IN VITRO ASSESSMENT OF METABOLIC DRUG-DRUG INTERACTION POTENTIAL OF APIXABAN THROUGH CYTOCHROME P450 PHENOTYPING, INHIBITION, AND INDUCTION STUDIES

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Abbreviations used: CYP, cytochrome P450; DMEM, Dulbecco's Modified Eagle Medium; DMSO, Dimethyl sulfoxide; EDTA, Ethylene diaminetetraacetic acid, tetrasodium salt; HIM, human intestinal microsomes; HKM, human kidney microsomes; HLM, human liver microsomes; LDH, lactate dehydrogenase; LC/MS, liquid chromatography/mass spectrometry; MCM, Modified Chee's Medium; NADPH, β-nicotinamide adenine dinucleotide phosphate (reduced form); V, the rate of metabolite formation; $K_m$, Michaelis-Menten constant; $K_i$, inhibition constant; $S$, substrate concentration; $V_{max}$, the maximal rate of metabolite formation.

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ABSTRACT

Apixaban is an oral, direct and highly selective factor Xa (fXa) inhibitor in late stage clinical development for the prevention and treatment of thromboembolic diseases. The metabolic drug-drug interaction potential of apixaban was evaluated in vitro. The compound did not show CYP inhibition (IC\textsubscript{50} values >20 \(\mu\text{M}\)) in incubations of human liver microsomes with the probe substrates of CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6 or 3A4/5. Apixaban did not show any effect at concentrations up to 20 \(\mu\text{M}\) on enzyme activities or mRNA levels of selected P450 enzymes (CYP1A2, 2B6, and 3A4/5) that are sensitive to induction in incubations with primary human hepatocytes. Apixaban showed a slow metabolic turnover in incubations of human liver microsomes with formation of O-demethylation (M2) and hydroxylation products (M4 and M7) as prominent in vitro metabolites. Experiments with human cDNA-expressed CYP enzymes, CYP chemical inhibitors, and correlation with P450 activities in individual human liver microsomes demonstrated that the oxidative metabolism of apixaban for formation of all metabolites was predominately catalyzed by CYP3A4/5 with minor contribution of CYP1A2 and 2J2 for formation of M2. The contribution of CYP2C8, 2C9, and 2C19 to metabolism of apixaban was less significant. Additionally, a human ADME study showed that more than half of the dose was excreted as unchanged parent (\(f_{\text{mCYP}} < 0.5\)), thus significantly reducing the overall metabolic drug-drug interaction potential of apixaban. Together with a low clinical efficacious concentration and multiple clearance pathways, these results demonstrate that the metabolic drug-drug interaction potential between apixaban and co-administered drugs is low.
INTRODUCTION

Apixaban [BMS-562247, 1-(4-methoxyphenyl)-7-oxo-6-(2-oxopiperidin-1-yl)phenyl-4,5,6,7-tetrahydro-1H-pyrazolo[3,4-c]pyridine-3-carboxamide, Figure 1], a novel and highly selective inhibitor of factor Xa (Pinto et al., 2007; Luetten et al., 2006; Wong et al., 2008), is under development for the treatment and prevention of thromboembolic disorders, prevention of stroke in patients with atrial fibrillation, and secondary prevention in patients with acute coronary syndromes. In addition to demonstrating high oral availability (Frost et al., 2007), clinical studies of apixaban have shown prevention of venous thromboembolic events in patients after knee replacement surgery (Lassen et al., 2007). Apixaban is efficacious and well tolerated in the treatment of patients with acute symptomatic deep vein thrombosis (DVT) (Büller et al., 2008). After an oral administration, apixaban was slowly metabolized and thus was mostly unchanged in circulation although apixaban was metabolized by multiple pathways in animals and humans (Zhang et al., 2009a; Zhang et al., 2009b). The primary metabolic pathways of apixaban in humans included $O$-demethylation (M2) and hydroxylation (M4 and M7). M2 was further conjugated by sulfation to form a sulfate metabolite (M1) (Raghavan et al., 2009; Wang et al., 2009). Other metabolites (M3, M5, and M6) previously identified as minor metabolites in animals and humans (Zhang et al., 2009a) were also very minor metabolites formed in the in vitro incubations in this study and were not further evaluated. Results of a clinical ADME study with $[^{14}C] $apixaban showed that more than 50% of dose was cleared through direct excretion of the parent drug (Raghavan et al., 2009).
Human P450 enzymes play a key role in the clearance of many drugs and alteration of activities of these enzymes is the major cause of metabolic drug-drug interactions. Three families of P450 enzymes (CYP1, CYP2, and CYP3) are involved in the metabolism of xenobiotics in humans, and CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, and 3A4/5 are responsible for the metabolism of the majority of drugs (Aithal et al., 1999; Crewe et al., 2002; Nebert et al., 2002). The objective of this study was to evaluate the metabolic drug-drug interaction potential of apixaban through investigating apixaban as 1) a victim for drug-drug interaction via P450 reaction phenotyping to identify major P450 enzymes involved in metabolism of apixaban, 2) as a perpetrator for drug-drug interaction via evaluating apixaban as an P450 enzyme inhibitor as well as an inducer. For reaction phenotyping, $[^{14}C]$apixaban was incubated with human cDNA-expressed P450 enzymes or human liver microsomes (HLM) to determine the catalytic turnover and the correlation between metabolite formation activities with the predetermined CYP activities of selected probe substrates. The P450 enzymes involved in apixaban metabolism were further investigated in HLM incubations with selective CYP chemical inhibitors. The kinetics of metabolite formation was determined with both HLM and cDNA-expressed P450 enzymes. The CYP inhibition and induction potential of apixaban were evaluated in HLM and primary human hepatocytes.
MATERIALS AND METHODS

