Atorvastatin Glucuronidation Is Minimally and Nonselectively Inhibited by the Fibrates Gemfibrozil, Fenofibrate, and Fenofibric Acid

Theunis C. Goosen, Jonathan N. Bauman, John A. Davis,1 Chongwoo Yu,2 Susan I. Hurst, J. Andrew Williams, and Cho-Ming Loi

Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, Ann Arbor, Michigan

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ABSTRACT:

Gemfibrozil coadministration generally results in plasma statin area under the curve (AUC) increases, ranging from moderate (2- to 3-fold) with simvastatin, lovastatin, and pravastatin to most significant with cerivastatin (5.6-fold). Inhibition of statin glucuronidation has been postulated as a potential mechanism of interaction (Drug Metab Dispos 30:1280–1287, 2002). This study was conducted to determine the in vitro inhibitory potential of fibrates toward atorvastatin glucuronidation. [3H]Atorvastatin, atorvastatin, and atorvastatin lactone were incubated with human liver microsomes or human recombinant UDP-glucuronosyltransferases (UGTs) and characterized using liquid chromatography (LC)/tandem mass spectrometry and LC/UV/β-radioactivity monitor/mass spectrometry. [3H]Atorvastatin yields a minor ether glucuronide (G1) and a major acyl glucuronide (G2) with subsequent pH-dependent lactonization of G2 to yield atorvastatin lactone. Atorvastatin lactonization best fit substrate inhibition kinetics ($K_m = 12 \mu M, V_{max} = 74 \text{ pmol/min/mg}, K_I = 75 \mu M$). Atorvastatin lactone yields a single ether glucuronide (G3). G3 formation best fit Michaelis-Menten kinetics ($K_m = 2.6 \mu M, V_{max} = 10.6 \text{ pmol/min/mg}$). Six UGT enzymes contribute to atorvastatin glucuronidation with G2 and G3 formation catalyzed by UGTs 1A1, 1A3, 1A4, 1A8, and 2B7, whereas G1 formation was catalyzed by UGTs 1A3, 1A4, and 1A9. Gemfibrozil, fenofibrate, and fenofibric acid inhibited atorvastatin lactonization with $IC_{50}$ values of 346, 320, and 291 µM, respectively. Based on unbound fibrate concentrations at the inlet to the liver, these data predict a small increase in atorvastatin AUC (1.2-fold) after gemfibrozil coadministration and no interaction with fenofibrate. This result is consistent with recent clinical reports indicating minimal atorvastatin AUC increases (1.2- to 1.4-fold) with gemfibrozil.

Atorvastatin calcium is the most widely used 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitor (statin) in the United States. Statins are considered first-line therapeutic agents for the prevention of coronary heart disease and atherosclerotic disorders related to hypercholesterolemia (Grundy et al., 2004). These drugs are generally well tolerated with established benefit as cholesterol-lowering agents (Newman et al., 2003; Bays, 2006; Guyton, 2006). Drug-drug interactions have been described during combination therapy between fibrates and various statins (Bottorff, 2006). Combination therapy with statins and fibrates is a promising approach in the treatment of patients with mixed hyperlipidemia as statins primarily reduce low-density lipoprotein, whereas fibrates reduce triglycerides and increase high-density lipoprotein levels with established reduction in cardiovascular morbidity (Rubins et al., 1999; Grundy et al., 2004; Vasudevan and Jones, 2006). Nevertheless, the relative potential for fibrates to interact with statins, particularly atorvastatin, necessitates further understanding.

Clinically, it has been shown that compared with monotherapy, gemfibrozil resulted in the most significant increases in hydroxy acid statin area under the curve (AUC) (>5-fold) when coadministered with cerivastatin (Backman et al., 2002), whereas AUC changes were moderate (2- to 5-fold) after coadministration with simvastatin (Backman et al., 2000), lovastatin (Kyrklund et al., 2001), and pravastatin (Kyrklund et al., 2003). Smaller changes in statin AUC (1.25– to 2-fold) were observed for rosvastatin (Schneck et al., 2004) and pitavastatin (Mathew et al., 2004). A similar pharmacokinetic interaction has not been observed with fluvastatin (Spence et al., 1995) and, except for a metabolite of pravastatin (Pan et al., 2000), is generally not observed during coadministration of statins with other fibrates.
fibrates (fenofibrate or bezafibrate) (Kyrklund et al., 2001; Martin et al., 2003; Bergman et al., 2004). The relative potential for gemfibrozil to have a pharmacokinetic drug-drug interaction with atorvastatin was, however, until recently, not known (Backman et al., 2005; Whitfield et al., 2005).

