

Mechanistic Studies on Metabolic Interactions between Gemfibrozil and Statins

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ABSTRACT

A series of studies were conducted to explore the mechanism of the pharmacokinetic interaction between simvastatin (SV) and gemfibrozil (GFZ) reported recently in human subjects. After administration of a single dose of SV (4 mg/kg p.o.) to dogs pretreated with GFZ (75 mg/kg p.o., twice daily for 5 days), there was an increase (~4-fold) in systemic exposure to simvastatin hydroxy acid (SVA), but not to SV, similar to the observation in humans. GFZ pretreatment did not increase the ex vivo hydrolysis of SV to SVA in dog plasma. In dog and human liver microsomes, GFZ exerted a minimal inhibitory effect on CYP3A-mediated SVA oxidation, but did inhibit SVA glucuronidation. After i.v. administration of [¹⁴C]SVA to dogs, GFZ treatment significantly reduced (2–3-fold) the plasma clearance of SVA and the biliary excretion of SVA glucuronide (together with its cyclization product

SV), but not the excretion of a major oxidative metabolite of SVA, consistent with the in vitro findings in dogs. Among six human UGT isozymes tested, UGT1A1 and 1A3 were capable of catalyzing the glucuronidation of both GFZ and SVA. Further studies conducted in human liver microsomes with atorvastatin (AVA) showed that, as with SVA, GFZ was a less potent inhibitor of the CYP3A4-mediated oxidation of this drug than its glucuronidation. However, with cerivastatin (CVA), the glucuronidation as well as the CYP2C8- and CYP3A4-mediated oxidation pathways were much more susceptible to inhibition by GFZ than was observed with SVA or AVA. Collectively, the results of these studies provide metabolic insight into the nature of drug-drug interaction between GFZ and statins, and a possible explanation for the enhanced susceptibility of CVA to interactions with GFZ.

Inhibitors of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase (“statins”) and fibric acid derivatives (fibrates) such as gemfibrozil (GFZ) are used widely for the treatment of hypercholesterolemia and hypertriglyceridemia, respectively (Farnier and Davignon, 1998; Fruchart et al., 1998; Rader and Haffner, 1999; Maron et al., 2000). Because of their complementary lipid-modifying effects, they are frequently prescribed together to treat patients with mixed hyperlipidemia (Shek and Ferrill, 2001). However, there have been reports of an increased risk of myopathy, including rhabdomyolysis, when GFZ and statins are coadministered (Murdoch et al., 1999). The basis of this increased risk of myopathy is not known, but it has been generally accepted that it is primarily of a pharmacodynamic origin because monotherapy with both fibrates and statins also is associated with a low incidence of myopathy. Recently, Backman et al. (2000) and Kyrklund et al. (2001) have demonstrated a pharmacokinetic interaction between GFZ and simvastatin (SV)/lova-

statin (LV) in healthy volunteers, thereby raising the possibility that the increased risk of myopathy with GFZ/statin combinations may have a pharmacokinetic component. Thus, after oral administration to humans of SV or LV (40 mg), GFZ (600 mg b.i.d. for 3 days) caused a minimal change in exposure to SV or LV, but an ~2- to 4-fold increase in the area under plasma concentration-time curve (AUC) and peak plasma concentration (C_{max}) of simvastatin hydroxy acid (SVA) or lovastatin hydroxy acid (LVA), active metabolites of SV or LV, respectively (Backman et al., 2000; Kyrklund et al., 2001). The authors hypothesized that the differential effect of GFZ on the hydroxy acid metabolites versus the lactone forms of SV or LV might be due to the effect of GFZ on non-CYP3A4-mediated metabolism of SVA or LVA. This hypothesis was based primarily on the observation that GFZ did not inhibit the 1'-hydroxylation of midazolam in human liver microsomes, a probe reaction for CYP3A4 activity (Backman et al., 2000), and that potent CYP3A inhibitors have been shown to cause a more pronounced increase in exposure to SV and LV, relative to the increase in their open

This work was conducted at Merck Research Laboratories, West Point, PA.

ABBREVIATIONS: HMG, 3-hydroxy-3-methylglutaryl; GFZ, gemfibrozil; SV, simvastatin; LV, lovastatin; AUC, area under plasma concentration-time curve; SVA, simvastatin hydroxy acid; LVA, lovastatin hydroxy acid; UGT, UDP-glucuronosyltransferase; AVA, atorvastatin; CVA, cerivastatin; UDPGA, UDP-glucuronic acid; HPLC, high-performance liquid chromatography; UGT, UDP glucuronosyltransferase; LC/MS/MS, liquid chromatography-tandem mass spectrometry; RSD, relative standard deviation.

acid levels (Neuvonen and Jalava, 1996; Kantola et al., 1998; Neuvonen et al., 1998). In humans, both SV and LV are cleared primarily by CYP3A-mediated pathways (Wang et al., 1991; Prueksaritanont et al., 1997).

Recently, SVA has been shown to undergo glucuronidation in human liver preparations *in vitro* and in animals *in vitro* and/or *in vivo* (Prueksaritanont et al., 2002). Therefore, the possibility exists that GFZ, which itself undergoes extensive glucuronidation (Okerholm et al., 1976), might serve as a competitive inhibitor of UDP-glucuronosyltransferase (UGT) isoforms and thereby increase the plasma AUC of the active hydroxy acid forms of statins. It should be noted that the metabolism of statins in both animals and humans involves open acid-lactone interconversion via various pathways (Prueksaritanont et al., 2002), including hydrolysis of the lactones by esterases or the newly identified paraoxonases (Vickers et al., 1990; Billecke et al., 2000; Draganov et al., 2000). In principle, therefore, induction of the paraoxonase/esterase-catalyzed hydrolysis of statin lactones to their respective hydroxy acids could also result in an increase in the plasma AUC of statin hydroxy acids and should be considered as a second potential mechanism of the observed GFZ-statin pharmacokinetic interaction.

Unlike SV and LV, all other marketed statins are administered as the pharmacologically active hydroxy acid forms. In humans, P450-mediated oxidative metabolism, catalyzed primarily by CYP3A (atorvastatin, AVA) and CYP2C subfamilies (cerivastatin, CVA, and fluvastatin), has been regarded as a major pathway of biotransformation of these drugs (Corsini et al., 1999; Igel et al., 2001). However, recent studies from this laboratory (Prueksaritanont et al., 2002) have provided evidence that glucuronidation is a common

metabolic pathway for the hydroxy acid forms of several statins in humans and animals. Considering that an increased risk of myopathy was observed for all statins when coadministered with GFZ, there may also be a potential for pharmacokinetic interactions between GFZ and these statins via metabolic interactions at the level of glucuronidation, non-CYP3A-mediated oxidation, or lactone hydrolysis.

