EFFECTS OF FIBRATES ON METABOLISM OF STATINS IN HUMAN HEPATOCYTES

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ABSTRACT:
This study investigated the metabolic interaction between fibrates and statin hydroxy acids in human hepatocytes. Gemfibrozil (GFZ) modestly affected the formation of β-oxidative products and CYP3A4-mediated oxidative metabolites of simvastatin hydroxy acid (SVA) but markedly inhibited the glucuronidation-mediated lactonization of SVA and the glucuronidation of a β-oxidation product (IC50 ~50 and 15 μM, respectively). In contrast, fenofibrate had a minimal effect on all the metabolic pathways of SVA. GFZ also significantly inhibited (IC50 ~50-60 μM) the oxidation of cerivastatin (CVA) and rosuvastatin (RVA), but not of atorvastatin (AVA), while effectively decreasing (IC50 ~30 to 60 μM) the lactonization of all three statins. As was observed previously with other statin hydroxy acids, RVA underwent significant glucuronidation to form an acyl glucuronide conjugate and lactonization to form RVA lactone in human liver microsomes and by UGT 1A1 and 1A3. While GFZ is not an inhibitor of CYP3A4, it is a competitive inhibitor (Ki = 87 μM) of CYP2C8, a major catalyzing enzyme for CVA oxidation. These results suggest that 1) the pharmacokinetic interaction observed between GFZ and statins was not likely mediated by the inhibitory effect of GFZ on the β-oxidation, but rather by its effect primarily on the glucuronidation and non-CYP3A-mediated oxidation of statin hydroxy acids, and 2) there is a potential difference between fibrates in their ability to affect the pharmacokinetics of statins, and among statins in their susceptibility to metabolic interactions with GFZ in humans.

Fibrates, lipid-regulating agents, and hydroxymethylglutaryl-coenzyme A reductase inhibitors or so called “statins”, cholesterol lowering agents, are frequently prescribed together to treat patients with mixed hyperlipidemia (Shek and Ferrill, 2001). There have been reports of increased risk of myopathy, including rhabdomyolysis with this coadministration (Murdock et al., 1999). Despite being generally accepted as a class effect for all fibrate-statin combinations, this increased risk has been observed at varied incidences with different fibrates and statins. More documented cases for myopathy have been reported with gemfibrozil (GFZ1)-statin combined therapy than with other fibrate-statin combinations (Shek and Ferrill, 2001). Recently, cerivastatin (CVA) was withdrawn from the market due to disproportionate numbers of fatal rhabdomyolysis cases (compared with other marketed statins), many of which occurred in patients receiving concomitant GFZ (Farmer, 2001).

Although it has generally been accepted that the increased risk of myopathy is due primarily to a pharmacodynamic drug-drug interaction, recent studies have suggested that the increased risk might also have a pharmacokinetic origin. In recent clinical studies, increases in the exposure mainly to statin hydroxy acids, but minimally to the lactone form of statins, were observed following coadministration of GFZ and statins (Backman et al., 2000; Kyrklund et al., 2001). Subsequently, using simvastatin (SV) and its active metabolite simvastatin hydroxy acid (SVA) as model compounds, and in vitro dog and human liver microsomes and in vivo dogs as study models, we have shown that the observed pharmacokinetic interaction was mediated at least in part by the inhibitory effect of GFZ on the glucuronidation, and not the CYP3A4-mediated oxidation of SVA (Prueksaritanont et al., 2002b). Glucuronidation is a previously unrecognized but common metabolic pathway for several statin hydroxy acids, including CVA and atorvastatin (AVA) (Prueksaritanont et al., 2002a).

Consistent with the above severe interactions reported with GFZ, CVA was shown to be more susceptible than SVA or AVA to metabolic interaction with GFZ at the level of glucuronidation and P450-mediated oxidation in human liver microsomes (Prueksaritanont et al., 2002b). The differential susceptibility of the oxidative metabolism of statins has been proposed to be due to the inhibitory effect of GFZ on CYP2C8 activity since CYP2C8 is an important enzyme involved in the oxidation of CVA but not SVA or AVA. To date, evidence for the effect of GFZ on CYP2C8 activity or substrates has not been available. Thus far, GFZ has been shown to be a potent inhibitor of CYP2C9 and possibly also CYP2C19 (Wen et al., 2001). Based on these results, there is also a potential for GFZ to interact with other statins via the glucuronidation and CYP2C9-mediated oxidation. In this regard, rosuvastatin (RVA), a new statin, has been reported to undergo slow oxidative metabolism, primarily by CYP2C9 and CYP2C19, in human hepatocytes, although not in human liver microsomes (McTaggart et al., 2001). To date, there have been no published reports on the glucuronidation of RVA.

