Characterization of Efflux Transporters Involved in Distribution and Disposition of Apixaban

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Running title: Efflux transporters in apixaban disposition

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Text pages (not including references): 25

# of Table: 4
# of Figures: 4
# of references: 45
# of words in abstract: 250
# of words in introduction: 450
# of words in discussion: 1500

[1]Abbreviations used: BCS, Biopharmaceutical Classification Systems; BCRP, breast cancer resistance protein; CsA, cyclosporin A; DMSO, dimethyl sulfoxide; DMEM, Dulbecco's Modified Eagle's Medium; ER, efflux ratio - permeability of basolateral to apical versus that of apical to basolateral; HBSS, Hank's balance salt solution; HEPES, N-(2-Hydroxyethyl) piperazine-N'-(2-ethanesulfonic acid); keto, ketoconazole; LC/MS, liquid chromatography and mass spectrometry; MDR1, multiple drug resistance protein (P-gp); NSAID, non-steroidal anti-inflammatory drugs; Pc_{A-B}, permeability of apical to basolateral direction; Pc_{B-A}, permeability of basolateral to apical direction; TEER, trans-epithelial electrical resistance

Abstract

The studies reported here were conducted to investigate the transport characteristics of apixaban and to understand the impact of transporters on apixaban distribution and disposition. In human P-gp- and BCRP-cDNA transfected cell monolayers as well as Caco-2 cell monolayers, the apparent efflux ratio of basolateral to apical (Pc_{B-A}) versus apical to basolateral permeability (Pc_{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 the 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. Co-incubation 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 NSAID 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, low fetus exposures, and milk excretion in rats.

Introduction

Efflux transporters are ATP Binding Cassette (ABC) proteins containing multi-trans-membrane spanning domains with homologous ATP-binding sites. Several members of this family are primary drug transporters which 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; Shitara et al., 2006; Zhou, 2008; Koshiba et al., 2008; Xia et al., 2005a; Giacomini et al., 2010). P-Glycoprotein (P-gp, encoded by MDR1, ABCB1), a member of the ABC transporter superfamily, is expressed in the human intestine, liver, brains, 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 to the current treatments including use of warfarin.

Apixaban is currently approved for the prevention of venous thromboembolism (VTEp) and the prevention of stroke in patients with atrial fibrillation (AF) (Lassen et al., 2007; Connolly et al., 2011; Granger et al., 2011). Apixaban has balanced elimination pathways including renal excretion, metabolism, intestinal/biliary excretion in humans (Raghavan et al., 2009; Zhang et al., 2010; Wong et al., 2011). Metabolism was responsible for 25% of apixaban 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; Elsby et al., 2008; Englund et al., 2006). 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, 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 radiolabeled [^{14}C]apixaban (102 μCi/mg) were synthesized at Bristol-Myers Squibb. The structure of apixaban is shown in Figure 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 (20 mM), nitrofurantoin (1 mM), ketoconazole (5 mM), and cyclosporin A (5 mM) were prepared in DMSO. A stock solution of naproxen (10 mM) was prepared in 10 mM HEPES assay buffer (pH 7.4). [^{14}C]Apixaban (102 μCi/mg) was prepared in DMSO (2.2 mM and 100 μCi/mL). [^{3}H]Digoxin (0.3 mCi/mg) was prepared in the transport assay buffer (5 μM and 1.3 μCi/mL). [^{3}H]Prazosin (0.2 mCi/mg) was diluted in ethanol containing 0.01 M HCl (13 mM and 1 mCi/mL). [^{14}C]Mannitol (0.1 mCi/mg) was diluted in ethanol:water (9:1) (20 mM, 0.4 mCi/ml). None of these 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$_1$ cells and control LLC-PK$_1$ cells (BD Gentest^{TM}, MA) contained the vector without human P-gp cDNA (Mock). The cells were seeded onto a collagen-coated polycarbonate filter membrane (port size 1 μ, diameter 6.5 mm) on the transwell inserts (0.7 cm^2)

(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-transwell plates, at 37°C, 5% CO_2 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 MDCKII (Madin Darby Canine Kidney) cells and control MDCKII cells (SOLVO Biotechnology, Hungary) contained the vector without human BCRP cDNA (Mock). Cells were cultured in DMEM Medium (Carbrex, NJ) at 37°C in an atmosphere of 5% CO_2 in cell culture flasks. Cells (50,000 cells/well) were seeded onto 24-well transwell inserts (0.7 cm^2). 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 transwell Corning 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 cm^2) at a density of 45,000 or 70,000 cells/well and grown in culture medium consisting of DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 0.5 mM HEPES, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin (all from Gibco BRL, Gaithersburg, MD). The culture medium was replaced every two days and the cells maintained at 37°C, 95% relative humidity, and 5% CO_2. Caco-2 cell passage numbers of 17-40 were used for these studies and were monitored for P-gp expression with positive

substrates within five days of projected use in the assay, usually 13 - 22 days post-seeding.

Monolayer integrity was evaluated by pre-experimental trans-epithelial electrical resistance (TEER) measurements performed using the EVOM resistance meter from World Precision Instruments or post-experimental Lucifer Yellow apical to basolateral flux determinations for each cell monolayer. After all samples were collected, Lucifer 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-min incubation on an orbital shaker (50 rpm) at 37°C, with ambient humidity and CO_2, receiver samples were removed to measure percent Lucifer Yellow flux. Monolayer integrity was also evaluated by determining permeability of mannitol and comparing with the permeability values of mannitol without co-assay compound. For P-gp or BCRP cDNA-expressed cells and Caco-2 cells, [^{3}H]digoxin (at 5 μM) or [^{3}H]prazosin (at 5 μM) was measured in a bi-directional assay and the efflux ratios were determined.

