Evaluation of cynomolgus monkeys for the identification of endogenous biomarkers for hepatic transporter inhibition and as a translatable model to predict pharmacokinetic interactions with statins in humans

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Running title: Endogenous biomarkers and OATP-related DDIs in monkeys

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Nonstandard abbreviations used:
OATP, Organic anion-transporting polypeptide; DBILI, Direct (conjugated) bilirubin; TBILI, Total bilirubin; UBILI, Unconjugated bilirubin; T3, Triiodothyronine; FT3, Free triiodothyronine; T4, Thyroxine; FT4, Free thyroxine; LCA, Lithocholic acid; UDCA, Urosodeoxycholic acid; HDCA, Hyodeoxycholic acid; CDCA, Chenodeoxycholic acid; DCA,
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Deoxycholic acid; DHCA, Deoxyhydrocholic acid; CA, Cholic acid; GCDCA, Glycochenodeoxycholic acid; GCA, Glycholic acid; TDCA, Taurodeoxycholic acid; TCDCA, Taurochenodeoxycholic acid; TCA, Taurocholic acid, DDIs, drug-drug interactions.
Abstract

Inhibition of hepatic transporters such as organic anion transporting polypeptides (OATPs) 1B can cause drug-drug interactions (DDIs). Determining the impact of perpetrator drugs on the plasma exposure of endogenous substrates for OATP1B could be valuable to assess the risk for DDIs early in drug development. As OATP1B orthologs are well conserved between human and monkey, we assessed in cynomolgus monkeys endogenous OATP1B substrates potentially suitable to assess DDI risk in humans. The effect of rifampin (RIF), a potent inhibitor for OATP1B, on plasma exposure of endogenous substrates of hepatic transporters was measured. From the eighteen biomarkers tested, RIF (18mg/kg, PO) caused significant elevation of plasma unconjugated and conjugated bilirubin, which may be attributed to inhibition of cOATP1B1 and cOATP1B3 based on in vitro to in vivo extrapolation analysis. To further evaluate if cynomolgus monkeys are a suitable translational model to study OATP1B-mediated DDIs, we determined the inhibitory effect of RIF on in vitro transport and pharmacokinetics of rosuvastatin (RSV) and atorvastatin (ATV). RIF strongly inhibited uptake of RSV and ATV by cOATP1B1 and cOATP1B3 in vitro. In agreement with clinical observations, RIF (18 mg/kg PO) significantly decreased plasma clearance and increased the area under the plasma concentration curve (AUC) of intravenously administered RSV by 2.8- and 2.7-fold, and increased AUC and maximum plasma concentration of orally administered RSV by 6- and 10.3-fold, respectively. In contrast to clinical findings, RIF did not significantly increase plasma exposure of either intravenous or orally administered ATV, indicating species differences in the rate-limiting elimination pathways.
Introduction

Transporters in the sinusoidal and canalicular membrane of hepatocytes play important roles in the disposition and elimination of many clinically used drugs and endogenous compounds (Hagenbuch and Stieger, 2013). Inhibition of these transporters by perpetrator drugs has the potential to alter systemic and liver exposure of victim drugs, metabolites, and endogenous substances, and may lead to clinically significant drug-drug interactions (DDIs) (Giacomini et al., 2010; Chu et al., 2013b; Corsini and Bortolini, 2013; Pfeifer et al., 2014). The organic anion-transporting polypeptides 1B1 and 1B3 (OATP1B1 and OATP1B3) are major uptake transporters localized in the sinusoidal membrane of human hepatocytes involved in the uptake of a range of endogenous substrates and drugs (Niemi et al., 2011). Drugs known to inhibit OATP1B can cause clinically significant DDIs with a number of OATP1B substrates, such as statins (Yoshida et al., 2012). As such, OATP1B1 and OATP1B3 have been recommended as important transporters to be evaluated in drug development (European Medicine Agency (EMA), 2012; CDER, 2012.)

Recently, regulatory agencies have proposed decision trees to predict the risk for DDIs by inhibition of OATP1B based on in vitro inhibition and maximum plasma exposure of inhibitors in vivo. However, such predictions are not mechanistic and often difficult for compounds with poor solubility and/or high non-specific binding. Furthermore, quantitative prediction of OATP1B-mediated DDIs using physiologically based pharmacokinetic (PBPK) modeling is challenging due to the lack of confidence in in vitro to in vivo extrapolation (IVIVE) and the multiplicity of transporters involved in the elimination of OATP1B substrates, particularly statins (Jones et al., 2012; Jamei et al., 2013).

Some endogenous compounds are substrates of drug transporters. Determining the impact of perpetrator drugs on the plasma or urinary exposure of these endogenous biomarkers in vivo in...
humans could be a valuable approach to assess the DDI liability of drug candidates, especially in early drug development (e.g., phase I clinical trials) where the clinically efficacious dose is not known yet and the design of relevant clinical DDI studies therefore is difficult. For example, the endogenous metabolite N-methylnicotimide was proposed as an endogenous probe to assess DDI risk for organic cation transporters (Ito et al., 2012), and 6β-hydroxycortisol and thiamine have been suggested as endogenous biomarkers for inhibition of renal organic anion transporter 3 and multidrug and toxin extrusion proteins, respectively (Imamura et al., 2014; Kato et al., 2014).

Many endogenous compounds are transported by hepatic transporters. For instance, in humans, unconjugated bilirubin is, at least in part, actively taken up into hepatocytes by OATP1B1 and OATP1B3, followed by glucuronidation by UGT1A1 to form conjugated bilirubin (monoglucuronosyl and bisglucuronosyl bilirubin), which is predominantly excreted into bile via multidrug resistance protein 2 (MRP2) (Keppler, 2014). Under conditions of cholestasis or inhibition of MRP2, the basolateral efflux transporter MRP3 transports bilirubin glucuronides into sinusoidal blood from where they are subsequently eliminated into the urine or re-taken up into hepatocytes by OATP1B1 or OATP1B3. The enterohepatic circulation of bile acids is mediated by a number of transporters in enterocytes and hepatocytes (Rodrigues et al., 2014). Sodium-taurocholate co-transporting polypeptide (NTCP), OATP1B1, and OATP1B3 are responsible for uptake of bile acids from portal blood into hepatocytes, while the bile salt efflux pump BSEP does transport bile acids into the bile. Some thyroid hormones, eicosanoids, porphyrins, estradiol17β-D-glucuronide, and estrone sulfate are also in vitro substrates for OATP1B (Niemi et al., 2011), but these compounds have not been evaluated as potential biomarkers for hepatic transporter inhibition in vivo.

OATP1B1 and -1B3 are poorly conserved between human, rodents and dogs (Chu et al., 2013a). Cynomolgus monkey cOATP1B1 and cOATP1B3, however, show a high degree of amino acid
sequence identity with human orthologs (Shen et al., 2013), but higher hepatic protein amounts as compared to humans (Wang et al., 2015). cOATP1B1 and cOATP1B3 are functionally similar to their human orthologs, both in terms of \textit{in vitro} transport activity and inhibition potency with selected substrates and inhibitors tested (Shen et al., 2013). Importantly, \textit{in vivo} DDIs of rosuvastatin (RSV) and pitavastatin with rifampin (RIF), a potent inhibitor of OATP1B, have been reported in cynomolgus monkeys (Shen et al., 2013; Takahashi et al., 2013). It is therefore conceivable that cynomolgus monkeys could be an appropriate preclinical model to identify endogenous biomarkers which are also sensitive to OATP1B inhibition in humans.

Here, using cynomolgus monkey as a preclinical model, we describe \textit{in vivo} and \textit{in vitro} studies to 1) identify endogenous biomarkers indicative for the inhibition of hepatic transporters such as OATP1B; and 2) evaluate whether the cynomolgus monkey is a suitable translational model to predict OATP-related DDIs in humans using RSV and atorvastatin (ATV) as model compounds.
Materials and Methods

Chemicals and Reagents

[^3]H Estradiol-17β-D-glucuronide (E217βG) (34.3 Ci/mmol),[^3]H cholecystokinin octapeptide (CCK-8) (80 Ci/mmol),[^3]H estrone-3-sulfate (ES) (45.6 Ci/mmol), and[^3]H taurocholic acid (TCA) (5 Ci/mmole) were purchased from PerkinElmer Life Sciences (Boston, MA).[^14]C Ethacrynic acid glutathione conjugate (EA-SG) (0.057 Ci/mmol) was synthesized by the Labeled Compound Synthesis Group, Merck Research Laboratories (Rahway, NJ).[^3]H Atorvastatin (ATV) (20 Ci/mmole),[^3]H rosuvastatin (RSV) (10 Ci/mmole),[^3]H rosuvastatin lactone (RSV lactone) (10 Ci/mmole) as well as unlabeled ATV, RSV and RSV lactone were purchased from American Radiolabeled Chemicals (St. Louis, MO).[^3]H Bilirubin (10 Ci/mmole) was purchased from Moravek Biochemicals (Brea, CA). Rifampin (RIF), cyclosporin A (CsA), Uridine 5′-diphosphoglucuronic acid (UDPGA), alamethicin, estradiol, nicardipine, and labetalol were purchased from Sigma-Aldrich (St. Louis, MO). Bromosulfophthalein (BSP) was purchased from MP Biomedicals (Solon, OH). Synthetic bile acids, chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), glycocholic acid (GCA), lithocholic acid (LCA), sodium glycochenodeoxycholate (GCDCDA), sodium glycodeoxycholate (GDCA), taurochenodeoxycholic acid (TCDCDA), taurodeoxycholic acid (TDCA), sodium taurocholate (TCA) and internal standard taurocholic acid-2,2,3,4,4-d5 ([^7]H5-TCA) were purchased from Sigma-Aldrich. Sodium glycolithocholate (GLCA) and hyodeoxycholic acid (HDCA) were purchased from Steraloids Inc (Newport, RI). All other reagents were commercially obtained with the highest analytical purity grade.