Materials. Two [$^{14}$C]apixaban labels were used: the molecule with $^{14}$C labeled at C$_{32}$ is denoted as label 1 and had a specific activity of 76.01 $\mu$Ci/mg and a radiochemical purity 96% and the molecule with $^{14}$C labeled at C$_{4}$ is denoted as label 2 and had a specific activity of 122.1 $\mu$Ci/mg and a radiochemical purity 99.27% (Figure 1). Label 1 was used for enzyme kinetic studies and the label 2 was used in all other experiments described in this study. Pooled human liver microsomes (HLM, 20 subjects), pooled human intestinal microsomes (HIM, 6 subjects), human cDNA-expressed CYP enzymes (in baculovirus-insect cells) CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, and 3A7 were purchased from BD Biosciences (Woburn, MA). Human intestinal S9 (HIS9), pooled human intestinal microsomes (HIM, 3 subjects), pooled human kidney microsomes (HKM, 8 subjects), and individual HLM from young donors (age of 1 to 8 months) were purchased from XenoTech, LLC (Lenexa KS). Additional individual HLM from young donors (age of 1.5 to 6 years) were purchased from CellzDirect (Durham, NC). The human CYP reaction phenotyping kit (version 7) was purchased from XenoTech, LLC. Furafylline, 4-methylpyrazole, tranylcypromine, sulfaphenazole, quinidine, ketoconazole, l-aminobenzotriazole (ABT), troleandomycin, $\beta$-nicotinamide adenine dinucleotide phosphate-reduced form ($\beta$-NADPH), 3'-phosphoadenosine 5'-phosphosulfate (PAPS), flufenamic acid, 4'-hydroxydiclofenac, phenacetin, (R)-(++)-propranolol, phenytoin, acetaminophen, dextromethorphan, $\alpha$-hydroxytriazolam, testosterone, 6$\beta$-hydroxytestosterone, 4-hydroxybutyranilide, sulfaphenazole, diclofenac, dextorphan, ketoconazole, and $\alpha$-napthoflavone, 6,7-dihydroxycoumarin, coumarin, 7-hydroxycoumarin, orphenadrine, trazodone, bupropion, and paclitaxel were obtained.
from Sigma-Aldrich Co. (St. Louis, MO). Midazolam, 1-hydroxymidazolam, (S)-
mephenytoin, (S)-4'-hydroxymephenytoin, (+)-N-3-benzylirvanol, hydroxybupropion
and 6α-hydroxypaclitaxel were from BD Biosciences (Woburn, MA). Montelukast was
purchased from Sequoia Research Products (Pangbourne, UK). All other media and
culture reagents were purchased from Invitrogen (Grand Island, NY). All organic
solvents and water were of HPLC grade. Stock solutions of [14C]apixaban at 0.5 and 5
mM were prepared in acetonitrile:water (1:1, v/v). Stock solutions of CYP inhibitors
were prepared in acetonitrile.

**Incubations with HLM, HIM, HIS9, HKM, and cDNA-expressed enzymes.** HLM of
young subjects were pooled by age: <1 year (age of 1 to 8 months, n = 4) or >1 and <6
years (age of 1.5 to 6 years, n = 3). [14C]Apixaban was incubated in triplicate with pooled
HLM (adult subjects or young subjects), HIM, HKM, HIS9, or human cDNA-expressed
P450 enzymes (CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5,
and 3A7). CYP3A7, an enzyme expressed in preborn and infant livers but not in adults
(Schuetz et al., 1994), was also tested to metabolize apixaban to support its potential use
in infants or pregnant women. The incubation mixtures (0.5 mL) contained phosphate
buffer (0.1 M, pH 7.4), CYP (50 pmole/mL), HLM (1 mg protein/mL), HIM (1 mg
protein/mL), HKM (1 mg protein/mL), or HIS9 (3.2 mg protein/mL), and [14C]apixaban
(2.5 or 25 μM), and NADPH (1.2 mM). The final acetonitrile content in incubations was
0.25% (v/v). Reactions were initiated with the addition of NADPH and incubations
continued for 60 min at 37°C with shaking (90 rpm). After incubation, ice-cold
acetonitrile (0.5 mL) was added to stop the reaction. After centrifugation at 3000 g for 5
min, an aliquot of 20-50 μL of supernatant was used for LC/MS analysis, and an aliquot
of 15 or 20 μL of supernatant was used for HPLC profiling. Similar sample treatment and
analytical procedures were used for all samples. The metabolite formation was investigated in incubations with different HLM protein concentrations or amounts of expressed enzymes CYP3A4, 3A5 or 1A2 for up to 1.5 hours at 2.5 and 25 μM apixaban concentrations. The metabolite formation was linear under these conditions.

**HLM incubations in the presence of CYP chemical inhibitors.** The incubation mixtures (0.5 mL, in triplicate) contained phosphate buffer (0.1 M, pH 7.4), HLM (1 mg/mL), [14C]apixaban (2.5 or 25 μM), NADPH (1.2 mM), and a single CYP chemical inhibitor. The chemical inhibitors used were furafylline (10 μM) for CYP1A2, tranylcypromine (30 μM) for CYP2A6, montelukast (3 μM) for CYP2C8, sulfaphenazole (10 μM) for CYP2C9, benzylbirtanol (1 μM) for CYP2C19, quinidine (1 μM) for CYP2D6, 4-methylpyrazole (20 μM) for CYP2E1, ketoconazole (1 μM) for CYP3A4/5, troleandomycin (20 μM) for CYP3A4/5, and 1-aminobenzotriazole (ABT, 1 mM) for all CYP enzymes. Both the competitive inhibitor ketoconazole and the mechanism-based inhibitor troleandomycin were used to evaluate their inhibition potential for apixaban metabolism. Furafylline is a specific mechanism-based inhibitor for CYP1A2. Metabolism-dependent inhibitors, furafylline, troleandomycin or ABT, were pre-incubated with HLM in the presence of NADPH for 15 min before the substrate was added. After substrate addition, the samples were then incubated at 37°C for 60 min with shaking. The final volume of acetonitrile in the incubation mixtures was 0.5% (v/v).

Control incubations (without inhibitors, NADPH, or HLM) were performed under similar conditions.

**Incubations with individual HLM for correlation analysis.** [14C]Apixaban at 5 μM was incubated in triplicate with individual HLM from 16 different donors. The activities
of CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4/5, and 4A11 had been determined by the vendor using marker substrates specific for each enzyme (XenoTech, Technical Information for Reaction Phenotyping Kit version 7), namely, 7-ethoxyresorufin O-dealkylation or phenacetin O-deethylation for CYP1A2, coumarin 7-hydroxylation for CYP2A6, S-mephenytoin N-demethylation or bupropion hydroxylation for CYP2B6, paclitaxel 6α-hydroxylation for CYP2C8, diclofenac 4’-hydroxylation for CYP2C9, S-mephenytoin 4’-hydroxylation for CYP2C19, dextromethorphan O-demethylation for CYP2D6, chloroxazone 6-hydroxylation for CYP2E1, testosterone 6β-hydroxylation or midazolam 1’-hydroxylation for CYP3A4/5, and lauric acid 12-hydroxylation for CYP4A11. The reaction mixtures (0.5 mL, in triplicate) contained phosphate buffer (0.1 M, pH 7.4), HLM (1 mg/mL), apixaban (5 μM), and NADPH (1.2 mM). The final acetonitrile in the incubation mixtures was 0.25% (v/v). Incubations were conducted for 60 min at 37°C in a shaking water bath.

Apixaban concentration-dependent metabolite formation. The incubation mixtures (0.5 mL, in duplicate) contained phosphate buffer (0.1 M, pH 7.4), NADPH (1.2 mM), HLM (1 mg protein/mL), CYP3A4 (120 pmole/mL), CYP3A5 (60 pmole/mL), or CYP1A2 (120 pmole/mL), and apixaban. Fourteen apixaban concentrations (range from 1 to 300 μM) were evaluated. The final concentration of acetonitrile in these incubation mixtures was 0.25% (v/v). The incubation was conducted at 37°C for 20 min in a shaking water bath.