1 Abbreviations used are: GFZ, gemfibrozil; CVA, cerivastatin; SV, simvastatin; SVA, simvastatin hydroxy acid; AVA, atorvastatin; RVA, rosuvastatin; RV, the lactone form of rosuvastatin; UDPGA, UDP-glucuronic acid; BOA, 2-bromooctanoic acid; HPLC, high performance liquid chromatography; ACN, acetonitrile; LC/MS/MS, liquid chromatography-tandem mass spectrometry; AV, the lactone form of atorvastatin; CV, the lactone form of cerivastatin; MS, mass spectrometry; UGT, UDP glucuronol transferase.

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In addition to the newly identified glucuronidation and the well-known P450-mediated oxidation, β-oxidation has also been observed for several statin hydroxy acids (Prueksaritanont et al., 2001). Like the glucuronidation, the β-oxidation occurs at the dihydroxy heptanoic or heptenoic acid side chain, a structural feature common to all statin hydroxy acids. In theory, inhibition of the β-oxidation of statin hydroxy acids could also lead to an increase in statin hydroxy acid exposure. However, the effect of GFZ on the β-oxidation pathway was not determined in our previous study (Prueksaritanont et al., 2002b) since at the time of study, there was no evidence in vitro or in vivo for the formation of β-oxidation products of SVA in humans. In rodents, the β-oxidation products of SVA have been well characterized (Prueksaritanont et al., 2002b), but in humans they only recently have been observed in our hepatocyte studies.

Thus, the aims of the present investigation were to 1) determine the effect of GFZ on the β-oxidation pathway of SVA, 2) compare the inhibitory potential of GFZ versus fenofibrate, another commonly prescribed fibrate, on SVA metabolism, 3) compare the susceptibility of RVA versus other stains to the inhibitory effect of GFZ, and 4) characterize the glucuronidation of RVA and examine the effect of GFZ on CYP2C8 activity in humans. To accomplish the first three objectives, human hepatocyte was chosen as a study model since all major metabolic pathways, including the β-oxidation of SVA and the oxidation of RVA, could be monitored in this system. AVA and CVA were also included in the study for a direct comparison with RVA and to confirm our previous liver microsomal observations (Prueksaritanont et al., 2002b).

Materials and Methods

Materials. SV, SVA, and [14C]SVA (Fig. 1), with specific activity of 50 μCi/μmol, were synthesized at Merck Research Laboratories (Rahway, NJ). AVA and CVA (Fig. 1) were extracted from commercial sources, while RVA (Fig. 1) and its lactone RV was synthesized at Merck Research Laboratories. The identity and purity of AVA, CVA, RVA, and RV were confirmed by infrared and NMR spectroscopy. GFZ, fenofibrate, fenofibric acid, Brij 58, alamethicin, UDPGA, 2-bromooctanoic acid (BOA), ketoconazole, paclitaxel, and sulfaphenazole were obtained from Sigma-Aldrich (St. Louis, MO), 6α-hydroxy paclitaxel was purchased from PanVera Corp. (Madison, WI), and human liver microsomes were obtained from Xenotech (Kansas City, KS) and BD Gentest. Human hepatocytes from four to six different donors were purchased from In Vitro Technologies (Baltimore, MD) in fresh suspensions. The cells were resuspended in 10 mM HEPES buffer for a final concentration of 3 × 10^6 cells/ml, and cell viability was determined prior to use.

In Vitro Metabolism Studies in Human Hepatocytes. A typical incubation mixture, in a final volume of 0.5 ml, contained 1.5 × 10^6 hepatocytes, and metabolic inhibitors (10–200 μM GFZ; 75 μM fenofibrate; 1 μM ketocazole; 20 μM sulfaphenazole; 200 μM BOA; 50 μM troleandomycin) or vehicle used to prepare the inhibitors [50% acetonitrile (ACN) in water]. For all experiments and for each hepatocyte preparation, incubations were done in triplicate. The reaction was started by the addition of statins (20-μM final concentration) following a 3-min preincubation at 37°C, and the reaction was incubated for up to 60 min for SVA, AVA, and RVA, and 120 min for RVA. Control experiments were performed by excluding hepatocytes from the incubation mixtures. The reaction was terminated by the addition of ACN containing pronase as an internal standard. Following centrifugation, the supernatant was dried, reconstituted in 20% ACN in water, and analyzed by HPLC and liquid chromatography-tandem mass spectrometry (LC/MS/MS), as described below.

