Identification of Human Liver Cytochrome P450 Enzymes Involved in the Metabolism of SCH 530348 (Vorapaxar), a Potent Oral Thrombin Protease-Activated Receptor 1 Antagonist

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ABSTRACT:

Vorapaxar (SCH 530348), a potent oral thrombin protease-activated receptor 1 antagonist, is being developed as an antiplatelet agent for patients with established vascular disease. The objective of this study was to identify the human liver cytochrome P450 (P450) enzyme(s) responsible for the metabolism of SCH 530348. Human liver microsomes metabolized SCH 530348 to M19, an amine metabolite formed via carbamate cleavage, and M20 (monohydroxy-SCH 530348). Recombinant human CYP3A4 exhibited the most activity (11.5% profiled radioactivity) for the formation of M19, followed by markedly less substrate conversion with CYP1A1 and CYP2C19. Trace levels of M19, a major excreted human metabolite, were detected with CYP1A2, CYP3A5, and CYP4F3A. Formation of M19 by human liver microsomes was inhibited 89% by ketoconazole, 75% by astemizole (a CYP2J2 inhibitor), and 43% by CYP3A4 monoclonal antibody. These results suggest that CYP3A4 and CYP2J2 are both involved in the formation of M20 metabolite.

Introduction

Vorapaxar (SCH 530348; [ethyl[(1R,3aR,4aR,6R,8aR,9S,9aS)-9-{[(E)-2-[5-(3-fluorophenyl)-2-pyridinyl]ethenyl]-dodecahydro-1-methyl-3-oxonaphtho[2,3-c][furan-6-yl]carbamate}] (Fig. 1), an analog of the natural product himbacine, is a potent antagonist of protease-activated receptor 1 (PAR-1), the primary thrombin receptor on human platelets. Vorapaxar is under investigation for the treatment and prevention of acute coronary syndrome, including acute MI, and is a major contributor of acute coronary syndrome, peripheral artery disease, or conditions with a high risk of embolism (Baigent et al., 2002; Dogné et al., 2002). Thrombin is the most potent activator of human platelets, stimulating them primarily through the interaction with PAR-1 (Andersen et al., 1999; Anderluh and Dolenc, 2002). Thus, a PAR-1 antagonist is anticipated to demonstrate a higher level of efficacy than either aspirin or adenosine diphosphate antagonists. The effects of aspirin, adenosine diphosphate receptor antagonists, and PAR-1 antagonists are also expected to be complementary because they inhibit different molecular targets on the platelet. With the failure of the oral GpIIb/IIIa antagonists in clinical trials, the issue of interindividual variability in response and resistance to the effects of certain antiplatelet agents (e.g., aspirin), and evidence that generation of thrombin continues during long-term treatment with antiplatelet agents (Eikelboom et al., 2002), there exists a need for additional therapies for chronic use. Vorapaxar is being developed to address this need. The safety and tolerability of vorapaxar was recently demonstrated in patients who underwent urgent percutaneous coronary interventions or coronary angiographies with planned percutaneous coronary interventions (Becker et al., 2009).

ABSTRACT: Vorapaxar (SCH 530348), a potent oral thrombin protease-activated receptor 1 antagonist, is being developed as an antiplatelet agent for patients with established vascular disease. The objective of this study was to identify the human liver cytochrome P450 (P450) enzyme(s) responsible for the metabolism of SCH 530348. Human liver microsomes metabolized SCH 530348 to M19, an amine metabolite formed via carbamate cleavage, and M20 (monohydroxy-SCH 530348). Recombinant human CYP3A4 exhibited the most activity (11.5% profiled radioactivity) for the formation of M19, followed by markedly less substrate conversion with CYP1A1 and CYP2C19. Trace levels of M19, a major excreted human metabolite, were detected with CYP1A2, CYP3A5, and CYP4F3A. Formation of M19 by human liver microsomes was inhibited 89% by ketoconazole, 75% by astemizole (a CYP2J2 inhibitor), and 43% by CYP3A4 monoclonal antibody. These results suggest that CYP3A4 and CYP2J2 are both involved in the formation of M20 metabolite.

Introduction

Vorapaxar (SCH 530348; [ethyl[(1R,3aR,4aR,6R,8aR,9S,9aS)-9-{[(E)-2-[5-(3-fluorophenyl)-2-pyridinyl]ethenyl]-dodecahydro-1-methyl-3-oxonaphtho[2,3-c][furan-6-yl]carbamate}] (Fig. 1), an analog of the natural product himbacine, is a potent antagonist of protease-activated receptor 1 (PAR-1), the primary thrombin receptor on human platelets. Vorapaxar is under investigation for the treatment and prevention of acute cardiac events in patients with acute coronary syndrome, those with histories of myocardial infarction (MI) or stroke, and those with peripheral arterial disease. Thrombin caused by ruptured or eroded atherosclerotic plaque may result in partial or complete occlusion to coronary and other small arteries. This process is the final underlying mechanism of acute coronary syndrome, including acute MI, and is a major contributing process in ischemic stroke (Davies et al., 1976; Yeghiazarians et al., 2002; Lip et al., 2002).