Metabolite profile. Metabolites in incubation samples were analyzed using a Shimadzu LC-10AT system equipped with a photodiode array ultraviolet (UV) detector (Shimadzu Scientific Instruments, Kyoto, Japan). Samples were injected onto an ACE 3 C18 column.
The mobile phase consisted of two solvents: A) 0.4% formic acid in water, pH 3.2, and B) 0.1% formic acid in acetonitrile. The gradient was as follows: Solvent B started at 0%, then linearly increased to 10% at 5 min, to 25% at 20 min, held at 25% for 30 min, to 50% at 60 min, to 100% at 65 min, held at 100% for 5 min, and then decreased to 0% at 72 min. The HPLC effluent (0.7 mL/min) was collected into Deepwell LumaPlate™-96 plates (PerkinElmer Life and Analytical Sciences, Shelton, CT) at 0.25 min intervals for 75 min with a Gilson Model 204 fraction collector (Gilson Medical Electronics, Middleton, WI). The plates were dried with a Savant Speed-Vac System (Global Medical Instrumentation, Inc., Ramsey, MN) and counted for 10 min per well with a TopCount analyzer (PerkinElmer Life and Analytical Sciences, Shelton, CT). Biotransformation profiles were prepared by plotting the resulting net CPM values vs HPLC time and radiochromatograms were reconstructed from the Topcount data using Microsoft® Excel software.

**Metabolite identification.** To identify the metabolites formed in incubations, LC/MS/MS analyses were performed on a LTQ mass spectrometer (ThermoFisher Scientific, San Jose, CA) with an ESI probe and a HEWLETT® PACKARD Agilent 1100 series HPLC system equipped with two pumps, an autoinjector, and a UV detector (Hewlett Packard, Wilmington, DE). The HPLC separation of the samples was performed using an ACE C18 column (3 μm, 4.6 x 150 mm). Samples were analyzed in the positive ionization mode and the capillary temperature was set at 280°C. The flow rate of nitrogen gas, spray current, and voltages were adjusted to give maximum sensitivity for the apixaban. The HPLC mobile phases and running conditions were the same as listed in the metabolite profile section.
Assessment of potential of apixaban to inhibit P450 enzymes. To assess the potential of apixaban to inhibit the major CYP enzymes, probe substrates specific for each enzyme were used to assay the activity of each CYP enzyme in HLM in the presence and absence of apixaban. IC$_{50}$ values for apixaban for each CYP enzyme were determined as described previously (Yao et al., 2007). Seven apixaban concentrations (0.0045-45 μM) were used. Briefly, to determine the IC$_{50}$ values, a mixture (180 μL) containing phosphate buffer (100 mM, pH 7.4) EDTA (1 mM), HLM (0.05~0.25 mg protein/mL), and probe substrate was incubated in a 96-Well reaction plate (300 μL, Axygen Scientific, Union, CA). The concentrations of P450 probe substrate were 45 μM for phenacetin, 100 μM for coumarin, 25 μM for bupropion, 5 μM for paclitaxel, 10 μM for diclofenac, 55 μM for S-mephenytoin, 10 μM for dextromethorphan, 5 μM for midazolam, and 75 μM for testosterone, which were near their respective Km values. After pre-incubation at 37°C for 5 min, 20 μL of NADPH (10 mM) was added to initiate the reaction. A positive control for each assay was run together with the test compound. The organic solvent concentration in the final incubation mixture was 0.17 % (v/v). The plate was incubated at 37°C for 5 to 10 min (40 min for the CYP2C19 assay). Meanwhile, a volume of 240 μL of acetonitrile (360 μL of methanol for CYP2C8) containing internal standard was pre-loaded into a filter plate. The standard and quality control samples (QCs) were prepared and run together without NADPH during the incubation. After incubation, 120 μL of the reaction mixture of apixaban or positive control was transferred into the filter plate which contained either acetonitrile or methanol to stop the reaction. A volume of 108 μL of the mixture in the reaction plate containing standard or
QC sample was transferred into the filter plate and 12 μL of NADPH was added to each well of standard and QC. Then the filter plate was stacked onto a 2 mL 96-well receiver plate (BD Bioscience, San Jose, CA), vortexed for 30 seconds, and the mixtures were passed through a 96-well filter plate (Millipore Co., Billerica, MA) with hydrophobic PTFE membrane (or hydrophilic PTFE membrane for CYP1A2 and CYP2A6 assay) by centrifugation for 5 minutes at a speed of 2000 g into the receiver plate, which was pre-loaded with 360 μL of 0.1% formic acid. Finally the receiver plate was vortexed again, and sealed with a polypropylene film. For quantification, 10-25 μL of sample was injected onto the LC/MS/MS.

Two LC/MS/MS systems were used for quantification of metabolites of the probe substrates. A TSQ Quantum mass spectrometer (ThermoFisher Scientific, San Jose, CA) was used for the CYP1A2, 2C9, 2C19, 2D6 and 3A4 assays. LC/MS/MS data for CYP1A2, 2C9, 2C19, 2D6 and 3A4 assays were acquired and analyzed using Xcalibur software (version 1.3, ThermoFisher Scientific, San Jose, CA). A 4000 Q trap LC/MS/MS system (Applied Biosystems/MDS Sciex, Ontario, Canada) was used for other assays. Shimadzu HPLC systems (Shimadzu Scientific Instruments, Columbia, MD, USA) equipped with LEAP CTC-PAL autosampler (LEAP Technologies, Carrboro, NC) were used for analyses. HPLC analytical columns were Agilent Zorbax SB-C18, 150 x 2.1 mm, 5 μm, (for CYP3A4 assay), YMC AQ C18 column, 50 x 2.1 mm, 3 μm (for CYP1A2, 2A6, 2C9, 2C19, and 2D6 assay), Phenomenex Luna Phenyl-hexyl column, 150 x 2 mm, 5 μm (for CYP2B6 assay), Waters XBridge, 50 x 2 mm, 5 μm (for CYP2C8 assay). LC/MS/MS data for CYP2A6, 2B6, and 2C8 assays were acquired and analyzed using Analyst software (version 1.4.1, Applied Biosystems/MDS Sciex, Ontario,
Cananda. Within the quantitation portion of the software, the chromatographic peaks were integrated and areas were determined. The concentration of marker metabolites in each sample was then quantified using the appropriate calibration curve and stable-isotope labeled metabolites as internal standards.

**Assessment of potential of apixaban to induce P450 enzymes.** The potential of apixaban to induce P450 enzymes was investigated by assessing the enzyme activity and mRNA levels of CYP1A2, 2B6 and 3A4/5 in primary cultures of human hepatocytes following a 3-day treatment with apixaban. Human hepatocytes were isolated as previously described (Mudra and Parkinson, 2001; Quistroff et al., 1989). Briefly, human liver tissue was perfused at 50 mL/min for 20 min with calcium-free buffer and then digested with a buffer containing 2.0 mM of CaCl₂ and collagenase (180 mg/L, approximately 90 units/mL). Hepatocytes were suspended in Dulbecco’s Modified Eagle Medium (DMEM, pH 7.4) containing 4.7% fetal bovine serum (FBS), insulin (5.89 μg/mL), penicillin (47 units/mL), streptomycin (47 μg/mL) and dexamethasone (0.94 μM). Hepatocytes were isolated using differential centrifugation in supplemented DMEM containing 18-24% (v/v) isotonic Percoll®. The viability of cells was analyzed using trypan blue exclusion with a hemacytometer.

