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Induction of P450 3A1/2 and 2C6 by gemfibrozil in Sprague-Dawley rats

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Abstract:
Fibrates are a group of peroxisome proliferator-activated receptor α agonists used in the treatment of dyslipidemia; however, they have been reported to cause species-related hepatocarcinogenesis and clinical myotoxicity. Gemfibrozil is one of the most commonly used fibrates, and it shows the highest risk for myotoxicity among the fibrates. The inhibitory drug-drug interaction mechanism associated with gemfibrozil has been explored recently, and the induction of human P450 3A4 and 2C8 has been reported. In this study, *in vivo* induction of rat P450 by gemfibrozil was studied in Sprague-Dawley rats. After the rats were dosed with gemfibrozil by oral gavage, microsomes were prepared. The metabolic activities of P450 3A1/2, 2C6, and 2D2 were assayed using probe substrates, and the systemic concentration of gemfibrozil during its administration was determined. P450 3A1/2 and 2C6 activities were induced 32–77% in the rats by gemfibrozil when the exposure concentration was in the clinical range. These data indicate that the inducibility of homologous P450 isoforms by gemfibrozil is similar in Sprague-Dawley rats and in humans. Inductive drug-drug interactions and inhibitory actions are involved in the co-administration of gemfibrozil with other drugs, which suggests the relevance for a fibrate-toxicology investigation.

Key words:
induction, rat, P450, gemfibrozil

Abbreviations: ALT – alanine aminotransferase, AST – aspartate aminotransferase, CK – creatine kinase, C_{max} – peak concentration in plasma following a dosage of an agent, CRE – creatinine, LC-MS/MS – high performance liquid chromatography-tandem mass spectrometry, MYO – myoglobin, NADPH – reduced nicotinamide adenine dinucleotide phosphate, P450 – cytochrome P450, PB – phosphate buffer, PBS – phosphate buffered saline, PPARα – peroxisome proliferator-activated receptor α

Introduction

Fibrates have long been used to treat dyslipidemia. Pharmacologically, they activate the peroxisome proliferator-activated receptor α (PPARα), change the adipokine release, and modulate glucose homeostasis [8, 11, 12]. However, species-related differences exist

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for fibrates between rodents and primates in terms of hepatotoxicity, PPARα expression and pharmacokinetics [7, 9]. Fibrates can also cause clinical myotoxicity with synergism in their combinations with statins [6]. Among the fibrates prescribed clinically, gemfibrozil possesses the highest risk for myotoxicity as a monotherapy and accounts for most of the deaths caused by the co-administration of cerivastatin and fibrates [4, 6, 23].

The mechanisms of the drug-drug interactions between gemfibrozil and statins have been explored recently due to severe myotoxicity, which caused the withdrawal of cerivastatin in 2002. The uptake of cerivastatin by the hepatocyte transporter organic anion-transporting polypeptide 2 is inhibited by both gemfibrozil and its metabolite (gemfibrozil-1-O-glu) [21]. For cytochrome P450 (P450) isoforms, gemfibrozil most potently inhibits P450 2C9, and gemfibrozil-1-O-glu is a mechanism-dependent inhibitor of P450 2C8 [16]. Gemfibrozil can also inhibit UDP-glucuronyl transferases 1A1 and 1A3, which are largely responsible for the glucuronidation and elimination of statins [19]. Clinically, gemfibrozil greatly increases the area under the concentration-time curve, the peak concentrations and/or the elimination half-lives of simvastatin, pravastatin, and cerivastatin [1, 2, 13, 20].

Besides gemfibrozil's inhibitory nature in pharmacokinetic processes, the inductive potential of gemfibrozil and other PPARα agonists have been evaluated in *in vitro* studies. Gemfibrozil induces human P450 3A4 and 2C8 in human hepatocytes with the exception of pregnane X-receptor activation, but the mechanisms remain unknown [18]. For phase II metabolism, UDP-glucuronyl transferase 2B4 is induced by the PPARα agonists fenofibric acid and Wy14643 in HepG2 cells, human hepatocytes, and Huh7 cells [3]. The species differences of P450 between rats and humans have been reviewed in terms of abundance, activity spectra, inducibility, substrates and inhibitor specificity [25]. Understanding gemfibrozil's induction of P450 in both rats and primates may provide a basis for the rat as a pharmacological model. However, little is known about the induction of rat P450 isoforms by gemfibrozil.

