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The herbal medicine compound falcarindiol from Notopterygii Rhizoma suppresses dendritic cell maturation

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Running title: Falcarindiol suppresses dendritic cell maturation

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Number of text pages: 20
Number of tables: 0
Number of figures: 6
Number of references: 28
Number of words:
Abstract: 204
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Abbreviations: ANOVA, analysis of variance; BM-DC, bone marrow-derived DC; DC, dendritic cell; DTH, delayed-type hypersensitivity; FACS, fluorescence-activated cell sorter; FITC, fluorescein isothiocyanate; IL, interleukin; LPS, lipopolysaccharides; mAb, monoclonal antibodies; MAPK, mitogen activated protein kinase; MHC, major histocompatibility complex; NF-κB, nuclear factor-κB; OVA, ovalbumin; Treg, regulatory T cells.

Section: Cellular and molecular
Abstract

Dendritic cells (DCs) are important for regulating the immune response. We report an herbal medicine compound, falcarindiol, that affects DC function. Ethanol extracts of 99 crude drugs that are the main components of 210 traditional Japanese medicines (Kampo medicine) approved by the Ministry of Health, Labor, and Welfare in Japan were prepared and screened using the murine epidermal-derived Langerhans cell line XS106. Notopterygii Rhizoma strongly suppressed major histocompatibility complex class II (MHC II) expression in XS106. Activity-guided fractionation led to the isolation and identification of falcarindiol as a principal active compound in Notopterygii Rhizoma. Falcarindiol (1-5 μM) dose-dependently suppressed MHC II expression in XS106. Fresh-isolated bone marrow-derived DCs were examined for the production of MHC II, CD80, CD86, interleukin (IL)-12p70, and IL-10. Treatment of bone marrow-derived DCs with 5 μM falcarindiol significantly inhibited lipopolysaccharides-induced phenotype activation and cytokine secretion, and inhibited MHC II expression by CD40 ligation, but not phorbol myristate acetate+ionomycin or IL-12. Falcarindiol inhibited DC maturation by blocking the canonical pathway of nuclear factor-κB and phosphorylated-p38. Topical application of 0.002% and 0.01% falcarindiol before sensitization dose-dependently suppressed delayed-type hypersensitivity to ovalbumin (p<0.01). Falcarindiol induces immunosuppressive effects in vitro and in vivo and might be a novel therapy for autoimmune or allergic diseases.
Introduction

Hyperactive immune responses cause allergies and various autoimmune disorders. Steroids, immunosuppressants, and non-steroidal antiinflammatory drugs are routinely used to treat such diseases. New treatment modalities that elicit antigen-specific immunosuppression with minimum adverse reactions are in particularly high demand. Accumulating evidence suggests that CD4+CD25+Foxp3+ regulatory T cells (Treg) have a significant role in maintaining self-tolerance. Treg are required for tolerance to allogeneic and exogenous antigens and the prevention of autoimmune disorders (Yamazaki and Steinman, 2009). Treg are mainly induced by immature or semi-mature dendritic cells (DCs). Regulatory DCs are indispensable for immunologic tolerance (Yamazaki and Steinman, 2009).

The aim of our study was to screen the constituents of herbal medicines as a first step toward developing drugs that suppress abnormal immune responses by inducing regulatory DCs. In our previous study, we screened 99 herbal compounds contained in common traditional Japanese medicine formulations based on the antigen-presenting function of XS106 cells, an established DC line (Fukui et al., 2007). The 99 extracts had various effects on major histocompatibility complex class II (MHC II) expression, ranging from enhancement to suppression. We identified Amomi Semen (seeds of *Amomum xanthioides*), Polyporus (sclerotiums of *Poria cocos*), and Plantaginis Semen (seeds of *Plantago asiatica*), as potent activators of DCs and the effects of Amomi Semen were investigated in detail (Fukui et al., 2007). Two other extracts significantly suppressed the expression of MHC II molecules on DCs. Of the two extracts, Notopterygii Rhizoma had lower cytotoxicity and the active ingredient was successfully isolated and identified. Notopterygii Rhizoma is a crude drug derived from the rhizome and roots of *Notopterygium incisum* or *N. forbesii*. In traditional Japanese medicine, Notopterygii Rhizoma is used in formulations to treat pain, sensory paralysis, and poor circulation.

