Solitary Inhibition of the Breast Cancer Resistance Protein Efflux Transporter Results in a Clinically Significant Drug-Drug Interaction with Rosuvastatin by Causing up to a 2-Fold Increase in Statin Exposure

Robert Elsby, Paul Martin, Dominic Surry, Pradeep Sharma, and Katherine Fenner

ABSTRACT

The intestinal efflux transporter breast cancer resistance protein (BCRP) restricts the absorption of rosvastatin. Of the transporters important to rosvastatin disposition, fomastatnin inhibited BCRP (IC50 = 50 nM) and organic anion-transporting polypeptide 1B1 (OATP1B1; IC50 > 10 μM), but not organic anion transporter 3, in vitro, predicting a drug-drug interaction (DDI) in vivo through inhibition of BCRP only. Consequently, a clinical interaction study between fomastatnin and rosvastatin was performed (and reported elsewhere). This confirmed the critical role BCRP plays in statin absorption, as inhibition by fomastatnin resulted in a significant 1.96-fold and 1.88-fold increase in rosvastatin area under the plasma concentration-time curve (AUC) and Cmax, respectively. An in vitro BCRP inhibition assay, using polarized Caco-2 cells and rosvastatin as probe substrate, was subsequently validated with literature inhibitors and used to determine BCRP inhibitory potencies (IC50) of the perpetrator drugs eltrombopag, darunavir, lopinavir, clopidogrel, ezetimibe, fenofibrate, and fluconazole. OATP1B1 inhibition was also determined using human embryonic kidney 293-OATP1B1 cells versus estradiol 17β-glucuronide. Calculated parameters of maximum enterocyte concentration ([put max]), maximum unbound hepatic inlet concentration, transporter fraction excreted value, and determined IC50 value were incorporated into mechanistic static equations to compute theoretical increases in rosvastatin AUC due to inhibition of BCRP and/or OATP1B1. Calculated theoretical increases in exposure correctly predicted the clinically observed changes in rosvastatin exposure and suggested intestinal BCRP inhibition (not OATP1B1) to be the mechanism underlying the DDIs with these drugs. In conclusion, solitary inhibition of the intestinal BCRP transporter can result in clinically significant DDIs with rosuvastatin, causing up to a maximum 2-fold increase in exposure, which may warrant statin dose adjustment in clinical practice.

Introduction

Breast cancer resistance protein (BCRP; encoded by ABCG2) is one of the two major clinically relevant human ATP-binding cassette (ABC)–transporter proteins (the other being P-glycoprotein; ABCB1) that use ATP hydrolysis to efflux drugs and xenobiotics out of cells (Giacomini et al., 2010). BCRP is ubiquitously expressed in the intestine (apical brush border membrane of enterocytes), liver (canalicular membrane of hepatocytes), and kidney (apical brush border membrane of renal proximal tubule cells) and can affect the absorption and elimination of drugs that are substrates of this transporter, ultimately defining their exposure (Mao and Unadkat, 2015). The important role that BCRP plays in drug disposition has been demonstrated clinically in pharmacogenetic studies investigating the ABCG2 single nucleotide polymorphism c421C>A (Q141K, rs2231142), in which the impaired transport function phenotype (421CA or AA) resulted in increased plasma exposures of several BCRP substrate drugs, including rosuvastatin, atorvastatin, fluvastatin, and diltromocan (Sparreboom et al., 2004; Keskiitalo et al., 2009; Giacomini et al., 2013). Such observations also suggest that interindividual differences in BCRP function due to pharmacogenetics likely contribute to variability in bioavailability (absorption), exposure [area under the plasma concentration–time curve (AUC) and maximum plasma concentration (Cmax)], and efficacy of drugs that are BCRP substrates (Giacomini et al., 2010, 2013).

ABBREVIATIONS: ABC, ATP-binding cassette; AUC, area under the plasma concentration–time curve; B-A, basolateral to apical; BCRP, breast cancer resistance protein; DDI, drug-drug interaction; DMISO, dimethylsulfoxide; HBSS, Hank’s balanced salt solution; HEK293, human embryonic kidney 293; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopyrazino-[1,2′:1,6′]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethyl ester; LC-MS/MS, liquid chromatography–tandem mass spectrometry; NTCP, sodium taurocholate cotransporting peptide; OAT3, organic anion transporter 3; OATP1B1, organic anion-transporting polypeptide 1B1; R406, N4-(2,2-dimethyl-3-oxo-4-pyrid[1,4] oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine.
Indeed, it has recently been reported that the observed ethnic difference and variability in exposure of the BCRP substrates rosuvastatin and atorvastatin between Caucasian and Asian populations can be partly explained by the higher frequency of the \textit{ABCG2} c.421C\textgreater{}A polymorphism, alongside a hypothesized lower intrinsic BCRP activity, in Asian populations. The resulting overall increase in statin absorption gives rise to the higher plasma exposures observed clinically in Asian compared with Caucasian subjects (Birmingham et al., 2015a).

Aside from genetic polymorphisms, concomitant administration of a drug that is an inhibitor of BCRP can also modulate the pharmacokinetics of a transported substrate, resulting in a drug-drug interaction (DDI), which may lead to toxicity due to increased exposure, or altered efficacy. DDIs attributed to BCRP have been observed clinically between topotecan and elacridar, resulting in increased topotecan absorption (Kruijtzer et al., 2002); between methotrexate and benzimidazoles, resulting in delayed renal elimination of methotrexate (Breedveld et al., 2004); and, in part, between rosuvastatin and cyclosporine, resulting in increased rosuvastatin absorption (Simonson et al., 2004; Tweedie et al., 2013). As highlighted earlier, there is a persuasive body of evidence that supports the clinical importance of the BCRP efflux transporter in drug development.