In this study, we evaluated the induction of P450s by gemfibrozil in Sprague-Dawley rats. After the rats were dosed with gemfibrozil by oral gavage for 20 days, the systemic concentration of gemfibrozil during its administration was determined. Microsomes were prepared and the metabolic activities of P450 isoforms were assayed using probe substrates to evaluate the inducibility of P450 in rats by gemfibrozil.

Materials and Methods

Chemicals and reagents

Gemfibrozil was obtained from Hunan Qianjin Xiangjiang Pharmaceutical Co. Ltd (Zhuzhou, China). Sodium carboxymethylcellulose was purchased from Shanghai RichJoint Chemical Reagents Co. Ltd. (Shanghai, China). HPLC-grade solvents (acetonitrile, methanol, and formic acid) were purchased from Dima Technology (Richmond Hill, USA). Bezafibrate, midazolam, 1-hydroxymidazolam, α-hydroxytriazolam, diclofenac, 4'-hydroxydiclofenac, flufenamic acid, dextromethorphan, dextrorphan, (R)-(+)-propranolol, and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Biochemistry analysis kits for alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine (CRE), and creatine kinase (CK) were purchased from Shanghai Kehua Bioengineering Co. Ltd. (Shanghai, China). Myoglobin (MYO) analysis kits and the standard for TruCal Myoglobin were purchased from DiaSys Diagnostic Systems GmbH (Holzheim, Germany). Purified water was produced by a Millipore Elix (Bedford, USA) system. All other reagents were of analytical grade from commercial sources.

Animals and treatments

The animal protocols were reviewed and approved by the Institutional Laboratory Animal Care and Use Committee of the Medical School at Ningbo University. Male and female Sprague-Dawley rats (5 weeks old, 120 ± 10 g for males and 110 ± 10 g for females) were provided by the Experimental Animal Center at the Southern Medical University and maintained under specific pathogen-free conditions in the Animal Center of Ningbo University. Rats were housed in plastic cages (3 per cage) and were maintained under a standard 12 h light/12 h dark cycle with free access to purified water and commercial rodent chow. Before each experiment, rats acclimated to the animal facility for at least 7 days. Twenty-four rats were divided into control and gemfibrozil groups randomly (6 male and 6 female for each group).

Gemfibrozil administration and sample collection

Dose formulation was prepared in 0.5% (weight/volume in purified water) aqueous sodium carboxymethylcellulose. The rats were dosed with gemfibrozil (1,320 mg/kg/day) for 20 days by an individualized volume, and the control group was dosed with vehicle only. The dosing volume was updated when the body weights were determined every 2 days. Drug administration was conducted by oral gavage.

Plasma samples were collected by orbital-sinus blood sampling the morning before dosing, every 2 days. The serum was separated and stored at –80°C for drug exposure analysis. Euthanasia was carried out by exsanguination under anesthesia 12 h after the last dose. Livers, hearts, spleens, lungs, and kidneys were dissected and weighed to calculate organ indices (ratio of the organ weight to the body weight). For each rat, liver tissue (5 g) was dissected and transferred to –80°C for preparation of microsomes for P450 activity analysis.

Toxicology and histopathology

ALT, AST, and CRE were monitored to detect drug-related hepatotoxicity and nephrotoxicity using standard methods. CK and MYO were evaluated to monitor myotoxicity. The remaining livers, kidneys, and skeletal muscles from the limbs were fixed in 10% phosphate-buffered formalin for at least 24 h, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. Toxicological reactions, including degeneration, necrosis, apoptosis, and fibrosis, were blindly examined under light microscopy by a pathologist.

Evaluation of the systemic concentration of gemfibrozil

To evaluate the systemic concentration of gemfibrozil during its administration, plasma samples that were collected during dosing were subjected to a validated method for gemfibrozil determination. Linearity, accuracy, extraction recovery, and precision were evaluated when the method was prepared.