Oral antiplatelet drugs are at the forefront of antithrombotic agents under investigation for chronic use because antiplatelet drugs are of proven benefit to subjects with histories of unstable angina, MI, stroke, peripheral artery disease, or conditions with a high risk of embolism (Baigent et al., 2002; Dogné et al., 2002). Thrombin is the most potent activator of human platelets, stimulating them primarily through the interaction with PAR-1 (Andersen et al., 1999; Anderluh and Dolenc, 2002). Thus, a PAR-1 antagonist is anticipated to demonstrate a higher level of efficacy than either aspirin or adenosine diphosphate antagonists. The effects of aspirin, adenosine diphosphate receptor antagonists, and PAR-1 antagonists are also expected to be complementary because they inhibit different molecular targets on the platelet. With the failure of the oral GpIIb/IIIa antagonists in clinical trials, the issue of interindividual variability in response and resistance to the effects of certain antiplatelet agents (e.g., aspirin), and evidence that generation of thrombin continues during long-term treatment with antiplatelet agents (Eikelboom et al., 2002), there exists a need for additional therapies for chronic use. Vorapaxar is being developed to address this need. The safety and tolerability of vorapaxar was recently demonstrated in patients who underwent urgent percutaneous coronary interventions or coronary angiographies with planned percutaneous coronary interventions (Becker et al., 2009).

ABBREVIATIONS: SCH 530348, ethyl[(1R,3aR,4aR,6R,8aR,9S,9aS)-9-{[(E)-2-[5-(3-fluorophenyl)-2-pyridinyl]ethenyl]-dodecahydro-1-methyl-3-oxonaphtho[2,3-c][furan-6-yl]carbamate}; PAR-1, protease-activated receptor 1; MI, myocardial infarction; P450, cytochrome P450; LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC, high-performance liquid chromatography; HLMs, human liver microsomes; FSA, flow scintillation analyzer.
SCH 530348, administered as a bisulfate salt, is rapidly absorbed and highly bioavailable (>90%) after oral administration. SCH 530348 is the major circulating drug-derived component after a single dose in plasma (essentially 100%), and it is slowly eliminated (mainly as metabolites) in urine and feces. The parent drug is not eliminated in urine, and less than 2% of orally administered SCH 530348 is eliminated unchanged in the feces. The drug is primarily eliminated as the amine metabolite (M19) formed via carbamate cleavage. Minor amounts of mono- and dihydroxy metabolites and glucuronide and sulfate conjugates are also formed. However, after multiple dose administrations, a hydroxylated metabolite, M20, becomes a predominant circulating metabolite and represents ~23% of the parent drug.

The appreciable accumulation of M20 (M + 16 metabolite), an equipotent active plasma metabolite in human, was only uncovered after long-term clinical dosing with vorapaxar. The objective of this in vitro study was to identify the cytochrome P450 (P450) enzyme(s) capable of metabolizing SCH 530348. Because the in vitro conversion of SCH 530348 to M20 was initially too low, the enzymology of M20 was determined using unlabeled SCH 530348 and then quantitatively studied using highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Materials and Methods

Chemicals. Glucose-6-phosphate dehydrogenase, monosodium D-glucose 6-phosphate, NADP, magnesium chloride, Trizma base, ammonium acetate, and quinidine were purchased from Sigma-Aldrich (St. Louis, MO). Ketocanazole was purchased from Oxford Biomedical Research (Oxford, MI). Acetonitrile acetic acid and methanol were high-performance liquid chromatography (HPLC) grade and purchased from Thermo Fisher Scientific (Waltham, MA); distilled water was prepared using a Milli-Q water purification system from Millipore Corporation (Billerica, MA); and Tris buffer was purchased from BD Biosciences (Woburn, MA). Unlabeled SCH 530348 and M20 were obtained from Schering-Plough (Kenilworth, NJ). Radiolabeled SCH 530348 (14C, radiochemical purity >97%, specific activity 126 μCi/mg) (Fig. 1) was prepared by the radiochemistry group at Schering-Plough Research Institute (Kenilworth, NJ). Single donor (H0029), pooled human liver microsomes (n = 10), intestine and lung microsomes, and a Reaction Phenotyping Kit were purchased from Xenotech, LLC (Lexena, KS). P450 Supersomes and CYP3A4 monoclonal antibodies were purchased from BD Biosciences.