Based on the above-mentioned considerations, we set out to explore possible underlying mechanisms for the pharmacokinetic interaction between statins and GFZ, using SV as a model compound. The investigation focused primarily on metabolic interactions mediated at the level of hydrolysis, glucuronidation, and oxidation of SVA, and involved *in vivo* studies in dogs and *in vitro* metabolism studies in dogs and humans. The dog was chosen as an animal model because our preliminary studies indicated that SV and SVA exhibited similar pharmacokinetics in dogs and humans and because GFZ is known to undergo extensive glucuronidation in dogs, as it is in humans (Okerholm et al., 1976). In addition, *in vitro* studies were conducted to compare different statins for their metabolic interaction potentials with GFZ. AVA and CVA were chosen for the latter study because they undergo extensive metabolism (Le Couteur et al., 1996; Boberg et al., 1998), via a glucuronidation pathway similar to SVA (Prueksaritanont et al., 2002), and oxidation by P450 isoforms similar to and different from SVA, respectively.

Experimental Procedures

Materials. SV, SVA, [^{14}C]SVA with a specific activity of 50 $\mu\text{Ci}/\mu\text{mol}$ (Fig. 1), [$^{13}\text{CD}_3$]SV, and [$^{13}\text{CD}_3$]SVA were synthesized at Merck (Rahway, NJ). AVA and CVA (Fig. 1) were extracted from commer-

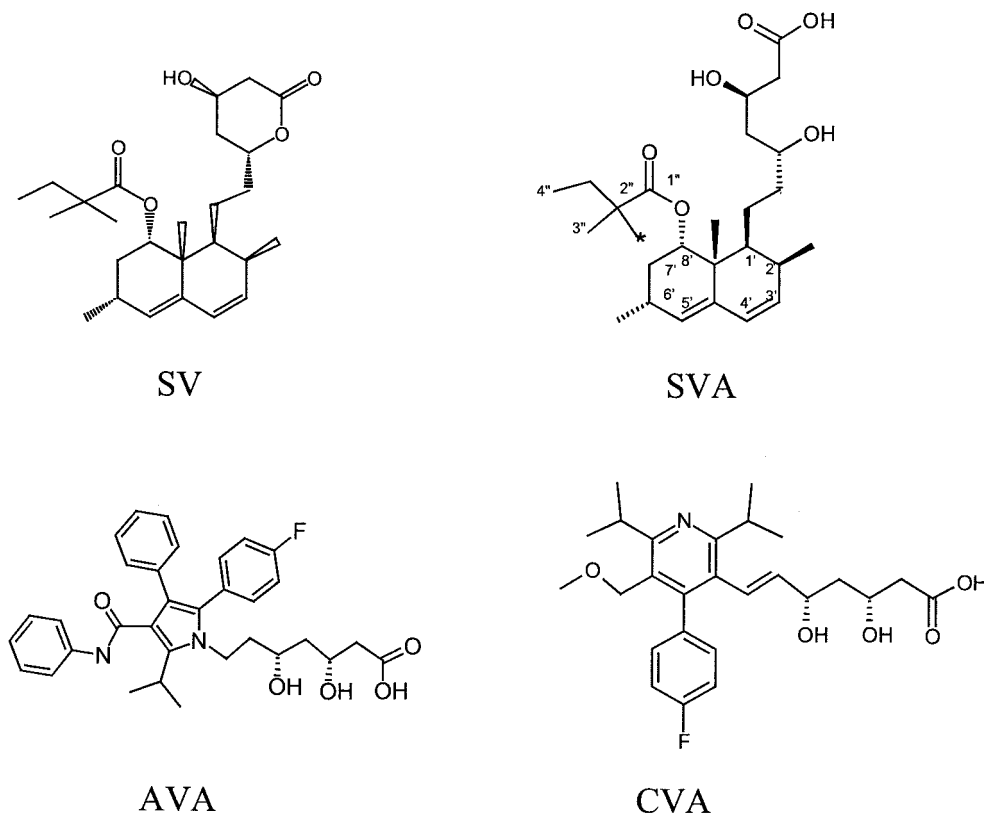


Fig. 1. Structure of SV, SVA, AVA, and CVA. *, position of the ^{14}C label.

cial sources and their identity and purity confirmed by infrared and NMR spectroscopy (Prueksaritanont et al., 1999). Brij 58, alamethicin, GFZ, clofibrac acid, and UDPGA were obtained from Sigma-Aldrich (St. Louis, MO). Sulfaphenazole and ketoconazole were purchased from Ultrafine (Manchester, England) and Research Diagnostics (Flanders, NJ), respectively. All other reagents were analytical or HPLC grade. Human recombinant UGTs were obtained from GENTEST (Woburn, MA) and Panvera (Madison, WI). Human liver microsomes were purchased from Xenotech (Kansas City, KS) and GENTEST, whereas those from beagle dogs (9–11 kg) were prepared in house as described previously (Prueksaritanont et al., 1997) and were pooled from four to six animals before use.

In Vivo Studies. All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use Committee. The in vivo studies were carried out in a crossover manner, with at least a 7-day washout period. Beagle dogs ($n = 4-5$) were pretreated with either vehicle (0.5% methylcellulose suspension) or GFZ (75 mg/kg p.o., in 0.5% methylcellulose suspension), twice daily for 5 days. The animals were fasted overnight before SV administration on day 5. On the morning of day 5, SV was administered at 4 mg/kg p.o. to dogs and blood samples were collected at 0, 30, 60, 90, 120, 180, 240, 360, 480, 600, 720, and 1440 min after SV administration. Plasma samples were separated immediately at 10°C and kept frozen at -20°C. On the morning of day 5 after treatment with vehicle or GFZ but before SV administration, blood samples (~15 ml/dog) were collected for ex vivo studies (see below).

Similar studies were conducted in dogs after i.v. administration of SVA, instead of p.o. administration of SV. In the first period, [¹⁴C]SVA or SVA was infused via a femoral vein at 1.2 mg/kg for 20 min to dogs with or without bile duct cannulation, respectively. In period II, the same animals were pretreated with GFZ (75 mg/kg p.o., twice daily) for 5 days before administration of [¹⁴C]SVA (1.2-mg/kg i.v. infusion for 20 min). For each period, blood samples were collected after SVA administration at 0, 10, 20 (end of i.v. infusion), 30, 50, 70, 90, 120, 180, 240, 360, 480, 600, and 1440 min. Plasma samples were separated immediately at 10°C and kept frozen (-20°C) before analysis. In the bile duct-cannulated animals, bile was collected in a bag containing 0.5 M ammonium acetate buffer, pH 4.5 (~10% of total bile volume), continuously every hour over a period of 10 h, and then during the 10- to 24-h period. The bile samples were frozen immediately on dry ice and kept at -20°C before analysis.

Ex Vivo Hydrolysis of SV. Blood samples from vehicle- and GFZ-pretreated animals were collected on the morning of day 5, approximately 15 min after administration of the last dose of vehicle or GFZ, and were centrifuged at 10°C. Plasma samples were used on the same day for ex vivo measurement of SV hydrolysis. SV was added to 0.4 ml of dog plasma, to obtain 0.1 and 10 μM final concentrations, and the plasma sample was incubated at 37°C for 2 h. At various times during the incubation, the reaction was stopped by the addition of 0.3 ml of ice-cold 0.1 M ammonium acetate buffer, pH 4.5. The samples then were extracted immediately using the liquid-liquid extraction method described below and analyzed for SV and SVA by LC/MS/MS. Control incubations were performed in plasma from animals that had not been pretreated with either vehicle or GFZ and in 0.1 M sodium phosphate buffer, pH 7.4.