In this method, 20 μl of plasma was first diluted by adding 100 μl of methanol/water (50/50). Protein precipitation was then performed on the diluted plasma by adding acetonitrile and bezafibrate (as an internal standard) to a final volume of 1 ml. To prepare the standard samples (final concentration 1–200 mg/l), a series of concentrated gemfibrozil stock solutions were prepared in the 100 μl methanol/water and added to blank rat plasma. These samples were centrifuged at 16,000 × g at 4°C for 30 min, and the supernatant (25 μl) was further diluted to 1 ml with methanol/water (50/50). Following centrifugation, 10 μl of the supernatant was subjected to a LC-MS/MS determination system (Applied Biosystems, Foster City, USA).

The separation was performed on a CAPCELL PAK C18 column (5 μm, 2.0 mm ID × 50 mm, Shiseido Co., Ltd., Tokyo, Japan) at room temperature with a flow rate of 0.2 ml/min. The liquid chromatography gradient was applied as the following: 50% B (methanol containing 0.1% formic acid) for 0.3 min, a linear gradient to 100% B for 1.5 min and held for 1.5 min, and 50% B for 0.3 min for equilibration. The mass spectrometer was operated in negative-ion mode using multiple reactions monitoring the transitions m/z 249.1/121.1 for gemfibrozil and 360.1/274.3 for bezafibrate. The turbo ion spray was maintained at 350°C with a 4,500 V applied to the spray needle. Nitrogen was used as the nebulizer, curtain, and collision gases. All the raw data were processed using the Analyst software 1.4.2 (Applied Biosystems, Foster City, USA).

Preparation of microsomes and induction assay

The thawed livers were homogenized in 2 volumes (w/v) of cold 0.25 M sucrose/0.1 M PB (1 mM EDTA, pH 7.4). Enzyme fractions were prepared at 4°C by differential ultracentrifugation. The supernatant from the first centrifugation was run at an average speed of 9,000 g for 60 min to obtain the microsomal fraction in a pellet. The pellet was homogenized in 10 ml of 0.1 M phosphate buffered saline (PBS, 1 mM EDTA, pH 7.4) and centrifuged at an average speed of 100,000 × g for 60 min. This was repeated twice to clean the hemoglobin. The microsomal preparations were re-suspended in 0.25 M sucrose/0.1 M PB (1 mM EDTA, pH 7.4), divided into small aliquots, and stored in Eppendorf tubes at –80°C until use. The protein concentrations were determined by the Bradford assay using commercial kits (Pierce, Rockford, IL).

The enzyme-activity assays were performed according to validated methodologies with some modifications [24], and one substrate concentration was used for activity comparison. Briefly, the microsomal

Tab. 1. HPLC conditions and MS/MS parameters for the three P450 isoform reactions

Reactions	HPLC gradient min (B%)	MS/MS detection parameters											
		Compound parameters							Gas/Source parameters				
		Q1/Q3 m/z (amu)	DT (s)	DP (V)	FP (V)	EP (V)	CE (eV)	CXP (V)	NEB (u)	CUR (u)	CAD (u)	IS* (kV)	TEP (°C)
P450 3A1/2													
1'-Hydroxymidazolam (M)	0 (30) → 0.2 (30) → 1.6 (95)→ 2.6 (95) → 2.8 (30) → 4.0 (30)	342/324	0.2	48	250	9	30	23	10	9	9	4	350
α-Hydroxytriazolam (IS)		359/341	0.2	50	250	8	30	25					
Midazolam (S)		326/291	0.2	48	250	9	37	20					
P450 2C2													
4'-Hydroxydiclofenac (M)	0 (40) → 1.0 (100) → 3.0 (100) 3.1 (40) 4.5 (40)	312/231	0.2	33	180	9	28	24	11	8	10	4.5	350
Flufenamic acid (IS)		282/264	0.2	30	160	8	20	25					
Diclofenac (S)		296/250	0.2	32	180	8	19	25					
P450 2D6													
Dextrorphan (M)	0 (25) → 1.0 (90) → 2.5 (9 0) 2.6 (25) → 3.5 (25)	258/157	0.2	30	150	10	50	15	11	9	9	3.5	350
Propranolol (IS)		260/183	0.2	30	150	9	25	20					
Dextromethorphan (S)		272/171	0.2	40	200	10	52	20					

