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**Isoflavones enhance interleukin-17 gene expression via retinoic acid
receptor-related orphan receptors α and γ**

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Abstract

The retinoic acid receptor-related orphan receptors α and γ (ROR α and ROR γ), are key regulators of helper T (Th)17 cell differentiation, which is involved in the innate immune system and autoimmune disorders. In this study, we investigated the effects of isoflavones on ROR α/γ activity and the gene expression of interleukin (IL)-17, which mediates the function of Th17 cells. In doxycycline-inducible CHO stable cell lines, we found that four isoflavones, biochanin A (BA), genistein, formononetin, and daidzein, enhanced ROR α - or ROR γ -mediated transcriptional activity in a dose-dependent manner. In an activation assay of the *Il17a* promoter using Jurkat cells, these compounds enhanced the ROR α - or ROR γ -mediated activation of the *Il17a* promoter at concentrations of 1×10^{-6} M to 1×10^{-5} M. In mammalian two-hybrid assays, the four isoflavones enhanced the interaction between the ROR α - or ROR γ -ligand binding domain and the co-activator LXXLL peptide in a dose-dependent manner. In addition, these isoflavones potently enhanced *Il17a* mRNA expression in mouse T lymphoma EL4 cells treated with phorbol myristate acetate and ionomycin, but showed slight enhancement of *Il17a* gene expression in ROR α/γ -knockdown EL4 cells. Immunoprecipitation and immunoblotting assays also revealed that BA enhanced the interaction between ROR γ t and SRC-1, which is a co-activator for nuclear receptors. Taken together, these results suggest that the isoflavones have the ability to enhance IL-17 gene expression by stabilizing the interactions between ROR α/γ and co-activators. This also provides the first evidence that dietary chemicals can enhance IL-17 gene

expression in immune cells.

Key words: interleukin 17; isoflavone; luciferase assay; retinoic acid receptor-related orphan receptor; Th17.

Abbreviation list

AF, activation function; ANOVA, analysis of variance; BA, biochanin A; CD-FBS, charcoal-dextran treated FBS; CHO, Chinese hamster ovary; CNS, conserved noncoding sequence; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DZ, daidzein; EAE, experimental autoimmune encephalomyelitis; EDTA, ethylenediamine tetra-acetic acid; FBS, Fetal bovine serum; FGF, fibroblast growth factor; FN, formononetin; G6Pase, Glucose-6-phosphatase; Gal, galactocidase; GE, genistein; HRP, horse-radish peroxidase; IL-17, interleukin 17; LBD, ligand-binding domain; LUC, luciferase; NCOA1, nuclear receptor co-activator-1; NCOR1, nuclear receptor co-repressor-1; NR, nuclear receptor; PCR, polymerase chain reaction; PMA, phorbol 12-myristate 13-acetate; RIP140, receptor interacting protein 140; ROR, retinoic acid receptor-related orphan receptor; RORE, ROR response element; PGC-1 α , peroxisome proliferator-activated receptor γ co-activator-1 α ; RT-PCR, reverse transcriptase-PCR; shRNA, short hairpin RNA; SD, standard deviation; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide-gel electrophoresis; SEM, standard error of mean; SRC-1, steroid receptor co-activator-1; Th17, T-helper 17; TRE, tetracycline responsive element; UAS, upstream activating sequence;

1. Introduction

The retinoic acid receptor-related orphan receptors, ROR α - γ (NR1F1-3), are members of the nuclear receptor superfamily and function as ligand-dependent transcription factors that are involved in the regulation of a wide range of physiological processes, including immune response, lipid/glucose homeostasis, and circadian rhythm (Jetten, 2009; Jetten et al., 2013; Takeda et al., 2014a; Takeda et al., 2014b). They are considered to be orphan receptors because their endogenous ligands have not yet been agreed upon definitively, and they have been identified with distinct tissue distributions and biological activities (Jetten, 2009). In the thymus, ROR α and two isoforms, γ 1 and γ 2 (also referred to ROR γ t), have been identified (He et al., 1998), with ROR γ t differing from the ROR γ 1 isoform in that it lacks the amino terminus of ROR γ 1. Recent studies have shown that the ROR α and ROR γ regulate the transcription of interleukin 17 (IL-17) and other pro-inflammatory cytokines in T helper 17 (Th17) cells (Ivanov et al., 2006; Yang et al., 2008). As Th17 cells play important roles in host defense against bacterial and fungal infections, as well as in a wide variety of autoimmune diseases, including psoriasis and rheumatoid arthritis (Harrington et al., 2005; Park et al., 2005; Weaver et al., 2007; Stockinger and Veldhoen, 2007), the identification of ligands that regulate the RORs has been the focus of significant interest due to their potential for clinical use (Huh and Littman, 2012; Solt and Burris, 2012).

Unlike most members of the nuclear receptor superfamily, RORs bind as monomers to ROR response element (RORE), consisting of an AGGTCA element preceded by an

AT-rich sequence, in the promoter regulatory region of the target genes. Subsequent transcriptional regulation by RORs is mediated through constitutively recruiting co-repressors and co-activators, including NCOR1, RIP140, NCOA1 and PGC-1 α (Jetten, 2009). Recent studies have shown that a synthetic chemical SR1001, digoxin derivatives, and ursolic acid act as ROR α and/or ROR γ inverse agonists to inhibit basal transcription mediated by ROR itself, and ameliorate the severity of experimental autoimmune encephalomyelitis (EAE), which is dependent on Th17 cell function (Huh et al., 2011; Solt et al., 2011; Xu et al., 2011). In our previous paper, we reported that some azole-type fungicides, such as imibenconazole, inhibited *Il17a* mRNA expression via ROR α/γ in mouse lymphoma EL4 cells, and that some environmental chemicals can also act as modulators of IL-17 gene expression in immune cells (Kojima et al., 2012). Thus, extensive study has been undertaken on inhibitory small molecules, including several ROR inverse agonists, and some of them may have potential applications to drug-therapy for autoimmune diseases in the future. On the other hand, there have been few reports on chemicals that enhance ROR activity, although some hydroxycholesterols, cholesterol sulfate and a synthetic chemical SR1078 have been reported to act as ROR agonists (Kallen et al., 2002; Wang et al., 2010). The identification of ROR agonistic compounds would aid in the development of therapeutic means for fighting certain bacterial or fungal infections and cancers through the augmentation of ROR and Th17 cell activity (Huh and Littman, 2012; Solt and Burris, 2012).

Isoflavones are naturally occurring plant chemicals, and their plant-based dietary intake may play a beneficial role in the treatment/prevention of obesity, cancer, osteoporosis, and cardiovascular disease (Setchell and Cassidy, 1999). Two of the major isoflavones found in humans are genistein (GE) and daidzein (DA), which are metabolized from their plant precursors, biochanin A (BA) and formononetin (FN), respectively. These isoflavones share a common diphenolic structure that resembles that of the potent synthetic estrogens diethylstilbestrol and hexestrol (Fig. 1). Therefore, the effects of isoflavones on human health have been the focus of much attention due to their estrogenic activity via estrogen receptors (Takeuchi et al., 2009). To date, there have been no reports on the effects of isoflavones on Th17 cell function, although the effects of GE on immunity have been extensively studied (Yellayi et al., 2002).