Permeability determination procedures

Permeability studies were performed in triplicate at pH 7.4 in Hank's balanced salt solution (Gibco BRL, Gaithersburg, MD) containing 10 mM HEPES, and pH adjusted with NaOH. For apical-to-basolateral (A to B) permeability (absorptive direction), the donor solution was placed in the apical compartment, and for basolateral-to-apical (B to 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 (35-100 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 (C_0) 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 (50 rpm) at 37°C, with ambient humidity and CO_2 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 min) and replaced by an equal volume of receiver solution. Samples from the donor chambers were taken at one time point of 120 min. To determine the extent of non-specific 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.

[^{14}C]Mannitol (50 μM) and [^{3}H]digoxin (5 μM) were used as the low permeability control and P-gp substrate control, respectively. [^{14}C]Apixaban was assayed at six concentrations (2.5, 5.0, 10, 25, 50, 100 μM) bi-directionally in both the P-gp transfected and the vector carrying LLC-PK_1 cell monolayers. Samples from the donor and receiver

chambers were taken at one time point of 90 min. Apixaban was also assayed bi-directionally 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 ketoconazole and cyclosporin A in both the donor and receiver chamber.

Permeability determination in BCRP-cDNA expressed cells

Incubations with BCRP-cDNA tranfected MDCKII cell monolayers were carried out in modified Krebs-Henseleit buffer plus 5 mM glucose at 37°C. Cells were pre-incubated in buffer for 10 min 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 [^{3}H]prazosin was also evaluated in the presence of 1 μM of 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 min. The concentration-dependent transport of apixaban was also evaluated at 1, 5, 25, and 100 μM in the A to B and B to 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-drug interaction study with apixaban. The high concentration of naproxen was selected because gastrointestinal concentration of naproxen could reach 5-10 mM following oral administration of 500 mg naproxen based on 250 mL of gastro-fluid volume in humans. The bi-directional permeability of apixaban (5 μM) was also determined in the presence of ketoconazole (20 and 50 μM) and naproxen (3 and 8 mM).

Permeability determination in Caco-2 cell monolayers

For Caco-2 cell monolayers, apical compartments received 200 µL, and basolateral compartments received 600 µL of permeability solutions. The cell transwells were then placed on an orbital shaker (50 rpm) and incubated for 2 hours at 37°C. Following incubation, samples were removed for analysis. Donor solutions were prepared for permeability comparators of a low permeability control [^{14}C]mannitol (50 µM, 1 µCi/mL) and a control P-gp substrate [^{3}H]digoxin (5 µM). The bi-directional permeability values of [^{14}C]apixaban at 3.0 or 30 µM were also determined in the presence of the two transporter inhibitors, ketoconazole and cyclosporin A (at 50 µM) as well as naproxen (at 0.2, 1 or 6 mM) present in both donor and receiver chambers. The bi-directional permeability of apixaban (3.0 µM), digoxin (5.0 µM), nitrofurantoin (5.0 µM) was also determined in the presence of ketoconazole (20 µM), cyclosporin A (20 µM), and Ko134 (1 and 11 µM) separately or in a combination. The efflux inhibition of digoxin (5 µM) was also evaluated in the presence of naproxen (8 mM). Probenecid (200 µM and 1 mM) was co-incubated with apixaban at 20 µM in Caco-2 monolayers to measure apixaban permeability (Loe et al., 1996; Horikawa et al., 2002).

The potential inhibition of P-gp by apixaban was explored by co-incubation of apixaban (up to 50 µM) with digoxin (5 µM), or apixaban (200 µM) with Rhodamine 123 (20 µM) in Caco-2 monolayers to determine the permeability of digoxin or Rhodamine 123, a P-gp substrate (van der Sandt et al., 2000). Rhodamine 123 was quantified using a fluorescence plate reader (HTS 7000, PerkinElmer, Norwalk, CT) with excitation of 492 nm and emission of 530 nm. Additional experiments using a range of apixaban concentrations in Caco-2 cells were conducted to assess the potential inhibition of apixaban on P-gp-mediated transport of digoxin. In this study, apixaban at 0.1 to 50 µM

was tested and Cyclosporin A was also tested at the same range of concentrations (0.1 to 50 μM) and served as a positive inhibitor. To investigate the involvement of paracellular transport, palmitoyl-L-carnitine, a paracellular absorption enhancer, was co-incubated with apixaban in the Caco-2 monolayers (Hochman et al., 1994).

Permeability in rat intestinal segments

Rat intestinal permeability was examined using excised segments from various intestinal sites of 8 week-old male rats as described previously (Kilic et al., 2004; Sinko et al., 1995). The intestinal segments were mounted on side-by-side diffusion chambers (Corning Incorporated, Acton, MA). Donor solution consisted of 200 μM apixaban and 1% DMSO in Tyrode's buffer (pH 7.4). Permeability studies were conducted at 37°C either in the mucosal (m) → serosal (s) or s → m direction using 4 mL of donor solution and 4 mL of receiver solution (pH 7.4, Tyrode's buffer containing 4% BSA). Samples were taken at 30, 60, 90, and 120 min from receiver compartments. Donor compartments were sampled at 120 min. Apixaban was quantified by LC/MS/MS.