In the present study, we examined the effects of Notopterygii Rhizoma on DCs and the potential of Notopterygii Rhizoma and its active compound, falcarindiol, to induce regulatory
DCs. The mechanisms of action of falcarindiol were also analyzed. Finally, the in vivo effects of falcarindiol were assessed in an animal model.

Methods

Animals

Female 6 to 9-week-old C57BL/6J (H-2b) mice and female 7-week-old BALB/c mice were purchased from Japan SLC (Hamamatsu, Japan). The mice were treated according to institutional guidelines, and all procedures using mice were performed in accordance with an institutionally approved protocol. The mice within a single experiment were age-matched. Each group comprised at least 5 mice. The results were obtained from at least three sets of independent experiments.

Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (HL3), FITC-conjugated anti-I-A/I-E (2G9), FITC-conjugated anti-CD80 (16-10A1) and FITC-conjugated anti-CD86 (GL1) were purchased from BD Biosciences Pharamingen (San Diego, CA). Phycoerythrin-conjugated anti-B7-DC/PD-L2 (TY25), phycoerythrin-conjugated anti-B7-H1/PD-L1 (MIH5), phycoerythrin-conjugated anti-B7RP-1/ICOSL (HK5.3), biotin-conjugated anti-B7-H3 (M3.2D7), and biotin-conjugated anti-B7-H4/B7S1 (clone 9) were purchased from e-Bioscience (San Diego, CA). FITC-conjugated streptavidin was purchased from EMD Biosciences Inc. (San Diego, CA). Phorbol myristate acetate and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO). CD40 ligand was purchased from PeproTech (Rocky Hill, NJ). IL-12 was purchased from R&D Systems (Minneapolis, MN).

Herbal medicine extraction

Ninety-nine crude drugs, most of which are contained in the 210 traditional Japanese medicines approved by the Ministry of Health, Labor, and Welfare of Japan, were purchased from Tsumura (Tokyo, Japan) (Fukui et al., 2007). Herbal medicines (5 g) were extracted
with 50 ml ethanol (EtOH)/water (70/30, v/v) for 24 h at room temperature, and filtered. The process was repeated three times in sequence and the extracts pooled. The pooled filtrate was concentrated in vacuo, the residue was dissolved in H₂O, and the aqueous solution was lyophilized. The lyophilisate was redissolved in dimethylsulfoxide. To investigate cytotoxicity, cells were cultured for 24 h in the presence of the herbal compound extracts at concentrations of 1 and 10 μg/ml, and cell survival was assessed using the XTT assay (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s protocol. No cytotoxicity was detected (data not shown).

Isolation of falcarindiol

Notopterygii Rhizoma (100 g; Tsumura, Tokyo, Japan) was extracted with 1 L EtOH for 24 h at room temperature and filtered. The filtrate was evaporated under reduced pressure to give EtOH extract (12.6 g). The EtOH extract was suspended with 300 mL of EtOH/water (70/30, v/v), and partitioned with hexane (300 mL × 3) to give an upper layer (3.0 g), insoluble material (1.0 g), and a lower layer (6.6 g). The lower layer was the most active fraction and was therefore subjected to further purification. The lower layer was suspended with 180 mL water, and partitioned with ethyl acetate (EtOAc, 180 mL × 3) to give the EtOAc fraction (5.6 g) and H₂O fraction (1.2 g). The EtOAc fraction was subjected to silica gel (Fuji Silysia FL100D) column chromatography (hexane-EtOAc=10:1 → EtOAc) and 7 fractions (fr.1 to fr.7) were collected. Fr.5 was subjected to ODS column chromatography (Cosmosil 140C18-OPN (Nacalai Tesque, Kyoto, Japan), water-methanol (MeOH)=1:1 → MeOH) to obtain 3 fractions [Fr.5(1) to Fr.5(3)]. The obtained active fraction, Fr.5(2), was further separated by preparative high performance liquid chromatography. The high performance liquid chromatography was run with a Shimadzu LC-10ADvp pump with a Shimadzu SPD-M10Avp detector with the following conditions; column: TOSOH TSK-GEL ODS-80TS (4.6 mm × 250 mm), flow rate: 1.0 mL/min; column temperature: 40°C; detector: UV 200-400 nm, and eluent: water with a gradually increasing MeOH concentration (1/1, v/v to MeOH).
Nuclear magnetic resonance measurement