Incubations with HLMs and S9. Pooled HLms (1 nmol P450/ml) were incubated with [14C]SCH 530348 (1–50 μM) for 120 min in the presence of an NADPH-generating system as described previously (Ghosal et al., 2006, 2007). All incubation mixtures contained 3 mM magnesium chloride in 0.5 ml of 100 mM potassium phosphate buffer, pH 7.4. Before the addition of drug, the incubation mixture was preincubated for 2 min at 37°C. Reactions were initiated by the addition of drug or NADP+, allowed to proceed for 120 min at 37°C, and then terminated by the addition of 0.5 ml of methanol. The incubation mixtures were vortexed and centrifuged (10,000g) at 4°C for 10 min, and the supernatants were analyzed by an HPLC-flow scintillation analyzer (FSA). Only samples from substrate concentrations of 10 and 50 μM were analyzed by LC-MS. Boiled human liver microsomes and incubations without NADPH served as negative controls. [14C]SCH 530348 (10 μM) was incubated with human liver S9 (1.6 mg protein/ml) using the procedures described above.

Optimization and Kinetic Parameters using Human Liver Microsomes from a Donor with High CYP3A4 Activity. Initial incubations of [14C]SCH 530348 with pooled human liver microsomes showed a low level of conversion to metabolites. Therefore, all subsequent incubations were performed using human liver microsomes from a single donor with high CYP3A4 activity. In vitro incubations of [14C]SCH 530348 with human liver microsomes were performed using various P450 concentrations (0.25–1 nmol P450/ml) with 10 μM [14C]SCH 530348 for 30, 60, 90, and 120 min. Substrate concentrations of 1 to 100 μM were used subsequently to optimize the concentration of the drug and to calculate Km and Vmax. Reactions were initiated and terminated as described above. The quantitation of M19 (confirmed by LC-MS) was based on radiometric detection and a standard curve (linear least-square fit) of seven substrate concentrations (0.05, 0.1, 0.2, 0.5, 1, 2, and 5 μM).

Screening of Human P450 Supersomes. Screening of human P450 Supersomes (CYP1A1, CYP1A2, CYP2A6, CYP1B1, CYP2B6, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, CYP4F2, CYP4A11, CYP4F3A, CYP4F3B, and CYP4F12) was initially conducted using a constant amount of P450 (0.2 nmol/ml) and [14C]SCH 530348 (10 μM) for 120 min. The screening was also performed under in vitro steady-state conditions of 25 μM [14C]SCH 530348 (~Km concentration). All incubations were performed in 100 mM potassium phosphate buffer, pH 7.4, as described above. For CYP2C9 and CYP2A6 Supersomes, incubations were performed in 100 mM Tris buffer (pH 7.4) as recommended by the supplier. Insect microsomes without cDNA of human P450 were used as control. For LC-MS analysis, supernatants were concentrated under nitrogen at room temperature. Because the rate of M20 formation was low in human liver microsomes and below the detection limit in some cases, kinetic parameters were not determined for M20 formation.

Kinetic parameters of CYP3A4 Supersomes were determined as described previously. Km and Vmax were determined using substrate concentrations from 1 to 100 μM.

Inhibition with Selective P450 Inhibitors and Inhibitory Antibodies. Inhibition of SCH 530348 metabolism was initially evaluated using selective chemical inhibitors of cytochrome P450 enzymes (ketocanazole for CYP3A4, quinidine for CYP2D6, sulfaphenazole for CYP2C9, omeprazole/ticlopidine/tranylcypromine for CYP2C19, orphenadrine/ticlopidine for CYP2B6, astemizole for CYP2J2, α-naphthoflavone for CYP1A1/CYP1A2, and inhibitory monoclonal antibodies against CYP2B6 and CYP3A4). Human liver microsomes (pooled or individual donor, 1 nmol/ml) were preincubated separately with various inhibitors for 15 min at room temperature followed by the addition of buffer, cofactor, and substrate (10 and 25 μM [14C]SCH 530348) for M19 formation. The inhibition of M20 formation was also studied with 25 μM unlabeled SCH 530348. All incubations were performed as described previously. Incubation volumes were 0.5 ml, and the final concentration of the organic solvents in the incubation system was less than 1% (v/v).