Rosuvastatin calcium is an 3-Hydroxy-3-methylglutaryl–coenzyme-A reductase inhibitor (statin) that has been developed to treat dyslipidemia by reducing low-density lipoprotein cholesterol (Crestor; AstraZeneca, Cambridge, UK). The critical disposition pathways of rosuvastatin and their individual contributions to overall clearance (fraction excreted values; \( f_e \)), determined by Elsby et al. (2012) and derived from clinical human mass balance, pharmacogenetic, and DDI evidence, are shown in Fig. 1. These critical pathways include the intestinal BCRP efflux transporter as the rate-determining barrier to rosuvastatin absorption (fraction absorbed = 0.5; therefore, BCRP \( f_e = 0.5 \)), the hepatic uptake transporter organic anion-transporting polypeptide 1B1 (OATP1B1) responsible for hepatic elimination, and the renal uptake transporter organic anion transporter 3 (OAT3) responsible for the active renal secretion of rosuvastatin (Elsby et al., 2012). Metabolism of rosuvastatin by CYP2C9 constitutes only a minor pathway to overall disposition (\( \leqslant 10\% \)).

![Fig. 1. Critical disposition pathways of rosuvastatin as described by Elsby et al. (2012).](image-url)
Fostamatinib is an oral spleen tyrosine kinase inhibitor that has been investigated as a treatment of rheumatoid arthritis (Weinblatt et al., 2014) and, due to patient comorbidities in this disease population, was likely to be coadministered with statins, such as rosuvastatin and atorvastatin. Fostamatinib is a prodrug (R788; chemical name/structure provided in Sweeny et al., 2010) that is rapidly and essentially completely metabolized by dephosphorylation in the enterocytes of the intestine to the active systemic metabolite R406 (N4-(2,2-dimethyl-3-oxo-4-pyrid[1,4]oxazin-6-yl)-5-fluoro-N2-(3,4,5-trimethoxyphenyl)-2,4-pyrimidinediamine) (Sweeny et al., 2010; Baluom et al., 2013). Therefore, the first aim of this study was to investigate fostamatinib and R406 as inhibitors of the principal rosuvastatin transporters BCRP, OATP1B1, and OAT3 in vitro and, on the basis of inhibition predictions, to use fostamatinib as a mechanistic in vivo tool in a...
Inhibition of Intestinal BCRP Increases Rosuvastatin Exposure

TABLE 1

<table>
<thead>
<tr>
<th>Transporter</th>
<th>IC50</th>
<th>[I]/[K]</th>
<th>Iinlet max u</th>
<th>R-value</th>
<th>Potential for DDI ([I]/[K] ≥ 0.1, [I]/[K] ≥ 10, R-value ≥ 1.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>1.6μM</td>
<td>0.050 μM</td>
<td>32</td>
<td>13,820</td>
<td>NA</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>1.6μM</td>
<td>0.031 μM</td>
<td>NA</td>
<td>0.16</td>
<td>0.284</td>
</tr>
</tbody>
</table>

In Vitro BCRP Inhibition Assay of Fostamatinib and R406. Uptake of the BCRP probe substrate [3H]estradiol 3-sulfate (1 μM) was determined using 24-well plates according to a previously validated method (Sharma et al., 2010) in HEK293-OATP1B1 cells in the absence and presence of R406 (0.1–10 μM) and the positive control inhibitor rifamycin SV (0.001–100 μM). Incubations (pools of 10 oocytes per condition) were conducted at room temperature for 60 minutes. Uptake solutions contained ≤2% (v/v) dimethylsulfoxide (DMSO).

Materials and Methods

Materials. Estrone 3-sulfate, sulfa-salazine, estradiol 17β-glucuronide, rifamycin SV, Triton X, novobiocin, Ko143 [3(S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4-dioxopropyrazino-[1,2-b,1,6]pyrido [3,4-bjnilol-3-propanoic acid 1,1-dimethyl ethyl ester], cyclosporin A, pantoprazole, diclofenac, nifedipine, clofuregol, fluconazole, and fenofibrate were purchased from Sigma-Aldrich (St. Louis, MO; Poole, Dorset, UK). Ethylbropapag, darunavir, and lopinavir were purchased from Selleck Chemicals (Houston, TX; Shanghai, China). Fluvastatin was obtained from Sequoia Research Products (Pangbourne, Reading, UK). [3H]estrone 3-sulfate, [3H]estradiol 17β-glucuronide, and Ultima Gold and Optiphase Supermix scintillation cocktails were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA; Buckinghamshire, UK). [3H]methotrexate and methotrexate were purchased from American Radiolabeled Chemicals (St. Louis, MO). Fostamatinib and its active metabolite R406 were supplied by AstraZeneca R&D (Alderley Park, Macclesfield, UK). Metoprolol, rosuvastatin, atorvastatin, and ezetimibe were supplied by AstraZeneca R&D Group. [3H]rosuvastatin was custom synthesized by Quotient Bioresearch (Cardiff, UK). Hanks’ balanced salt solution (HBSS; containing CaCl2 and MgCl2) and HEPES were purchased from Life Technologies (Paisley, UK; Grand Island, NY). All other chemicals, solvents, and reagents were purchased from Sigma-Aldrich.

