Characterization of Efflux Transporters Involved in Distribution and Disposition of Apixaban

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ABSTRACT

The studies reported here were conducted to investigate the transport characteristics of 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) and to understand the impact of transporters on apixaban distribution and disposition. In human permeability glycoprotein (P-gp)- and breast cancer resistance protein (BCRP)-cDNA-transfected cell monolayers as well as Caco-2 cell monolayers, the apparent efflux ratio of basolateral-to-apical (P_{B,A}) versus apical-to-basolateral permeability (P_{A,B}) of apixaban was >10. The P-gp- and BCRP-facilitated transport of apixaban was concentration- and time-dependent and did not show saturation over a wide range of concentrations (1–100 μM). The efflux transport of apixaban was also demonstrated by the lower mucosal-to-serosal permeability than that of the serosal-to-mucosal direction in isolated rat jejunum segments. Apixaban did not inhibit digoxin transport in Caco-2 cells. Ketoconazole decreased the P-gp-mediated apixaban efflux in Caco-2 and the P-gp-cDNA-transfected cell monolayers, but did not affect the apixaban efflux to a meaningful extent in the BCRP-cDNA-transfected cell monolayers. Coincubation of a P-gp inhibitor (ketoconazole or cyclosporin A) and a BCRP inhibitor (Ko134) provided more complete inhibition of apixaban efflux in Caco-2 cells than separate inhibition by individual inhibitors. Naproxen inhibited apixaban efflux in Caco-2 cells but showed only a minimal effect on apixaban transport in the BCRP-transfected cells. Naproxen was the first nonsteroidal antiinflammatory drug that was demonstrated as a weak P-gp inhibitor. These results demonstrate that apixaban is a substrate for efflux transporters P-gp and BCRP, which can help explain its low brain penetration, and low fetal exposures and high milk excretion in rats.

Introduction

Efflux transporters are ATP-binding cassette (ABC) proteins containing multitransmembrane-spanning domains with homologous ATP-binding sites. Several members of this family are primary drug transporters that pump substrates out of cells by using ATP as the energy source, thus significantly modulating the absorption, distribution, metabolism, and elimination of endogenous compounds, drugs, and other xenobiotics (Leslie et al., 2005; Xia et al., 2005a; Shitara et al., 2006; Koshiba et al., 2008; Zhou, 2008; Giacomini et al., 2010). Permeability glycoprotein (P-gp; encoded by multiple drug resistance protein 1, ABCB1), a member of the ABC transporter superfamily, is expressed in the human intestine, liver, brain, and other tissues, and plays an important role in oral bioavailability and tissue distribution of drug molecules that are substrates for this transporter (Zhou 2008). The breast cancer resistance protein (BCRP, ABCG2), another ATP-binding cassette efflux drug transporter (Doyle and Ross, 2003; Mao and Unadkat, 2005; Krishnamurthy and Schuetz, 2006), is highly expressed in various normal tissues, such as placenta, small intestine, liver, and mammary glands (Maliepaard et al., 2001). BCRP can transport a broad spectrum of substrates, including chemotherapeutic agents, organic anions, and xenobiotics (Doyle and Ross, 2003; Mao and Unadkat, 2005), and plays an important role in drug disposition (Koshiba et al., 2008).

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] is a highly selective, oral, direct inhibitor of factor Xa, a protease enzyme that plays a pivotal role in the coagulation cascade. Direct and selective inhibition of factor Xa represents a superior approach to anticoagulation therapy compared with the current treatments including use of warfarin. Apixaban is currently approved for the prevention of venous thromboembolism and the prevention of stroke...
in patients with atrial fibrillation (Lassen et al., 2007; Connolly et al., 2011; Granger et al., 2011). Apixaban has balanced elimination pathways, including renal excretion, metabolism, and intestinal/biliary excretion in humans (Raghavan et al., 2009; Zhang et al., 2009; Wong et al., 2011). Metabolism was responsible for 25% of apixaban (1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H-pyrrozolo[3,4-c]pyridine-3-carboxamide) clearance in humans and urinary excretion was an important elimination pathway (~27%). Tissue distribution studies in rats showed that there were low exposures in rat brain and fetal tissues; in addition, apixaban was highly excreted into milk (Wang et al., 2011). These characteristics of apixaban seem to indicate a role of active transport in disposition of this compound.

Interaction with transporters such as P-gp and BCRP has been widely studied using direct cell-based assays in Caco-2 or drug transporter cDNA-transfected cell lines derived from porcine or canine kidney cells (Taipalensuu et al., 2001; Englund et al., 2006; Elsby et al., 2008). The Caco-2 cell line is derived from a human colon adenocarcinoma, and the cell monolayers differentiate in culture to resemble the epithelial lining of the human small intestine and express a number of transporters including P-gp, BCRP, and MRP2 (Elsby et al., 2008). In comparison, cDNA-transfected cell lines are characterized by selective expression of P-gp or BCRP and are recommended in vitro systems to test transporter properties of a compound by the International Transporter Consortium (Giacomini et al., 2010). This study was conducted to evaluate apixaban as a potential substrate of common efflux transporters.