LC/MS analysis of apixaban and nitrofurantoin

The LC-MS/MS system consists of binary Shimadzu 10ADvp pumps controlled by a SCL-10Avp controller, a Leap HTS autosampler, and a Sciex 4000 QTrap hybrid triple quadrupole – linear ion trap mass spectrometer (Applied Biosystems, Forest City, CA). MS/MS detection was in positive mode for apixaban (m/z 460.1 to 443.2) and in a negative mode for nitrofurantoin (m/z 237.1 to 152.1). The internal standard was the stable labeled [$^{13}CD_3$]apixaban (m/z 464.1 to 447.2) for apixaban and tobutamide (m/z 269.2 to 169.9) for nitrofurantoin. Standard curves contained a four-point calibration curve (at 5, 50, 500 and 5000 nM) for apixaban and a eight-point curve (at 5, 10, 50,

100, 500, 1000, 2500, 5000 nM) for nitrofurantoin prepared in 1:1 Hank's buffer:acetonitrile. Study samples or calibration standards (50 µL) were first extracted with 50 µL of internal standard in methanol, and then 5 µL of the mixture was injected onto a Mac-Mod Halo C18, 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 min, then held at 100% B for 0.25 min before decreased to 2% B in 0.1 min. For nitrofurantoin, the mobile phases were (A) 2 mM ammonium acetate containing 0.2% formic acid and 2% actonitrile and (B) acetonitrile containing 0.2% formic acid and 2% water; the gradient was 5% to 100% B in 0.5 min 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 from the calibration curve.

Sample analysis for radioactive samples

Samples of 100 µL were removed to scintillation vials, and 8 mL of EcoLite™ scintillation fluid (Irvine, CA) added to each vial. Vials were counted on either a ^{3}H DPM program or a ^{14}C DPM program (with background subtraction) on a PerkinElmer Tri-Carb Scintillation Counter Tri-Carb 3100TR (PerkinElmer Life Sciences, Boston, MA).

Data analysis

The apparent permeability coefficient (Pc, nm/sec) was calculated according to the following equation: $Pc = dA/ (dt \cdot S \cdot C_0)$, where dA/dt is the flux of compound across the

monolayer (nmole/sec), S is the surface area of the cell monolayer, and C_0 is the initial concentration (μM) in the donor compartment. Pc_{B-A} is the Pc value measured in the B to A direction and Pc_{A-B} is the Pc value measured in the A to B direction. The efflux ratio was calculated as: Efflux Ratio = Pc_{B-A}/ Pc_{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); 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 x t), where v is the rate of P-gp or BCRP facilitated drug transport (pmol/cm^2/hr), IA is the intrinsic activity (pmol transported during the assay), and A 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., 2006). The percent inhibition of apixaban efflux is calculated as:

Percent Inhibition = 1-[(Pc_{B-A} of test compound in presence of inhibitor) - (Pc_{A-B} of test compound in presence of inhibitor)] / [(Pc_{B-A} of test compound in absence of inhibitor) - (Pc_{A-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

Pre-experimental TEER measurements and post-experimental 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 p ositive control inhibitors. TEER values were in agreement with the historical range of > 500 $\Omega \bullet cm^2$. The Lucifer Yellow flux was <2% with individual values < 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-PK_1-P-gp cells, the efflux ratios of digoxin (5 μM), a known P-gp substrate, were > 7, which were inhibited >80% by ketoconazole (at 20 or 50 μM), a known P-gp inhibitors. For MDCKII-BCRP cells, prazosin (5 μM), a known BCRP substrate, showed an efflux ratio of approximately 10, and the efflux was inhibited by Ko134 (1 μM), an analogue of fumitremorgin C, and a potent and selective inhibitor of BCRP (Allen et al., 2002), yielding an efflux ratio 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 non-specific binding to the cells or absorption to the transwell apparatus did not affect the assay under the conditions used.

Permeability in cell models

The bi-directional permeability of apixaban was studied in LLC-PK_1-P-gp cell monolayers. Digoxin at 5 μM showed an efflux ratio of 7.4, reduced >90% by ketoconazole (30 μM) (Figure 2A) or cyclosporin A (10 μM) (Data not shown). Apixaban was subject to active basolateral to apical transport with efflux ratios in LLC-PK_1-P-gp cells ranging from 23 to 38 compared to ratios of 1.4 to 4.4 in control cells (Table 1). Apixaban transport was linear over the time course (60, 90, 120 min) (Table 1). P-gp facilitated transport of apixaban was concentration-dependent and did not show saturation over the concentration range (2.5-100 μM) tested. 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 efflux ratios 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 ketoconazole IC_{50} values of 2.9 - 5.4 μM (Figure 2B). This finding indicates P-gp facilitated transport of apixaban across LLC-PK_1-P-gp cell monolayers.

The bi-directional permeability of [^{3}H]prazosin (5 μM) and [^{14}C]apixaban (1 to 100 μM) was studied in vector-containing MDCKII cell and BCRP cDNA-transfected MDCKII

cells (Table 2, Figure 3A and B). Prazosin efflux was completely inhibited by 1 μM of Ko134. Ketoconazole showed marginal inhibition on prazosin efflux at 20 μM but at 50 μM ketoconazole showed more robust inhibition. In the same experiment, naproxen showed a very low level of inhibition of prazosin efflux with a minor increase in Pc_{A-B} and decrease in Pc_{B-A} at the highest concentration (8 mM) tested, which was also accompanied by a slightly higher mannitol permeability of 30 nm/sec. The apixaban efflux ratio was 1.4 to 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 (Figure 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 (Figure 3B); however, ketoconazole 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 bi-directional 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 efflux ratio of apixaban ranged between 12 and 37 with the apparent permeability coefficient (Pc_{A-B}) values of approximately 6 to 16 nm/sec in the apical-to-basal direction and 140 to 387 nm/sec in the basal-to-apical direction (Table 3, Figure 4A). Both cyclosporin A (50 μM) and ketoconazole (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, Cyclosporin A and ketoconazole

completely inhibited 98% of efflux of digoxin at 5 μM (a well-characterized substrate of P-gp) (Table 3).

The apparent permeability (Pc) of apixaban in Caco-2 cells in either direction was not affected by probenecid, 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 para-cellular absorption enhancer, significantly increased the Pc of apixaban from 10 ± 0.5, 28 ± 2.3, 37 ± 5.1, 47 ± 5.2 nm/sec, approximately 1, 3.5, 5, and 6 folds at concentrations of 0, 0.2, 0.3, 0.5 mM, respectively, suggesting that apixaban showed potential paracellular as well as intra-cellular transport in Caco-2 cells.

