Investigating the Enteroenteric Recirculation of Apixaban, a Factor Xa Inhibitor: Administration of Activated Charcoal to Bile Duct-Cannulated Rats and Dogs Receiving an Intravenous Dose and Use of Drug Transporter Knockout Rats

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ABSTRACT

The study described here investigated the impact of intestinal excretion (IE), enteroenteric recirculation (EER), and renal tubule recirculation (RTR) on apixaban pharmacokinetics and disposition. The experimental approaches involve integrating apixaban elimination pathways with pharmacokinetic profiles obtained from bile duct-cannulated (BDC) rats and dogs receiving i.v. doses together with oral administration of activated charcoal (AC). Additionally, the role of P-gp (P-glycoprotein; abcb1) and BCRP (breast cancer resistance protein; abcg2) in apixaban disposition was evaluated in experiments using transporter inhibitors and transporter knockout (KO) rats. Approximately 20–50% of an apixaban i.v. dose was found in feces of BDC rats and dogs, suggesting IE leading to fecal elimination and intestinal clearance (IC). The fecal elimination, IC, and systemic clearance of apixaban were increased upon AC administration in both BDC rats and dogs and were decreased in BDC rats dosed with GF-120918, a dual BCRP and P-gp inhibitor. BCRP appeared to play a more important role for absorption and intestinal and renal elimination of apixaban than P-gp in transporter-KO rats after oral and i.v. dosing, which led to a higher level of active renal excretion in rat than other species. These data demonstrate that apixaban undergoes IE, EER, and RTR that are facilitated by efflux transporters. Intestinal reabsorption of apixaban could be interrupted by AC even at 3 hours post-drug dose (late charcoal effect). This study demonstrates that the intestine is an organ for direct clearance and redistribution of apixaban. The IE, EER, and RTR contribute to overall pharmacokinetic profiles of apixaban. IE as a clearance pathway, balanced with metabolism and renal excretion, helps decrease the impacts of intrinsic (renal or hepatic impairment) and extrinsic (drug-drug interactions) factors on apixaban disposition.

Introduction

Apixaban (Fig. 1), 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide, a highly selective, oral, direct inhibitor of Factor Xa, a protease enzyme that plays a pivotal role in the coagulation cascade, is a newly approved anticoagulant (Lassen et al., 2007; Connolly et al., 2011; Granger et al., 2011). Following oral administration of [14C]apixaban in humans, both metabolism (25%) and urinary excretion (27%) were important clearance pathways (Raghavan et al., 2009; Zhang et al., 2009a). Approximately 20–50% of an apixaban i.v. dose was found in feces of BDC rats and dogs, suggesting IE leading to fecal elimination and intestinal clearance (IC). The fecal elimination, IC, and systemic clearance of apixaban were increased upon AC administration in both BDC rats and dogs and were decreased in BDC rats dosed with GF-120918, a dual BCRP and P-gp inhibitor. BCRP appeared to play a more important role for absorption and intestinal and renal elimination of apixaban than P-gp in transporter-KO rats after oral and i.v. dosing, which led to a higher level of active renal excretion in rat than other species. These data demonstrate that apixaban undergoes IE, EER, and RTR that are facilitated by efflux transporters. Intestinal reabsorption of apixaban could be interrupted by AC even at 3 hours post-drug dose (late charcoal effect). This study demonstrates that the intestine is an organ for direct clearance and redistribution of apixaban. The IE, EER, and RTR contribute to overall pharmacokinetic profiles of apixaban. IE as a clearance pathway, balanced with metabolism and renal excretion, helps decrease the impacts of intrinsic (renal or hepatic impairment) and extrinsic (drug-drug interactions) factors on apixaban disposition.

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ABBREVIATIONS: AC, activated charcoal; apixaban, 1-(4-methoxyphenyl)-7-oxo-6-[4-(2-oxopiperidin-1-yl)phenyl]-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide; AUC, area under the plasma concentration versus time curve; BCRP (abcg2), breast cancer resistance protein; BDC, bile duct cannulation; CL, clearance; CLint, intestinal clearance; CLmet, metabolism clearance; CLr, biliary clearance; EER, enteroenteric recirculation; EHR, enterohepatic recirculation; F, bioavailability; GF-120918, N-[4-[2-(3,4-dihydro-6,7-dimethoxy-2(1H)-isoquinolinyl]ethyl][phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridinecarboxamide; GI, gastrointestinal tract; HP, β-CD, hydroxypropyl-β-cyclodextrin; HPLC, high-performance liquid chromatography; IC, intestinal clearance; IE, intestinal excretion; KO, knockout; LC/MS, liquid chromatography-mass spectrometry; MRT, mean residence time; P-gp, permeability-glycoprotein; RTR, renal tubule recirculation; T_{max}, time to reach peak concentration; WT, wild type.
transporters and drug-metabolizing enzymes are heterogeneously expressed in the intestine, affecting drug absorption and bioavailability (Suzuki and Sugiyama, 2000; Kaminsky and Zhang, 2003; Paine et al., 2006). P-gp (P-glycoprotein; abcb1) and BCRP (breast cancer resistance protein; abcg2), two members of the ABC transporter superfamily, are highly expressed at the luminal brush border membranes of intestinal enterocytes, proximal tubular membranes in the kidney, and canalicular membranes of hepatocytes in the liver and play an important role in drug absorption, tissue distribution, and drug elimination (Maliepaard et al., 2001; Doyle and Ross, 2003; Unadkat et al., 2004; Mao and Unadkat, 2005; Krishnamurthy and Schuetz, 2006; Huls et al., 2008; Koshiba et al., 2008; Zhou, 2008; Vlaming et al., 2009; Giacomini et al., 2010). In human, the MDR1 (P-gp) gene encodes a single drug transporting P-gp, where in mice and rats, the mdr1a and mdr1b genes encode two P-glycoprotein isoforms, but mdr1b is not expressed in mouse intestine (Thiebaut et al., 1987; Schinkel et al., 1994). The expression of intestinal efflux transporters is region dependent, with the expression of P-gp generally reported to increase from proximal duodenum to distal ileum in rats and humans (Mouly and Paine, 2003; Englund et al., 2006; Berggren et al., 2007; Hilgendorf et al., 2007; MacLean et al., 2008; Haslam et al., 2011). Both BCRP expression and its efflux function reached to peak in the ileum in rat and mouse intestines (Enokizono et al., 2007; Haslam et al., 2011); however, BCRP appeared to have the greatest expression in the duodenum along the human gastrointestinal tract (Gutmann et al., 2005). The expression of P-gp and BCRP is organ and species dependent, with a lower expression of BCRP in the kidney of human than rat and a higher expression of BCRP than P-gp in human intestines (Taipalensuu et al., 2001). Similar pharmacokinetic results in the P-gp-knockout (KO) and BCRP-KO rat and mouse models were recently reported for different P-gp and BCRP substrates (Bundgaard et al., 2012). In addition, the knockout of P-gp or BCRP did not affect expression of the other transporter in the mouse (Agarwal et al., 2012).