**Materials and Methods**

**Materials.** Apixaban and radiolabeled [14C]apixaban (102 μCi/mg), were synthesized at Bristol-Myers Squibb. The structure of apixaban is shown in Fig. 1. Other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). All chemicals were of analytical grade. Stock solutions of apixaban (10 mM), Ko134 (4.4 mM), digoxin (10 mM), prazosin (5 mM), mannitol [2R,3R,4R,5R]pentahexan-1,2,3,4,5,6-hexol; 20 mM], nitrofurantoin ((E)-1-[(5-nitro-2-furyl) methylideneaminol]imidazolidine-2,4-dione; 1 mM), ketocanazole (keto; 5 mM), and cyclosporin A (CsA; 5 mM) were prepared in dimethylsulfoxide (DMSO). A stock solution of naproxen [(+)-(S)-2-(6-methoxynaphthalen-2-yl); 10 mM] was prepared in 10 mM HEPES assay buffer (pH 7.4). [3H]Apixaban (102 μCi/mg) was prepared in DMSO (2.2 mM and 100 μCi/mg). [3H]Digoxin (0.3 mCi/mg) was prepared in transport assay buffer (5 μM and 1.3 μCi/ml). [3H]Prazosin (0.2 mCi/ml) was diluted in ethanol containing 0.01 M HCl (13 mM and 1 mCi/ml). [14C]Mannitol (0.1 mCi/mg) was diluted in ethanol/water (9:1) (20 mM, 0.4 mCi/ml). None of the chemicals used affected the pH of the transport buffer at the applied concentrations. The final concentration of organic solvent in the assay buffer was 1% (v/v).

**Transporter Cell Lines.** Human P-gp cDNA-transfected cells were prepared using porcine kidney-derived LLC-PK (porcine kidney proximal tubule cell line) cells, and control LLC-PK1 cells (BD Gentest, Woburn, MA) contained the vector without human P-gp cDNA (Mock). The cells were seeded onto a collagen-coated polycarbonate filter membrane (port size 1 μm, diameter 6.5 mm) on Transwell inserts (0.7 cm²) (Millipore, Billerica, MA) at a density of 50,000 cells/well and cultured in medium 199 supplemented with 0.05 mg/ml gentamycin and 7% fetal bovine serum in BD Falcon 24-well plates, at 37°C, 5% CO2, and 95% relative humidity for 7 days, with medium change once every 3–4 days.

**Human BCRP cDNA-transfected cells** were prepared using canine kidney-derived [Madin–Darby canine kidney II (MDCKII)] cells and control MDCKII cells (SOLVO Biotechnology; http://www.solvo biotech.com) contained the vector without human BCRP cDNA (Mock). Cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Carlsbad, CA) at 37°C in an atmosphere of 5% CO2 in cell culture flasks. Cells (50,000 cells/well) were seeded onto 24-well Transwell inserts (0.7 cm²). Transfected and control MDCKII cells were cultured on the inserts for 4 days prior to assay. Medium was changed daily and supplemented with 10 mM sodium butyrate 24 hours before the experiment.

Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD). Caco-2 cells were maintained in flasks and seeded onto 24-well Corning Transwell or BD Falcon plates for culture and assays. Caco-2 cells were seeded onto the filter membrane of Transwell inserts (0.33 or 0.7 cm²) at a density of 45,000 or 70,000 cells/well and grown in culture medium consisting of Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 0.5 mM HEPES, 1% nonessential amino acids, 1% t-glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin (all from Gibco BRL, Gaithersburg, MD). The culture medium was replaced every 2 days and the cells maintained at 37°C, 95% relative humidity, and 5% CO2. Caco-2 cell passage numbers of 17–40 were used for these studies and were monitored for P-gp expression with positive substrates within 5 days of projected use in the assay, usually 13–22 days postspeeding.

Monolayer integrity was evaluated by preexperimental transepithelial electrical resistance (TEER) measurements performed using the EVOM resistance meter from World Precision Instruments (Sarasota, FL) or postexperimental luminal yellow apical-to-basolateral flux determinations for each cell monolayer. After all samples were collected, luminal yellow was added to each monolayer at a final concentration of 100 μM. The inserts were placed in a new receiver plate containing the transport buffer. After 30 minutes incubation on an orbital shaker (50 g) at 37°C, with ambient humidity and CO2, receiver samples were removed to measure percent luminal yellow flux. Monolayer integrity was also evaluated by determining permeability of mannitol and comparing with the permeability values of mannitol without coassembly compound. For P-gp or BCRP-cDNA-expressed cells and Caco-2 cells, [3H]digoxin (at 5 μM) or [3H]prazosin (at 5 μM) was measured in a bidirectional assay and the efflux ratios (ERs) were determined.

**Permeability Determination Procedures.** Permeability studies were performed in triplicate at pH 7.4 in Hanks’ balanced salt solution (Gibco BRL) containing 10 mM HEPES, and pH adjusted with NaOH. For apical-to-basolateral (A→B) permeability (absorptive direction), the donor solution was placed in the apical compartment, and for basolateral-to-apical (B→A) permeability (secretive direction), the donor solution was placed in the basolateral compartment. Donor solutions of test compounds were prepared at the desired concentrations for each step of the study by diluting aliquots of the test compound stock solution into transport buffer with the receiver buffer prepared by adding the same concentration of an organic solvent (1%). After incubations, the media from the cell culture was aspirated, and both apical and basolateral portions of the Transwell plate were washed three times with the transport buffer. The transport buffer solution with the test compound was then transferred to appropriate wells. After incubation at 37°C, aliquots (50–100 μl) were taken from the receiver chambers to determine the translocated amount of a compound. Samples were taken from the donor compartments before and after incubation to determine the initial concentration (C0) and recovery of apixaban and other test compounds. Radioactive samples were removed to scintillation vials.

