In Vitro Assessment of Metabolic Drug-Drug Interaction Potential of Apixaban through Cytochrome P450 Phenotyping, Inhibition, and Induction Studies

Lifei Wang, Donglu Zhang, Nirmala Raghavan, Ming Yao, Li Ma, Charles A. Frost, Brad D. Maxwell, Shiang-yuan Chen, Kan He,1 Theunis C. Goosen, W. Griffith Humphreys, and Scott J. Grossman


Received July 24, 2009; accepted November 24, 2009

ABSTRACT:
Apixaban is an oral, direct, and highly selective factor Xa 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 cytochrome P450 inhibition (IC50 values >20 μM) 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 μM 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 P450 enzymes and P450 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 predominantly catalyzed by CYP3A4/5 with a minor contribution of CYP1A2 and CYP2J2 for formation of M2. The contribution of CYP2C8, 2C9, and 2C19 to metabolism of apixaban was less significant. In addition, a human absorption, distribution, metabolism, and excretion study showed that more than half of the dose was excreted as unchanged parent (fM CYP <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 coadministered drugs is low.

Supplemental material to this article can be found at: http://dmd.aspetjournals.org/content/suppl/2010/03/11/dmd.109.029694.DC1

This work was supported by Bristol-Myers Squibb and by Pfizer.

1 Current affiliation: UniTris Biopharma Co., Shanghai, China.

Article, publication date, and citation information can be found at http://dmd.aspetjournals.org doi:10.1124/dmd.109.029694.

ABBREVIATIONS: apixaban, BMS-562247, 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; P450, cytochrome P450; HLM, human liver microsome(s); HIM, human intestinal microsome(s); HIS, human intestinal S9; HKM, human kidney microsome(s); ABT, 1-aminobenzotriazole; HPLC, high-performance liquid chromatography; LC, liquid chromatography; MS/MS, tandem mass spectrometry; QC, quality control; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide.
are responsible for the metabolism of the majority of drugs (Nebert and Russell, 2002; Guengerich, 2007). The objective of this study was to evaluate the metabolic drug-drug interaction potential of apixaban through investigation of apixaban 1) as a victim for drug-drug interaction via P450 reaction phenotyping to identify major P450 enzymes involved in metabolism of apixaban and 2) as a perpetrator for drug-drug interaction via evaluation of apixaban as a P450 enzyme inhibitor as well as an inducer. For reaction phenotyping, \(^{14}\text{C}\)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 P450 activities of selected probe substrates. The P450 enzymes involved in apixaban metabolism were further investigated in HLM incubations with selective P450 chemical inhibitors. The kinetics of metabolite formation was determined with both HLM and cDNA-expressed P450 enzymes. The P450 inhibition and induction potential of apixaban were evaluated in HLM and primary human hepatocytes.

