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Immunotoxicological Investigation of 1-furan-2-yl-3-pyridin-2-yl-propenone in Female BALB/c 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 and tumor necrosis factor-α. In the present study, adverse effects of FPP-3 on immune functions were determined in female BALB/c mice. When mice were administered orally with FPP-3 at 125, 250 or 500 mg/kg for 7 consecutive days, FPP-3 suppressed the number of antibody-forming cells and reduced thymus weight at 500 mg/kg. In addition, FPP-3 administered mice exhibited reduced splenic cellularity and numbers of splenocyte subsets, such as $CD3^+$ cells, $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells and macrophages. IL-4 mRNA expression was significantly suppressed by FPP-3 treatment. Moreover, the number of $CD4^+IL\text{-}4^+$ cells was reduced following the treatment of mice with 500 mg/kg of FPP-3. These results suggested that FPP-3 at 500 mg/kg might be immunotoxic, and that FPP-3-induced immunotoxicity might be mediated, at least in part, through the inhibition of cytokine production, such as IL-4.

Keywords: 1-Furan-2-yl-3-pyridin-2-yl-propenone, Immunotoxicity, Antibody-forming cells, Cytokines

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). These results indicated that FPP-3 might have a potent anti-inflammatory activity. Therefore, further toxicological investigations for the development of FPP-3 as an anti-inflammatory drug were essential. Particularly, its immunotoxic potential would be critical, because FPP-3 affects immune systems for its pharmacological action.

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In our previous study of hepatotoxicity evaluation, FPP-3 was hepatotoxic at a very high dose (Jeon *et al.,* 2009). To our knowledge, however, toxicological effects of FPP-3 on immune functions in mice have not been reported yet. Thus, this study was conducted for the first time to examine whether exposure to FPP-3 has the potential to adversely affect immune functions. To evaluate the immunotoxic effects of FPP-3, we have chosen some parameters. For examples, the weights of body and lymphoid organs, such as spleen and thymus, antibody response to sheep

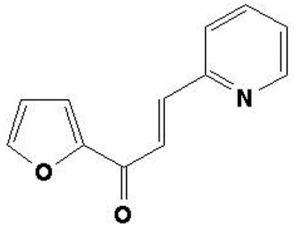

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Fig. 1. Structure of FPP-3 used for this study.

red blood cells (SRBCs), spleen cellularity (nucleated cells), quantitation of the numbers of splenic B-cells, T-cells and macrophages, and lymphoproliferation by LPS or concanavalin A (Con A) were determined. In addition, to elucidate the mechanism(s) of FPP-3-induced immunotoxicity, the *in vivo* effects of FPP-3 on the expressions of cytokine mRNA and/or the ability of spleen cells to produce intracellular cytokines, such as IL-2 and IL-4, were measured.

MATERIALS AND METHODS

Animals

Specific pathogen-free female BALB/c mice were obtained from the Orient (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) and tap water *ad libitum.* Mice of 6-7 weeks old (20 ± 2 g) were used in the present study. The animal quarters were strictly maintained at 23 ± 3°C and 50 ± 10% relative humidity. A 12-hr light/dark cycle was used with an intensity of 150-300 Lux.

Materials

FPP-3 (purity, ＞99%) used in this study was chemically synthesized in our group (Jahng *et al.,* 2004; Lee *et al.,* 2004). Alserver's solution, DEAE-dextran, agar and corn oil were obtained from Sigma Chemical Company (St. Louis, MO). 2-Mercaptoethanol (2-ME), LPS, Con A, fetal bovine serum (FBS), Earle's balanced salt solution (EBSS), guinea pig complement, L-glutamine, penicillin G-streptomycin solution, and RPMI 1640 media were purchased from GIBCO (Grand Island, NY). CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay kit was purchased from Promega (Madison, WI). BD Cytofix/Cytoperm Plus™ (with GolgiPlug™) kit was obtained from BD Biosciences (San Diego, CA). All antibodies used for the flow cytometry were purchased from Pharmingen (San Diego, CA). SRBCs were obtained from the College of Animal Sciences at Yeungnam University. All other chemicals used were of reagent grade commercially available.