In this study, we investigated the potential ROR α and ROR γ activities of isoflavones using Chinese hamster ovary (CHO)-K1 and Jurkat T cell-based reporter gene assays. As a result, we found that isoflavones, such as BA and FN, enhanced the constitutive activation of ROR α and ROR γ , and also enhanced the interactions between RORs and the co-activator NCOA1. In addition, these compounds were found to enhance *Il17a* gene expression in EL-4 cells in a ROR-dependent manner. Thus, we here provide the first evidence that dietary isoflavones might increase IL-17 gene transcriptional activity through their actions as ROR α and ROR γ agonists.

2. Materials and Methods

2.1. Chemicals and antibodies (Ab).

Formononetin (FN, >99% pure), biochanin A (BA, >99% pure), daidzein (DA, >99% pure) and genistein (GE, >98% pure) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A synthetic ROR inverse agonist, T0901317 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma-Aldrich. Ionomycin was purchased from LKT Laboratories (St. Paul, MN, USA). Dimethylsulfoxide was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and used as a vehicle. All compounds tested were dissolved in DMSO at a concentration of 1×10^{-2} M. Anti-FLAG and anti-SRC-1 Abs were obtained from Sigma-Aldrich and Santa Cruz Biotechnology (Santa Cruz, CA, USA), respectively.

2.2. Cell line and cell culture materials.

CHO-K1 cells, human lymphoma T Jurkat cells, mouse lymphoma EL4 cells, and human cervix carcinoma HeLa cells were obtained from the American Type Culture Collection. Fetal bovine serum (FBS) and charcoal-dextran treated FBS (CD-FBS) were obtained from Hyclone (Logan, UT, USA). Dulbecco's modified Eagle's medium (DMEM), DMEM plus Ham's F-12 nutrient mixture (DMEM/F-12) and RPMI-1640 medium were obtained from GIBCO-BRL (Invitrogen Rockville, MD, USA). Glutamine and penicillin-streptomycin (antibiotics) solutions were obtained from Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan), and 0.25% trypsin/0.02% ethylenediamine tetra-acetic acid (EDTA) disodium salt solution was obtained from

Life Technologies (Paisley, UK). CHO-K1 cells were maintained in DMEM/F-12 containing 5% FBS and antibiotics. EL4 and Jurkat cells were maintained in RPMI-1640 medium containing 10% FBS and antibiotics. HeLa cells were maintained in DMEM containing 10% FBS and antibiotics.

2.3. ROR-reporter gene assays using CHO Tet-on cells.

Doxycycline-inducible ROR stable cell lines were generated by transfecting a pTRE2 expression vector (Clontech, Mountain View, CA, USA) containing ROR α or ROR γ , into CHO Tet-on cells (Clontech) followed by transfection with the pGL4.27 luciferase (LUC) reporter vector (Promega, Madison, WI, USA) driven by 5xRORE. Single clones of pGL4.27-5xRORE- and pTRE2-ROR-expressing cells were selected from a medium containing hygromycin (Invitrogen, Grand Island, NY, USA) and puromycin (Sigma-Aldrich), respectively. CHO Tet-on cell lines were cultured in F12 medium supplemented with 10% FBS, and suitable for use in the Tet-on system (Clontech). To induce ROR expression, cells were treated for 20 h with 1 μ M doxycycline in the presence or absence of a dilution series of the isoflavones. All the assays were performed in triplicate and repeated independently at least twice. RORE-mediated activation of the LUC reporter was measured with a Luciferase Assay Substrate Kit (Promega). cAMP-based cell viability was evaluated by CellTiter-Glo Luminescent Cell Viability Assay (Promega).

2.4. Activation assay of the *III7a* promoter in Jurkat cells.

Jurkat cells were co-transfected with a pCMV- β -Gal plasmid (Clontech),

pCMV10-3xFlag-ROR α or pCMV10-3xFlag-ROR γ plasmid, and a pGL4.14 reporter plasmid (Promega) under the control of human *Il17a*-3kb-CNS promoter (Zhang et al., 2012), using Lipofectamine 2000 (Invitrogen), and then treated with the vehicle, or 0.1, 1, 10 μ M of the isoflavones. After 24 h, the *firefly* luciferase and β -galactocidase activities were measured using a Luciferase Assay Substrate Kit (Promega) and a Luminescent β -galactosidase Detection Kit II (Clontech), respectively. The *firefly* luciferase activity was normalized against β -galactocidase activity. All transfections were performed in triplicate and repeated at least twice.

2.5. Mammalian two-hybrid assay.

CHO-K1 cells were co-transfected with a pGL4.27-(UAS)₅ reporter plasmid, containing 5 copies of the Gal4 upstream activating sequence (UAS) in the reporter vector pGL4.27 (Promega), pCMV- β -Gal plasmid, pM-EBIP96(LXXLL) plasmid, and VP16-ROR α (LBD) or VP16-ROR γ (LBD) plasmid, containing the ligand-binding domain (LBD) of ROR (Kurebayashi et al., 2004; Takeda and Jetten, 2013), using Lipofectamine 2000 (Invitrogen), and then treated with the vehicle, or 0.1, 1, 10 μ M of the isoflavones. After 24 h, the *firefly* luciferase and β -galactocidase activities were measured as described above and their ratios calculated. All transfections were performed in triplicate and repeated at least twice.

2.6. *Il17a* mRNA expression in EL4 cells or ROR α/γ -knockdown EL4 cells.

We plated host EL4 cells or ROR α/γ -knockdown EL4 cells (Kojima et al., 2012) in 12-well microtiter plates (Nalge, Nunc, Denmark) at a density of 2×10^6 cells/ml in

RPMI-1640 medium containing 10% FBS. Next day, the cells were treated with the vehicle or both PMA (5 ng/ml) and ionomycin (1 μ M) for the induction of *Il17a*. At the same time, the cells were treated with the vehicle, or 0.1, 1 or 10 μ M of the isoflavones, and incubated at 37°C for 6 h. The cells were harvested and homogenized in 1 mL of TRIzol Reagent (Molecular Research Center, Cincinnati, OH, USA), followed by total RNA isolation. The complementary DNA (cDNA) was synthesized using 1 μ g of total RNA by Revertra Ace reverse transcriptase (TOYOBO, Osaka, Japan). The cDNAs were then stored at -20°C until analysis.

2.7. Real-time quantitative PCR for *Il17a* and β -actin.

We measured *Il17a* and β -actin mRNA levels using TaqMan[®] Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). cDNA prepared from the total RNA of the EL4 cells was subjected to real-time quantitative PCR on a 7900HT Fast Real Time PCR system (Applied Biosystems). The following probes were designed by Applied Biosystems: for *Il17a*, 5'-FAM d(CTTCATCTGTGTCTCTGATGCTGTT) NFQ-3'; and for β -actin, 5'-FAM d(ACTGAGCTGCGTTTTACACCCTTTC) NFQ-3'. The amount of *Il17a* mRNA was normalized against that of β -actin mRNA.

