Small Intestinal Metabolism of the 3-Hydroxy-3-methylglutaryl-Coenzyme A Reductase Inhibitor Lovastatin and Comparison with Pravastatin

WOLFGANG JACOBSEN, GABRIELE KIRCHNER, KATRIN HALLENSLEBEN, LAVIERO MANCINI, MICHAEL DETERS, INGELORE HACKBARTH, KAREN BANER, LESLIE Z. BENET, KARL-FRIEDRICH SEWING, and UWE CHRISTIANS

Department of Biopharmaceutical Sciences, School of Pharmacy, University of California, San Francisco, California (W.J., L.M., K.B., L.Z.B., U.C.); Institut für Allgemeine Pharmakologie, Medizinische Hochschule Hannover, Hannover, Germany (G.K., K.H., I.H., K.-F.S.); Institut für Pharmakologie und Toxikologie, Universität Rostock, Rostock, Germany (M.D.); and Department of Cardiothoracic Surgery, Stanford University Medical School, Stanford, California (U.C.)

Accepted for publication May 3, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

We compared the intestinal metabolic metabolism of the structurally related 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors lovastatin and pravastatin in vitro. Human small intestinal microsomes metabolized lovastatin to its major metabolites 6'-β-hydroxy (apparent \( K_m \) = 11.2 ± 3.3 \( \mu M \)) and 6'-exo-methylene (apparent \( K_m \) = 22.7 ± 9.0 \( \mu M \)) lovastatin. The apparent \( K_m \) values were similar for lovastatin metabolism by human liver microsomes. 6'-β-Hydroxyllovastatin formation by pig small intestinal microsomes was inhibited with the following inhibition constants: cyclosporine, 3.3 ± 1.2 \( \mu M \); ketoconazole, 0.4 ± 0.1 \( \mu M \); and troleandomycin, 0.8 ± 0.9 \( \mu M \). \( K_i \) values for 6'-exo-methylene lovastatin were similar. Incubation of pravastatin with human small intestinal microsomes resulted in the generation of 3'α,5'β,6'β,6'-trihydroxypravastatin (apparent \( K_m \) = 4560 ± 1410 \( \mu M \)) and hydroxypravastatin (apparent \( K_m \) = 5290 ± 1740 \( \mu M \)). In addition, as in the liver, pravastatin was metabolized in the small intestine by sulfation and subsequent degradation to its main metabolite 3'α-isopravastatin. It was concluded that lovastatin is metabolized by cytochrome P-450 3A enzymes in the small intestine. Compared with lovastatin, the cytochrome P-450-dependent intestinal intrinsic clearance of pravastatin was >5000-fold lower and cannot be expected to significantly affect its oral bioavailability or to be a significant site of drug interactions.

Received for publication June 8, 1998.

1 This study was supported by Deutsche Forschungsgemeinschaft Grants SFB265/A7, SFB260/A8, and Ch95/6-2 and National Institutes of Health Grant GM26981.

Lovastatin and pravastatin are structurally related (Fig. 1) inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting step in de novo cholesterol synthesis. In the long term, HMG-CoA reductase inhibitors slow the progression or even cause regression of coronary atherosclerosis, resulting in fewer new lesions and total occlusions compared with untreated hypercholesterolemic patients (MAAS Investigators, 1994; Jukema et al., 1995). The oral bioavailability of lovastatin is approximately 5% and highly variable (Henwood and Heel, 1988). In the liver, lovastatin is metabolized by cytochrome P-450 (CYP) 3A enzymes (Wang et al., 1991), and more than 90% is eliminated as metabolites in the bile (Henwood and Heel, 1988). One of the major side effects of HMG-CoA reductase inhibitors is skeletal muscle toxicity (Hsu et al., 1995); the incidence of lovastatin skeletal muscle toxicity in patients with HMG-CoA reductase inhibitor monotherapy is 0.1% (Bradford et al., 1991) but increases up to 30% when lovastatin is combined with a drug that is a CYP3A substrate/inhibitor, such as cyclosporine (Tobert, 1988). In clinical studies, itraconazole and cyclosporine, both of which are known to interfere with the CYP3A-dependent metabolism ofLovastatin, increased lovastatin blood concentrations by 20-fold (Neuvonen and Jalava, 1996; Olbricht et al., 1997). In skeletal muscle cells, high concentrations of HMG-CoA reductase inhibitors cause damage as a result of a reduced formation of HMG-CoA metabolites such as geranylgeraniol (Flint et al., 1997).

Until recently, poor oral bioavailability was mainly attributed to poor solubility in gastrointestinal fluids, poor permeability through the mucosal membrane, and/or extensive hepatic first-pass metabolism (Benet et al., 1996). CYP-dependent drug interactions were generally assumed to take place mainly in the liver. It has recently been recognized that CYP3A enzymes in the small intestinal mucosa are a significant drug metabolism and drug interaction site and, in combination with intestinal countertransporters, may play a ma-

ABBREVIATIONS: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; CYP, cytochrome P-450; MS, mass spectrometry; m/z, mass/charge; PAPS, adenosine 3'-phosphate 5'-phosphosulfate.
ior role in the low and variable oral bioavailability of several drugs that are CYP3A substrates (Lampen et al., 1995, 1996; Wu et al., 1995; Benet et al., 1996; Paine et al., 1997).