Animals

The in vivo studies were carried out by Maccine Pte Ltd (4 Lorong Chencharu, Singapore). The studies were approved by Maccine and Merck’s Institutional Animal Care and Use Committee (IACUC). Male cynomolgus monkeys (Macaca fascicularis) 7-10 years of age were used in
these studies. For the duration of the study, animals were housed in individual cages in a purpose-built outdoor housing facility (temperatures range approximately 23-32°C, humidity ranging from 60 to 100%). The animals were fasted for a minimum of 12 hr before treatment or blood sample collection with food restrictions not exceeding 16 h and water provided ad libitum.

**In Vitro Systems**

Human Embryonic Kidney 293 (HEK293) cells, HEK293 cells stably transfected with cynomolgus OATP1B1 (HEK293-cOATP1B1), cynomolgus OATP1B3 (HEK293-cOATP1B3), cynomolgus OATP2B1 (HEK293-cOATP2B1) and cynomolgus NTCP (HEK293-cNTCP) were generated at Solvo Biotechnology (Budapest, Hungary). Briefly, sequences verified cDNA encoding cOATP1B1 (Slco1b1), cOATP1B3 (Slco1b3), cOATP2B1 (Slco2b1) and cNTCP (Slc10a1) were synthesized and subcloned into lentiviral plasmids. HEK293 cells were then transduced with transporter containing lentiviral particles, and stable transduced clones were isolated and screened for optimal transport activity with probe substrates.

Membrane vesicles isolated from baculovirus infected Spodoptera frugiperda (Sf9) cells containing cynomolgus MRP2 (cMRP2) or control membrane vesicles (with no inserted transport proteins) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Pooled male cynomolgus monkey liver microsomes (Lot 226394FMU) were purchased from BD Biosciences Discovery Labware (Woburn, MA).

**In Vivo Studies**

The oral dosing solution of RIF was prepared in Polyethylene Glycol 400 (PEG400). The intravenous and oral dosing solution of both RSV and ATV were prepared in 2.5% v/v dimethylsulfoxide (DMSO) in 10% w/v captisol, and 0.5 % methylcellulose, respectively. To assess the effect of RIF on endogenous biomarkers and pharmacokinetics of RSV following RSV oral administration, a total of forty cynomolgus monkeys were divided into 4 dosing groups with
10 animals randomly assigned to each group (n=10/group): RIF 18 mg/kg oral, RSV 3 mg/kg oral, RIF 18 mg/kg oral plus RSV 3 mg/kg oral, and vehicle control. The animals were dosed with RIF and RSV by oral gavage at time points -1 and 0 hr, respectively. This dose regimen is based on *in vivo* DDI studies between RIF and RSV in cynomolgus monkey by Shen et al (Shen et al., 2013). Blood samples were collected from the saphenous femoral vein at 0, 0.25, 0.5, 1, 2, 3, 5, 7, and 24 hr. The plasma samples were obtained following centrifugation and used for the measurement of selected endogenous biomarkers, including direct (conjugated) bilirubin (DBILI), total bilirubin (TBILI), unconjugated bilirubin (UBILI), triiodothyronine (T3), free triiodothyronine (FT3), thyroxine (T4), free thyroxine (FT4), lithocholic acid (LCA), hyodeoxycholic acid (HDCA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), cholic acid (CA), glycochenodeoxycholic acid (GCDCA), glycholic acid (GCA), glycodeoxycholic acid (GDCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), glycolithocholic acid (GLCA), and taurocholic acid (TCA). Plasma concentrations of RSV and RIF were also measured in this study as described in pharmacokinetic studies below.

The effect of RIF (oral, 18 mg/kg) on pharmacokinetics of RSV or ATV following intravenous (i.v.) and oral (p.o.) administration were evaluated in cynomolgus monkeys, respectively. In this study, RSV and ATV were dosed by p.o. (3 mg/kg for RSV, 5 mg/kg for ATV) and i.v. (1 mg/kg for both RSV and ATV) administration. RIF (18 mg/kg oral) was administered 4 hr and 1 hr before i.v. and p.o. administration of statins tested, respectively to ensure that RIF concentrations reach a maximum level at early time points after i.v. and p.o. dosing of RSV or ATV (Shen et al., 2013; Takahashi et al., 2013). The animals were divided into various treatment groups with four animals per each group: RIF p.o, RSV or ATV i.v., RSV or ATV p.o, RSV or ATV i.v. plus RIF p.o., RSV or ATV p.o plus RIF p.o., and vehicle control. Blood samples were collected from the saphenous femoral vein at 0, 0.25, 0.5, 1, 3, 5, 7, and 24 hr after p.o. dosing of the statins, and at 0, 0.25, 0.75, 2, 4, 7, and 24 hr after i.v. dosing of the statins.
The plasma samples were obtained following centrifugation and 1M ammonium acetate buffer (pH 5.0) was immediately added at a ratio of 5µl buffer/100 µl plasma and used for the measurement of RIF, RSV, RSV lactone, ATV, and its major metabolites.

**Uptake and inhibition studies in cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP transfected cells**

cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP-mediated uptake were determined in HEK293 cells stably transfected with cOATP1B1, cOATP1B3, cOATP2B1 or cNTCP cDNAs. Briefly, cells were dislodged with trypsin EDTA and resuspended in Hank's buffered salt solution (HBSS) plus 10 mM HEPES. Cells were then suspended in 96 deep well glass coated plates at a density of 0.4 x 10^6 cells/well. Uptake was initiated by the addition of the probe substrate and the inhibitor at various concentrations. Cells were then incubated for the indicated time at 37°C and uptake was stopped by the addition of ice-cold phosphate buffered saline (PBS), followed by immediate centrifugation for 1 min at 3000 rpm at 4°C (Eppendorf, Model 5180R; Hamburg, Germany), and washing of the cell pellets with PBS. Cell pellets were resuspended in 50% acetonitrile, scintillation fluid (Ultima Gold; Perkin Elmer, Waltham, MA) was added, and radioactivity was determined by liquid scintillation counting in a LS6500 Multipurpose Scintillation Counter (Beckman Coulter, Brea, CA). [3H] E217βG (1 µM) was used as probe substrate for cOATP1B1, [3H] CCK8 (5nM) and [3H] E217βG (1 µM) for cOATP1B3, [3H] ES (0.1 µM) for cOATP2B1, and [3H] TCA (1 µM) for cNTCP, respectively. Uptake of [3H] RSV (0.1 µM), [3H] RSV lactone (0.1 µM) and [3H] ATV (0.1 µM) was also conducted in transporter transfected and control cells. Uptake of [3H] bilirubin (40 nM) was conducted in cOATP1B transfected cells using the method described above with some modifications. Due to the low solubility, high non-specific binding, and instability, [3H] bilirubin was prepared at 40 nM in uptake buffer and used immediately after the preparation. In addition, all studies were
conducted under dim light in the presence of 0.1% BSA to minimize degradation during the experiment and reduce high non-specific binding. Inhibitory effects of RIF (tested at seven concentrations ranging from 0 to 5 μM for cOATP1B1 and cOATP1B3, 0 to 750 μM for cOATP2B1 and 0 to 200 μM for cNTCP) on uptake of the above probe substrates were evaluated in cOATP1B1, cOATP1B3, cOATP2B1 and cNTCP transfected cells at time point that showed linear uptake (data not shown). The probe substrate concentrations used for inhibition studies were at least 5-10-fold lower than their respective $K_m$ values (data not shown).

**Vesicular uptake and inhibition studies**

Time- and ATP-dependent uptake of multidrug resistance protein 2 (MRP2) probe substrate $[^{14}C]$ EA-SG (1 μM) (Chu et al., 2004), and the inhibitory effect of RIF on uptake of $[^{14}C]$ EA-SG were conducted in cMRP2 and control vesicles. Membrane vesicles (10 μl) were added to a glass coating 96-deep well plates (Arctic White LLC, Bethlehem, PA) at 25 μg/well. The probe substrate (20 μl) with and without various concentrations of inhibitors were dissolved in transport buffer (0.25 M sucrose, 10 mM MgCl$_2$, 10 mM Tris-HCl buffer (pH 7.4)) and added into the wells containing vesicles. The mixtures of vesicle and dosing solution were pre-incubated for 3 min at 37°C. Uptake was initiated by the addition of 20 μl ATP regenerating reagent or AMP reagent (final concentration of 5 mM ATP or AMP, 10 mM creatine phosphate and 13 unit/mL creatine phosphokinase in transport buffer), followed by incubation at 37°C for the indicated time. Uptake was stopped by the addition of 200 μl ice-cold stop buffer (0.25 M sucrose, 0.1 M NaCl, 10 mM Tris-HCl buffer (pH 7.4)) followed by rapid filtration of the reaction mixture onto pre-wetted 96-well glass fiber type B filter plate (1.0 μm) (Millipore, Billerica, MA). Filters containing the membrane vesicles were washed with 200 μl ice-cold stop buffer, five times. The filter plate was dried at room temperature overnight and 100 μl scintillation fluid (Optiphase Supermix, Perkin Elmer, Boston, MA) was added to each sample.
Radioactivity was determined by liquid scintillation counting in a MicroBeta Wallac Trilux Scintillation Counter (Perkin Elmer, Boston, MA).