The nuclear magnetic resonance spectra were recorded in deuterated chloroform (CDCl₃) using a Brucker ABANCE 600.

Cell line

The murine epidermal-derived DC line XS106 (Timares et al., 1998; Matsue et al., 1999; Ozawa et al., 1999) was kindly provided by Akira Takashima from the Department of Medical Microbiology and Immunology at the University of Toledo Medical College and was used for screening and further analysis. The XS106 cells were expanded in complete RPMI 1640 (RPMI1640/10% fetal bovine serum/50 mM HEPES/5 mM sodium pyruvate/0.5 mM minimal essential medium non-essential amino acids solution/5x antibiotic-antimycotic, Sigma-Aldrich) in the presence of 1 ng/ml murine recombinant granulocyte-monocyte colony-stimulating factor and 10% fibroblast culture supernatant, as previously described (Matsue et al., 1999).

Generation of murine bone marrow (BM)-DCs

Murine BM-DCs were generated as described previously with minor modification (Inaba et al., 1992). Briefly, BM cells from the femurs and tibiae of C57BL/6J mice were flushed and seeded at 1×10⁶ cells in 1 mL of complete RPMI1640. Cultures were initiated with recombinant murine granulocyte macrophage-colony stimulating factor (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel) at 10 ng/ml. On days 3 and 5, nonadherent cells were depleted, and the medium was replaced with fresh medium containing 10 ng/ml of recombinant murine granulocyte macrophage-colony stimulating factor. On day 7, nonadherent cells were harvested and used as immature BM-DCs. The percentage of CD11c⁺ DCs among the nonadherent population averaged 80% when monitored by fluorescence activated cell sorter (FACS) analysis.

FACS analysis

Cells were suspended in FACS washing buffer (2% bovine serum albumin and 0.02% NaN₃ in phosphate buffered saline), blocked with rat or mouse IgG (Jackson ImmunoResearch
Laboratories, Inc., West Grove, PA) on ice for 10 min before antibody staining, and were then washed twice in FACS washing buffer, followed by incubation with monoclonal antibodies (mAb) on ice for 30 min. Cells were washed twice and analyzed by FACSCalibur (BD Immunocytometry Systems, Mountain View, CA). Staining was performed with 5 μg/ml mAbs.

**Cytokine assay**

BM-DCs were preincubated with 2.5 or 5 μM falcarindiol for 45 min, followed by 100 ng/mL lipopolysaccharide (LPS) for another 24 h. The supernatants were then collected and IL-12p70 and IL-10 expression levels were determined by an enzyme-linked immunosorbent assay (R&D Systems).

**Nuclear factor (NF)-κB translocation assay**

XS106 cells were preincubated with 1, 2.5, or 5 μM falcarindiol for 45 min, followed by 100 ng/mL LPS for 24 h. After incubation, the amount of active NF-κB in the nuclear extracts (2 μg/well) was evaluated using a Trans AM NF-κB Family kit (Active Motif Japan, Tokyo, Japan) according to the manufacturer’s protocol.

**Mitogen activated protein kinase (MAPK) activity analysis**

XS106 cells were preincubated with 1, 2.5, or 5 μM falcarindiol for 45 min, followed by 100 ng/mL LPS for 24 h. After incubation, phospho-ERK 1/2, phospho-JNK 1/2, and phospho-p38 activity was measured in the cell lysates (20 μg/well) using a Cytometric Bead Array (BD BioSciences Pharmingen, San Diego, CA).