In Vitro Metabolism Studies of RVA in Liver Microsomes. A typical incubation mixture, in a final volume of 0.2 ml, contained 0.4 mg of liver microsomes, preincubated with Brij 58 or alamethicin (at 10% mg/mg microsomal protein) for about 15 min, 10 mM MgCl2, 5 mM UDPGA, and 0.05 M Tris buffer, pH 7.0. Unless otherwise specified, the reaction was started by the addition of RVA following a 3-min preincubation at 37°C, and the reaction was incubated for 60 min. Control incubations were performed by excluding either the microsomes or UDPGA from the incubation mixtures. The reaction was terminated at appropriate time intervals by the addition of ACN, and the supernatant was analyzed by HPLC, as described below.

In Vitro Metabolism Studies of RVA and Fibrates by UGTs. Incubations with human recombinant UGTs were performed using the same conditions as described herein for UDPGA-dependent metabolism in human liver microsomes, except that the mixture contained 0.3 mg of UGTs, the concentration of the substrates was 250 μM for both RVA and fibrates. Control incubations using microsomes isolated from the same cell line containing the vector, but without a cDNA insert, also were included. Formation of the glucuronide conjugates of GFZ and fenofibric acid was determined by HPLC, using the same method as described below for SVA, with a UV detection at 272 nm for GFZ and 240 nm for fenofibric acid.

Inhibitory Effect of GFZ on CYP2C8 Activity in Human Liver Microsomes. Activities of CYP2C8 (paclitaxel 6α-hydroxylation) were determined using paclitaxel as a marker substrate (Rahman et al., 1994); over a concentration range of 1 to 50 μM, GFZ (40–250 μM final) was coincubated with the marker substrate and human liver microsomes (0.06 mg of microsomal protein/0.2 ml incubation) before the reaction was initiated with NADPH (1 mM). The incubation was performed at 37°C for 15 min, and the reaction was terminated with the addition of ACN. The formation of 6α-hydroxy-paclitaxel was determined by HPLC, with the following conditions: Waters C18-Symmetry column (150 × 4.6 mm, 5 μm) and a linear gradient of ACN and 5 mM formic acid (35% ACN to 65% ACN in 6 min) and a UV detector set at 230 nm.

Analytical Procedures for Statins and Metabolites. Quantitation of levels of SVA, CVA, AVA, and RVA and their metabolites (statin lactones and oxidative metabolites) in in vitro incubations was performed using HPLC with UV detection and/or by an on-line IN/US detector (PerkinElmerSciex Instruments, Tampa, FL), as described previously (Prueksaritanont et al., 2002a,b). For confirmation purposes and because of better sensitivity, especially for the statin lactones, an LC/MS/MS method was also used for the determination of statin and their metabolite levels. The analytes were separated through a Betasil C18 column (50 × 2.1 mm, 5 μm; Thermo Hypersil, Keystone Scientific Operations, Bellefonte, PA) using a gradient of ACN/water (90:10) and ACN/0.02% acetic acid, pH 4.5 (10:90), and were detected by a PE Sciei API 3000 tandem mass spectrometer with a turbo ionspray interface (PerkinElmerSciex Instruments, Boston, MA). The statin hydroxy acids, their corresponding oxidative metabolites, and the internal standard were detected in the negative ion mode, whereas statin lactones were detected in the positive ion mode. The precursor → product ions monitored were m/z 435 → m/z 319 (SVA); m/z 451 → m/z 335 (3’-hydroxy SVA); m/z 469 → m/z 353.
(dihydrodiol SVA), m/z 419 → m/z 303 (SV), m/z 391 → m/z 275 (β-oxidation product of SVA, B1), m/z 375 → m/z 259 (β-oxidation product of SVA, B2), m/z 347 → m/z 231 (β-oxidation product of SVA, B3), m/z 523 → m/z 175 (B3-glucuronide), m/z 557 → m/z 453 (AVA), m/z 573 → m/z 466 (hydroxy AVA), m/z 541 → m/z 422 (AV), m/z 458 → m/z 396 (CVA), m/z 444 → m/z 382 (demethylated CVA), m/z 474 → m/z 412 (hydroxy CVA), m/z 442 → m/z 345 (CV), m/z 480 → m/z 418 (RVA), m/z 466 → m/z 404 (demethylated RVA), m/z 464 → m/z 258 (RV), and m/z 423 → m/z 321 (the internal standard pravastatin). Due to unavailability of metabolite standards, concentrations of SVA metabolites were derived based on radiochromatograms. However, no attempt was made to estimate the absolute concentrations of metabolites of other statins. To determine the effect of GFZ and other inhibitors on the formation of statin metabolites, peak area ratios between each metabolite of statins and the internal standard obtained in the presence of inhibitors were compared to those obtained without inhibitors. Preliminary studies showed that these peak area ratios were linear upon dilution over the studied range and that various concentrations of GFZ did not affect the ionization signals of the ions monitored. In general, the effect of GFZ quantified based on the LC/MS method was in good agreement with that obtained by HPLC-UV.