Intestinal permeability

The intestinal absorption of apixaban was studied using isolated rat duodenum, jejunum, ileum, and colon segments (Kilic et al., 2004; Sinko et al., 1995). The mucosal-to-serosal Pc values of apixaban were lower at 33± 12, 73± 28, 51± 38, and 31± 22 nm/sec in the duodenum, jejunum, ileum, and colon segments, respectively, than the Pc values of apixaban in the serosal-to-mucosal direction of 170 ±23 nm/sec 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 ketoconazole and cyclosporin A at concentrations of 20 μM partially inhibited apixaban efflux compared to nearly complete inhibition of digoxin efflux in Caco-2 cells (Figure 4B). Although Ko134 at 1 μM 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 μM) inhibited the efflux of apixaban, digoxin, and nitrofurantoin by 40, 50, and 78%, respectively. At a concentration of 20 μM, ketoconazole or cyclosporin A did not inhibit the efflux of nitrofurantoin in the Caco-2 cells. With a combination of a P-gp inhibitor (ketoconazole or cyclosporin A) with the BCRP inhibitor (Ko134) at their 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 (Figure 4B).

The effect of naproxen on the bi-directional 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 bi-directional Caco-2 permeability assay through decreasing B to A permeability and increasing A to B permeability of digoxin (data not shown).

The efflux ratio 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, Cyclosporin A 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

In order 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 (Pc) values in the basolateral-to-apical direction than the apical-to-basolateral direction with efflux ratios >10 in LLC-PK_1-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 bi-directional permeability of apixaban was tested in the presence of transporter inhibitors. In transfected cell monolayers (LLC-PK_1-P-gp or MDCKII-BCRP), the P-gp inhibitor ketoconazole and the BCRP inhibitor Ko134 extensively inhibited apixaban efflux. However, in Caco-2 cells, cyclosporin A or ketoconazole (at 20 or 50 μM) only partially inhibited apixaban efflux (Table 3) compared to 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 LY-335979 (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 (Figure 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 transporters-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 cyclosporin A with Ko134 (>85% inhibition) appeared to be more effective than the combination of ketoconazole 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 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 considerably higher than that of P-gp and MRP2 (Taipalensuu et al., 2001). Although ketoconazole 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 cells, which are known to express endogenous canine P-gp (Gupta et al., 2007). This result is consistent with apixaban being a substrate of P-gp as well as BCRP. The low efflux ratios (2-4) of apixaban in LLC-PK_1 and MDCKII control cell monolayers suggested expression of endogenous transporters. While the expression of endogenous transporters may confound the ability to study the interaction of a compound with the transporter due to the interference in the control cells, the high efflux ratios of apixaban (>10) in the corresponding transporter-transfected cells (LLC-PK_1-P-gp, MDCKII-BCRP) clearly demonstrate a signal above the interference of endogenous transporters in these cells.

In general, a compound with a permeability coefficient Pc_{A-B} (absorptive direction) greater than 100 nm/sec is considered highly permeable and would predict high (>70%) in vivo absorption based on the biopharmaceutical classification system (FDA Guidance for Industry, 2000; Chong et al., 1997). Apixaban demonstrates a Pc_{A-B} value that would predict low absorption, however, the value is certainly limited by efflux processes. Apixaban has high intrinsic permeability with Pc values in the PAMPA assay of >200 nm/sec and relatively high Pc values in Caco-2 cells in the presence of P-gp and BCRP inhibitors. The good intrinsic permeability of apixaban led to good oral bioavailability in rats (30%), dogs (70%), and humans (50%) (Wong et al., 2011; Zhang et al., 2009; Shantsila and Lip, 2008) despite the efflux effect on its absorption.

Apixaban is a substrate for the efflux transporters P-gp and BCRP, which helps understand 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 (Tsui et al., 2005; Loschner and Potschka, 2005). BCRP itself might play a minor role in efflux of several BCRP substrates (as determined by in vitro bi-directional permeability) at the blood-brain barrier in mice (Zhao et al., 2009), but could play a significant role when co-expressed 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 (Prouillac and Lecoeur, 2010; Unadkat et al., 2004; Evseenko et al., 2006) that can pump compounds from the fetal compartment to the maternal circulation (Lankas et al., 1998; Jonker et al., 2005; Evseenko 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), 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 NSAID 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 bi-directional permeability of digoxin (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 liter, 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 now identified as the first NSAID as a weak P-gp inhibitor relative to ketoconazole and cyclosporin A. Clinical results suggest that the naproxen co-administration increased the Cmax and AUC of apixaban by approximately 50% without affecting its elimination phase, suggesting the pharmacokinetic interaction of apixaban was at absorption (data not shown). 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.

Ketoconazole at 20 μM only slightly affected the efflux ratio 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 ketoconazole is not a potent inhibitor of BCRP. However, ketoconazole 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 co-administration of ketoconazole might result in an interaction at absorption and drug metabolism of apixaban given that a clinical Cmax value of >10 μM of ketoconazole (Kaeser et al., 2009) and IC50 values of <5 μM for ketoconazole inhibition of both P-gp and CYP3A4. Indeed, a clinical drug-drug interaction study has shown that ketoconazole increased apixaban AUC and Cmax by 100% and 54%, respectively. Apixaban is not an inhibitor of P-gp or BCRP at clinical 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, 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 ketoconazole 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.

Acknowledgments. Authors thank Brad Maxwell for preparation of radiolabeled apixaban, Berend Oosterhuis and Greg Loewen from Solvo Biotechnology and

XenoTech, and Dr. Elke S. Perloff from BD Gentest for conducting some of these transporter experiments. Authors also thank Dr Bruce D. Car, and John H. Lawrence for helpful discussions.