In Vitro Metabolism Studies. All incubations were performed in triplicate. Statin glucuronidation was assayed in 0.3 ml of an incubation mixture, containing 0.45 mg of human or dog liver microsomal proteins, preincubated with 0.045 mg of Brij 58 (or 0.025 mg of alamethicin) for 15 min; 20 mM MgCl₂; 5 mM UDPGA; and 0.05 M Tris buffer, pH 7.0. For oxidative metabolism studies, a typical incubation mixture (in a final volume of 0.5 ml) contained 0.05 to 0.25 mg of liver microsomal protein, 50 μmol of sodium phosphate buffer, pH 7.4, 5 μmol of MgCl₂, and 0.5 μmol of NADPH. Five-microliter aliquots of 50% aqueous acetonitrile (control) or various concentrations of GFZ in 50% aqueous acetonitrile were coinubated with the statin substrates. Statins were prepared in 50% aqueous

acetonitrile solutions from which 5-μl aliquots were taken to generate final concentrations of 10 to 20 μM; these values were below or close to the respective K_m value for each statin. Incubations were conducted at 37°C and were terminated after 10 min (SVA and AVA) or 18 min (CVA) in studies of oxidative metabolism, or after 45 min in studies of glucuronidation, by the addition of acetonitrile. The rates of formation of all metabolites of statins were linear during these incubation periods. The acetonitrile extracts were evaporated to dryness and reconstituted for analysis by HPLC with UV detection (see below).

To identify the UGT isoforms responsible for the glucuronidation of GFZ and SVA, incubations were performed with various human recombinant UGTs using the same conditions as described herein for human liver microsomes, except that the mixture contained a UGT (0.3 mg of protein) and GFZ (250 μM, final concentration) or SVA (100 μM, final concentration), and was incubated in the absence of inhibitors for up to 60 min. Control incubations using microsomes isolated from the same cell line, containing the vector but without a cDNA insert, also were included.

Analytical Procedures for Statins and Metabolites. SVA, CVA, and AVA and their metabolites in incubation mixtures were analyzed using published HPLC methods (Prueksaritanont et al., 1999), with minor modifications. In brief, samples, held in an autosampler set at 5°C, were chromatographed on a Zorbax C₁₈ column (150 × 4.6 mm, 5 μm; Waters, Milford, MA), preceded by a C₁₈ guard column, with a linear gradient of acetonitrile and 10 mM ammonium acetate, pH 4.5. The eluate was monitored by UV absorption at 240 nm (SVA and AVA) or 280 nm (CVA). Due to the absence of authentic standards for glucuronide conjugates of statins, quantitation of these metabolites in the in vitro incubation mixtures was accomplished using standard curves for their respective parent statins, assuming identical extraction recoveries and extinction coefficients between the parent drug and the corresponding glucuronide conjugate. For the three statins, standard curves showed satisfactory linearity and precision (<15% coefficient of variation).

Levels of total radioactivity in bile and urine samples were determined directly by liquid scintillation counting (Packard Instrument Company, Inc., Downers Grove, IL). Concentrations of SV, SVA glucuronide, and SVA in bile samples were estimated based on total radioactivity and metabolite-profiling studies (using the above-mentioned HPLC conditions with an on-line IN/US β-RAM radioactivity detector).

For plasma samples, quantitation of SV and SVA was accomplished using LC/MS/MS as described previously (Zhao et al., 2000). In brief, SV and SVA were extracted at 4°C from dog plasma using Chem Elut cartridges (Varian, Palo Alto, CA), chromatographed using a Kromasil C₁₈ column (2 × 50 mm, 4 μm; Keystone Scientific, Bellefonte, PA) with a mobile phase of acetonitrile/ammonium acetate (75:25, 1 mM, pH 4.2), and detected by turbo ionspray on a PE Sciex (Ontario, Canada) API 365 tandem mass spectrometry with within-run polarity switching between negative ion (for SVA) and positive ion (for SV) monitoring. Stable isotope-labeled analogs of the compounds of interest ([¹³CD₃]SV and [¹³CD₃]SVA) were used as internal standards. The precursor/product ion transitions monitored were m/z 439.2 (M - H)⁻→319.1 (for [¹³CD₃]SVA), m/z 435.2 (M - H)⁻→319.1 (for SVA), m/z 423.1 (M + H)⁺→199.1 (for [¹³CD₃]SV), and m/z 419.1 (M + H)⁺→199.1 (for SV). The linear calibration range was 0.1 to 100 ng/ml. Interday and intraday precision (%RSD) and accuracy were <10% RSD and 98 to 106% for both SV and SVA. The interconversion between SV and SVA during sample preparation was found to be ≤0.2% for SVA→SV and ≤0.3% for SV→SVA. Both analytes were found to be stable after three cycles of freeze (-70°C)/thaw (4°C) and after 24 h under bench-top storage condition (4°C) in dog plasma, and after 24 h after reconstitution in HPLC mobile phase under autosampler storage condition (4°C).

A liquid-liquid cartridge extraction-LC/MS/MS method was used for the determination of GFZ in dog plasma. The analyte was extracted from 0.5-ml aliquots of dog plasma using the Chem Elut

cartridges and methyl *tert*-butyl ether. The analyte and internal standard (clofibrilic acid) were separated through a Metasil Basic column (50 × 2 mm., 3 μm; Metachem Technologies, Torrance, CA) using a mobile phase of 70:30 (v/v) acetonitrile/ammonium acetate (1 mM; pH 5.0), and were detected by tandem mass spectrometry with a turbo ionspray interface. Both GFZ and the internal standard were detected in the negative ion mode. The precursor → product ions monitored were *m/z* 248.9 → *m/z* 121.0 (for GFZ) and *m/z* 213.0 → *m/z* 126.8 (for clofibrilic acid). The method showed good reproducibility with an inter- and intra-assay precision of <10% (%RSD), as well as excellent accuracy with an inter- and intra-assay accuracy between 99 and 101%. This method has a lower limit of quantitation of 1.0 ng/ml with a linear calibration range from 1.0 to 250 ng/ml.

The concentrations of active and total HMG-CoA reductase inhibitors in plasma were determined according to an enzymatic assay described previously (Rogers et al., 1999). The enzyme assay measures the sum total of HMG-CoA reductase inhibitory activity from all active metabolites, using SVA as a standard. "Active" and "total" inhibitors refer to activity measured before and after base hydrolysis, respectively, of plasma samples. The values for active inhibitors represent the net inhibitory activity that is inherently present in plasma, whereas those for total inhibitors provide an estimate of the total inhibitory activity potential that is present in plasma because the active hydroxy acid metabolites coexist with their inactive lactones.

Identification of statin metabolites was accomplished by using liquid chromatography (HP-1050 gradient system; Hewlett Packard, San Fernando, CA)-tandem mass spectrometry (LCQ ion trap mass spectrometer; Finnigan-MAT, San Jose, CA) as described previously (Prueksaritanont et al., 1999).

