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

Jasminder Sahi, Ralph H. Stern, Mark A. Milad, Kelly A. Rose, Gordon Gibson, Xianxian Zheng, Linda Stilgenbauer, Nalini Sadagopan, Summer Jolley, Darryl Gilbert, and Edward L. LeCluyse

Departments of Pharmacokinetics, Dynamics and Metabolism (J.S., K.A.R., L.S., N.S.), Experimental Medicine (R.H.S.), Clinical Pharmacokinetics and Pharmacodynamics (M.A.M.), and Molecular Biology (X.Z.), Pfizer Global Research and Development, Ann Arbor, Michigan; Clinical Studies Centers, LLC, Little Rock, Arkansas (G.G.); and Division of Drug Delivery and Disposition, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina (S.J., D.G., E.L.L.)

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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, tienicid 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 Bereckinridge, 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, as much 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)pheny]acetallylsulfamate, was obtained from Pfizer (Ann Arbor, MI) (Scheme 1).

Human hepatic microsomes were obtained from BD Gentest (Woburn, MA). Collagen type I, insulin/transferin/elenium (ITS+), HepatoStim culture media, and Matrigel 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-phosphosphate/nitrobluetetrazolium (BCIP/NBT) phosphatase substrate was obtained from Kirkegaard and Perry Laboratories (Gaithersburg, MD). Glucose 6-phosphate, glucose-6-phosphate dehydrogenase, 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 K\textsubscript{i} values. All incubations were performed with 100 μM 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 (0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM) in 50 mM 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-D\textsubscript{3}/CH\textsubscript{3}CN after 25 min. The standards (4-acetaminophenol, singlet) and quality controls (triplet) were obtained from Sigma-Aldrich and 6β-acetaminopheno/CH\textsubscript{3}CN (500 μl) after 30 min. The standards (4' hydroxymercaptofen) and quality controls were prepared for LC/MS/MS analysis above. 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)/(+)-mephentoyin, 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-acetamidopheno/CH\textsubscript{3}CN (500 μl) after 10 min. The standards (4'-hydroxymephenytoin) and quality controls were prepared for LC/MS/MS analysis. A Phenomenex Max RP, 50 × 2.0 mm column (Phenomenex, Torrance, CA) was used. The mobile phase was 50:50 [aceto

Scheme 1
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 (Ameri-
sham Biosciences Inc., Piscataway, NJ). Images were scanned using a VersaDuc 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 pufropurin, 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 tripolridine (20 μg/ml) and chlor-
propamide (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.

The amount of hydroxybupropion and 4-hydroxytolbutamide in the resulting supernatant fraction was determined by reverse-phase HPLC (Zimmermann 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 cm reverse phase column (Supelco) preceded by a Palliguard 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 from 0 to 15 min, returning to 0% 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 chlor-
propamide, 10 min. Calibration standards were prepared by adding a known amount of 4-hydroxytolbutamide (0–50 μM) to the incubation mixture. The amount of 4-hydroxytolbutamide formed in each incubation mixture was calculated from the peak area ratio using least-squares linear regression and weighting 1/ν. The lower limit of quantification was 11 pmol.

CYP1A2 activity was determined by measuring the extent of O-dealkylation of 7-ethoxyresorufin 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.

Microarray Analysis of mRNA. RNA was extracted with TRIzol reagent following the method recommended by Invitrogen. Two hepatocyte preparations were used: the wells of hepatocyte monolayers were treated with drug or 0.1% DMSO vehicle. The microarray was fabricated as described before (Kane et al., 2001; Wen et al., 2002). Briefly, between three and eight oligonucleo-
tides for each mRNA were designed, and amino-modified 50-mer oligos were spotted onto SuModic slides (GE Health Care, Piscataway, NJ) using a Gen III robotic spotter (Amersham Biosciences Inc.). The oligos were spotted in duplicate on a single slide, and two different slides were used for analysis of mRNA from each hepatocyte sample well. Yeast control expression plasmids for each mRNA were designed, and amino-modified 50-mer oligos were spotted onto SuModic slides (GE Health Care, Piscataway, NJ) using a Gen III scanner (Amersham Biosciences Inc.) 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, CYP2C9, 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 the average of two different hepatocyte preparations. Determina-
tion 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 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, CYP2C9A, and CYP2C9.

Clinical Warfarin Study. The warfarin drug interaction study was con-
ducted 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 Ratio 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 the study 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 excluded 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 measure-
ments 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 avasimibe dose. Plasma samples were assayed for R- and S-warfarin by an HPLC assay with fluorescence detection at Bioassay Laboratory Inc., Houston, TX. The analytical range was 3 to 1000 ng/ml for both analytes. Concentra-
tions of S- and R-warfarin were log-transformed before use of an analysis of covariance to calculate the 90% confidence intervals for the ratio of on treatment to baseline concentrations.

Results

Inhibition of P450 Enzymes by Avasimibe in Human Hepatic Microsomes. The overall inhibition profiles of avasimibe toward three human P450 isoenzymes are shown in Fig. 1. Avasimibe exhib-
ted the greatest inhibition effect on CYP2C9 with an IC50 of 2.88 ± 0.14 μM. The positive control, sulfaphenazole, had an IC50 of 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 ± 0.18 μM). CYP1A2 activity was inhibited by avasimibe with an IC50 of 13.9 ± 5.0 μM. In comparison, the prototypic 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 \( \mu \text{M} \). Rifampin (10 and 50 \( \mu \text{M} \)) 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 \( \mu \text{M} \). 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 \( \beta\)-NF (50 \( \mu \text{M} \)) and 3-methylcholanthrene (3-MC) (7.5 \( \mu \text{M} \)) 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 \( \mu \text{M} \) (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 \( \mu \text{M} \)) induced CYP2C mRNA expression 3- and 2-fold, respectively, compared with 3.5- and 3.2-fold for \( \beta\)-NF and rifampin, respectively. CYP2C8 mRNA levels were induced to a similar extent by rifampin and 1 \( \mu \text{M} \) avasimibe (2.8 fold). As expected, the CYP1A inducers \( \beta\)-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 \( \beta\)-NF (50 \( \mu \text{M} \)) and 3-MC (8 \( \mu \text{M} \)) (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 1 μM avasimibe.

**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 (Hostetler 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 $C_{\text{max}}$ 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 inhibition and induction studies at various concentrations to elucidate the effect of avasimibe on CYP2C9. The in vitro IC50 of 2.8 μM for CYP2C9 using human hepatic microsomes revealed inhibition within the therapeutic concentrations of avasimibe ($C_{\text{max}}$ 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
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 these cases, highly bound compounds would have very low free concentration results in a better prediction of in vivo inhibition. In other words, the predominant clinical outcome upon multiple dosing with avasimibe is induction of CYP2C9.

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 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-
cellularly 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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Address correspondence to: Dr. Jasminder Sahi, Pfizer Global Research and Development, 2800 Plymouth Road, Ann Arbor MI 48105. E-mail: Jasminder.Sahi@Pfizer.com