Identification of statin metabolites was accomplished by using LC/MS techniques (Rheos 4000 pump; LEAP Technologies, Carrboro, NC; Finnigan MAT LQ C ion trap mass spectrometer, Thermo Finnigan MAT, San Jose, CA). Mass spectral analyses were performed using electrospray ionization in the negative ion mode (for statin glucuronide conjugates and oxidative metabolites) or positive ion mode (for statin lactones). The electrospray ionization voltage was set at 4 kV, with the heated capillary temperature held at 230°C.

An LC-NMR technique was used for identification of the acyl glucuronide conjugate of RVA. All NMR spectra were acquired under stopped-flow conditions, using an Inova (11.7 T/500 MHz) 51-mm narrow-bore spectrometer (Varian Inc., Palo Alto, CA) equipped with a 60-μl flow probe (Varian, Inc.), as described previously (Prueksaritanont et al., 2002a). 1H chemical shifts (in parts per million) are referenced relative to residual CD2HNC resonance at 1.99 ppm.

**Data Analysis.** The effects of GFZ on metabolism of the statins were expressed as percentages of metabolites (statin lactones and other metabolic products) formed in the presence of inhibitor relative to the corresponding values obtained in the absence of inhibitor (control) on the same day. The concentration of GFZ producing a 50% decrease in the metabolism of statins (IC50) was determined using nonlinear regression analysis, as previously described (Prueksaritanont et al., 1999).

**Results**

**Metabolism of SVA in Human Hepatocytes.** Since SVA metabolism in this system has not been reported previously, we first characterized the human hepatocyte metabolism of SVA. Figure 2, A and B, illustrates typical HPLC-UV and radioactivity chromatograms, respectively, derived from an incubate of SVA with human hepatocytes. In this system, major metabolites observed included those typically associated with oxidation, β-oxidation and glucuronidation processes. As was reported previously with SVA and SV in human liver microsomes (Prueksaritanont et al., 1997; 2002b), there were two major oxidative products of SVA, identified based on MS/MS, UV absorption spectra and HPLC retention time as the 3'-hydroxy SVA and the 3',5'-dihydrodiol metabolite ([M − H]− ions at m/z 451 and 469, respectively). In addition, three major β-oxidation products were observed, two of which possessed [M − H]− ions at m/z 391 (B1) and 375 (B2). B1 and B2 have been identified previously as 1'-[5-hydroxy-pentanoic acid] and [1'-pentanoic acid] derivatives of SVA, respectively (Prueksaritanont et al., 2001). The third product (B3) was detected at m/z 347, corresponding to a loss of two additional carbons from B2 and showed a MS/MS spectrum consistent with 1'-propanoic acid derivative of SVA, a product that had undergone an additional cycle of β-oxidation at the hydroxy acid side chain of B2 (data not shown). SV also was detected in the human hepatocyte incubate, but was minimal in the absence of hepatocytes, suggesting that the lactonization of SVA was mediated primarily by an enzymatic reaction. Under the studied conditions, SVA glucuronide ([M − H]+ at m/z 611) was barely detectable, consistent with the finding that the glucuronide conjugate of SVA readily undergoes spontaneous cyclization at physiological pH (Prueksaritanont et al., 2002a). However, a glucuronide conjugate of B3, with a [M − H]− ion of m/z 523 and MS/MS showing a loss of 176 from B3, was observed in low levels for all metabolites detected, formation rates were linear for up to 2-h incubation time.

**Effect of GFZ and Fenofibrate on SVA Metabolism in Human Hepatocytes.** Formation of all three β-oxidation products of SVA (B1, B2, and B3) was minimally affected by GFZ (IC50 >200 μM; Fig. 3, Table 1). As was observed earlier in the liver microsomal system (Prueksaritanont et al., 2002b), GFZ also showed modest inhibitory effect in human hepatocytes on the formation of the two hydroxylated metabolites of SVA (3'-hydroxy and 3',5'-dihydrodiol SVA) known to be mediated primarily by CYP3A (IC50 >200 μM; Fig. 3, Table 1). However, GFZ inhibited the lactonization of SVA (SV) and the glucuronidation of B3, in a concentration-dependent manner (Fig. 3), with IC50 values of ~50 and 15 μM, respectively (Table 1). Unlike GFZ, fenofibrate at the concentration of 75 μM did not appreciably affect any of the metabolic pathways of SVA (Table 2). Control experiments showed that BOA, a known β-oxidation inhibitor (Schulz, 1987), inhibited almost completely the three β-oxidation products of SVA as well as the glucuronide conjugate of B3, whereas only moderately (~55%) inhibited the lactonization of SVA and minimally affected the CYP3A-mediated oxidative metabolites (Table 2). As expected, ketoconazole and troleandomycin, known inhibitors of CYP3A (Newton et al., 1995), inhibited markedly the formation of CYP3A-mediated oxidative but not all other metabolites of SVA (Table 2). Ketoconazole and troleandomycin also inhibited the metabolism of SVA, a known CYP3A substrate, as evident by a marked increase in SV formation (Table 2). Sulfaphenazole, a potent inhibitor of human CYP2C9 (Newton et al., 1995), minimally affected all the metabolic pathways of SVA in human hepatocytes (Table 2).