Animal treatment

For the antibody response to SRBCs, mice were treated orally with FPP-3 at 125, 250 or 500 mg/kg in 10 ml corn oil once daily for 7 consecutive days. FPP-3 exposed mice were immunized intraperitoneally with 5×10^8 SRBCs per mouse in 0.5 ml of EBSS 4 days prior to enumerating the numbers of antibody-forming cells (AFC). In a subsequent study, mice were administered with the same doses of FPP-3 without an immunization, followed by the assays for splenic lymphocyte phenotyping, reverse transcription-polymerase chain reaction (RT-PCR), intracellular interleukins (ILs) production, and *ex vivo* mitogen-stimulated proliferation. The control animals received corn oil only at 10 ml/kg. 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) in 1989 and Yeungnam University.

Splenic antibody response to the T-dependent antigen, SRBCs

On the day of necropsy, spleens were removed and weighed. Single cell suspensions were prepared in 3 ml of EBSS, washed and resuspended in 3 ml of EBSS. Spleen cells were then diluted 30-fold by resuspending a 100 μl aliquot of each suspension in 2.9 ml of EBSS. The number of AFC was determined using a modified hemolytic Jerne plaque assay (Kaminski *et al.,* 1990). The results were expressed as AFC/10^6 spleen cells and AFC/spleen.

Quantitation of splenic and thymic lymphocyte subpopulations

The lymphocyte subpopulations in spleen and thymus were quantitated using a modified method of Jeon *et al.* (2005). To enumerate B-cells, T-cells, $CD4^+$ and $CD8^+$ T-cell subsets, and macrophages, splenic and thymic cells were collected and suspended in a staining buffer containing 2% FBS and 0.1% sodium azide in phosphate-buffered saline (PBS), pH 7.4, to a cell density of 1.0×10^6/tube. The cells were incubated with anti-mouse CD16/CD32 Fc receptor (1 μg/tube, clone 2.4G2, Pharmingen, San Diego, CA) for 15 min at 4°C to prevent nonspecific binding and then labeled with an appropriate monoclonal antibody (mAb) conjugated to a fluorescent probe. For spleen cells, anti-mouse CD3e mAb (clone, 145-2C11) conjugated to peridinin chlorophyll-a protein was used to enumerate T-cells. For T-cell subsets, in spleen and thymus, fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 (clone, GK1.5) and phycoerythrin (PE)-conjugated anti-mouse CD8a (clone, 53-6.7) were used. B-cells and macrophages were stained with anti-mouse CD45R/B220 mAb (clone, RA3-6B2) conjugated to PE and FITC-conjugated anti-mouse CD11b (clone, M1/70), respectively. An isotype control was used for each antibody. Cell suspensions were incubated with appropriate antibodies at 1 μg/tube for at least 30 min on ice in the dark. After incubation, the 1× FACS Lysing solution (Becton Dickinson,

San Jose, CA) was treated for 10 min for hemolysis of erythrocyte. The cells were washed twice, resuspended in PBS containing 2% FBS and 0.1% sodium azide in total volume of 0.5 ml, and analyzed using a FACSCalibur® flow cytometry with CellQuest® software (Becton Dickinson).

Mitogen-induced lymphocyte proliferation assay

Effects of FPP-3 on *ex vivo* lymphoproliferative responses in splenocyte cultures isolated from the mice exposed to FPP-3 for 7 consecutive days were determined. Spleen cell suspensions from FPP-3-treated mice were prepared in EBSS, washed and resuspended in RPMI 1640 medium containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μM 2-ME. The cell number was adjusted to 1.11×10^6 cells/ml of culture media. Then the cell suspension (180 μl) was transferred to each well of a 96-well tissue culture plate (Costar). The cultures were incubated in a humidified 5% CO_2 incubator at 37°C in the presence of given concentrations (20 μl) of either LPS (10 μg/ml) or Con A (2 μg/ml) for 72 hr. After incubation, 20 μl of CellTiter 96® AQueous Non-Radioactive Cell Proliferation kit was added in each well, and then the plate was incubated in the incubator for additional 4 hr. The plate was read on a micro ELISA reader (TECAN A-5082, Austria) at a wavelength of 490 nm. Results were expressed as mean absorbance minus naive control's absorbance.