**Materials and Methods**

**Materials.** Two \(^{14}\text{C}\)apixaban labels were used: the molecule with \(^{14}\text{C}\)labeled at C32 is denoted as label 1 and had a specific activity of 76.01 \(\mu\text{Ci/mg}\) and a radiochemical purity of 96% and the molecule with \(^{14}\text{C}\) labeled at C4 is denoted as label 2 and had a specific activity of 122.1 \(\mu\text{Ci/mg}\) and a radiochemical purity 99.27% (Fig. 1). Label 1 was used for enzyme kinetic studies and label 2 was used in all other experiments described in this study. Pooled HLM (20 subjects), pooled human intestinal microsomes (HIM) (6 subjects), human cDNA-expressed P450 enzymes (in baculovirus-insect cells) CYP1A2, 2A6, 2B6, 2C8, 2C9, 2C18, 2C19, 2D6, 2E1, 2J2, 3A4, 3A5, and 3A7 were purchased from BD Biosciences (San Diego, CA). Human intestinal S9 (HIS9), pooled HIM (3 subjects), pooled human kidney microsomes (HKM) (8 subjects), and individual HLM from young donors (ages 1–8 months) were purchased from XenoTech, LLC (Lenexa, KS). Additional individual HLM from young donors (ages 1.5–6 years) were purchased from CellzDirect (Durham, NC). The human P450 reaction phenotyping kit (version 7) was purchased from XenoTech, LLC. Furafylline, 4-methylpyrazole, tranylcypromine, sulfaphenazole, quinidine, ketoconazole, 1-aminobenzotriazole (ABT), troleandomycin, \(\beta\)-NADPH, 3’-phosphoadenosine 5’-phosphosulfate, flufenamic acid, 4’-hydroxydiclofenac, phencetin, (R)-(+)propranolol, phenytoin, acetaminophen, dextromethorphan, \(\alpha\)-hydroxytestolzolam, testosterone, 6\(\beta\)-hydroxytestosterone, 4-hydroxybutyralilide, sulfaphenazole, diclofenac, dextrophan, \(\alpha\)-naphthofo- vone, 6,7-dihydroxyxoumarin, coumarin, 7-hydroxyxoumarin, orphenadrine, trazodone, bupropion, and paclitaxel were obtained from Sigma-Aldrich (St. Louis, MO). Midazolam, 1-hydroxymidazolam, (S)-mephenytoin,
(S)-4'-hydroxyxymethoxetionin, (+)-N-3-benzylxirvanol, hydroxybropipion, and 6a-hydroxyoxaptaclxol were from BD Biosciences. Montelukast was purchased from Sequoia Research Products (Pangbourne, UK). All other media and culture reagents were purchased from Invitrogen (Carlsbad, CA). All organic solvents and water for use were from 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 P450 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 (ages 1–8 months, n = 4) or >1 and <6 years (ages 1.5–6 years, n = 3). [14C]Apixaban was incubated in triplicate with pooled HLM (adult subjects or young subjects). HIM, HKM, or HIS9, or human cDNA-expressed P450 enzymes (CYP1A2, 2A6, 2B6, 2C9, 2C19, 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), NADPH (1.2 mM), HLM (1 mg of protein/ml), CYP3A4 (120 pmol/ml), and lauric acid 12-hydroxylation for CYP4A11. The reaction was conducted for 60 min at 37°C in a shaking water bath. The final concentration of acetonitrile in the 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 UV detector (Shimadzu Scientific Instruments, Kyoto, Japan). Samples were injected onto an ACE 3 C18 column (3 μm, 4.6 × 150 mm; Mac-Mod Analytical, Inc., Chadds Ford, PA). 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, increased to 25% at 20 min, was held at 25% for 30 min, increased to 50% at 60 min, increased to 100% at 65 min, was 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 LuminaPlate-96 plates (PerkinElmer Life and Analytical Sciences, Waltham, MA) 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 with a well counter (PerkinElmer Life and Analytical Sciences). Biotransformation profiles were prepared by plotting the resulting net counts per minute values versus 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 (Thermo Fisher Scientific, Waltham, MA) with an electrospray ionization source and a Hewlett Packard 1100 series HPLC system equipped with two pumps, an autoinjector, and a UV detector (Hewlett Packard Analytical Direct, Wilmington, DE). HPLC separation of the samples was performed using an ACE C8 column (3 μm, 4.6 × 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 under Metabolic Profile.

Assessment of Potential of Apixaban to Inhibit P450 Enzymes. To assess the potential of apixaban to inhibit the major P450 enzymes, probe substrates specific for each enzyme were used to assay the activity of each P450 enzyme in HLM in the presence and absence of apixaban. IC50 values for apixaban for each P450 enzyme were determined as described previously (Yao et al., 2007). Seven apixaban concentrations (0.005–35 μM) were used. In brief, to determine the IC50 values, a mixture (180 μl) containing phosphate buffer (100 mM, pH 7.4), EDTA (1 mM), HLM (0.05–25 μg of protein/ml), and probe substrate was incubated in a 96-well reaction plate (300 μl). The concentrations of P450 probe substrates were 45 μM for phenacetin, 100 μM for coumarin, 25 μM for bupropion, 5 μM for paclitaxel, 10 μM for montelukast, 55 μM for S-mephentoin, 10 μM for dextromethorphan, 5 μM for midazolam, and 75 μM for testosterone, which were near their respective Ki values. After 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 preloaded into a filter plate. The standard and quality control (QC) samples were prepared and run together without NADPH during the incubation.