Inhibition of [14C]SCH 530348 metabolism to M19 was further evaluated using selective chemical inhibitors of cytochrome P450 enzymes (ketocanazole for CYP3A4, ticlopidine for CYP2B6/CYP2C19, and tranylcypromine for CYP2C19). Human liver microsomes from donor H0029 (0.5 nmol/ml) were preincubated separately with each of the inhibitors for 15 min at room temperature followed by the addition of buffer, cofactor, and substrate (25 μM [14C]SCH 530348). All incubations were performed as described above. The IC50 of ketocanazole (0.1–2 μM) for M19 formation was determined using human liver microsomes with high CYP3A4 activity under the conditions described previously.

Correlation Analysis. A reaction phenotyping kit consisting of 10 individual human liver microsomal preparations from individual donors was used for correlation analysis. The ability of human liver microsomes from each donor to metabolize SCH 530348 to its metabolite M19 was correlated with the
P450-specific enzyme activities for each sample. The assays were performed as described previously, with 25 μM substrate and an incubation period of 120 min. Because the rate of M20 formation was low in human liver microsomes and below the detection limit in some cases, correlation study was not performed for M20 formation.

Detection and Identification of In Vitro Metabolites of SCH 530348.

Samples from the initial microsomal incubations were analyzed for the identification of metabolites using an LC-MS system comprising a TSQ Quantum mass spectrometer (Thermo Fisher Scientific) operated in a positive electrospray ionization mode, an Alliance 2695 HPLC module (Waters, Milford, MA), and a model C525F00 FSA (PerkinElmer Life and Analytical Sciences, Waltham, MA). A Luna Phenyl-Hexyl 250 × 4.6 mm column (5-μm particle size; Phenomenex, Torrence, CA), which was connected to a Luna Phenyl-Propyl 4.0 × 3.0 mm guard column (5 μm particle size), was used for metabolite-profiling analyses. The mobile phases, which consisted of 95% 20 mM ammonium acetate (pH adjusted to 6.0 by glacial acetic acid) and 5% acetonitrile (phase A), and 95% acetonitrile and 5% 20 mM ammonium acetate (pH adjusted to 6.0 by glacial acetic acid) (phase B), were maintained at a constant flow rate of 1 ml/min. The temperature of the column was kept at 40°C. Separation was achieved using programmed linear changes in mobile phase composition starting with 10% of phase B for 2 min, increasing to 50% from 2 to 25 min, increasing to 98% from 25 to 52 min, and then maintaining at 98% from 52 to 57 min. The HPLC column effluent was divided so that 18% of the flow was diverted to the UV/FSA detector, and 82% was diverted to the FSA. The mass spectrometer was operated in positive electrospray ionization mode, with the spray voltage set at 3.7 to 4.8 kV, the tube lens offset at 29 to 133 V, the capillary temperature set at 300°C, the sample flow rate set at 0.21 ml/min, and the sheath and auxiliary gases set at 35 to 50 and 15 to 25 arbitrary units, respectively.

Intrinsic clearance.

Human liver microsomes with high CYP3A4 activity. The metabolic conversion of [14C]SCH 530348 to metabolites was low (3–6%) after incubation of the drug (1, 10, and 50 μM) with human liver microsomes and S9 (data for 1 and 50 μM are not shown). LC-MS analysis demonstrated that SCH 530348 was converted primarily to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS analysis. SCH 530348 was converted to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS analysis. SCH 530348 was converted primarily to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS analysis. SCH 530348 was converted primarily to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS analysis.

Results

Incubation with Pooled Human Liver Microsomes and S9. The metabolic conversion of [14C]SCH 530348 to metabolites was low (3–6%) after incubation of the drug (1, 10, and 50 μM) with human liver microsomes and S9 (data for 1 and 50 μM are not shown). LC-MS analysis demonstrated that SCH 530348 was converted primarily to an amine metabolite (M19) by carbamate cleavage (Table 1). The structure of M19 was confirmed by LC-MS analysis.

Optimization and Kinetic Parameters using Human Liver Microsomes from a Donor Expressing High CYP3A4 Activity. Human liver microsomes from a single donor expressing high CYP3A4 activity were incubated with 10 μM [14C]SCH 530348 at various concentrations of cytochrome P450 P450.5 (0.25–1 nmol/ml) for various times (30–120 min). The P450 concentration of 0.5 nmol/ml and an incubation time of 120 min were found to be optimal for the assay (data not shown). The effects of various substrate concentrations on the metabolite formation (M19), including their kinetic parameters were determined by the GraFit 5.00.1 program.

<table>
<thead>
<tr>
<th>Metabolite Label</th>
<th>Name</th>
<th>m/z</th>
<th>HLM</th>
<th>S9</th>
<th>CYP1A1</th>
<th>CYP1A2</th>
<th>CYP2B6</th>
<th>CYP2C9</th>
<th>CYP2C19</th>
<th>CYP2D6</th>
<th>CYP3A4</th>
<th>CYP3A5</th>
<th>Insect Control Microsomes</th>
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<tbody>
<tr>
<td>M18</td>
<td>Monooxy-M19</td>
<td>437</td>
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<td>–</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
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<td>–</td>
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</tbody>
</table>

- M19 and M20a were not separated in the radiochromatogram; however, based on LC-MS response of each, the contribution to the radioactive peak was determined.