M – metabolite, S – substrate, IS – internal standard, DT – dwell time, DP – declustering potential, FP – focusing potential, EP – entrance potential, CE – collision energy, CXP – collision cell exit potential, NEB – nebulizer gas, CUR – curtain gas, CAD – collision gas, IS* – ionspray voltage, TEP – temperature. Phase A: CH_3OH:H_2O:HCOOH 10:90:0.1 (v/v/v); Phase B: CH_3OH:H_2O:HCOOH 90:10:0.1 (v/v/v)

preparations from all rats were normalized to a final concentration of 0.2 mg/ml. The selected marker-substrate concentrations were the Km for the corresponding human P450 isoforms. After the microsomes and probe substrate (10 μM) were pre-incubated at 37°C for 5 min, 1 mM NADPH was added to start the reaction. Incubations for P450 3A1/2, 2C6, and 2D2 activities were performed at 37°C for 5, 7, and 8 min, respectively. Ice-cold acetonitrile with internal standards α-hydroxytriazolam, flufenamic acid, and (R)-(+)-propranolol, respectively, were used as stop solutions. Metabolites 1-hydroxymidazolam (P450 3A1/2), 4'-hydroxydiclofenac (P450 2C6), and dextrorphan (P450 2D2) were determined using LC-MS/MS.

The LC-MS/MS method for substrate metabolite determination was performed similar to referenced methods [24]. An ABI3000 triple, quadrupole mass spectrometer (Applied Biosystems, USA) coupled with HPLC (SHIMADZU 10A, Japan) and an MPS3C autosampler (GERSTEL, Germany) controlled by Analyst 1.4.2 workstation software (Applied Biosystems, Foster City, USA) were used for quantification. Compounds were separated on a CAPCELL PAK C18 column (5 μm, 2.0 mm × 50 mm) at room temperature with a flow rate of 0.2 ml/min. Chromatographic conditions and analytical parameters for the activity assays are summarized in Table 1.

Statistical analyses

P450 activities were calculated as the ratios between the metabolites and their internal standard. Toxicity-related changes were defined as a two-fold increase in ALT, AST, CRE, CK, and MYO. All data were expressed as the mean ± SD. SPSS 13 for Windows (Chicago, USA) was used for the data analysis, and differences were considered significant when $p < 0.05$. A general, linear model for repeated measures was used to analyze body weight during gemfibrozil treatment. A two-tailed, independent-sample t-test was used to analyze the organ indices and the activity induction.

