TITLE

Inhibition of intestinal OATP2B1 by the calcium receptor antagonist ronacaleret results in a significant drug-drug interaction by causing a two-fold decrease in exposure of rosuvastatin

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Abbreviations: AUC, area under the curve; BCRP, human breast cancer resistance protein; Cmax, maximal plasma concentration; DDI, drug-drug interaction; MRP, multidrug resistance protein; NTCP, sodium-taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein; Tmax, time to maximal plasma concentrations; t1/2, apparent half-life
ABSTRACT

Rosuvastatin is a widely prescribed anti-hyperlipidemic which undergoes limited metabolism, but is an in vitro substrate of multiple transporters (OATP1B1, OATP1B3, OATP1A2, OATP2B1, NTCP, BCRP, MRP2, MRP4, OAT3). It is therefore frequently used as probe substrate in clinical drug-drug interaction (DDI) studies to investigate transporter inhibition. While each of these transporters is believed to play a role in rosuvastatin disposition, multiple pharmacogenetic studies confirm that OATP1B1 and BCRP play an important role in vivo.

Ronacaleret, a drug development candidate for treatment of osteoporosis (now terminated), was shown to inhibit OATP1B1 in vitro (IC$_{50}$ 11 µM), while it did not inhibit BCRP. Since a DDI risk through inhibition of OATP1B1 could not be discharged, a clinical DDI study was performed with rosuvastatin before initiation of Phase II trials. Unexpectedly, co-administration with ronacaleret decreased rosuvastatin exposure by approximately 50%, while time of maximal plasma concentration and terminal half-life remained unchanged, suggestive of decreased absorption and/or enhanced first pass elimination of rosuvastatin. Of the potential in vivo rosuvastatin transporter pathways, two might explain the observed results: intestinal OATP2B1 and hepatic MRP4. Further investigations revealed that ronacaleret inhibited OATP2B1 (in vitro IC$_{50}$ 12 µM) indicating a DDI risk through inhibition of absorption. Ronacaleret did not inhibit MRP4, discharging the possibility of enhanced first pass elimination of rosuvastatin (reduced basolateral secretion from hepatocytes into blood). Therefore, a likely mechanism of the observed DDI is inhibition of intestinal OATP2B1, demonstrating the in vivo importance of this transporter in rosuvastatin absorption in human.
INTRODUCTION

Statins are potent inhibitors of HMG-CoA reductase and widely prescribed for the treatment of hypercholesterolemia (Cholesterol Treatment Trialists et al., 2010). Statin use is generally safe and well tolerated, although adverse events associated with myopathy have been reported, which can range from mild muscle pain to, in rare cases, fatal rhabdomyolysis (Maghsoodi and Wierzbicki, 2016). The risk of developing statin-related myopathy is increased by factors that increase statin plasma and muscle concentrations, such as dose and decreased function of transporters which mediate statin disposition. Pharmacogenomic studies have identified common variants in OATP1B1 and BCRP with reduced functional activity that are associated with increased plasma exposure of several of the statins and increased risk of statin-induced myopathy (Generaux et al., 2011; Elsby et al., 2012; Talameh and Kitzmiller, 2014). Co-administered drugs which are inhibitors of OATP1B1 and BCRP can have this effect as well (Elsby et al., 2012; Eng et al., 2016).

Statins are among the most commonly prescribed drugs in middle-aged and elderly populations; consequently the risk of drug-drug interactions with statins often needs to be addressed for drugs in development, e.g., to support co-med guidance for late phase clinical trials and to inform the prescribing practice post regulatory approval. In regulatory DDI guidances, rosuvastatin, pravastatin and pitavastatin have been recommended as clinical probe substrates to assess interactions with OATP1B1 inhibitors, since these statins are known to be sensitive to OATP1B1 modulation and they exhibit minimal metabolism by cytochrome P450 enzymes, reducing the risk of confounding metabolism interactions. Among these three statins, rosuvastatin has been used most frequently as probe substrate, likely due to the fact that it is also widely prescribed.
In vitro, rosuvastatin is a substrate of multiple uptake and efflux transporters: OATP1B1, OATP1B3, NTCP, OATP2B1, OATP1A2, BCRP, MRP2, MRP4 and OAT3 (Ho et al., 2006; Windass et al., 2007; Kitamura et al., 2008; Varma et al., 2011; Pfeifer et al., 2013b; Shen et al., 2013; Liu et al., 2015). While pharmacogenetic data is available to demonstrate the clinical relevance of hepatic OATP1B1 and intestinal BCRP for rosuvastatin, the clinical relevance of other transporters is less well established and relies mainly on demonstration of the compound as an in vitro substrate, in combination with in vitro inhibition and in vivo pharmacokinetic data. After intravenous administration, 8 mg over 4 hours to healthy male volunteers, 72% of rosuvastatin is eliminated via hepatic clearance and 28% via renal clearance (Elsby et al., 2012). Uptake into hepatocytes is mediated mainly via OATP1B1 and OATP1B3 expressed on the basolateral membrane, although NTCP may play a limited role as well (Ho et al., 2006). Biliary excretion likely involves apically expressed BCRP and MRP2, whereas MRP4 has been proposed to mediate basolateral secretion from hepatocyte into blood (Pfeifer et al., 2013a; Pfeifer et al., 2013b). In the kidney, active tubular secretion is responsible for > 90% of renal clearance and is believed to be primarily mediated by the basolateral proximal tubule uptake transporter OAT3, since rosuvastatin is not an in vitro substrate of OAT1 (Windass et al., 2007). Efflux from the proximal tubule cells likely involves BCRP, MRP2 and MRP4.