2.8. Interaction assay between ROR γ t and NCoA-1 using immunoprecipitation and immunoblotting.

The expression plasmids FLAG-ROR γ t and FLAG-ROR γ t Δ AF2 were constructed according to our previously described method (Matsuda et al., 2001). HeLa cells were plated on 6-cm dishes at 1×10^6 cells/dish, and transfected with FLAG-ROR γ t or

FLAG-ROR γ t Δ AF2 using jetPEI (Polyplus Transfection, Strasbourg, France), according to the manufacturer's instructions. At 36 h after transfection, the cells were left untreated or were treated with BA (1 μ M) for an additional 15 min. Immunoprecipitation and western blotting assays were performed as described previously (Matsuda et al., 2001). The immunoprecipitates from the cell lysates and the cell lysates were resolved on *G6Pase* and *FGF21* and transferred to polyvinylidene difluoride transfer membrane (PerkinElmer, Boston, MA, USA). The membranes were then immunoblotted with anti-FLAG or anti-SRC-1 Ab. Immunoreactive proteins were visualized using ImmobilonTM Western Chemiluminescent HRP Substrate (Millipore).

2.9. Data analysis.

We used a one-way analysis of variance (ANOVA) followed by Bonferroni correction to evaluate the differences in transcriptional levels between the control group and each of the chemical groups in the reporter gene assays and quantitative RT-PCR assays. Statistical significance was set at $p < 0.05$. Data were presented as means $\pm$ SD of three triplicate experiments.

3. Results

3.1. Effects of isoflavones on transcriptional activity in doxycycline-inducible Tet-on CHO cell line for ROR α and ROR γ

We examined the effects of isoflavones on ROR α - and ROR γ -mediated transcriptional activity using CHO Tet-on cells. This system consists of the CHO cells stably integrated with a doxycycline-inducible ROR α or ROR γ expression vector and a RORE-responsive-LUC reporter (Slominski et al., 2014). The system allows a sensitive detection of RORE-mediated LUC activation by the inducible expression of ROR on addition of doxycycline. Figure 2A and B show the transcriptional activity of the four isoflavones in CHO Tet-on cells expressing ROR α and ROR γ , respectively. In the ROR α assay, the isoflavones functioned as agonists of ROR α -mediated activation in a dose-dependent manner from 1×10^{-8} to 1×10^{-5} M (Fig. 2A). The order of the relative potency of ROR α agonistic activity was BA, FN > GE > DZ. In the ROR γ assay, these compounds acted as ROR γ agonists in a dose-dependent manner from 1×10^{-8} to 1×10^{-5} M (Fig. 2B). The order of the relative potency of ROR γ agonistic activity was FN > BA > GE, DZ.

Based on cytotoxicity testing using CellTiter-Glo, none of the compounds tested showed any cytotoxic effects at concentrations from 1×10^{-8} to 1×10^{-5} M (Fig. 2C and D).

3.2. Effects of isoflavones on the activation of the *Il17a* promoter in Jurkat T cells

ROR α and ROR γ have been reported to directly regulate the transcription of the IL-17 gene (Jetten, 2009; Yang et al., 2008). To examine the effect of isoflavones on the activation of the *Il17a* promoter, we cotransfected Jurkat cells with the ROR α or ROR γ expression plasmid and pGL4.14 reporter plasmid under the control of the *Il17a* promoter. As shown in Fig. 3A, we found that all four isoflavones enhanced the activation of the *Il17a* promoter via ROR α in a dose-dependent manner, with the activation by BA and GE, in particular, observed at a concentration of 1×10^{-6} M. In the cells transfected with ROR γ , these isoflavones significantly enhanced the activation of the *Il17a* promoter at a concentration of 1×10^{-5} M, with the activation by BA and FN again observed at the lower concentration of 1×10^{-6} M (Fig. 3B).

3.3. Effects of isoflavones on the interaction of ROR-LBD with the LXXLL-peptide EBIP96 assessed using a mammalian two-hybrid assay

Transcriptional activation by RORs is mediated through their interaction with co-activators, which interact with the activation domain (AD) of ROR through LXXLL-like motifs (Jetten, 2009). Therefore, we examined the effects of isoflavones on the interaction of the LBD of ROR α or ROR γ with the LXXLL-peptide EBIP96 using a mammalian two-hybrid system (Kurebayashi et al., 2004; Takeda and Jetten, 2013). In the ROR α -LBD assay, the isoflavones enhanced the interaction between the LBD of ROR α and the LXXLL-peptide in a dose-dependent manner (Fig. 4A). The order of the relative potency was FN > BA > GE, DZ. In the ROR γ -LBD assay, the isoflavones also

enhanced the interaction between the LBD of ROR γ and the LXXLL-peptide in a dose-dependent manner (Fig. 4B). The order of the relative potency was FN > BA, GE, DZ.

3.4. Effects of isoflavones on the in vitro expression of *Il17a* mRNA in mouse T lymphoma EL4 cells

Our previous study showed that several azole-fungicides, showing ROR α/γ inverse agonistic activity in CHO-reporter gene assays, inhibited *Il17a* gene expression in PMA/ionomycin-stimulated EL4 cells (Kojima et al., 2012). In this study, we examined whether isoflavones affect *Il17a* gene expression level in EL4 cells. Figure 5 shows the effects of isoflavones and T0901317 on *Il17a* gene expression in PMA/ionomycin-stimulated EL4 cells. Real-time PCR analysis revealed that four isoflavones significantly enhanced *Il17a* mRNA expression at concentrations from 1×10^{-7} to 1×10^{-5} M in PMA/ionomycin-stimulated EL4 cells. Of the four isoflavones, GE (1×10^{-5} M) induced the most potent increase of *Il17a* mRNA expression, and BA (1×10^{-5} M) followed it. In addition, treatment with FN and DZ (each 1×10^{-6} M) significantly enhanced *Il17a* mRNAs in comparison with vehicle treatment. In contrast, ROR inverse agonist T0901317 (1×10^{-5} M) potently suppressed *Il17a* mRNA expression in PMA/ionomycin-stimulated EL4 cells, as described in our previous paper (Kojima et al., 2012).

3.5. Effects of biochanin A on *Il17a* mRNA expression in the *RORα/γ* knockdown EL4 cells

We previously reported the establishment of EL4/shRorac and EL4/shNC cells as endogenous *RORα* and *RORγ* double-knockdown EL4 and control EL4 cell lines, respectively (Kojima et al., 2012). *RORα* and *RORγ* mRNA expression in EL4/shRorac cells respectively decreased at about 50 and 30% of those in EL4/shNC cells when the cells were treated with PMA/ionomycin (Kojima et al., 2012). In this study, we examined whether the enhancement of *Il17a* gene expression by isoflavones was *RORα/γ*-dependent using PMA/ionomycin-stimulated EL4/shRorac and EL4/shNC cells. Figure 6 show the respective effects of BA and T0901317 (each 1×10^{-5} M) on *Il17a* mRNA levels in EL4/shRorac and EL4/shNC cells treated with PMA/ionomycin. *Il17a* mRNA expression in the EL4/shRorac cells was found to be markedly decreased in comparison with that in EL4/shNC cells. Although BA induced a potent increase in *Il17a* mRNA expression in EL4/shNC cells, this compound showed slight enhancement of *Il17a* mRNA expression in EL4/shRorac cells. In contrast, T0901317 showed inhibitory effects on *Il17a* mRNA expression in both cell lines as described by our previous paper (Kojima et al., 2012)

3.6. Effects of biochanin A on the protein interaction between *RORγt* and co-activator SRC-1

The steroid receptor co-activator-1 (SRC-1) is one of co-activators able to

physically interact with ROR γ and enhance ROR γ -mediated transcriptional activation (Kurebayashi et al., 2004). We investigated the effect of BA on the protein-protein interaction between FLAG-ROR γ t or FLAG-ROR γ t Δ AF2 and endogenous SRC-1 using immunoprecipitation and immunoblotting. As shown in Fig.7, the presence of BA (1×10^{-6} M) enhanced the recruitment of SRC-1 by ROR γ t. On the other hand, deletion of the carboxyl terminal amino acids (477-495) of ROR γ t containing the activation function 2 (AF2) (ROR γ t Δ AF2) resulted in the total loss of BA-induced SRC-1 recruitment by ROR γ t.