RNA preparation and mRNA analysis by RT-PCR

For *in vitro* activation, single cell suspensions (2.5×10^6 splenocytes/ml) from mice administered with FPP-3 were cultured with 2 μg/ml of Con A in the presence of recombinant IL-2 (rIL-2, Roche Applied Science, Mannheim, Germany) at 37°C in 5% CO_2 for 24 hr. Following incubation, total RNA was prepared using RNAzol™ B reagent (Tel-Test, Inc.). First-strand cDNA was prepared from 2 μg of total RNA in 20 μl with oligo $(dT)_{18}$ using the First Strand cDNA Synthesis Kit (Fermentas Inc.). PCR was performed with 4 μl of the generated cDNA using 0.5 U of Taq polymerases (Solgent Inc., Korea). The following primer pairs were purchased from Solgent. The sense and anti-sense primer sequences used were: IL-2, 5'-GTGC-TCCTTGTCAACAGCGC-3', 5'- GAGCCTTATGTGTTGT-AAGC-3'; IL-4, 5'-ACAAAAATCA CTTGAGAGAGATCAT-3', 5'-AGTAATCCATTTGCATGATGCT CTT-3'; and β-actin, 5'-CAGGTCCCGGCCAGCCAGGT- 3', 5'-CACCCGCCA-CCA GTTCGCCA-3'. PCR was performed using the TGRADIENT (Biometra, Germany). The denaturation, annealing, extension, and cycle conditions were as follows: IL-2, 94°C for 30 s, 58°C for 30 s, 72°C for 30 s and 35 cycles; IL-4, 94°C for 30 s, 60°C for 30 s, 72°C for 30 s and 40 cycles; β-actin, 94°C for 30 s, 63°C for 30 s, 72°C for 30 s and 25 cycles. The PCR products were electrophoresed through a 1.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. The gel images were captured on an Image Analysis System (SLB, Seoul, Korea) and the PCR product yields were normalized to β-actin after a quantitative estimation using Labworks software (UVP, Inc., Upland, CA). Prior to analysis, the band intensities of PCR products were checked to ensure that they had not reached the saturation intensity.

Determination of intracellular IL-2 and IL-4 production

For *in vitro* activation, single cell suspensions (1×10^6 splenocytes/ml) from mice administered with FPP-3 were cultured with 2 μg/ml of Con A in the presence of recombinant IL-2 (rIL-2, Roche Applied Science, Mannheim, Germany) at 37°C in 5% CO_2 for 12 hr. After incubation, the splenocytes were stimulated again with the rIL-2 and Con A for 5 hr. Brefeldin A (10 μg/10^6 cells) was added in this stage. Then the cells were washed with staining buffer, and blocked nonspecific binding through adding anti-mouse CD16/CD32 Fc receptor (1 μg/10^6 cells/tube) for 20 min on ice. The $CD4^+$ cells were identified using FITC-conjugated anti-mouse CD4 (0.5 μg/tube; clone, GK1.5) mAb, which was suspended in staining buffer and incubated for 30 min on ice in a dark condition. To simplify the fixation and permeabilization of cells for immunofluorescent staining of intracytoplasmic ILs, the BD Cytofix/Cytoperm Plus™ (with GolgiPlug™) kit was used. To fix and permeabilize the cells, Cytofix/Cytoperm solution (100 μl/tube) was added and incubated on ice for 20 min at dark. And then, 1× Perm/Wash solution (1 ml/tube) was added for washing. To stain intracellular cytokines, the fixed/permeabilized cells were incubated with 100 μl of Perm/Wash solution containing PE-conjugated anti-mouse IL-2 mAb (0.25 μg/tube; clone, JES6-5H4) or IL-4 mAb (0.25 μg/tube; clone, 11B11) on ice for 30 min at dark. After washing with 1× Perm/Wash solution twice, the cells were resuspended in staining buffer (300 μl /tube) and performed the flow cytometric analysis.

Statistics

The results were expressed as the mean ± S.E. and the statistical differences between the different dose groups and the vehicle control were determined by one-way analysis of variance 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.

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

FPP-3 (mg/kg)	Body weight (g)	Absolute weights (mg)		Relative weights (% body weight)	
		Spleen	Thymus	Spleen	Thymus
VH	22.4 ± 0.7	141 ± 6	72 ± 5	0.63 ± 0.02	0.32 ± 0.02
125	23.1 ± 0.7	167 ± 11	69 ± 4	0.72 ± 0.03*	0.30 ± 0.01
250	22.8 ± 0.3	152 ± 6	64 ± 5	0.67 ± 0.02	0.28 ± 0.03
500	22.6 ± 0.5	145 ± 7	67 ± 4	0.65 ± 0.03	0.29 ± 0.02

Female BALB/c mice were treated orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Four days before necropsy, mice were immunized with SRBCs intraperitoneally. Each value represents mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p < 0.05$ (*).