Data Analysis. The AUC was calculated from time 0 to the last detectable sampling time using the linear trapezoidal rule. The peak plasma concentration (C_{\max}) and the time at which this peak occurred (T_{\max}) were determined by observation. Apparent clearance values for SVA were calculated as the i.v. dose divided by the AUC from time 0 to infinity ($AUC_{0-\infty}$).

The inhibitory effects of GFZ on the *in vitro* metabolism of statins were expressed in terms of the turnover of the substrates to products in the presence of GFZ relative to the corresponding values obtained in the absence of GFZ (control) on the same day. Due to the apparent instability of the acyl glucuronides of the three statins studied, the sum of the acyl glucuronide and the corresponding lactone was used to calculate rates of total glucuronidation for each statin (Prueksaritanont et al., 2002). For the oxidative pathway, formation rate of each major oxidative metabolite (CVA) or the sum of major metabolites (SVA and AVA) was used to calculate the oxidation rate of statins. Mean values from triplicate incubations were used for all calculations. The concentration of inhibitors producing a 50% decrease in the metabolism of statins (IC_{50}) was determined using nonlinear regression analysis, as described previously (Prueksaritanont et al., 1999).

Results

Effects of GFZ on Pharmacokinetics of SV in Dogs.

After oral administration of SV (4 mg/kg, single dose) to dogs pretreated with vehicle, the plasma profiles of SV and SVA and the corresponding pharmacokinetic parameters were comparable with one another (Fig. 2, A and B; Table 1). GFZ treatment caused increases in both the AUC_{0-12h} and C_{\max} of SVA by 3.8- and 2.6-fold (Fig. 2A; Table 1), but led to decreases in the AUC_{0-12h} and C_{\max} of SV by about 40 and 70%, respectively (Fig. 2B; Table 1). Marked intersubject variations in the fold increase of the AUC of SVA (range 1.0- to 9.3-fold) relative to that of SV (range 0.3- to 1.0-fold) were observed. Similar results were obtained using values for $AUC_{0-\infty}$ (data not shown). In addition, in GFZ-pretreated

dogs, there was a significant increase in T_{\max} values for both SVA and SV (Fig. 2, A and B; Table 1).

The systemic exposure to active HMG-CoA reductase inhibitors, which measures the sum total HMG-CoA reductase inhibitory activity resulting from SVA and all other active acid metabolites of SV (Cheng et al., 1994; Prueksaritanont et al., 1997), also was measured in the present study because this value is believed to be relevant to the adverse effects of this class of therapeutic agents (Rogers et al., 1999). As with plasma concentrations of SVA, apparent increases, although less pronounced, in plasma HMG-CoA reductase inhibitory activity were observed in GFZ-treated dogs. GFZ caused 2.2-fold (range 0.7- to 4.8-fold) and 1.6-fold (range 0.5- to 3.1-fold) increases in active and total inhibitors in plasma, respectively (Fig. 2, C and D; Table 1). In both the vehicle- and GFZ-treated animals, it was estimated that the relative contribution of SVA to the overall active inhibitors was low (~20%), supporting the presence of appreciable quantities of active metabolites of SV (other than SVA) in the systemic circulation. Similar to plasma levels of SVA and SV, the T_{\max} values for both the total and active inhibitors also increased significantly from ~1 h in control animals to ~3 h in GFZ-treated dogs (Fig. 2, C and D; Table 1).

At the GFZ dose used in these studies, plasma levels of GFZ reached a C_{\max} of $446 \pm 66 \mu\text{M}$ (range 332–492 μM) at ~1 h, and remained above 100 μM for about 5 h after dosing. Unlike the pharmacokinetics of SVA, there were only modest intersubject variations (<2-fold) in the GFZ plasma profiles. Values for AUC_{0-12h} of GFZ ranged from 1173 to 1664 μM·h, with a mean value of $1441 \pm 176 \mu\text{M}\cdot\text{h}$. These values were ~2-fold higher than the corresponding values reported after administration of 600 mg of GFZ b.i.d., to humans (Backman et al., 2000).

Ex Vivo Hydrolysis of SV in Dog Plasma. Studies were conducted *ex vivo* to examine whether the observed increase in SVA plasma levels in GFZ-treated dogs was due to induction of an enzyme(s) mediating the hydrolysis of SV. At SV concentrations of 0.1 and 1 μM, the rate of conversion of SV to SVA in plasma derived from vehicle-treated animals was about 20%/h (Table 2). Control experiments using pH 7.4 buffer indicated modest (<5%/h) chemical conversion of SV to SVA (Table 2), suggesting that the SV hydrolysis observed in dog plasma primarily was mediated enzymatically. No apparent increase in this rate of *ex vivo* hydrolysis was observed in plasma obtained from GFZ-treated animals (Table 2). In fact, the rate of SV to SVA conversion in plasma from dogs pretreated with GFZ seemed to be slightly lower than that in dogs pretreated with vehicle. However, a similar trend also was observed with plasma obtained from dogs without any pretreatment (Table 2), suggesting that this decrease probably was due to day-to-day variations in SV hydrolysis, and not to GFZ pretreatment.

Effect of GFZ on Glucuronidation and Oxidation of SVA in Dog and Human Liver Microsomes. As was observed previously, SVA underwent glucuronidation and lactonization in the presence of dog and human liver microsomes fortified with UDPGA (Prueksaritanont et al., 2002). In the presence of NADPH, SVA underwent oxidation in these preparations to afford at least two metabolic products, with UV and MS/MS characteristics indicative of metabolites formed by hydroxylation (3'-hydroxy; major product) and dehydrogenation (6'-exomethylene) processes, similar to