4. Discussion

RORs are orphan nuclear receptors, the endogenous ligands for which remain unknown, that are constitutively active and retain the ability to interact with a range of co-activator NR box peptides even in the absence of agonistic ligands (Jetten et al., 2001). Therefore, it may be relatively difficult to identify compounds that further increase ROR activity beyond the plateau of regulation induced by endogenous ligand (Huh and Littman, 2012). Indeed, most of the small molecules identified as ROR modulators are ROR α and/or ROR γ inverse agonists, and reports on newly identified ROR agonists are limited. To our knowledge, there has been only one report, by Wang et al. (2010), on the identification of ROR agonists among exogenous agents. They reported that the synthetic chemical SR1078 acts as a ROR α/γ dual agonist, and stimulates the expression of two ROR target genes, *G6Pase* and *FGF21*, in hepatocarcinoma cells (Wang et al., 2010). More recently, this chemical was reported to be useful as an anti-cancer therapeutic agent through a novel mechanism for the stabilization of p53 protein expression by ROR α (Wang et al., 2012), and as an insulin-inducing agent through the activation of insulin transcription by ROR α (Kuang et al., 2014). Thus, the identification of ROR α/γ agonists would be of great value with regard to not only the augmentation of the innate immune system via Th17 cell activity, but also other beneficial physiological processes.

In this study, we demonstrated for the first time that dietary isoflavones act as agonists of ROR α - and ROR γ -mediated transactivation. We used several cell-based

assays to demonstrate that isoflavones act as ROR agonists. The CHO Tet-on assay system is useful for the evaluation of ROR agonists and inverse agonists among a large range of chemicals using doxycycline-inducible ROR α or ROR γ . In these assays, four isoflavones were found to enhance RORE activation in a ROR-dependent manner (Fig. 2A and B). FN and BA, in particular, both of which possess a methoxy group at 4' position, showed higher agonistic activity to ROR α and ROR γ than did GE and DZ, which possess a hydroxyl group at the same position (Fig. 1), suggesting that isoflavones with a hydrophobic rather than a hydrophilic structure at that position can preferentially enhance RORE activation via ROR α/γ . In addition, using Jurkat cells, we found that isoflavones enhance *Il17a* promoter activation as well as RORE activation (Fig. 3A and B). These findings suggest that isoflavones might act as ROR agonists in the up-regulation of IL-17 gene expression in immune cells.

Transcriptional activation by nuclear receptors is mediated through interaction with nuclear co-factors that are part of a larger co-activator complex (McKenna and O'Mally, 2002). This implies that RORs contain an activation function (AF-2) located at the C terminus of its LBD, and agonist binding induces the AF-2 helix to adopt a conformation that encourages interactions with the LXXLL motifs of co-activator proteins such as SRCs (Fujita-Sato et al., 2011; Jin et al., 2010; Kurebayashi et al., 2004). On the basis of the results from two-hybrid assays, we showed that the LBDs of ROR α and ROR γ can interact with the LXXLL motifs of EBIP96 even in the absence of any exogenous ligands (Fig. 4A and B). Interestingly, four isoflavones, including BA,

were found to further enhance these high basal interactions between the LBD of ROR α or ROR γ and EBIP96 in a dose-dependent manner. In addition, based on the results of an immunoprecipitation assay, we demonstrated that BA remarkably enhanced the protein binding of ROR γ t with SRC-1, but failed to do so for ROR γ t lacking AF2 (Fig. 7). These results suggest that isoflavones enhance ROR α - and ROR γ -mediated transcriptional activity through the stabilization of the interactions between the AF2 of ROR and its co-activators, such as EBIP96 and SRC1, in immune cells.

In our previous study, we used PMA/ionomycin-stimulated EL4 cells to evaluate the influence of azole-type fungicides on *Il17a* gene expression, and also established ROR α/γ knockdown EL4 (EL4/shRorac) cells using shRNA technology (Kojima et al., 2012). Similarly, in this study, we examined whether isoflavones affect *Il17a* gene expression in EL4 or EL4/shRorac cells. As a result, we found that four isoflavones enhanced *Il17a* mRNA expression in PMA/ionomycin-stimulated EL4 cells (Fig. 5). In addition, BA was found to potently enhance *Il17a* expression in EL4/shNC cells, but slightly affect in EL4/shRorac cells (Fig. 6). This implies that the remaining ROR α/γ is involved in the slight enhancement of *Il17a* mRNA expression by BA. Taken together, these results suggest that isoflavones, including GE and BA, can positively modulate IL-17 production in a ROR-dependent manner. However, the isoflavone dose-dependency observed in *Il17a* expression assays using EL4 cells (Fig. 5) differs from the dose-dependency observed in reporter gene assays using CHO-K1 or Jurkat cells (Figs. 2-4). This may indicate a difference in response between endogenous RORs

in EL4 cells and over-expressed RORs in CHO-K1 or Jurkat cells. In addition, it has been reported that the enhancement of *Il17a* gene expression might involve not only ROR-mediated activation, but also other additional mechanisms, such as the activation of aryl hydrocarbon receptor (AhR) and signal transducer and activator of transcription 3 (STAT3) (Rutz et al., 2013). Interestingly, GE was reported to be able to activate mouse AhR and STAT3 (Zhang et al., 2003; Chau et al., 2007). These findings appear to support our data showing that GE induced the most potent increase in *Il17a* mRNA expression in EL4 cells. Figure 5 also shows that the effects of FN and DZ on *Il17a* mRNA expression in EL4 cells were not dose-dependent. Although we can't clearly explain this, their effects may indicate unknown inhibitory effects on *Il17a* mRNA expression by high doses of FN and DZ, and may involve specific structural requirements such as the lack of a hydroxyl group at position 5 in isoflavones (Fig. 1).

Isoflavones are mainly found in legumes, especially in plants from the family *Fabaceae*, such as soy and red clover (Coward et al., 1993), although they are also found in trace amounts in several fruits and plant seeds such as sesame and sunflowers (Liggins et al., 2000). According to many epidemiological studies, the daily intake of isoflavones among Southeast Asians ranges between 15 and 47 mg, while the Western population consumes only 0.15 to 1.7 mg of isoflavones per day (Medjakovic et al., 2010). Although the physiological concentrations reached during exposure to these isoflavones might be lower than the concentrations at which ROR activity is affected, an epidemiological study has reported that the mean concentration of GE in serum

samples from Japanese men was 492.7 nM, compared with 33.2 nM in those from men in the United Kingdom (Morton et al., 2002). Many studies have provided evidence regarding multiple health and toxic effects of isoflavones (Setchell and Cassidy, 1999). Thus, isoflavones are speculated to be of great value with regard to the augmentation of the innate immune system through the enhancement of Th17 cell activity as well as possessing health benefits when used as supplements for the amelioration of symptoms associated with menopause.

