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,1 Paul Martin, Dominic Surry,2 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, fostamatinib inhibited BCRP (IC_{50} = 50 nM) and organic anion-transporting polypeptide 1B1 (OATP1B1; IC_{50} > 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 fostamatinib and rosvastatin was performed (and reported elsewhere). This confirmed the critical role BCRP plays in statin absorption, as inhibition by fostamatinib resulted in a significant 1.96-fold and 1.88-fold increase in rosvastatin area under the plasma concentration–time curve (AUC) and C_{max}, 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 (IC_{50}) 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 ([I_{gut,max}]), maximum unbound hepatic inlet concentration, transporter fraction excreted value, and determined IC_{50} 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 rosvastatin, 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 c.421C>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 diflomotecan (Sparreboom et al., 2004; Keskitalo 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 (C_{max})], 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; DMSO, dimethylsulfoxide; HBSS, Hanks’ 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[4,3-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-pyridinyl]-3-(3,4,5-trimethoxyphenyl)-1,4-pyrindinediamine.
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>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 a 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 (\( \leq 10\% \)).

Fig. 1. Critical disposition pathways of rosuvastatin as described by Elsby et al. (2012).
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

**Fig. 2.** Inhibition of BCRP-, OATP1B1-, and OAT3-mediated probe substrate transport activity by fostamatinib, R406, and positive control inhibitors. Data are expressed as the mean (n = 3 wells or n ≥ 5 oocytes).

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TABLE 1
Summary of fostamatinib (100 mg) dose-related concentration and inhibitory kinetic parameters for calculation of [I]/Kᵢ ratios and R-value

<table>
<thead>
<tr>
<th>Transporter</th>
<th>Iₑ</th>
<th>Iₑ/Kᵢ</th>
<th>Iₑ/Kᵢ</th>
<th>Iₑ/Kᵢ</th>
<th>Iₑmax u</th>
<th>R-Value</th>
<th>Potential for DD (Rₑ/Kᵢ ≥ 0.1, Iₑ/Kᵢ ≥ 10, R-value ≥ 1.25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCRP</td>
<td>1.6⁺</td>
<td>691</td>
<td>0.050⁰</td>
<td>32</td>
<td>13,820</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>1.6⁺</td>
<td>NA &gt; 10</td>
<td>0.16</td>
<td>NA</td>
<td>0.284</td>
<td>1.03</td>
<td>No</td>
</tr>
</tbody>
</table>

Rₑ, mean steady-state maximum plasma concentration (Cmax) value for total systemic drug (R406); Iₑ, maximal theoretical gastrointestinal concentration of R788 prodrug [calculated from dose (mol)/250 ml]; Iₑmax u, maximum unbound liver inlet concentration, calculated as (Iₑ × Iₑ/Kᵢ + Fₑ × kₑ × dose/Qh), where Iₑ is the fraction unbound (R406 0.18), Fₑ is the fraction absorbed (as default taken to be 1.0), kₑ is the absorption rate constant (as default taken to be 0.01 min⁻¹), and Qh is hepatic blood flow (1500 ml/min); kₑ absolute inhibition constant (equates to IC₅₀ in these assays as probe substrate concentration used is < < < kₑ); NA, not applicable.

⁺Derived from plasma concentration data determined from multiple clinical studies.

*Fostamatinib (R788).

**R406.

Inhibition of Intestinal BCRP Increases Rosuvastatin Exposure

Materials and Methods

Materials. Estrone 3-sulfate, sulfasalazine, estradiol 17β-glucuronide, rifamycin SV, Triton X, novobiocin, 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-dimethylthyl ester), cyclosporin A, pantoprazole, diclofenac, nefopam, clofuroxolone, and fenofibrate were purchased from Sigma-Aldrich (St. Louis, MO; Poole, Dorset, UK), Ektrombopag, darunavir, and lovopin were purchased from Selleck Chemicals (Houston, TX; Shanghai, China). FhuVastatin was obtained from Sequoia Research Products (Pangbourne, Reading, UK). [3H]rosuvastatin was custom synthesized by Metoprolol, rosuvastatin, atorvastatin, and ezetimibe were supplied by AstraZeneca (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 Polyt-lysine 24-well multiwell plates, human BCRP (ABCC2) membrane vesicles, human OAT (SLC22A)–expressing Xenopus laevis oocytes, and control (water-injected) oocytes were supplied by BD Biosciences Discovery Labware (Woburn/Bedford, MA; Oxford, UK). UniFilter-96 GF/B filter plates and TopSeal A adhesive sealing film were supplied by PerkinElmer Life and Analytical Sciences (Boston, MA; Buckinghamshire, UK). [3H]methotrexate and methotrexate were purchased from American Quotient Bioresearch (Cardiff, 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 etizolame were supplied by AstraZeneca Compound Management 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 Technolog-ies (Paisley, UK; Grand Island, NY). All other chemicals, solvents, and reagents were purchased from Sigma-Aldrich.

