EFFECTS OF AVASIMIBE ON CYTOCHROME P450 2C9 EXPRESSION IN VITRO AND IN VIVO

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ABSTRACT:

Avasimibe, an acyl-CoA:cholesterol acyltransferase inhibitor, has been previously shown to be a potent inducer of CYP3A4 and multiple drug resistance protein 1. We have further characterized the drug interaction potential of avasimibe by studying the inductive and inhibitory effect of this compound on major drug-metabolizing enzymes. Enzymes known to be involved in the metabolism of drugs likely to be coadministered with avasimibe, such as CYP1A1/2, CYP2C, and CYP2B6, were evaluated further by microarray analysis, Western immunoblotting, and activity assays, using rifampicin and β-naphthoflavone as positive controls. No change was observed in CYP1A1/2 mRNA or activity levels after avasimibe treatment. Differential induction of CYP2C9- and CYP2B6-immunoreactive protein and activity was observed depending on drug concentration and donor. Microarray analysis showed a similar increase in CYP2C and CYP2B6 mRNA levels. The inhibition potential of avasimibe on the major drug-metabolizing enzymes was assessed using pooled human liver microsomes. Avasimibe inhibited CYP2C9 (IC50 2.9 μM), CYP1A2 (IC50 13.9 μM), and CYP2C19 (IC50 26.5 μM). A clinical drug interaction study was conducted to determine whether avasimibe might interact with the CYP2C9 substrate warfarin. Volunteers received 750 mg of avasimibe and showed a 54.2% reduction in trough concentrations of S-warfarin and decreased prothrombin times by 12, 15, 19, and 21% on days 6 through 9, respectively. These results demonstrate that avasimibe’s inductive spectrum resembles that of rifampin.

Avasimibe is an inhibitor of acyl-CoA:cholesterol acyltransferase, the enzyme that catalyzes the intracellular esterification of cholesterol. In clinical studies, a reduction in avasimibe AUC was found after multiple dose administration, consistent with autoinduction of metabolism or transport (Vora et al., 1997). Clinical studies with midazolam (a CYP3A4 substrate) revealed 3- and 6-fold increases in midazolam oral clearance after 50 mg and 750 mg, respectively, of oral avasimibe daily for 7 days (Sahi et al., 2003). When digoxin (a P-glycoprotein substrate) was administered to healthy human volunteers, a 40% decrease in digoxin AUC was observed with 750 mg of avasimibe daily for 10 days (Sahi et al., 2003). These studies confirmed avasimibe to be an inducer of both CYP3A4 and MDR1. Induction of CYP3A4 and MDR1 is mediated predominantly through activation of the pregnane X receptor (PXR) (Blumberg et al., 1998; Lehmann et al., 1998). Earlier studies in our laboratory confirmed that avasimibe significantly activates PXR and is more potent than the prototypical CYP3A4 inducer, rifampin (1 μM avasimibe ≥ 10 μM rifampin) (Sahi et al., 2003).

The potent activation of PXR by avasimibe indicates that there is potential for further drug-drug interactions, inasmuch as PXR has been implicated also in the induction of the human phase 1 drug-metabolizing enzymes CYP2C9 and CYP2B6 (Drocourt et al., 2001; Watkins et al., 2002) and the phase 2 drug-metabolizing enzymes UDP-glucuronosyltransferase 1A1 (Xie et al., 2003), carboxylesterase (Zhu et al., 2000) in human, and glutathione S-transferase (Falkner et al., 2001) in rat. CYP2C9 is involved in the metabolism of several drugs, e.g., S-warfarin, phenytoin, tolbutamide, ticlopidic acid, and nonsteroidal anti-inflammatory drugs including diclofenac (Miners and Birkett 1998; Leemann et al., 1992). Drug-induced increases in hepatic CYP2C9 gene expression have been reported with phenobarbitone (Serlin and Bereczkinridge, 1983), carbamazepine (Baciewicz, 1986), and rifampin (Williamson et al., 1997) and represent the basis for harmful drug-drug interactions. Similarly, diverse drugs are metabolized by CYP2B6, e.g., cyclophosphamide, ifosfamide, tamoxifen, nevirapine, efavirenz, and bupropion (Parkinson, 2001), and changes in the activity of this enzyme could potentially result in adverse drug interactions.