Intrinsic clearance.

Kinetic Parameters for the formation of M19 following incubation of [14C]SCH 530348 with human liver microsomes and human CYP3A4 Supersomes

Human liver microsomes with high CYP3A4 activity.

- Kinetic parameters were determined by the GraFit 5.00.1 program.
- s.e. = S.E.

TABLE 2

Kinetic parameters for the formation of M19 following incubation of [14C]SCH 530348 with human liver microsomes and human CYP3A4 Supersomes

<table>
<thead>
<tr>
<th>Kinetic Parameters</th>
<th>Human Liver Microsomes</th>
<th>CYP3A4</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_m (μM)</td>
<td>23.5 ± 1.91b</td>
<td>22.9 ± 1.84a</td>
</tr>
<tr>
<td>V_max (pmol/nmol P450/min)</td>
<td>57.9 ± 1.75b</td>
<td>166 ± 4.92a</td>
</tr>
<tr>
<td>V_max/K_m (μmol P450/min)</td>
<td>2.46</td>
<td>7.25</td>
</tr>
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</table>

a Kinetic parameters were determined by the GraFit 5.00.1 program.
b ± S.E.
parameters, are shown in Table 2. The $K_m$ for formation of M19 was 23.5 μM, and its $V_{max}$ was 57.9 pmol/nmol P450/min (Table 2). A substrate concentration of 25 μM was chosen for further experiments with human liver microsomes considering the linearity, percentage of conversion, and detection sensitivity for M19. Representative radiometric profiles of metabolites from incubation of SCH 530348 (25 μM) with human liver microsomes are presented in Fig. 2. Metabolite M20 formation was below the detection limit of the radiometric detector. Therefore, a more sensitive LC-MS/MS system was used for quantitation of M20. In the absence of the NADPH-generating system, no metabolite formation was observed (data not shown). The total recovery of injected radioactivity from the HPLC column was 92%.

**Incubation of SCH 530348 with Microsomes from Human Liver, Intestine, and Lung Tissues.** Incubation of unlabeled SCH 530348 with microsomes from human liver, intestine, and lung tissues showed that M20 was generated in all microsomal fractions, and its formation is highest in the liver > intestine > pulmonary tissues (trace level) (Fig. 3). After incubation of SCH 530348 (25 μM) with human liver microsomes, the rate of M19 formation (~30 pmol/nmol P450/min; data not shown) was much higher compared to the rate of M20 formation (~0.4 pmol/nmol P450/min) (Fig. 4, A and B). Experimental variability for the incubations was 2 to 6%.

**Screening with cDNA-Expressed Human P450 Supersomes.** In vitro screening of [14C]SCH 530348 (10 μM) with recombinant human P450 Supersomes showed that only CYP1A1, CYP1A2, CYP2C19, CYP3A4, and CYP3A5 yielded metabolites (mainly M19), suggesting the possible involvement of these enzymes in the metabolism of SCH 530348. CYP3A4 exhibited the most activity (109.8 pmol/nmol P450/min), followed by markedly less substrate conversion with CYP2C19, CYP3A4, and CYP3A5 (Fig. 4A, insert). The screening was repeated under steady-state condition (25 μM), and the results confirmed that CYP1A1, CYP2C19, CYP3A4, and CYP3A5 yielded M19 (Fig. 4A). As expected, CYP3A4 exhibited the most activity (11.5% profiled radioactivity), followed by markedly less substrate conversion by CYP1A1, CYP2C19, and CYP3A5 (Fig.
4A). Trace levels of M19 were also detected after incubation of [14C]SCH 530348 with CYP1A2 and CYP4F3A. The formation of radioactive metabolites with recombinant CYP1A1, CYP1A2, CYP2C19, CYP3A4, CYP3A5, and CYP4F3A suggested involvement of these enzymes in the metabolism of SCH 530348.

The metabolite profiles obtained in various incubations are provided in Table 1. For most of the analyzed samples, the conversion (1–6%) was represented by various amounts of amine (M19, m/z = 421) and mono-oxy (M20a, m/z = 509) metabolites. CYP3A4 showed significantly higher conversion (27.5%), ~22.1% of which is represented by M19. Therefore, M19 represents 80.4% of all metabolites catalyzed by CYP3A4. Low amounts (~1%) of three other oxidative metabolites M20a (M22, m/z = 509) and a mono-oxy-M19 (M18, m/z = 437) were also detected. Trace levels of two additional mono-oxy-SCH 530348 metabolites (M23 and M24, m/z = 509) were detected by LC-MS/MS in selected incubations.