**Delayed-type hypersensitivity (DTH) assay**

Falcarindiol or Notopterygii Rhizoma were mixed with white petroleum to make falcarindiol (0.002%, 0.01%, 0.05%) or Notopterygii Rhizoma (0.5%) ointment. Falcarindiol or Notopterygii Rhizoma ointment (20 mg) was applied to the shaved abdominal skin of BALB/c mice. Mice were sensitized by 500 μg ovalbumin (OVA) in an emulsion of 100 μL phosphate buffered saline (Sigma-Aldrich) and 100 μL incomplete Freund’s adjuvant (Sigma-Aldrich), and DTH was elicited with 100 μg OVA (2 mg/mL) in phosphate buffered...
saline. DTH elicitation was performed by subcutaneous injection of antigens into the left footpads 7 days after sensitization, if not otherwise stated. Phosphate buffered saline (50 μl) was injected into the right footpads as a negative control. Footpad swelling was evaluated by the thickness of the left footpad minus that of the right footpad at 24, 48, or 72 h after DTH elicitation. Footpad thickness was measured using a digital gauge (Mitsutoyo Corporation, Kanagawa, Japan).

**Statistical analysis**

Values are presented as the mean ± SEM. Analysis of variance (ANOVA) and Bonferroni-type multiple t-test and Student’s t-test were used to compare values. Values of p<0.05 were considered statistically significant.

**Results**

**Isolation and identification of the active compound in Notopterygii Rhizoma that suppresses DCs.** A previous study of 99 herbal medicines led to the identification of two herbal medicines, Saussureae Radix and Notopterygii Rhizoma, that significantly reduced MHC II expression in XS106 cells (Fukui et al., 2007, see Fig. 1 therein). Because Saussureae Radix has cytotoxic effects, Notopterygii Rhizoma was selected for the present study.

The 70% EtOH extract exhibited a stronger suppressive effect on MHC II expression than the EtOH extract used for the initial screening, therefore the 70% EtOH extract was used as the starting material for activity-guided fractionation. Using various chromatographic methods, the extract was fractionated following an MHC II expression assay of XS106 cells to yield a single compound as an active constituent (Figure 1). The 1H- and 13C-nuclear magnetic resonance data were completely identical with those of falcarindiol isolated from Angelica dahuria (Lechner et al., 2004). Thus, the active compound was identified as falcarindiol. Falcarindiol was previously isolated from Notopterygii Rhizoma as an antimicrobial compound (Matsuda et al., 2000).
Falcarindiol suppressed MHC II expression of untreated and LPS-treated XS106 cells in a concentration-dependent manner within a range of 1 to 5 μM (Figure 2). No cytotoxic effects were observed in XS106 cells at concentrations of less than 10 μM (data not shown), although cytotoxic effects were observed at concentrations over 15 μM. Therefore, the mechanisms of action of falcarindiol were further analyzed.

**Effects of falcarindiol on murine BM-DCs.** To analyze the biologic activity of falcarindiol, experiments were performed using BM-DCs. Cytotoxicity was first assessed using propidium iodide, and falcarindiol was not cytotoxic to BM-DCs up to 20 μM (data not shown). When falcarindiol was added on day 6 of BM-DC induction, no significant changes were observed in the expression of cell surface molecules (MHC II, CD80/86, PD-L1/2, ICOSL, B7-H3, B7S1) after 24 h incubation (data not shown). The addition of LPS 24 h after adding 5 μM falcarindiol decreased the expression of MHC II and CD86 compared to that of the LPS-alone group, confirming that falcarindiol blocked the LPS-mediated induction of cell surface molecules (Figure 3).

We then investigated the effects of falcarindiol on DC cytokine production. We measured interleukin (IL)-12p70, which is produced by activated DCs, and IL-10, which is produced by suppressive DCs and is involved in immunologic tolerance. Falcarindiol (5 μM) suppressed LPS-induced production of both IL-12p70 and IL-10 (Figure 4A). Transforming growth factor-β production was not detected in any of the groups (data not shown). We next investigated the suppressive effect of falcarindiol on DC maturation induced by various stimuli. LPS, CD40 ligand, and phorbol myristate acetate-ionomycin increased the expression of MHC II on BM-DCs, and falcarindiol suppressed the effects of LPS and CD40 ligand (Figure 4B). As these two compounds act via the NF-κB and MAPK pathways, falcarindiol appears to inhibit DC maturation by blocking these two pathways.