**Microsomal inhibition studies**

The inhibitory effect of RIF on cUGT1A1-mediated estradiol 3-glucuronidation in cynomolgus monkey liver microsomes was evaluated. Cynomolgus monkey liver microsomes (0.2 mg/mL) were incubated at 37°C for 20 min in a 0.2 mL reaction mixture containing 70 μM estradiol (the concentration equal to the K_m value, data not shown) and various concentrations of RIF in 66 mM HEPES buffer (pH 7.0) with 7 mM MgCl_2, 5 mM UDPGA, and 25 μg/mL alamethicin. Nicardipine was used as a positive control inhibitor. The reactions were terminated by adding 0.2 mL of ice-cold methanol containing labetalol as the internal standard. The samples were centrifuged at 14,000 x g for 30 min at 4°C, and the supernatants were collected to measure the formation of estradiol 3-glucuronide as described below.

**Biochemical parameters analysis**

Bilirubin (TBILI and DBILI), thyroid hormones (T3, FT3, T4, FT4), and other standard clinical chemistry parameters, such as ALT, AST, were measured by an automated Roche clinical analyzer (Cobas c501, Roche, Switzerland). UBILI was calculated as the difference between TBILI and DBILI.

**LC-MS/MS analysis**

Plasma concentrations of bile acids, including CA, DCA, GCA, GCDCA, GDCA, GLCA, HDCA, LCA, TCA, TCDCA, TDCA, CDCA were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described previously with some modifications (Castro-Perez et al., 2011). The bile acids were quantified on a Waters UPLC system coupled to the Waters Xevo TQ-S system using a targeted multiple reaction monitoring (MRM) method with $^{2}$H_5-TCA.
as internal standards. The MRM transitions and the retention times for the bile acids were confirmed by their respective synthetic standards. The MRM transitions monitored were CA (407.3/407.3), DCA (391.28/345.4), GCA (464.3/73.95), GCDCA (448.3/73.95), GDCA (448.3/73.95), GLCA (423.38/73.95), HDCA (391.3/391.3), LCA (375.3/375.3), TCA (514.3/106.9), TCDCA (498.3/106.92), TDCA (498.3/106.92), CDCA (391.33/391.33) and the internal standard ²H₅-TCA (519.38/106.9).

Estradiol 3-glucuronide in the supernatants of the microsomal incubation was measured by LC-MS/MS using a PE Sciex API 5000 triple quadrupole mass spectrometer (Concord, ON, Canada) with a TurboIonspray ion source in the negative mode. A Thermo Scientific Transcend LX-2 system (Franklin, MA.) was coupled to the API 5000 with a flow rate of 800 mL/min to direct sample into the mass spectrometer. Chromatographic separation was achieved using a Waters Acquity UPLC C18 column (2.1 x 30 mm, 1.7 µm; PN# 186002349). Mobile phase A was composed of deionized water containing 0.1% formic acid and mobile phase B was composed of acetonitrile containing 0.1% formic acid. A gradient program was initiated starting at 5% B which was held for 0.17 min, then ramped from 5% B to 40% B in 1.16 min. After holding at 40% B for an additional 0.17 min the program was changed back to 5% B. The column was re-equilibrated with 5% B for 1.0 min prior to the next injection. Mass spectrometric detection was accomplished by MRM of transitions unique to each compound. The transitions of the deprotonated precursor ions to the selected product ions of estradiol 3-glucuronide and internal standard labetalol were m/z 447.1 / 113.0 and 327.2 / 176.0, respectively.

The plasma concentrations of RIF, RSV, ATV and their metabolites were measured by LC-MS/MS. Briefly, an aliquot of 50 µL of plasma sample and 20 µL of acetonitrile: water (1:1) was added into in a 96-well polypropylene plate. Standards and quality control (QC) samples were prepared by adding 20 µL of standard solutions that were 2.5-times as concentrated as the
desired plasma standard or QC to 96-well plate in which 50 μL of control plasma had been placed. To effect the protein precipitation and the extraction of analytes, a 300 μL aliquot of acetonitrile containing 200 nM of internal standard (IS) was added. The plates were then vortexed for 2 min and then centrifuged at 4000 rpm for 5 min. A 200 μL aliquot of the supernatant was transferred to a clean plate, from which a 5 μL aliquot was injected directly on to the LC-MS/MS. The LC portion of the instrumentation was a Thermo Scientific LX-2 System (Model # 2303LX15KPSI) which consisted of a pair of Transcend LC pumps, a LEAP autosampler, and a VIM controller. Chromatography was performed at ambient temperature on a Waters Acquity UPLC HSS T3 C18 column (2.1 x 50 mm, 1.8 μm particle size; Part No. 186003538, Waters Corp., Milford, MA). The aqueous mobile phase (A) was 0.005% acetic acid in water and the organic mobile phase (B) was 0.005% acetic acid in acetonitrile. The triple quadrupole mass spectrometer was an Applied Biosystems / MSD Sciex API 5000 with a Turbo V™ Ion Source, operating in the positive ion mode. The mobile phase flow was 0.75 mL/min throughout the gradient run. The gradient was as follows: 15 sec at 95% A and 5% B, 90 sec of linearly ramping the gradient to a composition of 5% A and 95% B, holding this composition for 20 sec, and then returning directly to the initial composition and holding it for 60 sec. The analytes were monitored simultaneously using MRM with the settings described in the supplemental Table 1.

Data analysis

The IC$_{50}$ values for inhibition of transporter-mediated uptake/efflux in transporter transfected cells were obtained by fitting the data to equation 1 by nonlinear regression analysis.

$$Control(\%) = \frac{100}{1 + I/IC_{50}}$$

(1)
Where control (%) represents transporter-mediated uptake/efflux or metabolism measured in the presence of various concentrations of inhibitor to that in the absence of inhibitor.

The risk of RIF to cause the inhibition of certain transporter *in vivo* (R) in cynomolgus monkeys and humans were estimated using equation 2 (Hirano et al., 2006; Giacomini et al., 2010), which represents the ratio of the transporter-mediated clearance in the absence of inhibitor to that in its presence:

\[
R = 1 + \frac{f_u \times I_{in,max}}{I_{C_{50}}} \tag{2}
\]

Where \(f_u\) represents the plasma unbound fraction of RIF (\(f_u=0.19\) for cynomolgus monkeys, and \(f_u=0.20\) for humans) (Shen et al., 2013), \(I_{in,max}\) represents the estimated maximum RIF concentration at the inlet to the liver, and \(I_{C_{50}}\) was obtained from *in vitro* transporter inhibition study in transporter transfected cell lines. \(I_{in,max}\) was calculated based on equation 3 (Hirano et al., 2006; Giacomini et al., 2010):

\[
I_{in,max} = I_{max} + \frac{F_a \times \text{Dose} \times k_a}{Q_h} \tag{3}
\]

where \(I_{max}\) is the maximum plasma concentration of RIF, \(F_a\) is the fraction of the dose of RIF that is absorbed, \(k_a\) is the absorption rate constant of RIF, and \(Q_h\) is the hepatic blood flow rate in cynomolgus monkeys (220 ml/min) and humans (1500 ml/min). To estimate the \(I_{in,max}\) value, \(F_a\) was set at 1, \(k_a\) was set at 0.1 min\(^{-1}\), and the blood-to-plasma concentration ratio was assumed to be 1.

\(K_{puu,\text{liver}}\) (unbound liver to plasma concentration ratio at steady-state) of RIF was estimated in human hepatocytes by measuring the hepatocyte-to-medium partition coefficient for unbound RIF concentration using the method and equations described by Yabe et al. (Yabe et al., 2011).

Pharmacokinetic parameters were calculated by non-compartmental methods using WinNonlin 5.2 (Pharsight Corporation, Mountain View, CA). The area under the plasma concentration–time
curve (AUC) from time 0 to last time point with measurable concentrations (AUC_{0-last}) was calculated by the log–linear trapezoidal method. The AUC from time 0 to infinity (AUC_{inf}) was calculated as the sum of AUC_{0-last} and C_t/\lambda, where \lambda is the apparent terminal rate constant and C_t is the concentration at last time point. \lambda was calculated by regression of the terminal log–linear portion of the plasma concentration–time profile for each animal, and the apparent terminal half-life (t_{1/2}) was calculated as the quotient of the natural log of 2 (ln [2]) and \lambda. The maximal plasma concentrations (C_{max}) and the time to reach C_{max} (T_{max}) were obtained from the observed data. The steady-state volume of distribution (V_{ss}) was calculated as the quotient of dose and the product of AUC_{inf} from intravenous administration and \lambda (dose/AUC_{inf} \times \lambda). The total plasma clearance (CL_{total}) was calculated as the intravenous dose divided by the plasma AUC after intravenous administration (AUC_{iv}). The bioavailability (F) was determined from the AUC after oral administration (AUC_{po}) divided by the AUC_{iv} after normalizing the iv. and p.o. dose assuming linear pharmacokinetics within the dose tested. The hepatic extraction ratio (E_h), hepatic availability (F_h), and the product of fraction absorbed (F_a) and intestinal availability (F_g), F_aF_g, were estimated as described below. As the urinary excretion of RSV in cynomolgus monkeys was minimal (NDA, 2003), non-hepatic contribution to the systemic clearance of RSV was negligible. Therefore, we assumed that total blood clearance of RSV was equal to total hepatic clearance.