It is well documented that intestinal BCRP plays an important role in limiting rosuvastatin absorption. However, little information is available on the role of intestinal OATPs in the absorption of statins, including rosuvastatin. Grapefruit juice and naringin had a differential effect on absorption of pravastatin and pitavastatin in rats. Grapefruit juice and naringin decreased the absorption of pravastatin while absorption of pitavastatin was increased. Both compounds are substrates of rat intestinal Oatp1b5 and Oatp1b2, whereas pitavastatin, but not
pravastatin, is also a substrate of rat P-gp. It was concluded that grapefruit juice and naringin decreased the absorption of pravastatin through inhibition of intestinal Oatps, whereas absorption of pitavastatin was increased through a dominant effect of P-gp inhibition compared to Oatps (Shirasaka et al., 2011a).

This work describes the results of a drug-drug interaction study carried out to investigate the effect of a drug development candidate, ronacaleret, on the pharmacokinetics of rosuvastatin. Ronacaleret inhibited OATP1B1-mediated uptake of the probe substrate estradiol-17β-D-glucuronide with an IC₅₀ value of 11 µM in vitro, indicating a DDI risk through inhibition of OATP1B1, which would be expected to increase rosuvastatin exposure. Surprisingly, the plasma exposure of rosuvastatin was decreased by 50% upon co-administration with ronacaleret, while the time of maximal plasma concentration (Tmax) and the terminal half-life (t_{1/2}) were unchanged. This PK profile is suggestive of decreased rosuvastatin absorption and/or increased first pass elimination. Since rosuvastatin is an in vitro substrate of OATP2B1 and BCRP, preferential inhibition of intestinal OATP2B1 over BCRP could result in decreased absorption.

Rosuvastatin is also an in vitro substrate of MRP4, which has been shown to mediate basolateral secretion from sandwich-cultured hepatocytes. Hence, inhibition of MRP4 could also result in decreased rosuvastatin exposure. Given the unexpected nature of the clinical drug interaction (i.e., a reduction rather than an increase in systemic exposure, as would have been expected from an OATP1B1 or OATP1B3 dominant mechanism), further in vitro studies were carried out to investigate the underlying mechanism of the interaction between ronacaleret and rosuvastatin. The results of such investigations suggest an important role for OATP2B1 in rosuvastatin disposition in humans.
MATERIALS AND METHODS

Chemicals and Reagents. The human embryonic kidney cells (HEK-MSRII) and human OATP1B3, OATP1A2 and OATP2B1 BacMam baculovirus transduction reagents were supplied by the Biological Sciences group (BSci), GlaxoSmithKline (Collegeville, PA, USA); human embryonic kidney 293 cells (HEK293) transiently over-expressing OATP1B1*1A were obtained from Corning® (Corning, NY, USA); Chinese Hamster Ovary cell line heterologously expressing human OATP1B1 was obtained from University of Zurich); ABC transporter vesicle reagent set and membrane vesicles expressing human MRP3 were purchased from GenoMembrane (Tsurumi, Yokohama, Japan); membrane vesicles expressing human BCRP and MRP4, and Ko134 were obtained from SOLVO Biotechnology (Budaors, Hungary); MK-571 sodium salt hydrate, β-Estradiol 17-(β-D-glucuronide) sodium salt, sodium butyrate, rifamycin SV, cyclosporin A, and ketoconazole were purchased from Sigma-Aldrich (St. Louis, MO); Dulbecco’s modified Eagle’s medium (DMEM)/F12, fetal bovine serum (FBS), Dulbecco’s phosphate buffered saline (DPBS) and geneticin were purchased from Life Technologies (Grand Island, NY); Hank’s Balanced Salt Solution with Ca²⁺ and Mg²⁺ (HBSS), HEPES and MEM non-essential amino acid solution were purchased from Corning Life Sciences (Bedford, MA); montelukast was obtained from Cayman Chemical Company (Ann Arbor, MI); rosuvastatin calcium was purchased from Sequoia Research Products (Pangbourne, Berkshire, UK); MicroScint™-PS was purchased from Perkin Elmer (Waltham, MA); [³H]estradiol-17β-D-glucuronide and [³H]estrone sulfate were purchased from Perkin Elmer Life and Analytical Sciences (Boston, MA); [³H]rosuvastatin calcium (Specific Activity of 10 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO).
Clinical Study Design. This study was an open-label, two-period, fixed sequence, crossover study performed in healthy, non-smoking, postmenopausal female subjects (42-67 years old, mean 55 years, body weight 56-90 kg, mean 70 kg, N=24). There are no known gender related differences in plasma concentrations of rosuvastatin (Martin et al., 2002). In session 1, subjects received 10 mg rosuvastatin alone, followed by a washout period of at least 5 days. In session 2, subjects were administered 400 mg ronacaleret (4 x 100 mg tablets as free-base, IR) once daily for 10 days, structure shown in Figure 1. To evaluate short-term drug interaction effects, subjects were administered 10 mg of rosuvastatin together with ronacaleret on day 2. To evaluate chronic drug interaction effects, subjects were administered 10 mg rosuvastatin together with ronacaleret on day 8. Blood samples for pharmacokinetic analysis were collected at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, 30-36 (1 sample), 48, and 72 hours post-dose. Plasma rosuvastatin concentrations were determined using high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS). The lower limit of quantification (LLQ) was 0.2 ng/mL, using a 100 µL aliquot of human EDTA plasma with a higher limit of quantification (HLQ) of 30 ng/mL. The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents.