To date, only the liver metabolism of lovastatin has been evaluated (Greenspan et al., 1988; Vyas et al., 1988, 1990a,b; Wang et al., 1991). Because lovastatin is a known CYP3A substrate in the liver (Wang et al., 1991), we hypothesize that the small intestine is also a lovastatin drug metabolism site and that intestinal lovastatin metabolism is involved in drug interactions. Thus, it was our primary goal to study the intestinal metabolism of lovastatin. As a secondary goal, we compared the small intestinal metabolism of lovastatin with that of the structurally related HMG-CoA reductase inhibitor pravastatin.

Experimental Procedures

HPLC Equipment. For HPLC-UV analysis of lovastatin and its metabolites, a model 1090 M liquid chromatograph equipped with a diode array detector and an autosampler (Hewlett-Packard, Waldbronn, Germany) was used. The HPLC system was controlled and data were processed using Hewlett-Packard ChemStation Version C.02.02.

HPLC-Mass Spectrometry (MS) Equipment. Lovastatin and pravastatin metabolites were identified and pravastatin and its metabolites were quantified by HPLC-electrospray/MS. A model 1090 M HPLC (Hewlett-Packard) was connected to a model 5989B mass spectrometer equipped with an Iris Hexapole Ion Guide (Analytica of Branford, Branford, CT) by a model 59987A electrospray interface. The mass spectrometer and interface were controlled, and data were processed using ChemStation Revision A04.02 (Hewlett-Packard).

Materials. Lovastatin was the kind gift of Merck, Sharp & Dohme (Rahway, NJ). Pravastatin and its metabolites 3'-iso-hydroxy- and 3',5',6'-trihydroxypravastatin were the kind gifts of Bristol-Myers Squibb (Princeton, NJ). Cyclosporine was the kind gift of of Novartis (Basel, Switzerland). Ketoconazole was purchased from Research Biochemicals Inc. (Natick, MA). Estrone, dehydroepiandrosterone, mevastatin, adenosine-3'-phosphate 5'-phosphosulfate (PAPS), and troleanodycin were obtained from Sigma Chemical Co. (Deisenhofen, Germany).

Analytical HPLC columns were packed with Hypersil C8 material of 3-μm particles (Shandon, Chadwick, UK). Extraction columns were filled with C18 material of 25- to 40-μm particles (LiChroprep; Merck/Recipe, Darmstadt, Germany). All solvents were of HPLC quality and were purchased from Merck (Darmstadt, Germany).

Tissue Samples. To study human small intestinal metabolism, samples were collected from two male and two female patients undergoing surgery for tumor resection at the Klinik für Abdominal- und Transplantationschirurige (Medizinische Hochschule Hannover, Hannover, Germany). For patient safety, it was necessary to remove healthy tissue beyond the tumor border. Portions of this...
healthy tissues were used for our study. All samples were collected from anatomically equivalent positions of the duodenum. Only patients who did not take drugs known to interfere with CYP3A enzymes for at least 2 weeks before surgery were included. Human liver samples were collected after liver transplantation in children who received part of an adult liver (Klinik für Abdominal- und Transplantationschirurgie).

Collection of tissue samples for in vitro metabolism studies was approved by the Ethics Committee of the Medizinische Hochschule Hannover. Pig liver and small intestine samples were obtained from the local slaughterhouse. Samples were collected from pigs between 4 and 5 months of age with an average weight of 110 kg. All animals were from farms specializing in animal mass husbandry and had been fed a standard liquid diet. The diet was rich in protein and low in carbohydrates and supplemented with the amino acid lysine. As required by law and regularly verified by random screening, pigs were maintained drug free, which excluded the presence of xenobiotics interfering with CYP3A concentration and activity in the microsomal preparations. For each pig microsomal preparation, five tissue samples from individual pigs were pooled to provide sufficient sample volume.

Isolation of Microsomes. Human and pig intestinal epithelial cells were isolated according to the methods of Porteous et al. (1979) and Pinkus (1981). Enterocytes and liver samples were frozen at −80°C until the preparation of microsomes. Each preparation step was carried out at 4°C. Enterocytes and the liver samples were taken up in 4 times their volume of a buffer containing 0.1 mM Na/K phosphate, 0.1 mM KCl, 1 mM EDTA, and 0.1 mM dithiothreitol. Samples were homogenized using a glass-Teflon manual homogenizer and were treated with an ultrasonic disintegrator (Branson, Danbury, CT; four 1-min pulses, 80 W). Microsomes were isolated by differential centrifugation as described by Guengerich (1982) with the following modification: Instead of Trits buffer, 0.1 mM phosphate buffer (pH 7.4) was used. After ultracentrifugation, the supernatant (cytosol) was collected to study the hepatic and intestinal pravastatin phase II metabolism. The residue (microsomes) was reconstituted in four times its volume of a buffer solution containing 0.1 mM Na/K phosphate buffer, 0.1 mM pyrophosphate, 1 mM EDTA, and 0.1 mM dithiothreitol and stored at −80°C. No protease inhibitors were used during the isolation of small intestinal microsomes. The protease inhibitor phenylmethylsulfonyl fluoride was found to inhibit lovastatin metabolism. The use of a protease inhibitor mixture of 1 μM leupeptin and 0.3 μM aprotinin had no beneficial effect on CYP3A activity; however, it was essential to maintain the microsomes at 4°C during isolation. Protein concentrations were determined using the biocinchonic acid method described by Smith et al. (1985) with BSA as standard. Protein concentrations of the microsomal suspensions were adjusted with 0.1 M phosphate buffer, pH 7.4. The CYP concentrations were determined using the method described by Omura and Sato (1964) following the protocol of Estabrook and Werringloer (1978).