Table II. Effects of FPP-3 without an immunization on weights of body and lymphoid organs in female BALB/c mice

FPP-3 (mg/kg)	Body weight (g)	Absolute weights (mg)		Relative weights (% body weight)	
		Spleen	Thymus	Spleen	Thymus
VH	21.2 ± 0.7	96 ± 6	75 ± 2	0.45 ± 0.02	0.35 ± 0.01
125	21.5 ± 0.2	105 ± 2	79 ± 4	0.49 ± 0.01	0.37 ± 0.02
250	21.8 ± 0.3	100 ± 5	73 ± 3	0.46 ± 0.02	0.33 ± 0.01
500	19.3 ± 0.5	88 ± 8	42 ± 7**	0.45 ± 0.03	0.22 ± 0.03**

Female BALB/c mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. The body and organ weights were on the day of necropsy. Each value represents mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p<0.01$ (**).

RESULTS

Following that FPP-3 treated mice were sensitized intraperitoneally with SRBCs, the antibody response to SRBCs was enumerated four days later. Although the weights of body and lymphoid organs were not changed, FPP-3 at 500 mg/kg significantly decreased in the number of AFC in response to SRBCs by 25% when compared with the vehicle control (Table I and Fig. 2).

In the subsequent study, to determine the cellular target of FPP-3-induced immunotoxicity, mice were administered with the same doses of FPP-3 without an immunization, followed by splenic and thymic lymphocytes phenotyping using a flow cytometry. FPP-3 significantly decreased in the absolute and relative thymus weights by 44% and 37% at 500 mg/kg when compared to the vehicle control, respectively, but did not alter the spleen weight (Table II). In the flow cytometry, the mice treated with 500 mg/kg of FPP-3 showed a significant decrease in the % of macrophages to total splenocytes (Table III). As shown in Fig. 3 and 4, FPP-3 significantly reduced the absolute numbers of splenocytes and thymocytes at 500 mg/kg by 26% and 74%, respectively. FPP-3 significantly decreased in the numbers of total T-cells, $CD4^+$ cells, $CD8^+$ cells and macrophages in spleen at 500 mg/kg. The % of $CD4^+$ and $CD8^+$ T-cell subsets to total thymocytes were altered following the treatment with 500 mg/kg FPP-3 (Fig. 4A). The numbers of immature $CD4^+CD8^+$ cells and $CD4^+CD8^-$ cells

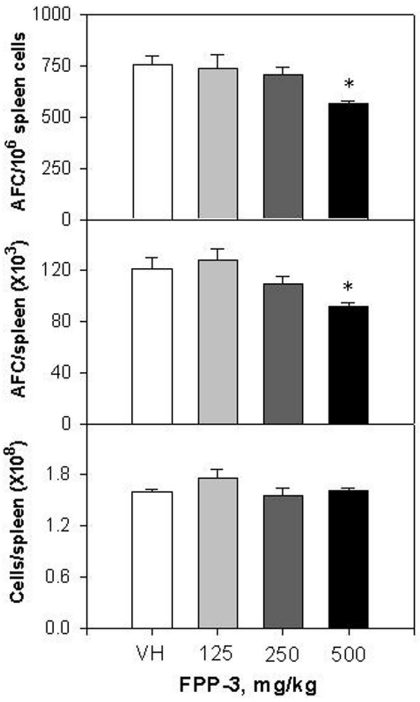

Fig. 2. Effect of FPP-3 on T-dependent antibody response in female BALB/c mice. Mice were treated orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Four days before enumerating the numbers of antibody-forming cells (AFC), mice were immunized with SRBCs intraperitoneally. Each value represents the mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p < 0.05$ (*).

Table III. Effects of FPP-3 without an immunization on phenotype percentage of splenocytes

Phenotypes		FPP-3 (mg/kg)			
		VH	125	250	500
Spleen (%)	T cells	46.87 ± 0.88	46.11 ± 0.70	47.11 ± 0.53	47.78 ± 1.66
	$CD4^+$ cells	29.25 ± 0.72	27.09 ± 0.51	28.73 ± 0.52	28.73 ± 2.00
	$CD8^+$ cells	10.86 ± 0.20	9.79 ± 0.25	10.01 ± 0.38	10.37 ± 0.43
	B cells	50.61 ± 0.75	50.57 ± 0.94	50.39 ± 1.01	53.01 ± 2.01
	Macrophages	3.73 ± 0.30	3.80 ± 0.33	4.09 ± 0.25	2.50 ± 0.42*
	$CD4^+/CD8^+$	2.70 ± 0.11	2.77 ± 0.05	2.89 ± 0.17	2.77 ± 0.13

Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Each bar represents the mean number of cells ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p < 0.05$ (*).