Previous data with apixaban demonstrated that the drug was a substrate for efflux transporters P-gp and BCRP (Zhang et al., 2013). In this study, we investigated the impacts of intestinal excretion (IE) and enteroenteric recirculation (EER) as well as renal tubule recirculation (RTR) (Fig. 2) on apixaban disposition through analysis of fecal excretion and pharmacokinetic profiles in bile duct-cannulated (BDC) rats, P-gp-KO and BCRP-KO rats, and dogs following i.v. administration of radiolabeled apixaban. Furthermore, the effect of AC on orally administered apixaban pharmacokinetics in the dog and human was considered.

Materials and Methods

Materials. Apixaban and [14C]apixaban (47.94 μCi/mg) (the structure is shown in Fig. 1) were prepared at Bristol-Myers Squibb (Princeton, NJ). The chemical and radioactive purity of [14C]apixaban were >98%. Activated charcoal (AC; EZChar) was purchased from Paddock (Minneapolis, MN). Chemicals used for dose formulations, propylene glycol, PEG-400, ethanol, and hydroxypropyl-β-cyclodextrin (HP-β-CD), N-methyl-2-pyrrolidone were obtained from Sigma-Aldrich (St. Louis, MO). Ultima Gold, Ultima Flo M, Monophasic S, Carbo-Sorb E, PermaFluor E; liquid scintillation cocktails, and Combust-o-cones and pads were obtained from PerkinElmer Life Sciences (Waltham, MA). Ecolite liquid scintillation cocktail was purchased from MP Biomedicals, Inc. (Irvine, CA). Solvents used for chromatographic analysis were high-performance liquid chromatography (HPLC) grade and purchased from EMD Chemicals (Gibbstown, NJ). All other reagents were of analytical grade.

Dose Preparations. The formulation vehicles are listed in Table 1. The oral dose formulation of apixaban for dogs was prepared at 1 mg/ml in 0.5% Tween 80 in Labrafri. The i.v. dose formulation of [14C]apixaban for dogs was prepared at 9.6 μCi/mg and 2 mg/ml in 30% HP-β-CD in 10 mM phosphate buffer (pH 7.2). The i.v. dose formulation of [14C]apixaban for rats was prepared at 47.94 μCi/mg and 2 mg/ml in 30% HP-β-CD in 10 mM phosphate buffer (pH 7.2). The oral dose formulation of apixaban for rats was prepared at 4 mg/ml in 30% HP-β-CD in 10 mM phosphate buffer (pH 7.2). The GF-120918 (elacridar) dose solution was prepared by dissolving 21 mg of the powder in 4.2 ml of dose vehicle consisting of N-methyl-2-pyrrolidone in saline water (1:1, v/v). AC dose suspension was prepared in water at 250 or 208 mg/ml. The mixture was subsequently shaked or homogenized using a polytron homogenizer for 10 minutes.

Animal Preparation, Dosing, and Sample Collection. All animal housing and care conformed to the standards recommended by the Guide for the Care and Use of Laboratory Animals. Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, IN). Wild-type (WT), P-gp (abcb1)-KO, or BCRP (abcg2)-KO Sprague-Dawley rats were purchased from SAGE Laboratory (Sigma-Aldrich). The rats were fed with Certified Rodent Diet #5002 (PMI Nutrition International, Brentwood, MO) and water was provided ad libitum. Non-naive male beagle dogs (7-11 kg) were purchased from Marshall Bioresources (North Rose, NY). The dogs were fed with Canine Diet #5006 (LabDiet; PMI Nutrition International) and water was provided ad libitum. Male Sprague-Dawley WT rats, P-gp-KO rats, and BCRP-KO rats, weighing 249–349 g at the time of dosing, were surgically implanted with catheters in the jugular and/or femoral veins and bile duct-cannulated for i.v. dosing, blood sampling, and bile collection (BDC). The BDC surgery of rats was performed 1 week prior to dosing. The bile duct-cannulated male dogs (9–14 kg) were maintained at the Bristol-Myers Squibb facility.

