LACTONIZATION IS THE CRITICAL FIRST STEP IN THE DISPOSITION OF THE 3-HYDROXY-3-METHYLGLUTARYL-COA REDUCTASE INHIBITOR ATORVASTATIN

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ABSTRACT:
In an in vitro study, we compared the cytochrome P450 (CYP)-dependent metabolism and drug interactions of the acid and lactone forms of the 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase inhibitor atorvastatin. Metabolism of atorvastatin acid and lactone by human liver microsomes resulted in para-hydroxy and ortho-hydroxy metabolites. Both substrates were metabolized mainly by CYP3A4 and CYP3A5. Atorvastatin lactone had a significantly higher affinity to CYP3A4 than the acid (Km; para-hydroxy atorvastatin, 25.6 ± 5.0 µM; para-hydroxy atorvastatin lactone, 1.4 ± 0.2 µM; ortho-hydroxy atorvastatin, 29.7 ± 9.4 µM; and ortho-hydroxy atorvastatin lactone, 3.9 ± 0.2 µM). Compared with atorvastatin acid, CYP-dependent metabolism of atorvastatin lactone to its para-hydroxy metabolite was 83-fold higher [formation CLint (Vmax/Km): lactone 2949 ± 3511 versus acid 35.6 ± 48.1 µl · min⁻¹ · mg⁻¹] and to its ortho-hydroxy metabolite was 20-fold higher [CLint lactone 923 ± 965 versus acid 45.8 ± 59.1 µl · min⁻¹ · mg⁻¹]. Atorvastatin lactone inhibited the metabolism of atorvastatin acid by human liver microsomes with an inhibition constant (K) of 0.9 µM while the K for inhibition of atorvastatin lactone was 90 µM. Binding free energy calculations of atorvastatin acid and atorvastatin lactone complexed with CYP3A4 revealed that the smaller desolvation energy of the neutral lactone compared with the anionic acid is the dominant contribution to the higher binding affinity of the lactone rather than an entropy advantage. Because atorvastatin lactone has a significantly higher metabolic clearance and the lactone is a strong inhibitor of atorvastatin acid metabolism, it can be expected that metabolism of the lactone is the relevant pathway for atorvastatin elimination and drug interactions. We hypothesize that most of the open acid metabolites present in human plasma are generated by interconversion of lactone metabolites.

Atorvastatin is a synthetic inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) 1 reductase (EC 1.1.1.88), which catalyzes the conversion of HMG-CoA to mevalonate, the rate-limiting step in de novo cholesterol synthesis (Gibson et al., 1996). Atorvastatin is administered as the calcium salt of the active hydroxy acid (Fig. 1). However, atorvastatin acid is converted to its lactone (Kearney et al., 1993, Fig. 1) and in clinical studies, the areas under the concentration time curves (AUCs) of atorvastatin lactone and acid were similar (Kantola et al., 1998).

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1 Abbreviations used are: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; AUC, area under the curve; CYP, cytochrome P450; HOMO, highest occupied molecular orbital; LC, liquid chromatography; MD, molecular dynamics; MM/PBSA, molecular mechanics/Poisson-Boltzmann surface area; MS, mass spectrometry; QM/MM, quantum mechanics/molecular mechanics.

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After incubation of atorvastatin acid with rat, dog, and human microsomes, para-hydroxy- and ortho-hydroxy atorvastatin (Fig. 1) were formed (Michniewicz et al., 1994; Christians et al., 1998). As shown by using specific cytochrome P450 (CYP) inhibitors and isolated CYP enzymes, CYP3A4 is the major enzyme involved in formation of the two metabolites. Atorvastatin has a relatively low affinity to CYP3A enzymes with Michaelis-Menten constants (Km) of 70 to 80 µM. Para- and ortho-hydroxy atorvastatin were detected as both lactone and acid in human plasma with the concentrations of the lactone metabolites exceeding those of the corresponding acid metabolites (Kantola et al., 1998). The metabolic pathway as proposed and generally accepted in the literature is shown in Fig. 1.

As of today, only the metabolism of atorvastatin acid, not of the lactone, has been studied. The hydrophobic substrate access channels of CYP enzymes are positioned near the membrane surface and hydrophobic compounds dissolved in the membrane can directly enter the access channels from the membrane (Peterson and Graham-Lorence, 1995). We hypothesize that the more lipophilic lactone is a better CYP enzyme substrate than atorvastatin acid. Our hypothesis is supported by in vitro drug metabolism data of the HMG-CoA reductase inhibitor lovastatin. Greenspan et al. (1988) found that the lactone lovastatin, rather than its open acid form, is subject to metabolism.

Therefore, in an in vitro study, we compared the metabolism of
atorvastatin acid and its lactone in regard to the metabolites formed, CYP enzymes involved, enzyme kinetics, and drug interactions, with the goal of identifying the relevant metabolic pathways. To better understand the differences in metabolism between atorvastatin and its lactone, in particular their regiospecificities in product formation, we performed molecular dynamics (MD) simulations of the two molecules complexed to CYP3A4. We calculated differences in binding free energies to CYP3A4 using the computational molecular mechanics/Poisson-Boltzmann surface area (MM/PBSA) approach (Srinivasan et al., 1998), which allowed for comparison with the Michaelis-Menten constants ($K_m$) measured in vitro. In addition, we used the MD trajectories in combination with a quantum mechanical frontier orbital approach to assess steric and electronic factors that influence the observed regiospecificity for aromatic hydroxylation.

