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AVASIMIBE INDUCES CYP3A4 AND MDR1 GENE EXPRESSION THROUGH ACTIVATION OF THE PREGNANE X RECEPTOR

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d) Abbreviations:

CYP450: Cytochrome P450

DEX: Dexamethasone

RIF: Rifampin

MDR1: Multiple Drug Resistance protein 1

ACAT: Acyl-CoA:Cholesterol Acyltransferase

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ABSTRACT

In vitro and clinical studies were conducted to characterize the potential of avasimibe, an ACAT inhibitor to cause drug-drug interactions. Clinically, 3- and 6-fold increases in midazolam (CYP3A4 substrate) oral clearance were observed following 50 mg and 750 mg avasimibe daily for 7 days, respectively. A 40% decrease in digoxin AUC (P-glycoprotein substrate) was observed with 750 mg avasimibe daily for 10 days. *In vitro* studies were conducted to define the mechanisms of these interactions. Induction was observed in CYP3A4 activity and immunoreactive protein (EC_{50} 200-400 nM) in primary human hepatocytes treated with avasimibe. Rifampin treatment yielded similar results. Microarray analysis revealed avasimibe (1 μM) increased CYP3A4 mRNA 20-fold, compared to a 23-fold increase with 50 μM rifampin. Avasimibe induced P-glycoprotein mRNA by about 2-fold and immunoreactive protein in a dose-dependent manner. Transient transfection assays showed that avasimibe is a potent activator of the human pregnane X receptor (hPXR) and more active than rifampin on an equimolar basis. Drug-drug interaction studies for CYP3A4 using pooled human hepatic microsomes and avasimibe at various concentrations, revealed IC50 values of 20.7 μM, 1.6 μM and 3.1 μM using testosterone, midazolam and felodipine as probe substrates, respectively. CYP3A4 inhibition is not observed in the clinical studies. Our results indicate that avasimibe causes clinically significant drug-drug interactions through direct activation of hPXR and the subsequent induction of its target genes CYP3A4 and MDR1.

PXR, mRNA, midazolam, human hepatocytes

Avasimibe is a sulfamic acid phenyl ester that inhibits acyl-CoA:cholesterol acyltransferase (ACAT), an enzyme that catalyzes the intracellular esterification of cholesterol, thereby reducing intracellular cholesterol ester content. This class of inhibitors reduces the absorption of dietary cholesterol, the secretion of hepatic very low density lipoproteins into the plasma and extent of atherosclerosis (Lee et al., 1996). Avasimibe is currently in clinical trials and the doses being administered to patients are between 50 to 750 mg daily. Avasimibe has been shown to reduce triglycerides at doses between 50 and 500 mg daily (Insull et al., 2001). The pharmacokinetics of avasimibe is characterized by less than proportional increases in systemic exposure with increasing dose, as measured by maximum plasma concentrations (Cmax) and the plasma concentration-time area under the curve (AUC). Cmax values of avasimibe were approximately 0.5 μM and 1.5 μM following multiple oral doses of 50 and 750 mg daily, respectively (Vora et al., 1997). The lack of dose proportionality was most likely due to the poor solubility of the compound. In general, a reduction in AUC following multiple dose administration is consistent with autoinduction of the metabolic pathways of a compound and/or induction of the MDR1 gene product P-glycoprotein.

Enzyme induction often results in decreasing plasma drug concentrations and the attenuation of the effect of concomitant medications (Smith, 2000). CYP3A4 is the most abundant CYP450 enzyme in the human liver and small intestine, and is involved in the metabolism of approximately 50% of marketed drugs (Parkinson, 2001). Drug-induced increases in hepatic CYP3A4 gene expression are caused by a variety of marketed drugs and herbal medicines, such as rifampin, dexamethasone, phenytoin, phenobarbital (Guzelian, 1988; Maurel, 1996) and St. John's wort (Mai et al., 2000; Perloff et al., 2001) and represent the basis for a number of potentially harmful drug-drug interactions. Induction of CYP3A4 is believed to be mediated predominantly through the activation of the nuclear orphan receptor, pregnane X receptor (PXR), also known as the steroid and xenobiotic receptor (SXR) (Blumberg et al., 1998; Lehmann et al., 1998). Recently, a number of other important genes involved in the elimination of drugs and other xenobiotics have been identified as target genes for this receptor, such as CYP2C9, MDR1, MRP2, and

OATP2 (Maurel, 1996; Geick et al., 2001). MDR1 is expressed in the apical membrane of mature enterocytes and the canalicular membrane of hepatocytes and a change in the concentration of this efflux protein could also contribute to the observed changing pharmacokinetics of avasimibe over time. For this reason, studies were conducted to study the effect of avasimibe on both CYP3A4 and MDR1.

Clinical studies were undertaken to determine avasimibe-induced changes in CYP3A4 and P-glycoprotein levels using midazolam and digoxin as probe substrates, respectively. In vitro studies were conducted to understand the mechanism of the observed interaction and to establish methods to predict such interactions for new chemical entities. Predictability of preclinical *ex vivo* and *in vitro* induction studies to patients is important for early drug discovery and pharmacotherapy. Human *in vitro* model systems were used to assess the ability of avasimibe to induce human hepatic gene expression at clinically relevant concentrations. Observing induction of enzyme activity in *in vitro* systems is often complicated by the fact that inducers can also be potent CYP3A4 inhibitors as has been observed for protease inhibitors (Gass et al., 1998), macrolide antibiotics (Wrighton et al., 1985) and imidazole antimycotic drugs (Hostetler et al., 1989). For this reason, we also explored the inhibition potential of avasimibe in human hepatic microsomes using the probe substrates testosterone, midazolam and felodipine, to assess potential drug interactions at three distinct substrate binding domains within the CYP3A4 binding site. Primary cultures of human hepatocytes were utilized to determine the potential of avasimibe to induce CYP3A4 and MDR1 gene expression. PXR activation assays were performed in Huh7 cells co-transfected with a PXR expression plasmid and a $(CYP3A4\ ER6)_2$-*tk*-luciferase reporter construct.

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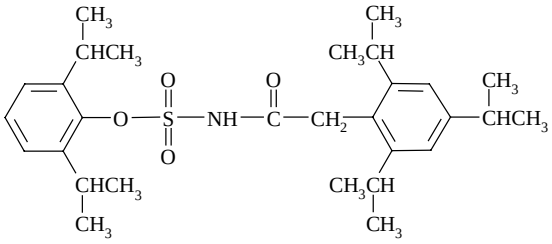
METHODS

In vitro Studies

Avasimibe (MW 502, Purity 99.9%), 2,6-bis(1-methylethyl) [[2,4,6,-tris (1 methylethyl)phenyl]acetyl]sulfamate, was from Parke-Davis Pharmaceutical Research, Ann Arbor, Michigan.