Pharmacokinetic Analysis. The pharmacokinetic analysis of concentration-time data for rosuvastatin was conducted using noncompartmental Model 200 of WinNonlin Professional Edition version 4.1.b (Pharsight Corporation, Mountain View, CA). The actual elapsed time from dosing was used to estimate all individual plasma PK parameters for evaluable subjects. When estimating the mean values for the concentration at a given time point, all not quantifiable (NQ) values were set to zero except when an individual NQ fell between two quantifiable values, in which case it was omitted.
Cell Preparation and Culture Conditions. HEK-OATP1B1*1A cells were thawed and resuspended in DMEM media supplemented with 10% fetal bovine serum and MEM non-essential amino acid solution. Cells were seeded at a density of 150,000 cells/well on poly-D-lysine-coated 48-well plates and incubated at 37°C, 5% CO₂ but no humidity for 4 h. Media was aspirated and cells were re-fed DMEM media supplemented with 5 mM sodium butyrate. Cells were incubated overnight at 37°C with 5% CO₂ but no humidity prior to use in inhibition experiments.

HEK-MSRII cells were transduced in DMEM Ham’s F-12 media containing OATP-BacMam virus (OATP1B3, OATP1A2 and OATP2B1), 10% fetal bovine serum, 0.4 mg/mL geneticin and 2 mM sodium butyrate. Transduced cells were seeded at a density of 200,000 cells/well on poly-D-lysine-coated 48-well plates and incubated at 37°C, 5% CO₂, and 95% humidity. At 48 h after plating, inhibition experiments were conducted.

CHO-OATP1B1 cells were seeded at a density of approximately 130,000 cells/well on polystyrene 24-well plates with DMEM media supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin (10,000 units/mL), 0.1% L-proline (50 mg/mL) and 0.7% geneticin (50 mg/mL). The cell monolayers were incubated for 48 h at 37°C, 5% CO₂, and 95% humidity. Transporter expression was then induced by addition of DMEM media containing sodium butyrate (final concentration 5 mM) for 24 hours prior to inhibition experiments.

Inhibition of OATP-Mediated Rosuvastatin Transport by Ronacaleret. Transduced cells were preincubated at 37°C for 15 to 30 min in 400 µL HBSS supplemented with 10 mM HEPES (OATP1B1 only) or DPBS containing ronacaleret at concentrations of 0, 0.03, 0.1, 0.3, 1, 3, 10, 30, 50 and 100 µM for OATP1B1 and OATP2B1; 0, 0.1, 0.3, 1, 3, 5, 10, 30, 50, and 100 µM for
OATP1B3; and 0, 0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 1, 3, 5, 10, 30, 50, and 100 µM for OATP1A2. A positive control inhibitor was included with each experiment with the following results: 30 µM rifamycin SV inhibited OATP1B1 and OATP1B3 by 82% and >95%, respectively; 10 µM ketoconazole inhibited OATP1A2 by 89% and 10 µM montelukast inhibited OATP2B1 transport by 71% and 43% at pH 7.4 and pH 6.0, respectively. The lesser inhibition at pH 6.0 is ascribed to the greater passive permeability of rosuvastatin at this pH (Varma et al., 2011). Following removal of preincubation solution, 400 µL of HBSS supplemented with 10 mM HEPES (OATP1B1 only) or DPBS containing ronacaleret at the above listed target concentrations and 1 µM (OATP1B1, OATP1B3, and OATP2B1) or 5 µM (OATP1A2) rosuvastatin were added to the wells in triplicate. Rosuvastatin substrate concentrations for these transporters in the present study were lower than the published Km values (van de Steeg et al., 2013; Bosgra et al., 2014; Lu et al., 2015). Cells were incubated at 37°C for 3 min for OATP1B1, OATP1B3 and OATP1A2 and for 10 min for OATP2B1. These incubation times were chosen based on preliminary experiments to determine the period during which uptake increased linearly with time (data not shown). The reaction was stopped with addition of ice-cold HBSS supplemented with 10 mM HEPES (OATP1B1 only) or DPBS prior to solubilization with water and subsequent analysis via LC-MS/MS. The OATP2B1 inhibition assay was conducted at two different pH values, pH 6.0 and pH 7.4.

**Inhibition of OATP-Mediated Transport of Prototypical Substrates by Ronacaleret.** Cell monolayers were preincubated in triplicate at 37°C for 15 to 30 min in 400 µL DPBS containing ronacaleret at concentrations of 0, 0.1, 1, 3, 10, 30 and 100 µM or positive control inhibitors, 10 µM rifamycin (OATP1B1, OATP1B3) 10 µM montelukast (OATP2B1) or 10 µM ketoconazole (OATP1A2). Following removal of preincubation solution, 400 µL of DPBS
containing positive control inhibitors or ronacaleret at the above listed target concentrations and
0.02 µM [³H]estradiol-17β-D-glucuronide ([³H]EG) (probe substrate for OATP1B1 and
OATP1B3) or 0.02 µM [³H]estrone sulfate ([³H]ES) (probe substrate for OATP1A2 and
OATP2B1) were added to the wells. Cells were incubated at 37°C for 5 min for OATP1B1,
10 min for OATP1B3, and 0.5 min for OATP1A2 and OATP2B1. These incubation times were
chosen based on prior experiments conducted in-house to determine the period during which
uptake increased linearly with time (data not shown). The reaction was stopped with addition of
ice-cold DPBS prior to lysis with 0.1% Triton-X and subsequent analysis via liquid scintillation
counting. The results for the positive control inhibitors were as follows: 10 µM rifamycin SV
inhibited OATP1B1 and OATP1B3 by 97 and 96%, respectively; 10 µM ketoconazole inhibited
OATP1A2 by 89% and 10 µM montelukast inhibited OATP2B1 transport by 88%.