E_h and F_h were estimated by the following equations:

\[ E_h = \frac{CL_{total}}{R_B \times Q_h} \quad (4) \]

\[ F_h = 1 - E_h \quad (5) \]

where the blood/plasma concentration ratio (R_B) for RSV was 0.61 (Yoshida et al., 2012), and the hepatic blood flow rate (Q_h) was 44 ml/min/kg (Hosea et al., 2009).

The product of F_aF_g was obtained by the equation below:
Statistical analysis

Student T-test was used to compare the pharmacokinetic parameters with and without the administration of RIF. A p-value of less than 0.05 was considered to be statistically significant.

A linear mixed-effects model (Pinheiro J.C. and Baes, 2004), which has become the standard statistical model for analyzing repeated measures, was used to assess the statistical significance of endogenous biomarkers by quantifying the mean profiles of treatments and both variation between-subject and variation within-subject over time using the equation below.

\[ Y = \text{Time} \ast \text{Treatment} + \text{ID} + \text{error}. \quad (7) \]

Where \( \text{Time} \ast \text{Treatment} \) is the mean response over time for each of the four groups separately (time and treatment interaction). It is also called the fixed effect of the model. Parameter \( \text{ID} \) is the random effect, which characterizes inherent differences among subjects (between-subject variation). The \( \text{error} \) is the residual after all fixed and random effects are removed. It is also known as the within-subject error term, or within-subject variation.

To test the drug effects, we compared the mean change-from-baseline of a treated group vs. that of vehicle control group using a linear contrast (combination) of \( \text{Time} \ast \text{Treatment} \) levels, where the mean effect and its standard error can be estimated using an \( R \)-package "gmodels", which was developed by Warnes et al. (http://cran.r-project.org/src/contrib/PACKAGES.html). To compare the treatment effects across different biomarkers, we further characterize the treatment effects using standardized effect size (\( ES \)) as shown in equation below.

\[ ES = \frac{\mu_1 - \mu_2}{\sigma} \quad (8) \]
Here $\mu_1$ and $\mu_2$ are the means of two treatments (i.e., treated and vehicle control) and $\sigma$ is the pooled standard deviation. Since the effect size is a relative measure expressed in multiples of standard deviations, we can compare and rank the sensitivities among biomarkers based on their effective sizes.
Results

Effects of RIF on plasma level of various endogenous biomarkers in cynomolgus monkeys

The plasma levels of biomarkers were measured in four treatment groups with 10 animals per group: RIF 18 mg/kg oral, RSV 3 mg/kg oral, RIF 18 mg/kg oral plus RSV 3 mg/kg oral, and vehicle control. In a pilot study, plasma level of bile acids (CA, DCA, GCA, GCDCA, GDCA, GLCA, HDCA, LCA, TCA, TCDCA, TDCA) and bilirubin (total and direct) were measured in 40 male cynomolgus monkeys at 1, 4, 8, and 11 hr for 11 days to assess the variability of these parameters between and within animals. Based on a power analysis, 10 animals per treatment group were needed to allow detection of significant changes (>2-fold difference with a power of 80%) (data not shown).

There were no abnormal treatment-related hematological signals observed in this study. Clinical chemistry parameters were measured at 24 hr (study day 1) and compared with the data pre- (day -14 and day 0 pre-study) and post-dose (day 7 post study). A weak increase (less than 1.5-2-fold) in CRP, AST, ALT, LDH, and CK was observed across all four dosing groups, but these parameters returned to normal at day 7 (data not shown). A follow-up study with vehicle alone indicated that the observed transient elevations of the above parameters were caused by cage squeeze and animal handling during repeat bleeds, and were independent of dosing with RIF or RSV. In addition, no hemolysis was observed in the treatment groups.

The plasma levels of various endogenous biomarkers were measured in ten animals following various treatments. A linear mixed-effect model was generated for each biomarker tested with the changes of plasma AUC0-24h from baseline of RIF vs. vehicle and RIF/RSV vs. vehicle. The effect sizes were used to rank these biomarkers. Figure 1 shows the ranking of the effects of RIF and RIF/RSV treatments on various endogenous biomarkers based on plasma AUC0-24h of these biomarkers. Plasma AUC of TBILI, DBILI, UBILI, GCA, GCDCA and GDCA in the RIF
group was significantly elevated as compared to the vehicle control (P<0.05). Plasma AUC of TBILI, DBILI, UBILI, and GCA in the RIF/RSV treatment group were also significantly higher than in the vehicle control. Plasma concentration-time profiles of selected biomarkers (TBILI, DBILI, UBILI, GCA, GCDCA, GDCA) in different treatment groups are shown in Figure 2. In the vehicle control group, the average plasma concentration of DBILI was approximately 3-fold lower than UBILI and TBILI. Lower base line level of DBILI has also been reported by others (Kim et al., 2005) and observed in other independent cynomolgus monkey studies (>300 animals) (unpublished observation) and is within a similar range as in mice (van de Steeg et al., 2012) and humans (unpublished observation). A significant elevation of conjugated, unconjugated and total bilirubin was observed in both the RIF and RIF/RSV treatment groups as compared to RSV alone and vehicle control groups, respectively. Average fold-change of AUC for TBILI, DBILI, and UBILI in RIF vs. vehicle control group was 2.8-, 2.4-, and 3.0-fold, respectively. Likewise, average fold-change of AUC for TBILI, DBILI, and UBILI in RIF/RSV vs. vehicle control group was 2.9-, 2.7-, and 3.0-fold, respectively. Bilirubin levels were still elevated at t = 24 hr following RIF or RIF/RSV administration. This may be explained by slow elimination of RIF in monkeys (Figure 5). At 24 hr, the RIF plasma unbound concentration only dropped to 1.1 µM (unbound C_{max} =2.6 µM), which was still ~3-fold higher than the IC_{50} value for cOATP1B1 (IC_{50}=0.38 µM) (Table 3). The plasma exposure of GCA, GCDCA, and GDCA showed higher variability in different treatment groups, despite a trend of weak increase in the RIF and RIF/RSV groups, especially at later time points. RIF did not cause statistically significant changes in T3, FT3, T4, FT4, as well as other bile acids tested (supplemental Figure 1 and 2).

**Effects of RIF on the pharmacokinetics of RSV and RSV lactone in cynomolgus monkeys**

The effects of RIF (p.o. administration) on the pharmacokinetics of RSV and RSV lactone, a
major metabolite of RSV, were investigated after i.v. and p.o. administration of RSV to cynomolgus monkeys (Figure 3, Table 1). After i.v. administration of RSV, RSV CL_total was decreased by 2.8-fold; AUC_{0-last} and AUC_{inf} increased by 2.7-fold with RIF treatment, which was accounted for by a decrease in CL_H. The V_{ss} and t_{1/2} of RSV were decreased and increased 1.4- and 2-fold, respectively, in the presence of RIF; however, these changes were not statistically significant. Following i.v. administration of RSV, AUC and C_{max} of RSV lactone were also increased by 2-3-fold in the presence of RIF, while no significant change in t_{1/2} and T_{max} was observed. After p.o. administration of RSV, AUC_{oral} and C_{max} of RSV was significantly increased by 5-6- and 10.3-fold, respectively, in the presence of RIF. T_{max} was decreased by 3-fold, whereas t_{1/2} remained unchanged. Further kinetic analysis indicated that inhibition of oral pharmacokinetics of RSV by RIF could be attributed to the inhibition of both hepatic clearance and intestinal absorption of RSV. RIF inhibited hepatic clearance of RSV as estimated by the product of F_{H}/F_{H}' (the ratio of RSV hepatic availability with and without RIF treatment) multiplied by CL_{total}/CL_{total}' (the ratio of RSV total clearance without and with RIF treatment) of 7.6-fold, whereas its impact on gut absorption and/or metabolism was relatively low with F_{a}F_{g}/F_{a}'F_{g}' (the ratio of RSV F_{a}F_{g} with and without RIF treatment) of 1.8-fold. After RIF treatment, the C_{max} of RSV lactone significantly increased by 8-fold. An increase in plasma AUC of RSV lactone was also observed (3.7-4.6-fold), but the effect was not statistically significant.

