Hepatotoxic Effects of 1-Furan-2-yl-3-pyridin-2-yl-propenone, a New Anti-Inflammatory Agent, in Mice

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Abstract — 1-Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) has recently been synthesized and characterized to have an anti-inflammatory activity through the inhibition of the production of nitric oxide. In the present study, adverse effects of FPP-3 on hepatic functions were determined in female BALB/c mice. When mice were administered with FPP-3 at 125, 250 or 500 mg/kg for 7 consecutive days orally, FPP-3 significantly increased absolute and relative weights of liver with a dose-dependent manner. In addition, FPP-3 administration dramatically increased the hepatotoxicity parameters in serum at 500 mg/kg, in association of hepatic necrosis. FPP-3 significantly induced several phase I enzyme activities. To elucidate the possible mechanism(s) involved in FPP-3 induced hepatotoxicity, we investigated the hepatic activities of free radical generating and scavenging enzymes and the level of hepatic lipid peroxidation. FPP-3 treatment significantly elevated the hepatic lipid peroxidation, measured as the thiobarbituric acid-reactive substance, and the activity of superoxide dismutase. Taken together, the present data indicated that reactive oxygen species might be involved in FPP-3-induced hepatotoxicity.

Keywords: FPP-3, Hepatotoxicity, Lipid peroxidation, *In vivo*

INTRODUCTION

1-Furan-2-yl-3-pyridin-2-yl-propenone (FPP-3) is a chemically synthesized novel compound with a propenone moiety (Fig. 1). A recent study demonstrated that FPP-3 could inhibit lipopolysaccharide (LPS)-stimulated production of nitric oxide (NO) and tumor necrosis factor (TNF)- α in the cultures of RAW 264.7 macrophages *in vitro* (Lee *et al.*, 2004). In addition, FPP-3 could not only inhibit cyclooxygenases (COXs) and 5-lipoxygenase activities but also inhibit COX-2 by 35-times more selectively than COX-1 (Jahng *et al.*, 2004). Furthermore, FPP-3 (0.5-50 mg/kg, p.o.) had more potent analgesic and anti-inflammatory effects than indomethacin, a conventional non-steroidal anti-inflammatory drug, in rats and mice without the ulcerogenic activity (Lee *et al.*, 2006). These results indicated that FPP-3 might have a potent anti-inflammatory activity. Therefore, further toxicological inves-

tigations for the development of FPP-3 as an anti-inflammatory drug were essential.

The objective of our present study was to evaluate the oral subacute hepatotoxicity induced by FPP-3 *in vivo*. Liver damage was assessed by quantifying activities of serum enzymes and hepatic lipid peroxidation as well as liver histopathology. In addition, *in vivo* effects of FPP-3 on the hepatic activities of free radical generating and scavenging enzymes were measured.

M.W., 199.21

Fig. 1. Structure of FPP-3 used for this study.

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MATERIALS AND METHODS

Materials

FPP-3 (purity, >99%) used in this study was chemically synthesized in our group (Jahng *et al.*, 2004; Lee *et al.*, 2004). Bovine serum albumin, *p*-nitrophenol, thiobarbituric acid (TBA), 5,5'-dithiobis-2-nitrobenzoic acid, NADPH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, ethoxyresorufin, methoxyresorufin, pentoxyresorufin, benzyloxyresorufin, erythromycin, D,L-glyceraldehyde and corn oil were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 4-Pyridine carboxyaldehyde was purchased from Acros Organics (Geel, Belgium). All other chemicals used were of reagent grade commercially available and used as received.

Animals and treatment

Specific pathogen-free female BALB/c mice were obtained from the Orient Co. (Seoul, Korea). The mice were received at 4-5 weeks of age and were acclimated for at least 2 weeks. Upon arrival, the animals were randomized and housed five per cage. All animals were maintained with gamma-irradiated LabDiet[®] (Purina Mills, MO, USA) and tap water *ad libitum*. Mice 6-7 weeks old (20 ± 2 g) were used in the present study. The animal quarters were strictly maintained at $23 \pm 3^\circ\text{C}$ and $50 \pm 10\%$ relative humidity. A 12 h light/dark cycle was used with an intensity of 150-300 Lux.