**Permeability Determination in P-gp-cDNA-Expressed cells.** Monolayers of P-gp-cDNA-transfected LLC-PK1 cells were incubated on an orbital shaker (50g) at 37°C, with ambient humidity and CO2 for the duration of the transport assay. The donor and receiver solutions were added to the apical (400 μl) or
basolateral (600–µl) chambers of the monolayers. The time-dependent transport of apixaban at 10 µM was evaluated, and samples from the receiver chambers were taken at three time points (60, 90, 120 minutes) and replaced by an equal volume of receiver solution. Samples from the donor chambers were taken at one time point of 120 minutes. To determine the extent of nonspecific binding of apixaban to the assay plate, apixaban donor solution was incubated under the conditions described above in a 24-well assay plate with no cells present.

[3H]Mannitol (50 µM) and [3H]Digitoxin (5 µM) were used as the low permeability control and P-gp substrate control, respectively. [3H]Apixaban was assayed at six concentrations (2.5, 5.0, 10, 25, 50, 100 µM) bidirectionally in both the P-gp-transfected and the vector-carrying LLC-PK1 cell monolayers. Samples from the donor and receiver chambers were taken at one time point of 90 minutes. Apixaban was also assayed bidirectionally at two concentrations (5.0 and 50 µM) in the P-gp-transfected cell monolayers in the presence of increasing concentrations (0, 1, 3, 10, 30 µM) of keto and CsA in both the donor and receiver chamber.

Permeability Determination in BCRP-cDNA-Expressed cells. Incubations with BCRP-cDNA-transfected MDCKII cell monolayers were carried out in modified Krebs-Henseleit buffer plus 5 mM glucose at 37°C. Cells were preincubated in buffer for 10 minutes to allow cells to adjust to the medium, then the buffer was added to the apical (400-µl) or basolateral chamber (800-µl). The transport of [3H]prazosin was also evaluated in the presence of 1 µM Ko134, a known inhibitor for BCRP transporter (Allen et al., 2002). Nitrofurantoin at 5 µM was used as a positive substrate for BCRP-mediated transport. The time-dependent transport of apixaban was evaluated at 15, 30, 60, and 120 minutes. The concentration-dependent transport of apixaban was also evaluated at 1, 5, 25, and 100 µM in the A-B and B-A directions in the BCRP-transfected and the vector-carrying MDCKII cells. The efflux inhibition of prazosin (5 µM) was evaluated in the presence of naproxen (8 mM), a potential inhibitor of efflux transporters based on results of a clinical drug–interaction study with apixaban. The high concentration of naproxen was selected because gastrointestinal concentration of naproxen could reach 5 µM following oral administration of 500 mg naproxen based on 250 ml of methanol, and then 5 µl of the mixture was injected onto a Mac-Mod HPLC system (Mac-Mod, Chadds Ford, PA) 2.7 µm, 2.1 × 30 mm at room temperature for analysis. For apixaban, the mobile phases were (A) 0.2% formic acid in water and (B) 0.2% formic acid in acetonitrile with a flow rate of 0.5 ml/min; the gradient started with 2% B, increased to 100% B in 0.5 minute, then held at 100% B for 0.25 minute before decreased to 2% B in 0.1 minute. For nitrofurantoin, the mobile phases were (A) 2 mM ammonium acetate containing 0.2% formic acid and 2% acetonitrile and (B) acetonitrile containing 0.2% formic acid and 2% water; the gradient was 5% to 100% B in 0.5 minute with a flow rate of 0.65 ml/min. Peak area ratios of apixaban or nitrofurantoin and internal standards were used for quantitation with a linear regression using a 1/x^2 weighting. The concentrations of study samples were then calculated for the calibration curve.

Sample Analysis for Radioactive Samples. Samples of 100 µl were removed to scintillation vials, and 8 ml Ecolite scintillation fluid (VWR, Bridgeport, NJ) was added to each vial. Vials were counted on either a 14H disintegrations per minute program or a 14C disintegrations per minute program (with background subtraction) on a PerkinElmer Tri-Carb Scintillation Counter Tri-Carb 3100TR.

Data Analysis. The apparent permeability coefficient (Pc, cm/s) was calculated according to the following equation: Pct = ΔA/Δt × S × C0, where ΔA/Δt is the change in radiocounts over time (pmol/cm² per hr). S is the surface area of the cell monolayer, and C0 is the initial concentration (µM) in the donor compartment. Pct, A→B is the Pc value measured in the B to A direction and Pct, B→A is the Pc value measured in the A-to-B direction. The ER was calculated as: Efflux ratio = Pct, B→A/Pct, A→B.