Sodium uptake buffer, ND96 buffer, BD Falcon HTS 96-well flat-bottom tissue culture treated plates, BD Biocoat Poly-t-lysin 24-well multwell plates, human BCRP (ABC22G) membrane vesicles, human OAT3 (SLC22A8)-expressing Xenopus laevis oocytes, and control (water-injected) oocytes were supplied by BD Biosciences Discovery Labware (Woburn/Bedford, MA; Oxford, UK). UniFilter-96 GF/F filter plates and TopSeal A adhesive sealing film were supplied by PerkinElmer Life and Analytical Sciences. Millicell-96 multwell cell culture insert plates (with polycarbonate membranes; 0.4 μm pore size, 0.12 cm² surface area) and Millicell 96-well transport companion plates were purchased from Millipore (Watford, Hertfordshire, UK). Ninety-six–well deep well (2 ml) polypropylene or shallow round-bottomed polypropylene plates were supplied by VWR International Ltd. (Lutterworth, Leicestershire, UK). HTS Transwell-96 permeable supports and associated 96-well plasticware were obtained from Corning Incorporated Life Sciences (Tewksbury, MA). A recombinant cell line expressing OATP1B1 [human embryonic kidney 293 (HEK293)–OATP1B1] was provided by AstraZeneca R&D. Caco-2 cells (HTB37, supplied at passage number 17) were purchased from American Type Culture Collection (Manassas, VA).

Rosuvastatin Clinical Interaction Study to Confirm the Impact that Solitary Inhibition of BCRP has on Rosuvastatin Exposure. The second aim was to validate a Caco-2 rosuvastatin BCRP inhibition assay, and then, using determined IC50 data and mechanistic static equations, to evaluate the role BCRP inhibition plays in the clinically DDI observed with rosuvastatin and a range of perpetrator drugs by comparing predicted theoretical fold increases in exposure to the clinically observed rosuvastatin AUC increases.

In Vivo OATP1B1 Inhibition Assay of R406. Uptake of the OATP1B probe substrate [3H]estradiol glucuronide (0.02 μM) was determined using 24-well plates according to a previously validated method (Sharma et al., 2010) in HEK293-OATP1B1 cells in the absence and presence of R406 (0.1–10 μM) and the positive control inhibitor sulfa-salazine (0.1–30 μM). Uptake solutions contained ≤2% (v/v) dimethylsulfoxide (DMSO).

In Vitro OAT3 Inhibition Assay of R406. Uptake of the OAT3 probe substrate [3H]methotrexate (10 μM) was determined according to a previously described method (Elshby et al., 2011b) using control and OAT3-expressing oocytes in the absence and presence of R406 (0.11–32 μM) and the positive control inhibitor sulfa-salazine (0.3–100 μM). Incubations (pools of 10 oocytes per condition) were conducted at room temperature for 60 minutes. Uptake solutions contained ≤2% (v/v) DMSO.

Data Analysis. In brief, the amount of radioactivity (disintegrations per minute) taken up by vesicles, HEK293-OATP1B1 cells, or oocytes was determined and used to calculate the amount (picomoles) of probe substrate, which was subsequently converted to uptake rate (pmol/min/mg protein or pmol/h/oocyte) as described previously (Sharma et al., 2010; Elshby et al., 2011b). Background condition uptake rate (uptake in BCRP vesicles minus ATP, uptake in HEK293-OATP1B1 cells in the presence of 100 μM positive control inhibitor where OATP1B1 is 100% inhibited, or uptake in control oocytes) was subtracted from that determined in transporter-expressing vesicles/cells/oocytes to give transporter-specific uptake rate. This was then converted to percentage (vehicle) control transport activity, which was subsequently plotted against nominal inhibitor concentration. Curves were fitted using XLfit 5.1 (ID Business Solutions Ltd., Guildford, Surry, UK) [four-parameter logistic model, Eq. 204, as previously described for P-glycoprotein inhibition (Elshby et al., 2011c)] to determine the concentration that produces half-maximal inhibition of probe substrate transport (IC50).

Clinical Interaction Study between Fostamatinib and Rosuvastatin. Recently, a clinical interaction study (NCT01725447, NCT00005039) between fostamatinib and rosuvastatin performed in healthy volunteers (62% Caucasian, 38% black ethnicity; 95% male and 5% female between the ages of 18 and 55 years, with body weight >50 kg and body mass index between 18 and 30 kg/m²) was fully described and reported elsewhere (Martin et al., 2016). In brief, the study was an open-label, fixed-sequence study that assigned eligible subjects to receive 20 mg of rosuvastatin over two treatment periods: alone for period 1 and in combination with fostamatinib at steady state (100 mg twice daily for 5 days) for period 2. Plasma concentrations of rosuvastatin and R406 were quantified by validated bioanalytical liquid chromatography–tandem mass spectrometry.
Validation of an In Vitro Caco-2 BCRP Inhibition Assay Utilizing Rosuvastatin as BCRP Probe Substrate. The following experimental properties were investigated in the validation of the Caco-2 BCRP inhibition assay as a method to identify inhibitors of BCRP: 1) the suitability of novobiocin as a positive control inhibitor, 2) interassay variability, and 3) acceptance criteria to qualify control future use of the assay. The basolateral-to-apical (B–A) transport of the probe substrate \([^{3}H\text{rosuvastatin}]\) was measured across polarized Caco-2 cell monolayers (triplicate wells per condition) in the absence and presence (in both donor and receiver compartments; added at the same time as rosuvastatin) of a range of concentrations (0.03–10, 0.1–30, or 0.3–100 \(\mu M\)) of the known BCRP inhibitors novobiocin, Ko143, cyclosporin A, pantoprazole, sulfasalazine, atorvastatin, diclofenac, fluvastatin, and nifedipine, and of the noninhibitor metoprolol, to determine an apparent \(IC_{50}\) value for the inhibition of BCRP-mediated rosvastatin transport. Experiments determining the inhibition of BCRP transport by the positive control inhibitor novobiocin were conducted on nine separate occasions spanning five cell passage numbers (24, 25, 26, 27, and 29) to ensure that the data generated were consistent over the passages routinely used for inhibition experiments (20–30). The remaining inhibitors were each assessed on three separate experimental occasions. The methodology used was a slight modification of the previously validated Caco-2 \(P_{app}\)-glycoprotein inhibition assay (Elsby et al., 2008), which is in agreement with the recommendations of the International \(P_{app}\)-glycoprotein IC\(50\) Working Group (Bentz et al., 2013). The main modifications were seeding of Caco-2 cells on 96-well multiwell insert plates at a density of 270,000 cells/ml (27,000 cells per well; 245,455 cells/cm\(^2\)), use of monolayers in transport studies (Bentz et al., 2013). The main modifications were seeding of Caco-2 cells on 96-well multiwell insert plates at a density of 270,000 cells/ml (27,000 cells per well; 245,455 cells/cm\(^2\)), use of monolayers in transport studies (14–21 days after seeding, and use of a single incubation time point (90 minutes) in the absence of shaking.