**Analysis of NF-κB in DCs.** When DCs are stimulated by LPS, NF-κB pathways are activated to induce cell maturation (Kim et al., 2005). Two signaling pathways are mediated by NF-κB: 1) the canonical pathway where the p50/p65 complex works as activated NF-κB;
and 2) the alternative pathway involving the p52/RelB complex (Youssef and Steinman, 2006). In the present study, the effects of falcarindiol on the intranuclear migration of p65, p50, p52, and RelB were investigated using XS106. When LPS were added, the intranuclear migration of these four molecules increased compared with the control (Figure 5A). Falcarindiol suppressed the LPS-induced increase in p65 and p50 translocation in a dose-dependent manner, but did not affect the LPS-induced increase in p52. Falcarindiol slightly decreased RelB translocation (Figure 5A). These results confirmed that falcarindiol blocks the maturation of XS106 cells by inhibiting the canonical pathway.

Analysis of MAPK in DCs. In addition to NF-κB, LPS induces DCs to activate the MAPK-mediated pathway (Agrawal et al., 2003; Matsuyama et al., 2003; Yu et al., 2004). A FACS array was used to analyze the intracellular expression of the activated (phosphorylated) forms of the following common MAPKs: ERK, JNK, and p38. Compared with the control group, the LPS group had significantly higher phosphorylated-p38 levels, but there were no marked changes in the p-JNK levels (Figure 5B). In addition, p-ERK expression was decreased. Falcarindiol suppressed the LPS-induced increase in phosphorylated-p38 expression, but it did not have a marked effect on p-JNK and p-ERK expression.

Suppressive effects of falcarindiol on DTH reactions in mice. The in vitro results indicate that falcarindiol might be therapeutically effective for treating immunologic abnormalities. Therefore, we investigated the effects of falcarindiol in an in vivo immune disease model. DTH models are often used to assess immune responses and immunologic tolerance (Isomura et al., 2006; Lee et al., 2007), and DCs are thought to have a large role as antigen-presenting cells during the sensitization and induction periods of DTH. We evaluated the effects of falcarindiol in a DTH model using OVA as the antigen.

Three days before sensitization, a petrolatum ointment containing falcarindiol was applied to the abdominal area of female BALB/c mice, and sensitization was induced by subcutaneous injection of OVA into the abdominal area. Seven days after sensitization, OVA was injected subcutaneously into the footpad, and footpad swelling was measured. Compared
with the normal group without induced sensitization, the control group had significantly greater footpad swelling, thus establishing the DTH model. The 0.01% falcarindiol ointment significantly suppressed sensitization 24 and 72 h after induction, and both the 0.002% and 0.01% falcarindiol ointments significantly suppressed sensitization 48 h after induction (Figure 6).

**Discussion**

In a previous study, 70% ethanol extracts were prepared from 99 crude drugs used in common traditional Japanese medicine formulations approved by the Ministry of Health, Labor and Welfare, Japan, and their effects on DCs were investigated (Fukui et al., 2007). Screening assays of these drugs were based on the amount of MHC II expression on the DCs. The objective of the present study was to identify specific compounds that enhance the function of regulatory DCs; therefore, the herbal medicines found to reduce MHC II expression were studied.

In the previous study, two herbal medicines, Saussureae Radix and Notopterygii Rhizoma, reduced MHC II expression (Fukui et al., 2007). Saussureae Radix is a dried root of *Saussurea lappa* or *S. costus* (Compositae) and contains essential oil constituents such as aplofaxene and costic acid. Saussureae Radix extracts and its components, however, are cytotoxic. Due to this toxicity, further analysis was not performed in the present study.