The present study also provides clues to the structural design of potent and selective ROR ligands with potential applications to the treatment of metabolic and immune disorders. The development of ROR agonists represents a promising therapeutic strategy in the treatment of immunosuppressive diseases (Huh and Littman, 2012; Solt and Burris, 2012). ROR α/γ agonists, such as isoflavones, enhance the beneficial functions of Th17 cells in fighting pathogens, and might be useful as lead compounds for the development of drugs for the treatment of immune dysfunction diseases involving the loss of Th17 cells. On the other hand, as the enhancement of ROR α/γ activity might adversely affect Th17 cell function in autoimmune diseases, the effect on Th17 cells associated with the intake of isoflavones needs to be assessed in *in vivo* studies using autoimmune animal models, such as EAE. Further studies, including *in vivo* tests using mice, are needed to fully elucidate the effect of isoflavones on IL-17 production in the immune system.

5. Conclusion

Our findings indicate that dietary chemicals, such as isoflavones, may act as ROR α / γ dual agonists to regulate Th17 cell function, leading to potential effects on the immune system in organisms. As the physiological roles of RORs are shown to more and more important, ROR agonists, such as isoflavones, might become valuable agents for the regulation of various diseases involving RORs.

Conflicts of interest

None.

Acknowledgements

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Figure Legends

Fig. 1. Chemical structures of the isoflavones used in this study.

Fig. 2. Effects of isoflavones on ROR-dependent transcriptional activation using CHO Tet-on cells. Reporter gene assay was performed using doxycycline-inducible Tet-on CHO cell line for ROR α (A) and ROR γ (B) under treatment for 20 h with 1 μ M doxycycline in the presence or absence of a dilution series of isoflavones, such as biochanin A, genistein, formononetin, and daidzein. Relative LUC activity driven by 5xRORE was determined as described in Materials and Methods. Reporter activation was plotted as a percentage of the control value [presence of vehicle]. Values represent the means $\pm$ SEM (n=3) and are presented as percentage induction, with 100% activity defined as the constitutive activity induced by the vehicle control (DMSO). Significant differences from the vehicle control are indicated by asterisks (* P < 0.05 in all 4 isoflavones; 1-way ANOVA). Cytotoxicity of the isoflavones was evaluated by the total amount of ATP in each cells using CellTiter-Glo Luminescent Cell Viability Assay in ROR α -expressing cells (C) and ROR γ -expressing cells (D).

Fig. 3. Isoflavone-induced enhancement of ROR α - and ROR γ -mediated *Il17a* promoter activation in Jurkat cells. Cells were co-transfected with the pCMV- β -Gal and pCMV10-3xFlag-ROR α (A) or pCMV10-3xFlag-ROR γ (B) and pGL4.14 reporter

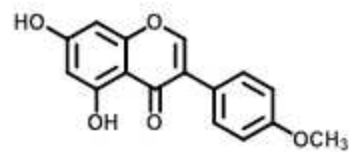
plasmid under the control of the *III7a* promoter and treated with increasing concentrations of the isoflavones at 0.1, 1, and 10 μ M. After 24 h, relative LUC activity was determined as described in Materials and Methods. The *firefly* luciferase activity was normalized against β -galactosidase activity. BA, GE, FN, and DZ represent each isoflavone, biochanin A, genistein, formononetin, and daidzein, respectively. Values represent the means $\pm$ SEM (n=3). Significant differences from the vehicle control (DMSO) plus ROR α or ROR γ are indicated by asterisks (* P < 0.05; 1-way ANOVA).

Fig. 4. Effects of isoflavones on interactions between ROR-LBD and the co-activator LXXLL peptide in mammalian two-hybrid assays. The analysis was performed by co-transfecting CHO-K1 cells with a pGL4.27-(UAS)₅ reporter plasmid, pCMV- β -Gal, pM-EBIP96 peptide, and either VP16-ROR α (LBD) (A) or VP16-ROR γ (LBD) (B). Cells were treated in the presence of the vehicle (DMSO), or increasing concentrations of the four isoflavones as indicated. After 24 h, relative LUC activity was determined as described in Materials and Methods. The *firefly* luciferase activity was normalized against β -galactosidase activity. BA, GE, FN, and DZ represent each isoflavone, biochanin A, genistein, formononetin, and daidzein, respectively. Values represent the means $\pm$ SEM (n=3) and are presented as the mean n-fold induction over the vehicle control. Significant differences from the vehicle control (DMSO) are indicated by asterisks (* P < 0.05; 1-way ANOVA).

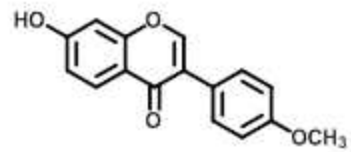
Fig. 5. Enhancement of *Il17a* mRNA expression by isoflavones in EL4 cells treated with PMA and ionomycin. EL4 cells were treated with PMA (5 ng/ml) and ionomycin (1 μ M) for *Il17a* gene induction. At the same time, the cells were treated with the vehicle, or 0.1, 1 or 10 μ M of the isoflavones, and incubated at 37°C for 6 h. *Il17a* mRNA expression in the cells was amplified by real-time RT-PCR and normalized against the expression of the β -actin housekeeping gene. BA, GE, FN, and DZ represent each isoflavone, biochanin A, genistein, formononetin, and daidzein, respectively. Values are expressed relative to the vehicle control with PMA/ionomycin stimulation (taken as 1) and represent the mean $\pm$ SD of three experiments. Significant differences from the vehicle control with PMA/ionomycine stimulation are indicated by asterisks (* P < 0.05 and ** P < 0.01; 1-way ANOVA).

Fig. 6. Comparison of enhancement of *Il17a* mRNA expression by biochanin A using EL4/shRorc cells and EL4/shNC cells. *Il17a* mRNA expression was measured in EL4/shRorac cells and EL4/shNC cells in the presence or absence of biochanin A (1×10^{-5} M) with PMA/ionomycin stimulation, and normalized against the expression of the β -actin housekeeping gene. Values are expressed relative to the vehicle control with PMA/ionomycin stimulation in EL4/shNC cells (taken as 1) and represent the mean $\pm$ SD of three experiments. Significant differences from the vehicle control with PMA/ionomycine stimulation are indicated by asterisks (* P < 0.05 and ** P < 0.01; 1-way ANOVA). ND, not detected (< 0.01).

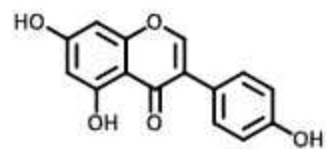
Fig. 7. Effects of biochanin A on protein interactions between ROR γ t and NCoA-1 (SRC-1). (A) The schematic diagram shows the amino acids structures of ROR γ t and ROR γ t Δ AF2, including DNA binding domain (DBD) and ligand binding domain (LBD). (B) HeLa cells in a 6-cm dish were transfected with expression plasmid FLAG-ROR γ t or FLAG-ROR γ t Δ AF2. At 36 h after transfection, the cells were left untreated or were treated with BA (1×10^{-6} M) for 15 min. The cells were lysed, immunoprecipitated with control Immunoglobulin (Ig) or anti-NCoA-1 (anti-SRC-1) Ab, and immunoblotted with anti-FLAG or anti-SRC-1 Ab. An aliquot of each total-cell lysate was analysed by immunoblotting with anti-FLAG Ab.