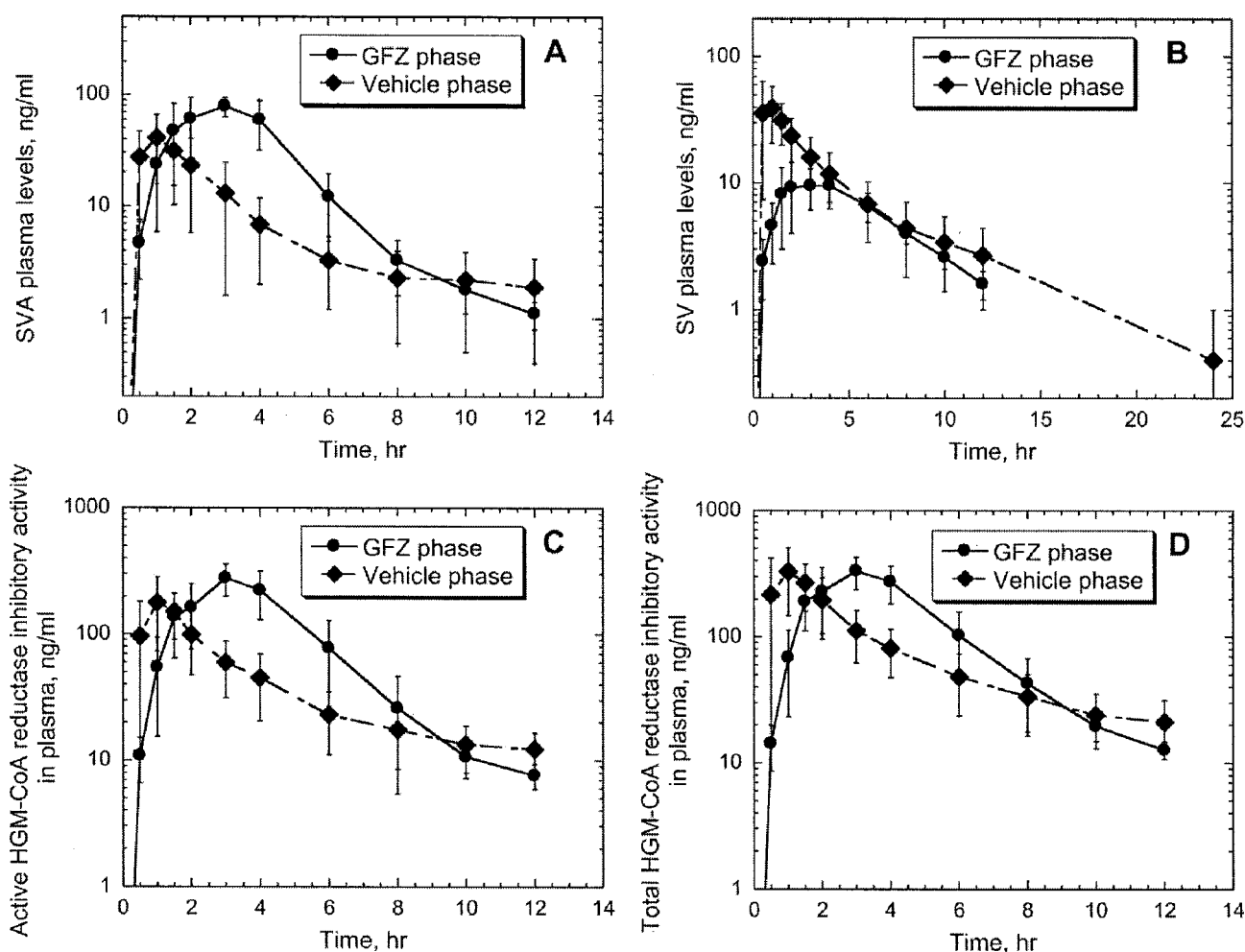


Fig. 2. Plasma profiles of SVA (A), SV (B), active HGM-CoA reductase inhibitor activity (C), and total HGM-CoA reductase inhibitor activity (D) after SV administration (4 mg/kg p.o., single dose) to dogs ($n = 5$) pretreated with vehicle or GFZ (75 mg/kg p.o., twice daily for 5 days). Results are expressed as means \pm S.D. ($n = 5$).

those observed previously for the major oxidative metabolites of SV (Prueksaritanont et al., 1997). In dog liver microsomes, GFZ caused marked inhibition of the glucuronidation of SVA ($IC_{50} = 195 \mu M$), but had a minimal effect on the formation of both oxidative metabolites (IC_{50} of $\sim 1000 \mu M$) (Fig. 3A). Similar effects of GFZ also were observed in human liver microsomes (Fig. 3B), where GFZ had a more pronounced

effect on the glucuronidation of SVA ($IC_{50} = 354 \mu M$) than on the oxidative pathways ($IC_{50} > 800 \mu M$). In human liver microsomes, the effect of GFZ on the glucuronidation of SVA seemed to be due to competitive inhibition (data not shown), with apparent K_i values of $\sim 400 \mu M$, which is in a comparable range to the apparent K_m value observed previously (Prueksaritanont et al., 2002). Control experiments showed

TABLE 1

Pharmacokinetic parameters after oral administration of SV (4 mg/kg) to dogs pretreated with vehicle or GFZ (75 mg/kg p.o.) twice daily for 5 days

Results represent means \pm S.D., $n = 5$.

Compound Measured	Vehicle Phase			GFZ Phase			Ratio (Range) GFZ/Vehicle	
	AUC_{0-12h}	C_{max}	T_{max}	AUC_{0-12h}	C_{max}	T_{max}	AUC	C_{max}
	ng/ml · h	ng/ml	h	ng/ml · h	ng/ml	h		
SV	136 \pm 54	47.5 \pm 26	0.8 \pm 0.3	66 \pm 16 ^a	11.3 \pm 3.9 ^b	3.0 \pm 1.0 ^b	0.6 \pm 0.3 (0.3–1.0)	0.3 \pm 0.2
SVA	108 \pm 63	43.8 \pm 26	1.0 \pm 0.4	288 \pm 108 ^a	80.8 \pm 17 ^a	3.0 \pm 0.7 ^b	3.8 \pm 3.2 (1.0–9.3)	2.6 \pm 2.0
Total inhibitor	1008 \pm 454	328 \pm 178	1.1 \pm 0.2	1372 \pm 355	338 \pm 75	3.2 \pm 0.4 ^b	1.6 \pm 1.0 (0.5–3.1)	1.4 \pm 0.9
Active inhibitor	534 \pm 258	182 \pm 102	1.2 \pm 0.3	1066 \pm 342 ^a	289 \pm 58	3.2 \pm 0.4 ^b	2.2 \pm 1.6 (0.7–4.8)	2.0 \pm 1.3

^a Statistically significant difference from control ($p < 0.08$).

^b Statistically significant difference from control ($p < 0.05$).

TABLE 2

Ex vivo hydrolysis of SV to SVA in dog plasma and buffer

Incubations were performed, in duplicate or triplicate, using plasma from dogs pretreated with vehicle (period I) or GFZ (period II) (treatment group, $n = 5$), and 0.1 and 1 μM SV, at 37°C for 60 min. Control hydrolysis experiments also were conducted on the same days in plasma from a different group of dogs without prior treatment (untreated group, $n = 3$), or in buffer. Results (percentage of SVA formed/h) are means \pm S.D. ($n = 3-5$).

Sample	Animal	0.1 μM SV		1 μM SV	
		Period I	Period II	Period I	Period II
Plasma	Treatment group ($n = 5$)	21.9 \pm 1.7	18.7 \pm 0.9	21.4 \pm 2.7	18.3 \pm 2.5
	Untreated group ($n = 3$)	20.4 \pm 0.4	16.7 \pm 0.3	19.0 \pm 0.5	18.1 \pm 2.4
Buffer		2.3 \pm 0.4	2.5 \pm 0.3	4.0 \pm 0.4	3.5 \pm 0.3

that the formation of the oxidative metabolites of SVA were inhibited by >80% by 25 μM troleanomycin and 1 μM ketoconazole, known CYP3A inhibitors (Table 3), but were minimally affected by 10 μM sulfaphenazole and 25 μM quinidine, potent CYP2C9 and CYP2D6 inhibitors, respectively (data not shown). Interestingly, under the experimental conditions used in these studies, GFZ was metabolized rapidly ($\geq 70\%$ at the end of the incubation) in the presence of UDPGA, but minimally in the presence of NADPH. Thus, it is possible that in both dog and liver microsomes, the IC_{50} (and K_i) values for the inhibition of statin glucuronidation by GFZ might be overestimated.