Human hepatic microsomes were from Gentest Corporation (Woburn, MA). Collagen type I, ITS+, hepatostim culture media and Matrigel® were from Collaborative Biomedical Research (Bedford, MA). Collagenase type IV was from Sigma (St. Louis, MO). Petri dishes were from NUNC (Naperville, IL). All other media and culture reagents were from Gibco BRL (Grand Island, NY). BCIP/NBT phosphatase substrate was from Kirkegaard & Perry Laboratories, Gaithersburg, MD. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 11β-hydroxy-testosterone, 6β-hydroxytestosterone, testosterone, β-naphthoflavone, NADP and dexamethasone were from Sigma Chemical Co. (St. Louis, MO) and 6β-hydroxytestosterone from Steraloids, Inc. (Wilton, NH). Antibodies were from Chemicon Inc. (Temecula, CA). All solvents and other chemicals used were of HPLC grade or the highest purity available.

Clinical midazolam study

This study was conducted in accordance with the ethical principles stated in the Declaration of Helsinki following approval by the Community Research Clinic Investigational Review Board, Ann Arbor, MI and obtaining informed consent from volunteers. Healthy men and women of non childbearing potential, not taking medications (including oral contraceptives and estrogen replacement), between the ages of 18 and 60 years old, and weighing 45 kg or greater were recruited. Subjects were prohibited from altering their

usual level of exercise and ingesting products containing grapefruit. All subjects (n=16) received oral midazolam 2 mg (Versed® solution) with a low fat breakfast before (reference) and on the seventh data of avasimibe 50 mg (n=8, $Test_{50mg}$) or 750 mg (n=8, $Test_{750mg}$) daily with a similar meal. Subjects received the midazolam dose with water (4 oz) 15 min after starting breakfast and fasted for 2 hours following the dose. A standardized low fat lunch was served 4 hours postdose. Plasma samples were collected for determination of midazolam concentrations prior to and 0.5, 1, 1.5, 2, 3, 4, 5, 6, and 8 hours after administration. Urine samples were collected for 24 hours after administration.

Clinical digoxin study

This study was conducted with the same principles and guidelines outlined above. Similar criteria were used for recruitment of volunteers who in addition, were required to have a creatinine clearance of 60 mL/min or greater. Subjects (n=12) received 0.25 mg digoxin tablets (Lanoxin®, Lot 8E5186) daily from Days 1 through 20 with a low fat breakfast and 750 mg avasimibe daily with breakfast on Days 10 through 20. Identical lunches and identical dinners were served on Days 10 and 20 at 4 hours and 10 hours postdose. Plasma samples were collected on Days 10 and 20 for determination of digoxin concentrations prior to and at 0.5, 1, 1.5, 2, 3, 4, 6, 12, and 24 hours after digoxin administration. Urine samples were collected for 24 hours after administration.

Analytical methods for midazolam and digoxin

Quantitation of midazolam in plasma and urine was by solid phase extraction and HPLC with tandem mass spectroscopy detection. The lower limit of detection was 0.5 ng/mL in plasma and 0.25 ng/mL in urine. The coefficient of variation of the plasma assay was less than 9%. Quantitation of digoxin in plasma and urine was by radioimmunoassay. The lower limit of

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detection was 0.15 ng/mL in plasma and 1.0 ng/mL in urine. The coefficients of variation of the plasma and urine assay were less than 14% and 9%, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameter values were calculated for each treatment, day, and subject using noncompartmental analysis of concentration-time data (WinNonlin Professional, Version 3, Pharsight Corp., Mountain View, CA). Maximum concentrations (Cmax) and times (tmax) were recorded as observed. Average of concentrations from the predose and 24 hr samples was reported as the minimum concentration (Cmin). Area under concentration-time profile (AUC) values was estimated using the linear trapezoidal rule. AUC(0-tldc) values were calculated from time zero to the time for the last detectable concentration. AUC(0-24) values were calculated from time zero to 24 hours. Oral clearance (CL/F) was calculated as dose/AUC(0-∞) for the midazolam study and dose/AUC(0-24) for the digoxin study. The percent of dose excreted unchanged in the urine, Ae%, was calculated by dividing Ae by the dose, where Ae is the amount excreted in the urine unchanged from 0 to 24 hours calculated by multiplying the concentration in urine by the volume of urine. Renal clearance, CLr, was calculated by dividing Ae by AUC(0-∞) for the midazolam study or AUC(0-24) for the digoxin study.

Isolation and culture of human hepatocytes

Tissues were obtained through qualified medical staff, with donor consent and approval of the UNC Hospitals ethics committee. Hepatocytes were isolated from human liver tissue procured through the Department of Surgery, University of North Carolina by the two-step collagenase digestion method of MacDonald *et al.* (MacDonald et al., 2001). Encapsulated liver tissue (15-100 g) was perfused with calcium-free buffer containing 5.5 mM glucose, 0.5 mM EGTA, 50 mg/mL ascorbic acid and 0.5% BSA for 10-15 min at a flow rate of 10-30 mL/min, followed by Dulbecco's modified Eagle's medium

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(DMEM) containing 0.5% BSA, ascorbic acid (50 mg/mL) and collagenase (0.4-0.8 mg/mL) for 15-20 min at a flow rate of 15-30 mL/min.

Hepatocytes were dispersed 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 (1000, 400, and 100 micron mesh), and washed by low-speed centrifugation (70 x *g*, 4 min). Cell pellets were resuspended in 30 mL supplemented DMEM and 8-12 mL 90% isotonic Percoll and centrifuged at 100 x *g* for 5 min. Resulting pellets were washed once by low-speed centrifugation. Hepatocytes were resuspended in supplemented DMEM and viability determined by trypan blue exclusion. Cell yields and viability varied between 10 and 30 million cells per gram of wet tissue and 75-95%, respectively.

Hepatocytes were cultured according to the method of LeCluyse *et al.* (1996). Briefly, 4-4.5 million hepatocytes were added to 60-mm NUNC Permanox® culture dishes coated with a simple collagen substratum in 3 ml of serum-free modified Chee's medium containing 0.1 μM dexamethasone, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium (ITS+) and allowed to attach for 2-4 hours at 37°C in a humidified chamber with 95%/5%, air/CO_2. Culture medium containing unattached cells was aspirated and fresh ice-cold medium containing 0.25 mg/ml Matrigel® was added to each dish. Medium was changed daily and cells were maintained for 36-48 hours before initiating treatment with test compounds.

Induction studies

Groups of hepatocyte cultures (n = 3-5 dishes per treatment group) were treated for 3 consecutive days with drug at concentrations outlined in the **RESULTS** section or vehicle (0.1% DMSO). At the end of each treatment period, cells were harvested for microsomal preparation. Cells were rinsed twice with ice-cold PBS, homogenization buffer (50 mM Tris-HCl, pH 7.0, 150 mM KCl, 2 mM EDTA) was added to each dish (0.5 mL/dish) and cells were scraped, pooled and sonicated with a Vibra-Cell probe sonicator

(Sonics & Materials, Danbury, CT). Cell lysates were centrifuged at 9,000 x *g* for 20 minutes at 4°C and supernatants were collected and centrifuged at 100,000 x *g* for 60 minutes at 4°C. The final microsomal pellets were resuspended in 0.2-0.4 mL 0.25 M sucrose. An aliquot from each fraction was taken for protein determination and samples subsequently stored at -80°C.