Optimization with human CYP3A4 Supersomes was performed with 25 µM [14C]SCH 530348 at various concentrations of cytochrome P450 (0.1–0.3 nmol/ml) and for various time periods (30–120 min). A P450 concentration of 0.2 nmol/ml and incubation time of 120 min was found to be optimal for the assay (data not shown). $K_m$ for the formation of M19 from CYP3A4 Supersomes was 22.9 µM.
whereas its $V_{max}$ was 166 pmol/nmol P450/min (Table 2). Similar $K_m$ values for M19 formation determined from incubation with human liver microsomes (23.5 $\mu$M) and CYP3A4 (22.9 $\mu$M) strongly suggest the involvement of CYP3A4 in its formation from SCH 530348. A representative radiometric profile of metabolites from incubation of [14C]SCH 530348 (25 $\mu$M) with CYP3A4 Supersomes is presented in Fig. 2.

The accumulation of M20, an active metabolite in human plasma, was established only after chronic administration of vorapaxar. The amount of circulating M20 formed was too low for detection by flow scintillation analyzation after a single radioactive dose of vorapaxar to humans. Therefore, the in vitro enzymology of M20 was investigated separately with unlabeled compound using a highly sensitive LC-MS/MS method. Screening of unlabeled SCH 530348 with human P450 Supersomes showed that CYP2J2 is the major enzyme generating M20 followed by CYP3A4 (Fig. 4B). Other P450s generated M20 in trace levels. However, the rate of M19 formation (0.7 pmol/nmol P450/min) was much higher compared to the rate of M20 formation (0.7 pmol/nmol P450/min) mediated by CYP3A4. The rate of M20 formation by CYP2J2 was 30.6 pmol/nmol P450/min.

Further screening of M20 (25 $\mu$M) with human liver microsomes and 19 P450s after 120-min incubation showed that M20 was further metabolized by CYP3A4 and CYP2J2 to downstream metabolites M16 (m/z 523) and M19 (m/z 421) (data not shown). Proposed P450-mediated in vitro biotransformation pathways for SCH 530348 is provided in Fig. 8.

Inhibition of M19 with Selective P450 Inhibitors. The results of chemical inhibition studies using pooled human liver microsomes with selective chemical inhibitors showed that ketoconazole (a CYP3A4 inhibitor) at 2 $\mu$M inhibited the formation of M19/M20a by 74% (Fig. 5A). Tranylcypromine (50 $\mu$M) and orphenadrine (300 $\mu$M) inhibited M19/M20a formation by 16 and 41%, respectively. In contrast, sulfaphenazole (3 $\mu$M), quinidine (5 $\mu$M), omeprazole (10 $\mu$M), and $\alpha$-naphthoflavone (10 $\mu$M) showed no inhibition. Because orphenadrine, a reported CYP2B6 inhibitor, showed 41% inhibition, CYP2B6-specific antibody was used to explore the contribution of CYP2B6 in the metabolism of SCH 530348. CYP3A4-specific inhibitory monoclonal antibody inhibited M19 by 89%, whereas the CYP2B6-specific inhibitory antibody had no effect (Fig. 5A). Experimental variability was 2 to 7%. These study results indicate that the effect of orphenadrine may be due to inhibition of CYP3A4. Guo et
al. (1997) have reported that orphenadrine inhibits multiple P450 enzymes, including CYP3A4, in human liver microsomes. Our studies also suggest that CYP3A4 is inhibited by orphenadrine, whereas CYP2B6 has minimal effects on vorapaxar metabolism.

The results of the inhibition studies using human liver microsomes expressing high CYP3A4 activity showed that ketoconazole (2 µM) inhibited the formation of M19 by 89% (Fig. 5A). The IC₅₀ value of ketoconazole was 0.73 µM (Fig. 6). These inhibition studies suggest that CYP3A4 is primarily responsible for the metabolism of SCH 530348. Tranylcypromine at a higher concentration of 100 µM and ticlopidine at 20 µM inhibited M19 formation by 34 and 5%, respectively (Fig. 5A). Ticlopidine is an inhibitor of CYP2B6 and CYP2C19 (Turpeinen et al., 2004). The very low level of inhibition by ticlopidine and no metabolite formation by CYP2B6 Supersomes again suggests no involvement of CYP2B6 in the metabolism of SCH 530348. However, moderate inhibition by tranylcypromine suggests the minor involvement of CYP2C19.