BCRP and MRP4 Inhibition Assays. Human BCRP or MRP4 vesicles were rapidly thawed at
37°C and added to a reaction buffer consisting of 50 mM MOPS-Tris, 70 mM KCl and 7.5 mM
MgCl₂ for a final vesicle concentration of 1 mg protein/mL. 14.5 µL aliquots were preincubated
at 37°C for 10 minutes in the presence and absence of ronacaleret or positive control inhibitors,
Ko134 (BCRP) or MK571 (MRP4). The final concentration range of ronacaleret in the
incubations was 0, 0.01, 0.3, 1, 10, 30 and 100 µM. Following pre-incubation, reactions were
initiated by the addition of 10 mM MgATP solution containing 1 µM of the probe substrate
[³H]Rosuvastatin and 200 mM glutathione. Rosuvastatin concentration in the BCRP and MRP4
vesicle assays was lower than published Km values (Huang et al., 2006; Pfeifer et al., 2013a).
Additional incubations were performed in the absence of inhibitor and in the presence of 10 mM
MgAMP solution containing 1 µM [³H]Rosuvastatin and 200 mM glutathione solution to
determine passive uptake. After a 2 minute incubation, reactions were terminated by the addition
of chilled stopping buffer (40 mM MOPS-Tris, 70 mM KCl). Incubation mixtures were
transferred to a 96-well glass fiber plate and incubation media was removed by vacuum
suctioning. The filter plate was washed 3 times with stopping buffer and dried in a 50ºC oven
for 30 min. After the addition of 100 µL MicroScint™-PS, samples were analyzed using
microplate scintillation. Positive control inhibitors, 1 µM Ko143 and 10 µM MK571, inhibited
rosuvastatin uptake by 100% and 96%, respectively.

**MRP3 substrate assay.** Transport of rosuvastatin by MRP3 was investigated using human
MRP3 vesicles. Vesicles were rapidly thawed at 37ºC and added to a reaction buffer consisting
of 50 mM MOPS-Tris, 70 mM KCl and 7.5 mM MgCl₂ for a final vesicle concentration of 1 mg
protein/mL. 14.5 µL aliquots were preincubated at 37ºC for 10 minutes in the presence and
absence of MRP3 inhibitor, 300 µM MK571. Following pre-incubation, reactions were initiated
by the addition of 10 mM MgATP solution containing 1 µM [³H]Rosuvastatin, 200 mM
glutathione and where necessary, inhibitor. After 0.5, 1, 1.5, 2, 5 and 10 min incubations,
reactions were terminated by the addition of chilled stopping buffer (40 mM MOPS-Tris, 70 mM
KCl). Incubation mixtures were transferred to a 96-well glass fiber plate and incubation media
was removed by vacuum suctioning. The filter plate was washed 3 times with stopping buffer
and dried in a 50ºC oven for 30 min. After the addition of 100 µL MicroScint™-PS, samples
were analyzed using microplate scintillation. Rosuvastatin was not a substrate of MRP3,
consistent with previous literature ((Pfeifer et al., 2013a))

**BCRP, MRP3 and MRP4 inhibition assays using prototypical substrate.** Ronacaleret, at
concentrations of 0, 0.01, 0.3, 1, 10, 30 and 100 µM (BCRP) and 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10,
30, 50, and 100 µM (MRP3 and MRP4), was incubated using the methods described above with
human BCRP, MRP3 or MRP4 vesicles and prototypical substrates 1 µM [³H]ES (BCRP) or
\[^3\text{H}\]EG (MRP3 or MRP4). The incubation times with probe substrate were 1 min for the BCRP vesicles and 5 min for MRP3 or MRP4 vesicles. These incubation times were chosen based on preliminary experiments to determine the period during which uptake increased linearly with time (data not shown). Positive control inhibitors, 3 µM Ko134, 100 µM MK571 and 30 µM MK571, inhibited BCRP transport by 96%, MRP3 transport by 92% and MRP4 transport by 83%, respectively.

**Sample Preparation and Analysis.** Samples from the cellular assays were prepared for bioanalysis by incubating the cell monolayers with 200 µL of water overnight at -20ºC to allow for cell lysis. After overnight storage, the entire contents of each well were collected and the concentration of rosuvastatin in the cell lysate was determined by ultra high-performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). Cell lysates containing rosuvastatin and the analytical internal standard (stable isotopically labeled rosuvastatin (25 ng/mL \[^2\text{H}_7\text{N}_2\]GSK977810) in 50:50 acetonitrile:water) were extracted by liquid-liquid extraction with tert-butyl methyl ether, followed by LC-MS/MS analysis.

The LC-MS/MS system consisted of an ACQUITY™ UPLC integrated system from Waters (Milford, MA, USA) and a triple quadruple mass spectrometer API 4000 (Applied Biosystems/MDS-Sciex, Concord, Ontario, Canada). Chromatographic separation was achieved using a gradient of 0.1% formic acid in water: acetonitrile on an Acquity BEH C18, 2.1 mm x 50 mm, 1.7 µm particle size column from Waters. An API4000 with Turbolonspray (TIS) was operated in the positive ionization mode, with the MRM transition of m/z 482-258 for rosuvastatin and 491-267 for its internal standard analysis at a mobile phase flow rate of 0.8 mL/min.
Samples from the vesicular assays were prepared for radiodetection by drying the filter plate in a 50°C oven for 30 min after stopping the reactions. 100 µL of Microscint (Perkin Elmer) was added to each well and total radioactivity was measured using a Perkin Elmer TriCarb liquid scintillation counter.