Western Blot Analysis

The CYP3A4 proteins in the microsomes and P-glycoprotein proteins in the cell lysates were visualized using Western immunoblotting (Parkinson and Gemzik, 1991). Microsomal and lysate protein samples (10-40 μg) were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Membranes were probed with specific polyclonal antibodies raised in rabbit to human CYP3A4 or P-glycoprotein, followed by an anti-rabbit IgG-biotinylated secondary antibody and streptavidin-horseradish peroxidase or alkaline phosphatase conjugate. Protein was visualized using BCIP/NBT phosphatase substrate.

PXR activation assay

Huh7 cells were plated at a density of 50K cells/well in 24-well plates in high glucose DMEM supplemented with 10% charcoal/dextran-treated FBS (HyClone Laboratories, Inc., Logan, UT). Transfection mixes contained 100 ng of hPXR expression vector (pCMV-SPORT, Life Technologies, Inc.), 100 ng of firefly luciferase reporter plasmid ($[CYP3A4\ ER6]_2$ -GL3-Promoter Vector, Life Technologies, Inc.) and 10 ng *Renilla* luciferase reporter vector (pRL-TK Vector, Life Technologies, Inc.) as internal control. Transfections were performed with Effectene (Qiagen, Inc.). Drug dilutions were prepared in medium supplemented with 10% charcoal-stripped, delipidated calf serum (Sigma). Cells were incubated for 24 h with drugs, and cell extracts prepared in lysis buffer (Promega). Reporter activity was determined using the Dual-luciferase Reporter Assay System according to the manufacturer's instructions (Promega).

Microarray analysis of CYP3A4 and MDR1 mRNA

Groups of hepatocyte cultures (n = 3-5 dishes per treatment group) were treated for 3 consecutive days with drug at concentrations outlined in the **RESULTS** section or vehicle (0.1% DMSO). At the end of each treatment period, RNA was extracted with Trizol reagent by following the method recommended by Gibco BRL (Grand Island, NY). The microarray was fabricated as described before (Kane et al., 2001; Yuan et al., 2002). Briefly, three oligonucleotide each for CYP3A4 and MDR1 were designed and amino-modified 50mer oligos were spotted onto SuModic slides using a Molecular Dynamic Gen III robotic spotter. Yeast control 100-600 expression plasmids from Incyte were used as spiking controls and synthetic transcripts were generated by *in vitro* transcription (MEGAscript, Ambion). A mixture of synthetic transcripts and each mRNA at a specific copy per cell were spiked into experimental RNA. Labeled cDNA target was generated with reverse transcription (Superscript II: Life Technology) in the presence of random primers (3.75 μM) and either Cy3- or Cy-CTP (0.16 mM). Two replicate hybridization reactions were carried out overnight at 42°C and florescent cDNA hybridization signals were detected using Molecular Dynamic Gen III scanner. Data was normalized based upon intensity values between the Cy3 and Cy5 channel of control transcripts spiked at a 1:1 ratio.

CYP3A4 inhibition studies

IC50 studies were conducted using testosterone, midazolam and felodipine as probe substrates to evaluate the effect of avasimibe on inhibition of the three known substrate binding domains of CYP3A4.

Testosterone: Incubations (7 minutes) were performed in duplicate with 50 mM potassium phosphate buffer (pH 7.4), 0.1 mg/mL human hepatocyte microsomal protein (pool of 15 donors), 50 μM testosterone, avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM) and 1 mM NADPH in a total volume of 500 μl.. Reactions were terminated by the addition of 500 μL of cold 250 ng/mL hydrocortisone/CH_3CN. The marker metabolite 6β-hydroxytestosterone was quantitated by LC/MS/MS analysis. Midazolam: Incubations (4 minutes) were performed in duplicate with 50 mM potassium

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phosphate buffer (pH 7.4), 0.04 mg/mL human hepatocyte microsomal protein (pool of 15 donors), 50 μM midazolam, avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM) and 1 mM NADPH in a total volume of 500 μl.. Reactions were terminated by the addition of 500 μL of cold 250 ng/mL triazolam/CH_3CN. The marker metabolite 1-hydroxymidazolam was quantitated by LC/MS/MS analysis.

Felodipine: Incubations (8 minutes) were performed with 50 mM potassium phosphate buffer (pH 7.4), 0.03 mg/mL human hepatocyte microsomal protein (pool of 15 donors), 1.5 μM felodipine, avasimibe (0, 0.3, 0.75, 1.5, 3, 7.5, 15, 30, and 40 μM) and 1 mM NADPH in a total volume of 750 μl. Reactions were terminated by the addition of 750 μL of cold 400 ng/mL [D_3]-dehydrofelodipine/CH_3CN. The marker metabolite dehydrofelodipine was quantitated by LC/MS/MS analysis.

Statistical Methods

For the induction studies, in vitro results are expressed as mean ± S.D. of three to five hepatocyte preparations. Within each experiment, assays were performed in duplicate. For the clinical studies, statistical comparisons were based on log-transformed Cmax and AUC for the probe drugs. Parameter values were evaluated by ANOVA, using a model incorporating subject and treatment effects for the midazolam study and subject effects for the digoxin study. Statistical tests were performed using the Type III sum of squares using WinNonlin Professional Version 3.0. Least-squares treatment mean values and 90% confidence intervals for the ratio (test /reference) were determined for each parameter. For the in vitro CYP3A4 inhibition studies, incubations were performed in duplicate and WinNonlin Software was used.

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RESULTS

Effect of avasimibe on midazolam pharmacokinetics in humans

After administration of 50 mg and 750 mg avasimibe daily, Cmax and AUC(0-tldc) of a single 2 mg dose of oral midazolam decreased significantly (Table 1). The decrease in Cmax and AUC(0-tldc) were greater following the 750 mg/day dose. Time to reach the maximum concentration was not altered. Due to the decreased midazolam concentration, terminal elimination rate constant could not be estimated from 5 of 8 concentration-time profiles following 750 mg avasimibe and 5 of 8 following 50 mg avasimibe, therefore AUC (0-∞) was not calculated.

Effect of avasimibe on digoxin pharmacokinetics in humans

During coadministration of digoxin (0.25 mg) with avasimibe 750 mg daily, the mean maximum concentrations, area under the curve from times 0 to 24 hours and minimum (trough) concentration of oral digoxin decreased significantly (Table 2). Time to achieve the maximum concentration was similar between treatments. Oral clearance increased significantly. Cumulative urinary excretion decreased significantly and renal clearance increased only slightly. These results are consistent with decrease absorption of digoxin.

Effect of avasimibe on CYP3A4 and MDR1 mRNA in human hepatocytes

Microarray analysis revealed that, the CYP1A inducers βNF (50 μM) and 3-MC (8 μM) used as negative controls, did not change CYP3A4 mRNA expression (Table 3). The positive control rifampin (50 μM) was a more potent inducer of CYP3A4 mRNA than phenobarbital at 2mM, increasing message level 2.7 times more and 1 μM avasimibe increased CYP3A4 mRNA expression close to levels induced by rifa. 3-MC did not change MDR1 mRNA expression and mild increases were observed in βNF, phenobarbital

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and rifampin-treated human hepatocytes. The largest increase in MDR1 mRNA expression was observed with 1 μM avasimibe ($p<0.05$).