After incubation, 120 μl of the mixture 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 samples. Then the filter plate was stacked onto a 2-ml 96-well receiver plate (BD Biosciences) and vortexed for 30 s, and the mixtures were passed through a 96-well filter plate (Millipore Corporation, Billerica, MA) with hydrophobic polystyrene membranes (or hydrophilic polystyrene membrane for CYP1A2 and CYP2A6 assay) by centrifugation for 5 min at a speed of 2000g into the receiver plate, which was preloaded with 360 μl of
0.1% formic acid. Finally the receiver plate was vortexed again and sealed with a polypropylene film. For quantification, 10 to 25 μl of sample was injected onto the LC-MS/MS system.

Two LC-MS/MS systems were used for quantification of metabolites of the probe substrates. A TSQ Quantum mass spectrometer (Thermo Fisher Scientific) was used for the CYP1A2, 2C9, 2C19, 2D6, and 3A4 assays. HPLC Scientific Instruments, Columbia, MD) equipped with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC) was used for analyses. HPLC 4000 Q trap LC-MS/MS system (Applied Biosystems/MDS Sciex, Concord, ON, Canada) was used for other assays. Shimadzu HPLC systems (Shimadzu Scientific Instruments, Columbia, MD) equipped with a CTC-PAL autosampler (LEAP Technologies, Carrboro, NC) were used for analyses. HPLC analytical columns were the following: Zorbax SB-C18, 150 x 2.1 mm, 5 μm (for CYP3A4 assay; Agilent Technologies, Santa Clara, CA), AQ C18, 50 x 2.1 mm, 3 μm (for CYP1A2, 2A6, 2C9, 2C19, and 2D6 assays; YMC, Inc., Wilmington, NC), Luna Phenyl-Hexyl, 150 x 2 mm, 5 μm (for CYP2B6 assay; Phenomenex, Torrance, CA), and XBridge, 50 x 2 mm, 5 μm (for CYP2C8 assay; Waters, Milford, MA). 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). 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 was 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 examining the enzyme activity and mRNA levels of CYP1A2, 2B6, and 3A4/5 in primary cultures of human hepatocytes after a 3-day treatment with apixaban. Human hepatocytes were isolated as described previously (Quistroff et al., 1989; Mudra and Parkinson, 2001). In brief, 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 CaCl2 and collagenase (180 mg/ml, approximately 90 units/ml). Hepatocytes were suspended in Dulbecco’s modified Eagle’s medium (DMEM) (pH 7.4) containing 4.7% fetal bovine serum, 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 to 24% (v/v) isotonic Percoll. The viability of cells was analyzed using trypan blue exclusion with a hemacytometer.

Hepatocytes were cultured as described previously (Robertson et al., 2000). In brief, 1 to 1.5 million hepatocytes in 3 ml of supplemented DMEM were seeded on 60-mm Permanox culture dishes coated with collagen and placed in a humidified incubator at 37°C with 95% 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/treatment) were treated daily for 3 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 P450 enzyme inducers, namely, omeprazole (100 μM), phenobarbital (750 μM), or rifampin (10 μM). Media samples were collected from all treatment groups before dosing on day 1 and at cell harvest at 72 h for determination of lactate dehydrogenase leakage. Approximately 24 h after the final treatment, microsomes and cell lysates were prepared from each culture, based on the methods described by 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.

### TABLE 1

Metabolite formation activities of [14C]apixaban in HLM from adult and pediatric donors and HIM at 2.5 and 25 μM drug concentrations

<table>
<thead>
<tr>
<th>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.</th>
<th>Formation of Metabolites</th>
<th>2.5 μM</th>
<th>25 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></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>
</tr>
<tr>
<td>HLM (1–6 years)</td>
<td>0.68 ± 0.39</td>
<td>Trace</td>
<td>0.71 ± 0.17</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>
</tr>
<tr>
<td>HIM (adult)</td>
<td>0.16 ± 0.02</td>
<td>0.13 ± 0.02</td>
<td>1.47 ± 0.07</td>
</tr>
</tbody>
</table>

![FIG. 2](https://example.com/figure2.png)
The probe substrate concentration and quantity of protein in each assay were as follows: 80 μM phenacetin and 0.008 mg of protein for CYP1A2; 500 μM bupropion and 0.024 mg of protein for CYP2B6; and 250 μM testosterone and 0.008 mg of protein for 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 236 to 238 for hydroxybupropion, and m/z 305 to 269 for 6β-hydroxytestosterone with stable isotope-labeled metabolites as internal standards. The mRNA levels of CYP1A2, CYP2B6, and CYP3A4 were quantified from cell lysates using a Quantigene High Volume Kit purchased from Genospectra (Fremont, CA) as described previously (Czerwinski et al., 2002).