Female BALB/c mice were administered orally with FPP-3 at 125, 250 or 500 mg/kg in 10 ml corn oil once daily for 7 consecutive days. All animals were sacrificed 24 h after the last FPP-3 administration. Each animal was euthanized by CO₂ asphyxiation. After the blood samples were removed, the abdominal cavity of each animal was opened and the liver removed and weighed. Serum was obtained after centrifugation of collected blood samples at $3,000 \times g$ for 10 min at 4°C and stored at -80°C prior to analysis. The control animals received corn oil only. All animal care and procedures were conducted according to the Guiding Principles in the Use of Animals in Toxicology, as adopted by the Society of Toxicology (Reston, VA, USA) in 1989.

Determination of hepatotoxicity parameters

The activities of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined using the spectrophotometric enzyme assay kits (Asan, Seoul, Korea) according to the instruction provided by the manufacturer. For the determination of TBA-reactive substance (TBARS) as lipid peroxidation, liver tissue was homogenized with four volumes of

ice-cold 0.1 M potassium phosphate buffer (pH 7.4), then centrifuged at $9,000 \times g$ for 20 min at 4°C . A 20% (w/v) homogenate was obtained and the TBARS was measured according to methods of Ohkawa *et al.* (1979). Aliquots of S-9 fractions were stored at -80°C prior to analysis. The protein content was determined according to the method reported by Lowry *et al.* (1951) using bovine serum albumin as a standard.

Assay of hepatic activities of drug-metabolizing enzymes in S-9 fraction

Ethoxyresorufin O-deethylase (EROD) activity was determined as described by Blank *et al.* (1987) with a slight modification. The reaction mixture consisted of 0.1 M potassium phosphate buffer, pH 7.4, containing 2 mg/ml of bovine serum albumin, 5 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 5 μM NADPH and 2.5 μM 7-ethoxyresorufin. The formation of resorufin was monitored fluorometrically at an excitation maximum of 550 nm and an emission maximum of 585 nm. Methoxyresorufin O-demethylase (MROD), pentoxyresorufin O-depentylase (PROD) and benzyloxyresorufin O-debenzylase (BROD) activities were determined by the method of Lubet *et al.* (1985) with a slight modification. All reaction components and assay procedures were exactly the same as the EROD assay, except that the substrates were 2.0 μM . *p*-Nitrophenol hydroxylase (PNPH) activity was determined as described by Koop (1986). The reaction mixture was composed of 0.1 M potassium phosphate buffer, pH 7.4, containing 100 μM *p*-nitrophenol, 1 mM NADPH and enzyme source. The amount of 4-nitrocatechol formed was measured spectrophotometrically at 512 nm. Erythromycin *N*-demethylase (ERDM) activity was determined by measuring the amount of formaldehyde formed, as described previously (Nash, 1953). Erythromycin at 400 μM was used as a substrate for assaying ERDM. The activities of carbonyl reductase (CBR) were determined using the following substrates: D,L-glyceraldehyde and 4-pyridine-carboxyaldehyde (Szotáková *et al.*, 2004). The cytosolic fraction was used as the enzyme source. Spectrophotometric determination of NADPH consumption at 340 nm in the reaction mixture served for the assessment of reductase activities.

Assays of free radical scavenging enzymes

Superoxide dismutase (SOD) activity in S-9 fraction was assayed by the method of Marklund and Marklund (1974). Catalase activity in mitochondrial fraction was assessed using a modified method of spectrophotometric method (Aebi, 1984; Lee *et al.*, 2007). Glutathione peroxidase (GPX) activ-

ity was measured spectrophotometrically using a technique based on method of Paglia and Valentine (1967). Glutathione reductase (GRE) activity was assayed according to the method of Carlberg and Mannervik (1975).