In brief, polarized Caco-2 cell monolayers were washed three times and then preincubated with warm transport buffer (HBSS containing 10 mM HEPES, pH 7.4) for 10–15 minutes at 37°C prior to the addition of donor and receiver solutions. Donor solutions of transport buffer containing \([^{3}H\text{rosuvastatin}]\) (1 \(\mu M\); approximately 10-fold lower than \(K_{m}\) for BCRP = 10.8 \(\mu M\); Huang et al., 2006) and the appropriate concentration of test inhibitor or DMSO (vehicle control; at the equivalent volume of solvent vehicle used for test inhibitor) were added to the basolateral compartment of the monolayer (total volume = 300 \(\mu l\)). Receiver solutions of transport buffer containing either the corresponding concentration of test inhibitor or DMSO (vehicle control) and the cell monolayer integrity marker Lucifer yellow (100 \(\mu M\)) were added to the apical compartments (total volume = 100 \(\mu l\)). Donor and receiver solutions contained 0.5% (v/v) DMSO. Following a 90-minute incubation at 37°C, the amount of \([^{3}H\text{rosuvastatin}]\) appearing in the receiver compartment was determined by sampling (50 \(\mu l\)), quantified by liquid scintillation counting, and used to calculate apparent permeability \((P_{app})\) as described previously (Elsby et al., 2008). The passive permeability of rosvastatin observed when BCRP is completely inhibited [derived from incubations containing the highest concentration (30 \(\mu M\)) of the positive control inhibitor novobiocin] was subtracted from the determined B-A \(P_{app}\) value in the absence or presence of test inhibitor, to give a corrected BCRP-mediated B-A \(P_{app}\), which was subsequently converted to percentage (vehicle) control transport activity. The resulting inhibition curves were plotted and fitted as described earlier to determine \(IC_{50}\) values, which are equivalent to \(K_{i}\) values (assuming competitive inhibition). Mass balance (percent recovery) of rosvastatin was calculated as described previously (Elsby et al., 2011c). The percentage mass (picomoles) of each coadministered Lucifer yellow across the cell monolayer was determined, and cell monolayer integrity was deemed acceptable if the transfer was \(\leq 1.5\%\).

Mechanistic Static Predictions of AUC Changes for Known Clinical DDIs with Rosuvastatin Based Upon Determined In Vitro Caco-2 BCRP Inhibitory Data. Eltrombopag, darunavir, lopinavir, clopidogrel, ezetimibe, fenofibrate, and fluconazole were assessed (over a range of six concentrations) in the validated Caco-2 BCRP inhibition assay (using both radiolabeled and a cross-validated LC-MS/MS endpoint) to determine inhibitory potencies toward BCRP-mediated rosvastatin transport. The \(IC_{50}\) (equating to \(K_{i}\)) values obtained for the aforementioned compounds and fostamatinib were incorporated into the following adapted Rowland-Matin mechanistic static equation, as described previously by Elsby et al. (2012), to predict the change in rosvastatin AUC based upon inhibition of a fraction excreted \((f_{e})\) value of 0.5 for intestinal BCRP:

\[
\text{Fold } \Delta \text{AUC} = \frac{1}{1 + \left(\frac{K_{i}}{[I]}\right)}(1 - f_{e})
\]

where \(K_i\) = absolute inhibition constant (equating to \(IC_{50}\) if the probe [\(S\)] \(<<<< K_{m}\) in the inhibition assay and assuming competitive inhibition, based on the Cheng-Prusoff equation; Cheng and Prusoff, 1973) and \([I]\) = maximum enteroocyte concentration \((I_{gut\, max})\) as described by Agarwal et al., 2013).

Additionally, the predicted change in rosvastatin AUC based upon inhibition of OATP1B1 \((f_{e}=0.38)\) was also determined using \(K_i\) values derived from either the aforementioned in vitro OATP1B1 inhibition assay (at 0.3, 1, 3, 10, 30, and 100 \(\mu M\) using LC-MS/MS as the analytical endpoint) or the literature, in context with the unbound maximum hepatic inlet concentration as \([I]\), to understand the contribution (if any) of OATP1B1 inhibition toward the overall magnitude of exposure change observed clinically through DDI.

**Results**

Assessment of Fostamatinib and R406 as Inhibitors of BCRP In Vitro. Inhibition of estrone 3-sulfate uptake by the positive control inhibitor sulfasalazine \((IC_{50} = 0.61 \mu M)\) passed the acceptance criteria set during the assay day (Elsby et al., 2011a) and therefore indicated that the vesicle test system was capable of detecting inhibitors of BCRP. Both fostamatinib and R406 inhibited BCRP-mediated transport of estrone 3-sulfate with \(IC_{50}\) values of 0.050 and 0.031 \(\mu M\), respectively (Fig. 2).