Effects of GFZ on SVA Metabolism in Vivo in Dogs. To substantiate the above-mentioned in vitro findings, the effects of GFZ on the metabolism of SVA were studied in vivo. After i.v. administration of SVA to dogs, SVA plasma levels declined rapidly, with plasma clearance and $t_{1/2}$ values of 19 ± 2 ml/min/kg and approximately 1 h, respectively (Fig. 4A). Treatment with GFZ caused a significant decrease (~ 2 -fold; $p < 0.05$) in the plasma clearance of SVA (9 ± 3 ml/min/kg). Because metabolites of SVA, including SVA glucuronide and SV, were shown to be eliminated primarily via biliary excretion in dogs (Prueksaritanont et al., 2002), only bile samples were collected in the present study. After i.v. administration of [^{14}C]SVA to dogs pretreated with vehicle or GFZ, the total radioactivity recovered in bile and urine was comparable (74 ± 8 and $67 \pm 8\%$ of the administered dose for the control and GFZ-treated animals, respectively). However, the biliary excretion of SVA glucuronide and SV was decreased by about 2- and 3-fold, respectively, in dogs pretreated with GFZ, compared with control animals (Fig. 4B). The sum of the biliary recovery of SVA glucuronide and SV accounted for $\sim 34\%$ of the i.v. dose, and was decreased ~ 3 -fold by GFZ pretreatment. In contrast, formation of a major oxidative metabolite of SVA, with a similar HPLC retention time, UV and LC-MS/MS characteristics to the 3'-hydroxy metabolite

observed in the above-mentioned liver microsomal study, was increased slightly by GFZ pretreatment (Fig. 4B). In the dog bile, the 6'-exomethylene product of SVA was present only in low levels. Values for the formation clearance of SVA glucuronide and SV, estimated as the product of the fraction excreted in bile and the SVA plasma clearance were decreased ~ 2 - to 5-fold, from 3.8 and 2.7 ml/min/kg in control animals to 7.0 and 0.5 ml/min/kg in GFZ-pretreated dogs, respectively. The formation clearance of the hydroxylated metabolite was decreased only slightly (1.9 ml/min/kg in control group and 1.5 ml/min/kg in GFZ-treated animals).

Identity of UGT Enzymes Catalyzing Glucuronidation of GFZ and SVA. Studies were conducted with recombinant UGTs to explore the metabolic interaction between SVA and fibrates at the level of individual UGT isoforms. Of the six human UGTs examined, UGT1A1, 1A3, 1A9, 2B7, and 2B15 were capable of catalyzing the glucuronidation of GFZ (Fig. 5). The glucuronidation of SVA was mediated by UGT1A1, 1A3, and 1A10 (Fig. 5). The involvement of UGT1A1 and 1A3 in the glucuronidation of SVA has been demonstrated recently (Prueksaritanont et al., 2002). The glucuronidation of GFZ and SVA therefore is catalyzed by at least two common human UGT isozymes, UGT1A1 and UGT1A3 (Fig. 5).

Effect of GFZ on Glucuronidation and Oxidation of Other Statins in Human Liver Microsomes. The potential for GFZ to interact with other statins at the level of metabolism was investigated using in vitro approaches with human liver preparations. As with SVA, both CVA and AVA underwent glucuronidation/lactonization and oxidative metabolism in human liver microsomes supplemented with UDPGA and NADPH, respectively. In liver microsomes supplemented with NADPH only, two major metabolites of each statin were observed. Based on previous studies (Boberg et al., 1997; Prueksaritanont et al., 1999), these metabolites

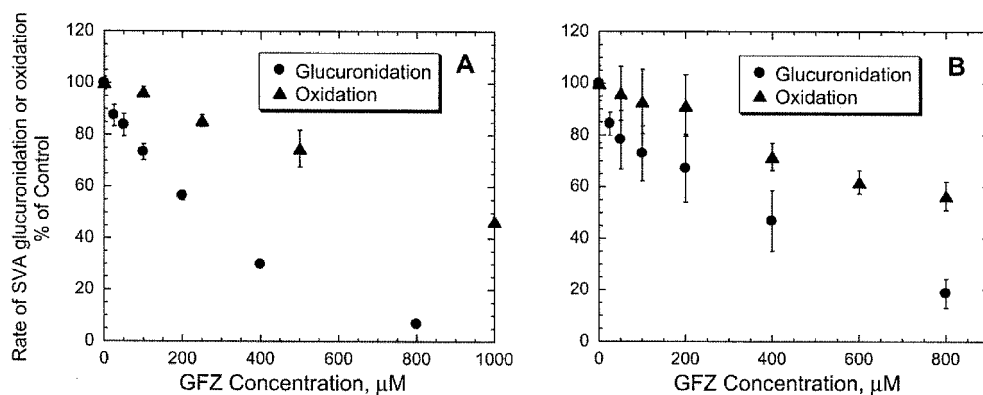


Fig. 3. Effect of GFZ on the glucuronidation and oxidation of SVA in dog (A) or human (B) liver microsomes. Results are expressed as the percentage of control values (means \pm S.D. of triplicate determinations) and were obtained after coinubation of SVA, GFZ, and UDPGA (glucuronidation) or NADPH (oxidation) at 37°C for 45 min (glucuronidation) or 10 min (oxidation) with liver microsomal preparations. Rates of glucuronidation were estimated from the sum of SVA glucuronide and SV, and rates of oxidation were calculated using the sum of the 3'-hydroxy and 6'-exomethylene metabolites of SVA.

TABLE 3

Inhibitory effects of GFZ (IC_{50} , μM) and ketoconazole (percentage of inhibition at 1 μM concentration) on glucuronidation and oxidation of statins in human liver microsomes

Incubations were performed in triplicate for each concentration of inhibitors, and involved coinubation of GFZ or ketoconazole with statins (10 μM) in the presence of NADPH (oxidation) or UDPGA (glucuronidation).

		Gemfibrozil	Ketoconazole
		IC_{50}	Inhibition
		μM	%
SVA	Glucuronidation	354	
	Oxidation	>800	>85
AVA	Glucuronidation	316	
	Oxidation	>750	>90
CVA	Glucuronidation	82	
	Oxidation (M1)	87	68
	Oxidation (M2)	220	~50

likely were two hydroxylated products of AVA and hydroxylated (M1) and *O*-demethylated (M2) metabolites of CVA.

As was noted with SVA, formation of AVA glucuronide was more susceptible to inhibition by GFZ than was the two oxidative metabolites of this statin (Fig. 6A); the IC_{50} values were 314 and >750 μM for the glucuronidation and oxidation reactions, respectively (Table 3). In contrast, the formation of both CVA glucuronide and oxidative metabolites of CVA was markedly inhibited by GFZ (Fig. 6B). In fact, the inhibitory potency of GFZ on CVA glucuronide formation (IC_{50} = 82 μM) was comparable with that on the generation of oxidative metabolite M1 (IC_{50} = 87 μM), and only slightly higher than that on M2 (IC_{50} = 220 μM) (Table 3). These IC_{50} values for CVA were much lower than those for the corresponding metabolic pathways for SVA and AVA (Table 3). Under the present in vitro incubation conditions, both oxidative metabolites of AVA were markedly inhibited (>90%) by the potent CYP3A inhibitor ketoconazole (1 μM). In contrast, ketoconazole, at 1 μM , inhibited the formation of the oxidative metabolites of CVA by less than or approximately 50% (Table 3). These results were consistent with previous reports that in humans, oxidative metabolism of AVA is catalyzed primarily by CYP3A4 (Yang et al., 1996), whereas that of CVA is mediated only in part by CYP3A4 (Boberg et al., 1997). For both statins, the potent CYP2C9 inhibitor sulfaphenazole (10 μM) had a minimal effect on their oxidative metabolism (data not shown).