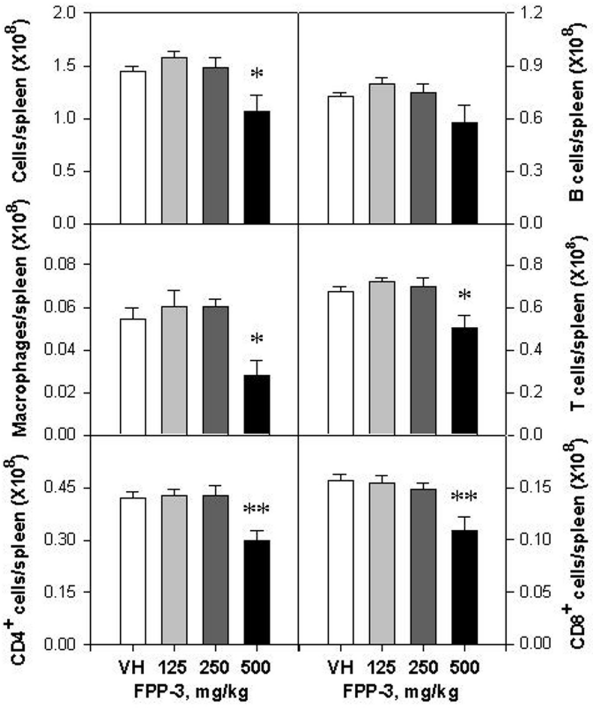

Fig. 3. Effect of FPP-3 on splenic lymphocyte subpopulations in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. Each bar represents the mean number of cells ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at either $p < 0.05$ (*) or $p < 0.01$ (**).

in thymus were remarkably decreased at 500 mg/kg FPP-3, whereas $CD4^-CD8^-$ cells were significantly increased at the same dose (Fig. 4B).

In the *ex vivo* mitogen-stimulated proliferation assay, splenocytes isolated from FPP-3 administered mice were cultured for 72 hr in the presence of either bacterial LPS or Con A. As shown in Fig. 5, the FPP-3 at 125, 250 and 500 mg/kg suppressed Con A-induced lymphoproliferation approximately 12%, 18% and 26% when compared with the vehicle control, respectively.