ABBREVIATIONS: AUC, area under the curve; MDR1, multiple drug resistance protein 1; PXR, pregnane X receptor; ITS+, insulin/transferrin/selenium; BCIP/NBT, 5-bromo-4-chloroindolyl-phosphatase/nitrobenzotetrazolium; HPLC, high-performance liquid chromatography; HLM, human liver microsomes; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; LC/MS/MS, liquid chromatography-tandem mass spectrometry; PT, prothrombin time; P450, cytochrome P450; β-NF, β-naphthoflavone; 3-MC, 3-methylcholanthrene.
In vitro drug-drug interaction studies using human hepatic microsomes revealed avasimibe to be a CYP2C9 inhibitor. Since CYP2C9 is involved in the metabolism of avasimibe (Robertson et al., 2001), and patients taking avasimibe are likely to be on warfarin (a CYP2C9 substrate) as well, a clinical drug interaction study was conducted. Because we had earlier established avasimibe to be an activator of PXR, and PXR is implicated in CYP2C9 and CYP2B6 induction, we explored the potential of avasimibe to induce these enzymes in primary human hepatocytes, inasmuch as we have obtained excellent in vitro/in vivo correlations using a primary hepatocyte model (Sahi et al., 2003). Other relevant enzymes (CYP1A1/2) that will possibly be involved in the clearance of coadministered compounds were studied also. Microarray analysis was conducted on mRNA extracted from primary human hepatocytes treated with avasimibe and prototypical positive controls to confirm the mechanism of induction and evaluate other drug-metabolizing enzymes that may be affected by avasimibe administration.

**Materials and Methods**

**In Vitro Studies.** Avasimibe (mol. wt. 502, purity 99.9%), 2,6-bis(1-methylthyl) [2,4,6-tris (1-methylthyl)phényl]acetyl)sulfamate, was obtained from Pfizer (Ann Arbor, MI) (Scheme 1). Human hepatic microsomes were obtained from BD Gentest (Woburn, MA). Collagen type I, insulin/transferrin/selenium (ITS), and Mitragel were obtained from Collaborative Biomedical Research (Bedford, MA). Collagenase type IV was obtained from Sigma-Aldrich (St. Louis, MO). Petri dishes were obtained from Nalge Nunc International (Naperville, IL). All other media and culture reagents were obtained from Invitrogen (Carlsbad, CA). 5-Bromo-4-chloroindolyl-phosphatase/nitroblue tetrazolium (BCIP/NBT) phosphatase substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and dexamethasone were obtained from Sigma-Aldrich and β-naphthoflavone (β-NF), NADP, and dexamethasone were obtained from Sigma-Aldrich and 6β-hydroxytestosterone from Steraloids (Wilton, NH). Antibodies were obtained from Chemicon International (Temecula, CA). All solvents and other chemicals used were of HPLC grade or the highest purity available.

**P450 Inhibition Studies.** Pooled human liver microsomes (HLM) from at least 15 donors were used for all inhibition assays. For IC₅₀ determinations, the substrate probes were used at their approximate in vitro Kᵢ values. All incubations were performed with 100 mM potassium phosphate buffer (pH 7.4) and 1 mM NADPH. Organic solvents were used to prepare stock solutions.

**CYP1A2.** Incubations were performed in a total volume of 0.5 ml, in duplicates with 0.1 mg/ml HLM, 30 μM phenacetin, 1 mM NADPH, and in the presence of avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM in 50 mM) in a potassium phosphate buffer at pH 7.4. After preincubation at 37°C for 7 min, NADPH was added to initiate the enzyme reaction. The reaction mixture was quenched with 500 μl of ice-cold 100 mg/ml paracetamol-D₃/CH₃CN after 25 min. The standards (4-acetamidophenol, singlet) and quality controls (triplicates for low, medium, and high) were prepared at room temperature. After mixing, 0.2 ml of the samples was transferred to another plate and submitted for LC/MS/MS analysis after centrifugation at 3000 rpm for 10 min. A Supelco Discovery Amide C₁₈, 100 × 2.1 mm (5-μm particle size) column (Supelco, Bellefonte, PA) was used. The mobile phase was isocratic, 40:60 [acetonitrile/formic acid, 0.1% (v/v)] at 0.2 ml/min.