Effect of avasimibe on CYP3A4 activity and immunoreactive protein in human hepatocytes

Protein induction of CYP3A4 protein by avasimibe in three preparations (HL132, HL143, HL144) treated with concentrations of avasimibe ranging from 0.05 to 10 μM showed a concentration dependent increase in CYP3A4 activity (Fig 1). The approximate EC_{50} value derived from these data was <0.5 μM, which was less than or equal to the EC_{50} values routinely observed for rifampin. In addition, the overall efficacy i.e. capacity to induce maximum CYP3A4 expression [Emax] of avasimibe was very similar to that of rifampin in every experiment at the highest concentrations tested. The induction of CYP3A4 protein by avasimibe was confirmed in three sets of microsomal samples by Western blot analysis (Fig. 2). Western blots of microsomal samples isolated from the three preparations of avasimibe- or rifampin--treated human hepatocyte showed similar concentration-dependent increases in CYP3A4 immunoreactive protein. These corresponded to the increases in testosterone 6β-hydroxylase activity.

Effect of avasimibe on P-glycoprotein immunoreactive protein

Induction of the *MDR1* gene product P-glycoprotein by avasimibe was evaluated using Western blot and densitometric analysis and compared with induction by rifampin. The results from two separate human hepatocyte preparations treated with concentrations of avasimibe ranging from 0.05 to 10 μM showed similar concentration-dependent increases in P-glycoprotein (Fig 3). Densitometric analysis of the immunoblots showed 1.5-2-fold increases in P-glycoprotein expression at 10 μM avasimibe depending on the preparation of hepatocytes. The overall activity (capacity to induce maximum P-glycoprotein expression [E_{max}]) of avasimibe was very similar to that of rifampin, which is a known inducer of human P-glycoprotein both *in vitro* and *in vivo*.

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Effects of avasimibe on hPXR activation

To determine whether the induction of CYP3A4 and P-glycoprotein by avasimibe is mediated through direct activation of PXR, 0.1-100 μM avasimibe was incubated with Huh7 cells co-transfected with a hPXR expression vector and a reporter gene construct containing multiple copies of the CYP3A4 proximal PXRE (Fig. 4). Rifampin (10 μM) was included as a positive control. The results demonstrate that avasimibe produced a dose-dependent increase in PXR activation that was maximal at a final concentration of 10 μM. The results also illustrate that avasimibe is approximately 10-fold more potent than rifampin as an activator of PXR because nearly identical reporter gene activities were observed at a final concentration of 1 μM avasimibe and 10 μM rifampin.

Inhibition of CYP3A4 by avasimibe in human hepatic microsomes

IC_{50} determinations illustrate the overall inhibition profile as a function of compound concentration. Since the CYP3A4 protein is highly complex and has multiple substrate binding domains, we used three probe substrates, each representing binding at a different site (Fig. 5). IC50 values were determined for the inhibition of CYP3A4 catalytic activity by avasimibe using pooled human liver microsomes and testosterone, midazolam and felodipine as probe substrates. All initial velocity measurements were compared to samples that contained only substrate at the approximate K_m value along with the inhibitor dissolution solvent (100% activity). As shown in Fig 5A, the IC50 for avasimibe, using testosterone as the probe substrate was 20.68 ± 5.26 μM, indicating that avasimibe is not a significant inhibitor. Using midazolam as a probe substrate, an IC50 of 1.64 ± 0.30 μM was obtained (Fig 5B). With the third probe substrate used, felodipine, avasimibe inhibited CYP3A4 activity with an IC50 of 3.14 ± 0.46 μM (Fig 5C). Ketoconazole run as a positive control, had

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IC50 values of 0.016 ± 0.002, 0.006 ± 0.001 and 0.021 ± 0.004 with testosterone, midazolam and felodipine, respectively.

DISCUSSION

Induction of drug metabolizing enzymes, especially CYP450 enzymes, has been observed in vitro for different drug classes. Whether this induction results in clinically significant drug-drug interactions, depends on the enzyme(s) induced, magnitude of the induction, concomitant inhibition and pharmacokinetic profile of co-administered drugs. A key factor in clinical CYP450 induction is the drugs dose, as significant induction is typically seen at higher doses (Smith, 2000). In humans, the major inducible CYP isozyme is CYP3A4. Since about 60 % of all marketed drugs are CYP3A4 substrate (Maurel, 1996), induction of this enzyme could result in potentially significant clinical drug-drug interactions. Avasimibe is metabolized by CYP3A4 (Robertson et al., 2001) and clinical pharmacokinetic studies revealed a reduction in the AUC following multiple dose administration as compared to a single dose (Vora et al., 1997). This indicated potential autoinduction of the metabolic clearance pathways of avasimibe. Confirmation of the role of CYP3A4 was obtained by studying the effects of avasimibe on the pharmacokinetics of the CYP3A4 substrate midazolam. Our results showed a dose-dependent reduction in midazolam Cmax and AUC establishing that CYP3A4 is induced by avasimibe. Our results resemble those obtained when rifampin was used as the inducer in clinical drug interaction studies and midazolam AUC decreased by 98% (Backman et al., 1996).

The effect of avasimibe on the P-glycoprotein substrate digoxin was also characterized. Cmax, AUC, and urinary excretion were all significantly reduced while renal clearance was minimally changed. These results indicate that the primary effect of avasimibe is decreased digoxin absorption. These results are similar to those with rifampin, wherein multiple dose rifampin treatment decreased digoxin AUC by about 30% (Greiner et al., 1999), in healthy volunteers. It is well established that P-glycoprotein transports many drugs that are metabolized by CYP3A4 and many

modulators of P-glycoprotein also modulate the CYP3A gene family (Geick et al., 2001). Furthermore, drugs that are inducers of both CYP3A4 and P-glycoprotein for the most part also are able to activate PXR (Moore and Kliewer, 2000; Moore et al., 2000; Geick et al., 2001). PXR is a key regulator of both CYP3A4 and MDR-1 gene expression in the mammalian liver (Geick et al., 2001; LeCluyse, 2001). We conducted experiments to characterize the effect of avasimibe on CYP3A4 enzyme activity, protein concentrations, and gene expression. We also determined whether P-glycoprotein gene expression in primary cultures of human hepatocytes was increased by avasimibe, and whether the autoinduction observed clinically was mediated by direct activation of the orphan nuclear receptor PXR.