Microsomal Metabolism of Lovastatin and Pravastatin. Microsomal protein was adjusted to the following concentrations: human liver, 0.25 g/l; human small intestine, 1.5 g/l; pig liver, 1.5 g/l; and pig small intestine, 1.5 g/l. Then 1 ml microsomal preparation and lovastatin [in acetonitrile/water (2:1, v/v); final concentrations, see below] were preincubated for 5 min. Of an NADPH-producing system containing 2 mM EDTA, 10 mM MgCl₂, 0.84 mM NADP, 18 mM isocitric acid, and 700 U/ml isocitrate dehydrogenase in 0.1 M phosphate buffer (pH 7.4), 0.5 ml was added. The assays were incubated for 20 min, and the reaction was stopped by protein precipitation after the addition of 0.5 ml of acetonitrile (liver microsomes) or methanol/0.2 M ZnSO₄ (7.3, v/v); small intestinal microsomes).

For pravastatin metabolism assays, the microsomal protein concentrations of human liver and small intestinal microsomes were adjusted to 2.5 g/l. Pravastatin dissolved in neutral water was added to the microsomal preparations (final concentrations, see below), and the assays were preincubated for 5 min. After the addition of the NADPH-producing system, assays were incubated for 60 min at 37°C. The reaction was stopped by the addition of 0.2 ml methanol/0.2 M ZnSO₄ (7.3, v/v).

Pravastatin Phase II Metabolism in Liver and Small Intestine. To study cytosolic sulfation of pravastatin in the cytosol of small intestinal mucosa cells and hepatocytes, the assay described for hepatic protein by Kitazawa et al. (1993) was used. Small intestinal cytosol preparations were based on isolated mucosa cells as described for the isolation of intestinal microsomes. Pravastatin (50 μM) was incubated with either human liver cytosol (20 mg protein) or human small intestinal mucosa cytosol (3 mg protein) and 0.5 M PAPS in 0.1 M Na/Na phosphate buffer at 37°C for 90 min. Reactions were stopped by protein precipitation after the addition of 500 μl of methanol/0.2 M ZnSO₄ (7.3, v/v). Negative controls contained heat-inactivated (10 min, 95°C) cytosol preparations or cytosol preparations were incubated with 0.1 M Na/Na phosphate buffer without PAPS.

Determination of Unbound Fraction. To compensate for non-specific binding during the microsomal incubation (Obach, 1997), the unbound fraction was determined using the Microcon YM-3 (Millipore, Bedford, MA) filtration system. Lovastatin and pravastatin were incubated with liver and intestinal microsomes and pravastatin in addition to cytosol from intestinal mucosa cells as described above but without NADP or PAPS, respectively. A 500-μl sample was transferred into the reservoir of the Microcon filter devices. After centrifugation at 10,900g for 30 min, the concentrations of pravastatin and lovastatin (after the addition of an equal volume of acetonitrile because lovastatin is water insoluble) were measured in the filtrate as described below.

Quantification of Lovastatin and Its Metabolites. After protein precipitation (see above), the internal standard mevastatin (600 ng) was added. The samples were centrifuged at 4°C (9400g, 5 min), and the supernatant was extracted with 3 ml of ethyl acetate/acetone (2:1, v/v). The samples were vortexed for 15 s, and 2.5 ml of organic phase was transferred into a glass centrifuge tube. The solvent was evaporated under a stream of nitrogen at 40°C. The residues were reconstituted in 100 μl of acetonitrile; 50 μl of water was added, and the samples were transferred into glass HPLC vials.

Next, 100 μl of the extracts was injected into the HPLC-UV system. Lovastatin and its metabolites were separated on a 250 × 4-mm analytical C₈ HPLC column using the following acetonitrile/sulfuric acid (pH 3) gradient: 0 min, 15% acetonitrile; 25 min, 50% acetonitrile; and 45 min, 50% acetonitrile. The column was washed with 95% acetonitrile for 7 min and reequilibrated to the start conditions within 5 min. The flow was 0.7 ml/min, and the column temperature was 40°C. The UV wavelengths of 239 and 273 nm were recorded in parallel. Lovastatin and its metabolites were quantified using an external lovastatin calibration curve after correction for losses during extraction using the internal standard mevastatin and for their different molar UV extinction coefficients. 6'-Hydroxyllovastatin, 21,400 mol⁻¹·l⁻¹·cm⁻¹; 6'-exomethylene-lovastatin, 32,200 mol⁻¹·l⁻¹·cm⁻¹; and lovastatin, 21,500 mol⁻¹·l⁻¹·cm⁻¹ as described by Vyas et al. (1990a).