**CYP2C9.** Incubations were performed under conditions similar to those above with HLM, 7.5 μM diclofenac, NADPH, and avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM). The reaction was quenched with 500 μl of 2.5 μg/ml 1³C₄,4'-hydroxydiclofenac/CH₃CN after 7 min. The standards (4'-hydroxydiclofenac), quality controls, and samples were prepared as above for LC/MS/MS analysis. A Phenomenex Synergi Max-RP, 50 × 2.0 mm column (Phenomenex, Torrance, CA) was used. The mobile phase was 50:50 [acetonitrile/0.1% formic acid in water (v/v)] at 0.27 ml/min (isocratic).

**CYP2C19.** Incubations were performed under conditions similar to those above with HLM, 50 μM (S)-(+) -mephenytoin, NADPH, and avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40) in potassium phosphate buffer. The reaction mixture was quenched with ice-cold 250 ng/ml 3-acetamidophenol/CH₃CN (500 μl) after 30 min. The standards (4'-hydroxymephenytoin) and quality controls were prepared for LC/MS/MS analysis as above. A Phenomenex Max-RP, 50 × 2.0 mm (5-μm particle size) column was used. Two mobile phases were used: mobile phase A, 90:10 [10 mM ammonium acetate with 0.1% acetic acid/CH₃CN (v/v)]; and mobile phase B: 10:90 [10 mM ammonium acetate with 0.1% acetic acid/CH₃CN (v/v)]. The gradient was 0–0.5 min, 100% A; 0.5–2.0 min, 100% B; 2.0–3.0 min, 100% A.

**Isolation and Cultivation of Human Hepatocytes.** Tissues were obtained through qualified medical staff, with donor consent and approval of the University of North Carolina Hospitals ethics committee. Hepatocytes were isolated from human liver tissue procured through the Department of Surgery, University of North Carolina, by two-step collagenase digestion (LeCluyse, 2001) and as described earlier (Sahi et al., 2003). Briefly, encapsulated liver tissue (15–100 g) was perfused with calcium-free buffer, followed by Dulbecco’s modified Eagle’s medium (DMEM) containing collagenase. Hepatocytes were digested from the digested liver in DMEM supplemented with 5% fetal calf serum, insulin (4 μg/ml), and dexamethasone (1.0 μM), passed through a series of fluorocarbon filters, and washed by low-speed centrifugation. Cell pellets were resuspended in supplemented DMEM with isotonic Percoll and centrifuged at 100g for 5 min. Resulting pellets were resuspended in medium and washed once by low-speed centrifugation. Hepatocytes were resuspended in supplemented DMEM and viability was determined by trypan blue exclusion. Cell yields and viability varied between 10 and 30 million cells per gram of wet tissue and 75 to 95%, respectively.

Hepatocytes were cultured according to the method of LeCluyse (2001). Briefly, 4 to 4.5 million hepatocytes were plated on collagen-coated culture dishes in 3 ml of serum-free modified Chee’s medium supplemented with 0.1 μM dexamethasone and ITS+ and incubated for 2 to 4 h at 37°C in a humidified chamber with 95%/5% air/CO₂ to allow for attachment. Culture medium was aspirated, and fresh ice-cold medium containing 0.25 mg/ml Matrigel was added to each dish. Medium was changed every 24 h thereafter, and treatment with test compounds was started on day 2 or 3.

**Induction Studies.** Groups of hepatocyte cultures (n = 3–5 dishes per treatment group) were treated for 3 consecutive days with vehicle (0.1% DMSO) or drug at concentrations outlined under Results. Cells were rinsed twice with ice-cold Hanks’ balanced salt solution at the end of each treatment period. Homogenization buffer (50 mM Tris-HCl, pH 7.0, 150 mM KCl, 2 mM EDTA) were added to each dish (0.5 ml/dish), and cells were scraped, pooled, and sonicated with a Vibra-Cell probe sonicator (Sonics and Materials, Danbury, CT). An aliquot of the cell lysates was stored at −80°C for Western blot analysis. The remaining cell lysates were centrifuged at 9000g for 20 min at 4°C, and supernatants were collected and centrifuged at 100,000g for 60 min at 4°C. The final microsomal pellets were resuspended in 0.2 to 0.4 ml of 0.25 M sucrose. A sample from each fraction was taken for protein determination and samples were subsequently stored at −80°C.

