary bile acids (produced
via intestinal metabolism) are not synthesized in the SCRH system and thus, were not quantified (Thomas et al., 2008). BEI values for endogenous TCA were comparable to those estimated following addition of [³H]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 [³H]TCA administration and 10-min PI exposure, LPV treatment (50 µm; 24-hr) significantly decreased the accumulation of endogenous TCA and α/β-TMCA in cells + bile (Figures 7 and 9). Addition of RTV to 50 µM LPV had little additive effect. However, addition of RTV to low dose LPV (5 µM) significantly reduced the accumulation of endogenously synthesized total bile acids and TCDCA in SCRH relative to LPV alone (Figures 6 and 8). The extent of the combined effect of LPV and RTV on Bsep inhibition differs considerably between 10-min and 24-hr exposure. In addition, RTV-mediated inhibition of CYP3A4 metabolism may increase cellular LPV or RTV concentrations, which may alter bile acid synthesis. The precise mechanism(s) responsible for PI-mediated decreases in endogenous bile acids are the subject of ongoing studies. These data also suggest that LPV and RTV alter the synthesis and biliary excretion of individual bile acids differentially.

The remarkable decrease in total measured bile acid content may be due to reduced bile acid synthesis. Consistent with this conclusion, 24-hr RTV (15-100 µM) exposure reportedly perturbed bile acid synthesis in a concentration-dependent manner by decreasing the activity of cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid catabolism (Zhou et al., 2006). 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 quantification of hepatocellular concentrations of *endogenous* bile acids may be required 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 relate to the hepatotoxicity observed clinically. At steady-state, LPV and RTV are 98-99% bound to plasma proteins (albumin and AAG). The average unbound fraction of LPV in plasma was 0.73% and ranged from 0.14-1.68% (Fayet et al., 2008). Total and unbound LPV plasma concentrations in HIV-infected patients ranged from 677-23,767 ng/ml (~1-38 µM), and 4.2-209.2 ng/ml (0.007-0.33 µM), respectively. PI concentrations selected for these studies exceeded reported unbound plasma concentrations because the clinically relevant unbound intracellular PI concentrations are unknown, but may exceed systemic concentrations due to transporter-mediated accumulation in hepatocytes and inhibition of intracellular metabolism and excretion.

In summary, 10-min LPV and RTV exposure reduced biliary excretion and, consequently, intracellular accumulation of TCA in SCRH. However, following 24-hr exposure to LPV and RTV, we were unable to demonstrate even additive toxicity. We observed a marked reduction in hepatocyte accumulation of endogenous bile acids (sum total of TCA, GCA, TCDC, GCDCA and α/β-TMCA), primarily attributed to decreased α/β-TMCA. These observations do not necessarily refute a role for bile acid transport inhibition in 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 hepatocytes to remain healthy in most patients. This adaptive response includes mechanisms that decrease hepatocyte content of bile acids, most likely involving reduced synthesis. Such responses 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.
DMD #47225

Authorship Contributions:

Participated in research design: Griffin and Brouwer.

Conducted experiments: Griffin, Perry and St. Claire.

Performed data analysis: Griffin, Perry, St. Claire, Watkins and Brouwer.

Wrote or contributed to the writing of the manuscript: Griffin, Watkins and Brouwer.
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Metab Rev 42:446-471.
DMD #47225


Footnotes

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Figure Legends.

**Figure 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 hr. Following incubation, LDH release (A) and cellular ATP (B) levels were measured. Data are presented as mean ± SEM (n =3).

**Figure 2.** Effect of LPV and RTV, alone and combined, on taurocholic acid (TCA) accumulation in SCRH. [³H]TCA 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; analysis of variance followed by a Bonferroni post test , versus cells + bile vehicle control, # vs. 5 µM LPV alone; p < 0.05).

**Figure 3.** Effect of LPV and RTV, alone and combined, on accumulation of chenodeoxycholic acid (CDCA) species in SCRH. [¹⁴C]CDCA 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 4.** Effect of LPV and RTV, alone and combined, on the Na⁺-dependent and Na⁺-independent uptake of [³H]TCA into freshly isolated suspended rat hepatocytes. [³H]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 ± SEM; n=3 livers in triplicate).

**Figure 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. Accumulation [\(^{14}\)C]CDCA species 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 6.** Accumulation of total measured bile acids (sum of TCA, GCA, TCDCA, GCDCA, and \(\alpha/\beta\)-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, #, versus 5 µM LPV, p < 0.05).