Method validation showed the following specifications for lovastatin: lower limit of quantification, 100 μg/l; upper limit of quantification, 2400 μg/l; linearity, r = 0.9997; interassay variability, 2.7% (n = 24, 3 days); recovery of lovastatin, its internal standard mevatatin, and its metabolites, >90%; and recovery of lovastatin acid, ranged from 50 to 60% and was significantly lower (p < .01, ANOVA). Extracted samples were stable for at least 48 h at room temperature (autosampler stability) and for at least 2 months when stored at −20°C.

Quantification of Pravastatin and Its Metabolites. After protein precipitation to stop the metabolism reaction, samples were centrifuged at 4°C (3400g, 5 min). The supernatant was drawn through extraction columns filled with C₁₈ material of 25- to 40-μm particles (Merck/Recipe) that had been primed by subsequently washing the columns with 2 ml of methanol and 2 ml of water. The
vacuum was adjusted to result in a flow rate of 1 ml/min. After the supernatants were loaded onto the extraction columns, the samples were washed with 2 ml of water. Pravastatin and its metabolites were eluted using 400 µl of acetonitrile/formic acid, pH 4 (2:1 v/v), and the eluates were transferred into glass HPLC microvials.

The extracted samples (50 µl) were injected into the HPLC-MS system. Pravastatin and its metabolites were separated on a 250×2-mm analytical column filled with Hypersil ODS2 C18 material of 5-µm particles (Shandon, Chadwick, UK). For the quantification of 3′,5′,6′-trihydroxypravastatin and hydroxypravastatin in the microsomal assays, the mobile phase consisted of formic acid (pH 4)/2-propanol (82:18 v/v); for quantification of 3′-iso-pravastatin in the cytosolic assays, the mobile phase was formic acid (pH 4)/2-propanol (70:30 v/v). The flow rate was 0.1 ml/min, and the column temperature was 40°C. After completion of analysis (20 min), the system was washed with 90% 2-propanol for 5 min with a flow rate of 0.25 ml/min. The electrospray interface was adjusted to the following parameters (nomenclature according to ChemStation software): nebulizer gas, nitrogen (purity, 5.0); 80 psi, drying gas, nitrogen (purity, 5.0); drying gas flow, 40 (arbitrary units); drying gas temperature, 350°C; V\text{cap} = -4000 V; V\text{inj} = -3500 V; V\text{cyl} = -6000 V; and capillary exit voltage, 160 V. The following parameters were used for mass spectrometry analysis: quadrupole temperature, 150°C; multiplier voltage, 1795 V; and X-ray, 10,000 V. Positive ions [M + Na]+ of pravastatin and its metabolites were recorded in the single ion mode: m/z 447, pravastatin and 3′-iso-pravastatin; m/z 463, hydroxypravastatin; and m/z 481, 3′,5′,6′-β-trihydroxypravastatin. The dwell time for each ion was 100 ms.

Method validation showed the following specifications for pravastatin: lower limit of quantification, 1 µg/l; upper limit of quantification, 1250 µg/l; linearity, r = 0.995; interassay variability, 8.3%; and recovery of pravastatin and its metabolites, >90%. Extracted samples were stable for at least 48 h at room temperature (autosampler stability) and for at least 3 weeks when stored at −20°C.

Identification of Lovastatin and Pravastatin Metabolites.

Lovastatin metabolites were identified by their characteristic UV absorption spectra (Vyas et al., 1990a) and HPLC-MS. The mass spectrometer was run in the scan mode (m/z 50–600). Isolated metabolite fractions were introduced into the mass spectrometer by flow injection using a manual injection valve connected between the analytical column and the electrospray interface. The electrospray interface and the mass spectrometer were adjusted as described for quantification of pravastatin and its metabolites above. For pravastatin, authentic standards of 3′,5′,6′-β-trihydroxypravastatin and 3′-iso-pravastatin were available. Pravastatin metabolite peaks in the ion-chromatograms were identified by comparison of mass spectra and HPLC retention times with the authentic standards.

Determination of Apparent K \text{m} and Apparent V \text{max} Values.

To determine the apparent K \text{m} and apparent V \text{max} values of lovastatin metabolite formation, microsomes were incubated with the following lovastatin concentrations (n = 4 for each concentration): human liver microsomes, 5, 10, 15, 20, 30, 40, and 50 µM; and human small intestinal, pig liver, and pig small intestinal microsomes, 10, 20, 30, 40, 50, 60, 70, and 100 µM. Human liver (n = 4) and small intestinal (n = 3) microsomes were incubated with pravastatin concentrations of 250, 500, 750, 1000, 1250, 1500, and 2000 µM. Heat-inactivated microsomes (95°C, 10 min) were used as negative controls. Apparent K \text{m} and V \text{max} values were determined after data fitting (SigmaPlot Version 4.0; Jandel Scientific, San Rafael, CA) using the Hanes-Woolf linearization method. Intrinsic clearances (V \text{max}/K \text{m}) were calculated after correction of the apparent K \text{m} values for the unbound fraction.