Uptake solutions contained 1% (v/v) DMSO.

In Vitro OAT3 Inhibition Assessment of R406. Uptake of the OAT3 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-OAT3B1 cells in the absence and presence of R406 (0.3–10 μM) and the positive control inhibitor rifamycin SV (0.001–100 μM). Incubations (triplicate wells per condition) were conducted in HBSS buffer (containing 10 mM HEPES, pH 7.4) at 37°C for 2 minutes, following a 15-minute preincubation with test inhibitor. Uptake solutions contained ≤1% (v/v) DMSO.

In Vitro OAT3 Inhibition Assessment of R406. Uptake of the OAT3 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-OAT3B1 cells in the absence and presence of R406 (0.3–10 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 ≤1% (v/v) DMSO.

Data Analysis. In brief, the amount of radioactivity (disintegrations per minute) taken up by vesicles, HEK293-OAT1B1 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; Elsby et al., 2011b). Background condition uptake rate (uptake in BCRP vesicles minus ATP, uptake in HEK293-OAT1B1 cells in the presence of 100 μM positive control inhibitor where OAT1B1 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 concentration that produces half-maximal inhibition of probe substrate transport [IC₅₀].

Clinical Interaction Study between Fostamatinib and Rosuvastatin. Recently, a clinical interaction study (NCT01725230, D4300C00039) 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.
into the following adapted Rowland-Matin mechanistic static equation, as 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 rosuvastatin AUC based upon inhibition of a fraction excreted \( f_e \) value of 0.5 for intestinal BCRP:

\[
\text{Fold } \Delta \text{AUC} = \frac{1}{1 + \frac{K_i}{[I]}} (1 - f_e)
\]

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 \( (\text{C}_{\text{max}} \text{ent}) \) as described by Agarwal et al., 2013.

Additionally, the predicted change in rosuvastatin 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 \( \mu \text{M} \) 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 \( (IC_{50} = 0.61 \mu \text{M}) \) passed the acceptance criteria set during the assay validation (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 \( \mu \text{M} \), 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 \( (IC_{50} = 0.02 \mu \text{M}) \) or sulfasalazine \( (IC_{50} = 4.3 \mu \text{M}) \) 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 \( IC_{50} > 10 \mu \text{M} \) (27% inhibition at 10 \( \mu \text{M} \); 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 \( C_{\text{max}} \) 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 \([I]/K_i\) ratio predicted that there was a potential for a DDI with BCRP substrate drugs in vivo through inhibition of intestinal BCRP. In contrast, R406 was unlikely to cause a DDI through inhibition of 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 rosuvastatin (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 \( C_{\text{max}} \) of rosuvastatin 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 \( \mu \text{M} \)) exhibited suitable BCRP-mediated efflux in the Caco-2 cell test system: mean ± S.D. \((n = 3 \text{ wells})\) A-B \( P_{\text{app}} = 0.39 ± 0.05 \) and B-A \( P_{\text{app}} = 15.6 ± 1.75 \times 10^{-6} \text{ cm/s} \), 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 Papp 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 IC₅₀ 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 IC₅₀ curves are shown in Fig. 4, which also shows that metoprolol did not inhibit BCRP-mediated transport of rosuvastatin. The mean IC₅₀ calculated for the positive control inhibitor novobiocin was 1.4 ± 0.5 μM (precision = 36%), giving an acceptance range of ±2.9 μM for future quality control of the assay (derived from the mean ± 3 S.D.). For all BCRP inhibitors tested, the IC₅₀ values determined from individual experimental occasions (Table 2) gave good interassay reproducibility, demonstrating that future assessment of compounds as inhibitors of BCRP in this test system can be acceptably performed just once, and need not be repeated multiple times. The validated inhibition assay was determined to be robust, reproducible, and fit for the routine evaluation of compounds as inhibitors of BCRP.