The mechanistic basis for the gemfibrozil-statin drug interaction is not clear, although it is generally accepted to comprise a pharmacodynamic and possibly also a pharmacokinetic component, because gemfibrozil related adverse events mirror those observed for statins, and combination therapy may result in increased statin exposure with apparent dose-related increases in adverse events (Law and Rudnicka, 2006). Because many statins that exhibit a pharmacokinetic interaction with gemfibrozil are at least partially cleared by CYP3A4 and gemfibrozil is not a CYP3A4 inhibitor, recent in vitro experiments published in the literature speculated that inhibition of statin glucuronidation by gemfibrozil but not fenofibrate may be involved (Prueksaritanont et al., 2002b,c). Furthermore, a novel mechanism for statin lactonization mediated through glucuronidation was described (Prueksaritanont et al., 2002a), although in vitro differential interaction of fibrates on atorvastatin glucuronidation is not clearly defined.

Pharmacokinetic interactions between statins and gemfibrozil are clearly complex because multiple clearance mechanisms for statins exist, including oxidative metabolism by cytochrome P450s, phase II metabolism by UDP-glucuronosyltransferases (UGTs), intestinal and hepatic uptake clearance by multiple transporters, and interconversion kinetics between statin hydroxy acids and their corresponding lactone forms (Shitara and Sugiyama, 2006). Atorvastatin is subject to first-pass metabolism, mainly mediated by CYP3A4, resulting in the formation of two hydroxylated active metabolites (Jacobsen et al., 2000; Lennernäs, 2003). Atorvastatin (active hydroxy acid form) is also biotransformed to atorvastatin lactone (Fig. 1), which could occur nonenzymatically at low intestinal pH (Kearney et al., 1993) or via a coenzyme A (Li et al., 2006) or acyl glucuronide intermediate pathway (Prueksaritanont et al., 2002a). In addition, atorvastatin is also a substrate for several transport proteins including the efflux transporter P-glycoprotein (Wu et al., 2000), hepatic uptake organic anion transporting polypeptide (OATP) 1B1 (also known as OATP-C or OATP2) (Chen et al., 2005), and a proton-monocarboxylic acid cotransporter (Wu et al., 2000). The relative importance of these clearance mechanisms for atorvastatin is not fully understood. Nevertheless, a significant role for OATP1B1 and CYP3A4 in atorvastatin clearance is implied on the basis of clinical drug-drug interactions with inhibitors such as single-dose rifampin and itraconazole, respectively (Backman et al., 2005; Bottorff, 2006; Lau et al., 2007).

To increase the current understanding of the potential differential inhibitory effect of fibrates on atorvastatin glucuronidation, the purpose of this study was to characterize atorvastatin glucuronidation in human liver microsomes, evaluate human recombinant UGTs that mediate atorvastatin glucuronidation, and determine the in vitro potential of gemfibrozil, fenofibrate, and its active metabolite, fenofibric acid, to modulate or inhibit atorvastatin glucuronidation.
Materials and Methods

Materials. Atorvastatin calcium and atorvastatin lactone were synthesized by the Department of Chemistry, Pfizer Inc. (Ann Arbor, MI). [3H]Atorvastatin (7.3 Ci/mmol, 99.4% pure) was synthesized by Pharmaceutical Sciences, Pfizer Inc. (Kalamazoo, MI). Fenofibric acid was supplied by Tyger Scientific, Inc. (Ewing, NJ), and euugenol was supplied by Alfa Aesar (Ward Hill, MA). Pooled human liver microsomes (mixed gender, N = 60) and recombinant baculovirus-derived microsomes expressing human UGT (vector control, Inc. (Ewing, NJ), and eugenol was supplied by Alfa Aesar (Ward Hill, MA). All other chemicals was commercially available and of analytical grade from Sigma-Aldrich (St. Louis, MO).

UGT-Catalyzed Metabolism of [3H]Atorvastatin in Human Liver Microsomes. Pooled human liver microsomes (1.0 mg/ml), 50 mM Tris-HCl buffer (pH 7.0 at 37°C), 5 mM MgCl2, and alamethicin (50 μg/mg protein) were preincubated (on ice for 15 min) before addition of [3H]Atorvastatin (10 μM, 2.1 μCi) in a final incubation volume of 200 μl and preincubated at 37°C for 3 min. Reactions were initiated with UDPGA (5 mM). After incubation for 60 min at 37°C, reactions were quenched on ice for 30 min with 100 μl of 5% glacial acetic acid in acetonitrile (final pH = 3.5) containing lovastatin as an internal standard. Quenched incubates were centrifuged at 12,000 rpm for 10 min before analysis of supernatants by LC-UV/μ-RAM/MS as described below. Stability of quenched incubates were evaluated by sequential injections from the autosampler held at ambient room temperature over a period of approximately 21 h. Product amounts were quantified on the basis of radioencoded equivalents and are expressed as a percentage of initial concentration. Glucuronides were identified by LC-MS/MS from incubations containing unlabeled atorvastatin (10 μM) or atorvastatin lactone (10 μM) as described below.