**Western Blot Analysis.** The CYP2C9 and CYP2B6 proteins in the cell lysates were visualized using Western immunoblotting (Parkinson and Gemzik, 1991). Lysate protein samples (20–40 μg) were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to nitrocellulose membranes. Membranes were then blocked for 1 h at room temperature in casein buffer, incubated overnight at room temperature with primary antibodies diluted in casein buffer, and then incubated at room temperature for 2 h with alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody diluted 1:800 in casein buffer or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody diluted 1:800 in casein buffer. Immunoreactive CYP2B6 and CYP2C9 protein were detected with chemiluminescence standards (data not shown) (Faucette et al., 2000). Dilutions for the primary antibodies were 1:5000 for polyclonal rabbit anti-human CYP2B6 and anti-human CYP2C9 (Chemicon International). Membranes probed with

![Scheme 1](image-url)
alkaline phosphatase-conjugated secondary antibody were developed using BCIP/NBT as substrate solution (Kirkegaard and Perry Laboratories), whereas those probed with horseradish peroxidase-conjugated secondary antibody were developed using an enhanced chemiluminescence detection reagent (Amer sham Biosciences Inc., Piscataway, NJ). Images were scanned using a Versa Doc 1000 imaging system (Bio-Rad, Hercules, CA).

CYP 2C9, CYP2B6, and CYP1A1/2 Activity Assays. Microsomal assays for CYP2C9 and CYP2B6 were conducted with 0.1 mg of microsomes, 500 μM tolbutamide or bupropion, respectively, 50 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 3 mM magnesium chloride, 1 mM NADP⁺, 5 mM glucose 6-phosphate, and 1 U/ml glucose-6-phosphate dehydrogenase. Total incubation volumes for the assays were 0.25 ml. Substrate stock solutions were prepared in methanol and added to individual reaction mixtures in volumes not exceeding 1% of total incubation volumes. Internal standards for the bupropion and tolbutamide assays were triprolidine (20 μg/ml) and chlorpropamide (75 μM), respectively. Reactions in microsomes were initiated at 37°C by addition of NADPH-generating system and terminated after 30 to 45 min with 150 μl of a mixture containing ice-cold acetonitrile and internal standard. Upon completion of the reaction, incubation mixtures were vortexed and centrifuged at 3000 rpm for 5 min to remove precipitated protein.

The amount of hydroxybupropion and 4-hydroxy tolbutamide in the resulting supernatant fraction was determined by reverse-phase HPLC (Leemann et al., 1993; Fauchet et al., 2000). CYP2C9 activity was determined by measuring tolbutamide 4-hydroxylation using reverse-phase HPLC. Briefly, 4-hydroxytolbutamide and chlorpropamide were separated on a Supelcosil C18 15 cm × 4.6 mm reverse phase column (Supelco) preceded by a Pelliguard C18 2.5 cm × 4.6 cm guard column (Supelco). Mobile phase A (80:20 v/v mixture of 0.01 M sodium phosphate dibasic, pH 4.3, and acetonitrile) and mobile phase B (50:50 v/v) were pumped at a flow rate of 1.0 ml/min using a gradient program. The linear gradient program was as follows: 100% mobile phase B to 0% mobile phase B from 0 to 15 min, return to 100% mobile phase B from 15 to 16 min. The column temperature was maintained at 40°C. Total analysis time was 20 min with the following retention times: 4-hydroxytolbutamide, 6.5 min; and chlorpropamide (75 μM), respectively. Reactions in microsomes were initiated at 37°C by addition of NADPH-regenerating system and terminated after 30 to 45 min with 150 μl of a mixture containing ice-cold acetonitrile and internal standard. Upon completion of the reaction, incubation mixtures were vortexed and centrifuged at 3000 rpm for 5 min to remove precipitated protein.

Microarray Analysis of mRNA. RNA was extracted with TRIzol reagent using the fluorometric method of Burke et al. (1985), with minor modifications (Rodrigues and Prough, 1991). Test drugs were washed from cells by replacing media and incubating cells for 15 min. Reaction was initiated by addition of 7-ethoxyresorufin (20 μM) to each well. Aliquots were removed at 15 min for fluorometric analysis.