The intrinsic activity (IA) of P-gp was calculated as the sum of P-gp- or BCRP-facilitated B-to-A transport in P-gp or BCRP cells relative to control cells and the negative effect of P-gp or BCRP on A-to-B transport in P-gp cells relative to control cells: IA = (d – c) – (b – a), where IA is the intrinsic activity (pmol of test compound transported during the assay); a is the A-to-B transport of test compound in control cells (pmol); b is the B-to-A transport of test article in control cells (pmol); c is the A-to-B transport of test article in P-gp- or BCRP-expressing cells (pmol); and d is the B-to-A transport of test compound in P-gp- or BCRP-expressing cells (pmol). The rate of P-gp- or BCRP-facilitated transport is then calculated as: v = IA/(A × S), where v is the rate of P-gp- or BCRP-facilitated drug transport (pmol/cm² per hr), IA is the intrinsic activity (pmol transported during the assay), and S is the surface area of the filter membrane.

Percent inhibition of apixaban efflux was determined by comparing the difference between the Pc values of apixaban incubated in the presence or absence of inhibitor (Balimane et al., 2008). The percent inhibition of apixaban efflux is calculated as: Percent inhibition = 1 – ([(Pct, A→B of test compound in presence of inhibitor)]/[(Pct, A→B of test compound outside of presence of inhibitor)]) x 100.
of test compound in absence of inhibitor) – \( (P_{CA,B}) \) of test compound in absence of inhibitor). Data are expressed as mean ± standard deviation. To estimate the IC_{50} value of transport inhibition, the data were fitted by means of nonlinear least-squares regression analysis using XL fit or WinNonlin (Scientific Consulting Inc., Cary, NC).

**Results**

**Cell Monolayer Integrity and Transporter Characteristics.** Preexperimental TEER measurements and postexperimental lucifer yellow A-to-B flux determinations were used to confirm monolayer integrity. The functionality of the cell monolayers was confirmed with the positive control substrates in the absence and presence of positive control inhibitors. TEER values were in agreement with the historical range of >500 Ω · cm². The lucifer yellow flux was <2% with individual values less than 3-fold higher than the mean. In addition, the permeability comparator mannitol showed permeability values of 6–30 nm/s that were within historical ranges. All of these data indicated that the cell monolayers were appropriate for use in experiments examining cellular permeability.

For Caco-2 and LLC-PK1-P-gp cells, the ERs of digoxin (5 μM), a known P-gp substrate, were >7, which were inhibited >80% by keto (at 20 or 50 μM), a known P-gp inhibitor. For MDCKII-BCRP cells, prazosin (5 μM), a known BCRP substrate, showed an ER of approximately 10, and the efflux was inhibited by Ko134 (1 μM), an analog of fumitremorgin C, and a potent and selective inhibitor of BCRP (Allen et al., 2002), yielding an ER of approximately 1. The results with the positive control substrates and inhibitors demonstrated that the efflux transporters in these cell models were functioning properly.

**Compound Recovery.** Recovery of apixaban and other compounds used in this study from the apical and basolateral chambers at the end of the assay (mass balance) as well as from the assay plate without cells was high (82–100%) indicating that the degree of nonspecific binding to the cells or absorption to the Transwell apparatus did not affect the assay under the conditions used.

**Permeability in Cell Models.** The bidirectional permeability of apixaban was studied in LLC-PK1-P-gp cell monolayers. Digoxin at 5 μM showed an ER of 7.4, reduced >90% by keto (30 μM) (Fig. 2A) or CsA (10 μM) (unpublished data). Apixaban was subject to active basolateral-to-apical transport with ERs in LLC-PK1-P-gp cells ranging from 23 to 38 compared with ratios of 1.4 to 4.4 in control cells (Table 1). Apixaban transport was linear over the time course (60, 90, 120 minutes) (Table 1). P-gp facilitated transport of apixaban was concentration-dependent and did not show saturation over the concentration range tested (2.5–100 μM). Therefore, the apparent \( K_m \) and \( V_{max} \) values could not be calculated. Ketoconazole (at 0, 1, 3, 10, and 30 μM) caused a concentration-dependent inhibition of apixaban transport in P-gp-expressing cells; the ERs were 27, 24, 17, 8.3, and 3.2 at 5 μM apixaban and 29, 23, 15, 7.3, and 3.2 at 50 μM apixaban, respectively. Efflux ratios were reduced by approximately 10-fold at the two apixaban concentrations with keto IC_{50} values of 2.9–5.4 μM (Fig. 2B). This finding indicates P-gp facilitated transport of apixaban across LLC-PK1-P-gp cell monolayers.

The bidirectional permeability of [³H]prazosin (5 μM) and [¹⁴C]apixaban (1–100 μM) was studied in vector-containing MDCKII cells and BCRP-cDNA–transfected MDCKII cells (Fig. 3, A and B; Table 2). Prazosin efflux was completely inhibited by 1 μM Ko134. Ketoconazole showed marginal inhibition on prazosin efflux at 20 μM but at 50 μM keto showed more robust inhibition. In the same experiment, naproxen showed a very low level of inhibition of prazosin efflux with a minor increase in \( P_{CA,B} \) and decrease in \( P_{CB,A} \) at the highest concentration (8 mM) tested, which was also accompanied by a slightly higher mannitol permeability of 30 nm/s. The apixaban ER was 1.4–2.4 in the control cells (Table 2), whereas the values in BCRP-cDNA-transfected cells were between 8 and 12. Ko134 strongly inhibited apixaban transport in the BCRP-transfected cell lines (Fig. 3B). These results indicate that apixaban is a substrate for the BCRP transporter. Ketoconazole had a minimal effect at 20 μM and a modest effect (33% inhibition) at 50 μM on apixaban transport (Fig. 3B); however, keto completely inhibited the low level of apixaban efflux in the vector-containing MDCKII cells (data not shown). Inhibition of apixaban efflux in BCRP-transfected cells by naproxen at 3 and 8 mM was not firmly established.