**Metabolism of Other Statins and Effect of GFZ on Their Metabolism in Human Hepatocytes.** As was observed previously in liver microsomes (Prueksaritanont et al., 1999; 2002b), two hydroxylated metabolites of AVA were detected, and the products of O-
demethylation and hydroxylation of CVA were observed in human hepatocytes. In addition, RVA underwent N-demethylation in human hepatocytes, consistent with a previous report in this system (McTaggart et al., 2001). As was the case for SVA, the lactones of the three statins also were observed in the incubates with human hepatocytes, while the glucuronide products of the parent statins AVA, CVA, and RVA were minimal under the studied conditions. β-Oxidation products of these statins were not monitored in the present study since their identities in human hepatocytes have not been well characterized.

For AVA, CVA, and RVA, GFZ inhibited statin lactonization in a concentration-dependent manner, similar to the observation with SVA (Fig. 4A). The IC₅₀ values for the inhibition of the lactonization of AVA, CVA, and RVA were ~40 to 60 μM (Table 1). As was the case for SVA oxidative metabolism, GFZ minimally affected (IC₅₀ >200 μM) the formation of hydroxylated metabolites of AVA (Fig. 4B; Table 1), known to be mediated primarily by CYP3A (Yang et al., 1996). In the case of CVA oxidation, GFZ markedly inhibited the formation of the hydroxy metabolite (IC₅₀ ~50 μM) but had a much lesser effect on the formation of the demethylated product (IC₅₀ ~200 μM) (Fig. 4B; Table 1). Like the observation with CVA, GFZ also inhibited the oxidative metabolism of RVA (Fig. 4B), with IC₅₀ value of 46 μM (Table 1).

Additional experiments showed that the oxidation of AVA was significantly inhibited by ketoconazole in human hepatocytes (Table 3), consistent with the fact that AVA oxidation is mediated primarily by CYP3A (Yang et al., 1996). As was the case with SV, a significant increase in AV formation in the presence of ketoconazole, a potent CYP3A inhibitor, is also consistent with the fact that AV is a CYP3A4 substrate (Jacobson et al., 2000). However, in the case of CVA, formation of the two oxidative metabolites was slightly or moderately (~20% for the hydroxy CVA and ~50% for demethylated CVA) inhibited by ketoconazole (Table 3). The results suggested that these metabolic pathways of CVA were mediated only in part by CYP3A4, consistent with previous reports (Boberg et al., 1997; Müller, 2000), and that CYP3A4 played a much lesser role in the formation of the hydroxy than the demethylated metabolites of CVA. Sulfaphenazole had a minimal inhibitory effect on the oxidation of AVA and CVA (Table 3), suggesting that CYP2C9 did not play a significant role in their oxidative metabolism in hepatocytes. In the case of RVA, the formation of the demethylated product was significantly inhibited by ketoconazole (~40–50%) and sulfaphenazole (~25 and 45% inhibition from two different subjects) (Table 3). These results suggest a possible involvement of both CYP3A and CYP2C9 in the oxidative metabolism of RVA, which are not consistent with the earlier claim that RVA oxidative metabolism in human hepatocytes is catalyzed primarily by CYP2C9 and not CYP3A4 (McTaggart et al., 2001).