Inhibition of Liver and Small Intestinal Lovastatin Metabolite Formation by CYP3A Antibodies and Inhibitors. Microsomal protein (100 µg) isolated from human intestinal mucosa was incubated with 0, 0.5, 1, 2.5, or 10 µl CYP3A antibody solution (1 µl = 10 µg protein; Gentest, Woburn, MA) on ice for 15 min. Then, 10 µM lovatstatin or 1000 µM pravastatin and the NADPH-producing system were added. Samples were incubated and extracted as described above.

The specific CYP3A inhibitors cyclosporine, troleandomycin, and ketoconazole (Guengerich, 1995) were dissolved in methanol; 10 µl was added to the microsomal assays and resulted in the following final concentrations: 0.1, 1, 10, and 100 µM for cyclosporine and 0.1, 1, 10, 100, and 500 µM for ketoconazole and troleandomycin. To the controls, 10 µl methanol was added. Cyclosporin and ketoconazole were preincubated with 1 ml of the microsomal preparation and 0.5 ml of the NADPH-producing system at 37°C for 15 min and troleandomycin for 20 min. The reaction was started by the addition of lovastatin. Lovastatin concentrations as described for the determination of apparent K \text{m} values were used. The inhibitors were added at the following concentrations: cyclosporine, 2.5, 5, and 10 µM; ketoconazole, 0.25, 0.5, and 1 µM; and troleandomycin, 25, 50, and 100 µM. To the controls, the same amount of inhibitor-free vehicle was added. The apparent K \text{m} values were determined using secondary plots after Lineweaver-Burk analysis. In secondary plots, the slope of the regression lines were plotted versus the inhibitor concentrations. The apparent K \text{m} values were the intersection of the fitted line of the secondary plot and the x-axis. Data were fitted using SigmaPlot Version 4.0.

Lovastatin Metabolism by Liver and Small Intestinal Microsomes. The two major lovastatin metabolites 6′-β-hydroxy- and 6′-exomethyleneLovastatin were formed during incubation with human liver and intestinal microsomes. Incubation with pig small intestinal microsomes also generated 3′-hydroxylovastatin.

Lovastatin metabolite formation was linear during the 60-min incubation with pig and human small intestinal microsomes and for 20 min with pig and human liver microsomes. The apparent K \text{m} and apparent V \text{max} values for the different microsomal preparations are listed in Table 1. Figure 2 shows the Hanes-Woolf plots of representative data sets after the incubation of lovastatin with human liver and small intestinal microsomes. As reported previously (Greenspan et al., 1988), in our study, lovastatin concentrations of >100 µM resulted in substrate inhibition. The apparent K \text{m} was lowest in human liver microsomes and similar for both lovastatin metabolites. In the human small intestine, apparent K \text{m} values were approximately 50% higher for 6′-β-hydroxylovastatin formation and about 3-fold higher for 6′-exomethyleneLovastatin formation than in the human liver. In the pig liver, the mean apparent K \text{m} value of 6′-β-hydroxylovastatin formation was 4-fold higher than in the human liver and that of 6′-exomethyleneLovastatin formation was 10-fold higher. The apparent K \text{m} values for the formation of lovastatin metabolites were similar in pig small intestinal and pig liver microsomes. CYP3A4/5 antibodies significantly inhibited the intestinal metabolism of lovastatin (Fig. 3A). At the highest antibody concentration (100 µg/100 µg microsomal protein), in comparison with uninhibited controls, 6′-exomethyleneLovastatin formation was reduced by 74.5% and 6′-β-hydroxylovastatin was reduced by 80.5%.

Pravastatin Metabolism by Liver and Small Intestinal Microsomes. Incubation of pravastatin with human small intestinal microsomes resulted in the formation of two metabolites. One metabolite could be identified as 3′,5′,6′-β-trihydroxypravastatin. The other metabolite was a hydroxylated pravastatin metabolite. Because no standard material for hydroxylated pravastatin metabolites was available, its exact structure was not identified. The metabolite pattern generated
by small intestinal microsomes equaled that after incubation with human liver microsomes. The apparent $K_m$ and apparent $V_{\text{max}}$ values are shown in Table 2. Apparent $K_m$ values of $3'\alpha,5'\beta,6'\beta$-trihydroxypravastatin in the human small intestine and in the liver were not significantly different. Compared with the liver, the mean $K_m$ value of hydroxypravastatin in the small intestine was 2.4-fold lower. The mean $V_{\text{max}}$ value of $3'\alpha,5'\beta,6'\beta$-trihydroxypravastatin was 1.5-fold and that of hydroxypravastatin was 2.5-fold lower in the human small intestinal microsomes than in the human liver microsomal preparations. This was at least in part due to the lower CYP concentrations/g microsomal protein. Although the mean CYP concentration in the liver microsomes was $0.116\pm0.09\ \text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$ (mean $\pm$ S.D., $n = 4$), the CYP concentrations in the small intestinal microsomes were below the detection limit.

Compared with lovastatin metabolites, the mean $K_m$ values of the pravastatin metabolites were approximately 400-fold higher and the $V_{\text{max}}$ values were 2.5-fold lower. Formations of $3'\alpha,5'\beta,6'\beta$-trihydroxypravastatin and hydroxypravastatin were significantly inhibited by CYP3A4/5 antibodies. However, at the highest antibody concentration (100 $\mu$g/100 $\mu$g microsomal protein), compared with uninhibited controls, $3'\alpha,5'\beta,6'\beta$-trihydroxypravastatin formation was inhibited by only 57.6% (Fig. 3B). Hydroxypravastatin formation was inhibited by 71.2%.