Inhibition of M20 with Selective P450 Inhibitors. Investigation of the effect of ketoconazole and astemizole on the formation of M20 from CYP3A4, CYP2J2 Supersomes, and human liver microsomes showed that ketoconazole inhibited M20 formation in human liver microsomes and CYP3A4 by ~90% and in CYP2J2 by ~51% (Fig. 5B). The present study with recombinant CYP3A4 and recombinant CYP2J2 demonstrated that ketoconazole is a potent inhibitor of CYP3A4 and a moderate inhibitor of CYP2J2 (Fig. 7). Astemizole inhibited M20 formation in human liver microsomes and CYP3A4 by ~76% and in CYP2J2 by ~87%. Hence, astemizole is an inhibitor of both CYP2J2 and CYP3A4 (Fig. 7). These data suggested that both CYP3A4 and CYP2J2 were involved in the formation of M20. In human intestine, ketoconazole and astemizole inhibits M20 by 80 and 77%, respectively, suggesting its potential formation via intestinal P450s (CYP3A4 and CYP2J2).

In human liver microsomes, CYP3A4-monoclonal antibody inhibited M20 by ~43%, suggesting the involvement of CYP3A4 (Fig. 5B). These data also suggest that ~43% of M20 formation is catalyzed by CYP3A4.

Correlation Analysis. The formation rate of M19 from [¹⁴C]SCH 530348 was measured in each of the 10 human liver microsomal sample provided in the reaction phenotyping kit. These values were then correlated with the biochemical activity data provided with the kit. Because the biochemical activities are mediated by specific P450 enzymes, a high correlation would suggest that similar enzymes were involved in the formation of metabolite(s) from SCH 530348. The highest correlation (r) between the reaction phenotyping kit assay data (n = 10) and the formation of M19 was noted for midazolam 1'-hydroxylation (r = 0.75) and testosterone 6β-hydroxylation (r = 0.92) (Table 3), which are catalyzed by CYP3A4 and CYP3A4/CYP3A5, respectively. There was poor correlation between the formation of M19 and phenacetin O-deethylation mediated by CYP1A2 (r = 0.22) and S-mephenytoin 4'-hydroxylation mediated by CYP2C19 (r = 0.22), suggesting that CYP1A2 and CYP2C19 have minor involvement. Results of correlation analysis between the enzyme activities and M19 formation confirmed that SCH 530348 is metabolized to M19 primarily by CYP3A4 in human liver microsomes.

Discussion

Clinical studies have shown that vorapaxar is extensively metabolized in humans. The major route of elimination of the drug is via an
amine metabolite (M19) formed by carbamate cleavage. However, after multiple dose administrations, metabolite M20 becomes a major (>10% total) circulating metabolite when steady state for the parent is achieved. Minor amounts of mono- and dihydroxy metabolites are also formed. To better predict the potential for possible drug-drug interactions after coadministration of SCH 530348 with other drugs, this study was undertaken to characterize the P450 enzymes responsible for the metabolism of vorapaxar. Characterization of the P450 enzyme(s) responsible for the metabolism of SCH 530348 to M19 and that whereas CYP3A4-specific inhibitory monoclonal antibodies inhibited M19 by 89%. The IC50 value of ketoconazole was determined to be 0.73 µM. These inhibition studies suggest that CYP3A4 is primarily responsible for the metabolism of SCH 530348. Orphenadrine, a reported inhibitor of CYP2B6 and CYP3A4 (Guo et al., 1997), inhibited M19/M20a formation 41%. However, further studies using monoclonal antibodies demonstrated that whereas CYP3A4-specific inhibitory monoclonal antibodies inhibited M19 by 89%, CYP2B6-specific inhibitory antibodies showed no inhibition. These results suggested that CYP3A4 is primarily responsible for the metabolism of SCH 530348 to M19 and that CYP2B6 has no contribution.

**TABLE 3**

<table>
<thead>
<tr>
<th>SCH 530348 = 25 µM</th>
<th>P450 Enzyme-Specific Reaction</th>
<th>P450 Involveda</th>
<th>r value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin O-deethylation</td>
<td>CYP1A2</td>
<td>0.22</td>
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<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2A6</td>
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<td>Bupropion hydroxylation</td>
<td>CYP2B6</td>
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<td>Paclitaxel 6a-hydroxylation</td>
<td>CYP2C8</td>
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<tr>
<td>Diclofenac 4‘-hydroxylation</td>
<td>CYP2C9</td>
<td>0.11</td>
<td></td>
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<tr>
<td>S-Mephenytoin 4‘-hydroxylation</td>
<td>CYP2C19</td>
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<tr>
<td>Dextromethorphan O-demethylation</td>
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<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>CYP2E1</td>
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<td></td>
</tr>
<tr>
<td>Midazolam 1’-hydroxylation</td>
<td>CYP3A4/CYP3A5</td>
<td>0.75b</td>
<td></td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>CYP3A4/CYP3A5</td>
<td>0.92c</td>
<td></td>
</tr>
<tr>
<td>Lauric acid 12-hydroxylation</td>
<td>CYP4A11</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

a Enzyme activities are from a reaction phenotyping kit (n = 10).

b p = 0.0127.

c p = 0.0001.