Discussion

The primary goal of the present investigation was to obtain insight into the mechanism of the clinical pharmacokinetic interaction between SV and GFZ. The results of this study suggest that GFZ has the potential to modulate the phar-

macokinetics of SVA by inhibition of SVA glucuronidation, a previously unrecognized pathway for elimination of the hydroxy acid forms of various statins. Furthermore, it seems that various statins may exhibit different degrees of susceptibility to the inhibitory effects of GFZ on metabolic clearance by glucuronidation and oxidation pathways. The evidence to support these conclusions was derived from a series of complementary in vivo pharmacokinetic studies using the dog as an animal model, and in vitro metabolism experiments in dog and human liver microsomes.

The results of the in vivo studies indicate that the dog is an appropriate animal model for humans for investigations of SV-GFZ interactions because the differential effects of GFZ on SVA and SV pharmacokinetics (increase in only SVA, but not SV levels), as well as the marked intersubject variability in the effect of GFZ on the AUC of SVA were similar to those reported recently in humans (Backman et al., 2000). Although the magnitude of the effect of GFZ on the AUC of both SVA (280% increase in dogs versus 185% increase in humans) and SV (40% decrease in dogs versus minimal change in humans) was greater in dogs than in humans, it was apparent that GFZ affected primarily SVA, and had a minimal effect on SV plasma levels, in both species. The apparent increase in T_{max} for both SVA and SV after administration of GFZ in the present dog study suggested a possible GFZ-mediated delay in the absorption of SV. Although such an effect of GFZ could not be ruled out, this hypothesis, although consistent with the decrease in the AUC of SV, is not consistent with the observed increase in plasma levels of SVA. However, considering that SV and SVA are relatively high-clearance compounds in dogs (Vickers et al., 1990), there is a possibility that GFZ might cause a delay in the T_{max} of SV and SVA by influencing the metabolism of SV/SVA during first pass.

As outlined in the Introduction, the differential effect of GFZ on SV and SVA could result from either induction of SV hydrolysis or inhibition of SVA metabolism by GFZ. The results of the ex vivo hydrolysis experiment effectively eliminated the former possibility. The finding that, in dog liver microsomes, GFZ was a more potent inhibitor of UGT enzymes than the P450 system suggests that the observed increase in the AUC of SVA in dogs treated with GFZ was not due to inhibition of hepatic SVA oxidation, but more likely was due to inhibition of the hepatic glucuronidation of SVA. This conclusion was supported by data from the in vivo experiments in dogs, which showed that GFZ caused a significant decrease in both the plasma clearance of SVA and the biliary excretion or formation clearance of SVA glucuronide, but not of the major hydroxylated metabolite of SVA. Considering that SVA glucuronide is converted readily to SV

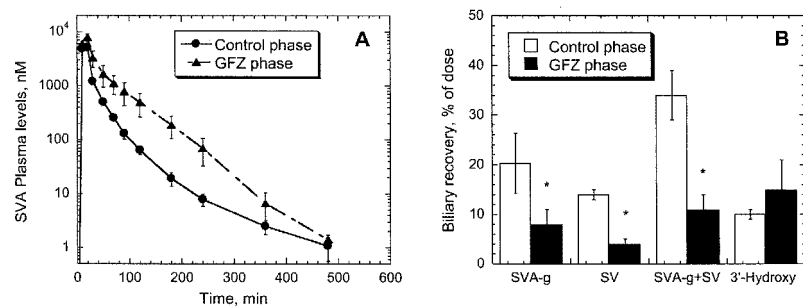


Fig. 4. Effect of GFZ on plasma clearance of SVA (A) and biliary excretion of SVA glucuronide (SVA-g), SV, and 3'-hydroxy SVA (B) after i.v. administration of SVA or [^{14}C]SVA (1.2 mg/kg i.v.) to dogs without ($n = 4$) or with ($n = 3$) bile duct cannulation, respectively. Results are means \pm S.D. ($n = 3-4$). *, statistical differences from control ($p < 0.05$).

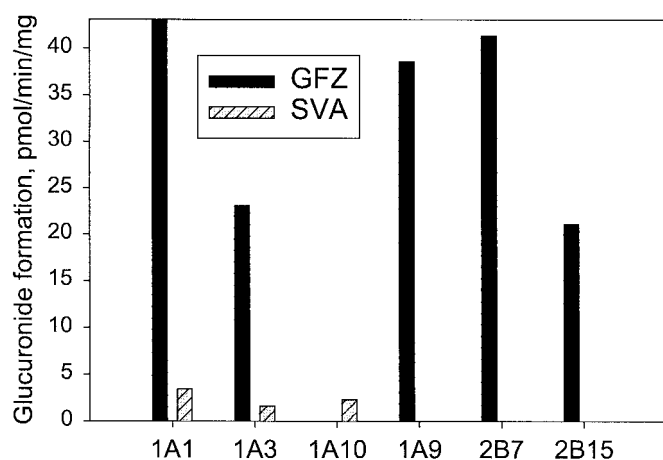


Fig. 5. In vitro glucuronidation of SVA and GFZ by six human UGT isozymes. Incubations were performed in duplicate using 100 μM SVA or 250 μM GFZ with recombinant UGTs, at 37°C for 60 min. Data points are average of duplicate incubations.

by spontaneous cyclization in vitro at physiological pH (Prueksaritanont et al., 2002), the decrease in SV levels in both dog plasma and bile also is consistent with an inhibition of SVA glucuronidation by GFZ. It is noteworthy that GFZ is highly bound (99%) to plasma proteins (Hamberger et al., 1986), and that the IC_{50} value of GFZ on SVA glucuronidation derived from the present in vitro study (~ 200 μM) is in the range of the total, and not unbound, plasma concentrations of GFZ in dogs (C_{max} of ~ 450 μM).