The bidirectional permeability of apixaban was studied in Caco-2 cell monolayers, which express a number of efflux transporters including P-gp and BCRP (Xia et al., 2005b). In this model, the ER of apixaban ranged between 12 and 37 with the apparent permeability coefficient \( (P_{CA,B}) \) values of approximately 6–16 nm/s in the apical-to-basal direction and 140–387 nm/s in the basol-to-apical direction (Fig. 4A; Table 3). Both CsA (50 μM) and keto (50 μM), known inhibitors of P-gp, partially reduced the efflux of apixaban at 3 μM by about 43 and 71% (Table 3), respectively, supporting that apixaban was a P-gp substrate, and potentially a substrate for other transporters. In the same experiment, CsA and keto completely inhibited 98% of the efflux of digoxin at 5 μM (a well-characterized substrate of P-gp) (Table 3).
The apparent permeability (P_{c}) of apixaban in Caco-2 cells in either direction was not affected by probenecid [4-(dipropylsulfamoyl) benzoic acid], an inhibitor for MRP2 (Table 3), suggesting that MRP2 was not involved in the efflux of apixaban in the Caco-2 monolayer. Palmitoyl-L-carnitine, a known paracellular absorption enhancer, significantly increased the P_{c} of apixaban from 10^6 0.5, 28 6 2.3, 37 6 5.1, 47 6 5.2 nm/s, approximately 1-, 3.5-, 5-, and 6-fold at concentrations of 0, 0.2, 0.3, 0.5 mM, respectively, suggesting that

### Table 1

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<thead>
<tr>
<th>Cell Line</th>
<th>Apixaban</th>
<th>Time</th>
<th>P_{c} Mean ± S.D.</th>
<th>Efflux Ratio Mean ± S.D.</th>
<th>P-gp-Facilitated Transport</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>A-B B-A B-A/A-B I/A pmol pmol/cm² per hour</td>
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<tr>
<td>P-gp 10</td>
<td>60 10 90 120</td>
<td>4.3 ± 0.2 130 ± 16 30 ± 3.3 146 ± 23 471 ± 76 31 ± 2.3 235 ± 20 505 ± 43</td>
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<tr>
<td>P-gp 5.0 90 90</td>
<td>5.5 ± 0.1 200 ± 11 36 ± 2.2 64 ± 12 139 ± 26 31 ± 1.9 167 ± 58 360 ± 125</td>
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<tr>
<td>P-gp 25 90 90</td>
<td>6.1 ± 0.6 220 ± 4.2 36 ± 4.3 548 ± 99 1179 ± 213 35 ± 4.6 1485 ± 69 3194 ± 148</td>
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<tr>
<td>100 90 90</td>
<td>5.7 ± 0.6 210 ± 9.3 36 ± 4.9 2772 ± 109 5962 ± 234</td>
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<td>Control 2.5–100 90</td>
<td>34–60.3 84–150 1.4–4.4 NA NA</td>
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A-B, apical-to-basolateral; B-A, basolateral-to-apical; I/A, intrinsic activity; NA, not applicable; P-gp, permeable glycoprotein; V, rate of transport.

The apparent permeability (P_{c}) of apixaban in Caco-2 cells in either direction was not affected by probenecid [4-(dipropylsulfamoyl) benzoic acid], an inhibitor for MRP2 (Table 3), suggesting that MRP2 was not involved in the efflux of apixaban in the Caco-2 monolayer. Palmitoyl-L-carnitine, a known paracellular absorption enhancer, significantly increased the P_{c} of apixaban from 10 ± 0.5, 28 ± 2.3, 37 ± 5.1, 47 ± 5.2 nm/s, approximately 1-, 3.5-, 5-, and 6-fold at concentrations of 0, 0.2, 0.3, 0.5 mM, respectively, suggesting that

Fig. 3. Permeability coefficients of prazosin (5 μM) (A) and apixaban (5 μM) (B) in MDCKII-BCRP cell monolayers in the presence or absence of Ko134 (1 μM), keto (20 and 50 μM), or naproxen (3 and 8 mM). The numbers after compounds are micromolar concentrations for keto and Ko134 and millimolar for naproxen (naprox).
Apixaban showed potential paracellular as well as intracellular transport in Caco-2 cells.

Intestinal Permeability. The intestinal absorption of apixaban was studied using isolated rat duodenum, jejunum, ileum, and colon segments (Sinko et al., 1995; Kilic et al., 2004). The mucosal-to-serosal $P_c$ values of apixaban were lower at 33 $\pm$ 6, 73 $\pm$ 28, 51 $\pm$ 38, and 31 $\pm$ 22 nm/s in the duodenum, jejunum, ileum, and colon segments, respectively, than the $P_c$ values of apixaban in the serosal-to-mucosal direction of 170 $\pm$ 23 nm/s in jejunum. These results are consistent with intestinal efflux transport in the jejunum segment. The efflux potential of apixaban in other segments of rat intestines is not known.