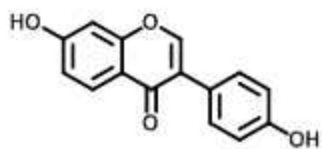
Biochanin A



Formononetin



Genistein



Daidzein

Figure 1

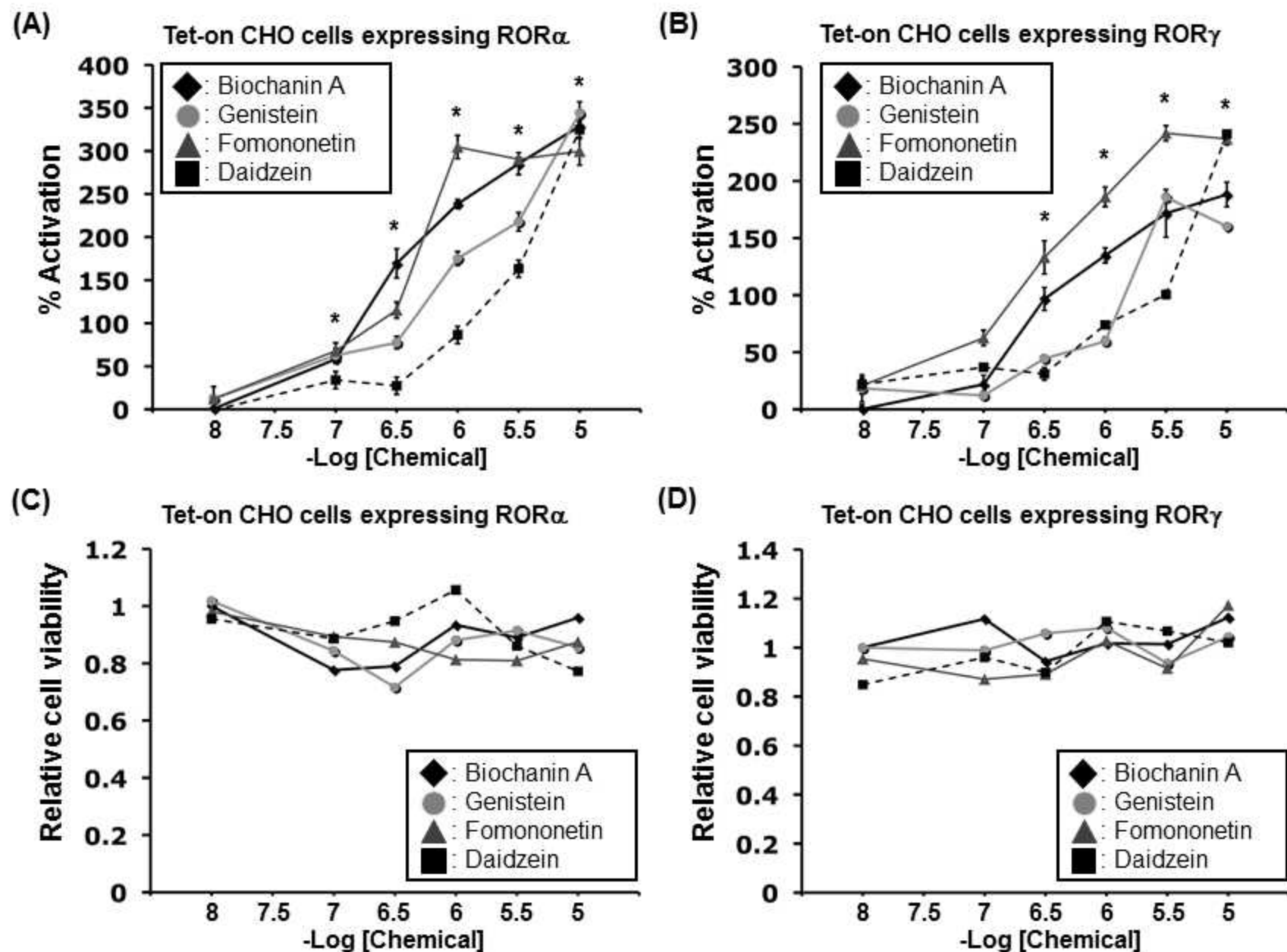