**Data Analysis.** IC₅₀ values were determined using the following equation:

\[
y = \frac{Range}{1 + \left( \frac{x}{IC_{50}} \right)} + \text{Background}
\]

(Eq. 1)

Where \(Y\) is the transport or uptake rate as a percentage of the uninhibited control, \(Range\) is the maximum rate of transport (100%) in the absence of inhibitor, Background is the uninhibitable transport rate as percentage of total rate, \(x\) is the concentration of inhibitor (µM) and \(s\) is the slope factor.
RESULTS

In a standard in vitro OATP inhibition screen, ronacaleret was shown to inhibit the OATP1B1 and OATP1B3 mediated uptake of the probe substrate estradiol-glucuronide with relatively low potency: IC$_{50}$ values of 11 and 60 µM respectively. At an anticipated clinical dose of 400 mg ronacaleret, with a plasma Cmax of approximately 3 µM (unbound plasma concentration of approximately 0.18 µM), a DDI resulting from inhibition of OATP1B1 could not be excluded. Therefore a DDI study was conducted to evaluate the effect of concomitant administration of ronacaleret on rosuvastatin (an OATP1B1 probe substrate) disposition in humans.

Clinical study results. Plasma concentration-time profiles (mean ± SD) of 10 mg rosuvastatin dosed orally, in the absence and presence of ronacaleret are shown in Figure 2. The corresponding pharmacokinetic parameters are listed in Table 1. Rosuvastatin AUC(0-∞) decreased an average of 48 to 50% and the maximum plasma concentration (C$_{max}$) decreased an average of 33 to 34% in the presence of both single and multiple doses of ronacaleret compared to rosuvastatin alone. The Tmax and t$_{1/2}$ remained unchanged across treatments. This result was unexpected and not consistent with inhibition of hepatic uptake transporters (e.g., OATP1B1), but rather with ronacaleret decreasing intestinal absorption and/or enhancing first pass hepatic elimination of rosuvastatin.

In vitro transporter inhibition studies. Ronacaleret was therefore investigated as a potential inhibitor of transporters which may mediate rosuvastatin absorption (OATP1A2 and OATP2B1) from the intestine or mediate basolateral secretion from hepatocytes into the blood (MRP4). Since MRP3 is also expressed on the basolateral membrane of hepatocytes and has been shown to be involved in basolateral secretion of fexofenadine in mice (Tian et al., 2008), we also
investigated inhibition of this transporter. MRP3 did not mediate uptake of rosuvastatin; furthermore, ronacaleret did not inhibit EG uptake mediated by MRP3. BCRP inhibition by ronacaleret was investigated as well. The in vitro inhibitory potency of ronacaleret is summarized in Table 2 (see also Supplemental Table 1). Ronacaleret inhibited OATP2B1-mediated rosuvastatin transport at pH 7.4 and pH 6.0 with IC$_{50}$ values of 16 µM and 12 µM, respectively. OATP1A2 was inhibited with an IC$_{50}$ of 1.8 µM (Supplemental Figure 3). Ronacaleret did not significantly inhibit BCRP or MRP4. Furthermore, ronacaleret did not inhibit P-gp-mediated transport of digoxin in human P-gp overexpressing MDCKII cells, data not shown. The inhibition profiles for OATP2B1 are shown in Figure 3. Rosuvastatin passive permeability is pH dependent, with greater permeability at lower pH (Varma et al., 2011). Consistent with the pH dependent passive permeability, a larger fraction of the permeability was inhibited at pH 7.4 compared to pH 6.0. The positive control inhibitor montelukast at 10 µM showed 72% inhibition of rosuvastatin uptake at pH 7.4, while the fitted maximal inhibition by ronacaleret was 71%. At pH 6.0 montelukast showed 43% inhibition of rosuvastatin uptake, while the fitted maximal inhibition by ronacaleret was 39%.

Ronacaleret-mediated inhibition of prototypical probe substrates was also investigated (Table 2) (see also Supplemental Table 2 and Supplemental Figures 4 through 7). Only in the case of OATP2B1 at pH 7.4 was there a substantial difference in IC$_{50}$ values between prototypical probe substrate and rosuvastatin. The reason for the less potent inhibition of [³H]ES transport via OATP2B1 by ronacaleret is not clear.
DISCUSSION

Ronacaleret is believed to be moderately well absorbed following oral dosing. It is excreted mostly as parent compound in the feces (63-66% of administered dose) and in urine (4% of administered dose). Ronacalaret is also the major circulating component in plasma (68-78% of plasma radioactivity). A notable metabolite accounted for 6-8% of plasma radioactivity (data on file) with the remaining radioactivity attributed to multiple minor circulating metabolites; therefore, the metabolites were not synthesized and not analyzed for their effect on transporter function. Even though ronacaleret is an organic acid, it was shown to not be a substrate of human OATP1B1 or OATP1B3. Furthermore, uptake in human hepatocytes was not affected by an inhibitor cocktail consisting of rifamycin SV, cyclosporine A, montelukast and quinidine (at 10µM each), and therefore it was concluded that hepatic uptake in human was most likely via a passive mechanism. Ronacaleret has moderate passive permeability across MDCKII cells in vitro (30 nm/s).

This study describes an unanticipated DDI between rosuvastatin and ronacaleret. Ronacaleret was initially identified as an in vitro inhibitor of OATP1B1 and OATP1B3 (Supplemental Figures 1 and 2, respectively). In the DDI study, the exposure of the OATP1B1 substrate rosuvastatin was decreased rather than unchanged or increased. Rosuvastatin AUC(0-∞) and Cmax decreased by about 50% and 34%, respectively in the presence of both single and multiple doses of ronacaleret compared to rosuvastatin alone. This could conceivably reduce the therapeutic effect of rosuvastatin, necessitating clinical monitoring of lipid levels and potentially a rosuvastatin dose adjustment or a recommendation of dose separation between ronacaleret and rosuvastatin, i.e., morning dosing of ronacaleret and evening dosing of rosuvastatin.
Since the t$_{1/2}$ was unchanged across treatments the observed decrease in AUC and Cmax is consistent with a decreased absorption or enhanced first pass elimination. The Tmax was also unchanged, indicating rosuvastatin intestinal transit time was not affected. In order to explain the mechanism of this DDI, we investigated inhibition by ronacaleret of transporters which, upon inhibition, might result in decreased absorption and/or enhanced first pass elimination of rosuvastatin.