Using our in vitro drug interaction data on CYP3A4, it would have been difficult to predict the clinical outcome, due to the complicated nature of the CYP3A4 protein that has multiple binding sites (Kenworthy et al., 2001; Lu et al., 2001). We found moderate interaction at one, i.e. the testosterone binding site, where avasimibe had an IC50 of 20.7 μM. The other two binding sites that we evaluated, using the prototypical probe substrates midazolam and felodipine, revealed inhibition within the therapeutic concentrations of avasimibe (between 50 and 750 mg in clinical trials, wherein Cmax is no more than 6 μg/ml), Taken in isolation, this data would indicate that when administered with other medications that are substrates for CYP3A4, avasimibe does have the potential to cause drug-drug interactions by changing the pharmacokinetics of the co-administered drug due to inhibition of CYP3A4 activity. However, when human hepatocytes were treated with avasimibe over three days, it was clear that induction was the predominant interaction, as a significant increase in CYP3A4 enzyme activity was observed at therapeutic concentrations. This validated the hypothesis that in the clinic, autoinduction of CYP3A4 was contributing to the drop in avasimibe concentration with repetitive dosing. The extent of this induction was characterized by incubating hepatocytes with different concentrations of avasimibe as well as moderate (phenobarbital) and potent (rifampin) inducers of CYP3A4. Our results suggest that avasimibe is more potent than either of these inducers of CYP3A with an EC_{50} between 0.5 and 1.0 μM.

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By comparison, rifampin and phenobarbital exhibit EC_{50} values of approximately 1 and 150 μM, respectively (Sahi et al., 2000).

Western blot analysis showed increased CYP3A4 immunoreactive protein that paralleled the increases in CYP3A specific activity. Because the observed increase in enzyme activity could be due to either stabilization of CYP3A protein or increased gene transcription, the levels of CYP3A mRNA were assessed using microarray analysis. Avasimibe produced a marked increase (~20 fold) in CYP3A4 mRNA in primary hepatocyte cultures, which was very similar in potency to rifampin and over twice that of phenobarbital. These results indicated that the induction of CYP3A4 activity was due to increased transcriptional activation of the CYP3A4 gene.

To understand the mechanism of increased transactivation of the CYP3A4 gene by avasimibe, we examined the ability of avasimibe to activate PXR in a transient transfection assay. PXR has been identified as the predominant regulator of drug-mediated CYP3A4 induction. Many drugs activate PXR, as its binding domain is larger than most related nuclear receptors and, consequently, fits more bulky and structurally diverse ligands (Watkins et al., 2001). Our results show fairly conclusively that the mechanism of avasimibe induction of CYP3A4 is through direct activation of PXR. Avasimibe not only activates PXR but also does so in a more potent (1 μM avasimibe ≈ 10 μM rifampin) and more effective manner than rifampin (10 μM avasimibe > 10 μM rifampin).

A drug interaction associated with inhibition is considered clinically significant when there is a doubling or more of plasma drug concentration and this increase has the potential to alter the drug response of the co-administered drug (Dresser et al., 2000). Similarly, a drug interaction associated with induction is considered clinically significant when there is a greater than 30% decrease in plasma drug concentrations and this decrease has the potential to alter the drug response (FDA industry guidelines). Multiple-dose

administration of avasimibe in healthy human volunteers produced dose-dependent increases in the clearance of midazolam, a benzodiazepine used as a CYP3A4 probe substrate because it is almost exclusively metabolized by CYP3A enzymes. Reductions in midazolam AUC were approximately 60% and 95% for 50 and 750 mg avasimibe, respectively. The induction seen with avasimibe is similar to that of other CYP3A inducers, which range from 93 to 95% for rifampin (Offermann et al., 1985), carbamazepine, and phenytoin (Kishi et al., 1997). Taken together with the *in vitro* hepatocyte data, these *in vivo* results suggest that avasimibe induces CYP3A4 through direct activation of PXR, consequently increasing the clearance of midazolam and decreasing bioavailability.

Since PXR is a key regulator of both *CYP3A4* and *MDR1*, these results led us to hypothesize that avasimibe might also induce *MDR1* gene expression, thereby leading to a dose-dependent increase in P-glycoprotein. Western immunoblots performed with homogenates from primary human hepatocytes after treatment with avasimibe confirmed this hypothesis. A dose-dependent increase in P-glycoprotein immunoreactive protein was observed which was very similar to that of CYP3A4 dose-response profiles. This further implied that changes in the overall pharmacokinetics of known P-glycoprotein substrates could be altered in patients receiving avasimibe treatment. Indeed, results from *in vivo* clinical studies using the P-glycoprotein substrate digoxin showed a 40% reduction in the AUC of digoxin in those patients receiving 750 mg of avasimibe. Combined, these findings show that avasimibe can cause sufficient induction of P-glycoprotein *in vivo* that lead to significant changes in digoxin bioavailability. The decrease in digoxin AUC was similar to that seen with rifampin (30.3%) (Greiner et al., 1999), which was shown to result in increased P-glycoprotein in enterocytes. Our results indicate that this interaction can be predicted with in vitro methods. In addition, these results present further evidence that drug-induced changes in *MDR1* gene expression have the potential of being clinically significant.

In conclusion, our findings indicate that avasimibe is an inducer of CYP3A4 enzyme activity at clinically relevant concentrations. Studies conducted using primary human hepatocyte cultures and the PXR

reporter gene assay system show that avasimibe increases CYP3A4 and MDR1 gene expression *in vitro* through activation of PXR. Consequently, avasimibe causes clinically significant changes in the pharmacokinetics of the CYP3A4 substrate midazolam and the P-glycoprotein substrate digoxin in healthy volunteers. The induction of CYP3A4 or P-glycoprotein may be the basis for the observed autoinduction of clearance for avasimibe itself. Moreover, the results from these studies show that studying drug drug interactions due to only inhibition in preclinical studies can be misleading, as induction can occur simultaneously, leading to a very different clinical outcome. We have also shown the utility of primary cultures of human hepatocytes for the study of drug-induced alterations in cytochrome P450 and P-glycoprotein expression *in vitro*.

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FIGURE LEGENDS

Figure 1. Concentration-dependent induction of CYP3A4 activity by rifampin and avasimibe in primary cultures of human hepatocytes.

Human hepatocytes were placed in primary culture for 36-48 hours prior to initializing treatment with: no treatment or DMSO for control (Con, open bars), 10 μM or 50 μM rifampin (RIF, hatched bars); or avasimibe at 0.05, 0.1, 0.5, 1, 5 and 10 μM concentrations (stippled bars). Seventy-two hours post treatment, cells were harvested, microsomal membranes made and testosterone 6β hydroxylation activity assayed, as per procedures in METHODS. A, B and C represent preparations of human hepatocytes made from three different donor livers – HL-132, HL-143 and HL-144 respectively.

Figure 2. Concentration-dependent induction of CYP3A4 protein concentration by rifampin and avasimibe in primary cultures of human hepatocytes.

Human hepatocytes were placed in primary culture for 36-48 hours prior to initializing treatment. Seventy-two hours post treatment, cells were harvested and microsomal membranes made and analyzed by Western blot hybridization for CYP3A4 as described in METHODS. The lanes shown represent the following: no treatment control (1); DMSO control (2); 10 μM rifampin (3); 50 μM rifampin (4); avasimibe at 0.05 (5), 0.1 (6), 0.5 (7), 1 (8), 5 (9) and 10 μM (10) concentrations. HL-132, HL-143 and HL-144 represent hepatocytes harvested from three donor livers.