Figure 2

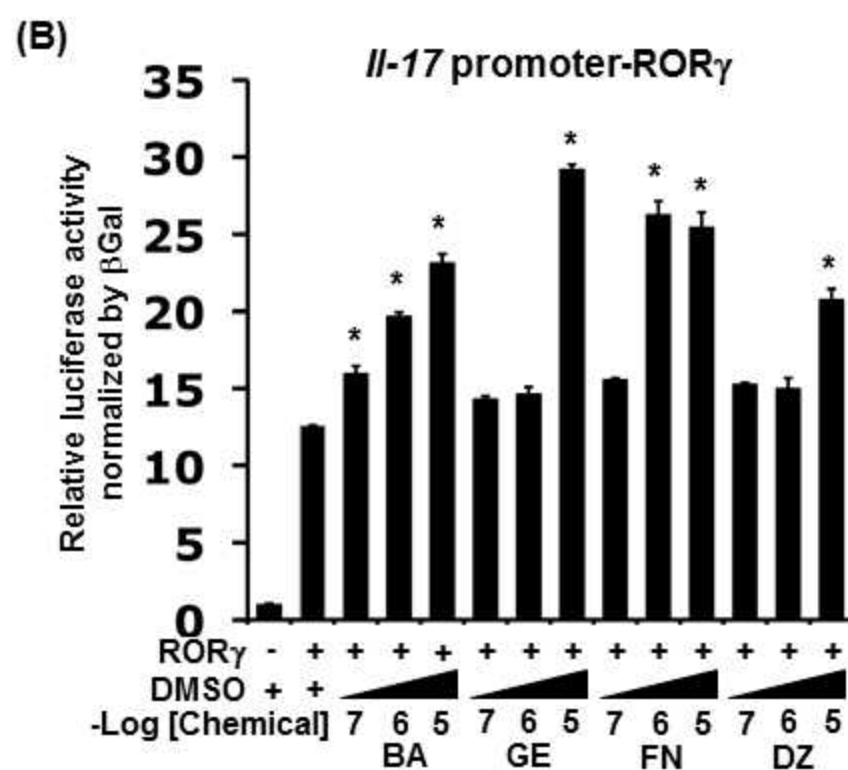
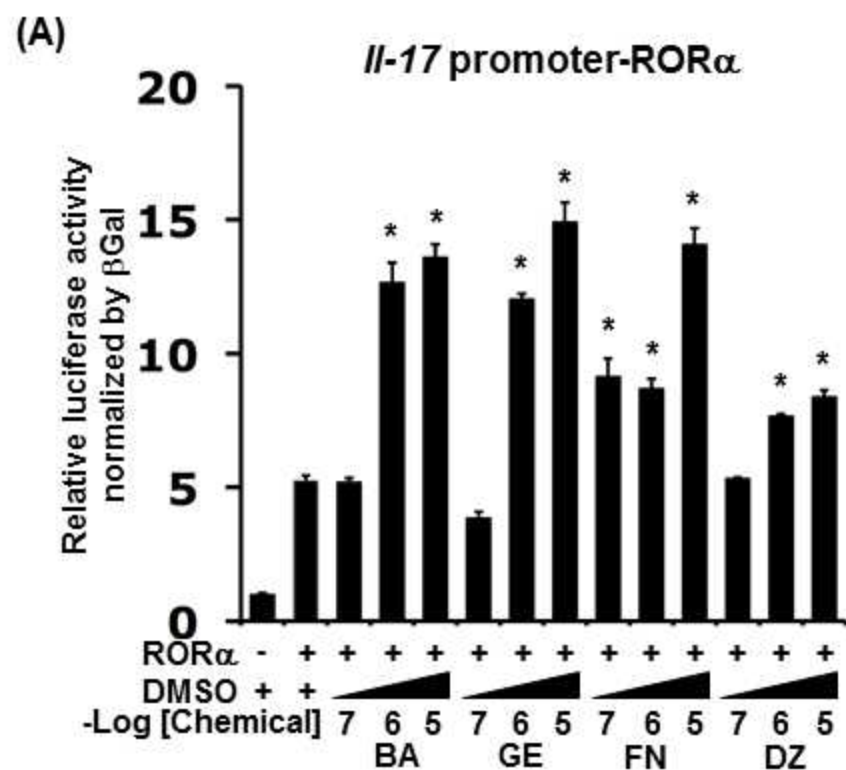
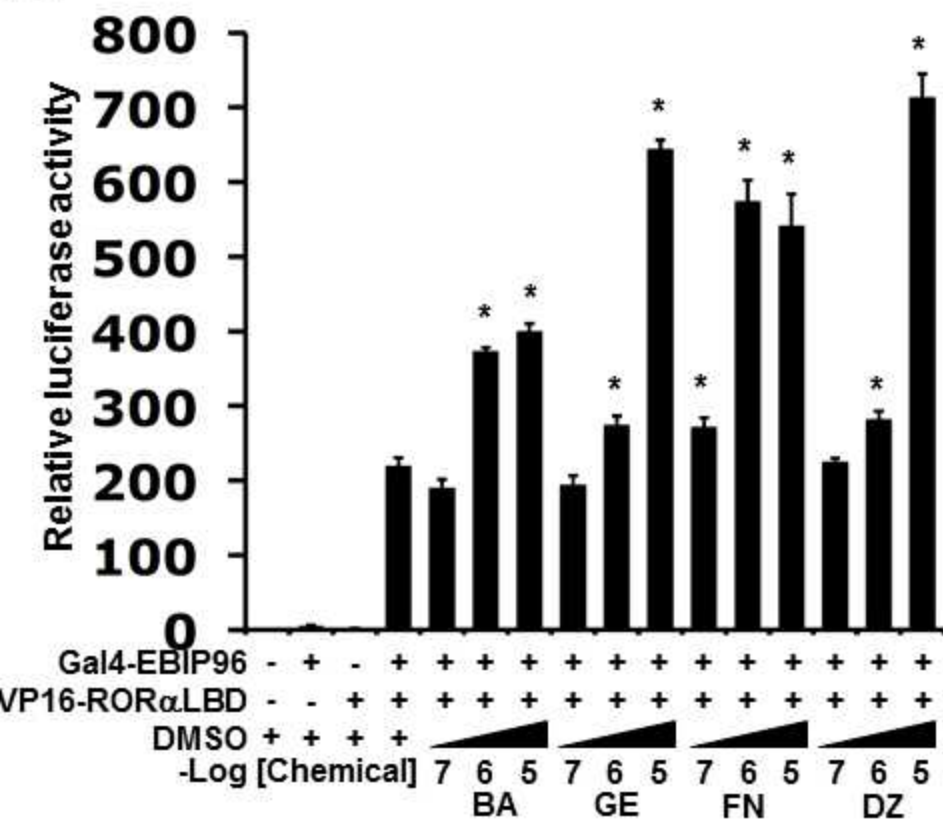


Figure 3

(A)



(B)