Hepatocytes were cultured as previously described (Robertson et al., 2000). Briefly, 1 to 1.5 million hepatocytes in 3 mL supplemented DMEM were seeded on 60 mm Permanox® culture dishes coated with collagen and placed in a humidified incubator at 37°C with 95-96% relative humidity and 95%:5%, air:CO₂, and culture medium was changed daily. After a 3 day adaptation period, culture groups of hepatocytes (n=3, per treatment) were treated daily for three consecutive days with vehicle (0.1% DMSO,
negative control) or one of three concentrations of apixaban (0.2, 2.0 or 20 µM) or one of three known human CYP enzyme inducers, namely, omeprazole (100 µM), phenobarbital (750 µM) or rifampin (10 µM). Media samples were collected from all treatment groups prior to dosing on day 1 and at cell harvest at 72 hours for determination of lactate dehydrogenase (LDH) leakage. Approximately 24 hours after the final treatment, microsomes and cell lysates were prepared from each culture, based on the methods described by Wortelboer (Wortelboer et al., 1990). The enzyme activity in microsomal samples was determined by incubating microsomal samples with probe substrates for 10 min at 37°C in a final volume of 0.4 ml. The probe substrate concentration and quantity of protein in each assay was as follows: phenacetin 80 µM and 0.008 mg protein for CYP1A2; bupropion 500 µM and 0.024 mg protein for CYP2B6; and testosterone 250 µM and 0.008 mg protein CYP3A4/5. The metabolites were quantified using HPLC/MS/MS analysis performed according to validated methods by following mass transitions of m/z 152 to 110 for O-dealkyl phenacetin, m/z 256 to 238 for hydroxy bupropion, and m/z 305 to 269 for 6β-hydroxy testosterone with stable-isotope labeled metabolites as internal standards. The mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were quantified from cell lysates using the Quantigene™ High Volume (HV) Kit purchased from Genospectra (Fremont, CA) as described previously (Czerwinski et al., 2002).

**Data Analysis.** Data are expressed as mean ± SD unless otherwise indicated. All data were graphed with Sigmaplot (version 10, SPSS Science, Chicago, IL) or KaleidaGraph (version 3.6, Synergy Software Reading, PA). K_m and V_max values were obtained by fitting the data to the Michaelis-Menten equation: V= V_max*S/(K_m+S) and a substrate inhibition model: V= V_max*S/[K_m+S(1+S/K_i)] using KaleidaGraph. IC_{50} data
were processed using Grafit (version 5.0, Erithacus Software Limited, London, UK). Pearson correlation analyses were performed with a linear regression analysis using Sigmaplot. The statistical test used for the correlation was t-test and the level of significance was set at $p<0.05$. Kruskal-Wallis ANOVA was performed for the non-parametrically distributed data sets. The ANOVA was followed by a Dunnett’s test to identify the group means that were significantly different from the controls ($p<0.05$ or 5% level of significance). This statistical test is designed for multiple comparisons with a mean. Statistical analyses were performed with Sigma Stat Statistical Analysis System (version 2.03, SPSS Science, Chicago, IL).
RESULTS

[$^{14}$C]Apixaban metabolism in HLM incubations. Biotransformation of [$^{14}$C]apixaban (at concentrations of 2.5 and 25 μM) was investigated in HLM incubations. Three prominent metabolites (M2, M4, and M7) were formed by HLM and the formation rate of metabolites followed the order of M7>M2>M4 (Table 1 and Figures 2 and 3). Addition of PAPS, the sulfation cofactor, and human liver cytosol to the HLM incubations did not change the rate or profile of apixaban metabolism. Apixaban was also metabolized by HLM isolated from young donors and the metabolite formation profiles were qualitatively similar to HLM from adults (Table 1). The HPLC retention times of metabolites and apixaban were 27.3 (M7), 28.5 (M2), 33.5 (M4), and 48.3 min (apixaban), respectively, and the molecular ions [M+H]$^+$ were at m/z 476, 446, 476, and 460, respectively. The major MS$^2$ fragment ions were m/z 459 for M7, m/z 429 for M2, m/z 459 for M4, and m/z 443 for apixaban. Based on the HPLC retention time, MS analysis, and comparison with previously identified metabolites in animals and humans (Zhang et al., 2009a; Zhang et al., 2009b), these three metabolites were identified as O-demethyl apixaban (M2) and two hydroxylated apixaban derivatives (M4 and M7).

[$^{14}$C]Apixaban metabolism by HIM, HIS9, HKM, and human cDNA-expressed enzymes. Three prominent metabolites (M2, M4, and M7) were formed by HIM and the formation rate of metabolites also followed the same order as in the liver microsomes, M7>M2>M4 (Table 1 and Figures 2-3). The human cDNA-expressed CYP3A4 and CYP3A5 enzymes showed a significant level of catalytic activity for formation of M2, M4, and M7, while CYP1A2, 2C8, 2C9, 2C19, and 2J2 all showed a relatively low level of activity for formation of M2 (Table 2 and Figures 3 and 4). Other P450 enzymes,
namely, CYP2A6, 2B6, 2C18, 2D6, and 2E1, did not significantly metabolize apixaban (Table 2 and Figure 5). CYP3A7 also catalyzed a low level of formation of M2, M4, and M7 (Table 2). Figures 2-4 show the representative metabolite profiles of [14C]apixaban with HLM and cDNA-expressed CYP3A4/5, 1A2, 2C9, 2C19, and 2J2. No significant metabolite peaks were observed in HPLC profiles of HIS9 and HKM incubation samples. The metabolite profiles with 2.5 μM [14C]apixaban in human cDNA-expressed P450 enzymes were similar to those at 25 μM of [14C]apixaban.

**P450 inhibition study.** The effects of chemical inhibitors on the formation of oxidative metabolites of apixaban were evaluated by radioactivity profiling in HLM incubations. The inhibition results are shown in Figure 6. 1-Aminobenzotriazole (ABT) inhibited the formation of M2, M4 and M7 by >90%. Ketoconazole and troleandomycin, CYP3A4/5 inhibitors, significantly inhibited the formation of M4 and M7 (by 80 to 100%), and inhibited the formation of M2 by 61 to 74%. Furafylline inhibited the formation of M2, M4 and M7 by 15 to 30%. The CYP2C19 inhibitor, benzynirvanol, showed inhibition of M4 formation at a low substrate concentration. The inhibitors of other P450 enzymes showed low levels of inhibition for formation of the primary oxidative metabolites of apixaban (Figure 6).

**Correlation study.** The Pearson correlation between the formation rates of M2, M4, and M7 and the vendor-predicted activities of ten CYP enzymes were evaluated using a panel of HLM samples from 16 donors at 5 μM apixaban. The correlation results are summarized in Table 3 and Figure 7. The best correlations for formation of M2, M4, and M7 (r = 0.76, 0.90, and 0.96, respectively) were observed with the predetermined CYP3A4/5 activity (testosterone 6β-hydroxylation formation rate) (Figure 7).
correlation for M2 formation was observed with CYP2C8 activity ($r = 0.65$) and 2C9 activity ($r = 0.51$), however a low correlation was observed with predetermined CYP1A2 activity ($r = 0.14$). A correlation of M7 formation was observed with CYP2B6 activity ($r = 0.50$) and 2C8 activity ($r = 0.61$). The correlation $r$ values were low (from 0.03 to 0.43) (Table 4) for M2, M4, and M7 formation and activities of CYP2A6, 2C19, 2D6, 2E1, and 4A11.