**Materials and Methods**

**Chemicals and Enzymes.** Atorvastatin, para-hydroxy atorvastatin (PD142542, BMS-241423-01) and ortho-hydroxy atorvastatin (PD152873, BMS243887-01), atorvastatin lactone, and the metabolite lactones (BMS241424-01 and BMS-2438883-01) were kind gifts from Parke-Davis (Ann Arbor, MI) and Bristol-Myers Squibb (Princeton, NJ). Fluconazole (Pfizer, Groton, CT), itraconazole (Janssen Pharmaceutica Inc., Titusville, NJ), saquinavir (Merck Sharp & Dohme, Rahway, NJ) were also kind gifts from the manufacturers. Mevastatin, NADP, isocitric acid, and isocitric dehydrogenase for the NADPH-generating system were purchased from Sigma Chemical Co. (St. Louis, MO). Human liver microsomes and recombinant CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP3A5 enzymes as well as antibodies against human CYP3A were from Gentest (Woburn, MA).

Mevastatin acid was generated from mevastatin (5 mg/ml) by incubation in phosphate buffer (pH 8.5)/acetonitrile (v/v, 1/1) at 40°C for 4 days. Conversion was controlled by HPLC/UV (detection wavelength 239 nm) and LC/MS, and the reaction was stopped when the purity of mevastatin acid was greater than 95%.

Stock solutions of atorvastatin acid and the open ring metabolites were prepared in methanol. Atorvastatin lactone and the metabolite lactones had to be dissolved in acetonitrile because, as confirmed by MS/MS, the lactones formed methyl esters when dissolved in methanol.

**Isolation of Microsomes.** The collection of human liver tissue samples for in vitro drug metabolism studies was approved by the Committee on Human Research, University of California, San Francisco. Microsomes were isolated by differential centrifugation as described by Guengerich (1982) with the following modifications: instead of Tris buffer, 0.1 M Na/K phosphate buffer (pH 7.4) was used. After ultracentrifugation, the supernatant was discarded, and the residue was reconstituted in 4 times its volume of a buffer solution containing 0.1 mM Na/K phosphate buffer/glycerol (4/1, v/v). Microsomal preparations were stored at −80°C. Protein concentrations were determined using the bicinchoninic acid method described by Smith et al. (1985). Bovine serum albumin was used as the standard. CYP concentrations were determined following the protocol of Estabrook and Werringloer (1978).

**Metabolism by Human Liver Microsomes.** Microsomal protein (0.05–0.2 mg), buffer, and either atorvastatin acid (in methanol, final concentration 12–120 μM) or atorvastatin lactone (in acetonitrile, final concentration 0.75–15 μM) were preincubated for 4 min. The reaction was started by adding 0.4 ml of an NADPH-generating system, containing 5 mM EDTA, 25 mM MgCl$_2$, 2.5 mM NADP, 45 mM isocitric acid, and 1.75 U of isocitrate dehydrogenase in 0.1 M Na/K phosphate buffer (pH 7.4). The mixtures were incubated for 2 to 10 min, and the reaction was stopped by protein precipitation after addition of 0.4 ml of ice-cold acetonitrile containing 400 ng of the internal standard mevastatin (for quantification of atorvastatin lactone) or 800 ng of mevastatin acid (for the quantification of atorvastatin acid).

Stability of atorvastatin acid and lactone during incubation was tested with active and heat-inactivated microsomes in the presence and absence of NADPH. A maximum of 3% atorvastatin lactone (in methanol, final concentration 12–120 μM) or atorvastatin lactone (in acetonitrile, final concentration 0.75–15 μM) were preincubated for 4 min. The reaction was started by adding 0.4 ml of an NADPH-generating system, containing 5 mM EDTA, 25 mM MgCl$_2$, 2.5 mM NADP, 45 mM isocitric acid, and 1.75 U of isocitrate dehydrogenase in 0.1 M Na/K phosphate buffer (pH 7.4). The mixtures were incubated for 2 to 10 min, and the reaction was stopped by protein precipitation after addition of 0.4 ml of ice-cold acetonitrile containing 400 ng of the internal standard mevastatin (for quantification of atorvastatin lactone) or 800 ng of mevastatin acid (for the quantification of atorvastatin acid).

Stability of atorvastatin acid and lactone during incubation was tested with active and heat-inactivated microsomes in the presence and absence of NADPH. A maximum of 3% atorvastatin lactone was nonenzymatically converted to the acid after 5 min of incubation at 37°C, while there was no detectable conversion of atorvastatin lactone to the acid. The amount of acid...
formed during incubation with atorvastatin lactone was negligible because, as shown below, atorvastatin acid at low concentrations does not significantly affect metabolism of the lactone.

Metabolism by cDNA-Expressed, Isolated CYP Enzymes. Microsomes from baculovirus-infected insect cells expressing the following human CYP enzymes were used: CYP1A2 (lot 6), CYP2B6 (lot 46), CYP2C8 (lot 6, 7), CYP2C9 (Arg144Ser) (lot 9), CYP2C19 (lot 3), CYP2D6 (lot 13), CYP2E1 (lot 5), CYP3A4 (lot 19, 20), and CYP3A5 (lot 8) (all Gentest). The metabolism assays were carried out as described for the human liver microsomes, and either 120 μM atorvastatin or 50 μM atorvastatin lactone was added to the assays. The incubation period was 60 min.

Extraction and Quantification of Atorvastatin Lactone, Atorvastatin Acid, and Their Metabolites. After protein precipitation by addition of 0.4 ml of ice-cold acetonitrile containing the corresponding internal standard to the reaction mixtures (vide supra), samples were mixed on a rotor for 10 s and centrifuged at 4°C and 10,900 g for 10 min. Supernatants were transferred into 1.8-ml HPLC brown vials (Hewlett-Packard, Palo Alto, CA) and were kept in the temperature-controlled autosampler at +4°C until analysis.