**Glucuronidation of RVA in Human Liver Microsomes.** In an incubate of RVA with human liver microsomes supplemented with UDPGA, two major products with a UV spectrum similar to that of RVA were observed (Fig. 5A). The nonpolar product, which eluted after RVA, was identified as the lactone RV, based on a comparison of UV spectrum and HPLC retention time with the authentic standard. LC/MS/MS studies of the more polar metabolite of RVA showed an [M – H]⁻ ion at m/z 656, which corresponded to an addition of 176 mass units to RVA (m/z 480) (Fig. 5B). In the negative ionization mode, MS³ spectra of this metabolite and RVA were similar; the ion at m/z 480 mainly underwent a neutral loss of 62, yielding a characteristic product ion of m/z 418 (Fig. 5C). These results suggested that the polar metabolite was the glucuronide conjugate of RVA. LC-NMR studies indicated that this conjugate is a β-1-O-acyl glucuronide of RVA. As was the case for SVA, CVA, and AVA (Prueksaritanont et al., 2002a), the anomeric proton of the glucuronide moiety appears at 5.54 ppm as a doublet with a scalar coupling constant of 8.2 Hz, which is indicative of a β configuration. The anomeric proton chemical shift is consistent with an acyl glucuronide rather than an alkyl ether glucuronide at the 3 or 5 position. Relative to the parent RVA, the methylene protons at position 2 (α to the carboxyl group) underwent concomitant downfield shift from 2.37 and 2.44 ppm to 2.51 and 2.58 ppm in the RVA glucuronide. The two metabolic products of RVA were not observed in the absence of UDPGA or liver microsomes, suggesting that they were mediated by UGTs. Based on the study with SVA glucuronide (Prueksaritanont et al., 2002a), RV was likely originated from RVA glucuronide following spontaneous cyclization. The formation rates of the acyl glucuronide conjugate and the lactone of RVA in human liver microsomes were best described by single-enzyme Michaelis-Menten kinetics, with values for apparent Km, Vmax, and resulting intrinsic clearance of 259 μM, 125 pmol/min/mg of microsomal protein, and 0.5 μl/min/mg of microsomal protein.
Effects of fibrates and known inhibitors of metabolizing enzymes on SVA metabolism in human hepatocytes

Results are expressed as percentage of control values (means ± S.D. n = 3–5 hepatocyte preparations), and were obtained following co-incubation of SVA (20 μM) and inhibitors at 37°C for 60 min with human hepatocytes (3 × 10^6 cells/ml).

<table>
<thead>
<tr>
<th>SVA Metabolite</th>
<th>75 μM GFZ</th>
<th>75 μM Fenofibrate</th>
<th>100 μM BOA</th>
<th>1 μM Ketoconazole</th>
<th>50 μM Troleandomycin</th>
<th>20 μM Sulfaphenazole</th>
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<tr>
<td>3'-Hydroxy</td>
<td>86 ± 12</td>
<td>96 ± 20</td>
<td>80 ± 3</td>
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<tr>
<td>Dihydrodiol</td>
<td>84 ± 12</td>
<td>97 ± 11</td>
<td>98 ± 4</td>
<td>28 ± 15</td>
<td>38 ± 2</td>
<td>80 ± 3</td>
</tr>
<tr>
<td>B1</td>
<td>99 ± 18</td>
<td>126 ± 23</td>
<td>6 ± 1</td>
<td>102 ± 15</td>
<td>92 ± 6</td>
<td>120 ± 25</td>
</tr>
<tr>
<td>B2</td>
<td>90 ± 12</td>
<td>120 ± 17</td>
<td>8 ± 3</td>
<td>114 ± 16</td>
<td>100 ± 2</td>
<td>121 ± 26</td>
</tr>
<tr>
<td>B3</td>
<td>108 ± 30</td>
<td>105 ± 20</td>
<td>3 ± 1</td>
<td>111 ± 17</td>
<td>70 ± 18</td>
<td>91 ± 16</td>
</tr>
<tr>
<td>B3-Glucuronide</td>
<td>19 ± 10</td>
<td>108 ± 25</td>
<td>2 ± 2</td>
<td>89 ± 10</td>
<td>95 ± 10</td>
<td>114 ± 30</td>
</tr>
<tr>
<td>SV</td>
<td>41 ± 15</td>
<td>92 ± 31</td>
<td>45 ± 3</td>
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Inhibitory Effect of GFZ on CYP2C8 Activity in Human Liver Microsomes. Inhibitory effects of GFZ on CYP2C8 activity were studied with human liver microsomes. GFZ at 20 μM reduced the activity of CYP2C8. The Ki value of GFZ for CYP2C8 was determined to be 12 μM. The results suggest that GFZ is a competitive inhibitor of CYP2C8.

Identity of UGT Enzyme Catalyzing the Glucuronidation of RVA and Fibrates. Similar to the previous observation with SVA, AVA, and CVA (Prueksaritanont et al., 2002a), both UGT1A1 and UGT1A3 catalyzed the formation of RVA acyl glucuronide conjugate. As was the case in the liver microsomal preparations, formation of the lactone and glucuronidation of RVA was higher for UGT1A1 (20 pmol/min/mg) than for UGT1A3 (5 pmol/min/mg), under the experimental conditions used, and was higher than those observed previously with other statins. UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B7, and UGT2B15, as well as the control microsomes, failed to produce either the glucuronide or the lactone to appreciable extent (≤1 pmol/min/mg).