Prior to dosing each animal (rat or dog) was weighed, randomized, and assigned a permanent identification number. During the acclimation period (at least 2 days prior to study initiation), the animals were housed in individual, suspended, stainless steel wire mesh cages. The animals were fasted overnight (at least 12 hour) prior to and through 4 hours following dose administration. The animal room was controlled to maintain a temperature of 75°F and 70% relative humidity, with a 12-hour light/dark cycle. The body weight of each animal was determined on the day before dosing. The doses administered were calculated based on the body weight of each animal. The formulations were administered to fasted animals from each group by oral gavage using a syringe with a gavage needle to deliver a dose at a target dose level or by intravenous doses. The amount of dose administered to each animal was determined by the difference in weights of the loaded dose syringe and needle prior to dose administration and the emptied syringe and needle after dose administration.

Apixaban (5 mg/kg, 1 mg/ml in 0.5% Tween 80 in Labrafri) was administered via oral gavage to 4 male beagle dogs followed by a 10 ml flush of water. AC (250 mg/kg, 208 mg/ml water suspension) was administered via oral gavage in three separate treatments in a parallel crossover design followed by a 10 ml flush of water at 0.25, 1, or 3 hours postdose. Male dogs (intact n = 3 or BDC n = 2) were dosed via i.v. infusion (5 minutes) of [14C]apixaban (1 mg/kg, 0.5 ml/kg). AC suspensions were administered to BDC dogs at 250 mg/kg via oral gavage at 10, 90, 180, and 420 minutes after the [14C]apixaban dosing. Apixaban (4 mg/kg) was administered to WT, GF-120918-pretreated WT, P-gp-KO, and BCRP-KO rats via oral gavage. [14C]Apixaban (2 mg/kg) was administered to BDC WT, GF-120918-pretreated WT, P-gp-KO, and BCRP-KO rats via i.v. bolus administration. GF-120918 was administered orally (10 mg/kg, 2 ml/kg, 5 mg/ml) at 1 hour before apixaban dose in intact rats or at 1 hour before and...
8 hours after \(^{14C}\)apixaban dosing in BDC rats. AC suspensions were also administered to BDC rats at 250 mg/kg via implanted duodenal catheters at 30 minutes before and 5, 90, and 180 minutes after the \(^{14C}\)apixaban dosing.

Blood was collected at various time points (K\(_2\)EDTA as an anticoagulant) via venipuncture of a peripheral blood vessel in dogs and femoral veins catheters from rats. These samples were placed on ice immediately after collection and centrifuged within 30 minutes of collection to harvest plasma. Urine, bile, and feces were collected as described in Table 2.

Radioactivity Determination. Triplicate aliquots of the diluted dose solutions, plasma, urine, bile, and cage wash were analyzed for radioactivity concentrations. Radioactivity determinations were made with a Tri-Carb Model 3100 TR liquid scintillation counter (PerkinElmer) using 5 ml of scintillation fluid (Ultima Gold) per sample or 15 ml of Ecolite.

Fecal samples were homogenized in four volumes of water. Triplicate aliquots (0.40–1.1 g) of fecal and gastrointestinal tract (GI) tract homogenates were placed into preweighed Combusto-cones and pads and allowed to air-dry overnight. The samples were then washed and analyzed for radioactivity. A model 307 Tri-Carb Sample Oxidizer, equipped with an Oximate-80 Robotic Automatic Sampler (PerkinElmer), was used for combustion of fecal homogenates. The \(^{14}CO_2\) generated samples from dosing of \(^{14C}\)apixaban was trapped in Carbo-Sorb E (7 ml) and mixed with PermaFluor E+ (8 ml) as the scintillator to determine radioactivity concentrations using a Tri-Carb Model 3100 TR liquid scintillation counter (PerkinElmer). The oxidation efficiency of the oxidizer was greater than 97%.

Metabolite Profiling. Metabolites in plasma, bile, and urine and fecal samples were profiled as described previously (Raghavan et al., 2009; Wang et al., 2011; Zhang et al., 2009a). Pooled plasma, urine, bile, or fecal homogenate samples were prepared by aliquoting appropriate volumes or weights of samples from each time interval. The pooled urine and bile samples were directly analyzed to obtain metabolite profiles following centrifugation at 1500g for 15 minutes at 4°C. The pooled fecal samples (about 1 ml) were mixed with 2 ml of methanol and shaken for 10 minutes. The mixtures were centrifuged at 1500g for 10 minutes at 4°C. The supernatants were transferred to clean tubes. The pellets were extracted twice with 2 ml of methanol (2 \times 2 ml). All supernatants were combined and evaporated to dryness under nitrogen, and the residues were reconstituted in 400 µl of 25% methanol in water. Metabolite profiling for pooled urine, bile, and feces, and dose analysis was performed on a Surveyor HPLC system equipped with a b-RAM radioactivity flow detector (Model 4; INUS Systems, Inc., Tampa, FL). The radioactivity detector, b-RAM, was equipped with a 100 µl flow cell and was operated using scintillation cocktail (Ultima Flo M) delivered at a flow rate of 1.0 ml/min. Chromatography was accomplished on a Phenomenex Luna C18, 4.6 \times 250 mm, 3 µm (Torrance, CA) for the apixaban studies. The mobile phase for apixaban sample profiling consisted of two solvents: A, 0.05% trifluoroacetic acid and B, 0.05% trifluoroacetic acid in acetonitrile; the gradient was as