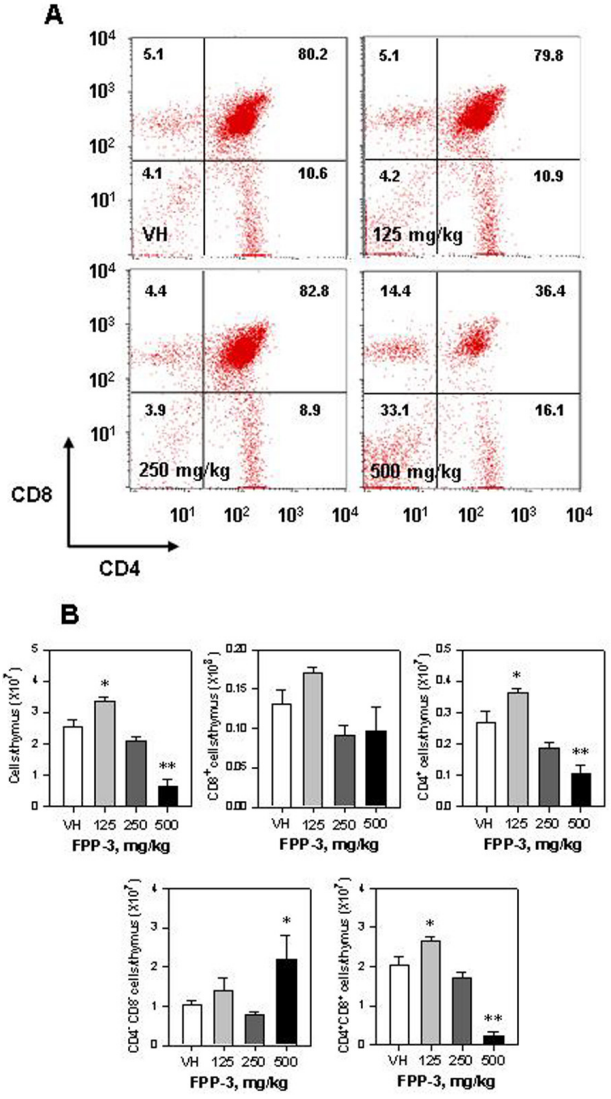

Fig. 4. Effect of FPP-3 on thymic lymphocyte subpopulations in female BALB/c mice. Mice were administered orally with FPP-3 in corn oil at 10 ml/kg for 7 consecutive days. (A) Percentage of thymocytes. (B) Absolute number of thymocytes. Each bar represents the mean number of cells ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at either $p < 0.05$ (*) or $p < 0.01$ (**).

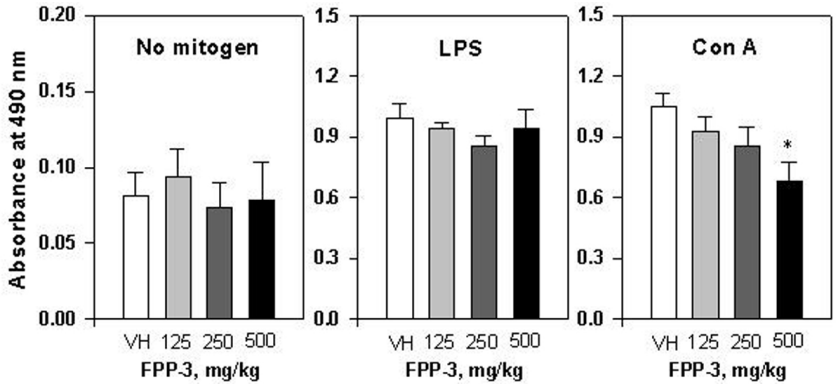

Fig. 5. Effect of FPP-3 on *ex vivo* lymphoproliferative responses in splenocyte cultures from female BALB/c mice. Splenocytes isolated from FPP-3 administered mice were cultured for 72 hr in the presence of either bacterial lipopolysaccharide (LPS) or concanavalin A (Con A). Detailed conditions were described in the Materials and Methods. Each bar represents the mean absorbance at 490 nm/well ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p < 0.05$ (*).