**Data Analysis.** Data are expressed as means ± S.D., unless otherwise indicated. All data were graphed with SigmaPlot (version 10; SPSS Science, Chicago, IL) or KaleidaGraph (version 3.6; Synergy Software, Reading, PA). Km and Vmax values were obtained by fitting the data to the Michaelis-Menten equation, \( V = V_{\text{max}} \times S/(K_m + S) \), and a substrate inhibition model, \( V = V_{\text{max}} \times S/[K_m + (S + S/K_m)] \) using KaleidaGraph. IC50 data were processed using Graphit (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 the r test, and the level of significance was set at \( p < 0.05 \). A Kruskal-Wallis analysis of variance was performed for the nonparametrically distributed data sets. The analysis of variance was followed by Dunnnett’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).

**Results**

**[14C]Apixaban Metabolism in HLM Incubations.** Biotransformation of [14C]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; Figs. 2 and 3). Addition of 3′-phosphoadenosine 5′-phosphosulfate, 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 metabolism formation profiles were qualitatively similar to those for 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 \([\text{M} + \text{H}]^+\) were at m/z 476, 446, 476, and 460, respectively. The major MS² 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 HPLC-MS/MS analysis, the metabolites were identified as M2, M4, and M7.

**Table 2**

<table>
<thead>
<tr>
<th>P450 Enzyme</th>
<th>M2</th>
<th>M2α</th>
<th>M4</th>
<th>M4α</th>
<th>M7</th>
<th>M7α</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</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>CYP2A6</td>
<td>0</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>
</tr>
<tr>
<td>CYP2B6</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>CYP2C8</td>
<td>0.04 ± 0.017</td>
<td>0.73 ± 0.53</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>CYP2C9</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>CYP2C18</td>
<td>0.01</td>
<td>1.03</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>CYP2C19</td>
<td>0.05 ± 0.0009</td>
<td>0.46 ± 0.08</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>CYP2D6</td>
<td>0.02 ± 0.0004</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>CYP2E1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>0.27 ± 0.06</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CYP3A5</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>CYP3A7</td>
<td>0.03 ± 0.024</td>
<td>0.03 ± 0.024</td>
<td>0.04 ± 0.03</td>
<td>0.04 ± 0.03</td>
<td>1.85 ± 0.74</td>
<td>1.85 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>0.01 ± 0.00</td>
<td>N.A.</td>
<td>0.01 ± 0.00</td>
<td>N.A.</td>
<td>0.02 ± 0.01</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

mpe, human liver microsomal protein-equivalent; N.A., not applicable because of unknown contents of CYP3A7.

α Normalized enzyme activity represents activity in the expressed enzyme (picomoles per minute per picomole) × concentration of the enzyme in HLM (picomoles of P450 per milligram of protein). Enzyme concentrations in HLM used for normalization were 37, 29, 7, 19, 60, 9, 7, 76, and 1 pmol/mg of 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).
and comparison with previously identified metabolites in animals and humans (Zhang et al., 2009, 2010), these three metabolites were identified as O-demethyl apixaban (M2) and two hydroxylated apixaban derivatives (M4 and M7).