Inhibition Studies in Caco-2 Cell Monolayers. Both keto and cyclosporin A at concentrations of 20 $\mu$M partially inhibited apixaban efflux compared with nearly complete inhibition of digoxin efflux in Caco-2 cells (Fig. 4B). Although Ko134 at 1 $\mu$M completely inhibited apixaban efflux in MCDKII-BCRP cells, it did not inhibit apixaban efflux in Caco-2 cells, minimally inhibited the efflux of nitrofurantoin, a selective BCRP substrate (Merino et al., 2005). Ko134 at a higher concentration (11 $\mu$M) inhibited the efflux of apixaban, digoxin, and nitrofurantoin by 40, 50, and 78%, respectively. At a concentration of 20 $\mu$M, keto or CsA did not inhibit the efflux of nitrofurantoin in the Caco-2 cells. With a combination of a P-gp inhibitor (keto or CsA)
with the BCRP inhibitor (Ko134) at concentrations (20 μM for P-gp and 1 μM for BCRP inhibitors) that only selectively inhibited their corresponding transporters, apixaban efflux was inhibited to a degree that was apparently higher than the sum of the inhibition generated by the inhibitors separately (Fig. 4B).

The effect of naproxen on the bidirectional permeability of apixaban and digoxin was examined in Caco-2 cell monolayers. The apixaban permeability at 3 μM was examined in the presence and absence of 0.2, 1, or 6 mM naproxen (Table 3). Naproxen at a concentration of 6 mM showed a 42% inhibition of efflux of apixaban and a lower level of inhibition at lower naproxen concentrations. Similarly, naproxen at 8 mM inhibited the digoxin efflux by 42–46% in the bidirectional Caco-2 permeability assay through decreasing B-to-A permeability and increasing A-to-B permeability of digoxin (unpublished data).

The ER of digoxin at a concentration of 5 μM was tested in the presence of apixaban, and apixaban at concentrations up to 50 μM did not inhibit the efflux of digoxin (<10% inhibition at all concentrations tested; Table 4). In contrast, CsA inhibited the efflux of digoxin by 98% at 50 μM with an IC50 of 2.9–3.2 μM. Apixaban did not inhibit the efflux of rhodamine 123 in the Caco-2 cells, another P-gp substrate (not shown) (Cygalova et al., 2009). These results suggest that apixaban is not an inhibitor of P-gp in Caco-2 cells.

**Discussion**

To better understand the disposition of apixaban, the drug was studied in various in vitro models that demonstrate permeability and transport properties. Apixaban showed markedly higher permeability coefficient ($P_c$) values in the basolateral-to-apical direction than the apical-to-basolateral direction with ERs >10 in LLC-PK1-P-gp, MDCKII-BCRP, and Caco-2 cell monolayers, suggesting active transport of apixaban by efflux transporters P-gp and BCRP. The transport of apixaban in the P-gp, and BCRP-transfected cell models showed no indication of saturation up to a concentration of 100 μM; therefore, the apparent $K_m$ and $V_{max}$ values for the transport could not be calculated. The lack of an apparent $K_m$ up to 100 μM gives reasonable evidence that apixaban is a low affinity substrate for the P-gp and BCRP transporters. This was further supported by the fact that apixaban was not an inhibitor of P-gp in Caco-2 cells. To further explore the interaction of apixaban with efflux transporters, the bidirectional permeability of apixaban was tested in the presence of transporter inhibitors. In transfected cell monolayers (LLC-PK1-P-gp or MDCKII-BCRP), the P-gp inhibitor keto and the BCRP inhibitor Ko134 extensively inhibited apixaban efflux. However, in Caco-2 cells, CsA or keto (at 20 or 50 μM) only partially inhibited apixaban efflux (Table 3) compared with the complete inhibition of the efflux of digoxin. Under similar experimental conditions, transport of apixaban in Caco-2 cells was not inhibited by a specific P-gp inhibitor LY335979 (Shepard et al., 2003) at a concentration of 5 μM, while it inhibited the efflux of rhodamine 123 (data not shown). Ko134 did not inhibit apixaban efflux at 1 μM, a concentration sufficient to block apixaban efflux in the BCRP-transfected cell lines, and did only partial inhibition at 11 μM (Fig. 4B). This result was consistent with the observation that apixaban is a substrate for both P-gp and BCRP, and inhibition of a single transporter would not effectively inhibit transport of apixaban in the multiple transporter-expressing Caco-2 cells. A combination of a P-gp inhibitor with a BCRP inhibitor provided more effective inhibition of apixaban efflux in Caco-2 cells. The combination of CsA with Ko134 (>85% inhibition) appeared to be more effective than the combination of keto with Ko134 (65–80% inhibition) although the reason is not known. It is difficult to estimate the relative contribution of P-gp and BCRP for apixaban transport in Caco-2 cells, and it is even more difficult to assess the effect of P-gp and BCRP on apixaban absorption even with knowledge of BCRP expression in the human jejunum is higher than that of P-gp and MRP2 (Taipalensuu et al., 2001). Although keto did not effectively inhibit the BCRP transport of prazosin in the MDCKII-BCRP cells, it inhibited a low level of apixaban efflux in vector-containing MDCKII