**Figure 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 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 9. Accumulation of α/β-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).
Table 1. Effect of lopinavir exposure for 24 hr, in the presence or absence of ritonavir, on sandwich-cultured rat hepatocyte viability. Day 3 sandwich-cultured rat hepatocytes were treated for 24 hr with lopinavir (LPV) in the absence or presence of 5 µM ritonavir (LPV/r). Data represent mean ± SEM (n=3 livers in triplicate).

<table>
<thead>
<tr>
<th>LPV Dosing Concentration (µM)</th>
<th>Viability (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LDH assay</td>
</tr>
<tr>
<td>5</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>25</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>50</td>
<td>98 ± 2</td>
</tr>
</tbody>
</table>
Table 2. Effect of lopinavir and ritonavir on the biliary excretion index and in vitro biliary clearance of [3H]taurocholic acid and [14C]chenodeoxycholic acid in sandwich-cultured rat hepatocytes. Data from Figures 2 and 3 were used to calculate the biliary excretion index (BEI) and in vitro biliary clearance (Cl\text{bile}), as described in the methods, in the absence or presence of 5 µM RTV (LPV/r);

<table>
<thead>
<tr>
<th></th>
<th>BEI (%)</th>
<th>Cl\text{bile} (ml/min/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>68 ± 3</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>5 µM RTV</td>
<td>21 ± 15</td>
<td>BLQ</td>
</tr>
<tr>
<td>5 µM LPV</td>
<td>49 ± 11</td>
<td>BLQ</td>
</tr>
<tr>
<td>5 µM LPV/r</td>
<td>9 ± 5</td>
<td>BLQ</td>
</tr>
</tbody>
</table>

Data represent mean ± SEM (n=3 livers in triplicate, analysis of variance followed by a Bonferroni post test; \text{a}, versus vehicle control; \text{b} versus 5 µM LPV alone, p < 0.05). BLQ: below limit of quantitation. Initial concentrations of TCA and CDCA in incubation medium were 1.07 µM and 5 µM, respectively.
Table 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. 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.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cells + bile</th>
<th>Cells</th>
<th>Medium</th>
<th>BEI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCA</td>
<td>5.14 ± 1.71</td>
<td>2.61 ± 1.78</td>
<td>0.651 ± 0.127</td>
<td>49</td>
</tr>
<tr>
<td>GCA</td>
<td>0.20 ± 0.06</td>
<td>0.13 ± 0.08</td>
<td>0.07 ± 0.03</td>
<td>35</td>
</tr>
<tr>
<td>TCDCA</td>
<td>1.07 ± 0.20</td>
<td>0.63 ± 0.20</td>
<td>0.017 ± 0.003</td>
<td>41</td>
</tr>
<tr>
<td>GCDCA</td>
<td>0.12 ± 0.08</td>
<td>0.07 ± 0.04</td>
<td>0.004 ± 0.003</td>
<td>42</td>
</tr>
<tr>
<td>α/β TMCA</td>
<td>168 ± 65</td>
<td>133 ± 72</td>
<td>1.59 ± 0.37</td>
<td>20</td>
</tr>
<tr>
<td>Total</td>
<td>174 ± 67</td>
<td>137 ± 74</td>
<td>2.34 ± 0.412</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1

A

LDH

Viability (% Control)

Concentration (μM)

B

ATP

Viability (% Control)

Concentration (μM)

LPV

RTV
Figure 2

[Diagram showing the accumulation of ³H]TCA (pmol/mg protein) for different treatments: Vehicle, 5 µM RTV, 5 µM LPV, and 5 µM LPV/r. Asterisks indicate statistical significance.]
Figure 3

Accumulation of [14C]CDCA (pmol/mg protein) for different treatments:
- Vehicle
- 5 µM RTV
- 5 µM LPV
- 5 µM LPV/r

The figure shows the accumulation of [14C]CDCA under various conditions, with bars representing the mean ± standard deviation.
Figure 4

A

\[ ^{3}H \text{TCA Uptake (pmol/mg protein)} \]

B

\[ ^{3}H \text{TCA Uptake (pmol/mg protein)} \]

C

\[ ^{3}H \text{TCA Uptake (pmol/mg protein)} \]

Time (s)
Figure 6

The bar graph illustrates the total BA accumulation (pmol/mg protein) for different treatments. The treatments include Vehicle, 5 μM RTV, 5 μM LPV, 50 μM LPV, and 50 μM LPV/r. The graph shows a significant increase in BA accumulation for treatments with 5 μM LPV and 5 μM LPV/r compared to the Vehicle control. The asterisk (*) indicates statistical significance, with ** and # representing different levels of significance between groups.
Figure 7

![Bar graph showing TCA Accumulation (pmol/mg protein) for different conditions.](image-url)
Figure 9

![Graph showing the accumulation of α/β TMCA (pmol/mg protein) for different treatments.](image-url)