Figure 3. Concentration-dependent induction of P-glycoprotein protein concentration by rifampin and avasimibe in primary cultures of human hepatocytes.

Human hepatocytes were placed in primary culture for 36-48 hours prior to initializing treatment. Seventy-two hours post treatment, cells were harvested and cell lysates made and analyzed by Western

blot hybridization for P-glycoprotein (P-gp) and densitometric analysis conducted as described in METHODS. The lanes shown represent the following: No treatment control (1); DMSO control (2); 10 μM rifampin (3); 50 μM rifampin (4); avasimibe at 0.05 (5), 0.1 (6), 0.5 (7), 1 (8), 5 (9) and 10 μM (10) concentrations. A and B represent hepatocytes harvested from two donor livers.

Figure 4. Concentration-dependent effects of avasimibe on hPXR activation

Huh7 cells were co-transfected with the hPXR expression vector and a reporter gene construct. Cells were incubated with rifampin or avasimibe for 24 hours, cell extracts prepared and reporter activity determined using the dual reporter assay system. Control cells received no treatment. Rifampin (10 μM) was used as a positive control.

Figure 5. Inhibitory profile of avasimibe towards hepatic microsomal CYP3A4 using testosterone (a), midazolam (b) and felodipine (c) as probe substrates

Human hepatic microsomes (pool of 15 donors) were incubated in duplicate as outlined in METHODS with NADPH and one of the following probe substrates: 50 μM testosterone (A), 50 μM midazolam (B) or 1.5 μM felodipine (C). The marker metabolites 6β-hydroxytestosterone, 1-hydroxymidazolam and dehydrofelodipine were monitored using LC/MS/MS as markers of CYP3A4 activity.

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Table 1. Effect of 50 and 750 mg avasimibe oral dosing on midazolam pharmacokinetic parameters. Sixteen subjects received 2 mg oral midazolam before and on the seventh data of avasimibe 50 mg or 750 mg daily (8 subjects each). Plasma samples were collected and analyzed for midazolam concentrations as described in METHODS.

Parameter	Midazolam	Midazolam + 50 mg Avasimibe			Midazolam + 750 mg Avasimibe		
	Least-Squares Mean	Least-Squares Mean	Ratio	90% CI	Least-Squares Mean	Ratio	90% CI
N	16	8			8		
Cmax (µg/ml)	5.92	2.52	42.6	33.8 to 53.7	1.06	17.9	14.2 to 22.5
Tmax (hours)	1.41	1.16	82.2	N. A.	0.781	55.6	N.A.
AUC(0-tldc) (µg/hr/ml)	21.2	5.74	27.1	19.1 to 38.4	1.38	6.49	4.57 to9.20

Cmax: maximum concentration; tmax: time of Cmax; AUC(0-tldc): area under the curve values from time zero to the time for the last detectable concentration. Ratio: Ratio of treatment mean values expressed as a percentage (100%·test/reference); 90% CI: 90% Confidence Interval estimate for the ratio (test/reference) of treatment mean values, expressed as a percentage of the reference mean. N.A: Not Applicable.

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Table 2. Mean steady-state digoxin pharmacokinetic parameter values before and during coadministration of 750 mg avasimibe.

Twelve subjects received 0.25 mg digoxin tablets daily from Days 1 through 20, and 750 mg avasimibe daily on Days 10 through 20. Plasma samples were collected on Days 10 and 20 for determination of digoxin concentrations as described in METHODS.

	Least-Squares Mean Values			
Parameter	Digoxin	Digoxin with Avasimibe	Ratio	90% Confidence Interval
N	11	10		
Cmax, ng/mL	1.52	1.10	72.8	65.8 to 80.5
tmax, hr	1.27	1.32	104	Not Applicable
AUC(0-24) ng·hr/mL	14.5	9.22	63.5	58.3 to 69.2
Cmin, ng/mL	0.504	0.278	55.1	43.3 to 70.2
CL/F, mL/min	324	490	151	130 to 173
Ae%	45.6	33.0	70.8	62.4 to 79.1
CLr, mL/min	137	150	110	101 to 119

Cmax: maximum concentration; tmax: time of Cmax; AUC(0-24): area under concentration-time profile (AUC) values from time zero to 24 hours; Cmin, minimum concentration; CL/F, oral clearance; Ae%, percent of dose excreted unchanged in the urine; CLr, renal clearance. Ratio: Ratio of treatment mean values expressed as a percentage (100%·test/reference); 90% Confidence Interval: 90% confidence interval estimate for the ratio (test/reference) of treatment mean values, expressed as a percentage of the reference mean.

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Table 3: Effect of avasimibe and prototypical inducers on CYP3A4 and MDR1 mRNA in primary human hepatocytes.

Compound	CYP3A4	MDR1
Rifampin (50 μM)	22.7±5.6	1.7±0.4
β -NF (50 μM)	No change	1.5±0.3
3-MC (8 μM)	No change	No change
Phenobarbital (2 mM)	8.4±0.7	1.4±0.6
CI-1011 (1 μg/ml)	19.8±4.0	2.3±0.3
CI-1011 (5 μg/ml)	5.9±0.9	1.7±0.2

Results are expressed as fold increase over control hepatocytes. Primary human hepatocytes (n=3 wells per treatment) were incubated for 48 h with 0.1% DMSO control or one of the positive controls: rifampin or phenobarbital or negative controls β-NF or 3-MC or avasimibe at 1 or 5 μg/ml. At the end of these treatments, the culture medium was discarded, RNA extracted, reverse transcribed, fluorescently labeled and microarray analysis was conducted as described in METHODS. Values represent fold increase over control. Each sample was analyzed in quadruplicate and each value represents the mean +/- S.D of two preparations.