### Table 3

<table>
<thead>
<tr>
<th>Test Conditions</th>
<th>$P_{A-B,app}$</th>
<th>$P_{B-A,app}$</th>
<th>% Inhibition of Efflux ± S.D.</th>
<th>Efflux Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin (5 μM)$^a$</td>
<td>23 ± 3</td>
<td>205 ± 7</td>
<td>NA</td>
<td>8.9</td>
</tr>
<tr>
<td>Digoxin (5 μM) + Cyclosporin A (50 μM)</td>
<td>86 ± 15</td>
<td>89 ± 17</td>
<td>98 ± 1</td>
<td>1.0</td>
</tr>
<tr>
<td>Apixaban (5 μM) + Ketoconazole (50 μM)</td>
<td>96 ± 3</td>
<td>95 ± 6</td>
<td>100 ± 0</td>
<td>1.0</td>
</tr>
<tr>
<td>Apixaban (3 μM)</td>
<td>16 ± 1</td>
<td>387 ± 54</td>
<td>NA</td>
<td>24.2</td>
</tr>
<tr>
<td>Apixaban (3 μM) + Cyclosporin A (50 μM)</td>
<td>67 ± 9</td>
<td>278 ± 25</td>
<td>43 ± 5</td>
<td>4.1</td>
</tr>
<tr>
<td>Apixaban (3 μM) + Ketoconazole (50 μM)</td>
<td>70 ± 8</td>
<td>177 ± 21</td>
<td>71 ± 5</td>
<td>2.5</td>
</tr>
<tr>
<td>Apixaban (3 μM) + Naproxen (0.2 mM)</td>
<td>12 ± 1</td>
<td>307 ± 13</td>
<td>21 ± 3</td>
<td>25.6</td>
</tr>
<tr>
<td>Apixaban (3 μM) + Naproxen (1 mM)</td>
<td>16 ± 3</td>
<td>279 ± 29</td>
<td>29 ± 7</td>
<td>17.4</td>
</tr>
<tr>
<td>Apixaban (3 μM) + Naproxen (6 mM)</td>
<td>35 ± 4</td>
<td>251 ± 25</td>
<td>42 ± 6</td>
<td>7.2</td>
</tr>
<tr>
<td>Apixaban (30 μM)</td>
<td>10 ± 2</td>
<td>292 ± 13</td>
<td>NA</td>
<td>29.2</td>
</tr>
<tr>
<td>Apixaban (30 μM) + Cyclosporin A (50 μM)</td>
<td>39 ± 4</td>
<td>182 ± 10</td>
<td>50 ± 6</td>
<td>4.7</td>
</tr>
<tr>
<td>Apixaban (30 μM) + Ketoconazole (50 μM)</td>
<td>69 ± 9</td>
<td>147 ± 21</td>
<td>79 ± 14</td>
<td>2.2</td>
</tr>
<tr>
<td>Apixaban (20 μM)$^b$</td>
<td>6 ± 0</td>
<td>140 ± 10</td>
<td>NA</td>
<td>23.3</td>
</tr>
<tr>
<td>Apixaban (20 μM) + Probepenicillic (200 μM)$^b$</td>
<td>7 ± 2</td>
<td>123 ± 6</td>
<td>14 ± 5</td>
<td>17.6</td>
</tr>
<tr>
<td>Apixaban (20 μM) + Probepenicillic (1 mM)$^b$</td>
<td>9 ± 0.5</td>
<td>137 ± 6</td>
<td>5 ± 2</td>
<td>15.2</td>
</tr>
</tbody>
</table>

*NA, not applicable.

$^a$ As a positive substrate.

$^b$ These experiments were done at a different time from other experiments in the table.

### Table 4

| Inhibition potential of digoxin transport by apixaban in Caco-2 cells |
|----------------|------------|------------|-------|
| **μM** | **μM** | $P_{A-B,app}$ | $P_{B-A,app}$ | % Inhibition of Digoxin Efflux |
| 5 | 0 | 6 ± 1 | 140 ± 9 | 134 |
| 5 | 5 | 18 ± 10 | 144 ± 6 | 126 | 5.9 |
| 5 | 25 | 5 ± 0.2 | 126 ± 33 | 121 | 9.9 |
| 5 | 12.5 | 7.5 ± 3.5 | 139 ± 10 | 132 | 1.5 |
transfected cells (LLC-PK1-P-gp, MDCKII-BCRP) clearly demonstrate. Apixaban has high intrinsic permeability with low absorption; however, the value is certainly limited by efflux (brain/blood ratio which helps explain the general properties of absorption and tissue-on its absorption. (50%) (Zhang et al., 2009; Wong et al., 2011) despite the efflux effect good oral bioavailability in rats (30%), dogs (70%), and humans (50%) (Zhang et al., 2009; Wong et al., 2011) despite the efflux effect on its absorption.

Apixaban is a substrate for the efflux transporters P-gp and BCRP, which helps explain the general properties of absorption and tissue-specific distribution. Apixaban had a very low brain penetration in rats (brain/blood ratio <0.1) following oral administration (Wang et al., 2011). P-gp and BCRP are expressed in the endothelial cells of the brain capillaries and in the epithelial cells of the choroid plexus (Löschner and Potschka, 2005; Tsuji, 2005). BCRP itself might play a minor role in efflux of several BCRP substrates (as determined by in vitro bidirectional permeability) at the blood-brain barrier in mice (Zhao et al., 2009) but could play a significant role when coexpressed with P-gp as supported by a synergistic effect of P-gp and BCRP for central nervous penetration of the tyrosine kinase inhibitor lapatinib in the P-gp- and BCRP-triple knockout mice (Polli et al., 2009).