Assessment of R406 as an Inhibitor of OATP1B1 and OAT3 In Vitro. Inhibition of probe substrate uptake by the positive control inhibitors rifampicin SV \((IC_{50} = 0.02 \mu M)\) or sulfasalazine \((IC_{50} = 4.3 \mu M)\) demonstrated that the transfected cell or oocyte test systems were able to detect inhibitors of OATP1B1 or OAT3, respectively. Although R406 did not inhibit OAT3-mediated transport, it did inhibit OATP1B1-mediated transport of estradiol 17β-glucuronide with an \(IC_{50} > 10 \mu M\) (27% inhibition at 10 \(\mu M\); Fig. 2).

Calculation of \([I]/K_{i}\) Ratios and R-Value to Predict the DDI Potential of Fostamatinib. The calculated \([I]/K_{i}\) ratios and R-value for the expected therapeutic dose of fostamatinib (100 mg) are shown in Table 1. The mean total \(C_{max}\) value of R406 (at steady state), used toward the calculation of hepatic inlet concentration, is derived from the plasma concentration data determined from multiple clinical studies. The \([I_{2}]/K_{i}\) ratio predicted that there was a potential for a DDI with hepatic OATP1B1 in vivo (as R-value < 1.25), nor through renal OAT3, as R406 was not an inhibitor of OAT3.

Effect of Fostamatinib on the Pharmacokinetic Parameters of the BCRP Substrate Rosuvastatin in Healthy Volunteers. The mean plasma concentration–time profiles (0–96 hours) of a single oral dose of rosvastatin (20 mg) when administered alone or concomitantly with 100 mg of fostamatinib at steady state (dosed twice daily for 5 days) are shown in Fig. 3 (reprinted from Martin et al., 2016). Fostamatinib increased the geometric least-squares mean AUC and \(C_{max}\) of rosvastatin by 96% (90% confidence interval, 78–115) and 88% (90% confidence interval, 69–110), respectively, indicating a clinical pharmacokinetic interaction (Martin et al., 2016).

Validation of an In Vitro Caco-2 BCRP Inhibition Assay Utilizing Rosuvastatin as BCRP Probe Substrate. Initial comparison of bidirectional apparent permeabilities confirmed that rosuvastatin \((1 \mu M)\) exhibited suitable BCRP-mediated efflux in the Caco-2 cell test system: mean ± S.D. \((n = 3\) wells\) A-B \(P_{app}\) = 0.39 ± 0.05 and B-A \(P_{app}\) = 15.6 ± 1.75 \(\times 10^{-6}\) cm/s, giving an efflux ratio of 40. BCRP is
solely responsible for rosuvastatin efflux in Caco-2 cells, as the efflux ratio returned to unity in the presence of the BCRP inhibitor novobiocin (30 μM; data not shown), which did not inhibit P-glycoprotein (up to 300 μM) in MDCK-MDR1 cells (R. Elsby, unpublished observation). The rosuvastatin B-A P_app values observed at cell passage numbers 24, 25, 26, 27, and 29 were 16.4 ± 1.80, 14.2 ± 1.50, 16.1 ± 3.10, 20.6 ± 1.9, and 12.5 ± 0.90 × 10^-6 cm/s, respectively, indicating consistent functional expression of BCRP over the range of cell passages used for inhibition experiments.

The determined interassay mean IC_{50} values for the inhibition of BCRP-mediated rosuvastatin transport by the positive control inhibitor novobiocin from nine separate occasions over five different cell passage numbers, and by Ko143, cyclosporin A, pantoprazole, sulfasalazine, atorvastatin, diclofenac, fluvastatin, and nifedipine are shown in Table 2, proving that the Caco-2 cell test system is able to correctly identify known literature inhibitors of BCRP. Mean IC_{50} curves are shown in Fig. 4, which also shows that metoprolol did not inhibit BCRP or OATP1B1 over the concentration ranges tested (data not shown).

Using the previously determined drug inhibitory affinities and their pharmacokinetic parameters given in Table 3, calculations were performed with mechanistic static equations to predict the theoretical fold increase in rosuvastatin AUC due to inhibition of BCRP and/or hepatic OATP1B1. The calculated theoretical fold increases in exposure due to inhibition of each transporter alone, and in combination, are shown in Table 4. For each individual drug highlighted, the theoretical fold increase in AUC due to inhibition of OATP1B1 alone was lower (ranging from 1.01- to 1.25-fold) than the corresponding increase due to sole inhibition of BCRP, which ranged from 1.15- to 2-fold (Table 4).

### Table 2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Determined Values from Different Occasions Mean</th>
<th>S.D.</th>
<th>CV (%)</th>
<th>Published IC_{50}/K (Probe Substrate Used)</th>
<th>Test System</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novobiocin</td>
<td>0.4, 1.5, 2.3, 1.6, 1.6, 1.5, 1.4, 1.1, 1.2</td>
<td>0.5</td>
<td>36</td>
<td>0.4 (methotrexate)</td>
<td>Vesicles</td>
<td>Saito et al. (2006)</td>
</tr>
<tr>
<td>Ko143</td>
<td>0.6, 0.2, 0.3</td>
<td>0.2</td>
<td>50</td>
<td>0.013 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Xie et al. (2007)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>5.5, 4.1, 1.6</td>
<td>2.0</td>
<td>54</td>
<td>6.7 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Xie et al. (2007)</td>
</tr>
<tr>
<td>Pantoprazole</td>
<td>8.5, 14.4, 5.9</td>
<td>4.4</td>
<td>46</td>
<td>13 (methotrexate)</td>
<td>Vesicles</td>
<td>Breedveld et al. (2004)</td>
</tr>
<tr>
<td>Sulfasalazine</td>
<td>2.7, 4.7, 1.4</td>
<td>1.7</td>
<td>59</td>
<td>0.74 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Elsby et al. (2011a)</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>0.5, 0.8, 0.4</td>
<td>0.2</td>
<td>33</td>
<td>14.3 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Hirano et al. (2005)</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>16.5, 19.1, 23.7</td>
<td>19.8</td>
<td>18</td>
<td>78 (methotrexate)</td>
<td>Vesicles</td>
<td>Lagas et al. (2009)</td>
</tr>
<tr>
<td>Fluavastatin</td>
<td>1.3, 1.1, 0.9</td>
<td>1.1</td>
<td>25</td>
<td>5.43 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Hirano et al. (2005)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>8.0, 11.1, 13.0</td>
<td>10.7</td>
<td>23</td>
<td>60 (methotrexate)</td>
<td>Vesicles</td>
<td>Saito et al. (2006)</td>
</tr>
</tbody>
</table>