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**TABLE 1**

<table>
<thead>
<tr>
<th>Species (Route)</th>
<th>Test Article</th>
<th>Vehicle</th>
<th>Dose Concentration</th>
<th>S.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog (oral)</td>
<td>Apixaban</td>
<td>0.5% Tween 80 in Labrafil</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>Dog (i.v.)</td>
<td>(^{14C})apixaban</td>
<td>30% HP-β-CD in 10 mM phosphate buffer (pH 7.2)</td>
<td>2</td>
<td>9.6</td>
</tr>
<tr>
<td>Rat (oral)</td>
<td>Apixaban</td>
<td>30% HP-β-CD in 10 mM phosphate buffer (pH 7.2)</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>Rat (i.v.)</td>
<td>(^{14C})apixaban</td>
<td>30% HP-β-CD in 10 mM phosphate buffer (pH 7.2)</td>
<td>2</td>
<td>47.94</td>
</tr>
<tr>
<td>Rat (oral)</td>
<td>GF-120918</td>
<td>30% HP-β-CD in 10 mM phosphate buffer (pH 7.2)</td>
<td>2</td>
<td>NA</td>
</tr>
<tr>
<td>Rat (i.v.)</td>
<td>GF-120918</td>
<td>N-methyl-2-pyridolone/saline (1:1)</td>
<td>5</td>
<td>NA</td>
</tr>
<tr>
<td>Rat, dog AC</td>
<td>Purified water</td>
<td>250/208</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

AC, activated charcoal; HP-β-CD, hydroxypropyl-β-cyclodextrin; NA, not applicable; S.A, specific activity.
follows: solvent B started at 5% and was maintained at 5% for 2 minutes, then linearly increased to 20% at 20.1 minutes, to 40% at 30 minutes, to 95% at 35 minutes, and to 5% at 36 minutes with a HPLC effluent of 0.7 ml/min.

An alternative HPLC system was a Hewlett Packard Agilent 1100 series system equipped with two pumps, an autosampler, a diode array detector, and an ACE 3 C18 column (4.6 mm × 150 mm). The mobile phase consisted of two solvents: A, 0.4% formic acid in water, pH 3.2, and B, 100% acetonitrile. The gradient was as follows: solvent B started at 5%, then linearly increased to 20% at 5 minutes, to 30% at 50 minutes, to 95% at 65 minutes, held at 90% for 2 minutes, and then decreased to 5% at 69 minutes. The HPLC effluent (0.7 ml/min) was collected into Deepwell LumaPlate-96 plates (PerkinElmer Life and Analytical Sciences, Shelton, CT) at 0.25-minute intervals for 75 minutes with a Gilson Model 204 fraction collector (Gilson Medical Electronics, Middleton, WI). The plates were dried with a Savant Speed-Vac (Savant Instruments, Farmingdale, NY). The column was reconstituted with deionized water using a Nano liquid chromatography (LC) system equipped with a Thermo TSQ Quantum double-focusing mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The mass analysis was performed on a liquid mass spectrometer operated under positive turbo-ionpray mode using the LTQ ion trap mass spectrometer (Thermo Scientific, San Jose, CA). Samples were analyzed with a noncompartmental method (Perrier and Gibaldi, 1982). The peak plasma concentration ($C_{\text{max}}$) and the time to reach peak concentration ($T_{\text{max}}$) were recorded directly from experimental observations. The area under the plasma concentration versus time curve from 0 to T ($AUC_{0-T}$), where T equals the time of the last measured plasma concentration, was calculated by a combination of conventional trapezoidal and log-trapezoidal methods by Kinetic v4.4 (Innolife Corp., Philadelphia, PA) or by WinNonlin (Pharsight, Mountain View, CA). AUC to infinity ($AUC_{\infty}$) was determined by the sum of $AUC_{0-T}$ and the extrapolated area. The extrapolated area was determined by dividing the last measured concentration by the slope of the terminal log-linear phase, which was <5% for all animal groups used in this study upon sample collection of sufficient data points. The terminal slope ($k$) of the plasma concentration-time profile was determined by the method of least squares (log-linear regression of at least three data points). The terminal half-life was estimated as $\ln 2/k$. The clearance via biliary excretion and metabolism ($CL_{\text{biliary+metabolism}}$) was estimated by using: $CL_{\text{biliary+metabolism}} = CL_{\text{total}} - CL_{\text{Int}} - CL_{R}$.

### Data Analysis

The plasma concentration versus time data for apixaban were analyzed with a noncompartmental method (Perrier and Gibaldi, 1982). The peak plasma concentration ($C_{\text{max}}$) and the time to reach peak concentration ($T_{\text{max}}$) were recorded directly from experimental observations. The area under the plasma concentration versus time curve from 0 to T ($AUC_{0-T}$), where T equals the time of the last measured plasma concentration, was calculated by a combination of conventional trapezoidal and log-trapezoidal methods by Kinetic v4.4 (Innolife Corp., Philadelphia, PA) or by WinNonlin (Pharsight, Mountain View, CA). AUC to infinity ($AUC_{\infty}$) was determined by the sum of $AUC_{0-T}$ and the extrapolated area. The extrapolated area was determined by dividing the last measured concentration by the slope of the terminal log-linear phase, which was <5% for all animal groups used in this study upon sample collection of sufficient data points. The terminal slope ($k$) of the plasma concentration-time profile was determined by the method of least squares (log-linear regression of at least three data points). The terminal half-life was estimated as $\ln 2/k$. The clearance via biliary excretion and metabolism ($CL_{\text{biliary+metabolism}}$) was estimated by using: $CL_{\text{biliary+metabolism}} = CL_{\text{total}} - CL_{\text{Int}} - CL_{R}$.