[^14C]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 > M4 > M2 (Table 1; Figs. 2 and 3). The human cDNA-expressed CYP3A4 and CYP3A5 enzymes showed a significant level of catalytic activity for formation of M2, M4, and M7, whereas CYP1A2, 2C8, 2C9, 2C19, and 2J2 all showed a relatively low level of activity for formation of M2 (Table 2; Figs. 3 and 4). Other P450 enzymes, namely, CYP2A6, 2B6, 2C18, 2D6, and 2E1, did not significantly metabolize apixaban (Table 2; Fig. 5). CYP3A7 also catalyzed a low level of formation of M2, M4, and M7 (Table 2). Figures 2 to 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[^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 Fig. 6. 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, benzylnirvanol, 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 (Fig. 6).
Correlation Study. The Pearson correlation between the formation rates of M2, M4, and M7 and the vendor-predetermined activities of 10 P450 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 Fig. 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) (Fig. 7). A correlation for M2 formation was observed with CYP2C8 activity (r = 0.65) and CYP2C9 activity (r = 0.51); however, a low correlation was observed with predetermined CYP1A2 activity (r = 0.14). A correlation of M7 formation with CYP2B6 activity (r = 0.50) and CYP2E1 activity (r = 0.61) was observed. The correlation r values were low (from 0.03 to 0.43) (Table 3) for M2, M4, and M7 formation and activities of CYP2A6, 2C19, 2D6, 2E1, and 4A11.

![Inhibition of formation of M2, M4, and M7 in the incubations of [14C]apixaban (2.5 or 25 μM) in HLM by selective P450 inhibitors. Human liver microsomes (1 mg/ml protein) were used in the presence of individual inhibitor at 37°C for 30 min. Percent 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 tranylcypromine, 1 μM for benzylnirvanol, 1 μM for quinidine, and 20 μM for 4-methylpyrazole. Experimental variations were <20%.

![The correlation between formation activities of M2, M4, and M7 and the predetermined activities of P450 enzymes in a panel of individual HLM samples. Individual human liver microsomes were incubated at 5 μM apixaban in the presence of NADPH at 37°C for 60 min. The Pearson correlation coefficients (r) of the predetermined CYP3A4/5 activities with the predetermined 2C8 and 2B6 activities were 0.66 and 0.59, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Correlation Coefficients (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>M2 Formation</td>
</tr>
<tr>
<td>CYP1A2</td>
<td>0.14</td>
</tr>
<tr>
<td>CYP2A6</td>
<td>0.24</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>0.36</td>
</tr>
<tr>
<td>CYP2C8</td>
<td>0.65*</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>0.51*</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>0.25</td>
</tr>
<tr>
<td>CYP2D6</td>
<td>0.12</td>
</tr>
<tr>
<td>CYP2E1</td>
<td>0.36</td>
</tr>
<tr>
<td>CYP3A4/5</td>
<td>0.76**</td>
</tr>
<tr>
<td>CYP4A11</td>
<td>0.26</td>
</tr>
</tbody>
</table>

* p < 0.05 (t test).
** p < 0.01 (t test).
kinetic parameters were calculated. The plots obtained from CYP3A4 ranged of apixaban concentrations (1–300 μM) were shown in Fig. 8. However, the plots obtained from HLM incubations indicated that formation of M2 and M4 was also not saturated in the range of 1 to 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 $V_i$ 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 2 times that by CYP3A5.

Assessment of Potential of Apixaban to Inhibit P450 Enzymes. For all P450 enzymes investigated, IC$_{50}$ values for apixaban were greater than the highest concentration evaluated (IC$_{50}$ > 45 μM), except for CYP2C19, for which the IC$_{50}$ was >20 μM (Table 4). IC$_{50}$ values for positive controls were within the expected range for all P450 enzymes.

Assessment of Potential of Apixaban to Induce the Activity and/or Expression of P450 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 P450 enzyme or DMSO (control). Table 5 summarizes the changes in the activity of each P450 enzyme under each condition. Apixaban had little or no effect on the activities of any of these three enzymes, whereas prototypical inducers produced expected increases in the activity of specific P450 enzymes. In addition, 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 $[^{14}C]$apixaban (at concentrations of 2.5 and 25 μM) 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-h 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 for a lower level of metabolic activities in the liver microsomal preparations from the young subjects than adults is not known, 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 CYP3A5 had higher activities to catalyze the formation of M4 and M7 than other P450 enzymes. In human intestinal microsomes, CYP3A4 is the most abundant P450 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 CYP3A5, 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 with the intestinal microsomes (Rodrigues, 1999; Paine et al., 2006). The expression level of CYP2J2 in human liver is still unknown. CYP3A4 is the major P450 expressed in the human intestine and liver, and the expression of CYP3A5 was relatively minor, accounting for 0.2% of total P450 enzymes on 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 to 60% of all P450 enzyme (Patki et al., 2003). CYP3A4 is expected to play a more significant role in clearance of apixaban in humans on average, although CYP3A5 could play a significant role in metabolism of apixaban in individuals expressing a polymorphically high level of CYP3A5. There are a 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 Supersomes compared with HLM cannot be generalized (Rostami-Hodjegan and Tucker, 2007; Zhang et al., 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 among different enzymes. To better estimate the contribution of each enzyme to the overall metabolism of apixaban in HLM, the activities of cDNA-expressed P450 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 (picomoles per minute per picomole) multiplied by concentration of the enzyme in HLM (picomoles of P450 per milligram of protein) (Shimada et al., 1994; Rodrigues, 1999; Rostami-Hodjegan and Tucker, 2007; Zhang et al., 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 P450-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; Fig. 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 formation of M2, M4, and M7 and the predetermined CYP3A4/5 P450-specific probe activities across a panel of HLM samples from 16 individuals. The positive correlation of apixaban metabolite formation with the activities of CYP2C8 and 2C9 might suggest a cross-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 enzyme responsible for formation of these metabolites.