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Inhibition of P450 Enzymes by Avasimibe in Human Hepatic Microsomes. The overall inhibition profiles of avasimibe toward three human P450 isozymes are shown in Fig. 1. Avasimibe exhibited the greatest inhibition effect on CYP2C9 with an IC50 of 2.88 ± 0.14 μM. The positive control, sulfaphenazole, had an IC50 of 0.339 ± 0.10 μM. Avasimibe was a less potent inhibitor of CYP2C19 (IC50 26.5 ± 2.8 μM), whereas the positive control used for CYP2C19 inhibition, ticlopidine, was significantly more inhibitory in nature (IC50 1.86 ± 0.12 μM). CYP1A2 activity was inhibited by avasimibe with an IC50 of 3.75 μM and 0.16 μM concentration of either of the two fluorescent probes Cy3 (for DMSO-treated samples) or Cy5 (for drug-treated samples). Hybridization reactions were carried out overnight at 42°C, and fluorescent cDNA hybridization signals were detected using a Gen III scanner (Amersham Biosciences Inc.). Data were normalized based upon the fluorescence intensity of the control transcripts labeled with Cy3 and Cy5 and spiked at a 1:1 ratio. The signal intensities for CYP1A1, CYP1A2, CYP2C8, CYP2C, and CYP2B6 were within the linear dynamic range. The mean of four fluorescent measurements was obtained for each individual well, followed by determining the mean for these data from the three wells. The data presented are average of two different hepatocyte preparations. Determination of inhibition of CYP2C9 mRNA levels was attempted, but could not be performed, since the only specific 50-mer oligo that could be designed for the CYP2C family for use in the gene array analysis was for CYP2C9. The homology between the other members of this family precluded the design of a specific probe, and the oligo utilized for these studies represents CYP2C18, CYP2C9, CYP2CA9, and CYP2C19.

Clinical Warfarin Study. The warfarin drug interaction study was conducted before it was known that avasimibe induced CYP2C9. It was performed because in vitro metabolism studies indicated that avasimibe might be a substrate for CYP2C9 in vivo and thus a potential competitive inhibitor of S-warfarin metabolism. The study, conducted in anticoagulated patients, was terminated when decreases in prothrombin time (PT) were observed.

The study was conducted in accordance with ethical principles stated in the Declaration of Helsinki following approval by the Arkansas Institutional Review Board, Little Rock, Arkansas, and obtaining informed consent. Men and women chronically anticoagulated with warfarin with a target International Normalized Ration of 2 to 3 were recruited. After demonstration of a stable PT, patients received avasimibe, 750 mg, once daily. PT was measured daily. The protocol provided for removal of patients from the study based on increases or decreases in coagulation tests or occurrence of a thrombotic event or bleeding complication. Planned enrollment was 12 patients and planned duration of dosing was 14 days. Eight subjects participated: one subject received 14 doses, one subject withdrew for personal reasons after receiving 4 doses, one was withdrawn due to an increase in PT after receiving 4 doses, and 5 subjects were withdrawn due to a decrease in PT after receiving 6 to 10 doses. Baseline PT was defined as the mean of three measurements obtained 1, 2, and 3 weeks before avasimibe dosing. Mean PT was determined for each day of dosing, and a repeated measures analysis of variance was used to calculate the 90% confidence intervals for the mean PT as a percentage of baseline. Prothrombin times were considered decreased if the 90% confidence interval for the percentage of baseline prothrombin time exceeded 100%.

Samples for R- and S-warfarin measurements were to be drawn before the daily warfarin dose at baseline and at completion of the study or early withdrawal. Baseline was defined as the mean of the warfarin concentrations obtained 1, 2, and 3 weeks before avasimibe dosing. For the one patient who received all 14 avasimibe doses, the mean of the concentrations on days 13, 14, and 15 were used for the on treatment measurement. On treatment measurements were not obtained for the patient with an increase in PT. On treatment measurements for the withdrawn subjects were obtained 1 day after the last dose for the patient who received 6 to 10 doses. Baseline PT obtained 1, 2, and 3 weeks before avasimibe dosing was determined for each patient. On treatment measurements were obtained 1 day after the last dose for the patient with an increase in PT. On treatment measurements for the withdrawn patients were obtained 1 day after the last dose for the patient who received 6 to 10 doses. Baseline PT was defined as the mean of three measurements obtained 1, 2, and 3 weeks before avasimibe dosing. Mean PT was determined for each day of dosing, and a repeated measures analysis of variance was used to calculate the 90% confidence intervals for the mean PT as a percentage of baseline. Prothrombin times were considered decreased if the 90% confidence interval for the percentage of baseline prothrombin time exceeded 100%.