### Results

#### AC Studies

Apixaban plasma concentration-time profiles in dogs following a 5 mg/kg oral dose with and without subsequent single administration of AC are shown in Fig. 3, A and B, and single-dose pharmacokinetic parameters are shown in Table 3. The apixaban pharmacokinetic data were compared in the same dog for the control and AC treatment separated by 2 weeks. When AC was administered 0.25 (15 minutes), 1, and 3 hours after apixaban, apixaban $AUC_{0-24}$ was 24, 19, and 37%, respectively, lower compared with apixaban alone. Apixaban $C_{\text{max}}$ was not markedly affected by administration of AC at any of the time points. Apixaban $T_{\text{max}}$ was 5.6 hours when administered alone, but decreased to <4 hours when AC was administered 1 and 3 hours after apixaban, respectively, with a corresponding increase of the systemic clearance (29–66%) and decrease in the mean residence time (MRT) and the C24 values ($C_{\text{24}}$).

#### BDC Dog Studies

Table 4 lists the radioactive dose recovery from BDC dogs following i.v. administration of [14C]apixaban (1 mg/kg) with and without administration of AC. Approximately 40–50% of the dose was recovered in feces of BDC dogs, indicating the drug...
was directly excreted into the gut and eliminated in the feces. Upon treatment with AC, the fecal recovery was increased in BDC dogs with no marked effect on the urinary elimination, and apixaban AUC was reduced by approximately 19–39% and apparent systemic clearance of apixaban was increased by 20–60% (Fig. 4A; Table 3). The increased fecal elimination with AC in BDC dogs corresponded to a >50% increase in intestinal clearance (IC), suggesting that AC prevents reabsorption of apixaban and resulted in the increases of IE through interruption of enteroenteric recirculation. In dogs, the peak-to-trough concentration ratio (C_{max}/C_{24}) after oral administration increased from 15 to >30 with AC treatments. The half-life (T_{1/2}) and MRT were lower in dogs after i.v. versus oral administration. Approximately 10–20% of dose was recovered in the 0-72 hours urine collection from BDC dogs. For comparison, urinary recovery was also similar in intact dogs following i.v. (Table 4) and oral administration of [14C]apixaban (Zhang et al., 2009a).

BDC Rat Studies. IE of apixaban was further investigated in BDC rats following i.v. administration of [14C]apixaban (2 mg/kg). Figure 4B describes the apixaban plasma concentration-time profiles in BDC rats with and without administration of AC or in the presence of GF-120918, a BCRP and P-gp inhibitor (Jonker et al., 2000; Matsson et al., 2009). The radioactive dose recovery values in bile, urine, and feces and pharmacokinetic parameters of apixaban in the BDC rats are listed in Tables 5 and 6, respectively. Approximately 21% of dose was found in the feces of BDC rats following i.v. administration of [14C]apixaban, suggesting direct IE of apixaban in rats, which led to IC representing 21% of the total systemic clearance. Administration of GF-120918 reduced the fecal dose recovery of apixaban by 34%, and the administration of AC increased the fecal recovery by approximately 36%. The AUC0-24 of apixaban was increased (100%) with GF-120918 and decreased (28%) with AC (Table 6). The IC and systemic clearance were decreased 66 and 50% by GF-120918 treatment and increased 80 and 42% by AC treatment, respectively. These data clearly demonstrated that inhibition of intestinal efflux transporters decreases the systemic clearance and IC of apixaban leading to the reduced IE, whereas AC increased both

**TABLE 3**

<table>
<thead>
<tr>
<th>Dosing Route</th>
<th>Oral Dose in Intact Dogs (n = 4) (5 mg/kg)</th>
<th>I.V. Dose in BDC Dogs (n = 2) (1 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC Treatment</td>
<td>Control</td>
<td>AC (0.25 h)</td>
</tr>
<tr>
<td>AUC0-24 (AUC0-∞ i.v.) (reduction) (μg·h/ml)</td>
<td>78.6 ± 7.0 (NA)</td>
<td>59.5 ± 4.6 (24%)</td>
</tr>
<tr>
<td>Cmax (μg/ml)</td>
<td>9.35 ± 1.33</td>
<td>8.42 ± 0.82</td>
</tr>
<tr>
<td>C0 (C24/Cmax) (μg/ml)</td>
<td>5.23 ± 1.02</td>
<td>2.82 ± 0.65</td>
</tr>
<tr>
<td>T1/2 (h)</td>
<td>5.7 ± 2.2</td>
<td>4.2 ± 1.0</td>
</tr>
<tr>
<td>CL/F, CL (ml/h/kg)</td>
<td>60 ± 16</td>
<td>82 ± 6.9</td>
</tr>
<tr>
<td>Vss/F, Vs (l/kg)</td>
<td>0.5 ± 0.1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>8.5 ± 2.3</td>
<td>6.4 ± 1.1</td>
</tr>
<tr>
<td>F*</td>
<td>93%</td>
<td>92%</td>
</tr>
<tr>
<td>Cmax/C24 (C0 i.v.)</td>
<td>15</td>
<td>30</td>
</tr>
<tr>
<td>CL/F (ml/h/kg),% total</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CL/F, (ml/h/kg),% total*</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>CL/F, (ml/h/kg),% total</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

AC: activated charcoal; AUC, area under the plasma concentration versus time curve; CL, clearance; F, bioavailability; MRT, mean residence time; NA, not available; ND, not determined.

*Fmax was last detectable time point for i.v. dosing or last determined time point for oral dosing.

Bioavailability might be underestimated because the AUC values of bile-duct cannulated animals were used.

Clearence was estimated from urinary dose recovery from 0-72 hour collections.

*The protein binding of apixaban in dog plasma was 92%; therefore, glomerular filtration rate × Fu = 368 × 0.08 = 29.4 ml/h/kg.
clearance values because of disrupted apixaban reabsorption. Importantly, GF-120918 did not affect the urinary or biliary excretion of apixaban in rats. The increase in intestinal recovery of the apixaban radioactive dose after AC corresponds to the decrease of the urinary excretion in rats, suggesting diversion of apixaban elimination from urinary to the IE route by AC. In rats, the volume of distribution, half-life, and MRT of apixaban were decreased after i.v. versus oral administration; the peak-to-last time point concentration ratio ($C_{\text{max}}/C_{10}$) increased from 15 after oral to 500 after i.v. administration.