**Substrate concentration-dependent metabolite formation.** In incubations of HLM and cDNA-expressed CYP3A4, the formation rates of M2, M4, and M7 depended on apixaban concentration as shown in Figure 8. However, the plots obtained from HLM incubations indicated that M2, M4, and M7 formation was not saturated over the range of apixaban concentrations (1-300 μM) (Figure 8), therefore no kinetic parameters were calculated. The plots obtained from CYP3A4 and CYP3A5 incubations indicated that formation of M2 and M4 was also not saturated in the range of 1-300 μM. However, M7 formation showed characteristics of apparent substrate inhibition by CYP3A4 and a reasonable saturation kinetic pattern by CYP3A5. The estimated $K_m$, $V_{max}$ and Ki values for M7 formation by CYP3A4 were 227 μM, 19.4 pmol/min/pmol, and 70.8 μM, respectively. The estimated $K_m$ and $V_{max}$ values for M7 formation by CYP3A5 were 106.8 μM and 5.1 pmol/min/pmol, respectively. The overall catalytic efficiency ($V_{max}/K_m$) for formation of M7 by CYP3A4 was approximately two times of that by CYP3A5.

**Assessment of the potential of apixaban to inhibit CYP enzymes.** For all CYP enzymes investigated, IC$_{50}$ values for apixaban were greater than the highest concentration evaluated ($IC_{50} > 45$ μM), except CYP2C19, for which the IC$_{50}$ was >20
µM (Table 4). IC₅₀ values for positive controls were within the expected range for all CYP enzymes.

**Assessment of potential of apixaban to induce the activity and/or expression of CYP enzymes.** The enzyme activities and mRNA levels of CYP1A2, CYP2B6, and CYP3A4/5 were determined in primary cultured human hepatocytes after treatment with apixaban (0.2, 2.0 or 20 µM), or prototypical inducers of each CYP enzyme, or DMSO (control). Table 5 summarizes the changes in the activity of each CYP enzyme under each condition. Apixaban had little or no effect on the activities of any of these three enzymes, while prototypical inducers produced expected increases in the activity of specific CYP enzymes. Also, apixaban produced an insignificant increase in mRNA levels of CYP1A2, CYP2B6 and CYP3A4/5. Assessment of lactate dehydrogenase leakage indicated that apixaban concentrations up to 20 µM had no detectable toxicity to human hepatocytes.
DISCUSSION

Biotransformation of \[^{14}\text{C}\]apixaban (at concentrations of 2.5 and 25 \(\mu\text{M}\)) was investigated with HLM, HIM, HIS9, HKM, and human cDNA-expressed P450 enzymes. Three prominent metabolites (M2, M4, and M7) formed in HLM each represented less than 5% of the parent compound after 1 hour incubations, indicating that apixaban has a slow metabolic turnover. The formation of metabolites was qualitatively similar between liver tissues from adult and young donors although the reason is not known for a lower level of metabolic activities in the liver microsomal preparations from the young subjects than adults, which suggested that pediatric patients can metabolize apixaban. Apixaban was also metabolized by human intestinal microsomes but not by human kidney microsomes, suggesting that the liver and intestines could be the major organs to metabolize apixaban in humans.

Identification of the human P450 enzymes involved in metabolism of apixaban was carried out with initial screening of the metabolic turnover by human cDNA-expressed P450 enzymes followed by evaluation of the effects of selective chemical inhibitors on metabolite formation in HLM incubations. Upon initial screening with human cDNA-expressed P450 enzymes, multiple P450 enzymes were found to be able to metabolize apixaban. CYP3A4/5, 1A2, and 2J2 were shown to catalyze the formation of M2 and CYP3A4 and 3A5 had higher activities to catalyze the formation of M4 and M7 than other P450 enzymes. In human intestinal microsomes, CYP3A4 is the most abundant CYP enzyme (80%), followed by CYP2C9 (15%), 2C19 (2.9%), 2J2 (1.4%), and 2D6 (1%) (Paine et al., 2006). CYP3A7 showed a lower level of apixaban metabolic activities than CYP3A4 and 3A5, which was consistent with reported metabolic activities of
several CYP3A enzymes (Williams et. al., 2002). In human liver microsomes, CYP3A4 is the major enzyme although there are more P450 enzymes expressed at significant levels compared to the intestinal microsomes (Paine et al., 2006; Rodrigues, 1999). The expression level of CYP2J2 in human liver is still unknown. CYP3A4 is the major CYP expressed in the human intestine and liver and CYP3A5 was relatively minor accounting for 0.2% of total P450 enzymes in average in the human liver (Shimada et al., 1994; Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007) although CYP3A5 could be present in individuals in significant quantities up to 20-60% of all P450 enzyme (Patki et al., 2003). CYP3A4 is expected to play a more significant role in clearance of apixaban in humans in average although CYP3A5 could play a significant role in metabolism of apixaban in individuals expressing a polymorphically high level of CYP3A5. There are several ways to extrapolate the metabolic activities of the expressed enzymes to HLM and none of them will fit all situations because of different assumptions used (Zhang et al., 2007). The observed increase of activity in the Supersomes compared to HLM can not be generalized (Rostami-Hodjegan and Tucker, 2007). Similar protein concentrations were used in all Supersome incubations so that activity determination in the expressed enzymes could not have been affected by different protein binding between different enzymes. To better estimate the contribution of each enzyme to the overall metabolism of apixaban in HLM, the activities of cDNA-expressed CYP enzymes were normalized to the content of each enzyme normally found in HLM according to a literature procedure: normalized enzyme activity was equal to the activity in the expressed enzyme system (pmol/min·pmol) multiplied by concentration of the enzyme in HLM (pmol P450/mg protein) (Zhang et al., 2007; Shimada et al., 1994; Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007). The results indicate that CYP3A4/5 was the major enzyme
responsible for formation of M2, M4, and M7 and CYP1A2 could significantly contribute to formation of M2 (Table 2). Other enzymes including CYP2C8, 2C9, and 2C19 might be minor contributors for metabolism of apixaban.

The apixaban metabolite formation activity was compared with CYP-specific probe activities across a panel of HLM samples from 16 individual donors. A good correlation was obtained between the formation of M2, M4, and M7 and the predetermined CYP3A4/5 activities (r = 0.76, 0.90, and 0.96, respectively) (Table 3, Figure 7). The formation of M2 showed a correlation with CYP2C8 and 2C9 activity (r = 0.51-0.65) but not with CYP1A2 activity (Table 3). In the reaction phenotyping kit, the enzyme activities of CYP3A4/5 correlated with those of CYP2B6 (r = 0.59) and 2C8 (r = 0.66) (XenoTech, Technical Information for Reaction Phenotyping Kit version 7). The reason for these apparent across correlations is not known. These apparent unknown correlations may be the reason for the apparent low level of correlation for formation of M2 with CYP2C8 and 2C9, and the formation of M7 with CYP2B6 and 2C8. The correlation of M2 formation with the activities of CYP2C8 and CYP2C9 might suggest an across correlation with CYP3A4/5. Therefore, lack of correlation of CYP1A2 activities with M2 formation suggested that CYP1A2 was not a major enzyme responsible for M2 formation. The fair correlation of CYP3A4/5 activities with formation of M2, M4, and M7 suggests that CYP3A4/5 was the major enzymes responsible for formation of these metabolites.