Atorvastatin, atorvastatin lactone, and their metabolites were quantified by HPLC/electrospray-MS in combination with an on-line column switching extraction step using a Hewlett-Packard series HP1100 liquid chromatograph consisting of a G1311A autosampler in combination with a G1330A thermostat, a G1312A binary pump, a G1322A degasser, a G1316A column thermocontroller, a G1311A HPLC pump (Hewlett-Packard, Palo Alto, CA). Solvent for the on-line extraction column was delivered by an additional quaternary G1311A HPLC pump. The HPLC/MS system was controlled, and data were processed using ChemStation software revision C06.02. (Hewlett-Packard). The column switching LC/LC-MS system was set up as described in detail by Christians et al. (2000). In brief, 15 μl of the extracted sample was injected into the LC/LC-MS system. Samples were loaded onto the 20 × 4 mm extraction column (Hewlett-Packard) filled with Hypersil MOS of 5 μm particle size (Shandon, Chadwick, UK) and washed using a mobile phase consisting of 2 mM ammonium acetate. The flow was 6 ml/min. After 1 min, the switching valve (model 7240, Rheodyne, Cotati, CA) was activated, and the analytes were eluted in the backflush mode from the extraction column onto a 100 × 2.1 mm analytical column filled with Hypersil ODS of 5 μm particle size (Hewlett-Packard). After 4 min, the switching valve was activated again, and the extraction column was cleaned with acetonitrile/2 mM ammonium acetate (9:1, v/v) (2 ml/min, 2 min) and re-equilibrated to the starting conditions.

The mobile phase of the analytical column consisted of 0.01% formic acid and acetonitrile. The following gradient was run: 0 min, 50% acetonitrile; 1 min, 50% acetonitrile; 1 min, 50% acetonitrile; 600 V; ion energy (octapole), 207 kPa); drying gas nitrogen, 5.0; flow, 7 l/min; 300°C; capillary nebulizer gas: nitrogen of grade 5.0 purity (Bay Airgas, Hayward, CA), temperature was 40°C. The mass selective detector was adjusted to the following conditions.

Atorvastatin, atorvastatin lactone, and their metabolites as well for the internal standards gave the best signal-to-noise ratio and were recorded in the single ion mode: m/z 559.2 for atorvastatin (retention time, 4.6 min); m/z 557.2 for both para- and ortho-hydroxy atorvastatin (retention time, 3.0/4.4 min); m/z 541.2 for atorvastatin lactone (retention time, 6.3 min); and m/z = 557.2 for both para- and ortho-hydroxy atorvastatin lactone (retention time, 4.0/5.8 min) (Fig. 2). The dwell time for each ion was 116 ms. The assay was validated according to the guidelines of good laboratory practice and had the following specifications: lower limit of quantitation, 0.5 μg/ml (atorvastatin and atorvastatin lactone) or 1 μg/l (their metabolites); linearity, 0.5 (1.0) to 500 μg/ml (r² > 0.998); interassay variability (3 days, three concentrations, each n = 9), ±9% for para-hydroxy atorvastatin, ±5.4% for ortho-hydroxy atorvastatin, ±11.1% for atorvastatin, ±10.9% for para-hydroxy atorvastatin lactone, ≤10% for ortho-hydroxy atorvastatin lactone, and ≤9.8% for atorvastatin lactone. The recovery of atorvastatin, atorvastatin-lactone, and their metabolites was >90%. Within-batch stability of extracted samples was established for at least 24 h at +4°C, and all samples were analyzed within this time period.

Determination of the Apparent Michaelis-Menten Constant (Km) and Vmax. To determine apparent Km and apparent Vmax of metabolite formation, liver microsomes as well as CYP enzyme preparations were incubated with the following concentrations (n = 4 for each concentration): 12, 24, 36, 48, 60, 72, 96, and 120 μM atorvastatin acid or 0.75, 1.5, 2, 3, 5, 7.5, and 15 μM atorvastatin lactone. Heat-denatured microsomes, control microsomes from insect cells (Gentest), and incubation mixtures without NADPH were used as negative controls. Km and Vmax were determined after data fitting using Hanes-Woolf plots. Data was fitted using SigmaPlot software (version 5.0; SPSS Inc., San Rafael, CA).

Identification of CYP Enzymes Involved in Atorvastatin Acid and Lactone Metabolism. The CYP enzymes involved were identified by incubation of atorvastatin acid and lactone using isolated, recombinant CYP enzymes. In addition, metabolism was inhibited by specific antibodies and chemical inhibitors.

To study the inhibition of atorvastatin acid and lactone metabolism by specific CYP antibodies, 100 μg of microsomal protein isolated from human liver (pool of 4) in 20 μl of 0.1 M Tris buffer (pH 7.4) was preincubated on ice with 0, 1, 2, 5, or 10 μl of CYP3A4/5 antibody solution (1 μl = 10 μg protein) (Gentest) for 25 min. Then, buffer (final volume 1.0 ml), either atorvastatin (final concentration: 120 μM) or atorvastatin lactone (final concentration: 50 μM), and the NADPH-generating system were added. Samples were incubated for 30 min and extracted as described above.

The effects of the CYP3A inhibitors/substrates itraconazole, fluconazole, and saquinavir (Guenicher, 1995; Fitzsimmons and Collins, 1997) on atorvastatin and atorvastatin lactone metabolism were assessed. Saquinavir was dissolved in methanol, fluconazole in acetonitrile, and itraconazole in acetonitrile/dimethyl sulfoxide (2:1, v/v). Inhibitor solution (10 μl) resulting in final concentrations of 0.1, 1, 5, and 20 μM (n = 4) for itraconazole, 1, 10, 100, and 200 μM (n = 4) for fluconazole, and 0.1, 1, 10, and 50 μM (n = 4) for saquinavir, was added to the liver microsomal preparations containing either 30 μM atorvastatin or 2 μM atorvastatin lactone. In addition, the impact of atorvastatin lactone on the metabolism of atorvastatin and of atorvastatin on the metabolism of atorvastatin lactone was studied: 0.1, 1, 5, and 20 μM atorvastatin lactone for the inhibition of atorvastatin metabolism and 0, 1, 10, 100, and 200 μM atorvastatin for the inhibition of the metabolism of atorvastatin lactone were added to the metabolism assays. Microsomes, to which 10 μl of the vehicle was added, were used as controls. The mixtures were incubated and analyzed as described above.