The pharmacokinetic properties of rosuvastatin are well described (Elsby et al., 2012). Rosuvastatin is dosed orally at 5-40 mg/day. Intestinal absorption of rosuvastatin amounts to ~50%. The in vitro passive membrane permeability of rosuvastatin is low (4-8x10$^{-6}$ cm/sec) to low/moderate at neutral pH (~ 5x10$^{-6}$ cm/sec), but increases to moderate/high (~ 13x10$^{-6}$ cm/sec) at pH 6.0 (Huang et al., 2006; Generaux et al., 2011; Varma et al., 2011). Rosuvastatin is an in vitro substrate of the intestinal efflux transporter BCRP. The c.421C>A single nucleotide polymorphism (SNP) in BCRP results in reduced activity (Furukawa et al., 2009; Ripperger and Benndorf, 2016) and pharmacogenetic studies have shown that in individuals carrying the AA haplotype, rosuvastatin exposure (AUC and Cmax) is up to ~2.4 fold greater than in individuals carrying the CC haplotype (Furukawa et al., 2009; Ripperger and Benndorf, 2016). This data demonstrates the important role of BCRP in limiting the absorption of rosuvastatin.

OATP2B1 is expressed in intestine, liver, placenta, heart and skeletal muscle (Kalliokoski and Niemi, 2009; Knauer et al., 2010). OATP2B1 is believed to be the main OATP expressed in the intestine, while conflicting data exists on expression levels of OATP1A2 (Tamai, 2012; Drozdzik et al., 2014). OATP2B1 is able to transport substrates at both neutral and intestinal pH (6.0). Several authors have proposed a role for intestinal OATPs, specifically OATP2B1, in the absorption of hydrophilic drugs, such as rosuvastatin and pravastatin, as well as fexofenadine.
and talinolol (Nozawa et al., 2004; Shirasaka et al., 2011a; Varma et al., 2011). This notion is supported by studies performed in animals and humans. The decrease in pravastatin absorption observed in rats on co-administration with grapefruit juice and naringin was ascribed to inhibition of intestinal Oatp-mediated transport of pravastatin (Shirasaka et al., 2011a). Similar results have been observed in rats for fexofenadine (Kamath et al., 2005) and talinolol (Shirasaka et al., 2009; Shirasaka et al., 2010). Oral bioavailability of fexofenadine in humans was decreased by grapefruit juice and naringin, which was at the time ascribed to preferential inhibition of OATP1A2 or OATP2B1 mediated uptake of fexofenadine into enterocytes versus P-gp mediated efflux from these cells (Bailey et al., 2007). However, given the recent data on expression levels of OATP2B1 and OATP1A2 in the intestine (Drozdzik et al., 2014), the effect of grapefruit juice and naringin on fexofenadine absorption in humans may be mainly mediated by inhibition of OATP2B1. Orange and apple juice greatly reduce the plasma concentrations of aliskiren, which was ascribed to inhibition of intestinal OATP2B1 (Tapaninen et al., 2011). The decrease in exposure of a therapeutic dose of celiprolol by grapefruit juice was also ascribed to OATP2B1 inhibition (Ieiri et al., 2012). The oral pharmacokinetics of fexofenadine and celiprolol showed a gene dose dependent decrease in individuals carrying the OATP2B1 c.1457C>T reduced function variant allele (Imanaga et al., 2011; Ieiri et al., 2012). A similar effect was reported for montelukast for the c.935G>A reduced function variant (Mougey et al., 2009; Mougey et al., 2011), although the role of OATP2B1 in montelukast absorption has recently been challenged (Chu et al., 2012; Brannstrom et al., 2015). Overall, evidence is accumulating for a role of intestinal OATP2B1 in the absorption of some drugs, including statins, although to the best of our knowledge, this has not yet been described for rosuvastatin.
A potential transport mechanism for rosuvastatin across the basolateral membrane of the enterocyte has not yet been established. MRP4 plays an important role in the basolateral efflux of adefovir generated from its prodrug adevofir dipivoxil in Caco-2 cells (Ming and Thakker, 2010). However it has been shown recently that MRP4 is not expressed in human intestinal tissues (Drozdzik et al., 2014). It is possible that, due to the OATP2B1-mediated uptake, there is enough of a concentration gradient between enterocyte and blood such that transport across the basolateral membrane could occur via passive diffusion.

Hepatic clearance is responsible for 72% of the clearance of an intravenous dose of rosuvastatin. About 10% of a rosuvastatin dose is metabolized by CYP2C9 to the N-desmethyl metabolite, the remainder is eliminated as parent compound. Pharmacogenetic studies have demonstrated the importance of OATP1B1 in rosuvastatin hepatic uptake. Homozygous carriers of the reduced function c.521T>C SNP exhibit a 1.7 fold increase in rosuvastatin exposure. OATP1B3 and NTCP are also believed to play a role. Whether OATP2B1 contributes significantly to hepatic uptake of statins is currently not clear. Following hepatocyte uptake, rosuvastatin is likely effluxed by BCRP and MRP2 across the cannalicular membrane resulting in biliary secretion, or by MRP4 across the basoateral membrane and thus may be subject to a repeated cycle of basolateral uptake and efflux, referred to as “hepatocyte hopping” (Pfeifer et al., 2013a; Pfeifer et al., 2013b). Inhibition of hepatic MRP4 could result in a greater fraction of rosuvastatin eliminated directly in the bile and could thus lead to a decrease in rosuvastatin exposure.