Enzyme Kinetics of Atorvastatin and Atorvastatin Lactone Glucuronidation. For determination of enzyme kinetic parameters, incubations containing 0.25 mg/ml HLMs were incubated with increasing concentrations (0.1–300 μM in 0.5% DMSO final) of atorvastatin or atorvastatin lactone for 60 min as described above. Preliminary experiments evaluating linearity of product formation with respect to human liver microsomal protein (0.1–1.5 mg/ml) and time (15–90 min) at a substrate concentration (1.8 μM) below the apparent Km indicated that product formation was linear with protein concentration up to 0.75 mg/ml for up to 90 min. Supernatants were analyzed by HPLC, and glucuronides were quantified by UV detection.

Substrate concentration [S] and velocity (V) data were fitted to the appropriate enzyme kinetic model by nonlinear least-squares regression analysis (SigmaPlot; SPSS, Chicago, IL) to derive the apparent enzyme kinetic parameters Vmax (maximal velocity) and Km or Km/S (substrate concentration at half-maximal velocity). The Michaelis-Menten model (eq. 1), the uncompetitive substrate inhibition model (eq. 2), the two-enzyme model (eq. 3), and the substrate activation model (eq. 4), which incorporates the Hill coefficient (n), were used:

\[ V = \frac{V_{\text{max}} \times S}{K_m + S} \]  
\[ V = \frac{V_{\text{max}} \times S}{K_{\text{m1}} + S} \times \left(1 + \frac{S}{K_m} \right) \]  
\[ V = \frac{V_{\text{max1}} \times S}{K_{\text{m1}} + S} + \frac{V_{\text{max2}} \times S}{K_{\text{m2}} + S} \]  
\[ V = \frac{V_{\text{max}} \times S^n}{S^n_{50} + S^n} \]

where Vmax is the maximal velocity, Km or K is the substrate concentration at half-maximal velocity, n is an exponent indicative of the degree of curve sigmoidicity, and K is an inhibition constant. The best fit was based on a number of criteria, including visual inspection of the data plots (Michaelis-Menten and Edie-Hofstee), distribution of the residuals, size of the sum of the squared residuals, and the standard error of the estimates. Selection of models other than Michaelis-Menten was based on the F test (P < 0.05) and the Akaike Information Criterion.

Role of UGT Enzymes in Atorvastatin Glucuronidation. Atorvastatin or atorvastatin lactone (1, 10, or 100 μM in 0.5% DMSO) was incubated with recombinantly expressed human UGTs (0.5 mg/ml) as described for HLMs. Incubations (200 μl) were quenched (60 min) with 50 μl of 5% glacial acetic acid in acetonitrile containing 125 ng/ml lovastatin on ice for 30 min. Supernatants were carefully removed for LC-MS/MS analysis as described below. Results were expressed as peak area of product/internal standard ratio obtained from LC-MS/MS analyses. Positive control incubations with recombinant enzymes were performed as above using β-estradiol (UGT1A1 and UGT1A3), trifluoperazine (UGT1A4), 7-hydroxy-4-trifluoromethylcoumarin (UGTs 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, and 2B15), and euugenol (UGT2B17) as substrates to confirm active enzyme in incubations.

Inhibition of Atorvastatin and Atorvastatin Lactone Glucuronidation by Fibrates. To determine inhibition of atorvastatin or atorvastatin lactone glucuronidation by gemfibrozil, fenofibrate, or fenofibric acid, HLMs (0.5 mg/ml) were incubated with atorvastatin (12 μM) or atorvastatin lactone (2.5 μM) at or below its respective Km concentration together with increasing concentrations (0.03–1000 μM in 0.5% DMSO) of gemfibrozil, fenofibrate, or fenofibric acid. Control incubations contained DMSO (0.5% final) without inhibitor. Quenched aliquots containing 125 ng/ml lovastatin (internal standard) were analyzed by LC-MS/MS, and results were compared with aliquots supplemented with internal standard (40 ng of lovastatin) analyzed by LC-UV. IC50 values calculated using both LC-MS/MS and LC-UV were comparable, and final data are presented from LC-MS/MS analyses because of superior analyte selectivity and sensitivity. IC50 estimates for inhibition of glucuronidation were determined by nonlinear curve fitting with GraphPad Prizm (Graph-Pad Software, San Diego, CA), and were defined as the concentration of inhibitor required to inhibit control glucuronidation reactions by 50%.

Analytical Procedures for Atorvastatin and Metabolites. Atorvastatin, atorvastatin lactone, and their corresponding glucuronide(s) were characterized by LC-MS/MS using a model API 4000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada). For LC-MS/MS profiling and identification of glucuronide(s) formed, aliquots (25 μl) of the supernatants from in vitro HLM incubations with either atorvastatin or atorvastatin lactone as substrate were injected onto a HPLC column (Xterra, MS C18, 5 μ, 2.1 × 100 mm; Waters, Milford, MA). The HPLC system consisted of an Agilent 1100 HPLC pump and CTC Analytics PAL autosampler maintained at 5°C. The mobile phase was 10 mM ammonium acetate pH 4.5 (A) and acetonitrile (B) at a flow rate of 0.25 ml/min. Initial solvent conditions were 30% B held for 1 min, increased linearly to 75% B over 16 min and run isocratically for 2 min, followed by a return to initial conditions in 1 min and reequilibration for 5 min. The mass spectrometer was operated using an ESI interface in the positive ion mode (5000 eV, 300°C).