Results

Inhibition of P450 Enzymes by Avasimibe in Human Hepatic Microsomes. The overall inhibition profiles of avasimibe toward three human P450 isozymes are shown in Fig. 1. Avasimibe exhibited the greatest inhibition effect on CYP2C9 with an IC50 of 2.88 ± 0.14 μM. The positive control, sulfaphenazole, had an IC50 of 0.339 ± 0.10 μM. Avasimibe was a less potent inhibitor of CYP2C19 (IC50 26.5 ± 2.8 μM), whereas the positive control used for CYP2C19 inhibition, ticlopidine, was significantly more inhibitory in nature (IC50 1.86 ± 0.12 μM). CYP1A2 activity was inhibited by avasimibe with an IC50 of 3.75 ± 0.10 μM. In comparison, the prototypical CYP1A2 inhibitor, furafylline, exhibited an IC50 value of 1.45 ± 0.19 μM.

In Vitro Effects of Avasimibe on CYP2C9, CYP2B6, and CYP1A2 activities in human hepatocytes. Induction of CYP2C9...
enzymatic activity was examined in two separate preparations of human hepatocytes treated with avasimibe at concentrations between 0.05 and 10 μM. Rifampin (10 and 50 μM) was used as a positive control. Rifampin increased the basal tolbutamide 4-hydroxylation in the two hepatocyte preparations by 4.8- and 5.6-fold (Fig. 2, a and b). In comparison, avasimibe increased CYP2C9 activity in a concentration-dependent manner, with maximum induction occurring between 5 and 10 μM. A parallel increase in CYP2C9-immunoreactive protein was observed in the corresponding Western immunoblots (Fig. 2, a and b). Although a marked increase in CYP2C9 protein and activity was observed in both hepatocyte preparations after treatment with avasimibe, the maximum induction was only 67 and 75% of that by rifampin.

CYP1A1/2 activity was measured in three preparations of human hepatocytes, and β-NF (50 μM) and 3-methylcholanthrene (3-MC) (7.5 μM) were used as positive controls. Data from two of these experiments are shown in Fig. 3. Whereas the positive controls increased enzyme activity between 12-fold and 66-fold in different preparations, no significant change in ethoxyresorufin O-dealkylase activity was observed in hepatocyte preparations from any of the donors that had been treated with avasimibe. For CYP2B6, concentration-dependent increases in enzyme activity were observed with avasimibe concentrations between 0.5 and 10 μM (Fig. 4, a and b). The increases in bupropion hydroxylation in the different preparations of hepatocytes were between 2.0- and 3.5-fold at the highest concentration tested, in comparison with 4- to 5-fold increases with rifampin. Western blot analysis revealed a similar concentration-dependent increase in CYP2B6-immunoreactive protein (Fig. 4).

Effect of Avasimibe on CYP2C, CYP2B6, and CYP1A mRNA in Human Hepatocytes. Determination of induction of CYP2C9 mRNA levels was attempted, but could not be performed, since the only specific 50-mer oligo that could be designed for the CYP2C family for use in the gene array analysis was for CYP2C8. The homology between the other members of this family precluded the design of a specific probe, and the oligo utilized for these studies represents CYP2C18, CYP2C9, CYP2C9A, and CYP2C19. The results showed that avasimibe (1 and 5 μM) induced CYP2C mRNA expression 3- and 2-fold, respectively, compared with 3.5- and 3.2-fold for phenobarbital and rifampin, respectively. CYP2C8 mRNA levels were induced to a similar extent by rifampin and 1 μM avasimibe (~2.8 fold). As expected, the CYP1A inducers β-NF and 3-MC did not change the CYP2C mRNA levels and served as negative controls. Microarray analysis revealed that CYP1A1 and CYP1A2 expression in primary cultures of human hepatocytes was increased markedly by the prototypical CYP1A inducers β-NF (50 μM) and 3-MC (8 μM) (Table 1). A small increase was found in
CYP1A2 but not CYP1A1 mRNA after treatment with phenobarbital (2 mM), and no change in CYP1A mRNA expression was observed with rifampin (50 μM) or avasimibe at either concentration (1 and 5 μM). CYP2B6 expression was induced to the greatest extent (~9-fold) by the positive control, phenobarbital (2 mM). Rifampin (50 μM) and, notably, β-NF also induced CYP2B6 mRNA expression. Avasimibe induced CYP2B6 mRNA to an extent similar to rifampin (4.0- and 3.6-fold, respectively) at the lowest concentration tested (1 μM). At a concentration of 5 μM, avasimibe induced CYP2B6 above control levels to a much lesser extent compared with rifampin or β-NF.