Statistics

The results were expressed as the mean $\pm$ S.E. and the statistical differences between the different dose groups and the vehicle control were determined by one-way analysis of variance (ANOVA) followed by the Dunnett's two tailed *post-hoc* test (SPSS program, ver 10.0). The significant values at either $*p < 0.05$ or $**p < 0.01$ were represented as asterisks.

RESULTS

Hepatotoxic effects of FPP-3 were studied in the present study. Female BALB/c mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. The body and liver weights were determined at 24 h after the last dosing. As shown in Table I, FPP-3 treatment significantly decreased in final body weight by 9% compared with vehicle control (VH) at the dose of 500 mg/kg, and dramatically increased in the relative liver weight by 25% and 84% at 250 and 500 mg/kg compared with control, respectively. Moreover, FPP-3 significantly increased the hepatic TBARS at 500 mg/kg. Furthermore, FPP-3 caused hepatotoxicity in mice, as measured by serum activities of ALT, AST and ALP, by 13.0-, 7.6- and 3.0-folds at 500 mg/kg treated group compared with control, respectively

(Fig. 2). FPP-3-induced hepatotoxicity as indicated by the biochemical data were confirmed by the conventional histopathological examination. Livers of mice in the control group showed a normal histological appearance (Fig. 3A), whereas livers of mice treated with FPP-3 at 500 mg/kg showed massive hepatic necrosis with mineralization (Fig. 3B). On the basis of histopathological findings, it was concluded that FPP-3 had hepatotoxic effects at 500 mg/kg treated group.

To elucidate the possible mechanism(s) of FPP-3 induced hepatotoxicity, the hepatic activities of drug-metabolizing enzymes and/or free radical generating and scavenging enzymes were determined following treatment with FPP-3. As shown in Fig. 4, FPP-3 treatment induced the

Table I. Effects of FPP-3 on body and liver weights and TBARS in female BALB/c mice

FPP-3 (mg/kg)	Body weight (g)	Organ weight		TBARS (nmoles/mg protein)
		Liver (g)	Liver (% of body weight)	
VH	21.2 $\pm$ 0.3	0.99 $\pm$ 0.05	4.68 $\pm$ 0.20	2.23 $\pm$ 0.10
125	21.5 $\pm$ 0.2	1.07 $\pm$ 0.03	4.99 $\pm$ 0.16	2.48 $\pm$ 0.12
250	21.8 $\pm$ 0.3	1.28 $\pm$ 0.04 ^b	5.86 $\pm$ 0.17 ^b	2.55 $\pm$ 0.14
500	19.3 $\pm$ 0.3 ^b	1.66 $\pm$ 0.07 ^b	8.59 $\pm$ 0.37 ^b	2.91 $\pm$ 0.26 ^a

Female BALB/c mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. The final body and liver weights were determined 24 hr after the last dosing. Each value represents mean $\pm$ S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at either ^a $p < 0.05$ or ^b $p < 0.01$.

Fig. 2. Effects of FPP-3 on activities of serum enzymes in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. The serum activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) were determined 24 hr later. Each bar represents the mean $\pm$ S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $**p < 0.01$.

Fig. 3. Histopathology of livers. (A) Normal liver treated with corn oil for 7 consecutive days. (B) Liver treated with 500 mg/kg of FPP-3 for 7 consecutive days.

hepatic PNPH and BROD activities. Meanwhile, the activities of EROD, PROD, BROD and ERDM were significantly suppressed at 500 mg/kg, probably by the hepatotoxicity induced by FPP-3. In addition, two well-known substrates for CBR, D,L-glyceraldehyde and 4-pyridine-carboxyaldehyde, were used for assaying the activity of CBR enzymes which has been proposed to metabolize FPP-3 (Lee *et al.*, 2008). The hepatic activities of CBRs were significantly induced by FPP-3 treatment (Fig. 5).