Quantification of atorvastatin and atorvastatin lactone and relative quantities of their corresponding glucuronides were determined after injection of supernatant (10 μl) from the same LC-MS/MS system described above except that the flow rate was 0.3 ml/min. Initial solvent conditions were 35% B followed by a linear increase to 50% B in 2 min, followed by an increase to 75% B in 0.01 min where these conditions were held for 1.99 min, followed by another increase to 95% B in 0.01 min where these conditions were held for 1.99 min, with a return to initial conditions in 0.01 min and a reequilibration time of 4.99 min. The column equilibration and multiple reaction monitoring transitions were as follows: atorvastatin (31 eV, 559.2 → 440.3), atorvastatin lactone (28 eV, 541.3 → 448.3), atorvastatin glucuronides (G1 and G2) (31 eV, 735.2 → 559.2), atorvastatin lactone glucuronide (G3) (28 eV, 717.3 → 624.3), and lovastatin (internal standard) (23 eV, 422.2 → 199.2). Atorvastatin and atorvastatin lactone were quantified on the basis of standard curves using authentic standards. Glucuronide amounts were expressed as an area ratio of analyte/ internal standard in the absence of authentic standards, except for determination of enzyme kinetic parameters for which LC-UV analyses were used as described below. Standards were prepared in HLMs or UGT cDNA matrix, containing all reagents included in incubation except UDPGA, with standard concentrations starting at the lower limit of quantification and ranging from 0.25 to 10 000 ng/ml for LC-MS/MS analyses and 5 to 15 000 ng/ml for LC-UV analyses, and with the range depending on the experiment of interest. Standards were quenched similar to samples as reported above.

LC-UV/μ-RAM/MS analyses were performed to evaluate relative glucuronide quantities, metabolite stabilities, and enzyme kinetic parameters. Aliquots of the supernatant (100 μl) from [3H]atorvastatin or unlabeled substrate incubations were monitored by UV absorption at 245 nm, an on-line β-RAM (DNUS Systems, Tampa, FL), and/or a LCQ Advantage ion trap mass spectrometer (Thermo Electron Corporation, San Jose, CA). Aliquots were injected onto a HPLC column (Luna C18(2), 3 μ, 4.6 × 150 mm; Phenomenex,
gemfibrozil and fenofibrate coadministration resulted in the formation of atorvastatin lactone as the major metabolite, and fenofibric acid terminal t\textsubscript{1/2} values of 2.4 and 22.7 h, estimates for gemfibrozil and fenofibrate aglycone were 1.2 and 1.1 min\textsuperscript{-1}, respectively.

Results

UGT-Mediated Atorvastatin Metabolism. Atorvastatin glucuronidation resulted in the formation of atorvastatin lactone as the major metabolite and three glucuronide conjugates (Fig. 1). [\textsuperscript{3}H]-Atorvastatin was glucuronidated and, after acetic acid quench (pH 3.5), yielded atorvastatin lactone as the major metabolite accounting for 64% of total product, a major atorvastatin acyl glucuronide conjugate (G2) and a minor ether glucuronide conjugate (G1), representing 32 and 4% of total product, respectively (Fig. 2). Atorvastatin lactone, in turn, is also glucuronidated and yields a single atorvastatin lactone ether glucuronide conjugate (G3), after incubation of atorvastatin lactone as substrate, as shown in Fig. 2. The rate of G3 formation was low after incubation with [\textsuperscript{3}H]-atorvastatin and, although chromatographically separated, was not detected in the radioactive trace, but could be detected by LC-MS/MS due to superior sensitivity. After incubation with atorvastatin lactone, atorvastatin and its glucuronides (G1, G2, and G3) were also detected by LC-UV and LC-MS/MS analyses, respectively. The nonenzymatic conversion of atorvastatin lactone to atorvastatin was confirmed under the physiological pH incubation conditions.