**Studies with Drug Transporter KO Rats.** To further assess the effects of intestinal efflux transporters on apixaban IE, [14C]apixaban was also dosed i.v. to P-gp-KO and BCRP-KO rats (Fig. 5; Tables 1 and 5). The urinary excretion was decreased from 50 to 35% in the BCRP-KO rats and from 50 to 44% in the P-gp-KO rats. The biliary excretion of apixaban, ranging 10–15% of dose, was similar in all rats. IE of apixaban increased compensatorily from 21 to 34% of dose, especially in the BCRP-KO rats compared with the wild-type rats. The increase in the P-gp-KO rats was less (from 21 to 25%) (Table 6). The apixaban AUC$_{0\rightarrow\infty}$ increased by 45 and 66%, respectively, in both P-gp-KO and BCRP-KO rats compared with the wild-type rats (Table 6). The contributions of P-gp and BCRP in governing apixaban disposition were also examined after oral doses to P-gp-KO and BCRP-KO rats or with a transporter inhibitor. As shown in Fig. 5 and Table 6, apixaban exposure (AUC) increased by 100% in the P-gp-KO rats but by 300% in the BCRP-KO rats compared with the wild-type rats after oral administration, predominately through an increase in $C_{\text{max}}$. GF-120918 increased apixaban exposures (AUC) by nearly 400%. Compared with the plasma-concentration-time profiles of apixaban in dogs (Fig. 3) and BCRP-KO rats (Fig. 5) after oral administration, apixaban showed a broad peak and even multiple peaks after oral administration in rats. The broad peak complexity of apixaban was more obvious in P-gp-KO rats than BCRP-KO rats. The apparent flip-flop pharmacokinetic profile of apixaban in rats (Table 5) as well as in dogs (Table 3) was characterized by a short $T_{1/2}$ value after i.v. than oral administration and the increase of $C_{\text{max}}/C_{\text{min}}$ ratios after oral versus i.v. administration, which was reduced in the P-gp-KO and BCRP-KO rats compared with the wild-type rats (Fig. 5; Table 5). The urinary excretion was approximately 40–45% of dose in both intact and BDC rats following i.v. administration of [14C]apixaban, indicating that bile duct cannulation did not affect the urinary excretion of apixaban. However, the urinary excretion after i.v. administration (40–45% of dose) was much higher compared with approximately 10% of dose after oral administration (Zhang et al., 2009a). Following oral administration in rats, P-gp-KO and BCRP-KO as well as GF-120918 pretreatments generally increased absorption, decreased volume of distribution and MRT, and decreased the apparent systemic clearance values of apixaban (Table 5).

**Discussion**

Approximately 20–50% of the i.v.-administered [14C]apixaban dose recovered in the feces of BDC rats and dogs confirmed that there was direct excretion of the drug from blood into the GI. The findings prompted further investigation of the mechanism of IE and the interplay of IE with EER and enterohepatic recirculation (her) (Fig. 2). A key to the finding of IE was an examination of efflux transporter involvement in the intestinal disposition of apixaban, which led to additional understanding of the involvement of RTR in apixaban disposition.
Direct IE led to significant fecal elimination in rats and dogs. Following i.v. administration of [14C]apixaban to BDC rats and dogs, approximately 20–50% of dose was recovered in feces, resulting in IC that represented 20–50% of the total systemic clearance. The reduction of i.v.-dosed apixaban AUC by AC in BDC rats and dogs with the increased fecal recovery and IC of apixaban clearly demonstrated that AC prevented reabsorption of apixaban that was excreted into intestinal lumen and so lead to interruption of EER. Oral administration of GF-120918, a dual P-gp and BCRP inhibitor, reduced intestinal lumen and so lead to interruption of EER. Oral administration of [14C]apixaban (i.v.) WT, BDC, intact, n = 3 49.5 ± 4.2 10.7 ± 1.1 21.1 ± 5.2 82.3 ± 2.2
[14C]apixaban (i.v.) and GF-120918 WT, BDC, n = 3 50.6 ± 3.1 14.7 ± 2.2 13.8 ± 2.4 79.1 ± 2.5
[14C]apixaban (i.v.) and Charcoal WT, BDC, n = 3 40.7 ± 2.6 15.7 ± 1.9 28.8 ± 2.5 85.2 ± 5.9
[14C]apixaban (i.v.) P-gp-KO, BDC, n = 3 44.3 ± 4.6 14.1 ± 2.7 24.9 ± 9.1 83.3 ± 3.4
[14C]apixaban (i.v.) BCRP-KO, BDC, n = 3 35.5 ± 0.4 12.7 ± 3.4 34.1 ± 4.3 82.3 ± 5.2
[14C]apixaban (oral)* WT, intact, n = 3 51.3 ± 5.1 NA 41.1 ± 1.9 92.4 ± 3.2
[14C]apixaban (oral)* WT, BDC, n = 3 13.4 NA 74.0 87.4

Oral dose was 30 mg/kg and collections were 0–168 h for 3 intact and 0–48 h for 3 BDC rats (Zhang et al., 2009a) and listed here for comparison.

<table>
<thead>
<tr>
<th>Compound and Dose Route</th>
<th>Rat Type</th>
<th>Dose Recovery (% of Dose in 0–24 h Collections)</th>
</tr>
</thead>
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<td>[14C]apixaban (i.v.)</td>
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BKR, breast cancer resistance protein; BDC, bile duct cannulation; KO, knockout; NA, not available; P-gp, P-glycoprotein; WT, wild-type.