![Proposed P450-mediated in vitro biotransformation pathways for SCH 530348.](image)
Tranylcypromine (100 μM), a CYP2C19 inhibitor, and ticlopidine (20 μM), an inhibitor of CYP2B6 and CYP2C19 (Turpeinen et al., 2004), inhibited M19 formation by 34 and 5%, respectively. The very low level of inhibition by ticlopidine again suggested the lack of involvement of CYP2B6 in the metabolism of SCH 530348. However, moderate inhibition by tranylcypromine suggested minor involvement of CYP2C19.

The results of correlation analysis between the enzyme activities and M19 formation confirmed that SCH 530348 is metabolized to M19 primarily by CYP3A4 in human liver microsomes. The highest correlation with the formation of M19 was noted for midazolam 1'-hydroxylation (r = 0.75) and testosterone 6β-hydroxylation (r = 0.92) catalyzed by CYP3A4 and CYP3A4/CYP3A5, respectively. There was no correlation between the formation of M19 and phenacetin O-deethylation mediated by CYP1A2 (r = 0.22) and S-mephénytoin 4'-hydroxylation mediated by CYP2C19 (r = 0.22). The poor correlation suggests that CYP1A2 and CYP2C19 may have only minor involvement in the formation of M19. The proposed biotransformation pathway of SCH 530348 to M19 (Fig. 8) involves oxidation primarily via CYP3A4 of the secondary carbon of the ethyl group, followed by loss of C2H2O and then CO2. Compared to CYP3A4, the contributions of CYP1A1, CYP1A2, CYP2C19, and CYP3A5 to the biotransformation of SCH 530348 in human liver microsomes are minor.

Screening of SCH 530348 with recombinant human P450 Superfamilies showed that CYP2J2 is the major enzyme generated M20, followed by CYP3A4. Other P450s generated M20 in trace levels. In the chemical inhibition study using human liver microsomes (expressing high CYP3A4 activity), formation of M20 was inhibited ~89% by ketoconazole and ~75% by astemizole. Based on the literature and our study, ketoconazole and astemizole are both capable of inhibiting CYP3A4 and CYP2J2 (Matsumoto et al., 2003). Other P450 inhibitors showed little or negligible inhibition, suggesting that their role is minor. In human liver microsomes, CYP3A4 monoclonal antibody inhibited M20 by ~43%, suggesting the involvement of CYP3A4. These data also suggest that ~43% of M20 formation is catalyzed by CYP3A4 and the rest is catalyzed by CYP2J2 and other P450s. CYP2J2 antibody is not commercially available to distinguish contribution of CYP3A4 and CYP2J2. These data suggest that both CYP3A4 and CYP2J2 contribute to the metabolism of SCH 530348 in human liver microsomes.

The overall role of CYP2J2 in drug metabolism has not been determined to date. Several antihistamine drugs, including terfenadine, ebastine, and astemizole, have been identified as good substrates for CYP2J2, a P450 isofrom predominantly expressed in the intestine and heart tissues, with low levels in the liver (Delozier et al., 2007). In fact, strong overlap in substrate recognition by CYP2J2 and CYP3A4 was observed among all the newly identified CYP2J2 substrates.

In human, the abundance of CYP3A4 is 82% in intestine and 40% in liver, whereas the abundance of CYP2J2 is 1.4% in intestine and 1 to 2% in liver (Paine et al., 2006). Hence, we can conclude that CYP3A4 will have a major contribution in the formation of M20. The contribution of CYP2J2 would likely be less than that for CYP3A4. In a clinical drug interaction study with ketoconazole (an inhibitor of CYP3A4 and CYP2J2), the combined effect was to increase the exposure only 2-fold.

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Authorship Contributions
Participated in research design: Ghosal, Lu, Gao, Ramanathan, Chowdhury, Kishnani, and Alton.
Conducted experiments: Ghosal, Lu, Ramanathan, Penner, and Gao.
Contributed new reagents or analytic tools: Ghosal and Lu.
Performed data analysis: Ghosal, Lu, Gao, Ramanathan, and Chowdhury.
Wrote or contributed to the writing of the manuscript: Ghosal, Gao, Chowdhury, Kishnani, and Alton.

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