The major atorvastatin acyl glucuronide (G2) was unstable under physiological pH and spontaneously lactonized to yield atorvastatin lactone. Atorvastatin lactonization was incomplete under our incubation conditions (pH = 7.0) and was quenched by addition of acetic acid (pH = 3.5) preventing further conversion of G2 to atorvastatin lactone before analysis. Under these conditions, formation of G2 was approximately 32%. For stability analyses, the glucuronidation products of atorvastatin, quenched with acetic acid to stop conversion to atorvastatin lactone, were stable for at least 21 h at ambient room temperature. The mean change (percent, percent coefficient of variation) in product amounts expressed as a percentage of initial concentration (mean of first two injections) directly after acetic acid quench were G1 (11.6, 9.2), G2 (−1.7, 4.9), atorvastatin (−0.1, 0.7), and atorvastatin lactone (8.0, 5.2). The product amounts did not change by more than 11.6% of initial amounts, and the coefficients of variation were less than 9.2%. The chemical conversion of atorvastatin to atorvastatin lactone incubated without UDPGA was minimal, typically less than 1%.

Prediction of Drug-Drug Interaction Potential. The effects of fibrate coadministration on atorvastatin AUC were predicted on the basis of observed in vitro inhibitory potencies and fibrate plasma exposures, using published methodology for predicting drug-drug interactions based on in vitro enzyme inhibition for cytochrome P450 enzymes (Kanamitsu et al., 2000; Brown et al., 2005). For predictions based on total (bound and unbound) or unbound fibrate maximal plasma concentration (C\textsubscript{max}) at steady state, a modified version of the equation developed by Rowland and Matin (1973) was used:

\[
\frac{\text{AUC}_{\text{F}}}{\text{AUC}} = \frac{1}{1 + \left(\frac{[I]}{[I]\text{max}} \cdot K_{i} \cdot \frac{f_{a} \cdot f_{\text{UGT}}}{1 - f_{a} \cdot f_{\text{UGT}}}\right)}
\]

where AUC/F represents the AUC ratio in the presence and absence of an inhibitor (predicted change in AUC), [I] is inhibitor concentration, K\textsubscript{i} is a constant describing affinity of inhibitor for the enzyme (assuming competitive inhibition K\textsubscript{i} = IC\textsubscript{50}/2, f\textsubscript{a} is the fraction of drug cleared by metabolism (as opposed to renal or biliary excretion of unchanged drug), and f\textsubscript{\text{UGT}} is the fraction of drug metabolized by UGT enzymes. Although f\textsubscript{\text{UGT}} was assumed to be 1 and K\textsubscript{i} was estimated from inhibitor pharmacokinetic data using the following equation:

\[
[I]_{\text{in}} = f_{\text{a}} \left(\frac{C\text{max}}{\text{K}_{\text{m}}} + k_{e} \cdot \frac{D}{\text{Q}_{\text{hep}}}ight)
\]

where [I]\textsubscript{in} is the maximum systemic plasma concentration (C\textsubscript{max}) of inhibitor drug, k\textsubscript{e} is the absorption rate constant, F\textsubscript{a} is the fraction of the dose absorbed from the gastrointestinal tract, D is the inhibitor dose, and Q\textsubscript{hep} is the hepatic blood flow (1450 ml/min). For unbound fractions, f\textsubscript{a} = 0.02 and f\textsubscript{a} = 0.01 were used for gemfibrozil and fenofibrin acid, respectively (Miller and Spence, 1998). Gemfibrozil and fenofibrin acid plasma concentrations and terminal elimination half-lives were from reported literature values (Whitfield et al., 2005). For these predictions, F\textsubscript{a} was assumed to be 1 and k\textsubscript{e} was estimated from inhibitor pharmacokinetic data using the following equation:

\[
t_{\text{max}} = \frac{\ln(k_{e}/k_{a})}{k_{a} - k_{a}}
\]

where t\textsubscript{max} is time to reach C\textsubscript{max} and k\textsubscript{a} is the elimination rate constant obtained from the elimination half-life (t\textsubscript{1/2} = 0.693/k\textsubscript{a}). By using gemfibrozil and fenofibrin acid terminal t\textsubscript{1/2} values of 2.4 and 22.7 h, estimates for gemfibrozil and fenofibrate k\textsubscript{a} were 1.2 and 1.1 min\textsuperscript{-1}, respectively.
**Glucuronide Structure Elucidation.** The structures of glucuronide conjugates as characterized by LC-MS/MS are depicted in Fig. 3. The collision-induced dissociation (CID) product ion spectrum for atorvastatin shows a protonated molecular ion of $m/z$ 559 (Fig. 3A). The major ions of interest in the spectrum include $m/z$ 466 representing the loss of the aniline moiety and $m/z$ 440 representing the loss of the phenyl-amino-carbonyl moiety.

The CID product ion spectrum of G2 shows a protonated molecular ion of $m/z$ 735 (Fig. 3C). The two atorvastatin glucuronides (G1 and G2) had nearly identical CID product ion spectra and because G2 was produced in greater quantities than G1, a representative CID spectrum for this metabolite is shown. As shown in Fig. 3C, the major fragment formed is the characteristic loss of 176 amu seen with glucuronides forming the aglycone, atorvastatin, at $m/z$ 541. The fragments of interest include $m/z$ 624, representing the loss of the aniline moiety, and $m/z$ 448, which is 176 amu less than $m/z$ 624 and represents the loss of glucuronide as well as a loss of aniline from G3.