Effects of RIF on pharmacokinetics of ATV and its metabolites in cynomolgus monkeys

The effects of oral dosing of RIF on the pharmacokinetics of ATV and its metabolites, were also evaluated after i.v. and p.o. administration of ATV (Figure 4, Table 2). ATV CL_{total} following i.v. administration was decreased 1.6-fold, whereas AUC_{iv} was increased 1.7-fold with RIF treatment. However, these changes were not statistically significant. Similarly, there was no
significant effect on the $V_{ss}$ and $t_{1/2}$ of ATV in the presence and absence of RIF. Likewise, all kinetic parameters for orally administered ATV in the presence of RIF were not significantly different from control, although a weak increase (less than 2-fold) of ATV AUC and $C_{max}$ was observed in the RIF treatment group. In contrast, RIF increased plasma AUC and $C_{max}$ of 2-hydroxy ATV (2OH-ATV), a major metabolite of ATV, by 3.1- and 3.4-fold, respectively, after i.v. administration of ATV and 8- and 20-fold following p.o. dosing of ATV, respectively. RIF showed a much more profound effect on 2-OH ATV with orally administered ATV. It is likely that some hydroxyl-metabolites are formed in the gut by intestinal CYP3A during the absorption phase when ATV is dosed orally. In fact, the AUC ratio of 2-OH ATV to ATV after p.o administration of ATV was increased nearly 2-fold compared to i.v. dosing (Table 2). Plasma exposure of ATV lactone and 4-hydroxy-ATV (4OH-ATV) was low and/or below the detection limit (data not shown). However, RIF treatment significantly increased plasma exposure of 4OH-ATV after both i.v. and p.o. administration of ATV (data not shown).

**Plasma concentration–time profiles of RIF in cynomolgus monkeys**

Plasma concentration–time profiles of RIF in cynomolgus monkeys after administration of RIF (18mg/kg, p.o.) at 4 hr and 1 hr prior to i.v. and p.o. administration of RSV are shown in Figure 5. $C_{max}$ of RIF followed by i.v. or p.o. administrations of RSV was 11.9 ± 2.8 µM and 13.9 ± 2.7 µM, respectively. This is comparable to a $C_{max}$ of RIF ($C_{max} = 12$ µM) in healthy human subjects after a single oral dose of RIF (600 mg) (Prueksaritanont et al., 2014). We also confirmed that the plasma exposure of RIF in biomarker and ATV DDI studies was comparable to the data described above (data not shown).

**Inhibitory effects of RIF on in vitro transport of prototypical substrates by cOATP1B1, cOATP1B3, cOATP2B1, cNTCP, cMRP2, and metabolism by cUGT1A1**
To understand the effect of RIF on the endogenous biomarkers mechanistically, *in vitro* transport and metabolism studies were conducted to assess the inhibitory effect of RIF on hepatic uptake transporters cOATP1B1, cOATP1B3, cOATP2B1, cNTCP, biliary efflux transporter cMRP2, and cUGT1A1.

Functional activity of cynomolgus monkey transporters in stably-transfected HEK 293 cells and membrane vesicles were initially evaluated using prototypical substrates: \[^{3}H\] E217βG (1 µM) for cOATP1B1, \[^{3}H\] CCK8 (5 nM) and \[^{3}H\] E217βG (1 µM) for cOATP1B3, \[^{3}H\] ES (0.1 µM) for cOATP2B1, \[^{3}H\] TCA (1 µM) for cNTCP, \[^{14}C\] EA-SG (1 µM) for cMRP2. As shown in Figure 6, uptake of the above probe substrates was significantly higher in the respective transporter cDNA transfected cell lines and cMRP2 containing vesicles (ATP-dependent uptake) compared to control cells or control vesicles, indicating that the transporters were functionally active in these *in vitro* systems.

Bilirubins (conjugated and unconjugated) are known to be substrates for human OATP1B1, OATP1B3, and MRP2 (Keppler, 2014). Some bile acids are transported by OATP1B1, OATP1B3, and NTCP (Xiang et al., 2009). The inhibitory effects of RIF on these transporters were therefore evaluated in order to start elucidating the mechanism explaining the changes of the biomarkers measured in the *in vivo* studies. The IC\textsubscript{50} values obtained with the cynomolgus monkey transporters were also compared to those measured for their human orthologs.

Inhibitory effect of RIF on cUGT1A1, the major UGT enzyme responsible for the glucuronidation of unconjugated bilirubin was also evaluated. cOATP1B1 and cOATP1B3-mediated uptake of \[^{3}H\] bilirubin was measurable, suggesting that unconjugated bilirubin is likely a substrate for cOATP1B1 and cOATP1B3. However, uptake activity was too low to use bilirubin as a probe substrate to determine IC\textsubscript{50} values. The low uptake window was most likely explained by poor solubility and high non-specific binding to cell membranes (data not shown).
Bilirubin mono/di-glucuronides were not available and were therefore not tested. Thus, in this study, \(^{[3]H}\)E\(_{217}\)\(\beta\)G (0.1 µM) was used as a surrogate probe for conjugated and unconjugated bilirubin to assess the inhibition by RIF of cOATP1B1, cOATP1B3, and \(^{[14]}\)C]EA-SG (1 µM) for inhibition of cMRP2-mediated transport. \textit{In vitro} IC\(_{50}\) values and estimated risk for \textit{in vivo} inhibition (R-values) were summarized in Table 3. RIF exhibited potent inhibition of cOATP1B1, cOATP1B3 with estimated R-values of 42.8 and 10.9 respectively. Similarly, RIF also strongly inhibited human OATP1B1- and OATP1B3-mediated E\(_{217}\)\(\beta\)G uptake with estimated R-values of 21.2 and 61.6, respectively. In contrast, RIF showed moderate to weak inhibition to cNTCP and human NTCP with R-values of 1.45 and 1.04, respectively. RIF showed weak inhibition of cMRP2 (IC\(_{50}\) =118 ± 14 µM, R=1.13), whereas RIF inhibited human MRP2-mediated EA-SG uptake with IC\(_{50}\) and R-value of 14.7 ± 0.8 µM (Prueksaritanont et al., 2014) and 1.82, respectively. RIF did not cause significant inhibition of estradiol 3-glucuronidation by cUGT1A1 in monkey liver microsomes (IC\(_{50}\) >100 µM, Table 3). In contrast, nicardipine, a positive control inhibitor, showed potent inhibition to cUGT1A1 with an IC\(_{50}\) value of 2.5 µM (data not shown).

\textit{In vitro} transport of RSV and ATV by cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP and inhibition by RIF

As shown in Figure 6, uptake of \(^{[3]H}\) RSV (0.1 µM) and \(^{[3]H}\) ATV (0.1 µM) in cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP transfected cells was time-dependent and significantly higher than in control cells, indicating that RSV and ATV were substrates for these transporters. Interestingly, uptake of \(^{[3]H}\) RSV lactone (0.1 µM) in cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP transfected cells was also significantly higher than in control cells (Supplemental Figure 3), indicating that RSV lactone was also a substrate for these transporters. As shown in Table 4, RIF was a potent inhibitor of cOATP1B1- and cOATP1B3-mediated RSV and ATV uptake (IC\(_{50}\) \ldots)
values of 0.25 ± 0.03 µM and 0.14 ± 0.01 µM for uptake of RSV and ATV by cOATP1B1; IC₅₀ values of 1.3 ± 0.1 µM and 0.75 ± 0.08 µM for uptake of RSV and ATV by cOATP1B3). However, RIF only showed moderate to weak inhibition of OATP2B1- and NTCP-mediated RSV and ATV uptake (IC₅₀ values of 61.7 ± 3.6 µM and 62.7 ± 10.7 µM for uptake of RSV and ATV by cOATP2B1; IC₅₀ values of 83.5 ± 7.6 µM and 93.4 ± 10.4 µM for uptake of RSV and ATV by cNTCP). R-value analysis indicated a potential *in vivo* inhibition to cOATP1B1 (R-values of 64.5 and 114.3 for RSV and ATV), cOATP1B3 (R-values of 13.2 and 22.2 for RSV and ATV), less or no potential for inhibition of cOATP2B1 (R-values of 1.26 and 1.25 for RSV and ATV) and cNTCP (R-values of 1.19 and 1.17 for RSV and ATV).
Discussion

In this study, we observed significant elevation of conjugated and unconjugated bilirubin in plasma of cynomolgus monkeys following oral administration of RIF, in which inhibition of cOATP1B1 and cOATP1B3 may play a major role. RIF showed potent inhibition of cOATP1B1 and cOATP1B3 with \textit{in vitro} IC\textsubscript{50} values 41.8- and 9.9-fold lower than its maximum unbound hepatic inlet concentration (15.9 µM). This is in agreement with its potent inhibition of human OATP1B1 and -1B3-mediated hepatic uptake both \textit{in vitro} and \textit{in vivo} (Prueksaritanont et al., 2014). RIF exhibited weak inhibition of cMRP2. Assuming that K\textsubscript{puu, liver} of RIF in humans (K\textsubscript{puu, liver}=3.3, unpublished observation) is comparable to monkeys, the IC\textsubscript{50} for cMRP2 (118 µM) is much higher than the estimated RIF unbound liver concentrations (C\textsubscript{u, liver}) in monkeys (8.7 µM), suggesting that the elevation of plasma conjugated bilirubin is not caused by the inhibition of cMRP2. However, RIF showed more potent inhibition of hMRP2 (IC\textsubscript{50}=14.7 µM, R= 1.82, estimated C\textsubscript{u, liver}=7.9 µM). Thus, inhibition of MRP2 may contribute to increased plasma conjugated bilirubin in humans. RIF is only a weak inhibitor for both cynomolgus (IC\textsubscript{50} >100 µM) and human UGT1A1 (IC\textsubscript{50} = 33 µM) (Chiou et al., 2014). Inhibition of hepatic UGT1A1 therefore will not be a likely contributor to the elevation of unconjugated bilirubin by RIF. Taking together, elevation of unconjugated and conjugated bilirubin in monkeys could be mainly attributed to inhibition of hepatic uptake/reuptake of unconjugated and conjugated bilirubin by cOATP1B.