The effect of the inhibitors and antibodies was statistically evaluated by analysis of variance (GLM procedure, SAS, version 6.12; SAS Institute, Cary, NC). Half-maximum inhibitor concentrations (IC50) were determined using the Microcal Origin software (version 3.5; Microcal Software Inc., Northampton, MA). Apparent inhibition constants (Ki) were estimated using the algorithm proposed by Cheng and Prusoff (1973).

Computational Approach. To theoretically predict enzymatic product distributions, one has to simulate the rate-limiting reaction step of the overall reaction, ideally using combined quantum mechanics/molecular mechanics (QM/MM) methods. However, because the structures of the CYP3A4/atorvastatin system are not known accurately enough to warrant the application of an elaborate QM/MM model we used a more qualitative approach in this study, which takes into account both electronic effects at the reaction site and interactions between CYP3A4 and the substrates. Different reaction mechanisms have been proposed for aromatic hydroxylation in CYP enzymes (Ortiz de Montellano, 1995; Darbyshire et al., 1996; Sarabia et al., 1997), but most recent experimental (Darbyshire et al., 1996) and theoretical results (Cnubben et al., 1992; Rietjens et al., 1993; Zakharieva et al., 1996, 1998) suggested that hydroxylation at an aromatic ring comprises an addition-rearrangement pathway, in which, in the first step, the electrophilic cytochrome P450-[Fe(IV)=O] cation radical attacks one of the aromatic carbon atoms to form a σ-adduct. Molecular orbital calculations (Zakharieva et al., 1996, 1998) showed that during the initial encounter, electrons flow from the substrate toward the porphyrin, which presumably explains why in another theoretical approach, the
electron densities of the highest occupied molecular orbital (HOMO) at different substitution sites could be used to predict the regioselectivity of hydroxylation in small substituted benzenes (Cnubben et al., 1992; Rietjens et al., 1993). Although regioselectivities of small substrates may be explained solely based on electronic factors, for larger compounds, such as atorvastatin, interactions between CYP3A4 and the ligand have to be taken into account. Because an essential condition for the reaction to occur is that the substrates orient in a reactive configuration we used the distances between the heme...
oxygen atom and possible aromatic carbon reaction centers of the substrate as criteria for correct orientation. Specific interactions between the enzyme and the substrates potentially influence the average distance to an aromatic reaction site, and we monitored those distances during our molecular dynamics simulation. Electronic criteria were assessed using the HOMO frontier orbital approach. We calculated the orbital population of the HOMO at each aromatic carbon atom to identify centers of increased electron density and hence reactivity for the attack of the cytochrome P450 [Fe(IV)/O] electrophile. Similar qualitative approaches considering both geometric and electronic criteria have been used successfully to rationalize the experimental product distribution for nonaromatic hydroxylation of CYP substrates (Collins et al., 1991; Paulsen and Ornstein, 1992; Paulsen et al., 1993; Fruetel et al., 1994; Harris and Loew, 1995).

Quantum Mechanical Calculation. Molecular orbital calculations to estimate the electronic effects on regiospecificity were performed for the mono-substituted benzene derivative acetalimide (R = NHCOCH₃), which was used as a model for atorvastatin. We did HOMO population analyses of the geometry-optimized acetalimide at the Hartree-Fock and density functional theory/B3LYP (Stephens et al., 1994) level, both using a cc-pVDZ basis set (Kendall et al., 1992).

Molecular Dynamics Simulation. The starting point for our MD simulations was a homology model of CYP3A4 by Szklarz and Halpert (1997), which is based on the crystallographic coordinates of four bacterial CYP enzymes. We first docked the two ligands, atorvastatin acid, the three-dimensional structure of which we obtained from the MDL Drug Data Report Database (San Leandro, CA), and its lactone, into the active site of the model using DOCK 4.0 (Ewing and Kuntz, 1997). For the subsequent molecular dynamics simulation, we used the standard para/94 force field of AMBER 5 (Cornell et al., 1995) together with new parameters for the ligands, which were derived as outlined previously (Fox and Kollman, 1998). Atomic partial charges for the ligands were obtained by semiempirical AM1 geometry optimization and subsequent single-point Hartree-Fock/6-31G* calculation of the electrostatic potential, to which the charges were fitted using the RESP procedure (Bayly et al., 1993). Force field parameters (Giannona, 1984; Collins et al., 1991) and partial charges (Harris and Loew, 1995) for the heme unit of CYP3A4, which consists of an iron-oxo porphyrin complex with an axial cysteinyl ligand from the protein, were taken from the literature. The two ligand-protein complexes were each solvated with a 25-Å sphere of TIP3P water molecules (Jorgensen et al., 1996), centered at the ferryl oxygen atom, and then equilibrated for a total of 200 ps at T = 300 K. After equilibration, we performed additional 200 ps of molecular dynamics during which we saved coordinates every 4 ps, yielding a representative set of structures (50 snapshots) for both complexes. The two complexes were analyzed for specific enzyme-substrate interactions to rationalize the observed regioselectivity and also postprocessed by the MM/PBSA approach to assess complex binding free energies. Throughout the simulations, we used a cutoff for nonbonded interactions of 14 Å and allowed van der Waals and internal energies (E_{vdw}) calculations, van der Waals (E_{vdw}), and internal energies (E_{int}).

\[
G = G_{\text{elec}} + G_{\text{np}} + G_{\text{MM}} - TS_{\text{nature}}. \quad (1)
\]

where \(G_{\text{elec}}\) is the electrostatic solvation free energy, which is computed by a continuum approach, usually using a finite-difference Poisson-Boltzmann (PB) model, and \(G_{\text{np}}\) is the nonpolar solvation free energy, which can be derived from the solvent accessible surface area (SA). \(E_{\text{MM}}\) denotes the sum of molecular mechanical (MM) energies of the molecule and can be further divided into contributions from electrostatic (\(E_{\text{elec}}\)), van der Waals (\(E_{\text{vdw}}\)), and internal energies (\(E_{\text{int}}\)).

\[
E_{\text{MM}} = E_{\text{elec}} + E_{\text{vdw}} + E_{\text{int}}. \quad (2)
\]

The last term in eq. 1, \(TS_{\text{nature}}\), is the solute entropy and is usually estimated by a combination of classical statistical formulae and normal mode analysis. Using eqs. 1 and 2, we calculated the binding free energy as

\[
\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{molecule}} + G_{\text{ligand}}), \quad (3)
\]

where the set of structures for the uncomplexed reactants was obtained from the coordinates of the complex by removing the protein or ligand atoms, respectively. In this approximation, \(E_{\text{int}}\) of eq. 2 cancels out. The detailed parameters for the MM/PBSA free energy evaluation were the same as those described previously (Kuhn and Kollman, 2000).