Falcarindiol was isolated from Notopterygii Rhizoma and identified as an active ingredient that decreases MHC II expression in DCs. Oral administration of a hot water extract of Notopterygii Rhizoma in mice suppresses contact dermatitis caused by picryl chloride (Sun and Xu, 2002). Although we did not assay the falcarindiol content in the water extract, it is highly possible that falcarindiol in a hot water extract suppresses contact dermatitis by suppressing DC maturation, thus suggesting *in vivo* effects of falcarindiol. Other studies have reported that falcarindiol protects the liver by blocking the production of nitric oxide in LPS-induced macrophages (Yoshikawa et al, 2006) and suppresses the expression of
inducible nitric oxide synthase by blocking the activation of NF-κB in rat primary stellate cells (Shiao et al, 2005). Inhibition of NF-κB suppresses DC maturation (Iruretagoyena et al, 2006; Yoon et al, 2006).

Based on the finding that falcarindiol reduced MHC II expression in XS106 cells, we conducted the same experiment using BM-DCs. Falcarindiol suppressed the LPS-induced maturation of BM-DCs. Falcarindiol inhibited the increase in MHC II and CD86 expression, but not CD80. In addition, falcarindiol suppressed LPS-induced production of IL-12p70 and IL-10; thus, falcarindiol appears to suppress not only the LPS-induced maturation of DCs (surface phenotype), but also the LPS-induced production of cytokines. IL-10 is an important cytokine for inducing Treg cells, and as falcarindiol suppressed the production of IL-10, it is likely that falcarindiol, rather than inducing tolerogenic DCs, inhibits DCs from maturing.

The NF-κB blocking effect of falcarindiol was previously reported in satellite cells (Shiao et al, 2005). In the present study, intracellular migration of NF-κB components in response to LPS stimulation was further analyzed in XS106 cells. Falcarindiol suppressed the migration of p65 and p50, but not p52. In particular, p65 migration was almost completely suppressed to a level comparable to that in unstimulated XS106 cells. The falcarindiol-sensitive subunits, p65 and p50, form a dimer that serves as activated NF-κB in the canonical pathway. In other words, falcarindiol inhibits the maturation of XS106 cells by blocking the canonical pathway. The canonical pathway is involved in inflammatory and immunologic reactions (Sha, 1998). In addition, the alternative pathway uses the p52/RelB complex as activated NF-κB. When the alternative pathway is activated, intranuclear transfer of the p50/p65 complex is blocked, thus suppressing immune responses involving CD4+ T cells (Ishimaru et al., 2006).

Compared with controls, 0.002% or 0.01% falcarindiol ointments suppressed footpad swelling, suggesting that falcarindiol arrests DC maturation to suppress DTH reactions. On the other hand, when a 0.05% falcarindiol ointment or 0.5% Notopterygii Rhizoma ethanol extract was applied, the swelling was not suppressed, indicating that falcarindiol has an optimal in vivo concentration. Cytotoxicity was observed in DCs at concentrations over 15
μM. The 0.002% and 0.01% falcarindiol ointments suppressed DTH, but the detailed mechanisms of action were not clarified in the present study. Because the ointments were applied before sensitization, falcarindiol appeared to have acted on DCs, but whether falcarindiol-induced immature DCs produced immunologic tolerance by inducing Treg cells and T cell unresponsiveness in the lymph nodes or falcarindiol negated the migration of immature DCs to the lymph nodes is not known. To elucidate the mechanisms of action of falcarindiol, further studies of the responses of falcarindiol-treated DCs and T cells and characterization of the T cells in the lymph nodes in animal models are needed.
References


Shiao YJ, Lin YL, Sun YH, Chi CW, Chen CF, and Wang CN (2005) Falcarindiol impairs the expression of inducible nitric oxide synthase by abrogating the activation of IKK and JAK


Footnotes

This work was financially supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.
Legends for Figures

Figure 1. Partition of EtOH extract of Notopterygii Rhizoma and the falcarindiol structure
Total activity = -(MFI of sample-treated group—MFI of untreated group) / (MFI of LPS-treated group – MFI of untreated group) x 100.
MFI: mean fluorescence intensity.