CV, coefficient of variation.
Fig. 4. Mean concentration-dependent inhibition of the BCRP-mediated transport of \([^{3}H]\)rosuvastatin (1 \(\mu\)M) by a range of known literature BCRP inhibitors, and the noninhibitor metoprolol, using polarized Caco-2 cell monolayers. Data are expressed as the mean (± S.D.) for \(n \geq 3\) experimental occasions per inhibitor.
TABLE 3

<table>
<thead>
<tr>
<th>Perpetrator Drug</th>
<th>Dose (mg)</th>
<th>MW</th>
<th>IC50 (μM)</th>
<th>IC50 (μM)</th>
<th>IC50/Ki Ratio</th>
<th>Cmax total (μM)</th>
<th>fα</th>
<th>[Iαmax] (μM)</th>
<th>fα</th>
<th>OATP1B1 Ki (μM)</th>
<th>Biliary BCRP ICαmax (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fostamatinib</td>
<td>100</td>
<td>578.52</td>
<td>691 0.05</td>
<td>13,820</td>
<td>58 1.9</td>
<td>0.018</td>
<td>0.289</td>
<td>&gt;10</td>
<td>9.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R406</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eltrombopag</td>
<td>75</td>
<td>442.5</td>
<td>678 2.1</td>
<td>323 29</td>
<td>17.7</td>
<td>0.01</td>
<td>0.236</td>
<td>2.7</td>
<td>0.11</td>
<td></td>
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<tr>
<td>Darunavir</td>
<td>600</td>
<td>547.7</td>
<td>4382 75</td>
<td>58 75</td>
<td>10.6</td>
<td>0.05</td>
<td>1.261</td>
<td>4.3</td>
<td>0.02</td>
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<tr>
<td>Lopinavir</td>
<td>400</td>
<td>628.8</td>
<td>2545 8.7</td>
<td>239 42</td>
<td>14.6</td>
<td>0.02</td>
<td>0.462</td>
<td>0.43</td>
<td>0.05</td>
<td></td>
<td></td>
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<tr>
<td>Clopidogrel</td>
<td>75</td>
<td>321.9</td>
<td>932 63</td>
<td>15 78</td>
<td>0.0099</td>
<td>0.02</td>
<td>0.311</td>
<td>1.8</td>
<td>0.005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>300</td>
<td>321.9</td>
<td>3726 63</td>
<td>59 311</td>
<td>0.130</td>
<td>0.02</td>
<td>1.245</td>
<td>1.8</td>
<td>0.02</td>
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<tr>
<td>Ezetimibe</td>
<td>10</td>
<td>409.4</td>
<td>98 2.9</td>
<td>34 2.4</td>
<td>0.014</td>
<td>0.10</td>
<td>0.050</td>
<td>2.2</td>
<td>0.02</td>
<td></td>
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<tr>
<td>Fenofibrate</td>
<td>67</td>
<td>360.8</td>
<td>743 170</td>
<td>4.4 62</td>
<td>25.8</td>
<td>0.01</td>
<td>0.398</td>
<td>20</td>
<td>NA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvonazole</td>
<td>200</td>
<td>306.3</td>
<td>2612 98</td>
<td>218 34.6</td>
<td>0.89</td>
<td>69.536</td>
<td>No inhibition</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Cmax total, mean steady-state maximum plasma concentration for total (bound plus unbound) drug measured in the clinical interaction study with rosuvastatin; fα, fraction unbound [taken from the drug label accessed via the Pharmapendium database (http://www.pharmapendium.com)]; IC50, maximal theoretical gastrointestinal concentration (calculated from [dose [mol]/250 ml]), Iαmax, maximum enterocyte concentration, calculated as [(Fa × kα × dose/Qent), where Fa is the fraction absorbed (as default taken to be 1.0), kα is the absorption rate constant (as default taken to be 0.1 min⁻¹), and Qent is the enterocyte blood flow (300 ml/min [Agarwal et al., 2013)]; Iαmax, maximum unbound liver inlet concentration, calculated as [IC50 × (Cmax total + (Fa × kα × dose/Qent)), where Fa is the fraction absorbed (as default taken to be 1.0), kα is the absorption rate constant (as default taken to be 0.1 min⁻¹), and Qent is hepatic blood flow (1500 ml/min); Kα, absolute inhibition constant (assuming competitive inhibition); equals to ICαmax in these assays as probe substrate concentration used is < < < < Kα; MW, molecular weight [taken from the drug label accessed via Pharmapendium database (http://www.pharmapendium.com)]; NA, not applicable. 