Results

In Vitro Metabolism of Atorvastatin and Atorvastatin Lactone by Human Liver Microsomes. When incubated with four different human liver microsomal preparations, two metabolites were detected by HPLC/MS in the scan and single-ion mode for both atorvastatin acid and lactone. Representative ion chromatograms are shown in Fig. 2. These peaks were detected neither in control incubations containing heat-inactivated microsomes nor after incubations with microsomes without NADPH. The metabolites with retention times of 3.0 and 4.4 min in atorvastatin acid reaction mixtures were identified as para-hydroxy and ortho-hydroxy atorvastatin acid, respectively, and the metabolites eluting at 4.0 and 5.8 min in atorvastatin lactone reaction mixture were identified as para-hydroxy and ortho-hydroxy atorvastatin lactone, respectively. The assignment of the metabolite structures was based on LC/MS analysis and on retention times compared with synthetic reference standards. Formation of atorvastatin metabolites by human liver microsomes/human cDNA-expressed CYP3A4 and CYP2C8 was linear over an incubation period of 10 min (CYP3A4: 6 min). Generation of atorvastatin lactone metabolites was linear only over a time period of 4 min for human liver microsomes and 2 min for cDNA-expressed, isolated CYP3A4 enzymes. Linearity was tested at conditions most likely to result in nonlinearity, including the lowest substrate concentration used in our study (0.2 μmol/l). The reason for nonlinearity at this concentration was metabolism of the substrate.

Enzyme Kinetics of Atorvastatin and Atorvastatin Lactone Metabolite Formation by Human Liver Microsomes and Human cDNA-Expressed CYP Enzymes. The atorvastatin metabolite profiles after incubation with human liver microsomes and cDNA-expressed human CYP3A4 or CYP3A5 were similar. In addition to CYP3A, to a minor extent CYP2C8 metabolized atorvastatin acid to its para-hydroxy metabolite (Table 1), whereas the formation of ortho-hydroxy atorvastatin could not be detected. All other CYP enzymes tested (CYP1A1, CYP2B6, CYP2C9-Arg, CYP2C19, CYP2D6, and CYP2E1) failed to catalyze the metabolism of atorvastatin to its para-hydroxy and/or ortho-hydroxy metabolite.

As observed for atorvastatin acid, the lactone metabolite patterns after incubation with human liver microsomes and cDNA-expressed human CYP3A4 or CYP3A5 were similar. However, incubation of the lactone with CYP2C8 did not result in detectable metabolite formation. None of the other CYP enzymes converted atorvastatin lactone to its metabolites. These results indicated that CYP3A enzymes were the CYP enzymes mainly responsible for the metabolism of atorvastatin acid and atorvastatin lactone. This was confirmed by the inhibition studies with specific CYP3A antibodies and chemical inhibitors (vide infra). The formation of para-hydroxy atorvastatin, ortho-hydroxy atorvastatin, para-hydroxy atorvastatin lactone, and ortho-hydroxy atorvastatin lactone by human liver microsomes as well as by human cDNA-expressed CYP3A4 and CYP2C8 followed Michaelis-Menten-type kinetics. Apparent \(K_{m}\) values, apparent \(V_{\text{max}}\) values, and the mean intrinsic clearances (\(V_{\text{max}}/K_{m}\)) for all metabolites are summarized in Table 1. In human liver microsomes, the \(K_{m}\) values for the formation of the atorvastatin lactone metabolites were 20-fold lower than those for the formation of the atorvastatin acid metabolites (Table 1). The \(V_{\text{max}}\) for the formation of para-hydroxy atorvastatin lactone was 3.1-fold greater than that for the corresponding acid.
metabolite. The $V_{\text{max}}$ values for the ortho-hydroxy metabolites were similar. These results translated into a mean intrinsic formation clearance ($CL_{\text{int}}$) of para-hydroxy atorvastatin lactone 83.1-fold greater than that of the para-hydroxy atorvastatin acid and a $CL_{\text{int}}$ of ortho-hydroxy atorvastatin lactone 20.2-fold greater than that of ortho-hydroxy atorvastatin acid. Comparison of atorvastatin lactone and atorvastatin acid metabolite formation by isolated CYP3A enzymes (Table 1) confirmed the results with human liver microsomes.

**ImmunoInhibition Studies.** Antibodies against human CYP3A enzymes inhibited metabolism of atorvastatin acid and lactone by human liver microsomes with an IC$_{50}$ of 40 µg of antibody and 50 µg of antibody per 100 µg of human liver microsomal protein, respectively (Fig. 3). Compared with uninhibited controls, the highest CYP3A antibody concentration tested (100 µg of antibody per 100 µg of human liver microsomal protein) reduced the formation of ortho-hydroxy atorvastatin acid by 71.8 ± 1.6% (mean ± S.D., n = 4), para-hydroxy atorvastatin acid by 68.2 ± 2.0%, ortho-hydroxy atorvastatin lactone by 64.3 ± 1.1%, and para-hydroxy atorvastatin lactone by 62.0 ± 1.5%.