Ronacaleret was an inhibitor of all of the OATPs investigated: OATP1B1, OATP1B3, OATP2B1 and OATP1A2. The EMA guidance criteria (EMA, 2012) for inhibition of OATP1B1, but not OATP1B3, were exceeded slightly, while the FDA criteria (Shirasaka et al., 2011b; FDA, 2012) were not, indicating an overall low risk for inhibition of OATP1B1 in vivo.
(Table 3). The I₂/IC₅₀ value for inhibition of OATP2B1 significantly exceeded the decision criteria, indicating a risk for inhibition of intestinal uptake of rosuvastatin and hence clinically significant DDI. Risk for OATP1A2 inhibition was not assessed since this transporter is not believed to play a significant role in the intestine. However, since ronacaleret is a more potent in vitro inhibitor of OATP1A2 than of OATP2B1, it is expected that if there is any uptake of rosuvastatin into enterocytes mediated by OATP1A2 this would also be inhibited by ronacaleret. While there was some inhibition of BCRP at 100 µM ronacaleret, an IC₅₀ could not be determined. A conservative assumption of an IC₅₀ of 300 µM would result in an I₂/IC₅₀ value for inhibition of BCRP just above the decision criteria. The maximum solubility of ronacaleret at pH 6 is 41.7 µM, well above the IC₅₀ for inhibition of OATP2B1. Taken together, the in vitro inhibition data is consistent with inhibition of intestinal OATP2B1.

There are only a small number of DDI studies published that report a decrease in rosuvastatin exposure > 20%. Erythromycin causes a decrease in rosuvastatin AUC₀-₄ and Cmax of 20% and 31% respectively (Cooper et al., 2003). The authors speculated that this might be due to an effect of erythromycin on intestinal motility. Erythromycin is not an inhibitor of OATP2B1 (Lan et al., 2009). Baicalin reduced rosuvastatin AUC by 42% and increased rosuvastatin clearance/F by 1.7 fold, which was ascribed to induction of OATP1B1. The anticonvulsant eslicarbazepine, which was approved in the US in 2013, decreased rosuvastatin exposure by 35% and increased clearance by 57%, an effect which was ascribed to possible induction of OATP1B1 and OATP1B3 (University of Washington DDI database).

Mechanistic interpretation of transporter DDI studies is often challenging due to the lack of specific probe substrates and inhibitors, as well as the fact that, for compounds which are not metabolized to a significant extent, absorption and elimination of parent compound frequently
involve uptake as well as efflux transporters working in opposite directions, such that if both are inhibited, the effect may cancel out. Given the multiple uptake and efflux transporters involved in the ADME of statins, mechanistic interpretation of a victim DDI result requires in vitro inhibition data on all transporters involved in disposition of the probe substrate.

Rosuvastatin is recommended as one of the clinical probe substrates to investigate inhibition of OATP1B1. It has been demonstrated recently that pitavastatin is a more sensitive and selective probe substrate for OATP1B1 than rosuvastatin (Prueksaritanont et al., 2014). Homozygous carriers of the reduced function c.521T>C SNP exhibit a 3 fold increase in pitavastatin vs a 1.7 fold increase in rosuvastatin exposure. Consistent with the pharmacogenetic data, pitavastatin exhibited a greater increase in exposure (7.6 fold) than rosuvastatin (3.3 fold) upon inhibition of hepatic OATP by co-administered intravenous rifampicin. Furthermore, oral rifampicin resulted in a greater increase than intravenous rifampicin in rosuvastatin, but not pitavastatin exposure, which was ascribed to inhibition of rosuvastatin intestinal efflux transporters (BCRP, Pgp and MRP2) and appears consistent with pharmacogenetic studies supporting a role for BCRP in limiting rosuvastatin, but not pitavastatin absorption. Since pitavastatin is also an in vitro substrate of OATP2B1 (Hirano et al., 2006; Shirasaka et al., 2011b), and thus not completely selective for OATP1B1 (or hepatic OATPs), a mechanistic interpretation of a pitavastatin DDI study with an oral perpetrator drug also requires knowledge of the in vitro OATP2B1 inhibition potential of the perpetrator drug.

In conclusion, ronacaleret causes a 50% decrease in exposure of rosuvastatin, without affecting the t1/2. A likely explanation of this observation is preferential inhibition of intestinal OATP2B1 over intestinal efflux transporters. Since pravastatin and pitavastatin are also OATP2B1 substrates, mechanistic interpretation of victim DDI study results using these drugs should
include in vitro inhibition data on OATP2B1. The current study highlights the need to carefully evaluate the interpretation of DDI studies with statins (and other drugs) as transporter probe substrates and also makes a case for the addition of OATP2B1 to be added to the list of clinically relevant transporters when assessing the DDI potential of a drug candidate during drug development.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: Johnson, Matheny, Patel, Ho, Chen and Ellens

Conducted experiments: Johnson, Patel

Contributed new reagents or analytic tools: NA

Performed data analysis: Johnson, Matheny, Patel, Chen and Ellens

Wrote or contributed to the writing of the manuscript: Johnson, Matheny, Chen and Ellens
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Generaux GT, Bonomo FM, Johnson M, and Doan KM (2011) Impact of SLCO1B1 (OATP1B1) and ABCG2 (BCRP) genetic polymorphisms and inhibition on LDL-C lowering and myopathy of statins. *Xenobiotica; the fate of foreign compounds in biological systems* 41:639-651.


FIGURE LEGENDS

**Figure 1.** Structure of ronacaleret.

**Figure 2.** Geometric mean (S.D.) plasma concentration versus time profiles for rosuvastatin in the absence and presence of co-administered ronacaleret. Circles: rosuvastatin 10mg (Session 1, Day 2); triangles: rosuvastatin 10mg + ronacaleret 400mg (Session 2, Day 2); squares: rosuvastatin 10mg + ronacaleret 400mg (Session 2, Day 8).