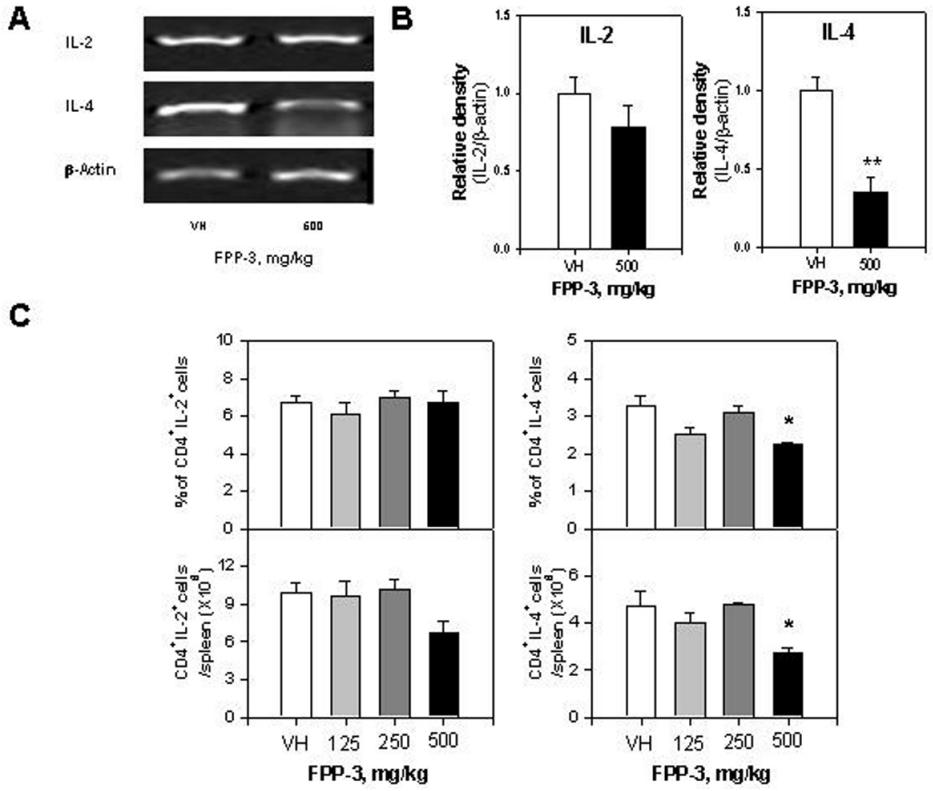

Fig. 6. Effects of FPP-3 on splenic cytokine gene expressions and intracellular IL-2 and IL-4 productions in female BALB/c mice. Splenocytes isolated from FPP-3 administered mice were cultured for 24 hr in the presence of Con A and recombinant IL-2. (A and B) RT-PCR. β-Actin was used as a control gene. (C) Flow cytometry. Experimental conditions were described in the Materials and Methods. Each bar represents the mean ± S.E. of 5 animals. The asterisks indicate the values significantly different from the vehicle control (VH) at $p < 0.05$ (*).

To clarify the possible mechanism(s) of FPP-3-induced immunosuppression, effects of FPP-3 on the splenic expression of cytokine mRNA and intracellular cytokine productions were investigated in this study. As shown in Fig. 6A and 6B, in RT-PCR, 500 mg/kg FPP-3 suppressed the expression of IL-4 mRNAs. The expression of IL-2 mRNA showed a weak suppression without a statistical significance. In the flow cytometry, the productions of splenic intracellular IL-4 in response to Con A were decreased in the cells isolated from mice administered with 500 mg/kg FPP-3. The numbers of $CD4^+$ $IL\text{-}4^+$ cells were reduced about 42%, following the administration of mice with 500 mg/kg of FPP-3 (Fig. 6C).

DISCUSSION

Investigating the immunotoxic potential of a candidate drug as a part of preclinical safety evaluation is very important. Particularly, a candidate acting on immune systems requires extensive studies on its adverse effects on immune functions. A key component of this investigation is the toxicologic profile generated through toxicity studies. The continuing efforts to develop anti-inflammatory agents from synthetic compounds led to synthesize a propenone compound FPP-3, which was demonstrated to inhibit the production of TNF-α and NO in the LPS-stimulated RAW264.7 macrophages (Jahng *et al.,* 2004; Lee *et al.,*

2004). In our preliminary study, FPP-3 might be moderately toxic in both ICR mice and Sprague-Dawley rats, because the estimated oral median lethal dose was about 1,000 mg/kg in both sexes (unpublished data).

The immunotoxic effects of certain chemicals may result in global impacts on immunological reactivity of an organism. The immune system is an intricate complex of interrelated cellular, molecular and genetic components, which provides a defense (i.e., immune response) against foreign organisms or substances and aberrant native cells. The immune responses are expressed as autoimmunity, chemical hypersensitivity or immunosuppression (Dean *et al.,* 1985). Traditional methods for toxicological assessment have implicated the immune system as a frequent target organ of toxic insult following exposure to certain drugs or environmental chemicals. A tiered approach, a screening battery, for characterizing potential immunotoxic compounds have been developed and validated in laboratory animals (Dean *et al.,* 1985; Luster *et al.,* 1988, 1992, 1993). These reports indicated that the highest associations with immunotoxicity were the T-dependent antibody response to SRBCs (78%) and the lymphocyte subpopulation analysis (83%) as determined by surface marker expression, and that the performances of only two or three immune tests are sufficient to predict immunotoxic compounds in rodents. Based on these literatures, some parameters of the immune function were measured in the present studies to investigate the immunotoxicological effects of FPP-3 in female BALB/c mice.

Because the AFC assay is the most sensitive indicator of the host's ability to mount an antibody response to a specific antigen (Luster *et al.,* 1993), the splenic antibody response to SRBCs was chosen. In the present study, gross indicators of immunotoxicity, such as changes in lymphoid organ weights, were not affected following FPP-3 treatment, whereas the number of AFC was significantly reduced at the highest dose of FPP-3 (Table I and Fig. 2). These results indicated a possible immunotoxic potential of FPP-3.

Flow cytometry is a unique technology useful in the examination of effects of immunotoxic agents on target cells of the immune system. The most common application of flow cytometry to immunotoxicity testings has been in the area of cell surface immunophenotyping of subsets of lymphoid cells found in the spleen or thymus of mice. To understand the cellular target in FPP-3-induced immunotoxicity, immunophenotyping of splenic and thymic cells isolated from FPP-3 treated mice was performed. The present results indicated that the number of splenic B cells was not significantly changed by FPP-3 treatment (Fig. 3). On the other hand, the numbers of total, $CD4^+$ and $CD8^+$ T cells and macrophages in spleens were significantly reduced by 500 mg/kg FPP-3. These results suggested that the T cells and macrophages in spleen might be relatively important cellular targets for FPP-3-induced immunotoxicity.