In the presence of PAPS, incubation of pravastatin with human liver and human small intestinal mucosa cytosol yielded $3'\alpha$-iso-pravastatin. $3'\alpha$-Iso-pravastatin was not detectable after incubations without the cofactor PAPS or with heat-inactivated cytosol. As shown in Fig. 4, the mean $3'\alpha$-iso-pravastatin formation rates in the liver (range, 0.3-1.2 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$) were 2-fold higher than those in the small intestine (range, 0-0.7 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1} \text{protein}$). Estrone and dehydroepiandrosterone, both substrates and inhibitors of sulfotransferases, inhibited the formation of $3'\alpha$-iso-pravastatin in liver and small intestinal cytosol. Compared with uninhibited controls, 200 $\mu$M estrone reduced $3'\alpha$-iso-pravastatin formation by 67% and 200 $\mu$M dehydroepiandros-

**TABLE 1**

<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>$6'\beta$-Hydroxylovastatin</th>
<th>$6'\beta$-Exomethylene Lovastatin</th>
<th>$3'\beta$-Hydroxylovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_m$ (µM)</td>
<td>$V_{\text{max}}$ (pmol/min·mg)</td>
<td></td>
</tr>
<tr>
<td>Human liver</td>
<td>$9.3 \pm 3.2$</td>
<td>$8.1 \pm 3.5$</td>
<td>N.D.</td>
</tr>
<tr>
<td>Human small intestine</td>
<td>$11.2 \pm 3.3$</td>
<td>$22.2 \pm 9.0$</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pig liver</td>
<td>$31.7 \pm 14.5$</td>
<td>$82.9 \pm 32.4$</td>
<td>N.D.</td>
</tr>
<tr>
<td>Pig small intestine</td>
<td>$34.2 \pm 9.7$</td>
<td>$80.7 \pm 42.3$</td>
<td>$53.9 \pm 20.3$</td>
</tr>
</tbody>
</table>

**Fig. 2.** Representative Hanes-Woolf plots of lovastatin metabolite formation by human liver (A) and small intestinal microsomes (B). The results of all experiments are summarized in Table 1.
terone by 93%. The unbound fraction of pravastatin in the liver cytosol preparations was 103.0 ± 6.2% of protein-free controls (mean ± S.D., n = 4), and it was 87.4 ± 2.8% in small intestinal mucosa cell cytosol preparations.

**Comparison of CYP-Dependent Metabolism of Lovastatin and Pravastatin.** To compare lovastatin and pravastatin metabolism, intrinsic metabolic clearances were calculated after correction of the \( K_m \) values for the unbound protein fraction. The unbound protein fractions for lovastatin were 59.7 ± 5.0% (n = 3) in liver microsomal preparations and 13.4 ± 0.8% in small intestinal microsomal preparations. The unbound fractions for pravastatin were 98.7 ± 5.0 and 80.1 ± 4.4%, respectively. Protein binding was linear over the protein and study drug concentration ranges used in this study.

The mean intrinsic metabolic clearance of 6'-exo-methylene lovastatin by liver microsomes was 4.2-fold and that of 6'-exo-methylene lovastatin was 8.3-fold higher than by small intestinal microsomes (Table 3). In comparison, the differences between intrinsic metabolic clearances of 3'-\( \alpha \),5'-\( \beta \),6'-\( \beta \)-trihydroxypravastatin (1.6-fold) and hydroxypravastatin (1.3-fold) in liver and small intestinal microsomes were smaller. The intrinsic clearances of lovastatin metabolites by liver microsomes was 10,000-fold and those of lovastatin metabolites by small intestinal microsomes were 5000-fold higher than metabolic intrinsic clearances of pravastatin metabolites (Table 3).

**Inhibition of Small Intestinal Metabolism of Lovastatin by Specific CYP3A Inhibitors Cyclosporin, Ketoconazole, and Troleandomycin.** Cyclosporin, ketoconazole, and troleandomycin were effective inhibitors of 6'-exomethylene lovastatin and 6'-\( \beta \)-hydroxylovastatin for...
mation in human liver and pig small intestinal microsomes (Table 4). Only ketoconazole also inhibited 3’-hydroxylovastatin formation in pig small intestinal microsomes. Ketoconazole was the most potent inhibitor of lovastatin metabolism with apparent \( K_m \) values in the liver and small intestine 4- to 30-fold lower than cyclosporine. Both cyclosporine and troleandomycin were better inhibitors of lovastatin metabolism in pig small intestinal than in human liver microsomes. In human liver microsomes, troleandomycin was a 3-fold weaker inhibitor than cyclosporine. In the pig small intestinal microsomes, however, troleandomycin \( K_m \) values were up to 50-fold lower than those in human liver microsomes.