In Vivo Effects of Avasimibe on Prothrombin Times and Warfarin Pharmacokinetics in Anticoagulated Patients. Clinical studies in eight anticoagulated patients were conducted to determine whether avasimibe might interact with the CYP2C9 substrate warfarin. Study participants received 750 mg of avasimibe, and mean PT was determined on each day of dosing. Although the PT was unchanged in one patient and was increased in a second patient, five patients exhibited a decrease in prothrombin time that led to their withdrawal from the study and discontinuation of the study. Mean baseline PT (S.E.) for the eight subjects was 18.7 (0.52). Mean PT decreased relative to baseline by 12, 15, 19, and 21% on days 6 through 9, respectively, of avasimibe administration. After repeated avasimibe treatment, R- and S-warfarin concentrations were reduced to 52.3 and 46.8% of baseline, respectively (Table 2).

Discussion

The prediction of drug interactions is increasingly complex, since potent CYP3A4 inhibitors have also been shown to induce CYP3A4 activity after chronic administration; e.g., protease inhibitors (Gass et al., 1998), macrolide antibiotics (Wrighton et al., 1985), and imidazole antimycotic drugs (Hostetter et al., 1989). Although it has become standard practice to study the inhibition potential of compounds on major drug-metabolizing enzymes early in the drug development process, the utility of assessing the induction potential at this stage is still being debated. Our earlier studies with avasimibe have clearly shown the utility of studying both inhibition and induction (Sahi et al., 2003).

Avasimibe is metabolized primarily by CYP3A4 and CYP2C9 (Robertson et al., 2001), and a dose-dependent reduction in midazolam C_{max} and AUC was observed after avasimibe treatment, indicating CYP3A4 induction (Sahi et al., 2003). Because avasimibe is metabolized also by CYP2C9, we have now conducted induction and inhibition studies at various concentrations to elucidate the effect of avasimibe on CYP2C9. The in vitro IC_{50} of 2.8 μM for CYP2C9 using human hepatic microsomes revealed inhibition within the therapeutic concentrations of avasimibe (C_{max} was ≤ 3 μM at doses between 50 and 750 mg). This finding indicates that, when administered with other medications that are substrates for CYP2C9, avasimibe might have the potential to cause drug-drug interactions by changing the pharmacokinetics of the coadministered drug due to inhibition of CYP2C9 activity. However, the clinical study clearly demonstrated that the metabolism of S-warfarin, a probe drug for CYP2C9, was stimulated rather than inhibited, with trough concentrations decreased by half. This was accompanied by a clinically

![Fig. 3. Concentration-dependent induction of CYP1A1/2 activity by avasimibe in primary cultures of human hepatocytes. Human hepatocytes were placed in primary culture for 36 to 48 h before initiating treatment as follows: no treatment or DMSO vehicle (open bars), 50 μM β-NF (shaded bars) or 3-MC (shaded bars), or avasimibe at concentrations between 1 and 20 μM (filled bars). After 72 h of treatment, cells were harvested, microsomal membranes were made, and ethoxyresorufin O-dealkylase activity was assayed, as per procedures under Materials and Methods. a and b represent preparations of human hepatocytes made from two different donor livers.](image1)

![Fig. 4. Concentration-dependent induction of CYP2B6 activity by avasimibe in primary cultures of human hepatocytes. Human hepatocytes were placed in primary culture for 36 to 48 h before initiating treatment as follows: no treatment or DMSO for control (open bars), 10 μM or 50 μM rifampin (Rif; shaded bars), or avasimibe at concentrations between 0.05 and 10 μM (filled bars). After 72 h of treatment, cells were harvested, microsomal membranes were made, and CYP2B6 activity was assayed by measuring hydroxylation of bupropion, as per procedures under Materials and Methods. Cell lysates were made and analyzed by Western blot hybridization for CYP2B6, and densitometric analysis was conducted as described under Materials and Methods. a and b represent preparations of human hepatocytes made from two different donor livers.](image2)
significant decrease in prothrombin time. The accompanying decrease in R-warfarin concentrations likely reflects induction of CYP3A4 and may have contributed to the decrease in prothrombin time. These results indicate that the predominant clinical outcome upon multiple dosing with avasimibe is induction of CYP2C9.