**Figure 3.** Concentration-dependent inhibition of human OATP2B1-mediated rosuvastatin uptake by ronacaleret at pH 7.4 and pH 6.0. Transporter inhibition was investigated by determining uptake of rosuvastatin (1 µM) in the HEK cell line overexpressing OATP2B1 at a pH of 7.4 (open triangles) and pH 6.0 (closed squares).
Table 1. Rosuvastatin and Ronacaleret Pharmacokinetic Parameters\(^a\)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>AUC(0-(\infty)) (ng.h/mL)</th>
<th>Cmax (ng/mL)</th>
<th>tmax (h)(^b)</th>
<th>t(_{1/2}) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosuvastatin alone</td>
<td>23</td>
<td>59.1 (50.1)</td>
<td>4.39 (54.1)</td>
<td>4.00 (2.00-6.00)</td>
<td>11.3 (22.1)</td>
</tr>
<tr>
<td>Rosuvastatin + Ronacaleret</td>
<td>20</td>
<td>31.3 (68.0)</td>
<td>2.91 (52.8)</td>
<td>4.00 (0.50-4.00)</td>
<td>11.0 (65.6)</td>
</tr>
<tr>
<td>(single dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rosuvastatin+ Ronacaleret</td>
<td>23</td>
<td>29.9 (71.6)(^c)</td>
<td>2.96 (77.0)</td>
<td>4.00 (1.00-6.00)</td>
<td>10.9 (35.8)(^c)</td>
</tr>
<tr>
<td>(multi-dose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ronacaleret (multi-dose)</td>
<td>24</td>
<td>13700 (78.7)</td>
<td>2320 (80.5)</td>
<td>1.5 (1.00-4.00)</td>
<td>5.08 (18.7)</td>
</tr>
</tbody>
</table>

\(^a\) Geometric Mean (CV\(^b\)%)

\(^b\) Median (Range)

\(^c\) n=22

CV\(_{b}\) = between subject variability
**Table 2. In vitro transporter inhibition by ronacaleret**

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Substrate</th>
<th>IC$_{50}$ (µM)</th>
<th>Substrate</th>
<th>IC$_{50}$ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP1B1</td>
<td>$[^3]$H</td>
<td>EG</td>
<td>11</td>
<td>rosvastatin</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>$[^3]$H</td>
<td>EG</td>
<td>60</td>
<td>rosvastatin</td>
</tr>
<tr>
<td>OATP1A2</td>
<td>$[^3]$H</td>
<td>ES</td>
<td>7.4</td>
<td>rosvastatin</td>
</tr>
<tr>
<td>OATP2B1</td>
<td>$[^3]$H</td>
<td>ES</td>
<td>NC$^a$</td>
<td>rosvastatin (pH 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>rosvastatin (pH 6.0)</td>
</tr>
<tr>
<td>BCRP</td>
<td>$[^3]$H</td>
<td>ES</td>
<td>No inhibition</td>
<td>rosvastatin</td>
</tr>
<tr>
<td>MRP3</td>
<td>$[^3]$H</td>
<td>EG</td>
<td>No inhibition</td>
<td>rosvastatin</td>
</tr>
<tr>
<td>MRP4</td>
<td>$[^3]$H</td>
<td>EG</td>
<td>NC$^c$</td>
<td>rosvastatin</td>
</tr>
</tbody>
</table>

$^a$. Ronacaleret inhibited OATP2B1 transport of $[^3]$H|ES in vitro by 49% at a concentration of 100 µM.

$^b$. Ronacaleret inhibited human BCRP transport of rosvastatin in vitro by 20% at the highest test concentration of 100 µM.

$^c$. Ronacaleret inhibited human MRP4 transport of $[^3]$H|EG in vitro by 25% at the highest test concentration of 100 µM.

$^d$. Ronacaleret inhibited human MRP4 transport of rosvastatin in vitro by 31% at the highest test concentration of 100 µM.

NC = not calculated; data insufficient to determine IC$_{50}$.
ND = rosvastatin uptake into MRP3 vesicles was not detected. IC$_{50}$ not determined.

### Table 3. DDI risk assessment

<table>
<thead>
<tr>
<th></th>
<th>R</th>
<th>R threshold</th>
<th>R value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OATP2B1</td>
<td>( R = 1 + \frac{[I]}{IC_{50}}; )</td>
<td>FDA: 11</td>
<td>299</td>
</tr>
<tr>
<td></td>
<td>( I = \text{dose in 250 mL} )</td>
<td>EMA: 11</td>
<td>299</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>( R = 1 + \frac{[I]}{IC_{50}}; )</td>
<td>FDA: 1.25</td>
<td>1.191</td>
</tr>
<tr>
<td></td>
<td>( I = I_{u, \text{inlet, max, b}}^a )</td>
<td>EMA: 1.04</td>
<td>1.191</td>
</tr>
<tr>
<td>OATP1B3</td>
<td>( R = 1 + \frac{[I]}{IC_{50}}; )</td>
<td>FDA: 1.25</td>
<td>1.098</td>
</tr>
<tr>
<td></td>
<td>( I = I_{u, \text{inlet, max, b}}^a )</td>
<td>EMA: 1.04</td>
<td>1.098</td>
</tr>
</tbody>
</table>

Ronacaleret MW: 447.5

\( I_{u, \text{inlet, max, b}} = (C_{\text{max, b}} + F_a * F_g * k_a * \text{dose/Qh}) * f_{u,b} \)

\( F_a * F_g = 1 \) (assumed)

\( k_a = 0.1 \text{ min}^{-1} \) (assumed)

\( Q_h = 97 \text{ L/h} \)

\( f_{u,b} = 0.067 \) (measured)
Figure 1
Figure 2

Plasma Rosuvastatin (ng/mL)

Time (h)
Figure 3