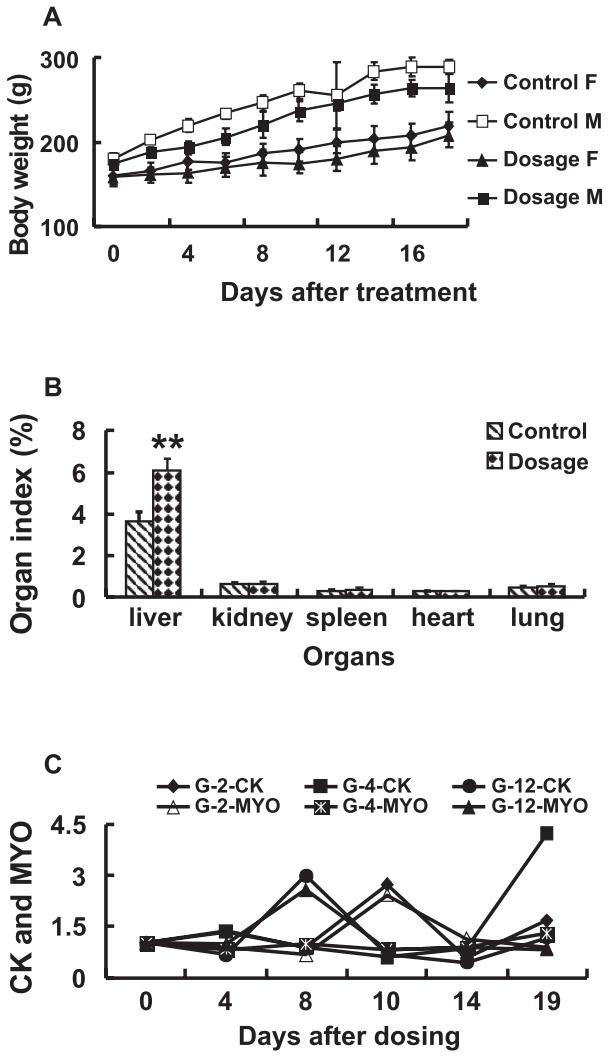

Fig. 1. Physical and biochemical effects in rats caused by gemfibrozil administration. (**A**) Body weight following gemfibrozil administration did not change significantly (n = 12, p = 0.161 compared with the control). (**B**) Among five organ indices, liver index increased significantly (n = 12, ** p < 0.01 compared to the control). (**C**) Myotoxicity biomarkers CK and MYO in rats 2, 4 and 12. Organ indices were calculated as the ratio of the organ weight to the body weight. F – female, M – male, CK – creatine kinase, MYO – myoglobin

Tab. 2. Hepatotoxicity associated with gemfibrozil treatment

	ALT	AST	Degeneration and necrosis	Apoptosis
Toxicity incidence	3/12	5/12	5/12	2/12

ALT – alanine aminotransferase, AST – aspartate aminotransferase. Note: A two-fold increase in the values over baseline was defined as toxicity-related changes. Toxicological reactions, including degeneration, necrosis and apoptosis, were defined by pathological analysis

Results

Physical and biochemical effects of gemfibrozil in rats

The rats dosed with gemfibrozil showed lower body weights than controls, but no significant differences were found between the groups (Fig. 1A, $p > 0.05$). No significant changes were found in the kidney, spleen, heart, and lung organ indices between treated and control animals (Fig. 1B, $p > 0.05$). However, a 65% increase in the liver index was noted in rats dosed with gemfibrozil compared to controls (Fig. 1B, $p < 0.01$).

From biochemical monitoring and pathological analyses, hepatotoxicity, and myotoxicity were detected in animals exposed to gemfibrozil. The biochemical and pathological results correlated well each other, and both indicated that toxicity occurred in liver. The obvious hepatotoxicity included degeneration, necrosis, and apoptosis (Tab. 2). Increases in CK and MYO were detected in 2 rats, which indicated myotoxicity; however, both biomarker concentrations returned to normal 2–4 days after their increase. Myotoxicity was also detected in another rat, but the increase in CK was detected when the rat was sacrificed (Fig. 1C). No toxicity was found in the skeletal muscles of either group by pathological analysis (data not shown). No toxicity was noted in the kidneys of either group, indicating gemfibrozil tolerance in the rat kidney (data not shown).

Systemic gemfibrozil concentration assay

By performing a method-validation procedure, a good linear relationship was obtained over the concentration range of 1–200 mg/l for gemfibrozil in rat plasma. The correlation coefficients for the weighted ($1/x^2$), least-squares linear-regression curves were over 0.994. This method's accuracy, extraction recovery, and precision are shown in Table 3, which verifies the suitability of this method for analyzing systemic gemfibrozil concentrations.

Using bezafibrate as an internal standard, gemfibrozil's average systemic-trough concentration in rats was determined as 2.9–10.9 mg/l when 1,320 mg/kg -day was dosed (Fig. 2). The overall, average trough concentration during gemfibrozil administration was

Tab. 3. The method's accuracy, extraction recovery and precision used to determine gemfibrozil in rat plasma

Gemfibrozil concentration mg/l	Accuracy		Extraction recovery	
	Recovery (%)	Precision (RSD, %)	Recovery (%)	Precision (RSD, %)
1	96.7	10.78	100.2	9.50
30	104.7	6.64	108.1	6.17
200	111.2	6.18	95.9	1.27

RSD – relative standard deviation

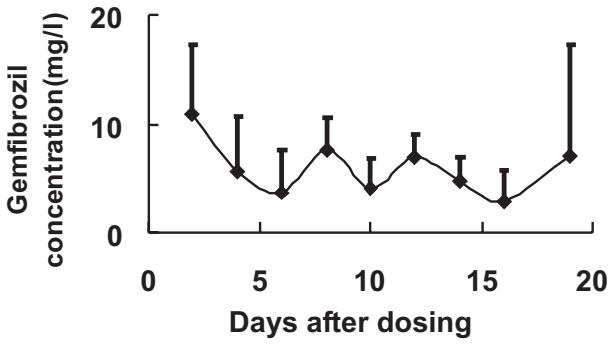

Fig. 2. Gemfibrozil trough concentrations in rats during drug administration. Data expressed as the mean ± SD (n = 12)

5.8 ± 5.0 mg/l. No accumulation was shown during the 20 days of gemfibrozil administration.