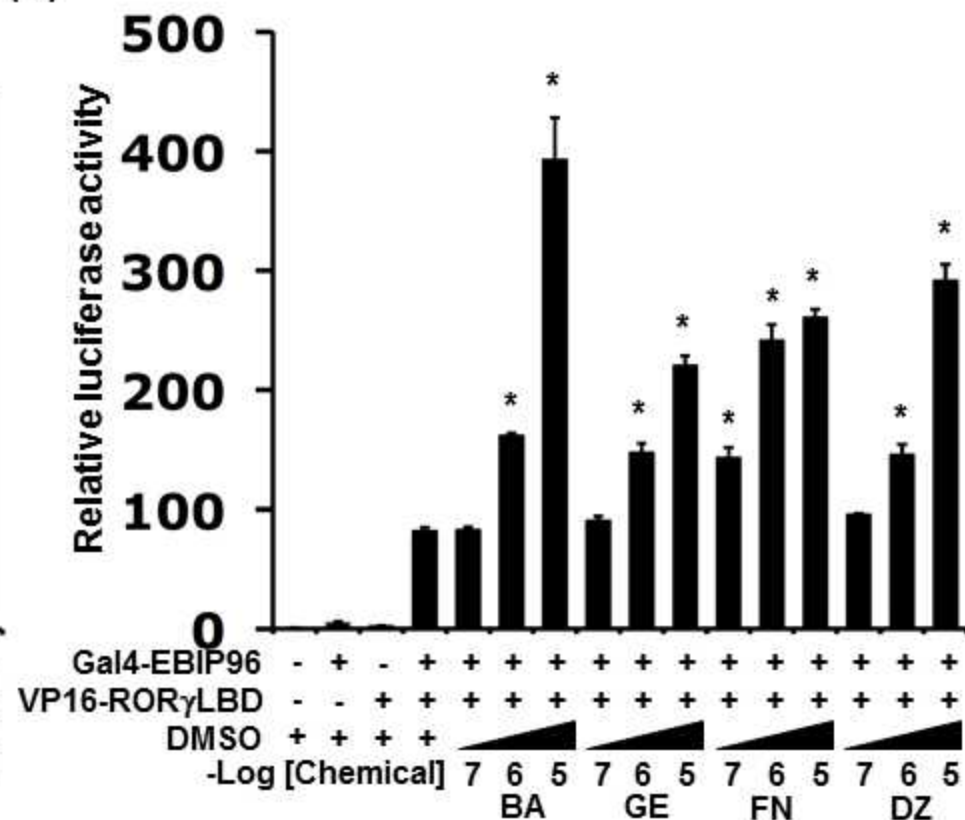


Figure 4

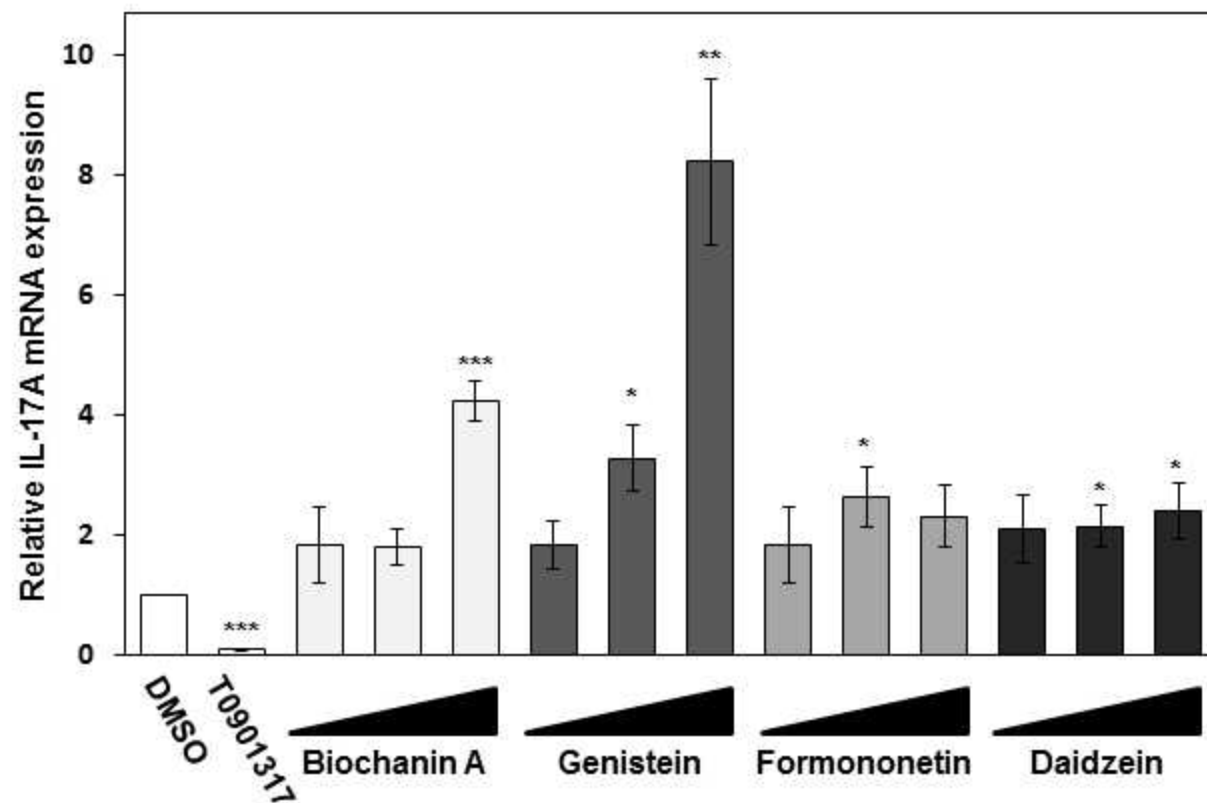


Figure 5

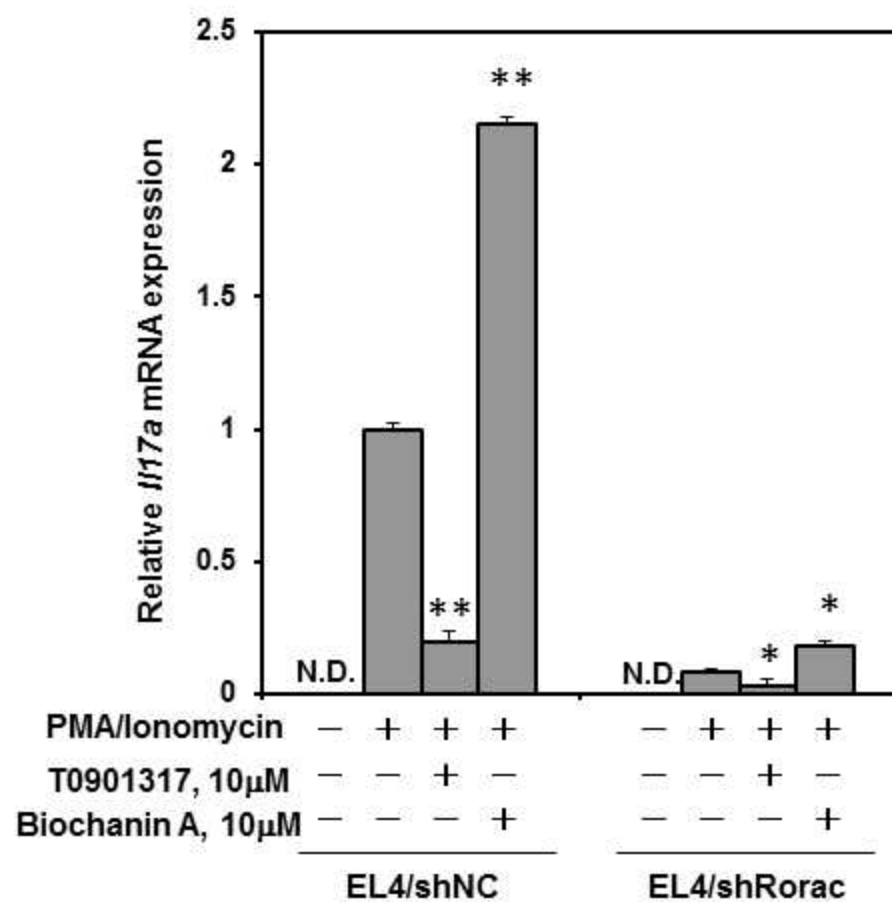


Figure 6

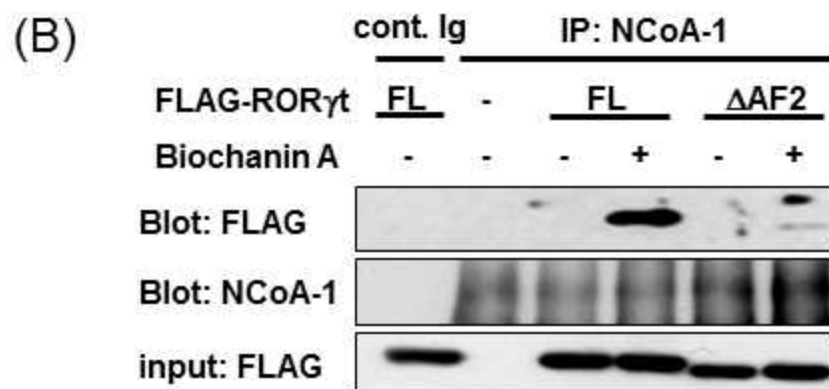
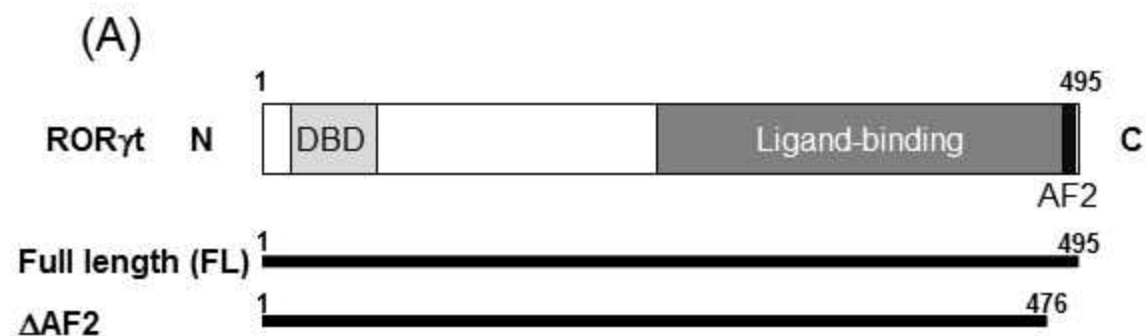


Figure 7