The above-mentioned in vitro-in vivo studies in dogs demonstrated the validity of in vitro metabolism approaches for studying GFZ-statin interactions. Further in vitro microsomal studies suggested that, as in dogs, GFZ also was a more potent inhibitor of glucuronidation ($\text{IC}_{50} = 354$ μM) than oxidative metabolism ($\text{IC}_{50} > 800$ μM) of SVA in humans. The observed inhibitory effect of GFZ on SVA glucuronidation in human liver microsomes also is consistent with the present finding that the glucuronidation of both GFZ and SVA was catalyzed by at least two common human UGT isozymes, and suggests that the drug interaction proceeds by way of competitive inhibition by GFZ of UGT1A1 and/or 1A3. The IC_{50} value for the inhibition of SVA glucuronidation in human liver microsomes was in a range of the C_{max} values reported for GFZ (up to 250 μM) after administration of GFZ (600 mg b.i.d.) to humans (Backman et al., 2000). In addition, as was the case in dogs, glucuronidation of SVA seems to be a metabolic clearance mechanism in humans in vivo because SVA glucuronide has been observed in bile and urine after administration of radiolabeled SVA to humans (Merck Research Laboratories, unpublished data). The present in vitro

results with ketoconazole and troleandomycin suggest that the metabolic oxidation of SVA in human liver microsomes is catalyzed primarily by CYP3A, as with SV (Prueksaritanont et al., 1997). This conclusion is fully consistent with the finding that GFZ had only a modest effect on SVA oxidation because GFZ is not a CYP3A inhibitor (Backman et al., 2000). Moreover, because sulfaphenazole did not affect the formation of oxidized metabolites of SVA, the present results also suggest that CYP2C9 does not play a significant role in the oxidative metabolism of SVA. Therefore, the effect of GFZ on plasma levels of SVA observed in humans (Backman et al., 2000) cannot be attributed to the inhibitory properties of GFZ on CYP2C9, as was speculated recently (Wen et al., 2001). Collectively, the results of this investigation support the hypothesis that the GFZ-mediated increases in systemic exposure to SVA after administration of SV to humans are due, at least in part, to the inhibitory effects of GFZ on SVA glucuronidation, and do not involve alterations in the CYP3A4-mediated metabolism of SVA or SV.

In subsequent in vitro experiments, we evaluated the susceptibility of statins other than SVA to the inhibitory effects of GFZ on glucuronidation and oxidation pathways. The finding that GFZ inhibited the glucuronidation of AVA to greater extent than oxidation of this statin is in agreement with the observation that AVA undergoes UGT1A1- and 1A3-mediated glucuronidation (Prueksaritanont et al., 2002) and CYP3A-mediated oxidation (Yang et al., 1996; Jacobson et al., 2000), similar to SVA. However, the finding that the glucuronidation of CVA was more sensitive to inhibition by GFZ than was the glucuronidation of either SVA or AVA, suggests that enzyme(s) other than UGT1A1 and 1A3 might also be involved in the conjugation of this statin. Interestingly, the oxidation of CVA, which has been shown to be mediated by both CYP3A4 and CYP2C8 (Boberg et al., 1997; Mück, 2000), was found to be markedly inhibited by GFZ in the present human liver microsomal study. The minimal inhibitory effect of sulfaphenazole on the oxidative metabolism of CVA ruled out the possibility that the inhibition of M1 and M2 formation by GFZ was due to inhibition of CYP2C9 activity. Because GFZ is not an inhibitor of CYP3A4, the marked inhibition on CVA oxidative metabolism observed in human liver microsomes probably is due to inhibition of CYP2C8 activity by GFZ. In view of the fact that glucuronidation and oxidation are two major metabolic pathways for CVA metabolism in humans, and because CVA undergoes extensive metabolism in humans, the present in vitro metabolism data suggest that CVA would be more prone to metabolic interactions with GFZ in humans than either SVA and AVA. In this regard, it is noteworthy that the incidence of reported cases of severe rhabdomyolysis after combination

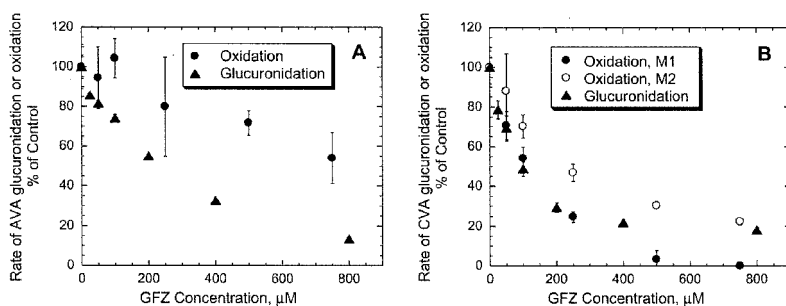


Fig. 6. Effect of GFZ on the glucuronidation and oxidation of AVA (A) and CVA (B) in human liver microsomes. Results are expressed as the percentage of control values (means \pm S.D. of triplicate determinations) and were obtained after coinubation of AVA or CVA, GFZ, and UDPGA (glucuronidation), or NADPH (oxidation) at 37°C for 45 min (glucuronidation) or 10 to 18 min (oxidation) with liver microsomal preparations. Rates of glucuronidation were estimated from the sum of glucuronide and the corresponding lactone of each statin, and rates of AVA oxidation were calculated using the sum of the two hydroxylated metabolites of AVA.

use of GFZ with CVA, which prompted a recent worldwide withdrawal of CVA from the market, is greater than that with the first-generation statins SV and LV (Farmer, 2001). Furthermore, considering that glucuronidation has now been recognized as a common metabolic pathway for several statin acids (Prueksaritanont et al., 2002) and because GFZ has been shown to be a potent inhibitor of CYP2C9 and CYP2C19 (Wen et al., 2001), it may be anticipated that GFZ will impair both the glucuronidation and oxidation of statins whose oxidative metabolism is catalyzed primarily by CYP2C9 and/or CYP2C19, including the new agent rosuvastatin (McTaggart et al., 2001).

To date, few examples of drug-drug interactions at the level of glucuronidation have been reported. Inhibitors of UGTs implicated in clinical pharmacokinetic interactions typically are administered at high doses and achieve high systemic exposure (Taburet and Singlas, 1996; Liston et al., 2001); this is true with GFZ, whose peak plasma concentrations in humans exceed 100 μM after the usual daily dose of 600 mg b.i.d. (Backman et al., 2000). It is noteworthy that, unlike studies with the P450 system, there are technical limitations associated with in vitro glucuronidation experiments, which typically require disruption of the endoplasmic reticulum membrane by detergents to activate UGTs (Trapnell et al., 1998; Fisher et al., 2000). As a consequence, the validity of quantitative extrapolations of in vitro glucuronidation data to the in vivo situation remains to be established (Rommel and Burchell, 1993). Although accurate quantitative predictions of drug interactions with statins in vivo currently are not feasible based on in vitro data alone, due largely to the above-mentioned reasoning together with the complexities associated with reversible lactone/hydroxy acid metabolism and incomplete information on statin metabolism in humans, the results of the present study nevertheless provide valuable insight into the mechanism(s) of clinically significant drug-drug interactions between GFZ and statins.

To conclude, these preclinical studies have demonstrated that GFZ-mediated elevations of SVA AUC after oral SV administration to humans are not due to inhibitory effects of GFZ on CYP3A-mediated metabolism of SVA or SV, but are due, at least in part, to the inhibitory activity of GFZ on UGT1A1- and/or UGT1A3-mediated glucuronidation of SVA. The present results also suggest that GFZ has the potential to modulate the pharmacokinetics of other statins, including AVA and CVA, by inhibition of statin hydroxy acid glucuronidation. Moreover, various statins exhibit differential susceptibility to the inhibitory effects of GFZ on their metabolic clearance via glucuronidation and/or non-CYP3A4-mediated oxidative pathways. CVA is more susceptible than SVA or AVA to interaction with GFZ at the level of glucuronidation and of CYP2C8-mediated oxidation in humans.

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