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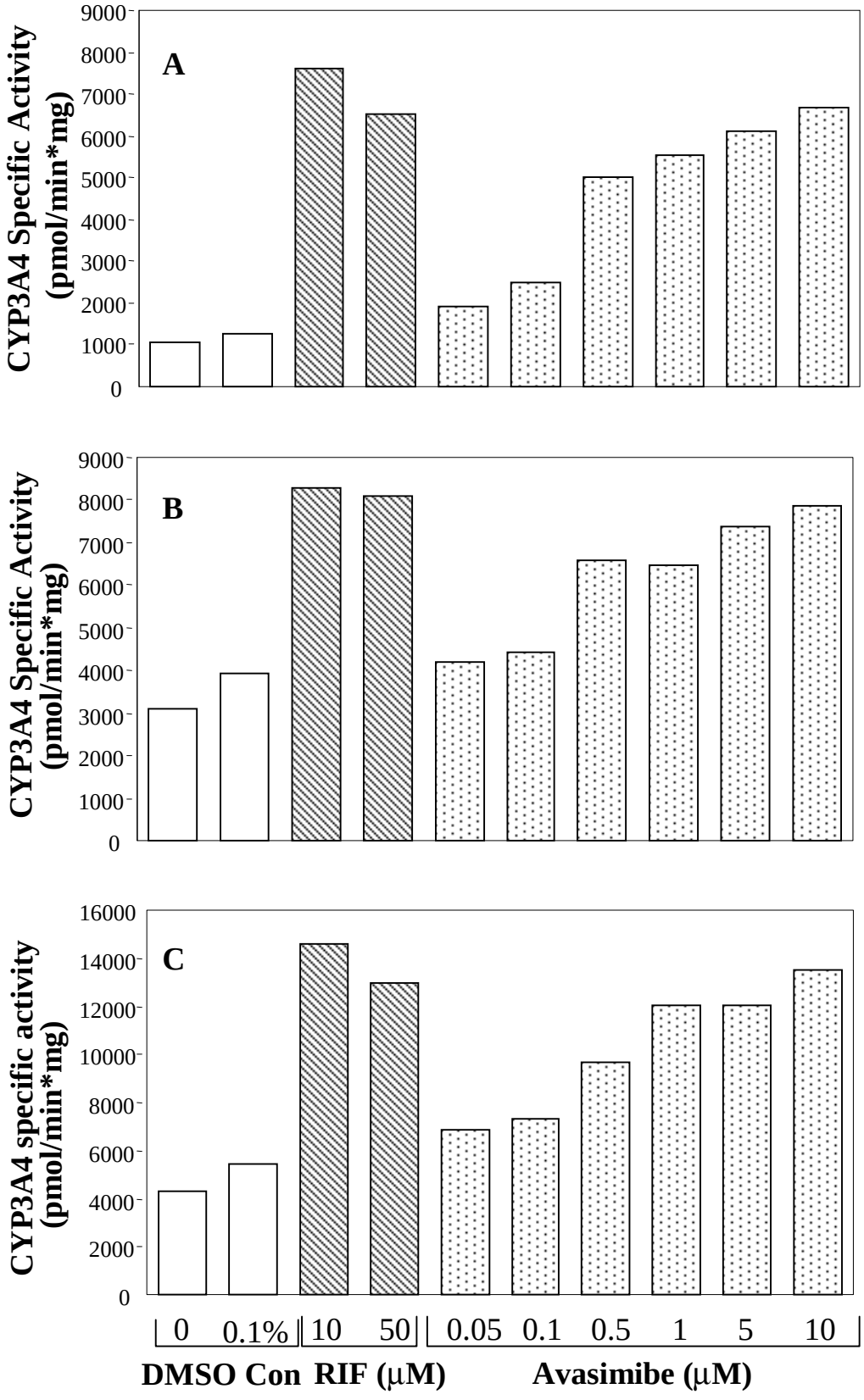

Figure 1

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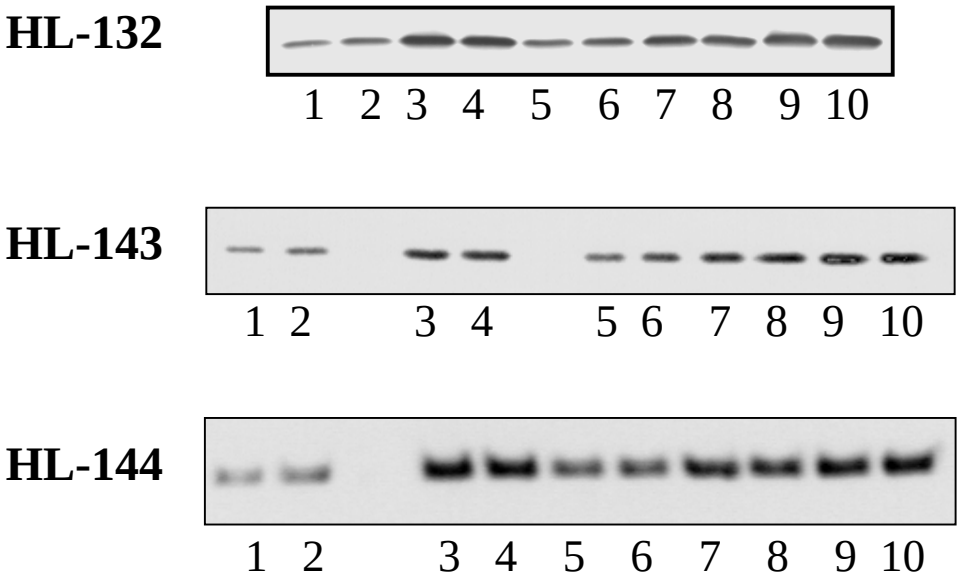

Figure 2

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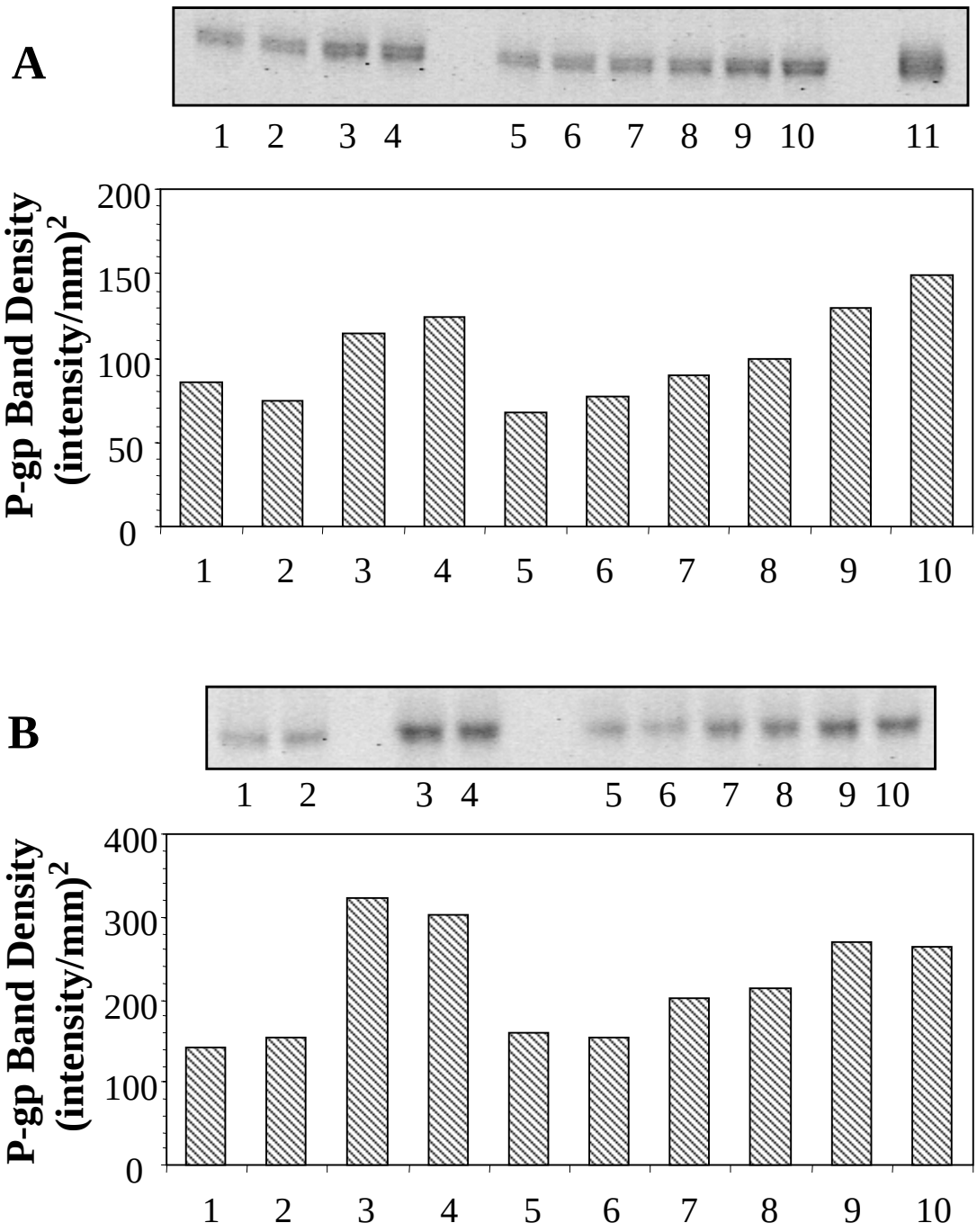