The results for cDNA-expressed P450 enzymes and correlation studies are generally supported by the studies of individual chemical inhibitors. ABT, a general inhibitor 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%). Benzylnirvanol, a CYP2C9 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 CYP2C9 inhibitors were probably due to nonspecific 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 of formation of M4 by the CYP2C19 inhibitor was not consistent with the results of the experiments with cDNA-expressed CYP2C19 because 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 catalyzed mainly by CYP3A4/5 with minor contributions of CYP1A2, 2J2, 2C8, 2C9, and 2C19 to metabolism of apixaban.

Reaction phenotyping is challenging for a compound such as apixaban that has a slow turnover and forms multiple metabolites in vitro and in vivo, especially when metabolite standards are not available. Separation and quantitation of all components provide multiple challenges and is resource-intensive. 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 used 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 because 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 seemed to display characteristics of saturation kinetics at a high apixaban concentration (>150 μM). This could be the reason for the higher formation rate of M7 than of 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 determine which type of kinetics these reactions follow, a problem that 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 14 concentrations of apixaban over a 1 to 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, or intestinal clearance was more important than metabolic clearance) (Raghavan et al., 2009; Zhang et al., 2009), indicates that alternative elimination routes are available, should the primary metabolic enzymes be inhibited. Therefore, although apixaban may be the victim of drug-drug interactions with drugs that modulate P450 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 the induction potential of a compound (Hewitt et al., 2007; Chu et al., 2009; Guidance for Industry, 1997). 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, pregnane X receptor, and constitutive androstane receptor. 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 with 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 induction potential (Bjornsson et al., 2003; Draft Guidance for Industry, 2006; Obach et al., 2006). Considering that the IC\(_{50}\) values were >20 μM and the efficacious plasma concentrations of apixaban are submicro-molar as well as the fact that no formation of glutathione adducts suggests no mechanism-based inhibition (data not shown), the potential (\( I/I_{IC_{50}} < 0.03 \)) for apixaban to inhibit metabolism of other coadministered P450 substrates would be predicted to be extremely low.

Because apixaban showed no significant inhibition or induction to important P450 enzymes, it is unlikely to alter the metabolism of coadministered drugs metabolized by P450 enzymes. The studies with cDNA-expressed enzymes, P450 chemical inhibitors, and correlation analysis showed that apixaban was metabolized mainly by CYP3A4/5 with relatively minor contribution from additional P450 enzymes. However, the drug-drug interaction potential from coadministration of CYP3A4/5 inhibitors is lessened because of the multiple routes of apixaban clearance with an \( I_{IC_{50,CYP}} < 0.5 \). These results predicted that the metabolic drug-drug interaction potential between apixaban and coadministered 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.

Acknowledgments. We thank Scott Tran for preparation of the \( ^{14} \)C-labeled apixaban.

References


Pollard JW and Walker JM eds) vol 5, Humana Press, Clifton, NJ.

Address correspondence to: Dr. Donglu Zhang, Pharmaceutical Candidate Optimization, Bristol-Myers Squibb, P.O. Box 4000, Princeton, NJ 08543. E-mail: donglu.zhang@bms.com