The study results of cDNA-expressed P450 enzymes and correlation studies are generally supported by the studies of individual chemical inhibitors. 1-Aminobenzotriazole (ABT), a general inhibitors for all P450 enzymes, potently inhibited the formation of M2, M4,
and M7 by >90% in HLM incubations. Ketoconazole (1 μM), a CYP3A inhibitor, significantly inhibited the formation of M2 (~74%), M4 (88-100%), and M7 (>98%) at 2.5 and 25 μM apixaban. Troleandomycin, a mechanism-based CYP3A inhibitor, blocked the formation of M2 (>61%), M4 (81-100%), and M7 (>98%) in HLM incubations. Furafylline, a specific CYP1A2 inhibitor, also showed some levels of inhibition for the formation of M4 (by 16-27%) and M7 (by 20-23%). Benzynirvanol, a CYP2C19 inhibitor, showed inhibition for formation of M4 (by 47%) at 2.5 μM apixaban but had no significant inhibition at 25 μM apixaban. These low levels of inhibition by CYP1A2 and CYP2C19 inhibitors were probably due to non-specific protein binding and larger experimental variations for a slow metabolic reaction. In contrast, the inhibitors for CYP2A6, 2C8, 2C9, 2D6, and 2E1 had no significant effect on the formation of M2, M4, and M7. The apparent inhibition for formation of M4 by the CYP2C19 inhibitor was not consistent with the results of the experiments with cDNA-expressed CYP2C19 since very low levels of metabolites were formed by these enzymes. Overall, the results generally support the conclusion that the formation of M2, M4, or M7 was mainly catalyzed by CYP3A4/5 with minor contribution of CYP1A2, 2J2, 2C8, 2C9, and 2C19 to metabolism of apixaban.

Reaction phenotyping is challenging for a compound like apixaban that has a slow turnover and forms multiple metabolites in in vitro incubations, especially when metabolite standards are not available. The separation and quantitation of all components provides multiple challenges and is resource intensive. The accurate determination of changes in enzyme activity is an example of these challenges when metabolites are at low concentrations in the absence or presence of P450 chemical inhibitors or antibody
inhibitors (data not shown). In these cases it is essential that the combined approaches using cDNA-expressed enzymes, selected enzyme inhibitors, and correlation studies with probe substrate activities of individual liver microsomal samples are employed to get the best possible picture of the metabolism of a new compound.

The kinetics of apixaban oxidation in liver microsomes as well as in the expressed enzymes (CYP3A4, CYP3A5 and CYP1A2) was unusual. The binding affinity of apixaban was exceptionally low and it was not practical in general to derive accurate $K_m$ values for formation of these metabolites in liver microsomal incubations and in the incubations with CYP3A4, CYP3A5, or CYP1A2 since the metabolite formation was not saturated over the range of apixaban concentrations (1-300 μM) that was limited by the compound solubility. The formation of M7 by CYP3A4 and CYP3A5 was an exception and appeared to display characteristics of saturation kinetics at high apixaban concentration (>150 μM). This could be the reason for the higher formation rate of M7 than M2 and M4 observed in liver microsomal incubation at the apixaban concentrations used. The unsaturated formation of M2, M4 and M7 in liver microsomal incubations makes it difficult to conclude which type of kinetics these reactions follow, which was at least partially due to different concentration dependent metabolite formation kinetics of CYP3A4 and CYP3A5. M2 formation in incubation with expressed CYP1A2 (120 pmol/mL) was also investigated with fourteen concentrations of apixaban over a 1-300 μM range. A similar unsaturated kinetic pattern was observed and no kinetic parameters could be derived (data not shown).

The relatively slow in vitro metabolism of apixaban is consistent with its relatively low in vivo clearance. Apixaban was metabolized by numerous pathways and enzymes,
primarily CYP3A4/5, with relatively minor contributions by CYP1A2, 2J2, 2C8, 2C9 and 2C19. This multiple-pathway metabolism of apixaban, in combination with the contribution of renal and biliary clearance as well as intestinal secretion to elimination (urinary/biliary/intestinal clearance was more important than metabolic clearance) (Raghavan et al., 2009; Zhang et al., 2009a), indicates that alternative elimination routes are available, should the primary metabolic enzymes be inhibited. Therefore, while apixaban may be the victim of drug-drug interactions with drugs that modulate CYP enzymes, such interactions are not likely to be of any significant magnitude.

Primary human hepatocyte systems have been shown to reasonably predict human in vivo induction potential and are recognized as an useful system for assessing induction potential of a compound (Hewitt et al., 2007; Chu et al., 2009; Guidance for Industry of FDA). The primary mechanisms for P450 enzyme induction in these systems is activation of gene transcription by nuclear receptors, the mostly common of which are aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), and constitutive androstane receptor (CAR). Both the measurement of mRNA levels and enzyme activities of commonly used P450 probes of CYP1A2, CYP2B6, and CYP3A4/5 were used to assess the P450 induction potential of apixaban in cultured primary hepatocytes isolated from multiple donors. There was no increase in either mRNA levels or enzyme activities in apixaban-treated human hepatocytes at apixaban concentrations up to 20 μM tested relative to vehicle controls compared to the expected increases of both parameters by positive control inducers.

Similar to hepatocyte systems for induction, liver microsomal systems yield acceptable inhibition data for prediction of clinical drug-drug interaction and were recommended
systems to test interaction potential (Obach et al., 2006, Draft FDA Guidance, 2007; Bjornsson et al., 2003). Considering that the IC₅₀ values were > 20 uM and the efficacious plasma concentrations of apixaban are sub-micromolar for apixaban as well as the fact that no formation of glutathione adducts suggesting no mechanism-based inhibition (data not shown), the potential ([I]/IC₅₀ < 0.03) for apixaban to inhibit metabolism of other co-administered P450 substrates would be predicted to be extremely low.

Since apixaban showed no significant inhibition or induction to important P450 enzymes it is unlikely to alter the metabolism of co-administered drugs metabolized by P450 enzymes. The studies with cDNA-expressed enzymes, CYP chemical inhibitors, and correlation analysis showed that apixaban was mainly metabolized by CYP3A4/5 with relatively minor contribution from additional CYP enzymes. However, the drug-drug interaction potential from co-administration of CYP3A4/5 inhibitors is lessened due to the multiple routes of apixaban clearance with an F_m(cyp) < 0.5. These results predicted that metabolic drug-drug interaction potential between apixaban and co-administered drugs that are P450 substrates or inhibitors is minimal, and that dose adjustment based on concomitant medication use is unlikely to be necessary with apixaban.