As shown in Table I, treatment with FPP-3 significantly increased the hepatic content of lipid peroxide as measured by TBARS at 500 mg/kg. Subsequently, the effects of FPP-3 on the activities of free radical scavenging enzymes, such as SOD, catalase, GPX and GRE, were determined in hepatic S-9 or mitochondrial fractions isolated from the mice treated with FPP-3 for 7 days (Fig. 6). FPP-3 treatment significantly increased in the hepatic activities of SOD and GPX at 500 mg/kg. Meanwhile, the activities of catalase and GRE were not altered following treatment with FPP-3.

Fig. 4. Effects of FPP-3 on hepatic cytochrome P450 (CYP)-associated monooxygenase activities in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Twenty-four h after the administration, liver S-9 fractions were prepared from individual animals. Each bar represents the mean $\pm$ S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at either * $p < 0.05$ or ** $p < 0.01$. EROD: CYP 1A-selective ethoxyresorufin O-deethylase, MROD: CYP 1A-selective methoxyresorufin O-demethylase, PROD: CYP 2B-selective pentoxyresorufin O-depentylase, BROD: CYP 2B-selective benzyloxyresorufin O-debenzylase, PNPH: CYP 2E1-selective *p*-nitrophenol hydroxylase, ERDM: CYP 3A-selective erythromycin *N*-demethylase.

Fig. 5. Effects of FPP-3 on hepatic carbonyl reductase activities in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Twenty-four hr after the administration, liver S-9 fractions were prepared from individual animals. Reactions for enzyme activities were carried out using 0.5 M D,L-glyceraldehyde (A) and 4-pyridine-carboxyaldehyde (B) for 20 min at 25°C. The reactions were initiated by the addition of 3 mM β -NADPH solution. Each bar represents the mean $\pm$ S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at either * $p < 0.05$ or ** $p < 0.01$.

DISCUSSION

Investigating the toxic potential of a candidate drug as a part of preclinical safety evaluation is very important. A key component of this investigation is the toxicological profile generated through preclinical studies. In our preliminary study, oral LD₅₀ of FPP-3 was approximately 1 g/kg in both ICR mice and Sprague-Dawley rats (unpublished data). However, other toxicological profile of FPP-3 has not been investigated until to date. Therefore, FPP-3 was selected as a test article in the present study to understand the toxicological effects of FPP-3 in experimental animals. The hepatotoxic effects of FPP-3 was investigated in mice using several toxicity parameter, such as serum ALT, AST and ALP levels, hepatic contents of TBARS, and hepatic activities of drug-metabolizing enzymes and/or free radical generating and scavenging enzymes. Female BALB/c mice were selected for this particular study, because this strain has widely been used as an animal model in numerous immunotoxicological studies (Luster *et al.*, 1992) and because the immunotoxicological effects of FPP-3 was also planned to be investigated in the near future.

The increases in the liver/body weight ratio and the lev-

Fig. 6. Effects of FPP-3 on hepatic activities of free radical scavenging enzymes in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. The hepatic activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione reductase (GRE) in S-9 fractions and catalase in mitochondrial fractions were determined. Each value represents mean $\pm$ S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at * $p < 0.05$.

els of serum hepatotoxic parameters due to FPP-3 treatment were observed in this study. Although the exact mechanism(s) for the increased liver weight following treatment with FPP-3 is unknown, these results can reflect the hepatocellular injury after treatment with FPP-3.

Induction of drug-metabolizing enzymes would be critical in developing new drug candidate, because the induction of drug-metabolizing enzymes by a drug candidate would cause drug interaction such as drug tolerance. Among the enzymes, CYP-dependent monooxygenases were determined following the 7-day exposure to FPP-3, because CYP enzymes are the major oxidation enzymes, which catalyze the detoxication and bioactivation of many endogenous and exogenous substrates including drugs and environmental pollutants (Lamba *et al.*, 2002). Furthermore, the reduction is also a significant step in the phase I biotransformation for a variety of aromatic, alicyclic and aliphatic compounds bearing a carbonyl group (Maser, 1995). Among the reducing enzymes, the CBR might be implicated to metabolize FPP-3 in our previous studies (Lee *et al.*, 2008). Therefore, we investigated the effects of FPP-3 on CYPs and CBR in the present studies