Figure 3

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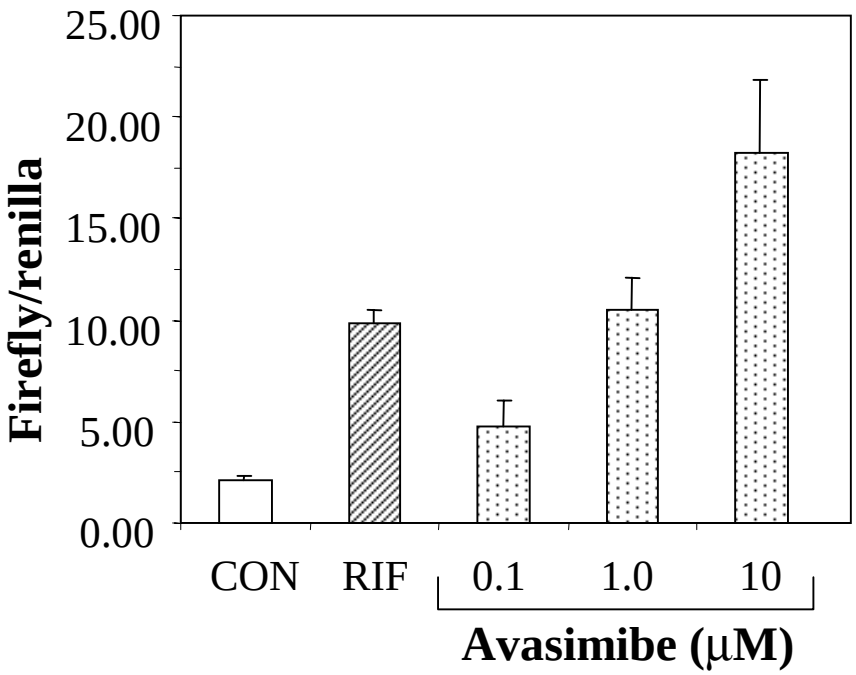

Figure 4

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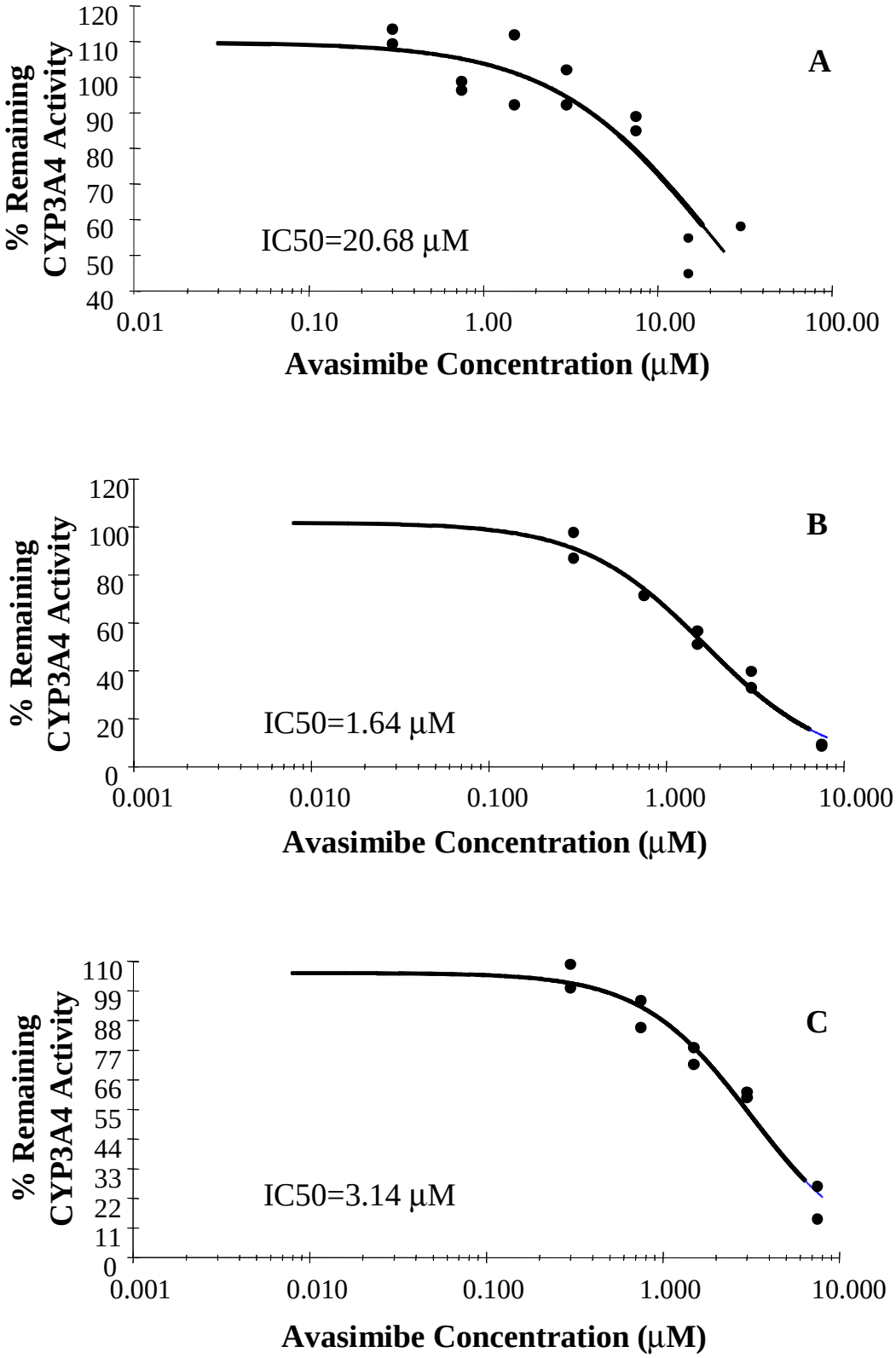

Figure 5