**Discussion**

We demonstrated that lovastatin is metabolized in the small intestine to its major metabolites and that the generated metabolism pattern is similar to that in the liver. As in the liver, lovastatin metabolism was inhibited by CYP3A inhibitors and antibodies, indicating that in the small intestine, CYP3A enzymes are involved in lovastatin metabolism and that the small intestine is a potential site of lovastatin drug interactions.

It has to be taken into account that only apparent enzyme kinetic parameters were determined for the following reasons: microsomes do not represent an isolated enzyme, but a mixture of different enzymes and more than one enzyme of the CYP3A subfamily may be involved in the metabolism of lovastatin or pravastatin. In addition, the inhibitors used are also CYP3A substrates and are metabolized during incubation. Nonspecific binding to microsomal proteins has been recognized as an important factor when comparing the metabolism of different substrates. Evidence has been reported that the results of in vitro upscaling based on the intrinsic metabolic clearance correlated better with the in vivo situation when \( K_m \) values were corrected for the unbound fraction (Obach, 1997). Accordingly, we compared intrinsic clearances of lovastatin and pravastatin after correction of the \( K_m \) values for the unbound fractions.

The highest CYP3A concentration in the gastrointestinal tract is present in the duodenum and decreases in the following sequence: duodenum > jejunum > ileum > colon (Kolars et al., 1994; Paine et al., 1997). To avoid interference with variability resulting from the different locations of small intestinal samples, it was essential to collect samples from equivalent anatomical positions of the duodenum. Furthermore, patients with a history of ingesting drugs known to influence the activity of CYP3A enzymes were excluded. Duodenal samples were chosen because they have the highest CYP3A concentration and the proximal small intestine is the primary location at which lovastatin is predominantly absorbed.

For lovastatin drug interaction studies in the small intestine, pig small intestinal microsomes were used because the quantities of human small intestinal samples available were insufficient. Because some differences in midazolam metabolism in pigs and humans have been reported (Ochs et al., 1987; Gorski et al., 1994), the metabolite patterns and enzymatic parameters of human and pig liver and small intestinal microsomes were cross-validated in our study. The results proved that pig small intestinal microsomes were a valid model for the study of lovastatin drug metabolism and drug interactions. This is supported by other studies evaluating drug metabolism and interactions of CYP3A substrates in the small intestine. Thus, Lampen et al. (1995, 1996) found similar \( K_m \) and \( V_{max} \) values for the formation of tacrolimus and cyclosporine metabolites in pig and human liver and small intestinal microsomes and almost identical \( K_m \) values for drug interactions in human liver and pig small intestinal microsomes. Lampen et al. (1995) showed that pig CYP3A enzymes in the liver and small intestine cross-react with a human CYP3A antibody, indicating structural homologies of the respective human and pig enzymes. Although the inhibition constants probably cannot be directly extrapolated from pigs to humans, effective and potent inhibitors of lovastatin metabolism in the pig small intestine can be expected to also be effective and potent inhibitors of lovastatin metabolism in the human gut mucosa.

Although the intrinsic metabolic clearance of lovastatin metabolites in human intestinal microsomes was 4.2- to 8.3-fold lower than that in liver microsomes, it is impossible to estimate the contribution of intestinal metabolism to lovastatin first-pass metabolism and drug interactions. The lower intrinsic clearance by intestinal than by liver microsomes in our study was mainly due to the lower concentration of microsomal protein in the intestinal microsomal preparations and the resulting lower \( V_{max} \) value. Despite a well-established lower metabolic capacity of the small intestine in comparison with the liver, recent results demonstrated that the small intestine may play a significant role in first-pass metabolism (Benet et al., 1996). The most important unresolved

---

**TABLE 3**

Comparison of intrinsic metabolic clearances (\( K_m/V_{max} \)) of lovastatin and pravastatin metabolites in human liver and small intestinal microsomes

<table>
<thead>
<tr>
<th></th>
<th>Lovastatin</th>
<th>Pravastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6β-Hydroxy-</td>
<td>6’-Exomethylene</td>
</tr>
<tr>
<td>( K_m/V_{max} )</td>
<td>( \mu M \cdot min^{-1} \cdot mg^{-1} )</td>
<td>( \mu M \cdot min^{-1} \cdot mg^{-1} )</td>
</tr>
<tr>
<td>Liver Mean</td>
<td>421.5</td>
<td>232.0</td>
</tr>
<tr>
<td></td>
<td>(130.2–924.9)</td>
<td>(78.3–482.3)</td>
</tr>
<tr>
<td>Small intestine Mean</td>
<td>99.9</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>(79.5–125.6)</td>
<td>(18.9–35.4)</td>
</tr>
</tbody>
</table>

All values are mean ± S.D. (n = 4). \( K_m \) values were corrected for the unbound fraction before calculation of intrinsic metabolic clearances. Ranges are shown in parentheses.