Induction of rat P450 activity

By validated LC-MS/MS methods, the activities of P450 3A1/2, 2C6, and 2D2 were evaluated using an internal standard method. P450 3A1/2's activity and inducibility showed gender-related differences. In control animals, 3A1/2 activity in female rats was 80% lower than in male rats. However, after gemfibrozil administration, its activity increased 65% and 32% in females and males, respectively (Fig. 3A; p = 0.001 and p = 0.007 for females and males, respectively).

The P450 2C6 activity in males was 50% higher than in females. After gemfibrozil administration, its activity in males and females increased 57% and 77%

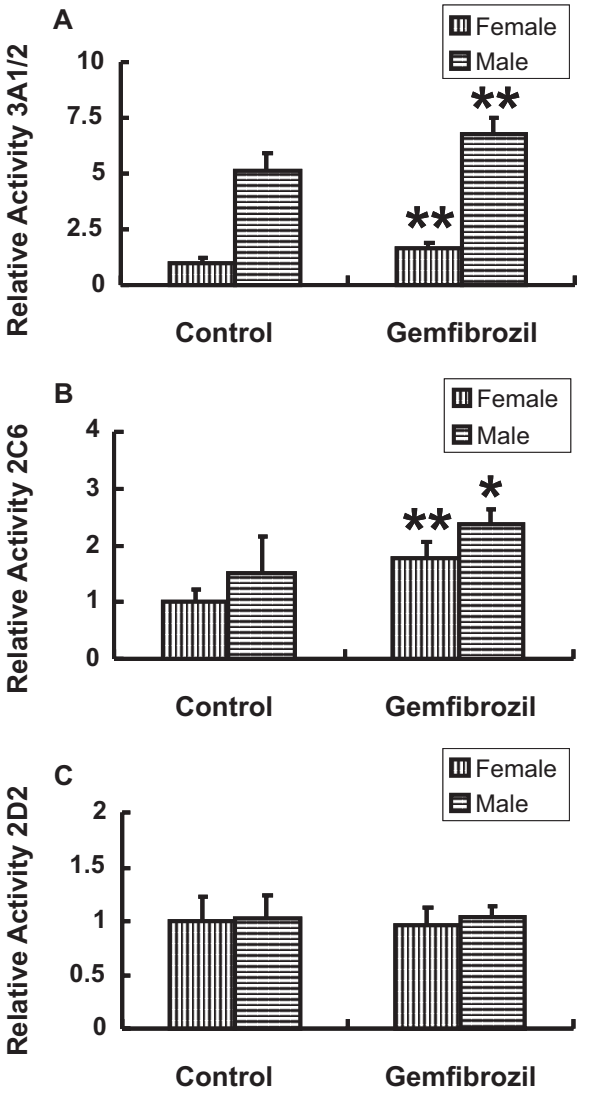

Fig. 3. Relative activities after in vivo induction by gemfibrozil. (**A**) Activity of 3A1/2 was induced significantly (** p < 0.01 for male and female compared with the control); (**B**) Activity of 2C6 induced significantly (* p < 0.05 and **p < 0.01 for male and female compared with the control); (**C**) Activity of 2D2 showed no difference between two genders and no induction was observed (p = 0.677 between male and female; p = 0.596 and p = 0.762 compared with the control for male and female, respectively)

(Fig. 3B; p = 0.021 and p = 0.000 for males and females, respectively). For both P450 3A1/2 and 2C6, the lower the isoform activity in the control animals was, the more it was induced by gemfibrozil.

It is known that P450 2D2 is usually not inducible. In this study, gemfibrozil administration did not influence its activity in males or females (Fig. 3C; p = 0.596 and p = 0.762, respectively). Additionally, in the control group, P450 2D2 activity was not different between the two genders (p = 0.677).