Our data suggest that conjugated and unconjugated bilirubins could be suitable biomarkers for inhibition of hepatic OATP1B. Although further studies are needed to assess if our findings are translatable to humans, some \textit{in vitro} studies have suggested that inhibition of OATP1B1 and OATP1B3 by several drugs, including RIF, correlated with drug induced unconjugated hyperbilirubinemia (Vavricka et al., 2002; Campbell et al., 2004; Chang et al., 2013; Chiou et al.,...
2014). Furthermore, individuals with an OATP1B1 (SLCO1B1) polymorphism showed significant elevation of both unconjugated and conjugated bilirubin (Zhang et al., 2007). A genome-wide association study suggested that OATP1B3 (SLCO1B3) variants contributed to idiopathic mild unconjugated hyperbilirubinemia (Sanna et al., 2009). Complete deficiency of OATP1B1 and OATP1B3 causes human Rotor syndrome by disruption of hepatic reuptake of conjugated bilirubin (van de Steeg et al., 2012). To explore bilirubin as biomarker for inhibition of OATP1B, studies are needed to assess the elevation of bilirubin by various OATP inhibitors in humans and their correlation with clinical DDIs for various OATP substrates at different dose levels. Despite that the increase in conjugated and unconjugated bilirubin by RIF is likely primarily caused by inhibition of OATP1B, a potential involvement of alternative pathways, contributing to hepatic uptake of bilirubins both in humans and/or monkeys, cannot be excluded.

Our studies suggest that bile acids are not sensitive biomarkers for hepatic uptake transporters in cynomolgus monkeys, despite a weak elevation of GCA, GCDCA, and GDCA by RIF. As RIF is only a moderate to weak inhibitor for cNTCP/NTCP, the elevation of these bile acids cannot be explained by the inhibition of cNTCP, but is possibly attributed to inhibition of cOATP1B. However, the contribution of OATP1B to uptake of these bile acids is not known. In fact, in humans, the effect of OATP1B1 (SLCO1B1) polymorphisms on plasma concentrations of bile acids is controversial (Xiang et al., 2009; Xiang et al., 2011). Likewise, there is no remarkable elevation of thyroid hormones, which is similar to the reports in Oatp1b2 knockout mice (Meyer zu Schwabedissen et al., 2011).

To further evaluate the utility of cynomolgus monkeys as a translational model to study OATP-mediated DDIs (Shen et al., 2013), we conducted in vitro studies to determine the inhibitory effect of RIF on transport of RSV and ATV by a range of hepatic uptake transporters. Given that RIF has the potential to impact both hepatic uptake and gut efflux transporters (Prueksaritanont
et al., 2014), we conducted a comprehensive pharmacokinetic analysis to assess the effect of oral RIF on the pharmacokinetics of intravenously and orally administered RSV and ATV and their metabolites in cynomolgus monkeys.

In humans, hepatic and renal clearance of RSV accounted for 72% and 28% of total clearance, with FaFg of 43% and minimal metabolism (Martin et al., 2003; Yoshida et al., 2012). The elimination of RSV in cynomolgus monkeys was similar to humans, except that the renal elimination was substantially lower (<5%) (NDA, 2003). E_h of RSV was higher (E_h=0.73) compared to that in humans (E_h=0.53), suggesting that hepatic uptake is the predominant pathway for elimination of RSV in monkeys. RIF increased RSV AUC_{iv} and decreased CL_{total} by 2.7- and 2.8-fold, respectively. The inhibition of hepatic uptake may play a significant role explaining these effects. RIF increased C_{max} and AUC_{oral} of orally administered RSV by 10.3- and 6.1-fold, respectively. The increase in AUC_{oral} was primarily due to a decrease in hepatic clearance (F_H/F_H × CL_{total}/CL_{total}' = 7.6). The increase in the fraction absorbed from the gut lumen, likely caused by inhibition of gut cBCRP (F_a/F_g × F_a/F_g' =1.8), might also contribute to an increase in the AUC_{oral} and C_{max} of RSV, which is in line with clinical observations (Prueksaritanont et al., 2014). In humans, RIF (600 mg single dose, p.o.) also markedly increased AUC_{oral} and C_{max} of RSV (5 mg, p.o.) by 4.4- and 9.9-fold, respectively (Prueksaritanont et al., 2014), which is similar to that in monkeys and consistent with the R-value analysis (Table 4). Higher fold change of AUC_{po} in monkeys (6.1-fold in monkey vs. 4.4-fold in human) might be explained by higher contribution of hepatic clearance to overall elimination of RSV in monkeys. Interestingly, RIF also increased AUC and C_{max} of RSV lactone. As RSV lactone was a substrate for cOATP1B1 cOATP1B3, cOATP2B1, and cNTCP, the increase in plasma exposure of RSV lactone is likely caused by the inhibition to these transporters.
In vivo disposition of ATV involves active hepatic uptake followed by extensive metabolism by CYP3A4. In humans, hepatic uptake was the rate-limiting step for hepatic elimination of ATV (Maeda et al., 2011), and hepatic OATP1B was the major contributor to its hepatic uptake (Vildhede et al., 2014). Multiple clinical DDI studies showed that RIF 600 mg (single dose p.o. and i.v.) increased plasma AUC of ATV by 8.5- to 12-fold (He et al., 2009; Maeda et al., 2011), and 7.3-fold (Lau et al., 2007), respectively. Such profound DDIs were attributed mainly to the inhibition of OATP1B. In vitro, RIF showed comparable inhibitory potency to ATV uptake by cOATP1B1 and cOATP1B3 as compared to humans. We therefore predicted that RIF would cause a DDI with ATV in monkeys similar as in humans. Surprisingly, RIF did not have significant impact on either intravenously or orally administered ATV in monkeys. In these studies, plasma exposure of RIF was similar to clinical DDI studies. In monkeys, CLh of ATV was 44.8 ml/min/kg (assuming R B = 1), which is comparable to Qh (44 ml/min/kg). Thus, the CLtotal of intravenously administered ATV was hepatic blood flow-limited (Eh=1). In contrast, ATV showed lower CLtotal (8.9 ml/min/kg) and moderate Eh (0.42) in humans (Lennernas, 2003). This could explain the weak impact of RIF on ATV AUCiv and CLtotal in monkeys, but not for the lack of DDIs with orally administered ATV, as the clearance following oral dosing (CL/F) should not be affected by hepatic blood flow. The mechanisms for lack of translation of such DDI are unclear. It was apparent that cOATP1B-mediated hepatic uptake may not be the rate-limiting step for hepatic elimination of ATV in monkeys. It is possible that uptake of ATV into monkey hepatocytes was not dominated by cOATP1B, but by other transporters, such as OATP2B1 or NTCP, which are not inhibited by RIF. Evaluation of relative contribution of these transporters to hepatic uptake of ATV in monkeys could help to test this hypothesis. Furthermore, high ATV clearance in monkeys is likely attributed to higher total CYP3A level in monkey gut and liver compared to humans (Martignoni et al., 2006). Therefore, it is possible that hepatic/gut metabolism is a more dominant pathway for the elimination of ATV in monkeys,
assuming that the permeation of ATV into the liver is not rate-determining. Interestingly, despite lack of impact on plasma exposure of ATV, RIF significantly increased plasma AUC and C\textsubscript{max} of 2-OH ATV (Figure 4, Table 2) and 4-OH ATV (data not shown). This is consistent with clinical observations (Lau et al., 2007). Like in humans, 2-OH- and 4-OH ATV are substrates for cOATP1B1 and cOATP1B3 (data not shown). Increase in plasma exposure of these metabolites by RIF is likely caused by inhibition of uptake by cOATP1B.

In conclusion, conjugated and unconjugated bilirubin could be sensitive endogenous biomarkers for inhibition of hepatic transporters, primarily OATP1B in cynomolgus monkeys. Clinical studies are ongoing to confirm if this finding is translatable to humans. Lack of translation of ATV-RIF DDI from cynomolgus monkeys to humans suggests that the utility of cynomolgus monkeys as a translational model to predict OATP-mediated DDIs is compound-dependent and monkeys are not suitable to predict DDI risk of drug candidates prospectively. Species differences in rate-limiting elimination pathways between monkeys and humans must be taken into consideration when applying these approaches.
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Authorship Contributions

Participated in research design: Chu, Shih, Hentze, Salituro, Castro-Perez, Palamanda, Liaw, Savage, and Evers.

Conducted experiments: Chan, Shaw, Owens, Salituro, Cai, Newton, Fernandis, and Ng.

Contributed new reagents or analytic tools: Salituro

Performed data analysis: Chu, Shin, Shaw, Hentze, Chan, Owens, Wang, Salituro, Newton, Palamanda, Liaw, Evers.

Wrote or contributed to the writing of the manuscript: Chu, Shih, Hentze, Chan, Owens, Wang, Liaw, Newton, Savage, Evers.
References


Figure Legends

Figure 1: Ranking of the effects of different treatments on the change of various endogenous biomarkers in cynomolgus monkeys. Panel A and B are the ranking of the changes on biomarker plasma AUC_{0-24} between RIF (18 mg/kg, p.o.) and vehicle control (A) or RIF (18 mg/kg, p.o) and RSV (3 mg/kg, p.o.), and vehicle control (B). The biomarkers were ranked by effect size and p-value (see Material and Methods for the details). The black bars indicate positive effect sizes and the white bars denote negative effect sizes.