The BCRP transporter plays an important role in clinically observed DDIs, the majority of which are attributable to inhibition of intestinal BCRP, resulting in increased absorption of substrates such as topotecan and rosvastatin (Kruijtzer et al., 2002; Elsby et al., 2012). Studying the potential for a drug to perpetrate a BCRP-mediated DDI in the clinic involves initial in vitro evaluation of the drug as an inhibitor of BCRP and, where warranted, a follow-up interaction study with a clinical BCRP probe substrate, such as rosuvastatin, to confirm interaction potential in vivo (Giacomini et al., 2010; Food and Drug Administration draft DDI guidance 2012, [http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf](http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf)). However, rosvastatin would not be a reliable probe for such a study, as BCRP is not the only pathway that is critical to the disposition of rosuvastatin in vivo, since both OATP1B1 and OAT3 pathways are also important (Elsby et al., 2012). Consequently, any clinical study using rosuvastatin would simply be a broad drug interaction study unless the “interacting” drug is proven not to inhibit OATP1B1 and OAT3 in vitro or, if an in vivo inhibitor, not predicted to inhibit in vivo, and only then would it become a mechanistic study for assessing BCRP inhibition.

On the basis of fulfilling these criteria, following assessment of IC50 values toward BCRP (R788; [Iα]/Kα = 13,820; DDI likely), OATP1B1 (R406; R-value = 1.03; no DDI potential), and OAT3 (R406; no inhibition), it was recognized that fostamatinib could be used as an in vivo tool for a mechanistic clinical BCRP interaction study with rosvastatin. Such a study would confirm the critical role BCRP plays in rosvastatin absorption by evaluating, through DDI, the impact solitary inhibition of BCRP has on statin exposure. Although rosvastatin was not used as the in vitro probe to assess OATP1B1 and BCRP inhibition potential, estradiol 17β-glucuronide is a surrogate for OATP1B1-mediated rosvastatin transport (Izumi et al., 2015), and for BCRP, even if a fostamatinib IC50 value using rosvastatin was 100-fold higher than that obtained versus estrone 3-sulfate, it would not change DDI potential or predictions. The data reproduced from the clinical interaction study by Martin et al. (2016) demonstrated that sole inhibition of BCRP by fostamatinib resulted in a clinically significant approximately 2-fold increase in rosvastatin exposure (AUC and Cmax). Examination of rosvastatin concentration–time profiles (Fig. 3) with and without fostamatinib suggested that there was no change in rosvastatin elimination rate (0.044 or 0.049 hour⁻¹, respectively), as elimination phases were parallel. Coupled with the finding that there was no change in rosvastatin half-life (15.7 vs. 14.2 hours, respectively), these data indicated that the DDI with fostamatinib was attributable to increased rosvastatin absorption due to inhibition of intestinal BCRP (Martin et al., 2016). Furthermore, the magnitude of the observed exposure change is consistent with a doubling in rosvastatin absorption, which not only suggests complete inhibition of intestinal BCRP (fα = 0.5) by fostamatinib (conforming to Kα being >1000 times lower than anticipated intestinal concentration), but also confirms that BCRP efflux restricts absorption of rosvastatin to 50% in humans.

Eltrombopag, darunavir, lopinavir, clopidogrel, ezetimibe, fenofibrate, and fluvonazole are drugs that are listed on the Crestor FDA label as perpetrating pharmacokinetic DDIs upon coadministration with rosvastatin. Of the rosvastatin disposition pathways, OAT3 inhibition is unlikely to contribute to these DDIs because darunavir and fluvonazole do not inhibit OAT3 in vitro, and eltrombopag (Kα = 7.8 μM), lopinavir (Kα > 10 μM), and fenofibric acid (Kα = 2.2 μM) are not predicted to inhibit OAT3 in vivo based on Cmax/Kα ratios being <0.1 (Chu et al., 2007; Yoshida et al., 2012). Additionally, inhibition of OATP1B3 is unlikely to play a role since, although eltrombopag (Kα = 7.8 μM) is unlikely to contribute to these DDIs because darunavir and fluvonazole do not inhibit OAT3 in vitro, and eltrombopag (Kα = 7.8 μM), lopinavir (Kα > 10 μM), and fenofibric acid (Kα = 2.2 μM) are not predicted to inhibit OAT3 in vivo based on Cmax/Kα ratios being <0.1 (Chu et al., 2007; Yoshida et al., 2012). Additionally, inhibition of OATP1B3 is unlikely to play a role since, although eltrombopag (Kα = 7.8 μM), lopinavir (Kα = 25.6 μM), darunavir (Kα = 4.51 μM), and ezetimibe (Kα > 4 μM) are inhibitors, R-value extrapolations indicate their potential for DDI as unlikely (<1.25), and lopinavir is not an inhibitor (De Bruyn et al., 2011, 2013; Takeuchi et al., 2011; Vildhede et al., 2014). Finally, inhibition of sodium/taurine cotransporting peptide (NTCP) can be ruled out as a contributory factor since 1) despite being an NTCP inhibitor in vitro (Kα = 25 μM; Dong et al., 2013), ezetimibe is not expected to cause a DDI via this mechanism in vivo (R = 1.00); 2) lopinavir does not inhibit NTCP (Vildhede et al., 2014); and 3) although it is unknown whether the
remaining drugs are NTCP inhibitors, even if they were to have similar potencies to those described earlier for OATP1B3, then R-values would still indicate that DDI potential by this mechanism is unlikely. Consequently, this reasoning indicates inhibition of BCRP and/or OATP1B1 as being the predominant transporter pathway(s) to focus on to decipher the underlying mechanisms behind observed DDIs. The IC_{50} values of these drugs toward BCRP were determined in the Caco-2 BCRP inhibition assay to obtain clinically relevant parameters that could be used, alongside OATP1B1 potencies, in calculations of maximum theoretical increases in rosuvastatin exposure to aid understanding of the mechanism(s) behind each DDI. Apart from flucanazole, which did not inhibit BCRP, all of the drugs were inhibitors of BCRP-mediated rosuvastatin transport and, other than fenofibrate, were predicted to cause a DDI through intestinal BCRP inhibition in vivo ([I_{2}]K, ratios > 10; Table 3). Interestingly, none of these inhibitors were anticipated to inhibit biliary BCRP in vivo (ratios of [I_{det max}]K were all ≤ 0.1; Table 3), supporting the notion that inhibition of intestinal BCRP is clinically important for BCRP-mediated DDIs. However, although all the drugs (except flucanazole) were OATP1B1 inhibitors, only darunavir, lopinavir, and clopidogrel (300 mg) gave R-values > 1.25 (= 1.29, 2.07, and 1.69, respectively), indicating potential for an interaction in vivo via OATP1B1. Collectively, these data suggested that inhibition of intestinal BCRP may be the principal cause of the clinically observed DDIs perpetrated by eltrombopag, darunavir, lopinavir, ezetimibe, and fenofibrate, as all predictions were within 10% of the clinical value (Table 4). Furthermore, the accuracy of the predictions not only support our earlier assumption that BCRP and (to a lesser extent) OATP1B1 are the dominant pathways involved in the majority of rosuvastatin clinical DDIs, but also that [I_{gut max}] appears to be the correct concentration parameter for use in predictions with rosuvastatin.