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Footnotes:

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Legends of Figures

Figure 1. Structure of apixaban

Figure 2. (A) Permeability coefficients of digoxin (5 μM) and apixaban (5 μM) in LLC-PK_1-P-gp cell monolayers in the absence and presence of ketoconazole. (B) Inhibition of apixaban efflux by ketoconazole at several concentrations in LLC-PK_1-P-gp cell lines (the numbers after compounds are concentrations in μM for keto = ketoconazole).

Figure 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), ketoconazole (20 and 50 μM), or naproxen (3 and 8 mM) (the numbers after compounds are concentrations in μM for keto = ketoconazole and Ko134 and mM for naprox = naproxen).

Figure 4. (A) Permeability coefficients of digoxin (5 μM) and apixaban (3 μM) in Caco-2 cell monolayers in the absence and presence of ketoconazole. (B) Inhibition of permeability efflux of digoxin (5 μM), nitrofurantoin (5 μM), and apixaban (3 μM) by individual and combined transporter inhibitors in Caco-2 cell monlayers (the numbers after compounds are concentrations in μM for keto = ketoconazole and CsA = cyclosporin A and mM for naprox = naproxen).

Table 1. T ime- and concentration-dependent transport of apixaban in human P-gp transfected porcine kidney cells (LLC-PK_1-P-gp) and control cells (LLC-PK_1, Mock)

Cell line	**Apixaban (µM)**	**Time (min)**	**Pcapp (nm/s) mean ± SD**		**Efflux Ratio mean±SD**	**P-gp facilitated transport**	
			A - B	**B - A**	**(B-A/A-B)**	**IA (pmol)**	**V (pmol/cm^2/hr)**
P-gp	10	60	4.3 ± 0.2	130 ± 16	30 ± 3.3	146 ± 23	471 ± 76
	10	90	5.1 ± 0.3	160 ± 9.3	31 ± 2.3	235 ± 20	505 ± 43
	10	120	4.4 ± 0.4	170 ± 9.2	38 ± 1.5	294 ± 13	474 ± 22
Control	10	60-120	31- 44.1	74-97	1.7 - 3.2	NA	NA
P-gp	2.5	90	9.4 ± 2.6	210 ± 9.3	23 ± 4.9	33 ± 13	71 ± 28
	5.0	90	5.5 ± 0.1	200 ± 11	36 ± 2.2	64 ± 12	139 ± 26
	10	90	6.2 ± 0.2	200 ± 11	31 ± 1.9	167 ± 58	360 ± 125
	25	90	6.1 ± 0.6	220 ± 4.2	36 ± 4.3	548 ± 99	1179 ± 213
	50	90	6.9 ± 0.6	240 ± 9.5	35 ± 4.6	1485 ± 69	3194 ± 148
	100	90	5.7 ± 0.6	210 ± 9.3	36 ± 4.9	2772 ± 109	5962 ± 234
Control	2.5-100	90	34 - 60.3	84 -150	1.4 - 4.4	NA	NA

NA = not applicable.

Table 2. Time- and Concentration-dependent transport of apixaban in human BCRP-transfected Canine kidney cells (MDCKII -BCRP) and control cells (MDCKII, Mock)

Cell line	**Apixaban (μM)**	**Time (min)**	**Pcapp (nm/s) mean ±SD**		**Efflux Ratio mean ±SD**	**BCRP facilitated transport mean±SD**	
			A - B	**B - A**	**(B-A/A-B)**	**IA (pmol)**	**V (pmol/cm^2/hr)**
BCRP	1	15	13.9 ± 6.0	148 ± 7.5	10.7 ± 0.4	2.2 ± 0.6	12.6 ± 3.4
	1	30				2.5 ± 0.5	7.1 ± 1.4
	1	60				12.9 ± 3.0	18.4 ± 4.3
	1	120				45.6 ± 2.1	32.6 ± 1.5
	5	15	14.1 ± 5.0	168 ± 12	12.0 ± 0.4	2.6 ± 3.8	14.8 ± 21.7
	5	30				41.5 ± 9.8	118.5 ± 28.0
	5	60				71.6 ± 50.6	102.2 ± 72.3
	5	120				251 ± 12	179.3 ± 8.6
	25	15	10.6 ± 3.4	149 ± 13	11.5 ± 0.3	36.1 ± 8.8	206.2 ± 50.3
	25	30				73.4 ± 2.4	209.7 ± 6.9
	25	60				335 ± 182	478.6 ± 260
	25	120				1568 ± 134	1120 ± 95.7
	100	15	16.1 ± 3.9	130 ± 18	8.1 ± 0.3	603 ± 28	3445 ± 160
	100	30				725 ± 32	2071 ± 91
	100	60				1100 ± 573	1571 ± 818
	100	120				5580 ± 90	3985 ± 64
Control	1-100		49.3 -74.9	89.5 - 112	1.4 - 2.4	NA	NA

NA = not applicable.