Studies also were conducted with recombinant UGTs to explore potential differences in the metabolic interaction between GFZ and fenofibrate at the level of individual UGT isoforms. In humans, both fenofibrate and GFZ undergo extensive glucuronidation (Miller and Spence, 1998). Of the six human UGTs examined, UGT1A1, UGT1A3, UGT1A9, and UGT2B7 were capable of catalyzing the glucuronidation of fenofibric acid, the major metabolite of fenofibrate (Fig. 6). As was reported earlier, the glucuronidation of GFZ was mediated by all the UGTs examined except UGT1A10 (Prueksaritanont et al., 2002b). The pattern of UGT isoform selectivity in the glucuronidation of fenofibric acid was different from that of GFZ; UGT1A1 and UGT1A3, the isoforms known to catalyze the glucuronidation of statins, including SVA, appeared to contribute to a lesser degree to the glucuronidation of fenofibric acid, as compared to that of GFZ (Fig. 6).

Inhibitory Effect of GFZ on CYP2C8 Activity in Human Liver Microsomes. Inhibitory effects of GFZ on CYP2C8 activity were determined using the human liver microsomes and GFZ at 20 μM. GFZ selectively inhibited the activity of CYP2C8, with a Ki value of 12 μM. The results suggest that GFZ is a competitive inhibitor of CYP2C8.
Discussion

In this study, we used human hepatocyte, which is a more complete system and closer to in vivo situation than the liver microsomes, as a study model to show effects of fibrates on all major metabolic pathways known to statin hydroxy acids (β-oxidation, glucuronidation, and P450-mediated oxidation). GFZ was not an inhibitor of CYP3A, based on its modest effect, compared to ketoconazole and troleandomycin, on the formation of both the hydroxylated products of SVA. GFZ also was not an inhibitor of β-oxidation since it had a minimal effect, in contrast to BOA, on the three β-oxidation products of SVA but not the glucuronide conjugate of B3. Due to the known instability of SVA glucuronide involving spontaneous cyclization to SV at physiological pH (Prueksaritanont et al., 2002a), SV formation was used as an indicator for SVA glucuronidation. In the present study, the contribution of SVA glucuronidation to SV formation was estimated to be greater than 50%, and the inhibitory activity of GFZ on SVA lactonization was attributed considerably to its effect on SVA glucuronidation. These conclusions were based on the following analysis; in hepatocytes, statin lactonization could be mediated via not only the glucuronidation but also the CoASH-dependent pathway (Prueksaritanont et al., 2001), and SV could also undergo hydrolysis and CYP3A4-mediated oxidation (Prueksaritanont et al., 1997). The relative contribution of SVA glucuronidation to SV formation was estimated based on the percent uninhibited of SV formation in the presence of BOA (−45%; Table 2), coupled with the finding that BOA did not inhibit the CYP3A4-mediated oxidative metabolites of SVA (Table 2). In addition, BOA had a minimal effect on SV hydrolysis in human plasma and liver microsomes and a slight inhibitory effect (~30% inhibition at 100 μM BOA) on the glucuronidation of SVA in human liver microsomes (data not shown). Similarly, GFZ modestly affected the β-oxidation of SVA (Table 1) and did not
affect the hydrolysis of SV (data not shown) and CYP3A4 activity. The marked inhibition of B3-glucuronide, but not B3, by GFZ in the present human hepatocyte study (Fig. 3) also is consistent with the conclusion that GFZ inhibited the glucuronidation process. Interestingly, the finding that fenofibrate had a much lesser effect than GFZ on the glucuronidation-mediated lactonization of SVA agrees with the observation that the glucuronidation of fenofibric acid, unlike GFZ, appeared to be catalyzed primarily by UGT1A9 and UGT2B7, which are not implicated in the glucuronidation of SVA (Prueksaritanont et al., 2002a).

Our results with other statins demonstrated that RVA, like several statin hydroxy acids, also undergoes substantial glucuronidation, suggesting that RVA may be subject to considerable but unrecognized (due to the instability of its acyl glucuronide conjugate) metabolism in vivo in humans. As was the case with SVA, the inhibitory effect of GFZ on the lactonization of these statins in human hepatocytes could also be attributed at least in part to the inhibitory effect of GFZ on the statin glucuronidation, considering that the acyl glucuronides of all statins studied undergo spontaneous cyclization to their respective lactones. Substantiating this proposal is also the fact that GFZ was found to inhibit the statin glucuronidation in human liver microsomes, with IC₅₀ values of ~80 (CVA) to ~300 μM (AVA and SVA) (Prueksaritanont et al., 2002b). It is noteworthy that the IC₅₀ value of GFZ obtained for the lactonization of CVA in this study might underestimate the actual inhibitory effect of GFZ on CVA glucuronidation for two reasons. 1) CV has potential to serve as a substrate of CYP2C8, based on the finding with CVA (Boberg et al., 1997; Mück, 2000), and 2) GFZ is a CYP2C8 inhibitor, as suggested by the present study with paclitaxel. The apparently much higher IC₅₀ values obtained for the glucuronidation of SVA and AVA in the previous study was not due to extensive binding of GFZ to liver microsomal proteins since <40% binding was observed under the employed incubation conditions (data not shown). The exact reason for this discrepancy is not known, but it remains a possibility that it might be related to the disruption of the endoplasmic reticulum membrane by detergents used to activate UGTs in the liver microsomal experiment (Soars et al., 2002) or to potential inhibitory activity of oxidative metabolites of GFZ formed in human hepatocytes.