using liver S-9 fraction. As shown in Fig. 3, 4, FPP-3 caused significant inductions of the activities of CYP 2E1-selective PNPH, CYP 2B1-selective BROD and CBRs. In addition, some enzymes were suppressed by the highest dose of FPP-3, indicating the hepatotoxicity at this dose (Fig. 3). The induction of PNPH and BROD indicate that further investigation would be required to see whether there may be significant drug interaction of FPP-3 with CYP 2B- and CYP 2E1-selective substrates *in vivo* in the near future. A recent report also suggested that FPP-3 could induce glutathione-S-transferase and NADPH:quinine oxidoreductase activities in MCF-7 cells (Hwang *et al.*, 2008). Regarding the possible drug interaction of FPP-3, there had been *in vivo* studies on the pharmacokinetics of theophylline and warfarin (Shanmugam *et al.*, 2006, 2008).

It has recently been recognized that under certain circumstances, CYPs can produce reactive oxygen species (ROS) that result in oxidative stress. CYP 2E1 is among the most active CYP producing ROS (Gonzalez, 2005). Oxidative stress is imposed on cells as a result of one of three factors: 1) an overproduction in oxidant generation, 2) a breakdown in antioxidant defenses, or 3) a failure to repair oxidative damage. ROS are either oxygen free radicals, reactive anions containing oxygen atoms, or molecules containing oxygen atoms that can either produce free radicals or are chemically activated by them. Examples are superoxide and hydroxyl radical (Hayes and McLellan, 1999; Nicholls and Budd, 2000). Under normal conditions, ROS are cleared from the cell by the action of antioxidant enzymes SOD, catalase or GPX. These enzymes limit the effects of oxidant molecules on tissues and are active in the defense against oxidative cell injury by means of their being free radical scavengers (Kyle *et al.*, 1987). If superoxide is not prevented from generation, it leads to the formation of highly reactive species, hydroxyl radicals, which may attack proteins and lipid membranes causing cell damages. Lipid peroxidation and hydrogen peroxide are, therefore, the markers for assessing the extent of damage in cells (Zhao *et al.*, 2000).

An increased SOD activity represents an adaptive response to a higher superoxide anion production (Wong *et al.*, 1989). Catalase activity plays a central role in defending cell against oxidative stress (Yu, 1994). The perfect balance between the antioxidant enzymes, such as SOD, catalase and GPX, is of great importance for the maintenance of the cellular integrity. The unbalance between these enzymes may be critical, causing an accumulation of superoxide anion or H₂O₂, which in the presence of Fe²⁺, can be converted to hydroxyl radical. An increased oxida-

tive stress caused by accumulation of hydroxyl radicals is the proposed pathogenic mechanism in hepatic oxidative damage.

In the present study, treatment with FPP-3 generated a significant elevation in the activity of SOD, but did not change level of catalase in mice (Fig. 6). In addition, FPP-3 significantly increased lipid peroxidation at high-dose group (Table I). These data suggested that the unbalanced SOD:catalase ratio may result in the increased formation of hydroxyl radicals, which could increase the hepatic oxidative damage.

An overproduction of ROS has been demonstrated to stimulate the expression and synthesis of various inflammatory cytokines. Among them, proinflammatory cytokines have been suggested to induce tissue damage, and are considered to be an important initiator of the inflammatory responses (Roy *et al.*, 1992; Larrea *et al.*, 1994). For example, TNF- α , a proinflammatory cytokine, is assumed to associate with the pathophysiology of liver disease. Normally, TNF- α participates in the regulation of inflammation and immunity to pathogens. However, unusually high amounts of hepatic TNF- α occur in many acute and chronic liver diseases, including fulminant hepatic failure, metabolic disease and biliary obstruction, as well as certain inflammatory bowel diseases (Larrea *et al.*, 1994; Luster *et al.*, 1999; Simeonova *et al.*, 2001; Thapa *et al.*, 2009). For this reason, effects of FPP-3 on hepatic gene expression of TNF- α are currently under investigation.

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