Figure 2: Mean plasma concentration-time profiles of TBILI (A), DBILI (B), UBILI (C), GCA (D), GCDCA (E) and GDCA (F) following oral administration of RIF (18 mg/kg) (black circle), RSV (3 mg/kg) (white square), RIF (18 mg/kg) and RSV (3 mg/kg) (black triangle), and vehicle control (white triangle) in cynomolgus monkeys. Values shown are mean ± SE for n=10 animals.

Figure 3: Effects of RIF (18 mg/kg p.o.) on mean plasma concentration-time profiles of RSV (A, B) and RSV lactone (C, D) following i.v. (A, C) and p.o. (B, D) administration of RSV in cynomolgus monkeys. RIF was administered 4 hr and 1 hr before i.v. (1 mg/kg) and p.o. (3 mg/kg) administration of RSV, respectively. Plasma concentrations of RSV and RSV lactone with (black circles) and without (white circles) the administrations of RIF were measured. Values shown are mean ± SE for n=4 (i.v.) and n=10 (p.o.) animals, respectively.

Figure 4: Effects of RIF (18 mg/kg p.o.) on mean plasma concentration-time profiles of ATV (A, B), and its major metabolite 2-OH ATV (C, D) following i.v. (A, C) and p.o. (B, D) administration of ATV in cynomolgus monkeys. RIF was administered 4 hr and 1 hr before i.v. (1 mg/kg) and p.o. (5 mg/kg) administration of ATV. Plasma concentrations of ATV and 2-OH
ATV with (black circles) and without (white circles) the administrations of RIF were measured. Values shown are mean ± SE for n=4 animals.

**Figure 5:** Mean plasma concentration-time profiles of RIF following p.o. administration of RIF (18 mg/kg) in cynomolgus monkeys. RIF was orally administered 4 hr and 1 hr before i.v. (1 mg/kg) and p.o. (3 mg/kg) administration of RSV. Plasma concentrations of RIF were measured after i.v. (1 mg/kg) (A) and p.o. (3 mg/kg) (B) administration of RSV. Values shown are mean ± SE for n=4 animals.

**Figure 6:** Transport of prototypical substrates by cOATP1B1, cOATP1B3, cNTCP-transfected HEK-293 cells and cMRP2 containing membrane vesicles. Uptake of [3H] E217βG (0.1 µM) or [3H] TCA (1 µM) into cOATP1B1 (A), cOATP1B3 (B), and cNTCP (C) transfected HEK-293 cells (black bars) and control HEK-293 cells (white bars) in the presence and absence of CsA (5 µM) or BSP (100 µM) at indicated time was measured. Panel D showed ATP-dependent uptake of [14C] EA-SG (1 µM) into cMRP2 (black bars), and control (white bars) membrane vesicles at indicated time. Values shown are mean ± SE of experiments performed in triplicate.

**Figure 7:** Uptake of [3H] RSV (0.1 µM) and [3H] ATV (0.1 µM) into cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP transfected cells. Time-dependent uptake of [3H] RSV (0.1 µM) (panels A to D) and [3H] ATV (0.1 µM) (panels E to H) into cOATP1B1 (A and E), cOATP1B3 (B and F), cOATP2B1 (C and G), and cNTCP (D and H) transfected HEK-293 cells were measured. Black bars represent uptake in transporter transfected HEK-293 cells, white bars represent uptake in control HEK-293 cells. Values shown are mean ± SE of experiments performed in triplicate.
TABLE 1
Pharmacokinetic parameters of RSV and RSV lactone after single intravenous (1 mg/kg) or oral (3 mg/kg) administration of RSV to cynomolgus monkeys with and without oral administration of rifampin (18mg/kg). Each value represents mean ± S.E. from 4-10 monkeys.

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<td>CL&lt;sub&gt;total&lt;/sub&gt; (L/hr/kg)</td>
<td>1.17 ± 0.17</td>
<td>0.42 ± 0.03</td>
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<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>1.56 ± 0.27</td>
<td>1.11 ± 0.24</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>2.93 ± 0.88</td>
<td>5.85 ± 1.48</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-last&lt;/sub&gt; (hr*µM)</td>
<td>1.80 ± 0.23</td>
<td>4.77 ± 0.44</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (hr*µM)</td>
<td>1.88 ± 0.24</td>
<td>4.99 ± 0.40</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>0.04 ± 0.23</td>
<td>0.45 ± 0.11</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>4.27 ± 0.63</td>
<td>1.25 ± 0.40</td>
</tr>
<tr>
<td>F&lt;sub&gt;H&lt;/sub&gt;</td>
<td>0.36</td>
<td>0.66</td>
</tr>
<tr>
<td>F&lt;sub&gt;M&lt;/sub&gt;</td>
<td>0.36</td>
<td>0.66</td>
</tr>
<tr>
<td>F&lt;sub&gt;F&lt;sub&gt;G&lt;/sub&gt;&lt;/sub&gt;</td>
<td>0.36</td>
<td>0.66</td>
</tr>
<tr>
<td>RSV lactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>1.62 ± 0.11</td>
<td>1.38 ± 0.09</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-last&lt;/sub&gt; (hr*µM)</td>
<td>1.43 ± 0.23</td>
<td>3.10 ± 0.31</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (hr*µM)</td>
<td>1.50 ± 0.23</td>
<td>3.16 ± 0.29</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>1.03 ± 0.33</td>
<td>3.05 ± 0.31</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>0.25 ± 0.00</td>
<td>0.25 ± 0.00</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Significantly different from control group (P<0.05).
**TABLE 2**

Pharmacokinetic parameters of ATV and 2-hydroxy-ATV after single intravenous (1mg/kg) or oral (5mg/kg) administration of ATV to cynomolgus monkeys with and without oral administration of RIF (18mg/kg). Each value represents mean ± S.E. from 4 monkeys.

<table>
<thead>
<tr>
<th></th>
<th>ATV i.v.</th>
<th></th>
<th></th>
<th>ATV p.o.</th>
<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>RIF p.o.</td>
<td>Ratio</td>
<td>Control</td>
<td>RIF p.o.</td>
<td>Ratio</td>
</tr>
<tr>
<td>ATV</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CL&lt;sub&gt;total&lt;/sub&gt; (L/hr/kg)</td>
<td>2.69 ± 0.39</td>
<td>1.66 ± 0.27</td>
<td>0.62</td>
<td>2.16 ± 0.32</td>
<td>1.22 ± 0.31</td>
<td>0.56</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>0.69 ± 0.07</td>
<td>0.93 ± 0.09</td>
<td>1.34</td>
<td>4.43 ± 1.26</td>
<td>5.07 ± 1.03</td>
<td>1.14</td>
</tr>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>0.71 ± 0.11</td>
<td>1.17 ± 0.20</td>
<td>1.65</td>
<td>0.42 ± 0.07</td>
<td>0.61 ± 0.21</td>
<td>1.45</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-last&lt;/sub&gt; (hr*µM)</td>
<td>0.71 ± 0.11</td>
<td>1.18 ± 0.20</td>
<td>1.65</td>
<td>0.45 ± 0.07</td>
<td>0.76 ± 0.25</td>
<td>1.67</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>0.30 ± 0.08</td>
<td>0.37 ± 0.16</td>
<td>1.24</td>
<td>1.00 ± 0.00</td>
<td>2.63 ± 1.60</td>
<td>2.63</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>3.00 ± 0.00</td>
<td>0.63 ± 0.22</td>
<td>0.21</td>
<td>4.50 ± 1.50</td>
<td>2.50 ± 1.50</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>2-hydroxy-ATV</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>t&lt;sub&gt;1/2&lt;/sub&gt; (hr)</td>
<td>0.70 ± 0.05</td>
<td>1.88 ± 0.24</td>
<td>2.70</td>
<td>20.78 ± 10.22</td>
<td>5.16 ± 1.72</td>
<td>0.25</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-last&lt;/sub&gt; (hr*µM)</td>
<td>0.39 ± 0.03</td>
<td>1.18 ± 0.26</td>
<td>3.05</td>
<td>0.24 ± 0.05</td>
<td>1.80 ± 0.18</td>
<td>0.25</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt; (hr*µM)</td>
<td>0.41 ± 0.03</td>
<td>1.25 ± 0.27</td>
<td>3.05</td>
<td>0.38 ± 0.06</td>
<td>1.90 ± 0.26</td>
<td>0.25</td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (µM)</td>
<td>0.21 ± 0.04</td>
<td>0.72 ± 0.13</td>
<td>3.41</td>
<td>0.02 ± 0.00</td>
<td>0.39 ± 0.07</td>
<td>0.25</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)</td>
<td>3.00 ± 0.00</td>
<td>0.63 ± 0.22</td>
<td>0.21</td>
<td>4.50 ± 1.50</td>
<td>2.50 ± 1.50</td>
<td>0.56</td>
</tr>
</tbody>
</table>

<sup>a</sup>: Significantly different from control group (P<0.05).
**TABLE 3.** *In vitro* inhibition of RIF to cOATP1B1/OATP1B1, cOATP1B3/OATP1B3, cNTCP/NTCP, cMRP2/MRP2, or cUGT1A1/UGT1A1 and risk for *in vivo* inhibition in cynomolgus monkeys and humans

<table>
<thead>
<tr>
<th>Transporters</th>
<th>Cynomolgus monkeys</th>
<th>Humans</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probe substrates</td>
<td>IC₅₀ (µM)</td>
</tr>
<tr>
<td>cOATP1B1/OATP1B1</td>
<td>E₂17βG (1 µM)</td>
<td>0.38 ± 0.02</td>
</tr>
<tr>
<td>cOATP1B3/OATP1B3</td>
<td>E₂17βG (1 µM)</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>cNTCP/NTCP</td>
<td>TCA (1 µM)</td>
<td>35.1 ± 6.3</td>
</tr>
<tr>
<td>cMRP2/MRP2</td>
<td>EA-SG (1 µM)</td>
<td>118 ± 14</td>
</tr>
<tr>
<td>cUGT1A1/UGT1A1</td>
<td>Estradiol (70 µM)</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

ⁿ: Risk for in vivo inhibition: estimated based on *in vitro* IC₅₀ values and unbound hepatic inlet concentrations.