---

**TABLE 4**

Apparent inhibition constants (\( K_i \)) for the inhibition of lovastatin metabolism by cyclosporine, ketoconazole, and troleandomycin in human liver and pig small intestinal microsomes

<table>
<thead>
<tr>
<th>Microsomal Preparation</th>
<th>6β-Hydroxylovastatin</th>
<th>6’-Exomethylene Lovastatin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( \mu M )</td>
<td></td>
</tr>
<tr>
<td>Cyclosporine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver</td>
<td>7.6 ± 2.3</td>
<td>10.3 ± 1.4</td>
</tr>
<tr>
<td>Pig small intestine</td>
<td>3.3 ± 1.2</td>
<td>1.6 ± 1.3</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.3</td>
</tr>
<tr>
<td>Pig small intestine</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>Troleandomycin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver</td>
<td>26.6 ± 18.5</td>
<td>31.0 ± 14.9</td>
</tr>
<tr>
<td>Pig small intestine</td>
<td>0.8 ± 0.9</td>
<td>0.6 ± 0.5</td>
</tr>
</tbody>
</table>

The apparent \( K_i \) values were calculated using Lineweaver-Burk analysis. All values are given as mean ± S.D. (n = 4 preparations from different individuals).
issue is the role of intestinal countertransport and its functional interaction with intestinal CYP enzymes. Lovastatin interacts with P-glycoprotein (Dimitroulakos and Yeger, 1996), an ATP-binding cassette transporter, that is involved in intestinal countertransport (Benet et al., 1996; Hunter and Hirst, 1997). It can be expected that countertransport limits the access of drugs to the CYP enzymes and may prevent CYP enzymes from being overwhelmed by the high drug concentrations present in the small intestine. On the other hand, with a drug being repeatedly transported out of the mucosa cells and being reabsorbed, repeated exposure to CYP3A enzymes may lead to more efficient metabolism (Benet et al., 1996; Gan et al., 1996). In addition, the intestinal transporter/CYP barrier may control substrate availability to liver CYP enzymes. Another factor that complicates the estimate of the relative role of intestinal in comparison with liver first-pass metabolism is the high interindividual variability of intestinal CYP3A enzymes and transporters as well as liver CYP3A enzymes (Kolars et al., 1994; Lown et al., 1997). Because in vivo the functional interaction between intestinal transporters and CYP3A enzymes seems important (Benet et al., 1996) and intestinal transporters control substrate availability to the CYP3A enzymes, it is unclear whether the intrinsic metabolite clearance, which was calculated based on isolated intestinal microsomal preparations, alone is a valid parameter to decide whether intestinal metabolism of lovastatin is clinically relevant and significantly involved in the low oral bioavailability of lovastatin and lovastatin drug interactions.

Pravastatin is the only HMG-CoA reductase inhibitor that is mainly eliminated unchanged (Everett et al., 1991; Quion and Jones, 1994). Its main metabolite, 3'α,5β,6β-trihydroxypravastatin, is inactive and has a terminal plasma half-life slightly shorter than that of pravastatin (Quion and Jones, 1994). Our study showed that small intestinal microsomes generated the same metabolite pattern as liver microsomes. As shown before (Jacobsen et al., 1999), CYP3A4 and CYP3A5 are involved in the formation of these metabolites in the liver and, as shown in our study using CYP3A antibodies and specific chemical inhibitors, also in the small intestine. However, as in the case of formation of 3',5α,5β,6β-trihydroxypravastatin, inhibition at the highest CYP3A antibody doses was incomplete, and the involvement of other microsomal enzymes in the formation of this metabolite may be possible. A similar result was described for 3',5α,5β,6β-trihydroxypravastatin in the liver (Jacobsen et al., 1999). When compared with lovastatin, however, the small intestinal intrinsic clearance (V_{max}/K_{m}) of pravastatin metabolites was approximately 5000-fold lower (Table 3). The difference in the intrinsic clearances indicated that in contrast to lovastatin and although both drugs are structurally related, CYP-dependent small intestinal metabolism of pravastatin cannot be expected to play a significant role in pravastatin pharmacokinetics. The results of our study also suggest that mechanisms other than CYP-dependent metabolism must be responsible for the low oral bioavailability of pravastatin of an average 15% (Quion and Jones, 1994). The major pravastatin metabolite 3',5α,5β,6β-trihydroxypravastatin is generated by both acidic degradation in the stomach (Triscari et al., 1995) and, in the liver, by sulfation at the 6β-hydroxy group by sulfotransferases, followed by a nucleophilic attack of hydroxy anions at the 3'α position (Kitazawa et al., 1993). Our study showed that the gut wall is an additional site of 3',5α,5β,6β-trihydroxypravastatin formation.

It is concluded from our study that lovastatin is metabolized in the small intestine by CYP3A enzymes, resulting in a similar metabolite pattern as in the liver. Thus, as described for other CYP3A substrates (Benet et al., 1996), gut wall metabolism is a potential factor in the low and variable oral bioavailability of lovastatin and its pharmacokinetic interactions with other drugs that are CYP3A substrates and/or inhibitors. In comparison, the gut intrinsic clearance of pravastatin is 5000-fold lower, and only minor pravastatin metabolites are generated. Based on our results, it is unlikely that intestinal CYP3A-mediated metabolism plays a clinically relevant role in pravastatin pharmacokinetics. However, as in the liver, pravastatin undergoes phase II metabolism in the small intestine, which results in formation of its major metabolite 3',5α,5β,6β-trihydroxypravastatin.

References

Jacobsen et al. 138

References


Lampen A, Christians U, Bader A, Hackforth I and Sewing KF (1996) Drug inter-