Table 3. Inhibition of digoxin and apixaban efflux in Caco-2 cells

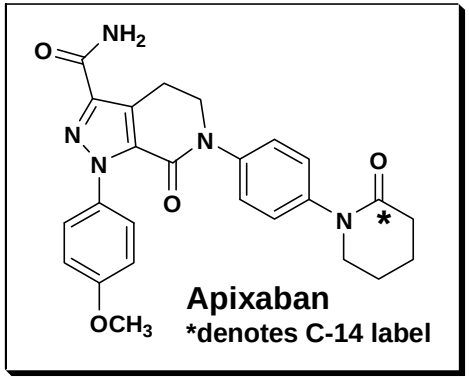
Test Conditions	$Pc_{A-B, app}$ (nm/sec)	$PC_{B-A, app}$ (nm/sec)	% Inhibition of Efflux ± SD	Efflux Ratio
Digoxin (5 μM)[a]	23 ± 3	205 ± 7	NA	8.9
Digoxin (5 μM)+ Cyclosporin A (50 μM)	86 ± 15	89 ± 17	98 ± 1	1.0
Digoxin (5 μM)+ Ketoconazole (50 μM)	96 ± 3	95 ± 6	100 ± 0	1.0
Apixaban (3 μM)	16 ± 1	387 ± 54	NA	24.2
Apixaban (3 μM) + Cyclosporin A (50 μM)	67 ± 9	278 ± 25	43 ± 5	4.1
Apixaban (3 μM) + Ketoconazole (50 μM)	70 ± 8	177 ± 21	71 ± 5	2.5
Apixaban (3 μM) + Naproxen (0.2 mM)	12 ± 1	307 ± 13	21 ± 3	25.6
Apixaban (3 μM) + Naproxen (1 mM)	16 ± 3	279 ± 29	29 ± 7	17.4
Apixaban (3 μM) + Naproxen (6 mM)	35 ± 4	251 ± 25	42 ± 6	7.2
Apixaban (30 μM)	10 ± 2	292 ± 13	NA	29.2
Apixaban (30 μM) + Cyclosporin A (50 μM)	39 ± 4	182 ± 18	50 ± 6	4.7
Apixaban (30 μM) + Ketoconazole (50 μM)	69 ± 9	147 ± 21	79 ± 14	2.2
Apixaban (20 μM)[b]	6 ± 0	140 ± 10	NA	23.3
Apixaban (20 μM)+ Probenecid (200 μM)[b]	7 ± 0.2	123 ± 6	14 ± 5	17.6
Apixaban (20 μM) + Probenecid (1 mM)[b]	9 ± 0.5	137 ± 6	5 ± 2	15.2

[a] as a positive substrate. [b]these experiments were done at a different time from rest of experiments in the table. NA, not applicable. SD = Standard Deviation.

Table 4. Inhibition potential of digoxin transport by apixaban in Caco-2 cells

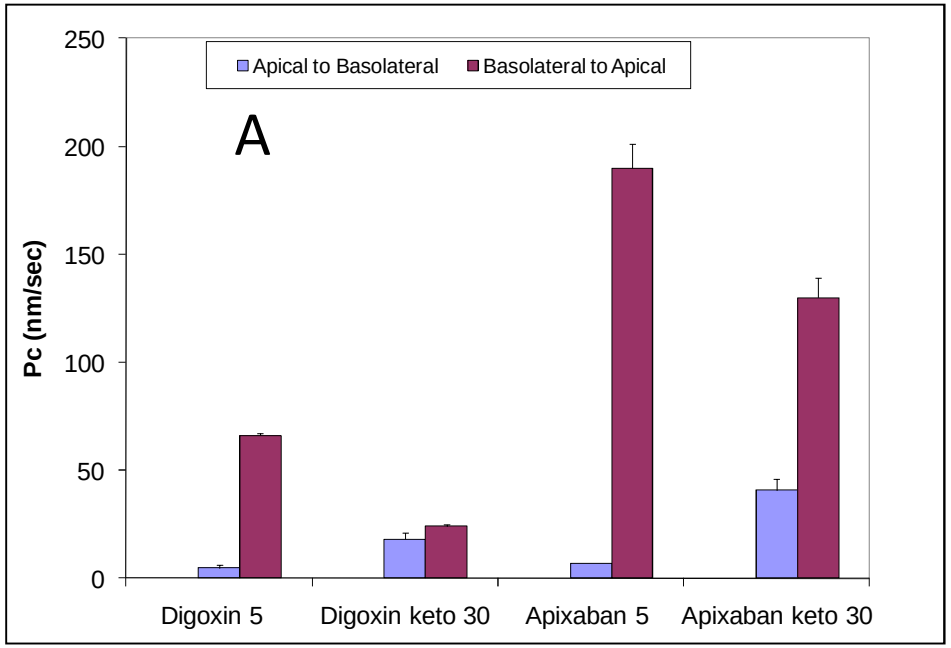
Digoxin (μM)	Apixaban (μM)	$Pc_{A-B, app}$ (nm/sec)	$PC_{B-A, app}$ (nm/sec)	PC Difference (nm/sec)	% Inhibition of digoxin efflux
5	0	6 ± 1	140 ± 9	134	0
5	50	18 ± 10	144 ± 6	126	5.9
5	25	5 ± 0.2	126 ± 33	121	9.9
5	12.5	7 ± 3.5	139 ± 10	132	1.5

Fig 1



Apixaban
***denotes C-14 label**

Fig 2


Apical to Basolateral
Basolateral to Apical
A
Pc (nm/sec)
250
200
150
100
50
0
Digoxin 5
Digoxin keto 30
Apixaban 5
Apixaban keto 30