ᵇ: Prueksaritanont et al., 2014; Izumi et al., 2013; Chiou et al., 2014
**TABLE 4.** *In vitro* inhibition of RIF of uptake of RSV (0.1µM) or ATV (0.01 µM) mediated by cOATP1B1, cOATP1B3, cOATP2B1, or cNTCP, and the risk for *in vivo* inhibition in cynomolgus monkeys

<table>
<thead>
<tr>
<th>Transporters</th>
<th>IC₅₀ (µM)</th>
<th>R-value for RSV</th>
<th>IC₅₀ (µM)</th>
<th>R-value for ATV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transformer</td>
<td>Probe substrate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV (0.1 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RSV (0.1 µM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cOATP1B1</td>
<td>0.25 ± 0.03</td>
<td>64.5</td>
<td>0.14 ± 0.01</td>
<td>114.3</td>
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<tr>
<td>cOATP1B3</td>
<td>1.3 ± 0.1</td>
<td>13.2</td>
<td>0.75 ± 0.08</td>
<td>22.2</td>
</tr>
<tr>
<td>cOATP2B1</td>
<td>61.7 ± 3.6</td>
<td>1.26</td>
<td>62.7 ± 10.7</td>
<td>1.25</td>
</tr>
<tr>
<td>cNTCP</td>
<td>83.5 ± 7.6</td>
<td>1.19</td>
<td>93.4 ± 10.4</td>
<td>1.17</td>
</tr>
</tbody>
</table>
Figure 2

A. Total Bilirubin (TBILI)  
B. Direct Bilirubin (DBILI)  
C. Unconjugated Bilirubin (UBILI)  
D. Glycholic Acid (GCA)  
E. Glycochenodeoxycholic Acid (GCDCA)  
F. Glycodeoxycholic Acid (GDCA)

The figure shows the changes in bilirubin levels and several bile acids over time in different groups. The graphs indicate the concentration of bilirubin and bile acids at different time points (0, 0.5, 1.0, 1.5, 2.0, etc.) and highlight the differences between the groups RIF/Vehicle, Vehicle/RSV, RIF/RSV, and Vehicle/Vehicle.
Figure 3

A. RSV Plasma Concentration (nM) over time with RIF treatment vs RSV alone.

B. RSV Lactone Plasma Concentration (nM) over time with RIF treatment vs RSV alone.

C. RSV Lactone Plasma Concentration (nM) over time with RIF treatment vs RSV alone.

D. RSV Lactone Plasma Concentration (nM) over time with RIF treatment vs RSV alone.
Figure 4

A

ATV Plasma Concentration (µM)

RIF treatment
ATV alone

Time (hr)

0 2 4 6 8

B

ATV Plasma Concentration (µM)

RIF treatment
ATV alone

Time (hr)

0 10 20 30

C

2-OH ATV Plasma Concentration (µM)

RIF treatment
ATV alone

Time (hr)

0 10 20 30

D

2-OH ATV Plasma Concentration (µM)

RIF treatment
ATV alone

Time (hr)

0 10 20 30
Figure 5

A

B

RIF Plasma Concentration (µM)

Time (hr)

0 10 20 30
Figure 6

A) [3H]E217/IG (1 µM) Uptake (pmole/10^6 cells/5 min) for HEK293-cOATP1B1 and HEK293-cOATP1B3.

B) [3H]E217/IG (1 µM) Uptake (pmole/10^6 cells/5 min) for HEK293-cNTCP and HEK293-cMRP2.

C) [3H]TCA (1 µM) Uptake (pmole/10^6 cells/5 min) for HEK293-cOATP1B1 and HEK293-cOATP1B3.

D) [14C]EA-SG (1 µM) Uptake (pmole/mg protein/5 min) for cOATP1B1 and cOATP1B3.

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Figure 7

A) cOATP1B1

B) cOATP1B3

C) cOATP2B1

D) cNTCP

E) ATP1B1

F) cOATP1B3

G) cOATP2B1

H) cNTCP

HEK293-cOATP1B1
HEK293-cOATP1B3
HEK293-cOATP2B1
HEK293-cNTCP
HEK293

[3H] RSV (0.1 µM) Uptake (pmole/10^6 cells)

[3H] ATV (0.1 µM) Uptake (pmole/10^6 cells)

Time (min)

This article has not been copyedited and formatted. The final version may differ from this version.
Supplementary Figure 1
Mean plasma concentration-time profiles of several bile acids following oral administration of RIF (18 mg/kg) (black circle), RSV (3 mg/kg) (white square), RIF (18 mg/kg) and RSV (3 mg/kg) (black triangle), and vehicle control (white triangle) in cynomolgus monkeys. Values shown are mean ± SE for n=10 animals.
Supplementary Figure 2

Mean plasma concentration-time profiles of triiodothyronine (T3) (A), free triiodothyronine (FT3) (B), thyroxine (T4) (C), and free thyroxine (FT4) (D) following oral administration of RIF (18 mg/kg) (black circle), RSV (3 mg/kg) (white square), RIF (18 mg/kg) and RSV (3 mg/kg) (black triangle), and vehicle control (white triangle) in cynomolgus monkeys. Values shown are mean ± SE for n=10 animals.
Supplementary Figure 3
Uptake of RSV lactone into cOATP1B1, cOATP1B3, cOATP2B1, and cNTCP transfected cells. Time-dependent uptake of RSV lactone (0.1 µM) into cOATP1B1 (A), cOATP1B3 (B), cOATP2B1 (C), and cNTCP (D) transfected HEK-293 cells were measured. Black bars represent uptake in transporter transfected HEK-293 cells, while bars represent uptake in control HEK-293 cells. Values shown are mean ± SE of experiments performed in triplicate.
Evaluation of cynomolgus monkeys for the identification of endogenous biomarkers for hepatic transporter inhibition and as a translatable model to predict pharmacokinetic interactions with statins in humans
Xiaoyan Chu, Shian-Jiun Shih, Rachel Shaw, Hannes Hentze, Grace H. Chan, Karen Owens, Shubing Wang, Xiaoxin Cai, Deborah Newton, Jose Castro-Perez, Gino Salituro, Jairam Palamanda, Aaron Fernandis, Choon Keow Ng, Andy Liaw, Mary J. Savage, and Raymond Evers

Drug Metabolism & Disposition

**Supplemental Table 1.**
The multiple reaction monitoring (MRM) conditions for measurement of RSV, ATV and their metabolites by LC-MS/MS

<table>
<thead>
<tr>
<th>Analyte</th>
<th>IS Used</th>
<th>Parent Ion (m/z, Da)</th>
<th>Daughter Ion (m/z, Da)</th>
<th>DP* (Volts)</th>
<th>CE** (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
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<td>281.2</td>
<td>193.1</td>
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<td>50</td>
</tr>
<tr>
<td>Diclofenac</td>
<td></td>
<td>296.2</td>
<td>215.0</td>
<td>51</td>
<td>29</td>
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<tr>
<td>Rifampin</td>
<td>Imipramine</td>
<td>823.4</td>
<td>791.7</td>
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<td>25</td>
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<td>Atorvastatin</td>
<td>Diclofenac</td>
<td>559.2</td>
<td>440.2</td>
<td>100</td>
<td>37</td>
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<td>2-OH-Atorvastatin</td>
<td>Diclofenac</td>
<td>575.2</td>
<td>440.2</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>4-OH-Atorvastatin</td>
<td>Diclofenac</td>
<td>575.21</td>
<td>440.2</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>Atorvastatin Lactone</td>
<td>Diclofenac</td>
<td>541.2</td>
<td>448.2</td>
<td>100</td>
<td>25</td>
</tr>
<tr>
<td>2-OH-Atorvastatin Lactone</td>
<td>Diclofenac</td>
<td>556.2</td>
<td>448.2</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td>4-OH-Atorvastatin Lactone</td>
<td>Diclofenac</td>
<td>556.21</td>
<td>448.2</td>
<td>100</td>
<td>21</td>
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<tr>
<td>Rosuvastatin</td>
<td>Imipramine</td>
<td>482.2</td>
<td>258.1</td>
<td>100</td>
<td>50</td>
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<tr>
<td>Rosuvastatin Lactone</td>
<td>Imipramine</td>
<td>464.2</td>
<td>258.1</td>
<td>100</td>
<td>50</td>
</tr>
</tbody>
</table>

*Declustering Potential

**Collision Energy