Figure 2. Falcarindiol downregulates MHC II expression in a dose-dependent manner (A). XS106 cells (2 × 10^5) were incubated with 0.5, 1, 2.5, or 5 μM falcarindiol for 24 h. XS106 were subsequently stained with FITC-conjugated anti-MHC II mAb and examined by FACS analysis. Falcarindiol downregulates MHC II expression in LPS-induced mature DCs in a dose-dependent manner (B). XS106 cells (2 × 10^5) were preincubated with 0.5, 1, 2.5, or 5 μM falcarindiol for 45 min followed by stimulation with LPS for 24 h. XS106 were subsequently stained with FITC-conjugated anti-MHC II mAb and examined by FACS analysis. Results are expressed as mean ± SEM (n=3) of one representative experiment of at least three. *p<0.05, ***p < 0.001 compared with the control group or LPS treated group without falcarindiol (ANOVA and Bonferroni’s correction for multiple comparisons t-test).

Figure 3. Falcarindiol downregulates MHC II and co-stimulatory molecule expression in LPS-induced mature BM-DCs. BM-DCs (1 × 10^6) were preincubated with 2.5 or 5 μM falcarindiol for 45 min followed by stimulation with LPS for 24 h. BM-DCs were subsequently stained with FITC-conjugated anti-MHC II, CD80, and CD86 mAb and examined by FACS analysis. Results are expressed as mean ± SEM (n=3) of one representative experiment of at least three. *p<0.05, **p < 0.01 compared with LPS-treated group (ANOVA and Bonferroni’s correction for multiple comparisons t-test).

Figure 4. Cytokine production by BM-DCs treated with falcarindiol (A). BM-DCs (1 ×
were preincubated with 2.5 or 5 μM falcarindiol for 45 min followed by stimulation with LPS for 24 h. The secretion of IL-12p70 and IL-10 in the supernatants was assayed by enzyme-linked immunosorbent assay. Results are expressed as mean ± SEM (n=3) of one representative experiment of at least three. ***p < 0.001 compared with LPS-treated group (ANOVA and Bonferroni’s correction for multiple comparisons t-test).

Effects of falcarindiol on MHC II expression of stimulated BM-DCs (B). BM-DCs (1 × 10^6) were preincubated with 5 μM falcarindiol for 45 min followed by stimulation with LPS (100 ng/ml), IL-12 (50 ng/ml), CD40L (2 μg/ml), or phorbol myristate acetate (10 ng/ml)+ionomycin (400 ng/ml) for 24 h. BM-DCs were subsequently stained with FITC-conjugated anti-MHC II, CD80, and CD86 mAb and examined by FACS analysis. Each column represents the mean florescence intensity± SEM of one representative experiment of at least three. $p < 0.09 vs. the nontreated group (Student’s t-test).

Figure 5. Falcarindiol inhibits NF-κB activation on XS106 (A). XS106 cells (4 × 10^5) were preincubated with falcarindiol for 45 min followed by stimulation with LPS for 24 h. Nuclear protein extracts of XS106 were prepared, and NF-κBp65, p50, p52, and RelB nuclear protein were examined. Each column is presented as representative data of three independent experiments.

MAPK activation of XS106 treated with falcarindiol and LPS (B). XS106 cells (4 × 10^5) were preincubated with falcarindiol for 45 min followed by stimulation with LPS for 24 h. The cell lysates of XS106 were prepared and measured by FACS array analysis. Results are expressed as mean ± SEM (n=3) of one representative experiment of at least three. **p<0.01, ***p < 0.001 compared with LPS treated group without falcarindiol (ANOVA and Bonferroni’s correction for multiple comparisons t-test).