**Enzyme Kinetics of Atorvastatin and Atorvastatin Lactone Glucuronidation.** The apparent enzyme kinetic parameters for glucuronidation of atorvastatin and atorvastatin lactone are summarized in Table 1 and are shown graphically in Figs. 4 through Fig. 6. Separate kinetic parameters were determined with either atorvastatin or atorvastatin lactone as substrates to best characterize kinetically the proposed glucuronidation pathways of atorvastatin, resulting in the formation of three glucuronides and atorvastatin lactone (Fig. 1).

The lactonization of atorvastatin (formation of G2 followed by spontaneous degradation to atorvastatin lactone) implies a single-step enzymatic reaction. Therefore, for atorvastatin lactonization kinetic parameters (particularly $V_{\text{max}}$), product amounts of G2 and atorvastatin lactone were summed. Atorvastatin lactonization best fit a substrate inhibition model as evidenced by the Eadie-Hofstee plot (Fig. 4, inset). Higher atorvastatin substrate concentrations ($>100 \mu M$) were not fitted to the kinetic model (Fig. 4) because atorvastatin contains low levels of atorvastatin lactone ($<1\%$). This leads to ambiguity in the amount of atorvastatin lactone formed enzymatically when greater than 100 $\mu M$ atorvastatin was incubated with HLMs. This observation was further complicated by apparent substrate inhibition kinetics for atorvastatin lactonization, which was clearly apparent when [S] against $V$ data are plotted for G2 formation up to 300 $\mu M$ atorvastatin.

**Fig. 3.** MS/MS spectra (ESI positive ion mode) of atorvastatin (A), atorvastatin lactone (B), atorvastatin glucuronide (C), and atorvastatin lactone glucuronide (D).
Kinetic parameters for the glucuronidation of atorvastatin and atorvastatin lactone after incubation with human liver microsomes

<table>
<thead>
<tr>
<th>Product</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg)</th>
<th>$k_i$ (μM)</th>
<th>$CL_{int}$ (μl/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactonization</td>
<td>12 ± 3.7</td>
<td>74 ± 13</td>
<td>75 ± 29</td>
<td>6.2</td>
</tr>
<tr>
<td>G2</td>
<td>28 ± 4.5</td>
<td>NA</td>
<td>36 ± 5.8</td>
<td>NA</td>
</tr>
<tr>
<td>G3</td>
<td>2.6 ± 0.3</td>
<td>11 ± 0.3</td>
<td>NA</td>
<td>4.2</td>
</tr>
</tbody>
</table>

NA, not applicable.

Lactonization is the major glucuronidation pathway for atorvastatin, mediated via acyl glucuronide (G2) formation, calculated from summation of G2 and atorvastatin lactone as products. G3 is the atorvastatin lactone glucuronide.

Lactonization and G2 formation exhibited substrate inhibition kinetics.

...(continued)
acetic acid, to inhibit atorvastatin or atorvastatin lactone glucuronidation in HLMs is summarized in Table 2 and Fig. 8. As shown in Fig. 8A, the formation of G1 increased approximately 1.6-fold in the presence of 600 μM gemfibrozil, compared with controls without gemfibrozil. The mean increase was maximal in the presence of 600 μM gemfibrozil with no difference from control at 1000 μM. The lactonization of atorvastatin (Fig. 8B) was inhibited by gemfibrozil with an IC₅₀ value of 346 μM. G3 formation from incubations with atorvastatin lactone as substrate was not significantly inhibited by gemfibrozil.

Fenofibrate and its active metabolite, fenofibric acid, similarly increased G1 glucuronidation of atorvastatin approximately 6.7- and 9.5-fold, respectively, at 1000 μM and 291 μM (Fig. 8A). The increased formation of G1 was inhibited by fenofibrate and fenofibric acid with IC₅₀ values of 329 and 291 μM, respectively. All inhibitors appeared soluble at higher concentration, which is supported by increasing inhibitor concentration, resulting in decreased enzyme activity through 1000 μM (Fig. 8, B and E).

In contrast to gemfibrozil, the in vitro glucuronidation of atorvastatin lactone (G3) was inhibited by fenofibrate and fenofibric acid (Fig. 8F). The mean inhibitions of G3 formation were 45 ± 0.7 and 50 ± 2.4% at fenofibrate and fenofibric acid concentrations of 1000 μM, respectively. The fitted IC₅₀ values for inhibition of atorvastatin lactone glucuronidation were therefore slightly higher than 1000 μM.

Prediction of Drug-Drug Interaction Potential. The predicted in vivo effects of fibrates on human atorvastatin AUC is summarized in Table 3. For both gemfibrozil and fenofibrate, the predicted increase in atorvastatin AUC was considered to be minimal.