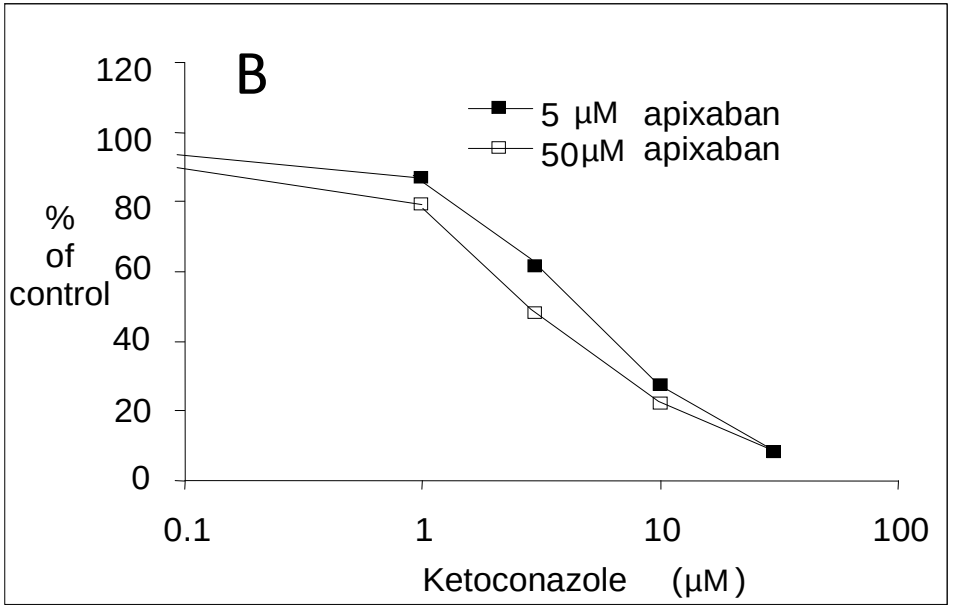

B
5 µM apixaban
50µM apixaban
% of control
120
100
80
60
40
20
0
0.1
1
10
100
Ketoconazole (µM)

Fig 3

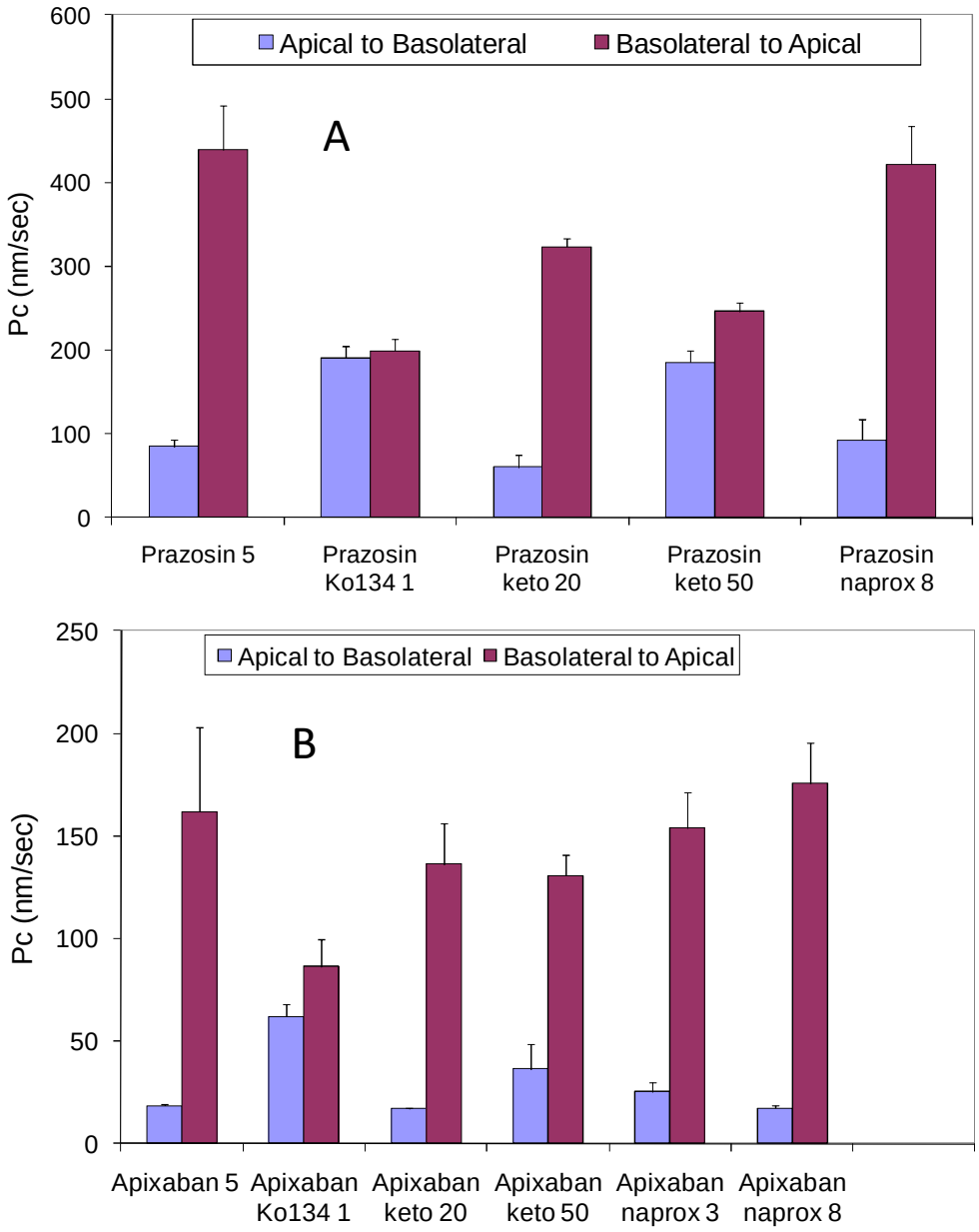
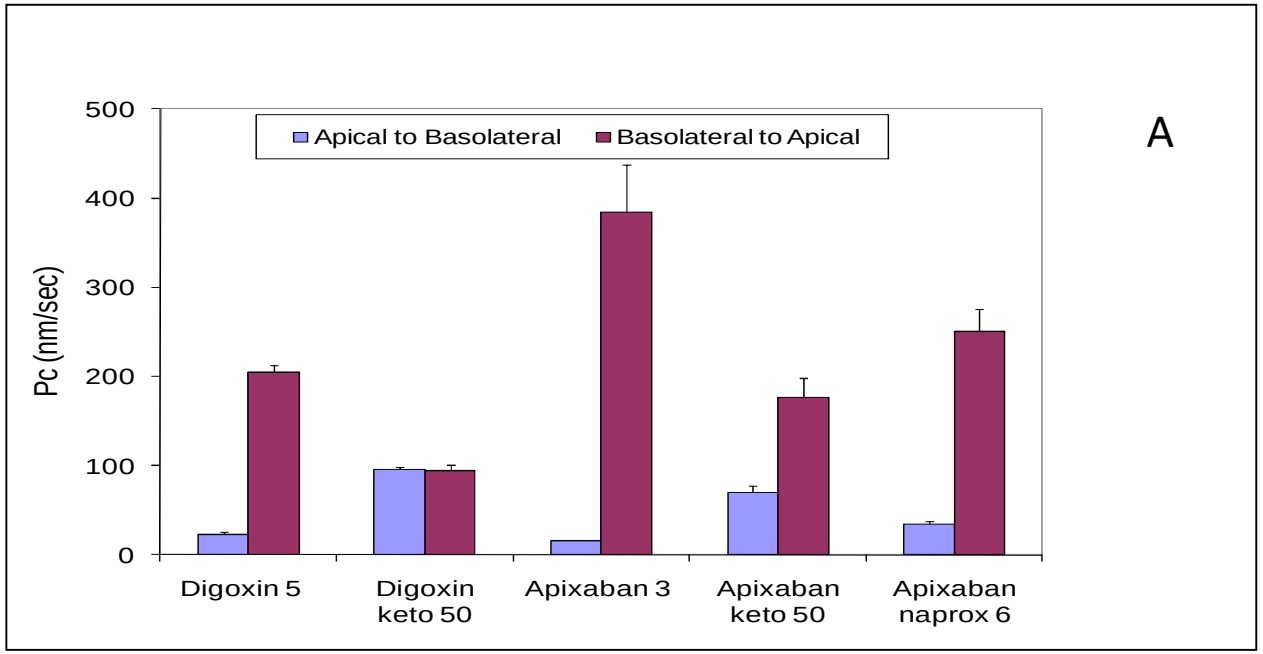
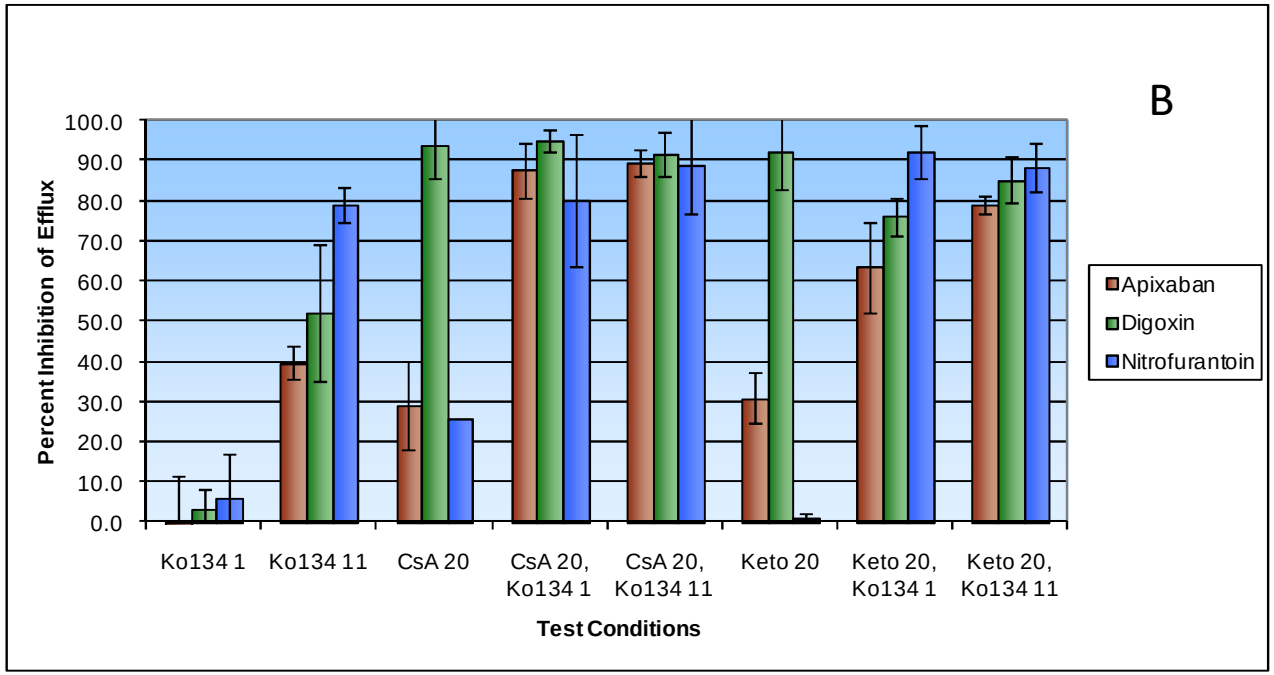

Apical to Basolateral
Basolateral to Apical
A
Pc (nm/sec)
600
500
400
300
200
100
0
Prazosin 5
Prazosin Ko134 1
Prazosin keto 20
Prazosin keto 50
Prazosin naprox 8
Apical to Basolateral
Basolateral to Apical
B
Pc (nm/sec)
250
200
150
100
50
0
Apixaban 5
Apixaban Ko134 1
Apixaban keto 20
Apixaban keto 50
Apixaban naprox 3
Apixaban naprox 8

Fig 4


A
Apical to Basolateral
Basolateral to Apical
Pc (nm/sec)
500
400
300
200
100
0
Digoxin 5
Digoxin keto 50
Apixaban 3
Apixaban keto 50
Apixaban naprox 6


B
Percent Inhibition of Efflux
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Ko134 1
Ko134 11
CsA 20
CsA 20, Ko134 1
CsA 20, Ko134 11
Keto 20
Keto 20, Ko134 1
Keto 20, Ko134 11
Test Conditions
Apixaban
Digoxin
Nitrofurantoin