The present results also suggest that statins differ in their susceptibility to the metabolic interactions with GFZ via the oxidative pathway; GFZ inhibited the oxidative metabolism of CVA and RVA but not of the CYP3A substrates SVA and AVA. Based on the studies with known P450 inhibitors and a known substrate of CYP2C8, the inhibition of CVA oxidation observed could be attributed largely to the inhibitory effect of GFZ on CYP2C8 activity. Interestingly, our data which showed the moderate effect of sulfaphenazole on the demethylation of RVA suggest that the observed inhibition of RVA oxidation could not be due mainly to the inhibitory potential of GFZ on CYP2C9 activity. Although the exact reason for this is not known, a possibility exists that the decreased RVA oxidation might be a consequence of reduced RV following inhibition of RVA glucuronidation by GFZ. This suggestion is based on a hypothesis that RV, but not RVA, undergoes oxidative metabolism. The hypothesis is substantiated by an earlier observation (McTaggart et al., 2001) that the oxidation of RVA was observed in human hepatocytes known to contain both UGTs and P450s but not in human liver microsomes (presumably under oxidative conditions).

Unlike the liver microsomal system, there is a transport process preceding metabolic events in the hepatocyte model. In theory, inhibition of statin uptake could also result in decreased metabolite formation. Considering that both RV and the RVA oxidative metabolite were decreased comparably in the present hepatocyte study, there is a possibility for GFZ to inhibit the uptake of RVA in to hepatocytes. In line with this observation, RVA has recently been shown to be a...
good substrate of organic anion transporter polypeptide (Brown et al., 2001), and GFZ has been shown to be an inhibitor, although not potent, of taurocholate uptake in rat hepatocytes (Sabordo and Sallustio, 1997). In contrast, decreased uptake of SVA, AVA, and CVA into hepatocytes by GFZ is not likely a major cause for the diminished statin lactonization observed since differential effects of GFZ were observed on the oxidation versus the lactonization of the three statins in this system.

Considering that the IC_{50} values of GFZ obtained for the lactonization of all statins studied and for the oxidation of CVA and RVA are lower than the therapeutic peak plasma concentrations of GFZ (up to 250 µM) reported in man (Backman et al., 2000), the present results suggest a potential for clinically significant interactions between fibrates and statins. In fact, the finding that both the lactonization and oxidation of CVA, while only the lactonization of SVA and AVA was markedly inhibited by GFZ, agrees well with the enhanced susceptibility of CVA, compared to SVA or AVA, to interactions with GFZ (Farmer, 2001). For these three statins, metabolism is the major eliminating pathway in humans (Cheng et al., 1994; Le Couteur et al., 1996; Boberg et al., 1998). The significant effect of GFZ observed on both the lactonization and oxidation of RVA, similar to CVA, in the present study suggest that there is also a potential for a pharmacokinetic interaction between the new statin RVA and GFZ. However, since it is presently not known whether these two metabolic pathways contribute appreciably to the in vivo disposition of RVA, the magnitude of this interaction remains to be determined in a clinical study.

Additionally, considering that the exposure to fenofibrate, primarily as fenofibric acid (C_{max} up to 50 µM), is much less than the exposure to GFZ at their respective therapeutic doses (Shepherd, 1994; Rašlova et al., 1997; Backman et al., 2000), the present results suggest that fenofibrate would be less likely than GFZ to inhibit SVA metabolism in humans. In a clinical pharmacokinetic study, fenofibrate did not increase SVA/SA exposure when coadministered with SV (Mercer Research Laboratories, manuscript in preparation), supporting the aforementioned conclusion.

In summary, results from the present investigation suggest that the pharmacokinetic interaction observed between SV and GFZ is not due to the inhibitory effect of GFZ on the β-oxidation of SVA and substantiate our previous finding (Prueksaritanont et al., 2002b) that GFZ interacts with SV via the glucuronidation, and not CYP3A-mediated oxidation of SVA. The present investigation also suggests a potential difference between GFZ and fenofibrate in their ability to alter the pharmacokinetics of statins and among various statins in their susceptibility to pharmacokinetic interactions with GFZ in humans, at the levels of statin glucuronidation-mediated lactonization and non CYP3A4-mediated oxidation. In addition, these results provide a possible explanation for the difference in the pharmacodynamic interactions observed among various statin-fibrate combinations.

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