Discussion

The findings presented here describe the relatively weak and nonselective inhibition of atorvastatin glucuronidation by gemfibrozil and fenofibrate, the major fibrates prescribed in the United States, as well as characterization of the in vitro glucuronidation of atorvastatin and its lactone metabolite. Based on these findings, the predicted change in human atorvastatin AUC during gemfibrozil or fenofibrate coadministration is minimal.

After administration of atorvastatin calcium, atorvastatin lactone is commonly detected in human plasma with comparable AUC exposure to atorvastatin (Kantola et al., 1998). The relative contribution of possible pathways responsible for atorvastatin lactone formation is not clear but may involve acid-catalyzed formation at low intestinal pH before absorption (Kearney et al., 1993) or enzymatic formation mediated through acyl glucuronide formation (Prueksaritanont et al., 2002a) or via a coenzyme A-dependent pathway (Li et al., 2006). Because the base-catalyzed formation of atorvastatin from the lactone would be essentially irreversible at higher pH, enzymatic formation of atorvastatin lactone would probably be dominant under physiological pH. The in vitro UGT-catalyzed metabolism of atorvastatin in HLMs presented here indicates that atorvastatin lactone is a major metabolite and that its formation is mediated through acyl glucuronide (G2) formation. Additionally, the formation of a single atorvastatin lactone glucuronide (G3) and a minor atorvastatin ether glucuronide (G1) was characterized. After administration of [¹⁴C]atorvastatin in patients with a T-tube, biliary excretion was the major elimination route with unchanged atorvastatin, ortho-hydroxy atorvastatin, para-hydroxy atorvastatin, ortho-hydroxy atorvastatin acyl glucuronide, and ortho-hydroxy atorvastatin ether glucuronide accounting for the majority of radioactivity (Le Couteur et al., 1996). However, the above-mentioned glucuronides (G1, G2, and G3) have not been detected in any in vivo samples, and the high instability of G2 under physiologic pH suggests that once formed, it probably contributes mainly to atorvastatin lactonization. In addition, in vitro intrinsic clearance values for atorvastatin and atorvastatin lactone glucuronidation were comparable.
but significantly lower than those obtained for oxidative metabolism (Jacobsen et al., 2000). However, because relative UGT expression levels in human liver are unknown, it complicates the scaling of phase I and phase II metabolism to overall clearance. Collectively, these findings suggest that glucuronidation may contribute to a significant portion of hepatic atorvastatin lactonization and overall atorvastatin clearance.

On the basis of experiments with recombinant enzymes, both atorvastatin and atorvastatin lactone are glucuronidated (G2 and G3) by multiple UGTs including UGT 1A1, 1A3, 1A4, 1A8, and 2B7. The highest rates of lactonization or atorvastatin lactone glucuronidation activity were mediated by UGT1A1 and UGT1A3 or UGT1A3 and UGT1A4, respectively (Fig. 7). The formation of the minor atorvastatin glucuronide (G1) was mediated by UGT1A3, UGT1A4, and UGT1A9. It has previously been shown that atorvastatin lactonization is mediated by UGT1A1 and UGT1A3, although only 6 of the 12 currently available UGTs were evaluated (Prueksaritanont et al., 2002a). UGT reaction phenotyping, compared with cytochrome P450, has received less attention because of a lower risk of UGT-mediated drug-drug interactions (Williams et al., 2004). Evidence for high turnover by individual recombinant UGTs (Fig. 7) does not necessarily imply relative contributions in human liver or extrahepatic tissues because enzyme preparations are standardized to protein content, and the tools available to determine relative hepatic or extrahepatic levels of individual UGTs in subcellular human tissue fractions are lacking. Currently, of the 18 known human nucleotide sequences encoding UGT enzymes, hepatic isoforms that are not available include UGT2B10, UGT2B11, and UGT2B28 (Miners et al., 2004). Nevertheless, our data suggest that at least six UGT enzymes could contribute to atorvastatin glucuronidation, which reduces potential drug-drug interaction risk through inhibition of a single enzymatic pathway (Williams et al., 2004).

Atorvastatin lactonization was inhibited by gemfibrozil as well as fenofibrate and its active metabolite, fenofibric acid, with similar apparent inhibitory potencies (Table 1). This result is in contrast to previous studies that reported no inhibition by fenofibrate (75 M), which was proposed as the explanation for a differential fibrate interaction with statins in the clinic (Prueksaritanont et al., 2002b). In the presence of increasing concentrations of gemfibrozil, fenofibrate, or fenofibric acid, the formation of G1 increased 1.6-, 6.7-, and 9.5-fold, respectively (Fig. 8). The increase in G1 formation may be attributed to metabolic shunting and/or heterotropic enzyme activation commonly observed with UGTs (Williams et al., 2002; Miners et al., 2004). The significantly larger increase in G1 formation in the pre-
ence of fenofibrate and fenofibric acid may reflect their ability to inhibit a parallel glucuronidation pathway (G3), whereas atorvastatin lactone glucuronidation was relatively insensitive to inhibition by gemfibrozil (Fig. 8).