Xenobiotics-induced thymus atrophy is apparently caused by reduced proliferation rates of the immature thymocyte precursor pool leading to a reduction of the $CD4^+CD8^+$ thymocytes, which contribute approximately 80% of the thymocytes (Kremer *et al.,* 1994). In the present study, the treatment with FPP-3 without an immunization significantly decreased in the weights and total cellularity of thymus. In addition, FPP-3 treatment resulted in the decreased percentages of immature $CD4^+CD8^+$ thymocytes and the increased percentages of $CD4^-CD8^-$ thymocytes at the highest dose (Fig. 4A). These results suggested that, in addition to the thymic hypocellularity, FPP-3 might inhibit the thymocyte maturation.

Lymphocyte stimulation with mitogens is a measure of cell-mediated and humoral immunity. Con A is well known activator of T cells and LPS is a B-cell activator (Whitehurst and Geppert, 1996). In the *ex vivo* lymphoproliferation assay, FPP-3 inhibited splenocyte proliferation in response to Con A, but not to LPS (Fig. 5). Inhibition of Con A-induced lymphocyte proliferation by FPP-3 suggested that FPP-3 might suppress the cellular immunity.

The effects of drugs or environmental chemicals on the immune system are produced mostly by the following potential mechanisms: (1) interaction with the activated aromatic hydrocarbon receptor (Okey *et al.,* 1984); (2) membrane perturbation effects (Pallardy *et al.,* 1992); (3) changed IL production (Myers *et al.,* 1988); (4) disruption of the concentration of intracellular calcium mobilization (Davila *et al.,* 1995); or (5) metabolic activation to reactive metabolites (Ginsberg *et al.,* 1989).

Immune responses are regulated by various cells that are components of innate immunity and by the helper T (Th) cells that are components of adaptive immunity (Kurt-Jones *et al.,* 1987; Swain *et al.,* 1991). Naive $CD4^+$ (antigen-inexperienced) Th0 cells may be divided into two cell subpopulations, termed as Th1 and Th2, according to differences in the activities of their secreted cytokines (Mosmann *et al.,* 1986). Th1 cells primarily secrete IFN-γ, IL-2 and TNF-α, which promote cellular immunity, whereas Th2 cells secrete a different set of cytokines, primarily IL-4, IL-10 and IL-13, which promote humoral immunity (Fearon and Locksley, 1996; Mosmann and Sad, 1996; Mullen *et al.,* 2001). Th1 and Th2 cytokines both promote the growth/differentiation of their subsets and inhibit the

growth/differentiation of the opposing subset (Seder *et al.,* 1992).

In the study, we investigated the expression of cytokine mRNA and the ability to produce intracellular cytokines, such as IL-2 and IL-4, in Con A-treated splenocytes isolated from FPP-3 treated mice. As shown in Fig. 6A and 6B, FPP-3 markedly suppressed the expression of IL-4 mRNA at the dose of 500 mg/kg FPP-3. In case of IL-2, however, FPP-3 did not show a significant difference. In addition, the numbers of IL-2 and IL-4 producing cells were determined flow cytometrically. *In vivo* administration of mice with FPP-3 and a subsequent *in vitro* Con A-activation resulted in a significant suppression of the intracellular IL-4 production at the highest dose (Fig. 6C). These results suggested that FPP-3 would suppress the Th2 activity, and that FPP-3 could alter the function of T-cells *in vivo,* which coincided with a decrease in the lymphoproliferation of T-cells (Fig. 5).

Controlling the cell cycle is critical for cell growth and normal organ development. Deregulation of cell cycle progression is a signal that can initiate the apoptosis (Shi *et al.,* 1994). In our present study, based on the flow cytometry analysis, FPP-3 did not cause any significant changes at the doses tested (data not shown).

In conclusion, our present study demonstrated that a subacute treatment with 500 mg/kg FPP-3, a dose much higher than the dose for anti-inflammatory activity, had some potential immunotoxic capabilities, including the inhibition of cytokine production and the ability to inhibit T cell proliferation.

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