* Oral dose was 30 mg/kg and collections were 0–168 h for 3 intact and 0–48 h for 3 BDC rats (Zhang et al., 2009a) and listed here for comparison.

Pharmacokinetic parameters of apixaban following a single oral dose of apixaban or i.v. dose of [14C]apixaban to male bile duct-cannulated Sprague-Dawley rats

<table>
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<tr>
<th>TABLE 6</th>
<th>Percent recoveries of radioactive dose (± S.D.) following a single i.v. dose of [14C]apixaban to male bile duct-cannulated Sprague-Dawley rats</th>
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AUC: area under the plasma concentration versus time curve; BCRP, breast cancer resistance protein; BDC, bile duct cannulation; CL, clearance; F, bioavailability; IC, intestinal clearance; KO, knockout; MRT, mean residence time; NA, not available; P-gp, P-glycoprotein; WT, wild-type.

* Samples were taken at different time points.

+ Bioavailability was underestimated because the AUC values of bile duct-cannulated animals were used where enterohepatic recirculation could not be considered. The >100% bioavailability in the BCRP-KO rats and after the treatments with GF-120918 reflected the increased absorption in transporter knockout rats and the increased urinary clearance of apixaban after i.v. administration of BDC rats compared with oral administration of intact rats in addition to elimination of potential low level of enterohepatic recirculation in intact rats, both of which reduced the apparent apixaban AUC after i.v. administration.

The protein binding of apixaban in rat plasma was 96%, therefore, glomerular filtration rate × Fu = 0.31 ± 0.04 = 0.012 l/h/kg.

A compound with high solubility and high intrinsic permeability such as apixaban can be absorbed rapidly after oral administration at the proximal intestine, partially escaping the barrier function of P-gp and BCRP that are highly expressed in the small intestines (Mori et al., 2008). Apixaban appears to fit that profile as it does have a relatively short $T_{\text{max}}$. AC administration at various times (0.25–6 hours) post-apixaban oral dose in dogs (solution formulation) and humans (tablet formulation) did not significantly affect the $C_{\text{max}}$ or $T_{\text{max}}$ of apixaban as apixaban was rapidly absorbed. The lack of an effect on $C_{\text{max}}$ led to the conclusion that the impact of AC is not on the initial absorption phase but on the IE/EER phase (Fig. 2). Timely administration of AC could sequester the drug that has been excreted into the lumen and prevention of reabsorption. Administration of AC 0.25 hour after drug dose in dogs was less effective at reducing the apixaban AUC versus 3 hours because AC did not have sufficient opportunity to mix with apixaban preabsorption and transited the GI tract prior to extensive IE; therefore, there was actually decreased effective contact with the drug when charcoal was dosed at 0.25 hour. Considering the small intestinal transit time of 2–4 hours for substances such as charcoal in dogs (Orisakwe and Ogbonna, 1993; Parrott and Lave, 2008; Abuasal et al., 2012), administration of AC at 2–4 hours after the drug dose, when apixaban initial absorption in the upper GI tract is complete and the IE phase is beginning, may be the most effective timing to remove the drug from the intestine. In the presence of AC, the shorter half-life and greater clearance in dogs and humans after oral dose of apixaban and in rats and dogs after i.v. administration of apixaban supports that AC increases the apparent systemic clearance of apixaban by 20–40% and IC by 20–60%. Therefore, AC removes apixaban from the body by preventing its reabsorption. AC has been shown to block EHR of some drugs in human and animal studies (Gadgil et al., 1982; Heimer and Englund, 1986; Roberts et al., 2002; Stass et al., 2005). Additionally, the shorter half-life and lower exposures of moxifloxacin and phenobarbital upon administration of AC in humans and rabbits following i.v. drug administration has been ascribed to AC blocking enteroenteric recycling of the drugs (Wakabayashi et al., 1994; Stass et al., 2005).

EER is a complex and dynamic process of concentration gradient-dependent transcellular passive diffusion, transporters-mediated transport, and segment-dependent reabsorption, operating in the same or opposite direction to intestinal mobility and intestinal transit. Apixaban molecules that undergo IE can be reabsorbed, although reabsorption might occur at a more distal segment of the intestine where there is a lower level of transporter expression. The drug secreted into the lumen serves as a secondary source of drug for absorption. Therefore, the intestine is also an organ for drug distribution and redistribution as well as absorption and clearance. Apixaban EER, along with EHR and RTR, are represented in Fig. 2. Due to the reabsorption, IE may or may not lead to a measurable level of fecal elimination. The interplay between drug metabolizing enzymes and transporters involved in EER as well as EHR and RTR could serve to enhance metabolism (Wang et al., 2010) (summary of product characteristics at http://www.ema.europa.eu).

Similar to intestinal excretion and reabsorption (EER), apixaban undergoes RTR (Fig. 2). Renal excretion is the result of glomerular filtration of unbound apixaban, tubular secretion, and tubular reabsorption (Fig. 2). Urinary elimination accounted for approximately 50% of the apixaban dose, and renal clearance represented approximately 50% of the systemic clearance in rats. On the basis of glomerular filtration rate and apixaban plasma protein binding (He et al., 2011), apixaban clearly shows active urinary excretion in rats. Conversely, urinary elimination only accounted for approximately 10% of the apixaban dose in dogs, and the renal clearance (~20 ml/h/kg) represents 13–19% of the systemic clearance, which would argue for a low level of net tubular reabsorption (glomerular filtration rate $\times \text{Fu} = 29$ ml/h/kg). Urinary elimination in humans was moderate (27% of dose) (Raghavan et al., 2009), and the renal clearance of apixaban is similar to the glomerular filtration rate likely due to the cancellation of active secretion versus reabsorption.