Figure 6. Topical falcarindiol suppresses OVA-induced DTH. Falcarindiol, EtOH extract of Notopterygii Rhizoma, or petroleum jelly were applied to the shaved abdominal skin of
BALB/c mice on day 0, then followed by sensitization by 500 μg OVA (day 1) and elicitation with 100 μg OVA on day 8. Footpad thickness was measured at 24, 48, and 72 h after elicitation. Each column represents the mean footpad swelling ± SEM of 6 mice. *p < 0.05 **p < 0.01 ***p < 0.001 vs. the control (sensitization) group (ANOVA and Bonferroni’s correction for multiple comparisons t-test).
Fig 1

Notopterygii Rhizoma (100 g)
extracted with EtOH at room temp.

Notopterygii Rhizoma EtOH extract (12.6 g) [11.1]

Partitioned with hexane and 70% EtOH

Upper layer (3.0 g)
Insoluble material (1.0 g)
Lower layer (6.6 g)

Partitioned with EtOAc and water

EtOAc fraction (5.6 g)
H₂O fraction (1.2 g)

Silica gel column chromatography
Hexane : EtOAc = 10 : 1 → EtOAc

fr.1 (238 mg)
fr.2 (449 mg)
fr.3 (405 mg)
fr.4 (967 mg) [11.5]
fr.5 (692 mg)
fr.6 (986 mg)
fr.7 (837 mg)

ODS column chromatography
MeOH : H₂O = 1 : 1 → MeOH

Fr.(1) (13.6 mg) [4.5]
Fr.(2) (80.0 mg) [11.8]
Fr.(3) (23.8 mg) [6.5]

HPLC ODS H-80 Column
MeOH : H₂O = 4 : 1

Falcarnindiol (7.3 mg) [18.5]

[Image of Falcarnindiol structure]
Fig 3

MHC class II

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<th>LPS (100 ng/mL)</th>
<th>Falcarindiol (μM)</th>
<th>Mean fluorescence intensity</th>
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CD80 (B7-1)

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<td>200</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>*</td>
</tr>
</tbody>
</table>

CD86 (B7-2)

<table>
<thead>
<tr>
<th>LPS (100 ng/mL)</th>
<th>Falcarindiol (μM)</th>
<th>Mean fluorescence intensity</th>
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<td>-</td>
<td>-</td>
<td>*</td>
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<td>+</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>+</td>
<td>2.5</td>
<td>150</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>*</td>
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</tbody>
</table>
Fig 5

A  

**Canonical**  

<table>
<thead>
<tr>
<th>LPS (100 ng/mL)</th>
<th>Falcarindiol (µM)</th>
<th>Absorbance (450nm)</th>
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</thead>
<tbody>
<tr>
<td>-</td>
<td>0</td>
<td>p65: 0.2, p50: 0.1</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>p65: 0.8, p50: 0.6</td>
</tr>
<tr>
<td>+</td>
<td>2.5</td>
<td>p65: 0.4, p50: 0.2</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>p65: 0.2, p50: 0.1</td>
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</table>

**Alternative**  

<table>
<thead>
<tr>
<th>Falcarindiol (µM)</th>
<th>p52</th>
<th>RelB</th>
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<tbody>
<tr>
<td>0</td>
<td>n.d.</td>
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</tr>
<tr>
<td>+</td>
<td>1</td>
<td>0.1</td>
</tr>
<tr>
<td>2.5</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>5</td>
<td>0.12</td>
<td>0.12</td>
</tr>
</tbody>
</table>

B  

<table>
<thead>
<tr>
<th>LPS (100 ng/mL)</th>
<th>Falcarindiol (µM)</th>
<th>Unit/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>0</td>
<td>p-ERK: 500, p-JNK: 1000, p-p38: 1500</td>
</tr>
<tr>
<td>+</td>
<td>1</td>
<td>p-ERK: 2500, p-JNK: 2000, p-p38: 1500</td>
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<tr>
<td>+</td>
<td>2.5</td>
<td>p-ERK: 2000, p-JNK: 1500, p-p38: 1500</td>
</tr>
<tr>
<td>+</td>
<td>5</td>
<td>p-ERK: 2000, p-JNK: 1500, p-p38: 1500</td>
</tr>
</tbody>
</table>

**Significance Levels**  

- **p-ERK**:  |
- **p-JNK**:  |
- **p-p38**:  |
Fig 6