Whole-body autoradiography showed that the radioactivity concentration in fetal tissues was much lower than those in the respective maternal tissues, suggesting that there was a placental membrane restriction to the transfer of apixaban into the fetus (Wang et al., 2011). The placental barrier contains efflux transporters such as BCRP, P-gp, and Mrp2 in the apical membrane of placental syncytiotrophoblasts (Unadkat et al., 2004; Esseenko et al., 2006; Prouillac and Lecoeur, 2010) that can pump compounds from the fetal compartment to the maternal circulation (Lankas et al., 1998; Jonker et al., 2005; Esseenko et al., 2006). BCRP is the most abundant transporter expressed in the placenta, and the mRNA level of BCRP was found to be close to 10 times greater than that of P-gp in human placenta (Maliepaard et al., 2001). The abundant expression in the placenta supports that BCRP plays an important role in limiting the transfer of apixaban to the fetus in rats.

Apixaban was extensively secreted into milk in lactating rats following oral administration (Wang et al., 2011). The high milk/plasma ratio [about 8 for Cmax and 30 for AUC (area under the plasma concentration-versus-time curve)], which is well above that predicted based on physiochemical properties of apixaban, strongly suggests that the active transport was involved in the lacteal secretion of apixaban. BCRP is strongly induced in the mammary gland of mice, cows, and humans during lactation and is responsible for the active secretion of clinically important substrates (Jonker et al., 2005). In comparison, other efflux transporters such as P-gp, Mrp1, and Mrp2 were found to be absent from breast tissue in lactating mouse, suggesting these efflux transporters may not be as important as BCRP.

Naproxen is a nonsteroidal anti-inflammatory drug used for the reduction of moderate to severe pain, fever, inflammation, and stiffness. This study investigated the effect of naproxen on the efflux of apixaban in MDCKII-BCRP cell monolayers. Naproxen did not affect prazosin efflux at the concentrations that did not disrupt cell monolayers of MDCKII-BCRP cells and, therefore, naproxen would at best be a very weak inhibitor of BCRP. The bidirectional permeability of doxorubicin (5 μM) and apixaban (3 μM) in Caco-2 cell monolayers was also examined in the presence of naproxen; apixaban efflux was inhibited up to 42% at a concentration of 6 mM. An oral dose of 500 mg of naproxen would provide an intestinal concentration of 6–10 mM given an upper gastrointestinal volume of 0.25 liters, and naproxen would potentially enhance absorption of a P-gp substrate through intestinal transporter inhibition. Naproxen has been identified as an inhibitor of organic cation transporters OAT1 and OAT3 (Khamdang et al., 2002), and it is the first nonsteroidal anti-inflammatory drug identified as a weak P-gp inhibitor relative to keto and CsA. Clinical results suggest that the naproxen coadministration increased the Cmax and AUC of apixaban by approximately 50% without affecting its elimination phase, suggesting the pharmacokinetic interaction of apixaban was at absorption (unpublished data). In addition, naproxen and its major metabolite (naproxen glucuronide) did not inhibit CYP3A4 (direct or time-dependent) at concentrations up to 300 μM, a clinically relevant concentration of naproxen. Naproxen glucuronide did not inhibit P-gp at a 1 mM concentration. Therefore, the clinical observation of increased absorption of apixaban could be explained by a mechanism of inhibition of intestinal efflux of apixaban.

Keto at 20 μM only slightly affected the ER of prazosin measured in experiments with MDCKII-BCRP cell monolayers and did not appear to greatly inhibit BCRP-mediated transport of nitrofurantoin in Caco-2 cells. These data suggest that keto is not a potent inhibitor of BCRP. However, keto is a strong inhibitor for both the efflux transporter P-gp and CYP3A, the main enzyme responsible for metabolism of apixaban (Wang et al., 2010). Although available data would not allow quantitative assessment of the relative contribution of P-gp and BCRP or quantitative prediction of clinical drug-drug interaction, the results would predict that coadministration of keto might result in an interaction at absorption and drug metabolism of apixaban given that a clinical Cmax value of >10 μM of keto (Kaeser et al., 2009) and IC50 values of <5 μM for keto inhibition of both P-gp and CYP3A4. Indeed, a clinical drug-drug interaction study has shown that keto increased apixaban AUC and Cmax by 100 and 54%, respectively. Apixaban is not an inhibitor of P-gp or BCRP at clinically relevant concentrations, which would predict an unlikely drug-drug interaction with substrates of P-gp and BCRP.

In summary, permeability studies in P-gp- and BCRP-expressed cell lines and Caco-2 cells demonstrated that apixaban is a substrate for efflux transporters P-gp and BCRP. These efflux transporters may play a role in the disposition of apixaban such as low brain penetration and low fetal exposure and milk excretion in rats. Inhibition of these transporters provides an explanation for the observed low level of drug-drug interactions with keto and naproxen. This study also demonstrates the application of multiple approaches, including in vitro models, probe substrates, and inhibitors of transporters that are needed to study a compound, especially when it is a substrate for multiple transporters.
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Authorship Contributions

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Contributed new reagents or analytic tools: Shou, Balimane, Han.

Performed data analysis: Zhang, He, Herbst, Kolb, Wang, Balimane, Gan, Humphreys.

Wrote or contributed to the writing of the manuscript: Zhang, Gan, Frost, Humphreys.

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