The renal elimination of apixaban was significantly reduced in the P-gp-KO and especially BCRP-KO rats following an i.v. dose, demonstrating that active transport is involved in apixaban renal elimination.

**Fig. 5.** Apixaban plasma concentration-time profiles in wild-type, P-gp-KO, and BCRP-KO rats ($n = 3$) following an intravenous dose at (2 mg/kg) and oral administration at 4 mg/kg of apixaban. (A) Linear scale; (B) log scale.
in rats. Knockout of a transporter would decrease active transport excretion and also would reduce the prevention of tubule reabsorption, leading to the reduced urinary elimination. The reduced renal elimination led to the increased compensatory intestinal elimination of apixaban because knockout of P-gp or BCRP still leaves an alternative transporter in the intestine for apixaban efflux in addition to passive diffusion through loosely packed intestinal epithelium layer (Meier et al., 2006). Due to interference of renal excretion changes in the P-gp-KO or BCRP-KO rats, it was not possible to evaluate the contribution of P-gp or BCRP to apixaban IE directly; however, when apixaban was dosed orally, BCRP-KO rats showed an approximately twofold higher effect to increase the apixaban exposure versus the P-gp-KO rats. Therefore, BCRP plays a more important role than P-gp in apixaban absorption. IE of apixaban was markedly reduced by application of the P-gp and BCRP inhibitor GF-120918 in rats. The low level of BCRP expression in the human tubular epithelium cells (Huls et al., 2008) would suggest less impact of the transporter on apixaban urinary elimination in humans than rats.

In contrast to EER and RTR, EHR would not be as high for apixaban because biliary excretion is a minor elimination pathway for apixaban in rat and dog and likely in human. The biliary elimination of apixaban was not markedly affected by the transporter knockout or the transporter inhibitor mainly due to the fact that liver uptake is the driving force for the biliary elimination (Watanabe et al., 2009; Fennner et al., 2012; Varma et al., 2012) and apixaban was not a substrate for hepatic uptake transporters OATP1B1/3 or OAT1/3 (unpublished data).

The efflux of apixaban by P-gp and BCRP in the upper GI tract would delay apparent absorption of a fraction of apixaban dose, which could be a mechanism limiting overall rate of absorption, so $C_{\text{max}}$ is low after oral administration of apixaban. The increased $C_{\text{max}}$ values in the P-gp-KO and BCRP-KO rats have demonstrated the importance of intestinal efflux transporters in limiting apixaban absorption. The fraction for the reabsorption of apixaban in the duodenum, jejunum, and upper ileum would also be limited by the high levels of P-gp and BCRP expression, which could contribute to the broad peaks of apixaban. Both the recycling mechanisms, IE-intestinal absorption (EER) and glomerular filtration-tubular reabsorption (RTR), prolong apparent elimination half-life through decreasing apparent clearance of apixaban. The recycling mechanisms together with the metabolic stability of apixaban slowed down the decline of $C_{\text{min}}$. Therefore, the low peak-to-trough plasma concentration ratio of apixaban is consistent with intestinal efflux-limited absorption that controls the $C_{\text{max}}$ and the recycling mechanisms coupled with metabolic stability that controls the $C_{\text{min}}$. Furthermore, the recycling mechanisms (EER and RTR) of apixaban contributed to a half-life of 12 hours for apixaban despite its small volume of distribution and being subject to glomerular filtration. Different from the absorption-limited flip-flop kinetics examples (Yanez et al., 2011), the apparent flip-flop kinetics of apixaban in rats is the result of the combined effects of intestinal BCRP/P-gp-limited absorption and RTR/EER recycling.

Taken together, AC application in BDC animals was a useful method to study IE and EER, which demonstrates that interruption of EER supports reduction of apixaban exposures by AC. IE has not been widely applied to understand dispositional profiles of drugs although studies have been reported to assess IE of drugs that are substrates of P-gp or BCRP using in vitro permeability assays, ex vivo segmental intestine excretion, and activated charcoal techniques (Mayer et al., 1996; Sparreboom et al., 1997; Gramatte and Oertel, 1999). With increasing use of physiologically based pharmacokinetic modeling to predict clinical profiles and drug-drug interaction potential that require accurate inputs of drug-related as well as physiological parameters (Bolger et al., 2009; Abuasal et al., 2012; Jones et al., 2012), full characterization of elimination pathways including IE, RTR, and EER is becoming increasingly important. The study clearly demonstrated that 1) IC represents 20–50% of the systemic clearance of apixaban; 2) the overall disposition of apixaban in rats and dogs was governed by a complex and dynamic interplay between absorption, intestinal/renal excretion, and reabsorption in EER and RTR processes; and 3) species-dependent urinary excretion is likely due to different expression of renal efflux transporters. The impact of AC in humans is consistent with IE as an important elimination pathway in addition to metabolism and urinary excretion each accounting for one-third of total clearance in humans. Moreover, apixaban IE and recycling mechanisms of EER and RTR coupled with efflux transporters-limited absorption and its metabolic stability contribute to the overall pharmacokinetic profile of apixaban, including the low peak-to-trough plasma concentration ratio, and help decrease the impacts of intrinsic (e.g., renal and hepatic impairment) and extrinsic (e.g., drug-drug interactions) factors on apixaban disposition.

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Authorship Contributions


References

from obsolete liver transporters to key determinants of hepatobiliary clearance. *Drug Metab Dispos* 38:231–232.