To predict the extent of a pharmacokinetic interaction between atorvastatin and gemfibrozil or fenofibrate, a similar approach previously used for cytochrome P450 enzymes was applied (Brown et al., 2005). Assuming that inhibition of glucuronidation plays a role in the mechanism of the pharmacokinetic interaction, clinical differences in the ability of these fibrates to influence atorvastatin pharmacokinetics probably relate to relative differences in hepatic fibrate exposure, because differential ability to inhibit in vitro glucuronidation was not observed in this study. After oral administration, total drug (bound and unbound) peak plasma concentrations (C_max) of gemfibrozil and fenofibric acid are on the order of 80 and 30 μM after administration of 600 mg b.i.d. or 160 mg q.d., respectively (Miller and Spence, 1998; Schneck et al., 2004). Initial predictions using total drug concentrations, as currently recommended by draft Food and Drug Administration guidance, predicted a small gemfibrozil and fenofibric acid interaction. However, both gemfibrozil and fenofibric acid are extensively bound to plasma proteins (>98%), resulting in maximum unbound plasma concentrations of approximately 1.6 and 0.3 μM, respectively (Miller and Spence, 1998). Circulating drug levels are therefore much lower than the IC_{50} values for atorvastatin lactonization obtained in this study (Table 1), consistent with previously reported IC_{50} values obtained for gemfibrozil in HLMs (316 μM) or hepatocytes (63 μM) (Pruksaritanont et al., 2002b). More sophisticated predictions for drug-drug interactions were obtained by incorporating unbound inhibitor concentration at the inlet to the liver and inhibitor absorption rate constant (Brown et al., 2005). When these values are used to predict the likelihood of a drug-drug interaction between atorvastatin and fibrates, a small increase in atorvastatin AUC is predicted with gemfibrozil (1.2-fold) and no interaction after fenofibrate coadministration (Table 3). In comparison, a small gemfibrozil interaction (1.3-fold) and lesser fenofibrate interaction (1.1-fold) are predicted using total drug peak plasma concentrations. The former method using unbound plasma fibrate concentration at the inlet to the liver is also believed to be of physiological relevance (Kanamitsu et al., 2000). Recently, small changes in atorvastatin AUC (1.35-fold) were reported after coadministration of atorvastatin (40 mg) with gemfibrozil (600 mg twice daily) and no significant change when administered with fenofibrate (160 mg daily) (Whitfield et al., 2005). The modest interaction between atorvastatin and gemfibrozil was also observed in another study indicating a minimal change in atorvastatin AUC (1.24-fold) at a lower atorvastatin dose (20 mg) after coadministration with gemfibrozil (Backman et al., 2005). The atorvastatin lactone/atorvastatin AUC ratio also decreased (~20–35%) during gemfibrozil coadministration, whereas AUC ratios for hydroxylated metabolites/atorvastatin increased (~25–50%), suggesting inhibition of glucuronidation (lactonization) and shunting toward oxidative metabolism (Backman et al., 2005; Whitfield et al., 2005). Therefore, on the basis of these predictions, inhibition of statin glucuronidation is likely to contribute to a lesser drug-drug interaction between atorvastatin and gemfibrozil, compared with the observed pharmacokinetic interaction between gemfibrozil and other statins.

In summary, gemfibrozil and fenofibrate are weak and nonselective inhibitors of atorvastatin lactonization. On the basis of the in vitro findings, the likelihood for a significant pharmacokinetic interaction between atorvastatin and these fibrates due to inhibition of glucuronidation is low (Williams et al., 2004). The low drug-drug interaction potential is based on the observation that multiple UGT enzymes contribute to atorvastatin glucuronidation, the possibility of metabolic shunting, weak in vitro inhibition potency and lack of differentiation in the ability of gemfibrozil or fenofibric acid to inhibit atorvastatin glucuronidation, and comparable clinical plasma exposure to gemfibrozil and fenofibric acid. However, on the basis of unbound fibrate concentrations at the inlet to the liver, the observed inhibition of atorvastatin glucuronidation by fibrates in HLMs predicts a small increase in atorvastatin AUC (~1.2-fold) when it is coadministered with gemfibrozil and there is no interaction with fenofibrate. These findings are consistent with clinical finding indicating minimal increases in atorvastatin AUC (~1.2- to 1.4-fold) observed during coadministration with gemfibrozil and no change with fenofibrate (Backman et al., 2005; Whitfield et al., 2005).

References


**Address correspondence to:** Dr. Theunis C. Goosen, Department of Pharmacokinetics, Dynamics and Metabolism, Pfizer Global Research and Development, 2800 Plymouth Rd., Ann Arbor, MI 48105. E-mail: theunis.goosen@pfizer.com