Predicted versus Observed AUC Changes for Known Clinical DDIs with Rosuvastatin Based upon Determined In Vitro Caco-2 BCRP Inhibitory Data. Eltrombopag, darunavir, lopinavir, clopidogrel, ezetimibe, and fenofibrate inhibited BCRP-mediated rosuvastatin transport with IC₅₀ values of 2.1, 75, 8.7, 63, 2.9, and 170 μM, respectively (Table 3). In HEK293-OATP1B1 cells, darunavir, lopinavir, clopidogrel, and ezetimibe inhibited OATP1B1-mediated estradiol 17β-glucuronide uptake with IC₅₀ values of 4.3, 0.43, 1.8, and 2.2 μM, respectively (Table 3). In contrast, fluconazole 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 that would occur as a consequence of these drugs’ inhibition of intestinal 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 IC₅₀ values from different occasions</th>
<th>Mean</th>
<th>S.D</th>
<th>CV (%)</th>
<th>Published IC₅₀/Ki (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>0.4</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>0.4</td>
<td>0.2</td>
<td>50</td>
<td>0.013 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Xia et al. (2007)</td>
</tr>
<tr>
<td>Cyclosporin A</td>
<td>5.5, 4.1, 1.6</td>
<td>3.7</td>
<td>2.0</td>
<td>54</td>
<td>6.7 (estrone 3-sulfate)</td>
<td>Vesicles</td>
<td>Xia et al. (2007)</td>
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<tr>
<td>Pantoprazole</td>
<td>8.5, 14.4, 5.9</td>
<td>9.6</td>
<td>4.4</td>
<td>46</td>
<td>13 (methotrexate)</td>
<td>Vesicles</td>
<td>Breedveld et al. (2004)</td>
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<tr>
<td>Sulfasalazine</td>
<td>2.7, 4.7, 1.4</td>
<td>2.9</td>
<td>1.7</td>
<td>59</td>
<td>0.74 (estrone 3-sulfate)</td>
<td>Vesicles</td>
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<td>Atorvastatin</td>
<td>0.5, 0.8, 0.4</td>
<td>0.6</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>3.6</td>
<td>18</td>
<td>78 (methotrexate)</td>
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<td>Lagas et al. (2009)</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>1.3, 1.1, 0.9</td>
<td>1.1</td>
<td>0.2</td>
<td>18</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>2.5</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 $[^3H]rosuvastatin$ (1 μM) 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.
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 rosuvastatin (Kruitzjer et al., 2002; Elsby et al., 2012). Studying the inhibitory properties and pharmacokinetic parameters of drugs coadministered with rosuvastatin in a clinical interaction study (Martin et al., 2016). Furthermore, the magnitude of the observed exposure change is consistent with a doubling in rosuvastatin absorption, as perpetrating pharmacokinetic DDIs upon coadministration with 

Table 3: Inhibitory properties and pharmacokinetic parameters of drugs coadministered with rosuvastatin in a clinical interaction study

<table>
<thead>
<tr>
<th>Perpetrator Drug</th>
<th>Dose</th>
<th>MW</th>
<th>[I]</th>
<th>BCRP Kᵢ</th>
<th>[I]/Kᵢ Ratio</th>
<th>Cmax total</th>
<th>Cmax u</th>
<th>fₘ</th>
<th>OATP1B1 Kᵢ</th>
<th>Bilary BCRP</th>
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<tr>
<td>Fostamatinib</td>
<td>100</td>
<td>578.52</td>
<td>691</td>
<td>0.05</td>
<td>13,820</td>
<td>58</td>
<td>1.9</td>
<td>0.018</td>
<td>0.289</td>
<td>&gt;10</td>
</tr>
<tr>
<td>R406</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
<td>1.0</td>
<td>0.003</td>
<td>0.236</td>
<td>2.7</td>
</tr>
<tr>
<td>Darunavir</td>
<td>600</td>
<td>547.73</td>
<td>4382</td>
<td>75</td>
<td>58</td>
<td>29</td>
<td>17.7</td>
<td>0.05</td>
<td>1.261</td>
<td>4.3</td>
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<tr>
<td>Lopinavir</td>
<td>400</td>
<td>628.8</td>
<td>2545</td>
<td>8.7</td>
<td>59</td>
<td>74</td>
<td>10.6</td>
<td>0.05</td>
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<td>0.02</td>
</tr>
<tr>
<td>Clopidogrel</td>
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<td>321.9</td>
<td>932</td>
<td>63</td>
<td>15</td>
<td>78</td>
<td>0.0099</td>
<td>0.02</td>
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<td>1.8</td>
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<tr>
<td>Emetine</td>
<td>10</td>
<td>409.4</td>
<td>98</td>
<td>2.9</td>
<td>34</td>
<td>2.4</td>
<td>0.014</td>
<td>0.10</td>
<td>0.050</td>
<td>2.2</td>
</tr>
<tr>
<td>Fenofibrate/fofenibrin acid</td>
<td>67</td>
<td>360.83</td>
<td>743</td>
<td>170</td>
<td>4.4</td>
<td>62</td>
<td>25.8</td>
<td>0.01</td>
<td>0.398</td>
<td>20</td>
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<tr>
<td>Fluconazole</td>
<td>200</td>
<td>306.3</td>
<td>2612</td>
<td>No inhibition</td>
<td>NA</td>
<td>218</td>
<td>34.6</td>
<td>0.89</td>
<td>69,536</td>
<td>NA</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; fₘ, fraction unbound (taken from the drug label accessed via the Pharmapendium database (http://www.pharmapendium.com)); [I]ᵢ, maximal theoretical gastrointestinal concentration (calculated from dose [mol]/250 ml); [I]inlet max, maximum enterocyte concentration, calculated as ([Cmax total] × dose/Qent), where Fᵢ is the fraction absorbed (as default taken to be 1.0), [I]ᵢ is the absorption rate constant (as default taken to be 0.1 min⁻¹), and Qent is the enterocyte blood flow (300 ml/min; Dong et al., 2013); [I]gut max, maximum unbound liver inlet concentration, calculated as [I]ᵢ × [(Cmax total) × (Fᵢ × [dose/Qent])], where Fᵢ is the fraction absorbed (as default taken to be 1.0), [I]ᵢ is the absorption rate constant (as default taken to be 0.1 min⁻¹), and Q₀ is hepatic blood flow (1500 ml/min); Kᵢ, absolute inhibition constant (assuming competitive inhibition); equates to IC₅₀ in these assays as probe substrate concentration used is < < < < < Kᵢ; MW, molecular weight (taken from the drug label accessed via Pharmapendium database (http://www.pharmapendium.com)); NA, not applicable.