**Drug Interaction with the Metabolism of Atorvastatin and Atorvastatin Lactone by Human Liver Microsomes.** The specific CYP3A substrate saquinavir and the CYP3A inhibitors iraconazole and fluconazole significantly affected atorvastatin and atorvastatin lactone metabolism formation. The formation of atorvastatin acid and atorvastatin lactone metabolites was almost completely inhibited by addition of 50 µM saquinavir [inhibition in comparison to control (mean ± S.D., n = 4): para-hydroxy atorvastatin acid, 97.9 ± 1.7%, ortho-hydroxy atorvastatin acid, 76.7 ± 9.4%; para-hydroxy atorvastatin lactone, 98.9 ± 0.6%, ortho-hydroxy atorvastatin lactone, 97.3 ± 1.4%], 20 µM itraconazole (para-hydroxy atorvastatin acid, 86.5 ± 4.6%, ortho-hydroxy atorvastatin acid 84.3 ± 7.0%; para-hydroxy atorvastatin lactone, 96.7 ± 2.4%, ortho-hydroxy atorvastatin lactone, 96.7 ± 3.0%) (Fig. 3), and 200 µM fluconazole (para-hydroxy atorvastatin acid, 83.4 ± 6.1%, ortho-hydroxy atorvastatin acid, 94.5 ± 3.4%; para-hydroxy atorvastatin lactone, 85.0 ± 0.5%, ortho-hydroxy atorvastatin lactone, 82.2 ± 1.3%).

Apparent inhibition constants ($K_i$) are shown in Table 2. In comparison to incubation with atorvastatin lactone, the apparent $K_i$ values for metabolite formation after incubation of atorvastatin acid with human liver microsomes in the presence of itraconazole or saquinavir were similar. Fluconazole inhibited the formation of atorvastatin acid metabolites with mean $K_i$ values 1.4-fold (para-hydroxy atorvastatin) and 2.5-fold (ortho-hydroxy atorvastatin) lower than the formation of the corresponding atorvastatin lactone metabolites.

Atorvastatin lactone was found to be a potent inhibitor for the metabolism of atorvastatin (Table 2) whereas, vice versa, atorvastatin acid had only a relatively small inhibitory effect on the formation of atorvastatin lactone metabolites. The addition of 200 µM atorvastatin reduced the formation of para-hydroxy and ortho-hydroxy atorvastatin lactone by 47.0 ± 4.3% and 29.4 ± 7.7% (mean ± S.D., n = 4), respectively.

**Computational Studies.** *Binding Free Energy Calculation.* To identify the physical factors contributing to the lower apparent $K_m$ values for the metabolism of atorvastatin lactone in comparison to its acid, we performed free energy of binding calculations using the computational MM/PBSA approach. The MM/PBSA results are listed in Table 3. Assuming that $K_m$ can be treated as a dissociation constant with no major contributions from other steps in the reaction process, the observed difference in the apparent dissociation constant $K_m$ corresponds to a difference in free energy of binding of
Attorvastatin and the neutral lactone. The sum of both polar contributions of atorvastatin and atorvastatin lactone metabolites served as data point represents the mean ± S.D. (n = 4).

\[ \Delta \Delta G_{\text{bind}} = -RT \ln \frac{K_{m2}}{K_{m1}} \]  

Based on the measurements in Table 1, the experimental \( \Delta \Delta G_{\text{bind}} \) values between the lactone and the acid in CYP3A4 were 1.3 and 1.8 kcal/mol for ortho- and para-hydroxylation (\( T = 310 \) K), respectively.

As expected, there was a considerable difference in the polar free energy contributions, \( E_{\text{es}} \) and \( G_{\text{pe}} \), between the negatively charged atorvastatin and the neutral lactone. The sum of both polar contributions (\( \Delta \Delta E_{\text{es}} + \Delta \Delta G_{\text{pe}} \)) indicated that the anionic atorvastatin has to pay a desolvation penalty ~5 kcal/mol greater than that for the lactone. Because the two substrates become completely buried in the CYP3A4 enzyme upon binding, both molecules have to find an adequate hydrogen bonding pattern in the enzyme for their polar functional groups, which are well solvated in aqueous solution, to keep the desolvation free energy contribution low. In the hydrophobic interior of CYP3A4 this is clearly more difficult for atorvastatin because of its three closely positioned hydroxy and carboxy groups. Inspection of the equilibrated complex showed that one of those hydroxy groups must point toward the hydrophobic CYP3A4 residues Met371 and Leu477 to accommodate favorable interactions of the other polar groups. Table 3 shows that van der Waals interactions between the lactone and CYP3A4 are slightly more favorable than those for atorvastatin acid. However, this is compensated for by a larger hydrophobic effect (\( \Delta \Delta G_{\text{wp}} \)) of the open acid form. The entropic advantage for lactone binding, arising from the fewer degrees of freedom of the ring form in solution compared with the more flexible acid form, was calculated to be only 1 kcal/mol.

**Prediction of the Regiospecificity of Hydroxylation.** Table 4A lists the calculated HOMO electron densities at the carbon atoms of the aromatic ring of acetanilide, which was used as a model for atorvastatin. Both quantum mechanical approaches suggested that little electron density is localized in the meta position relative to the aromatic substituent \( R = \text{NHCOCH}_3 \), but rather in the ortho and in particular in the para substitution sites. This is in agreement with the observation that this substituted orientates to para and ortho positions rather than to the meta position in an electrophilic aromatic substitution. Other substantial electron density in the HOMO was found at the aromatic carbon to which \( R \) is connected and at the nitrogen and oxygen atoms, but, as shown in our in vitro studies, those sites were not the target of metabolism reactions.