Our analysis of the inhibition data utilized the total plasma concentration and not the unbound/free plasma concentration. It is possible that for certain classes of compounds, the unbound plasma concentration results in a better prediction of in vivo inhibition. In these cases, highly bound compounds would have very low free plasma concentrations and would not be expected to be involved in inhibitory drug-drug interactions. Perhaps this is the case for avasimibe because it is highly protein-bound (99.8%) or, conversely, it is possible that in vivo induction overshadows the inhibitory effect. It was not possible to ascertain whether either of these hypotheses is valid from our studies.

To understand the effect of avasimibe on the induction of drug-metabolizing enzymes more completely, we used primary human hepatocytes that were treated with avasimibe for 3 consecutive days. Concentrations were chosen based on avasimibe clinical plasma concentrations and would not be expected to be involved in inhibitory drug-drug interactions. Perhaps this is the case for avasimibe because it is highly protein-bound (99.8%) or, conversely, it is possible that in vivo induction overshadows the inhibitory effect. It was not possible to ascertain whether either of these hypotheses is valid from our studies.

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Avasimibe is a potent PXR activator (Sahi et al., 2003), and PXR is known to regulate major drug-metabolizing enzymes. We further studied the effect of different concentrations of avasimibe on enzymes known to be regulated by this nuclear receptor. In addition to CYP2C9, CYP2B6 has been shown to be directly regulated by PXR in primary human hepatocytes (Drocourt et al., 2001; Gerbal-Chaloin et al., 2001; Goodwin et al., 2001). Since avasimibe was likely to be coadministered with theophylline, a CYP1A substrate, we also examined the effect of avasimibe on CYP1A1/2. The effect of 3 days of treatment with avasimibe on P450 activity was examined in two preparations of human hepatocytes. CYP2B6 activity was increased in both preparations of human hepatocytes but varied markedly, depending on the donor. No significant changes in CYP1A1/2-dependent activity were observed in hepatocytes treated with avasimibe relative to the positive controls 3-MC and β-NF. Microarray analysis revealed that after 3 days of treatment, avasimibe induced CYP2B6 and members of the CYP2C subfamily, including CYP2C8, but not CYP1A1 or CYP1A2 transcripts. These results indicate that the induction of CYP2C9 activity was likely due to increased transcriptional activation of the CYP2C9 gene.

A discrepancy between the mRNA and activity data was observed under the culture conditions used, since the transcript was induced more at the 1 μM than the 5 μM dose. One possible explanation is that hepatocytes used for RNA analysis were cultured without the Matrigel overlay, perhaps making them more susceptible to toxicity at this dose. Bell-shaped concentration-response profiles were observed in some preparations of human hepatocytes, indicating toxicity at higher doses (data not shown). Overall, the data using primary human hepatocytes indicate that CYP2C9 and CYP2B6 are up-regulated by avasimibe, whereas no change in CYP1A1/2 expression is observed. Inasmuch as these genes are target genes for human PXR, the results from this work are in keeping with the hypothesis that PXR mediates drug interactions between substrates of these enzymes and avasimibe in humans (Goodwin et al., 2001; Xie et al., 2003).

In conclusion, our findings demonstrate that avasimibe is an inducer, and less so an inhibitor, of CYP2C9 enzyme activity at clini-
ally relevant concentrations. As a result, avasimibe causes clinically significant increases in the clearance of the CYP2C9 substrate S-warfarin. These results clearly show that good qualitative predictions for CYP2C9-related clinical drug interactions due to enzyme induction can be made from in vitro studies. In addition, these studies demonstrate that drug-drug interaction assessment should not be considered complete without conducting complementary inhibition and induction experiments. Further research is required to predict more accurately the extent of induction, the implications of protein binding, and the net result of combined induction and inhibition in vivo.

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