Discussion

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 rosuvastatin (Kruitzjer 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 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). However, rosuvastatin 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; [I]ᵢ/Kᵢ = 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 rosuvastatin. Such a study would confirm the critical role BCRP plays in rosuvastatin absorption by evaluating, through DDI, the impact solitary inhibition of BCRP has on statin exposure. Although rosuvastatin was not used as the in vitro probe to assess OATP1B1 and BCRP inhibition potential, estradiol 17β-glucuronide is a surrogate for OATP1B1-mediated rosuvastatin transport (Izumi et al., 2015), and for BCRP, even if a fostamatinib IC₅₀ value using rosuvastatin 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 rosuvastatin exposure (AUC and Cmax). Examination of rosuvastatin concentration–time profiles (Fig. 3) with and without fostamatinib suggested that there was no change in rosuvastatin 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 rosuvastatin half-life (15.7 vs. 14.2 hours, respectively), these data indicated that the DDI with fostamatinib was attributable to increased rosuvastatin 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 rosuvastatin absorption, which not only suggests complete inhibition of intestinal BCRP (Kᵢ = 0.5 mM) by fostamatinib (conforming to Kᵢ being > 1000 times lower than anticipated intestinal concentration), but also confirms that BCRP efflux restricts absorption of rosuvastatin to 50% in humans. Eltrombopag, darunavir, lopinavir, clopidogrel, ezetimibe, fenofibrate, and fluconazole are drugs that are listed on the CRESTor FDA label as perpetrating pharmacokinetic DDIs upon coadministration with rosuvastatin. Of the rosuvastatin disposition pathways, OAT3 inhibition is unlikely to contribute to these DDIs because darunavir and fluconazole do not inhibit OAT3 in vitro, and eltrombopag (Kᵢ = 7.8 μM), lopinavir (Kᵢ > 10 μM), and fenofibrate acid (Kᵢ = 2.2 μM) are not predicted to inhibit OAT3 in vivo based on Cmax Kᵢ 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 (Kᵢ = 25.6 μM), darunavir (Kᵢ = 4.51 μM), and ezetimibe (Kᵢ > 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; Vldhede et al., 2014). Finally, inhibition of sodium/taurocholate cotransporting peptide (NTCP) can be ruled out as a contributory factor since 1) despite being an NTCP inhibitor in vitro (Kᵢ = 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 (Vldhede 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 fluconazole, 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]_{gut}/K_{i} ratios >10; Table 3). Interestingly, none of these inhibitors were anticipated to inhibit biliary BCRP in vivo (ratios of [I]_{inlet}/K_{i} 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 fluconazole) 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, clopidogrel, ezetimibe, and fenofibrate (Table 4).