The results of our MD simulations for atorvastatin and atorvastatin lactone are summarized in Table 4B, which shows the average distance between the heme oxygen of CYP3A4 and aromatic carbon atoms at the different substitution sites relative to \( R \). Although, in comparison to the other possible substitution sites, the aromatic carbon atoms in the meta position were on average closest to the heme oxygen, they were not involved in drug metabolism reactions because of the low electron density at this position (Table 4A). The average C-O distances for the para and ortho sites in both atorvastatin acid and lactone correlated well with the measured rate constants, with the para position in atorvastatin lactone having the shortest distance and highest rate constant (Table 4B). The electron density calculations in combination with the MD results explain that in atorvastatin acid the para product is preferred over the ortho product because of its more favorable electron density, which compensates for the slightly larger average C-O distance and hence lower probability for forming a reactive configuration.

**Discussion**

Lactonization of atorvastatin may not only change its affinity to CYP3A enzymes but may also affect the preferred metabolism positions, the affinity to other CYP enzymes, and drug-drug interactions.

We showed that lactonization did not result in generation of different metabolite patterns by human liver microsomes of isolated CYP3A4 enzymes. Both atorvastatin acid and lactone were metabolized to their corresponding para-hydroxy and ortho-hydroxy metabolites. A qualitative mechanism-based approach using molecular dynamics and quantum mechanical calculations explained the product distribution observed in our in vitro studies. Our calculations showed that both orientation and electronic factors have to be taken into account to predict the product distribution. This became especially apparent for meta-hydroxylation for which our simulations suggested that the substrates orient in a configuration favorable for reaction, but ultimately do not react because of the low electron density at the meta position (Table 4).

The good agreement between our qualitative predictions and the in vitro measurements further supports the postulated addition-rearrangement reaction mechanism. However, more extensive quantum mechanical calculations would be needed to test different mechanisms.

We are aware that the structural information of a homology model, on which we based our simulations, is limited and, hence, did not attempt to do more quantitative calculations of rate constants, such as...
because the CYP3A inhibitors saquinavir and itraconazole, as well as by cDNA-expressed CYP2C8 was 49-fold lower than that by cDNA-

S.E. For details see

relevance because the CLint of atorvastatin for CYP3A enzymes. The only difference was included that like atorvastatin, its lactone is mainly metabolized by

CYP enzymes (Nelson and Strobel, 1988, 1989), and CYP3A4 residues equivalent to known substrate contact residues of the reference bacterial proteins are located in the active site or the substrate access channel (Szklarz and Halpert, 1997). Because additional site-directed mutagenesis studies have confirmed key amino acid residues for substrate binding (Szklarz and Halpert, 1998), which were identified from docking different ligands into the homology model, we are confident that the CYP3A4 model gives a good structural representation of the substrate binding site. We found that the root-mean-square deviation of all the Cα atoms between the fully minimized structure of the docked complex and the structures of the molecular dynamics simulation is on average 2.7 ± 0.1 Å (2.0 ± 0.1 Å for the Cα atoms closer than 6 Å from the substrate), indicating that the complex remains both stable and relatively close to the initial structure during MD.

Based on the results of our studies with isolated CYP enzymes, with specific CYP3A antibodies, and with chemical inhibitors, we concluded that like atorvastatin, its lactone is mainly metabolized by CYP3A enzymes. In general, lactonization did not affect the specificity of atorvastatin for CYP3A enzymes. The only difference was that incubation with CYP2C8 yielded detectable amounts of atorvastatin acid but not of lactone metabolites. The CYP2C8 pathway had not been described before. However, it is most likely not of clinical relevance because the CLint of para-hydroxy atorvastatin formation by cDNA-expressed CYP2C8 was 49-fold lower than that by cDNA-expressed CYP3A4. The inhibition studies confirmed that CYP3A is the only relevant metabolic pathway of atorvastatin and its lactone, because the CYP3A inhibitors saquinavir and itraconazole, as well as specific CYP3A antibodies, almost completely inhibited atorvastatin metabolism by human liver microsomes.

As indicated by 20-fold lower apparent Km values, atorvastatin lactone had a significantly higher affinity to CYP3A enzymes than the open acid form. Free energy calculations using the computational MM/PBSA approach gave the qualitatively correct prediction that the lactone binds better than the open acid form and yielded interesting insights into the binding forces that distinguish both atorvastatin forms (Table 3). In particular, the higher affinity of the lactone in comparison with the acid form seems to be mainly due to a smaller desolvation free energy penalty for the neutral lactone rather than to an entropy advantage. However, our MM/PBSA results overestimated the binding free energy difference, ∆ΔGbind, between both atorvastatin forms by ~5 kcal/mol. Possible reasons for this difference include insufficient resolution of the homology model at the binding interface or deviations from a Michaelis-Menten or similar mechanism, which would have invalidated the assumption that Km represents a dissociation constant. It had also to be taken into account that the substrate binding in CYP enzymes involves a heme iron with a coordinated water molecule rather than the activated oxygen used in our simulations. However, because the two atorvastatin forms differ in their structure far from the active site, this should affect the calculated relative binding free energy only to a small extent. Despite those uncertainties, our simulations strongly suggest that the dominant factor for the higher affinity of atorvastatin lactone to CYP3A4 than the open acid is its smaller desolvation penalty.

The intrinsic clearances (CLint) for the formation of the para-hydroxy and ortho-hydroxy metabolites were 83- and 20-fold higher, respectively, for the lactone than for the open acid form. In addition, atorvastatin lactone, which was found in plasma at similar concentrations as the administered open acid form (Kantola et al., 1998), was a potent inhibitor of the metabolism of atorvastatin acid. On the other hand, atorvastatin acid only significantly affected the in vitro metabolism of atorvastatin lactone at the highest concentration tested (200 μM), which was far above the atorvastatin concentration range in patients. These results strongly suggest that in vivo atorvastatin lactone is the relevant substrate for CYP3A enzymes and not atorvastatin as depicted in Fig. 1. Based on our results, we propose the metabolic pathway shown in Fig. 4, where the first step in atorvastatin metabolism is lactonization. After metabolism by CYP3A enzymes, some of the resulting lactone metabolites are converted to the open acid forms.