The clinical role of intestinal BCRP inhibition in causing rosuvastatin DDIs implies that the magnitude of such DDIs may be reduced in individuals who are polymorphic for ABCG2 c.421C>A, due to these individuals having less functional BCRP activity to inhibit. This may translate to an ethnic difference in DDI susceptibility if the higher frequency of ABCG2 polymorphism in a particular group results (in part) in lower functional BCRP activity at the population level, as has been demonstrated in Asian compared with Caucasian populations (Sakiyama et al., 2014; Birmingham et al., 2015a,b). Interestingly, such an ethnic difference has been observed clinically with eltrombopag, where there was a larger 1.88-fold increase in rosuvastatin AUC in non-Asian subjects compared with only a 1.3-fold increase in Asian subjects following coadministration of eltrombopag (Allred et al., 2011). Thus, the much smaller magnitude of AUC change for the Asian group is consistent with this group having less functional BCRP activity to be inhibited by eltrombopag. It was for this reason that the non-Asian group AUC change was used as the clinically observed AUC change for comparison with predictions in this study, as it more accurately reflects the impact of inhibiting fully functioning BCRP.

Finally, taking into account the analyses from both this study and that of Elsby et al. (2012) toward understanding the likely mechanisms behind clinically observed DDIs with rosuvastatin, it is interesting to note that out of a total of 12 reported clinical DDIs (the first 10 listed on the Crestor label, plus clopidogrel and fostamatinib) for which increases

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**TABLE 4**

Predicted versus observed AUC changes of rosuvastatin with various coadministered drugs based upon inhibition of intestinal BCRP (f_{I} = 0.5) or hepatic OATP1B1 (f_{I} = 0.38) transport

<table>
<thead>
<tr>
<th>Perpetrator Drug</th>
<th>Dose</th>
<th>Predicted Fold Increase in AUC Due to Inhibition of Composite Pathways</th>
<th>Overall Predicted Fold Increase in AUC</th>
<th>Clinically Observed Fold Increase in AUC</th>
<th>Reference</th>
<th>Primary Mechanism of DDI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg</td>
<td>Intestinal BCRP (Theoretical Max = 2.0)</td>
<td>OATP1B1 (Theoretical Max = 1.6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fostamatinib/R406</td>
<td>100</td>
<td>2.00</td>
<td>1.01</td>
<td>2.02</td>
<td>Martin et al. (2016)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Eltrombopag^{a}</td>
<td>75</td>
<td>1.87</td>
<td>1.03</td>
<td>1.93</td>
<td>Allred et al. (2011)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Darunavir</td>
<td>600</td>
<td>1.33</td>
<td>1.09</td>
<td>1.45</td>
<td>Samineni et al. (2012)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Lopinavir</td>
<td>400</td>
<td>1.71</td>
<td>1.25</td>
<td>2.14</td>
<td>Kiser et al. (2008)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>75</td>
<td>1.38</td>
<td>1.06</td>
<td>1.46</td>
<td>Pinheiro et al. (2012)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>300</td>
<td>1.71</td>
<td>1.18</td>
<td>2.02</td>
<td>Pinheiro et al. (2012)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Ezetimibe</td>
<td>10</td>
<td>1.29</td>
<td>1.01</td>
<td>1.30</td>
<td>Koosoglou et al. (2004)</td>
<td>BCRP inhibition</td>
</tr>
<tr>
<td>Fenofibrate/ fenofibrinic acid</td>
<td>67</td>
<td>1.15</td>
<td>1.01</td>
<td>1.16</td>
<td>Martin et al. (2003)</td>
<td>NA</td>
</tr>
<tr>
<td>Flucanazole</td>
<td>200</td>
<td>None</td>
<td>None</td>
<td>1.10^{b}</td>
<td>Cooper et al. (2002)</td>
<td>CYP2C9 inhibition</td>
</tr>
</tbody>
</table>

NA, not applicable.

^{a} The clinically observed fold-increase in exposure used for comparison with the prediction is the mean AUC change derived from non-Asian subjects within the clinical interaction study.

^{b} Based upon inhibition of CYP2C9 (f_{I} = 0.10, flucanazole CYP2C9 K_{I} = 7 μM; Kunze et al., 1996).
In conclusion, solitary inhibition of the intestinal BCRP efflux transporter can result in a clinically significant DDI with rosuvastatin causing a maximum 2-fold increase in exposure, which may warrant statin dose adjustment or dose capping in clinical practice.

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Authorship Contributions

Participated in research design: Elsby, Surin, Martin.

Conducted experiments: Elsby, Sharma, Surin.

Performed data analysis: Elsby, Sharma, Surin, Martin, Fenern.

Wrote or contributed to the writing of the manuscript: Elsby, Martin, Surin, Sharma, Fenern.

References


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