Predictions of AUC change were performed using determined IC_{50} values and the adapted Rowland-Matin equation, as described by Elsby et al. (2012). Previously, a maximum 2-fold increase in exposure due to complete inhibition of intestinal BCRP efflux was predicted when ([I]_{gut}/K_{i}) >10. However, such an approach likely overpredicts the impact due to BCRP inhibition (particularly for those DDIs where the increase in observed AUC is <2-fold) because of high [I]_{gut} values, which may not reflect true concentrations once intestinal solubility is accounted for. An alternative parameter, maximum inhibitor concentration in the enterocyte ([I]_{gut} maximum), may be a more accurate representation of intestinal concentration as it is independent of solubility (Agarwal et al., 2013) (see Table 3 for equation). Consequently, in this study, we explored the use of ([I]_{gut} maximum) in the adapted Rowland-Matin equation, alongside a BCRP IC_{50} of 0.5, to establish whether taking into account varying degrees of intestinal BCRP inhibition (rather than assuming 100% inhibition) resulted in more accurate predictions of clinical exposure. Comparison of predicted increases in rosuvastatin AUC due to inhibition of composite transporter pathways BCRP and OATP1B1 confirmed intestinal BCRP inhibition to be the primary mechanism underlying each DDI; as for each individual drug, the AUC increase due to BCRP was higher than that attributed to OATP1B1 inhibition alone (Table 4). Additionally, all of the predicted AUC increases due to only OATP1B1 inhibition were ≤1.25. Overall, calculated fold increases in AUC (the product of the major AUC change from BCRP with the minor from OATP1B1) accurately predicted the clinically observed dose-dependent fold increases in rosuvastatin exposure due to clopidogrel and those increases due to 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} maximum) 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 (Alfred 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 fosmatatinib) 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 (mg)</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>Fostamatinib/R406</td>
<td>100</td>
<td>2.00</td>
<td>1.01</td>
<td>2.02</td>
<td>1.96</td>
<td>Martin et al. (2016)</td>
</tr>
<tr>
<td>Eltrombopag</td>
<td>75</td>
<td>1.87</td>
<td>1.03</td>
<td>1.93</td>
<td>1.88</td>
<td>Allred et al. (2011)</td>
</tr>
<tr>
<td>Darunavir</td>
<td>600</td>
<td>1.33</td>
<td>1.09</td>
<td>1.45</td>
<td>1.48</td>
<td>Samineni et al. (2012)</td>
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<tr>
<td>Lopinavir</td>
<td>400</td>
<td>1.71</td>
<td>1.25</td>
<td>2.14</td>
<td>2.10</td>
<td>Kiser et al. (2008)</td>
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<tr>
<td>Clopidogrel</td>
<td>75</td>
<td>1.38</td>
<td>1.06</td>
<td>1.46</td>
<td>1.40</td>
<td>Pinheiro et al. (2012)</td>
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<tr>
<td>Clopidogrel</td>
<td>300</td>
<td>1.71</td>
<td>1.18</td>
<td>2.02</td>
<td>1.96</td>
<td>Pinheiro et al. (2012)</td>
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<tr>
<td>Ezetimibe</td>
<td>10</td>
<td>1.29</td>
<td>1.01</td>
<td>1.30</td>
<td>1.21</td>
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<td>Fenofibrate/fenofibric acid</td>
<td>67</td>
<td>1.15</td>
<td>1.01</td>
<td>1.16</td>
<td>1.07</td>
<td>Martin et al. (2003)</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>200</td>
<td>None</td>
<td>None</td>
<td>1.10α</td>
<td>1.14</td>
<td>Cooper et al. (2002)</td>
</tr>
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</table>

NA, not applicable.

*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.

*Based upon inhibition of CYP2C9 (f_{i} = 0.10, fluconazole CYP2C9 K_{i} = 7 μM; Kunze et al., 1996).
In AUC were ≥1.2-fold, six (50%; Table 4) can be attributed to BCRP inhibition as the primary mechanism, two (17%; cyclosporine and atazanavir) can be attributed to complete inhibition of BCRP combined with inhibition of OATP1B1, and one (8%; gemfibrozil) can be attributed to the inhibition of both OATP1B1 and OAT3. The remaining three DDIs (tipranavir, dronedarone, and itraconazole) have not been studied, and their mechanisms wait to be determined. These findings confirm the importance of intestinal BCRP inhibition toward manifesting clinical DDIs with rosuvastatin.

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, Surry, Martin.
Conducted experiments: Elsby, Sharma, Surry.
Performed data analysis: Elsby, Sharma, Surry, Martin, Fener.
Wrote or contributed to the writing of the manuscript: Elsby, Martin, Surry, Sharma, Fener.

References

Dhillon@ (flucloxacilone) U.S. Food and Drug Administration drug label, revised March 2014; Pfizer Inc. Accessed via Pharmadup endpoint (www.pharmadup.com) on September 10, 2014.


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