Our results, indicating that the major atorvastatin elimination pathway is via the lactone and that the lactone has a more than 5-fold higher affinity to CYP3A enzymes than previously reported for lovastatin and simvastatin, may explain why in clinical drug interaction studies, the impact of CYP3A inhibitors on atorvastatin pharmacoki-

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Fluconazole</th>
<th>Saquinavir</th>
<th>Itraconazole</th>
<th>Atorvastatin</th>
<th>Atorvastatin lactone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent inhibition constants (Km) are given as mean ± S.D. (n = 4 different preparations of human liver microsomes). For the inhibition studies, atorvastatin and atorvastatin lactone concentrations close to their Km (Table 1) were chosen. Apparent Km values were estimated based on half-maximal inhibition concentrations (IC50), which were determined separately for para- and ortho-hydroxy metabolite formation, and the apparent Km values shown in Table 1 using the Cheng-Prusoff equation (Cheng and Prusoff, 1973).</td>
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</table>

<table>
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<tr>
<th>Inhibitor</th>
<th>para-Hydroxy</th>
<th>ortho-Hydroxy</th>
<th>para-Hydroxy</th>
<th>ortho-Hydroxy</th>
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</thead>
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<tr>
<td>Fluconazole</td>
<td>9.1 ± 2.8</td>
<td>7.5 ± 3.6</td>
<td>12.8 ± 3.7</td>
<td>18.5 ± 2.6</td>
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<td>Saquinavir</td>
<td>0.6 ± 0.4</td>
<td>0.6 ± 0.5</td>
<td>0.5 ± 0.4</td>
<td>0.4 ± 0.2</td>
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<td>Itraconazole</td>
<td>0.05 ± 0.03</td>
<td>0.04 ± 0.01</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.01</td>
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<td>Atorvastatin</td>
<td>0.9 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>90.0 ± 28.5</td>
<td>&gt;900</td>
</tr>
</tbody>
</table>

**TABLE 3**

Differences in MM/PBSA binding free energy contributions (in kcal/mol) between atorvastatin and atorvastatin lactone for the association with CYP3A4

Energies were averaged over 50 structures, except for the difference in solute entropy contribution (−TΔS) for which a single snapshot was used. Values are presented as means ± S.E. For details see Materials and Methods.

<table>
<thead>
<tr>
<th>Ligand</th>
<th>ΔΔGbind</th>
<th>ΔΔGdesolv</th>
<th>ΔΔGvdW</th>
<th>ΔΔGelec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin Lactone</td>
<td>−6.3 ± 1.4</td>
<td>−28.5</td>
<td>−2.6</td>
<td>−6.3 ± 1.4</td>
</tr>
<tr>
<td>− Atorvastatin</td>
<td>93.4 ± 1.3</td>
<td>98.5 ± 1.5</td>
<td>−1.8 ± 0.9</td>
<td>1.6 ± 1.0</td>
</tr>
</tbody>
</table>

MD simulations were performed at 310 K and 1 atm, and the complexes were equilibrated for 1 ns and simulated for 2 ns (50 structures) using the Amber 9.0 program. Free energy components were calculated using the MM/PBSA method. The relative free energy only to a small extent. Despite those uncertainties, our simulations strongly suggest that the dominant factor for the higher affinity of atorvastatin lactone to CYP3A4 than the open acid is its smaller desolvation penalty.

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The pharmacokinetics of these other HMG-CoA reductase inhibitors. In healthy volunteers, simvastatin AUC values were 18.8-fold and lovastatin AUC values were 20-fold higher when it was coadministered with itraconazole than when with placebo (Neuvonen and Jalava, 1996; Neuvonen et al., 1998). In comparison, itraconazole increased atorvastatin AUC values only 3.2-fold (Kantola et al., 1998). Drug interactions with the CYP3A-mediated metabolism of HMG-CoA reductase inhibitors are of great clinical importance because the resulting high plasma concentrations of active HMG-CoA reductase inhibitors are associated with a higher risk for myopathy and rhabdomyolysis (Christians et al., 1998).

Our results indicate that future studies assessing the metabolism and drug interactions of atorvastatin should include the lactone. This may also apply to other HMG-CoA reductase inhibitors, which are administered in their acid form.

Acknowledgments. We thank Dr. G. D. Szklarz (Department of Basic Pharmaceutical Sciences, West Virginia University, Morgan-

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### Table 4: Theoretical prediction of the product distribution taking both electronic (A) and orientation (B) factors into account

<table>
<thead>
<tr>
<th></th>
<th>ortho</th>
<th>meta</th>
<th>para</th>
<th>Rest of Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. HOMO electron densities</td>
<td></td>
<td></td>
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<tr>
<td>DFT</td>
<td>0.20</td>
<td>0.06</td>
<td>0.41</td>
<td>1.33</td>
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<tr>
<td>HF</td>
<td>0.20</td>
<td>0.08</td>
<td>0.49</td>
<td>1.23</td>
</tr>
<tr>
<td>B. Averge distances</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td></td>
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</tr>
<tr>
<td>C_{Heme,Sub}</td>
<td>4.1 ± 0.3</td>
<td>3.4 ± 0.2</td>
<td>4.3 ± 0.4</td>
<td></td>
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<tr>
<td>V_{max} (pmol · mg⁻¹ · min⁻¹)</td>
<td>5.376 ± 409</td>
<td>5.463 ± 169</td>
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<tr>
<td>Atorvastatin lactone</td>
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<tr>
<td>C_{Heme,Sub}</td>
<td>4.7 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.5 ± 0.3</td>
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<tr>
<td>V_{max} (pmol · mg⁻¹ · min⁻¹)</td>
<td>4.235 ± 205</td>
<td>14.312 ± 348</td>
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</table>

Fig. 4. Metabolic pathways of atorvastatin and atorvastatin lactone proposed on basis of our results.
town, WV) for kindly providing us with the homology model of cytochrome P4503A4.

References