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Figure 1. Chemical structures of C-14 labeled apixaban and metabolites M2, M4, and M7.

Figure 2. Representative HPLC-radiochromatographic profiles of a 60-min incubation of [14C]apixaban (2.5 or 25 μM) with HLM (1 mg/mL protein) or human cDNA-expressed CYP3A4 (50 pmole P450/mL).

Figure 3. Metabolite profiles in the incubations in liver microsomes of young subjects and human intestinal microsomes at 2.5 μM [14C]apixaban. The incubations were for 60 min at 1 mg/mL proteins and 37°C.

Figure 4. Metabolite profiles in the incubations of expressed CYP3A5, CYP1A2, 2C9, 2C19, and 2J2 at 2.5 μM [14C]apixaban (30 μM for CYP3A5). The incubations were for 60 min at 50 pmole P450/mL and 37°C.

Figure 5. Formation of M2, M4, and M7 in incubations of [14C]apixaban (2.5 or 25 μM) with human cDNA-expressed P450 enzymes. The incubations were for 60 min at 50 pmole P450/mL and 37°C.

Figure 6. Inhibition of formation of M2, M4, and M7 in the incubations of [14C]apixaban (2.5 or 25 μM) in HLM by selective CYP inhibitors. Human liver microsomes (1 mg/mL proteins) were used in the presence of individual inhibitor at 37°C for 30 min. % Inhibition = (metabolic activity in HLM - metabolic activity in the presence of inhibitor)/metabolic activity in HLM. Inhibitor concentrations were 1 mM for ABT, 1 μM for ketoconazole, 100 μM
for troleandomycin, 10 μM for furafylline, 3 μM for montelukast, 30 μM for tranilcypromine, 1 μM for benzylniirvanol, 1 μM for quinidine, and 20 μM for 4-methylpyrazole. Experimental variations were <20%.

Figure 7. The correlation between formation activities of M2, M4, and M7 and the predetermined CYP3A4/5 activities in a panel of human liver microsomes (HLM) from 16 individual donors. The incubations were for 60 min at 1 mg/mL proteins and 37°C.

Figure 8. Apixaban concentration-dependent metabolite formation in the incubations with HLM (1 mg/mL) or human cDNA-expressed CYP3A4 (120 pmole P450/mL), or CYP3A5 (120 pmole P450/mL). The formation of each metabolite was calculated by measuring the radioactive peak in sample profile. The incubations were for 20 min at 37°C.
Table 1. The metabolite formation activities of [14C]apixaban in human liver microsomes (HLM) from adult and pediatric donors and human intestinal microsomes (HIM) at 2.5 and 25 μM drug concentrations.

<table>
<thead>
<tr>
<th>Microsomes</th>
<th>Formation of metabolites (pmol/min/mg protein)</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2.5 μM</td>
<td>25 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>M2</td>
<td>M4</td>
<td>M7</td>
<td>M2</td>
<td>M4</td>
<td>M7</td>
</tr>
<tr>
<td>HLM (adult)</td>
<td>0.76±0.11</td>
<td>0.19±0.02</td>
<td>3.49±0.91</td>
<td>8.39±2.44</td>
<td>2.25±0.99</td>
<td>33.64±12.9</td>
</tr>
<tr>
<td>HLM (1-6 years)</td>
<td>0.68±0.39</td>
<td>Trace</td>
<td>0.71±0.17</td>
<td>3.44±0.93</td>
<td>1.05±0.53</td>
<td>9.84±3.62</td>
</tr>
<tr>
<td>HLM (&lt;1 year)</td>
<td>0.65±0.09</td>
<td>0.20±0.08</td>
<td>3.02±0.97</td>
<td>9.96±3.17</td>
<td>3.12±0.99</td>
<td>37.67±19.9</td>
</tr>
<tr>
<td>HIM (adult)</td>
<td>0.16±0.02</td>
<td>0.13±0.02</td>
<td>1.47±0.07</td>
<td>2.08±0.46</td>
<td>2.01±0.47</td>
<td>10.3±3.87</td>
</tr>
</tbody>
</table>

HLM from adult and pediatric donors and HIM at 1 mg/mL proteins were incubated with 2.5 or 25 μM apixaban in the presence of NADPH at 37°C for 60 min.
Table 2. Formation activities of oxidative metabolites of [14C]apixaban (25 μM, n=3) by human cDNA-expressed P450 enzymes

<table>
<thead>
<tr>
<th>CYP enzyme</th>
<th>M2 (pmol/min/ pmol CYP) Mean ± SD</th>
<th>M2a (pmol/min/mg mpe)</th>
<th>M4 (pmol/min/pmol CYP) Mean ± SD</th>
<th>M4a (pmol/min/mg mpe)</th>
<th>M7 (pmol/min/pmol CYP) Mean ± SD</th>
<th>M7a (pmol/min/mg mpe)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1A2</td>
<td>0.52±0.18</td>
<td>19.19±6.64</td>
<td>0.01±0.0098</td>
<td>0.45±0.36</td>
<td>0.01±0.004</td>
<td>0.50±0.15</td>
</tr>
<tr>
<td>2A6</td>
<td>0</td>
<td>0.01±0.001</td>
<td>0.41±0.04</td>
<td>0.01±0.003</td>
<td>0.42±0.08</td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>0.01±0.001</td>
<td>0.10±0.01</td>
<td>0.02±0.005</td>
<td>0.17±0.04</td>
<td>0.01±0.001</td>
<td>0.09±0.005</td>
</tr>
<tr>
<td>2C8</td>
<td>0.04±0.017</td>
<td>0.73±0.33</td>
<td>0.02±0.007</td>
<td>0.39±0.14</td>
<td>0.01±0.001</td>
<td>0.13±0.13</td>
</tr>
<tr>
<td>2C9</td>
<td>0.06±0.019</td>
<td>3.32±1.15</td>
<td>0.01±0.001</td>
<td>0.82±0.007</td>
<td>0.01±0.002</td>
<td>0.33±0.14</td>
</tr>
<tr>
<td>2C18</td>
<td>0.01±0.019</td>
<td></td>
<td>0.01±0.001</td>
<td>0.82±0.007</td>
<td>0.01±0.002</td>
<td>0.33±0.14</td>
</tr>
<tr>
<td>2C19</td>
<td>0.05±0.009</td>
<td>0.46±0.08</td>
<td>0.02±0.003</td>
<td>0.16±0.03</td>
<td>0.02±0.009</td>
<td>0.18±0.08</td>
</tr>
<tr>
<td>2D6</td>
<td>0.02±0.004</td>
<td>0.13±0.03</td>
<td>0.02±0.005</td>
<td>0.13±0.04</td>
<td>0.01±0.003</td>
<td>0.05±0.02</td>
</tr>
<tr>
<td>2E1</td>
<td>0</td>
<td></td>
<td>0.01±0.001</td>
<td>0.82±0.007</td>
<td>0.01±0.002</td>
<td>0.33±0.14</td>
</tr>
<tr>
<td>2J2</td>
<td>0.27±0.06</td>
<td>-</td>
<td>0.01±0.001</td>
<td>0.82±0.007</td>
<td>0.01±0.002</td>
<td>0.33±0.14</td>
</tr>
<tr>
<td>3A4</td>
<td>0.18±0.04</td>
<td>13.68±3.23</td>
<td>0.06±0.02</td>
<td>4.23±1.38</td>
<td>1.32±0.44</td>
<td>100.19±33.69</td>
</tr>
<tr>
<td>3A5</td>
<td>0.03±0.024</td>
<td>0.03±0.024</td>
<td>0.04±0.003</td>
<td>1.85±0.74</td>
<td>1.85±0.74</td>
<td></td>
</tr>
<tr>
<td>3A7</td>
<td>0.01±0.00</td>
<td>NA</td>
<td>0.01±0.00</td>
<td>NA</td>
<td>0.02±0.01</td>
<td>NA</td>
</tr>
</tbody>
</table>

Human cDNA-expressed P450 enzymes were incubated with 25 μM apixaban in the presence of NADPH at 37°C for 60 min.