Discussion

Clinically, gemfibrozil trough concentrations at steady-state have been reported to be 1.2–1.5 mg/l [20]. The average C_{max} reaches 25.6 mg/l with a concentration range of 22–49 mg/l [13, 20]. Through an *in vitro* approach, gemfibrozil's 50%-effective concentration to activate PPARα is 5–12.5 mg/l, and this activation is concentration dependent [5]. In this study, the average through concentrations ranged between 2.9–10.9 mg/l when rats were dosed with 1,320 mg/kg-day of gemfibrozil (Fig. 2). These exposure levels correlate with pharmacologically effective and clinically systemic concentrations. Gemfibrozil induces human P450 3A4 and 2C8 [18], and, in this study, rat homologues P450 3A1/2 and 2C6 were induced by oral doses of gemfibrozil. Therefore, the inducibility of rat P450 by gemfibrozil is similar to that of humans. This suggests the relevance for clinical drug-drug interaction and toxicological studies in rats.

Probe substrates and inhibitors of human P450 isoforms have already been identified and are included in the FDA guidelines. Diclofenac 4-hydroxylation and dextromethorphan O-demethylation are characterized as catalytic markers for rat P450 homologues 2C6 and 2D2. Midazolam 1-hydroxylation and midazolam 4-hydroxylation are both predominantly catalyzed by P450 3A1 and 3A2, and these two isoforms show similar midazolam 1-hydroxylation activity [10, 15]. In this study, midazolam, diclofenac, and dextromethorphan were selected as specific substrates using an internal standard method. Because the P450 2D subfamily is not inducible, it was used as a reference for the validation of the design and operation of this study. P450 2D2 was not induced in both males and females that were administered gemfibrozil in this study. In contrast, P450 3A1/2 and 2C6 activities were induced 32.4–77.3% over control, indicating a strong correlation of this study with human results.

Mechanisms have been recently elucidated for complicated drug-drug interactions associated with gemfibrozil, especially with concomitant usage of cerivastatin [22]. P450 2C8 is an important enzyme involved in the oxidation of cerivastatin, and P450 3A4 is the major enzyme for the oxidation of simvastatin and atorvastatin. In this study, P450 3A1/2 and 2C6 activities and inducibilities were different between males and females. For 3A1/2, its activity in males was 5 times greater than in females; however, its inducibility in males (32%) was only half of that in females (65%). For 2C6, its activity in males was 50% higher than in females, but its inducibility was similar in the two genders. In contrast, no activity or inducibility differences were observed for the 2D2 isoform between the two genders. Because the coadministration of gemfibrozil and statins causes high risk of clinical myotoxicity, gender-related differences in P450 activity and inducibility are important factors that cannot be ignored when animal models are used to investigate toxicity mechanisms.

Rat toxicity was evident in this study, both physiologically and biochemically. The body weights of the rats were decreased, and the liver organ index was increased significantly. Biochemical analyses and pathological examinations showed liver toxicity; however, no kidney toxicity was observed. These results could be attributed to the relatively high exposure level and the hepato-sensitivity of rats. This was in accordance with previous report that primate livers are more refractory to peroxisome proliferation and carcinogens than are rodents [7].

In our gemfibrozil myotoxicity study in Cynomolgus monkeys, both 600 and 300 mg/kg-day caused a high incidence of myotoxicity, and the trough concentration for the low dose was between 19–47 mg/l [14]. In this study, the 1,320 mg/kg-day dose was twice the lowest dose in the monkey study based on body surface area; however, the exposure level was much lower than in the monkeys. It has been reported that fenofibrate causes myotoxicity in rats with a dosing period of one month [17]. The temporal increases in CK and MYO in this study suggest that the myotoxicity in these rats dosed with gemfibrozil for 20 days does not last long or stabilize. In comparing these results with reported data in Cynomolgus monkeys, this can be partly attributed to lower exposure in terms of low systemic plasma concentrations, a shorter dosing period or species-related difference of sensitivity.

In this study, gemfibrozil administration in rats induced P450 3A1/2 and 2C6 when the plasma concentration was in the clinical exposure-level range. The inducibility of rat P450 3A1/2 and 2C6 by gemfibrozil is similar to homologous, human isoforms. Therefore, the induction caused by gemfibrozil may plays important roles in drug-drug interactions in rats, providing information for animal-model selection and mechanistic studies for toxicology investigations. Complicated drug-drug interactions may be involved in gemfibrozil's co-administration with other drugs in the clinic.

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