*Normalized enzyme activity = activity in the expressed enzyme (pmol/min/pmol)*conc. of the enzyme in HLM (pmol P450/mg protein). Enzyme concentration in HLM used for normalization were 37, 29, 7, 19, 60, 9, 7, 76, and 1 pmol/mg microsomal protein for CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4, and 3A5, respectively (Shimada et al., 1994; Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007). mpe-Human liver microsomal protein-equivalent.

NA, not applicable because of unknown contents of CYP3A7.
Table 3. The correlation between the activities for formation of M2, M4, and M7 and the predetermined activities of P450 enzymes in a panel of individual human liver microsomal (HLM) samples.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Correlation coefficients (r)</th>
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<tbody>
<tr>
<td></td>
<td>M2 formation</td>
<td>M4 formation</td>
<td>M7 formation</td>
<td></td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.14</td>
<td>0.14</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>CYP2A6</td>
<td>0.24</td>
<td>0.34</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.36</td>
<td>0.46</td>
<td>0.50*</td>
<td></td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.65*</td>
<td>0.42</td>
<td>0.61*</td>
<td></td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.51*</td>
<td>0.31</td>
<td>0.36</td>
<td></td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.25</td>
<td>0.43</td>
<td>0.36</td>
<td></td>
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<tr>
<td>CYP2D6</td>
<td>0.12</td>
<td>0.26</td>
<td>0.39</td>
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</tr>
<tr>
<td>CYP2E1</td>
<td>0.36</td>
<td>0.03</td>
<td>0.19</td>
<td></td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>0.76**</td>
<td>0.90**</td>
<td>0.96**</td>
<td></td>
</tr>
<tr>
<td>CYP4A11</td>
<td>0.26</td>
<td>0.07</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Individual human liver microsomes was incubated at 5 μM apixaban in the presence of NADPH at 37°C for 60 min.

t-test: *P<0.05; **P<0.01.

* The Pearson correlation coefficients (r) of the predetermined CYP3A4/5 activities with the predetermined 2C8 and 2B6 activities was 0.66 and 0.59, respectively.
Table 4. Inhibition effects of apixaban on P450 enzyme activities in human liver microsomes.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Probe substrate Assay</th>
<th>IC$_{50}$ for apixaban (µM)</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>Phenacetin O-deethylation</td>
<td>&gt;45</td>
<td>α-naphthoflavone 0.0068</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>Coumarin 7-hydroxylation</td>
<td>&gt;45</td>
<td>tranylcypromine 0.077</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>Bupropion hydroxylation</td>
<td>&gt;45</td>
<td>orphenadrine 464.7</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>Paclitaxel 6α-hydroxylation</td>
<td>&gt;45</td>
<td>montelukast 0.0882</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>Diclofenac 4'-hydroxylation</td>
<td>&gt;45</td>
<td>sulfaphenazole 0.528</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>(S)-Mephenytoin 4'-hydroxylation</td>
<td>&gt;20</td>
<td>N-3-benzylhirvanol 0.399</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>Dextromethorphan O-demethylation</td>
<td>&gt;45</td>
<td>quinidine 0.0526</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Midazolam 1'-hydroxylation</td>
<td>&gt;45</td>
<td>ketoconazole 0.0302</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>Testosterone 6β-hydroxylation</td>
<td>&gt;45</td>
<td>ketoconazole 0.0451</td>
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</tbody>
</table>

The concentrations of probe substrates used were at their Km values. The incubations were for 5-40 min at 0.05-0.25 mg/mL proteins and 37°C.
Table 5. Induction effects of apixaban on P450 enzyme activities in primary cultures of human hepatocytes.

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Concentration</th>
<th>CYP1A2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CYP2B6&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CYP3A4/5&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO 0.1%</td>
<td>1.00 ± 0.37</td>
<td>1.00 ± 0.30</td>
<td>1.00 ± 0.27</td>
<td></td>
</tr>
<tr>
<td>Apixaban 0.2 µM</td>
<td>0.973 ± 0.003</td>
<td>0.892 ± 0.049</td>
<td>0.984 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>Apixaban 2.0 µM</td>
<td>0.934 ± 0.028&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.934 ± 0.044</td>
<td>0.907 ± 0.134</td>
<td></td>
</tr>
<tr>
<td>Apixaban 20 µM</td>
<td>0.928 ± 0.037&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.12 ± 0.20</td>
<td>1.23 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>Omeprazole 100 µM</td>
<td>37.4 ± 1.2</td>
<td>11.0 ± 10.9</td>
<td>2.50 ± 1.34</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital 750 µM</td>
<td>2.03 ± 0.44</td>
<td>22.0 ± 22.4</td>
<td>7.36 ± 3.99</td>
<td></td>
</tr>
<tr>
<td>Rifampin 10 µM</td>
<td>2.19 ± 0.14</td>
<td>13.1 ± 6.8</td>
<td>8.97 ± 5.16</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Fold induction determined from rate for treatment group divided by rate for control. Values are the mean ± standard deviation of three human hepatocyte preparations. Fold inductions are rounded to three significant figures and standard deviation is rounded to the same degree of accuracy.

<sup>b</sup> Significance found among treatment groups (where 0.1% DMSO is the vehicle control) according to Kruskal-Wallis One Way Analysis on Ranks (p < 0.05) but unable to specify the groups that statistically differ from the other groups according to Dunnett's test with positive controls.

<sup>c</sup> Statistically significant according to Dunnett’s test (p > 0.05) without positive controls.
Figure 1.

Apixaban, * denotes $^{14}$C label site 1

M2 ($O$-demethyl apixaban)

Apixaban, * denotes $^{14}$C label site 2

M4 (hydroxy apixaban)

M7 (hydroxy apixaban)
Figure 2.
Figure 3.
Figure 4.
Figure 5.
Figure 7.

CYP3A4/3A5 activity (Testosterone 6β-hydroxylation: pmol/mg protein/min)

R = 0.96

R = 0.90

R = 0.76
Figure 8.

- Metabolite formation (pmol/min/mg protein)
- Metabolite formation (pmol/min/pmole CYP3A4)
- Metabolite formation (pmol/min/pmole CYP3A5)

Apixaban (µM)

HLM

CYP3A4

CYP3A5

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